EVOLUTIONARY BIOMECHANICS OF SOUND PRODUCTION AND RECEPTION

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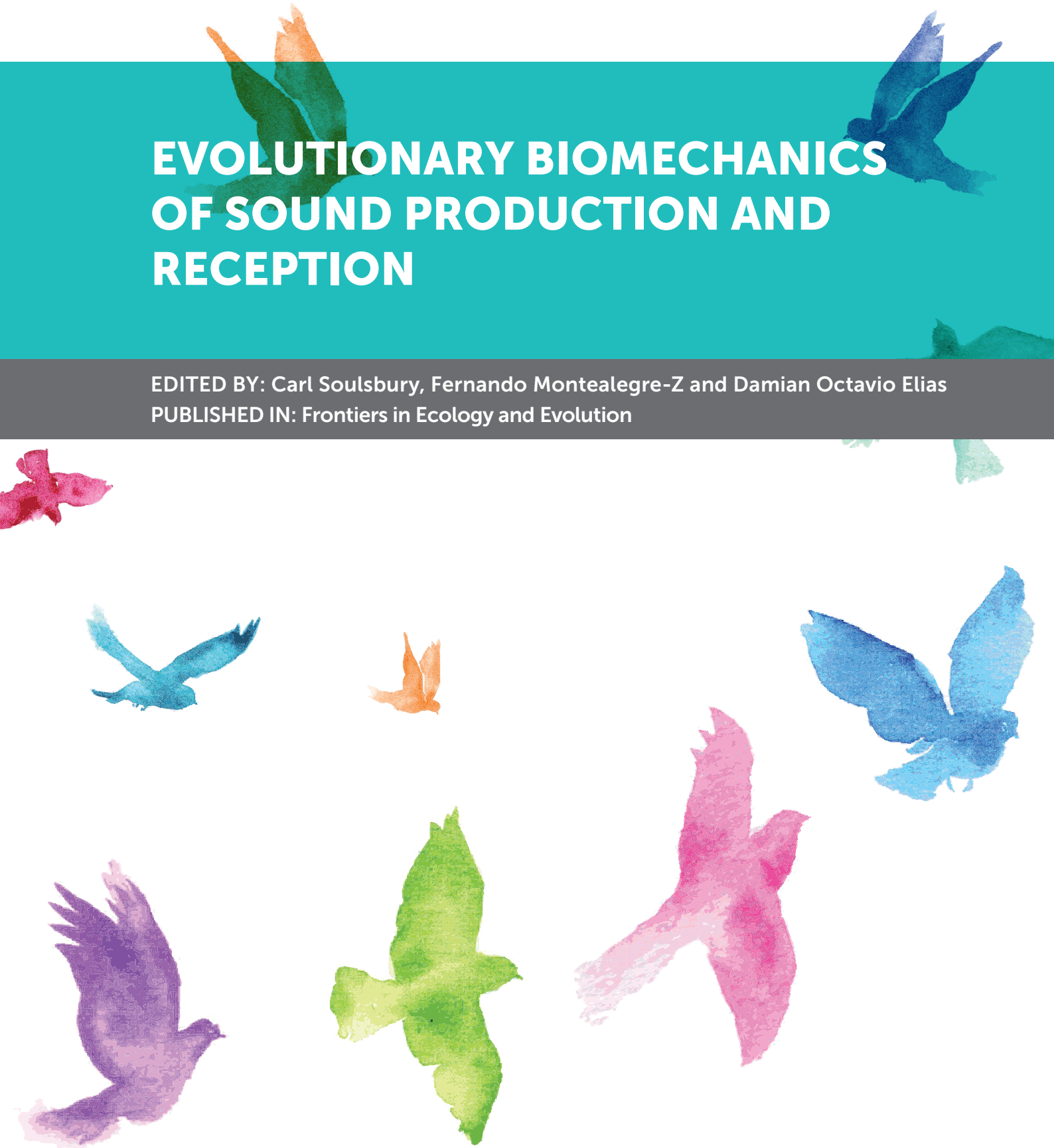

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EVOLUTIONARY BIOMECHANICS OF SOUND PRODUCTION AND RECEPTION

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EDITORIAL
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Editorial: Evolutionary Biomechanics of Sound Production and Reception

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Editorial on the Research Topic

Evolutionary Biomechanics of Sound Production and Reception

INTRODUCTION

Animals are capable of producing and detecting a broad range of vibrations transmitted through air, fluids such as water, or solids. The production of vibrations for communication (i.e., signals) in the animal kingdom involves three successive steps: (1) the production of vibrations, (2) modifications of vibrations to target specific functions, and (3) the coupling of these vibrations to the medium in which vibrations propagate. Although all three steps represent a challenge, producing signals and coupling them to the medium is exceedingly difficult in an energetic sense (particularly signals transmitted through air), and is perhaps the main reason why only two large groups of animals have evolved airborne sound and vibratory communication: Arthropods and Vertebrates, and within these groups only some taxa are capable of producing airborne sound signals (Bradbury and Vehrencamp, 1998). In Arthropods, acoustic communication is limited to crustaceans, arachnids and insects (Dumortier, 1963), with airborne sound detection being most prominent in some insect orders (Yack, 2004), with some reports in the literature for airborne sound production and detection in crustaceans (Popper et al., 2001), and Arachnids (Shamble et al., 2016). The detection of vibrations in the medium involves three successive steps: (1) a detection system to capture the vibrations, (2) the coupling of vibrations to the organism, and (3) the processing of sensory stimuli. In vertebrates airborne sound production and hearing (airborne sound detection) is more noticeable, although some taxa have lost at least one of these two features (Goutte et al., 2017). Airborne sounds, and vibratory communication (also known as biotremology) play a critical role in the day-to-day routines and survival of many species, for example in social communication (including mating), territoriality, detecting predators and in the detection and orientation of prey capture including echolocation.

This special issue covers various topics of acoustic communication (sound production and hearing) in animals from invertebrates to mammals primarily focusing on airborne sound but including one article on substrate-borne vibrations in webs (Miller and Mortimer). Each of the articles in this issue examines the biomechanics of the various forms of mechanisms that animals use for airborne sound production and detection (mechano-sensation). Therefore, articles are not centred on one specific topic but instead cover a range of systems that highlight recent advancements in animal bioacoustics.

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SOUND PRODUCTION IN ANIMALS

Animals produce vocal sounds when the acoustic vibrations originate in the respiratory system. But sound can also be produced mechanically by the interaction of body parts or by using external objects in the environment. Historically, most research has focused on vocal communication in animals, whereas other non-vocal sound mechanics (e.g., stridulation, percussion, tremulation) has attracted the attention of researchers and have advanced considerably in the last four decades.

Vocal sounds are restricted to vertebrate animals (Bradbury and Vehrencamp, 1998), although some insects use their respiratory system to eject air and produce sound (Drosopoulos and Claridge, 2005). Producing signals and coupling them to the medium is difficult, and there are physical and biomechanical constraints that shape both the production and detection of airborne sounds. Jakobsen et al., discuss the physical and physiological mechanisms that constrain the production, radiation and propagation of loud airborne sounds. Importantly, other factors can constrain the transmission of acoustic signals; Tanner and Bee demonstrate the critical role of ambient noise in affecting signal identity and hence sexual selection in frogs.

Non-vocal sounds are produced by many invertebrates and by some members of nearly all vertebrate classes and occur across a variety of behavioral contexts from signals that function to bring animals together (social signals e.g., mating signals) to those meant to keep them apart (asocial signals e.g., warning and aggression signals). A third type of signal is specific to animals that use echolocation ("auto-communication") and function to capture prey and navigate the environment. Non-vocal signals are generally poorly studied in vertebrates and their importance in some taxa needs greater exploration. In their opinion article, Verga and Ravignani, discuss the current and future study of non-vocal (percussive) signals in seals and the need for a holistic approach into the study of form and function in these signals. Similarly, there is a growing body of work on the drumming behavior of woodpeckers. Schuppe et al. synthesizes our current understanding of the evolutionary biomechanics of woodpecker drumming and highlight how the critical integration evolution, behavior, biomechanics and physiology is necessary to understand this signal. Invertebrates by contrast, have been extensively studied for non-vocal sound production. Even so, our understanding of sound production in well-studied models is incomplete. Jonsson et al. present the latest advancements in field crickets that explain how crickets synchronize wings vibrations to produce pure tone airborne sound signals.

Asocial signals can be used for a variety of functions including antipredatory and defensive signals. Low et al. present an overview of insect defensive signals and discuss their forms, function and evolutionary origins. More specifically moths have evolved sophisticated antipredatory behavior to counter detection by bat echolocation, including anti-bat sounds echolocation. This bat-moth story is one of the pillars of neuroethology (Conner and Corcoran, 2012), and in this special issue, O'Reilley et al. further this work by characterizing sound production in four microlepidopteran taxa which use wingbeat-powered ultrasound, and which may offer an anti-bat function.

SOUND RECEPTION IN ANIMALS

Mechanosensors activate and respond to stimuli representing different kinds of force such as touch, medium flow, airborne sound, substrate vibrations, and strain (see research article by Strauß et al.). According to Cocroft et al. (2014), biotremology and chemical signaling are the oldest forms of communication known, and both probably evolved from the original cell–cell mechanical and chemical interactions within early metazoans. Although biotremology and chemical signaling are some of the less well-known among all the sensory modes, the former has received more attention in the last decade. Miller and Mortimer present recent insights on how vibratory transmission constraints are mitigated to promote information transfer in spider webs.

Auditory airborne sensory organs are morphologically diverse across animals, with respect to their body location, accessory structures, and number of auditory units, but remarkably uniform in that most are innervated by specialized mechanosensory receptors. The evolution of hearing is a topic that is of interest to a broad audience, and there have been fascinating new insights over the past 30 years. Fossil evidence and modern comparative/evolutionary analyses have prompted a reinterpretation of the evolution of the middle ear bones, eardrum, and spaces around the inner ear (Köppl and Manley, 2014). In the same way, the basic units of hearing, the hair cells in tetrapods and the chordotonal organs of insects, share some common biophysical principles in spite of being separated by millions of years of evolution. These evolutionary insights have opened up an enormous amount of Research Topics in hearing, with plenty of new potential model organisms that should be considered. Warren and Nowotny, provide an important comparison between mammalian and insect ears, whilst Ketten et al. compare adaptations for echolocation in water vs. air. Both articles highlight the variety and shared specializations for hearing across insects and in terrestrial and aquatic mammals, from both evolutionary and biomechanical perspectives, including sound capturing, directional hearing, impedance conversion and frequency analysis. Further, directional hearing (assessing the location of different sound sources) is vital for many species to detect predators and prey and is especially challenging for small organisms such as insects. Mason revisits the sophisticated hyper-accurate ear of the small acoustic parasitoid fly *Ormia ochracea* and shows that they solve this problem using pure-time differences in neural responses to prey sound cues.

CONCLUSIONS

During the last 50 years, our understanding of sound production and sound reception has advanced considerably, and we now know much more on the biomechanics of sound production

and hearing, as well as the evolutionary diversity (including shared adaptations) of the systems used by animals. This special issue brings together a cutting-edge collection of papers that broadly synthesizes key areas in evolutionary biomechanics and critically provide a number of key areas of future research.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Species Recognition Is Constrained by Chorus Noise, but Not Inconsistency in Signal Production, in Cope's Gray Treefrog (*Hyla chrysoscelis*)

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Optimal mate choice based on the assessment of communication signals can be constrained by multiple sources of noise. One well-known impediment to acoustically guided mating decisions is the ambient noise created by multiple signaling individuals in large social groups, in which ambient noise can mask signals by impairing signal recognition and discrimination by receivers. Although studied far less often, another potential source of noise in communication systems stems from variability or inconsistency in how signalers produce their signals. Consistency is especially important in the context of mate choice because sexual advertisement signals are frequently produced repeatedly through time and are composed of constituent parts (e.g., notes and pulses) that are repeated within signals. Inconsistent signal production within individuals has the potential to mask between-individual differences that are often the target of receiver decision-making. In this study of Cope's gray treefrog, *Hyla chrysoscelis*, we tested the hypothesis that ambient noise and inconsistent signaling, both independently and synergistically, impair discrimination of species identity. We assayed female discrimination based on pulse rate, a signal of species identity, in quiet and at three levels of ambient noise designed to simulate a breeding chorus. We used synthetic advertisement calls that were invariant or generated with one of three experimental levels of inconsistency in pulse rate, chosen based on levels of within-individual variation observed in natural calls. Pulse rate discrimination was impaired by average and above-average levels of chorus noise, but not by inconsistency in signal production. Receivers spent slightly more time making decisions at the highest level of chorus noise, but response latencies were unaffected by inconsistency. There was no evidence of synergism between ambient noise and inconsistency. Our results suggest that ambient noise, but not inconsistency in signal production, may limit sexual selection on a signal of species identity in natural settings.

Keywords: acoustic communication, anuran, mate choice, noise, sexual selection, signaling, species recognition, within-individual variation

INTRODUCTION

"Noise" refers to any factor that causes signal detection or discrimination errors (Shannon, 1948; Brumm and Slabbekoorn, 2005; Wiley, 2015). As such, noise is a potent source of selection on animal communication systems (Brumm, 2013), particularly on acoustic signals and auditory perception (Brumm and Slabbekoorn, 2005). The potency of noise stems from its ability to impair receiver decisions with critical impacts on evolutionary fitness, such as mate choice, that depend on recognizing and discriminating among signals. Many acoustically communicating species, for example, produce high amplitude signals and breed in dense social aggregations, where the ambient noise generated by the sounds of conspecific and heterospecific signalers is an important aspect of the acoustic environment in which mate choice occurs (Gerhardt and Huber, 2002; Brumm and Slabbekoorn, 2005). In some frog and insect communication systems, for example, the ambient noise in breeding choruses is known to impede signal recognition and signal discriminability on the basis of temporal and spectral properties (Wollerman and Wiley, 2002; Bee, 2008a; Vélez and Bee, 2011; Römer, 2013; Ward et al., 2013a; Reichert and Ronacher, 2015; Lee et al., 2017; Tanner and Bee, 2019, 2020). Consequently, ambient noise can affect the strength and direction of sexual selection imposed by receivers on signalers by limiting female preference expression.

While the impacts of biotic, abiotic, and anthropogenic sources of ambient noise are increasingly recognized (Römer, 2013; Reichert and Ronacher, 2015; Wiley, 2015; Slabbekoorn et al., 2018; Dominoni et al., 2020), other potential sources of noise in animal communication are rarely explored. One such source is inconsistency in signal production (Gerhardt and Watson, 1995; Nehring et al., 2013; Tanner and Bee, 2019, 2020). In some systems, the degree of consistency in signal production might itself function as a signal if consistent motor performance is a reliable indicator of mate quality (Ballentine, 2009; Byers et al., 2010). However, inconsistent signal production is also an important source of noise to consider. As Nehring et al. (2013) note, when "intra-individual variation does not convey any information that is useful for the receiver, it is noise, since it potentially makes it harder for the receiver to identify and interpret the information" (p. 378). Noise in signal production is particularly important in the context of mate choice because it has potential to obscure the between-individual differences that are frequently considered the targets of mate selection by receivers (Gerhardt and Watson, 1995; Tanner and Bee, 2019, 2020). Acoustic signals, for example, are typically produced repeatedly during sexual advertisement or courtship and also comprise repeated constituent elements, such as pulses or notes, that are not produced identically upon every iteration. Substantial inconsistency in signal production, even over short time intervals such as a single bout of signaling, has been widely reported in diverse taxa (e.g., orthopterans, Shaw and Herlihy, 2000; fish, Amorim and Vasconcelos, 2008; anurans, Gerhardt, 1991; reptiles, Crews, 1975; mammals, Mitani and Brandt, 1994). Despite widespread documentation of inconsistent signal production, and the comparative wealth of data demonstrating female preferences with regard to between-individual differences, few studies have investigated if and how inconsistent signaling affects signal recognition and discriminability either using simulations (Lengagne et al., 2016) or empirically (Gerhardt and Watson, 1995; Tanner and Bee, 2019, 2020). Even fewer studies have investigated the potential interaction between ambient noise and inconsistent signaling. One likely reason for the dearth of previous work on inconsistent signaling is the historical primacy of investigating the criteria that receivers use to discriminate among signalers using experimental stimuli designed explicitly to remove natural levels of within-individual variation in signal production as a potential experimental confound.

Variation in advertisement call traits within and among males, and associated female preferences, have been particularly well-studied in North American treefrogs (Hylidae) (Gerhardt, 2001; Gerhardt and Huber, 2002), such as Cope's gray treefrog (*Hyla chrysoscelis*) (Gerhardt and Doherty, 1988; Gerhardt, 2001, 2008; Bush et al., 2002; Bee, 2008b; Ward et al., 2013b; Tanner et al., 2017). We recently showed that both ambient chorus noise and inconsistent signaling impair the ability of female *H. chrysoscelis* to discriminate among potential mates based on differences in their rate of call production ("call rate") (Tanner and Bee, 2020), a potential non-arbitrary signal of *male quality* due to the high metabolic cost of signaling (Taigen and Wells, 1985). In the present study, we investigated the effects of ambient chorus noise and inconsistent signaling on the ability of female *H. chrysoscelis* to discriminate among potential mates based on a signal of *species identity*. Male *H. chrysoscelis* produce advertisement calls that consist of a series of repeated constituent elements, that is, a sequence of about 12 to 43 sound pulses generated via independent contractions of the body wall (McLister et al., 1995; Girgenrath and Marsh, 1997). Based on analyses of 1000 calls (20 calls/male; 50 males) the temperature-corrected (20°C) mean rate of pulse production within individual calls was 48.8 pulses per second [pps], and across males in the population, temperature-corrected mean pulse rates ranged between 41 and 64 pps (Ward et al., 2013b). Female *H. chrysoscelis* rely upon pulse rate to distinguish between conspecific males and males of the closely related eastern gray treefrog (*Hyla versicolor*), a cryptic, tetraploid sister species that breeds syntopically across their shared range (Bush et al., 2002; Schul and Bush, 2002). Males of *H. versicolor* produce an acoustically similar call with a slower temperature-corrected (20°C) mean pulse rate of 21.5 pps (based on analyses of 368 calls from 13 males; 14 to 58 calls/male) (Ward et al., 2013b). Across males in the population, the temperature-corrected mean pulse rate of *H. versicolor* calls ranged between 17 and 35 pps. Female *H. chrysoscelis* reject calls with pulse rates substantially lower or higher than the species typical rate (Bush et al., 2002; Gerhardt, 2008; Ward et al., 2013a). Within the natural range of variation in conspecific pulse rates, females have directional preferences for faster pulse rates (Bush et al., 2002; Tanner et al., 2017). Mismating with *H. versicolor* is costly because resulting offspring are sterile triploids (Gerhardt et al., 1994; Tucker and Gerhardt, 2012).

Previous studies of *H. chrysoscelis* have measured the extent of within-individual variation in pulse rates (Ward et al., 2013b) and established that inconsistency does not signal body condition

or size (Tanner and Bee, 2019) and thus potentially acts as a source of noise in the communication channel in that it could obscure receiver estimates of meaningful call characteristics. Furthermore, while female *H. chrysoscelis* have preferences for more consistent (less variable) call rates, they do not discriminate among signals on the basis of inconsistency in pulse rate alone (Tanner and Bee, 2019). Here, we tested the hypothesis that ambient noise and inconsistent signaling, both independently and synergistically, impair the expression of female preferences for the pulse rates of male advertisement calls. Female preference functions were assayed across a biologically relevant range of mean pulse rates using two-alternative choice tests in which subjects were able to choose between two sequences of synthetic calls that simulated two calling males. Realistic levels of pulse rate inconsistency were introduced in both sequences by manipulating the within-individual coefficient of variation (CV_w) in this call property. We performed the experiment in quiet and at three levels of ambient noise designed to reflect variation in the background noise levels of gray treefrog choruses. We predicted that high levels of both ambient noise and inconsistency would impair signal discrimination on the basis of differences in mean pulse rate, and that a synergistic effect of both noise sources would further impair pulse rate discrimination. We further predicted that response latencies would increase in noisy conditions and when subjects discriminated between highly inconsistent and more similar pulse rates because, in these difficult discrimination tasks, subjects might benefit from increased assessment times.

MATERIALS AND METHODS

Subjects

Subjects were 289 gravid female *H. chrysoscelis* of the western mtDNA lineage (Ptacek et al., 1994) captured in amplexus. Amplectant pairs were collected by hand at night (2200-0200) during the breeding season (mid-May to early July) in 2015, 2016, and 2017. Gravid female treefrogs collected in amplexus are as discriminating as those captured prior to pair formation (Murphy and Gerhardt, 1996). Capture sites were located in east-central Minnesota and included Carver Park Reserve (44.52490, −93.43031; Carver County), Richardson Nature Center (44.84214, −93.37148; Hennepin County), Crow-Hassan Park Reserve (45.19471, −93.65368; Hennepin County), and Lake Maria State Park (45.32012, −93.94389; Wright County). Treefrog pairs were housed in plastic containers that were labeled with unique ID numbers and taken to the laboratory, where the frogs were placed in aged tap water and maintained at approximately 2–4°C for up to 36 h to delay the deposition of eggs. Prior to testing, we placed each pair in room-temperature aged tap water inside a temperature-controlled incubator at 20°C for at least 30 min, until they reached a body temperature of 20 ± 1°C. In empirical studies of pulse rate discrimination, temperature control is essential because male signal production and female mating preferences are temperature dependent (Gerhardt, 1978; Gerhardt and Huber, 2002). We released all treefrogs at their original capture sites within 3 days of collection. The subjects were not marked. Because females probably breed only once or at most twice during the relatively short breeding season in Minnesota (Ritke et al., 1990), and because we collected frogs from multiple, large wetlands over multiple years, the risk of recapturing the same individuals, and associated risk of pseudoreplication, is very low. We chose to tolerate this small risk of unknowingly testing the same individual twice rather than subjecting each individual to invasive marking procedures that carry some risk to the animals (e.g., toe-clipping) and typically do not last multiple years.

Acoustic Stimuli

We generated synthetic treefrog calls and ambient noise *de novo* using custom scripts in MATLAB® versions 2015a and 2016a (The Mathworks, Natick, MA, United States). All stimuli were generated at a sample rate of 44.1 kHz and a bit-depth of 16. Each of the two stimuli generated for a given two-alternative choice test consisted of a sequence of synthetic calls that simulated a male treefrog producing an advertisement call at a constant rate. A total of 1,820 unique stimuli were used in our phonotaxis tests, and no call sequence was assigned to more than one subject. We modeled the stimuli after natural advertisement calls produced by Cope's gray treefrogs in east-central Minnesota, using the mean trait values published in Ward et al. (2013b) to set the values of all call traits not under consideration. Calls comprised 30 pulses with a constant, 50% pulse duty cycle. We manipulated the mean pulse rates of calls and the within-individual variation in pulse rate across the calls in a given call sequence. Within each call, pulse duration and interpulse interval were equal and determined as functions of the experimentally determined pulse rate and the fixed 50% pulse duty cycle. Call duration was always fixed in terms of the number of pulses per call (30 pulses), but the absolute duration of each call, measured in milliseconds, was allowed to vary as necessary to accommodate different pulse rates (the study-wide slowest pulse rate of 31.68 pps yielded a call duration of 931.2 ms; the study-wide fastest pulse rate of 54.19 pps yielded a call duration of 544.4 ms). Individual pulses were constructed by adding two phase-locked sinusoids with frequency components at 1,250 Hz (−11 dB) and 2,500 Hz (0 dB) and the amplitude envelope of each pulse was shaped with species-typical onsets and offsets that were fixed proportions (0.36 and 0.64, respectively) of pulse duration. The amplitude envelope of each call was given a linear onset over the first 50 ms. Stimuli were played back at 85 dB (SPL re 20 μPa, fast RMS, C-weighted), measured at a distance of 1 m, to approximate the level of a natural call (Gerhardt, 1975). Previous studies have demonstrated that females respond readily in choice tests with similarly designed synthetic stimuli and playback methodology (Ward et al., 2013b; Tanner et al., 2017; Tanner and Bee, 2019).

We generated ambient, "chorus-shaped" noise by filtering white noise to have the average long-term spectral characteristics of a gray treefrog chorus following the procedures outlined in Vélez and Bee (2011). To ensure any effects of chorus-shaped noise were not artifacts of a particular realization of a randomly generated stimulus, we replicated the noise files (N = 34). No more than ten subjects were assigned the same noise replicate. We generated the noise replicates at a sample rate of 11.025 kHz

and then upsampled to 44.1 kHz; this was done to circumvent limited computing power during stimulus construction.

Experimental Design

We used a series of two-alternative choice tests to measure the shapes of female preference functions for pulse rate in the presence of ambient noise and signal inconsistency. Stimuli consisting of sequences of calls were constructed by randomly drawing a value for the pulse rate of each consecutive call within a sequence from a normal distribution having a mean and standard deviation that were specified to allow us to manipulate both mean pulse rate and inconsistency in pulse rate across treatments. The means of separate distributions were centered on pulse rates that were either −3, −2, or −1, or 0 *SD* from the population mean, generating *nominal* mean pulse rates of 35.6, 40.0, or 44.4, and 48.8 pps, respectively. We assayed female preferences between −3 and 0 standard deviations of the population mean pulse rate because, as noted previously, female *H. chrysoscelis* use pulse rate to discriminate between conspecific and heterospecific males (Schul and Bush, 2002). Males of the closely related *H. versicolor* produce acoustically similar calls with slower pulse rates and female *H. chrysoscelis* discriminate against slower-than-average conspecific pulse rates (Gerhardt et al., 1994; Bush et al., 2002; Ward et al., 2013a; Tanner et al., 2017). Note that a value of −2 *SD* (40.0 pps) corresponds to the lower end of the range of variation in conspecific (*H. chrysoscelis*) pulse rates (adjusted to 20°C), whereas −3 *SD* (35.6 pps) falls outside the range of conspecific pulse rates but approximates the upper end of the range of variation in heterospecific (*H. versicolor*) pulse rates (adjusted to 20°C).

To investigate the impacts of ambient noise, we replicated all two-alternative choice tests in quiet and at three levels of ambient noise (60, 70, and 80 dB SPL). In the quiet condition, no noise was broadcast. The three levels of broadcast ambient noise were chosen to approximate the mean (70 dB SPL) and ±1 *SD* (10 dB) of the sound pressure levels of background noise measured in east-central Minnesota gray treefrog choruses (Tanner and Bee, 2019).

We manipulated inconsistency in pulse rate by controlling in our synthetic signals the within-individual coefficient of variation in this property across calls within a sequence simulating a calling male ($CV_w = SD/\bar{X}$). That is, the pulse rate of each individual call within a stimulus sequence was fixed, but pulse rate was allowed to vary across calls within a sequence. In addition to calls that were invariant within a sequence (CVw = 0.000), we chose three *nominal* levels of within-individual (i.e., within-sequence) variation (CV_w = 0.004, 0.010, 0.030; **Figure 1**) to match the minimum, mean, and maximum CV_w previously measured in the population (Ward et al., 2013b). These population estimates of pulse rate inconsistency are based on analyses of single call bouts comprising 20 consecutively produced calls from each of 50 males (1,000 calls total); the average 20-call bout lasted 133.5 s (range: 70.4–291.3 s) (Ward et al., 2013b). To introduce realistic levels of inconsistency in pulse rate into our stimuli, we manipulated the standard deviation of the normal distributions centered on each target mean pulse rate to achieve the three desired levels of CV_w for each mean pulse rate. Hence, the pulse rates of consecutive calls in each sequence were independently chosen from a distribution that allowed pulse rate to vary inconsistently according to the nominal level of CV_w around the specified nominal mean pulse rate of the stimulus (see **Figures 1A,B**). Higher levels of inconsistency (larger CV_w) correspond to broader normal distributions and thus generated more variable synthetic signals. To ensure that randomly chosen pulse rates fell within a behaviorally relevant range, we excluded pulse rate values that were higher than the fastest *H. chrysoscelis* pulse rate reported in Ward et al. (2013b) or lower than three standard deviations slower than the mean *H. versicolor* pulse rate.

We designated separate experimental and control treatment groups. For subjects in the experimental group, we measured how female preferences for pulse rates at the population mean (48.8 pps) over slower pulse rates changed in the presence of ambient noise and inconsistency using a 3 × 4 × 4 factorial design in which we manipulated the mean pulse rate of the slower alternative stimulus (PR = 35.6 [−3 *SD*], 40.0 [−2 *SD*], 44.4 pps [−1 *SD*]; within subjects), ambient noise levels (quiet, 60, 70, and 80 dB SPL; within subjects), and levels of inconsistency (CV_w = 0.000, 0.004, 0.010, 0.030; between subjects). Thus, each subject in the experimental group was assigned to a single level of inconsistency and tested in up to 12 behavioral trials ($N_{subjects}$ = 246; N_{trials} = 2,641) in which the choice was between two equally inconsistent signals with different mean pulse rates (i.e., population mean versus −3, −2, or −1 *SD*) across four levels of ambient noise. Subjects in the control group ($N_{subjects}$ = 43; N_{trials} = 657) chose between two signals with equal nominal mean pulse rates set at the population mean of 48.8 pps (i.e., population mean versus −0 *SD*) across all 16 factorial combinations of ambient noise (quiet, 60, 70, and 80 dB SPL; within subjects) and inconsistency (CV_w = 0.000, 0.004, 0.010, 0.030; within subjects).

It is important to note that for both treatment groups, the two alternative stimuli in a given choice test *always* had the same experimentally specified, nominal level of inconsistency. Hence, all subjects in both treatment groups chose between two equally inconsistent alternatives; subjects in the experimental group were given choices of stimuli having different nominal mean pulse rates, whereas subjects in the control group chose between two stimuli with the same nominal mean pulse rate of 48.8 pps.

Testing Protocol

We conducted choice tests in a custom-built, temperature controlled, semi-anechoic chamber (2.8 m × 2.3 m × 2.1 m, length × width × height; IAC Acoustics, North Aurora, IL, United States) at 20 ± 1°C. The chamber walls and ceiling were acoustically insulated and covered in dark gray, perforated material (IAC Planarchoic™ panel system). The floor was covered with dark gray, low-pile carpet. The testing arena was circular (2.0 m × 0.6 m, diameter × height) and constructed from hardware cloth covered with black fabric. We placed an acoustically transparent release cage (9 cm × 2 cm, diameter × height) on the floor in the center of the arena. The cage could be operated by means of a rope-and-pulley system by an observer outside the chamber. We used two Mod1 Orb speakers (Orb Audio, New York, NY, United States) for

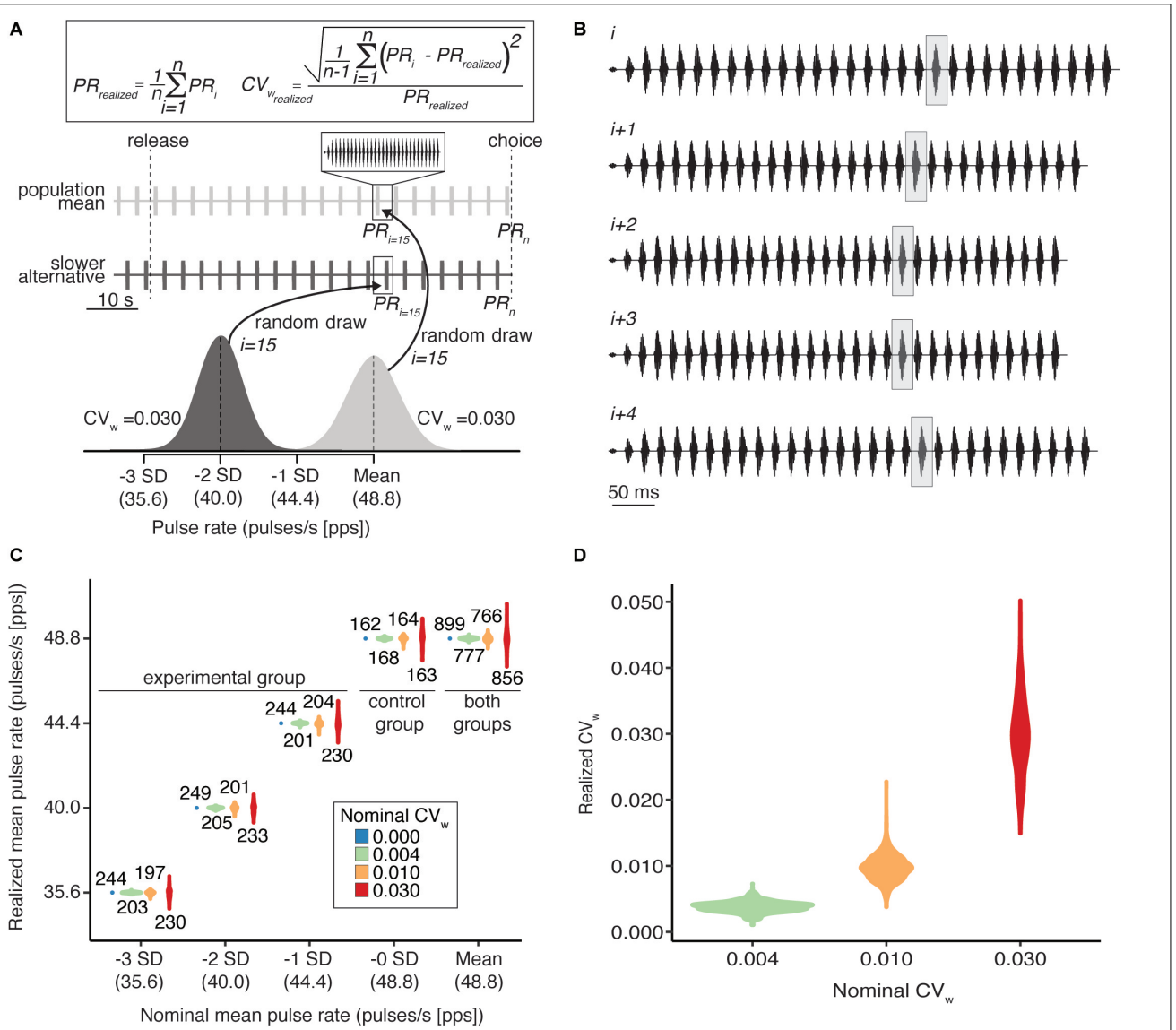

FIGURE 1 | Relationships between realized and nominal trait values in experimental stimuli. Nominal values represent experimentally specified levels while realized values reflect averages among the stimuli that were heard by the subject in each trial. The trait values of individual calls in stimuli with higher assigned CV_w were drawn from broader distributions, making these stimuli more variable. **(A)** Random draws from normal distributions representing the population mean (light gray) and *–2 SD* (dark gray) distributions used to generate two alternative signal sequences (waveforms) for a single behavioral trial. Equations (top) demonstrate the computation of realized mean pulse rate and realized CV_w for a given sequence. **(B)** Waveforms of five representative calls with randomly drawn pulse rates (*i* to *i* + 4) from a representative stimulus sequence whose nominal mean pulse rate was 0 *SD* (48.8 pps) in the CV_w = 0.030 inconsistency level. The 20th pulse in each call is highlighted in gray to aid in visualizing the variable pulse rate across calls. **(C)** Violin plots show the distributions of realized alternative pulse rates in experimental stimuli grouped by nominal mean pulse rate. Subjects assigned to the experimental group were given a choice between a stimulus whose nominal mean pulse rate matched the population mean of 48.8 pps (Mean) and an alternative stimulus with a nominally slower pulse rate (i.e., Mean versus –3, –2, or –1 *SD*). Subjects assigned to the control group were given a choice between two equivalent stimuli whose nominal mean pulse rates matched the population mean (i.e., Mean versus –0 *SD*). Text annotations show the sample size, in number of tests, at each combination of nominal mean pulse rate and coefficient of variation within stimuli (CV_w). Because in each test subjects across both groups chose between a stimulus with a mean pulse rate (Mean) and one whose pulse rate was –3, –2, –1, or –0 *SD* from the mean, the sample sizes of the Mean alternative are necessarily sums of the other four tests performed at the same nominal level of within-individual variation. **(D)** Distributions of realized CV_w across three nominal levels of within-individual variation. Invariant stimuli necessarily have a realized CV_w of 0 and are not depicted in **(D)**. In both panels **(C,D)**, colors represent nominal levels of within-individual variation.

sound playback. We positioned the two speakers 90° apart on the floor outside the arena wall. Phonotaxis trials were conducted under infrared light (Tracksys, Ltd., Nottingham, United Kingdom) and scored in real time by a trained observer from outside the chamber by means of a closed-circuit television system with an infrared-sensitive video camera (Panasonic WV-BP334; Panasonic Corporation of North America, Secaucus, NJ, United States) mounted from the chamber ceiling.

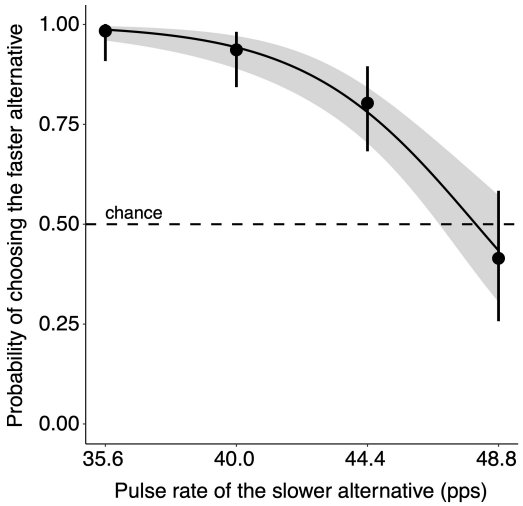

FIGURE 2 | Pulse rate preference function in quiet conditions and the absence of inconsistency ($CV_w = 0$). Subjects chose between a stimulus whose nominal mean pulse rate was equal to the population mean (0 *SD* = 48.8 pps) and a stimulus whose nominal mean pulse rate was 0, 1, 2, or 3 *SD* slower (48.8, 44.4, 40.0, or 35.6 pps, respectively). The proportion choosing the faster realized mean pulse rate declined as the two alternative pulse rates became more similar. Individual points show the proportion of tests in which subjects chose the faster stimulus calculated across all tests with the same nominal mean pulse rate. The solid black line shows the logistic regression line of best fit with gray shading illustrating standard error. The dashed line shows 0.5, or the probability of choosing the stimulus with the faster pulse rate by chance. The error bars show 95% Clopper–Pearson ("exact binomial") confidence intervals.

Synthetic signals and ambient noise were broadcast using Adobe Audition 3.0 (Adobe Systems, Inc., San Jose, CA, United States) on a Dell Optiplex 980 PC (Dell Computer Corporation, Round Rock, TX, United States). We output audio using a MOTU model 16A 16-channel sound card (MOTU, Inc., Cambridge, MA, United States) and amplified it using Crown XLS1000 High-Density Power Amplifiers (HARMAN Professional, Northridge, CA, United States). We calibrated sounds to their target SPLs using a Bruël and Kjær Type 4950 microphone (Bruël and Kjær, Nærum, Denmark) placed 1 m from the speaker at the approximate position of a subject's head at the beginning of a choice test. Signals and noise were broadcast from the same speaker (co-located). In a given choice test, we broadcast the same noise replicate from both speakers simultaneously and calibrated the playback level of the ambient noise with the microphone pointed directly between the speakers such that noise from both sides contributed equally to the summed SPL.

To prevent side bias and control for presentation order, we randomized for each subject which signal was played first during a choice test, the order in which tests were conducted, and which speaker played the stimulus with the nominally slower pulse rate. For subjects in the control group, we arbitrarily labeled the two nominally equivalent stimuli and then randomized the playback speaker. At the beginning of a choice test, we separated the subject from her mate and placed her in the release cage. We allowed each subject to acclimate in the quiet chamber for 60 s. At the end of the acclimation period, we began playback. When ambient noise was present, the noise began first and played for 30 s prior to the broadcast of signals. When ambient noise was absent, silence continued for a further 30 s to ensure subjects spent the same amount of time in the release cage at the beginning of each test, regardless of the ambient noise condition. The two alternative signals were played back in an alternating and non-overlapping temporal arrangement and spaced such that there was an equal period of silence before and after each call.

When the two alternating signals had each played twice, the lid of the release cage was lifted, and the subject was allowed to move freely within the arena. Tests continued until one of the following conditions was met: (1) the subject indicated a choice by entering a response zone defined as a 10 cm radius semi-circle in front of a playback speaker; (2) the subject touched the arena wall in the quadrant of the arena opposite to the quadrant separating the two playback speakers; (3) at the end of 3 min following the lifting of the lid, the subject had not left the release cage; or (4) at the end of 5 min, the subject had not entered a response zone.

We recorded the subject's binary choice and her latency to respond, measured as the time elapsed between release from the cage and entering a response zone. Female treefrogs usually make mating decisions in 1–3 min (Schwartz et al., 2004; Tanner et al., 2017); the mean response latency across all subjects in the present study was 88.0 ± 50.8 s. This corresponds closely to the duration of the individual calling bouts over which inconsistency in pulse rate was determined by Ward et al. (2013b). Between tests, subjects were housed with their mates and returned to the incubator for a "time out" of at least 3 min. When a choice was not indicated within 5 min, the outcome of the test was scored as "no response" and following a time out, the subject was re-tested in the same test. Subjects that scored no response twice for the same test (51 of 246 subjects in the experimental group and 4 of 49 subjects in the control group) were not tested further, but we included their responses from completed tests in our statistical analyses. On average, subjects that did not complete the entire battery of tests completed 6 of 12 tests (range 1 to 11) in the experimental group and 8 of 16 tests (range 2–12) in the control group. Responses from the two groups were combined for statistical analyses.

Statistical Analysis

Experimental stimuli had trait values drawn from normal distributions and were therefore intrinsically variable and dependent on the response latency of the subjects (**Figure 1**). In trials in which subjects listened longer before indicating a choice, *realized* mean pulse rates and *realized* levels of inconsistency more closely matched the *nominal* levels specified by our experimental design. It was therefore necessary to account for the trait values of experimental stimuli that were actually experienced by a given subject during a given trial. Otherwise, stochastic deviations from the nominal pulse rate and CV_w specified by our treatment levels would introduce error into analyses. To account for the trait values subjects actually experienced, we performed a *post hoc* calculation of the realized mean pulse rate and CV_w in the sequence of signals experienced by the subject

during the trial (**Figure 1**). We used the response latency to exclude calls from each sequence that the subject did not hear (i.e., that did not occur) between the beginning of playback and indicating a choice. We then calculated the average CV_w as the arithmetic mean of the realized CV_w values computed for the two alternative stimuli in each test. We did this to reduce the dimensionality of predictor variables. Because both stimuli in a given test always had the same nominal level of inconsistency, across the experiment we observed that the mean difference between the realized CV_w values for the two alternative stimuli in a given choice test was very small (9.07×10^{-5}; 95% of differences fell in the interval $[-6.04 \times 10^{-5}, 2.42 \times 10^{-4}]$; range $[-2.74 \times 10^{-2}, 2.58 \times 10^{-2}]$).

We fit two generalized estimating equation (GEE) models to examine female preference functions based on two response variables: binary choices (the probability that a subject chose the stimulus with the faster pulse rate) and response latencies (time elapsed prior to making a choice). GEE is an extension of generalized linear models (GLM) compatible with either binary or continuous response variables and designed for repeated measures of the same individual (Hardin and Hilbe, 2012). We specified exchangeable correlation structures, which assume that correlations between observations of the same subject are homogenous. Wald statistics with a significance criterion of $\alpha = 0.05$ were used for hypothesis testing. In each model, we included the following independent variables: main effects of the realized mean pulse rate of the stimulus with the slower pulse rate, average realized CV_w, and ambient noise level, and all two-way interactions.

RESULTS

Within the range of pulse rates tested, subjects showed pronounced preferences for calls with realized mean pulse rates (hereafter, "pulse rate") near the population mean over calls with slower pulse rates, but were less likely to choose the faster pulse rate when the two alternatives had similar rates ($\beta = -0.288$, $W = 85.90$, $p < 0.001$; **Figure 2** and **Table 1**). The probability of choosing the population mean pulse rate over a slower alternative was 0.770 in two-choice tests overall (2,540 of 3,298 tests) and 0.819 in tests performed in quiet with invariant stimuli (186 of 227 tests). Subjects were most likely to choose the population mean pulse rate when the pulse rate of the alternative was much slower (**Figure 2**); in tests with a nominal pulse rate of 35.6 pps (-3 *SD*), for example, subjects chose the faster, population-mean pulse rate with probability 0.946 (827 of 874 tests; **Figure 2**). Results from the control group confirmed that playback speaker assignment (and thus side of the testing arena) did not affect the outcome of trials (GEE: $\beta = -0.077$, $W = 0.04$, $p = 0.840$), suggesting no side bias was present in our experimental set-up.

Ambient noise presented at the highest experimental level impaired female expression of pulse rate preferences (**Figures 3A,B** and **Table 1**). Subjects were significantly less likely to choose the faster, population-mean pulse rate in the 80-dB condition (probability 0.723; 581 of 803 tests; $\beta = -5.187$, $W = 13.85$, $p < 0.001$) than in quiet (probability 0.795; 662 of 833 tests). There was a significant two-way interaction between the pulse rate of the slower stimulus and the highest level of ambient noise ($\beta = 0.107$, $W = 12.02$, $P < 0.001$), such that female preference functions for pulse rate were less steep when measured in the presence of ambient noise at 80 dB. In contrast, the level of inconsistency in the two alternative stimuli, measured as the average realized CV_w, had no effect on the probability that subjects chose the stimulus with the population-mean pulse rate ($\beta = -65.351$, $W = 1.66$, $p = 0.197$; **Figure 3B**). Subjects chose the population-mean pulse rate with probability 0.793 in tests with perfectly consistent stimuli, compared to 0.744 ($CV_w = 0.004$), 0.768 ($CV_w = 0.010$), and 0.771 ($CV_w = 0.030$) in tests using calls with inconsistent pulse rates (**Supplementary Figure S1**). Inconsistency and ambient noise did not interact synergistically to affect the probability of choosing the faster pulse rate (**Table 1** and **Supplementary Figure S2**).

The mean ($\pm SD$) response latency across all trials was 88.0 ± 50.8 s (range 6–300 s). Response latency modestly increased as a function of the pulse rate of the slower stimulus ($\beta = 1.156$, $W = 9.27$, $p = 0.002$; **Figure 3C**), such that on average, subjects spent longer making decisions when two alternatives had more similar pulse rates. When tests were performed in quiet with perfectly consistent signals, mean response latencies were 81.9 ± 40.6, 87.0 ± 47.9, 87.3 ± 48.3, and 94.0 ± 54.3 s when the nominal pulse rate of the designated slower alternative was 35.6 (-3 *SD*), 40.0 (-2 *SD*), 44.4 (-1 *SD*), and 48.8 (0 *SD*) pps, respectively. Accounting for the pulse rate of the slower alternative and inconsistency, ambient noise at both 70 dB ($\beta = 58.235$, $W = 9.90$, $p = 0.002$) and 80 dB ($\beta = 88.897$, $W = 15.21$, $p < 0.001$) significantly increased response latencies, such that subjects spent more time making decisions when listening in population-mean and above-average noise levels. The pulse rate of the slower stimulus interacted significantly with ambient noise at both 70 dB ($\beta = -1.530$, $W = 12.29$, $p < 0.001$) and 80 dB ($\beta = -1.888$, $W = 12.19$, $p < 0.001$) such that the effect of the slower mean pulse rate was reversed at these levels of ambient noise relative to the quiet and 60 dB conditions (**Figure 3C**). There was no significant effect of inconsistency on response latency ($\beta = 327.596$, $W = 0.21$, $p = 0.649$; **Figure 3D**). Mean response latencies were 89.2 ± 46.3 s ($CV_w = 0.000$), 89.6 ± 55.2 s ($CV_w = 0.004$), 87.7 ± 52.3 s ($CV_w = 0.010$), and 85.6 ± 49.9 s ($CV_w = 0.030$) within nominal levels of inconsistency. Inconsistency and ambient noise did not interact synergistically to affect the latency to respond (**Table 1**).

DISCUSSION

We examined the impact of ambient noise and inconsistent signal production on species recognition by female Cope's gray treefrogs. Our main results can be summarized as follows. Consistent with earlier studies, we found pronounced directional preferences for the population-mean pulse rate over slower pulse rates (Gerhardt, 2008; Ward et al., 2013a; Tanner et al., 2017). In general, subjects discriminated reliably between signals on

TABLE 1 | Output from two GEE models – one for the proportion of subjects choosing the population mean pulse rate over a slower pulse rate and one for response latency – examining the effects of the realized mean pulse rate (PR) of the slower pulse rate stimulus, arithmetic mean of the realized coefficients of variation in the two alternative stimuli (CV_w), and ambient noise level (Noise), including all two-way interactions ($N_{subjects}$ = 289, N_{trials} = 3,298).

Response variable	Term	Estimate	Standard error	Wald statistic	*P*
P(Chose faster alternative)	**Intercept**	**13.954**	**1.420**	**96.61**	**<0.001**
	PR	**−0.288**	**0.031**	**85.90**	**<0.001**
	CV_w	−65.351	50.696	1.66	0.197
	Noise at 60 dB	1.430	1.491	0.92	0.338
	Noise at 70 dB	−1.336	1.381	0.94	0.333
	Noise at 80 dB	**−5.187**	**1.394**	**13.85**	**<0.001**
	PR * CV_w	1.321	1.107	1.42	0.233
	CV_w * Noise at 60 dB	3.401	9.998	0.12	0.734
	CV_w * Noise at 70 dB	16.102	9.946	2.62	0.105
	CV_w * Noise at 80 dB	2.672	10.852	0.06	0.805
	PR * Noise at 60 dB	−0.033	0.033	1.01	0.315
	PR * Noise at 70 dB	0.021	0.030	0.48	0.488
	PR * Noise at 80 dB	**0.107**	**0.031**	**12.02**	**0.001**
Response latency	**Intercept**	**40.239**	**15.632**	**6.63**	**0.010**
	PR	**1.156**	**0.380**	**9.27**	**0.002**
	CV_w	327.596	719.170	0.21	0.649
	Noise at 60 dB	7.557	19.474	0.15	0.698
	Noise at 70 dB	**58.235**	**18.506**	**9.90**	**0.002**
	Noise at 80 dB	**88.897**	**22.791**	**15.21**	**<0.001**
	PR * CV_w	−7.158	15.646	0.21	0.647
	CV_w * Noise at 60 dB	−69.087	150.782	0.21	0.647
	CV_w * Noise at 70 dB	156.150	154.751	1.02	0.313
	CV_w * Noise at 80 dB	−59.859	232.372	0.07	0.797
	PR * Noise at 60 dB	−0.190	0.481	0.16	0.694
	PR * Noise at 70 dB	**−1.530**	**0.436**	**12.29**	**<0.001**
	PR * Noise at 80 dB	**−1.888**	**0.541**	**12.19**	**<0.001**

Significant model terms are shown in bold.

the basis of pulse rate, reflecting the robust nature of species recognition mechanisms in gray treefrogs. Consistent with our hypothesis, however, natural levels of ambient noise simulating a gray treefrog chorus had significant impacts on mate choice. Specifically, discrimination against slower-than-average pulse rates was reduced, and subjects spent longer making mating decisions, in high levels of ambient noise. In stark contrast to our hypothesis, inconsistency in signal production had no measurable effects on pulse rate discrimination, and it did not interact synergistically with ambient noise.

Ambient Noise

While mate choice was unaffected by noise presented at the lowest level, ambient noise presented at a high amplitude caused females to choose the slower, non-preferred signal more often than they did in quiet. In nature, the sound levels of gray treefrog choruses can be highly variable from one night to the next (Tanner and Bee, 2019) and the extent of the masking effect of ambient noise experienced by receivers is also variable according to the spatial relationship between the target signal and the noise source (Bee, 2007, 2008a; Nityananda and Bee, 2012; Ward et al., 2013a; Caldwell et al., 2016). Thus, both night-to-night and spatial variation in the intensity of selection imposed on males and signals by treefrog receivers are to be expected. As a population-level consequence, selection on pulse rate may be less intense than estimates made in quiet listening conditions (e.g., Tanner et al., 2017) would otherwise suggest. Individual *H. chrysoscelis* receivers should be at greater risk of making pulse rate discrimination errors on nights when the chorus is better attended and, thus, noisier. Errors in pulse rate discrimination can potentially lead to errors in species recognition in this system (e.g., Bee, 2008a), which would have potentially devastating consequences, because heterospecific matings produce sterile, triploid offspring (Gerhardt et al., 1994; Servedio and Noor, 2003; Tucker and Gerhardt, 2012). Such call trait discrimination errors are also expected to result in reduced receiver fitness if specific features of the call signal mate quality (Bee, 2008b; Ward et al., 2013b; Tanner and Bee, 2020).

Response latencies were also significantly higher in the presence of high levels of ambient noise; however, the estimated increase in response latencies reported here was modest in the context of a single breeding episode. At present, it is difficult to assess the extent to which such small noise-induced increases in response latency might materially affect individuals under natural mate searching conditions. This difficulty arises, in part, because we currently lack sufficient data on female sampling strategies

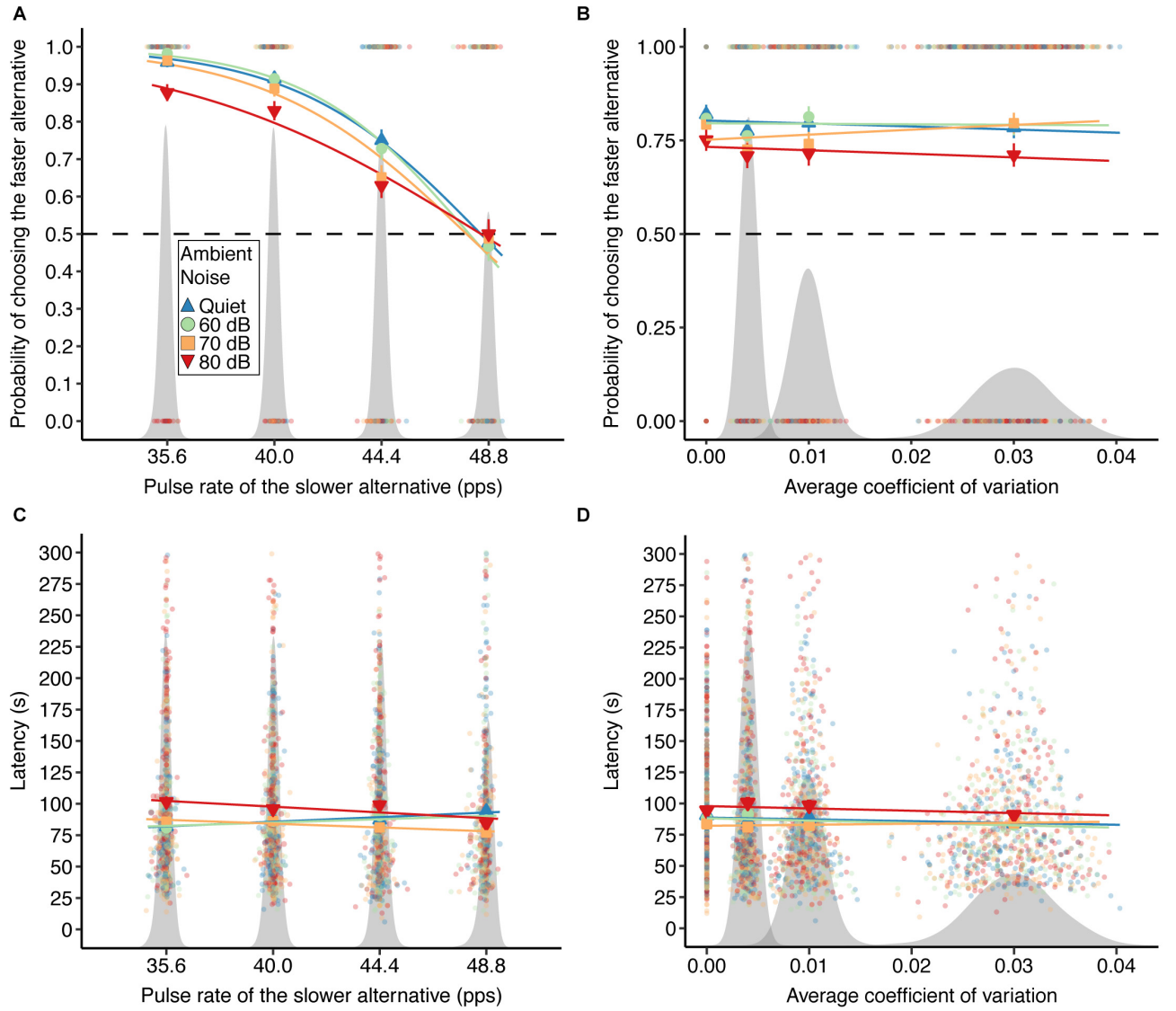

FIGURE 3 | The effects of pulse rate, ambient noise, and inconsistency on receiver responses. **(A)** The probability of choosing the preferred, faster pulse rate declined as a function of the realized pulse rate of the slower alternative. **(B)** The probability of choosing the faster stimulus was not affected by the average CV_w of the two alternative stimuli. In panels **(A,B)**, the dashed black line shows 0.5, the probability of choosing the stimulus with the faster pulse rate at random. Response latency as a function of **(C)** the realized pulse rate of the slower alternative and **(D)** the average coefficient of variation within males. In all panels, the results of individual tests are shown with pale, circular symbols. Colors and symbols shown in the legend depict mean response variables within combinations of predictors, while lines show linear model fits and error bars show standard errors. Different colors and symbols represent levels of ambient noise. Gray shading shows the distributions of experimental stimuli for each plot: realized mean alternative pulse rates are shown in panels **(A,C)**, and average realized coefficients of variation are shown in panels **(B,D)**.

prior to mate choice in frogs (Murphy and Gerhardt, 2002; Schwartz et al., 2004; Murphy, 2012) to definitively conclude that increases in response latency in the presence of ambient noise negatively impact females or their mating decisions under natural listening conditions. However, one viable and potentially relevant consequence of increased response latencies in the presence of ambient noise could be an increased cost of mate searching. For example, time spent searching for a mate may incur missed opportunity costs (i.e., time not spent foraging), or expose females to pond-dwelling predators (e.g., larger frogs, giant water bugs) and parasites (e.g., leeches) to which they might otherwise be less vulnerable (Crowley et al., 1991; Magnhagen, 1991; Grafe, 1997; Zuk and Kolluru, 1998; Martin and Wagner, 2010; Bonachea and Ryan, 2011; Beckers and Wagner, 2018). In addition, mate searching in natural environments may be further complicated, relative to controlled laboratory settings, by the potentially larger distances females must traverse in ponds to select a mate, the increased complexity of natural habitats, the vastly more numerous potential choices in a breeding chorus, and the fact that multicomponent advertisement signals vary

along multiple behaviorally relevant dimensions at the same time (Tanner et al., 2017). Thus, the effect of chorus noise on the time spent making decisions may be more important in natural populations. It follows from Signal Detection Theory that increased listening time may serve to prevent errors in trait discrimination because receivers are, in effect, sampling from a distribution of male calls to estimate the central trait value of the signaler and listening for longer is equivalent to drawing more values from the distribution (Wiley, 2006). Consistent with this idea, female túngara frogs may increase their response latencies as the trait values of two alternative signals become increasingly similar (Bosch et al., 2000) and errors in signal discrimination become more likely. We hypothesized that longer listening times might be especially important in the presence of ambient noise when calls were inconsistent, and thus, increased sampling could result in more accurate estimates of mean pulse rates. However, we ultimately found no evidence of synergistic effects between ambient noise and inconsistency.

Overall, our findings on the effects of ambient noise add to a growing body of evidence that suggests receivers of diverse taxa sometimes fail to express well-documented mating preferences in natural soundscapes (Wollerman and Wiley, 2002; Bee and Schwartz, 2009; Bee et al., 2012; Römer, 2013; Reichert and Ronacher, 2015; Lee et al., 2017; Tanner and Bee, 2020). Ambient noise thus provides at least a partial explanation for why between-individual variation in sexually selected traits is maintained in spite of apparently persistent sexual selection ("the lek paradox"; Kirkpatrick and Ryan, 1991; Møller and Pomiankowski, 1993; Pomiankowski and Møller, 1995). The impacts of ambient noise on female preference expression probably also explain, at least partially, why field experiments sometimes fail to show the same sexual selection measured in laboratory studies (Sullivan and Hinshaw, 1992; Friedl, 2006; Dawson and Ryan, 2009). Such findings suggest that experiments performed under ideal listening conditions, that is, in the absence of the ambient noise that is a feature of many natural signaling contexts, may tend to overestimate the intensity of sexual selection on signals because they artificially inflate signal discriminability.

Inconsistent Signaling

Pulse rate preferences were reliably expressed in spite of natural levels of inconsistency in signal production that effectively widened the signal distributions. This key finding is not consistent with our hypothesis and stands in stark contrast with results from an earlier study showing that female preferences for faster rates of call production during a bout of signaling eroded as a function of inconsistency in signaling (Tanner and Bee, 2020). In that study, inconsistency in call rate more profoundly limited expression of female mating preferences than the better-known effects of ambient noise. The striking difference between the impacts of inconsistency on call rate discrimination (Tanner and Bee, 2020) and those on pulse rate discrimination presented here might be explained by the different biological functions of discrimination based on differences in pulse rate versus call rate in *H. chrysoscelis*. Female *H. chrysoscelis* use pulse rate (and not call rate) to distinguish between conspecific males and males of the cryptic, tetraploid sister-species, *H. versicolor* (Schul and Bush, 2002), which produce an acoustically similar call. In contrast, call rate is considered a potential non-arbitrary signal of male quality due to the high energetic costs of calling (Taigen and Wells, 1985; Wells and Taigen, 1986). Compared with mate quality assessment (i.e., call rate discrimination), species recognition (i.e., pulse rate discrimination) may generally be more robust against the impacts of inconsistent signaling because the costs of mating with the wrong species are expected to far outweigh those of mating with a low-quality conspecific (but see Pfennig, 2007).

On the other hand, the difference between the results of the present study and those of Tanner and Bee (2020) may be attributable to the differing magnitudes of inconsistency in pulse rate versus call rate in natural populations, which informed our methodology. While both experiments introduced natural levels of inconsistency, pulse rate is far less variable than call rate within individuals (Gerhardt, 1991; Ward et al., 2013b). Consequently, the distributions of signal traits with different means did not overlap in the experiment described here, which limited the opportunity for mistakes in mean trait value estimation by receivers and thus might account for the absence of effects of inconsistent pulse rates on pulse rate discrimination. A potential linkage between the biological and methodological explanations for the difference in experimental outcomes is that, given the importance of pulse rate in species recognition, past selection may have acted to minimize the level of inconsistency in this call trait. The present study was not designed to test this hypothesis; future studies could do so by incorporating experimental stimuli with levels of inconsistency that exceed the current range of natural variation. An additional factor that may have contributed to these different results is that receiver estimation of pulse rate and call rate take place over different timescales, with pulse rate potentially being estimated within the space of a single call, while call rate must be estimated by listening to multiple calls.

Within-individual variation in pulse rate may play some role in communication in this species, but if so, that role remains unknown. Tanner and Bee (2019) showed that inconsistency in pulse rate did not signal male body size or condition. In the congener *Hyla avivoca*, males dynamically adjust their interpulse silent intervals (and consequently, the pulse rate) to avoid overlapping pulses with calling neighbors, and females prefer calls with interdigitated pulses to calls whose pulses overlap with those of another call (Martínez-Rivera and Gerhardt, 2008). However, no similar pulse rate adjustment occurs in the gray treefrogs, *H. chrysoscelis* and *H. versicolor* (Gerhardt, 1991; Ward et al., 2013b). In recent years, there has been increasing interest in understanding the causes and consequences of various aspects of within-individual variation, including individual plasticity (Nussey et al., 2007; Rodríguez et al., 2013), persistent individual differences in behavior ("animal personality"; Dingemanse and Wolf, 2010; Dingemanse et al., 2010, 2012), and between-individual differences in intraindividual variability (Stamps et al., 2012). However, the interplay of within- and between-individual variation in the context of signal discrimination and mate choice remains poorly understood. Further study is needed to understand how the ubiquitous within-individual variation in communication behaviors – particularly the inconsistency in

signal production that occurs within signalers – impacts receiver decision-making.

CONCLUSION

We conclude that ambient noise alters the landscape of receiver-mediated selection on signals in Cope's gray treefrog, and likely in other species that communicate in noisy social environments. We suggest that when receiver behavior is measured in quiet, simplified laboratory conditions, we are likely to overestimate the action of sexual selection in natural environments due to artificially high signal discriminability. Our data did not support the hypothesis that inconsistent production of a species recognition signal (pulse rate) acts, like ambient noise, to limit the expression of female preferences. This finding is in contrast to a similar assay performed in the same species manipulating call rate, in which female preferences were even more profoundly impacted by inconsistent signaling than by ambient noise. Thus, we note that the sources of noise that prevent expression of female preferences are variable across contexts and systems, and even between individual components of multicomponent signals (Tanner and Bee, 2020). A complete understanding of how communication systems evolve will require close examination of noise sources and their effects on receiver behavior in realistically complex environments.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. The data can be found here: doi: 10.13020/9bem-wj63.

ETHICS STATEMENT

All procedures described herein were carried out in accordance with the principles of the Basel Declaration and the Animal Behavior Society/Association for the Study of Animal Behaviour guidelines for the ethical treatment of animals. Our procedures met all legal requirements of the United States of America and were approved by the University of Minnesota Institutional Animal Care and Use Committee under protocol 1701-34456A.

AUTHOR CONTRIBUTIONS

JT collected, curated, and analyzed the data and wrote the first draft of the manuscript. Both authors designed the experiment, secured funding, contributed to the analysis and interpretation of the data, and edited the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2020.00256/full#supplementary-material

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REVIEW
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Control vs. Constraint: Understanding the Mechanisms of Vibration Transmission During Material-Bound Information Transfer

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Material-bound vibrations are ubiquitous in the environment and are widely used as an information source by animals, whether they are generated by biotic or abiotic sources. The process of vibration information transfer is subject to a wide range of physical constraints, especially during the vibration transmission phase. This is because vibrations must travel through materials in the environment and body of the animal before reaching embedded mechanosensors. Morphology therefore plays a key and often overlooked role in shaping information flow. Web-building spiders are ideal organisms for studying vibration information transfer due to the level of control they have over morphological traits, both within the web (environment) and body, which can give insights for bioinspired design. Here we investigate the mechanisms governing vibration information transfer, including the relative roles of constraints and control mechanisms. We review the known and theoretical contributions of morphological and behavioral traits to vibration transmission in these spiders, and propose an interdisciplinary framework for considering the effects of these traits from a biomechanical perspective. Whereas morphological traits act as a series of springs, dampers and masses arranged in a specific geometry to influence vibration transmission, behavioral traits influence these morphologies often over small timescales in response to changing conditions. We then explore the relative roles of constraints and control mechanisms in shaping the variation of these traits at various taxonomic levels. This analysis reveals the importance of morphology modification to gain control over vibration transmission to mitigate constraints and essentially promote information transfer. In particular, we hypothesize that morphological computation is used by spiders during vibration information transfer to reduce the amount of processing required by the central nervous system (CNS); a hypothesis that can be tested experimentally in the future. We can take inspiration from how spiders control vibration transmission and apply these insights to bioinspired engineering. In particular, the role of morphological computation for vibration control could open up potential developments for soft robots, which could use multi-scale vibration sensory systems inspired by spiders to quickly and efficiently adapt to changing environments.

Keywords: biotremology, sensory ecology, physical ecology, morphological computation, Araneae

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INTRODUCTION

Material-bound vibrations are an important sensory mode for many animals. These vibrations, which propagate through and along the surfaces of materials known as substrates, are ubiquitous in the environment and the study of the biological use of these vibrations is known as "biotremology" (Hill and Wessel, 2016). They can either be produced intentionally as a signal (e.g., in courtship) for communication, or as an unintentional cue (e.g., through the movement of a prey item) that can be used for information by receivers (Mortimer, 2017). Traditionally these vibrations have been overlooked as a potential source of biologically relevant information, with modern techniques unveiling the importance of this sensory modality. This is especially true for terrestrial arthropods, the context of this review, where material-bound vibration information transfer was likely used long before acoustic information transfer (or hearing) evolved (Cocroft and Rodríguez, 2005; Hill, 2009).

Like other sensory modalities, the biological use of material-bound vibrations for information transfer involves the generation, transmission and sensing (here used to refer to sensory transduction) of vibrations. This review focuses on the role of vibration transmission as an important, but relatively overlooked stage in this process, which is the stage encompassing everything between generating a vibration and transduction within sensory cells. This stage is interesting because although the theory of how vibrations propagate through materials is well developed, its application within this biological context is in its infancy and the role of natural selection in shaping or controlling this process is not well understood. In terrestrial arthropods, vibrations first propagate through the substrate before passing into the arthropod's body where its leg couples with the substrate. Following coupling, vibrations propagate to mechanosensors that are typically embedded in the leg (**Figure 1**; Pringle, 1955). Mechanical transmission of vibrations therefore involves both propagation of the vibration within a material in the environment (or substrate) and within the animal's body. Not covered here, at mechanosensors, mechanical stimuli (such as vibrations) lead to the generation of an action potential in the sensory neurons innervating the sensor, and the wave is transformed into an electrical signal that travels along this neuron (Barth and Pickelmann, 1975). For detail and discussion on the effect of neuronal filtering and sensory transduction on the transformation of vibrational information, we refer readers to an alternative review (Barth, 2019).

Vibration transmission is shaped by the interplay between natural selection, evolutionary, and physical constraints, yet the interactions between these processes in this context are not well understood. Both morphological and behavioral traits have been shaped by natural selection to influence vibration transmission (**Table 1**), which are under evolutionary constraints, e.g., trade-offs and developmental constraints. Each stage of transmission also varies in the degree to which physical constraints are acting. These physical constraints are imposed by their biomechanics and include damping (energy loss) and filtering of vibrational information (Mortimer, 2017). Control mechanisms can be used to mitigate these constraints (see section "Discussion").

In this review, we will use spiders as a case study to probe the mechanisms influencing vibration transmission during the process of vibration information transfer, building on previous reviews that discuss physical constraints acting during vibration transmission (Mortimer, 2017, 2019; Barth, 2019). This is the dominant sensory modality for many spiders (Barth, 2002a), and is used for communication with conspecifics during courtship (Schüch and Barth, 1985, 1990; Elias et al., 2003, 2005), as well as sensing prey and predators (Klärner and Barth, 1982; Masters, 1984b; Landolfa and Barth, 1996). As such, mechanisms to influence vibration transmission apply to varied biological contexts with spiders, including prey capture, and conspecific communication. The mechanosensors involved in spider vibration sensing are slit sensilla—some of which are grouped into distinct structures known as lyriform organs (Barth, 2002a). In particular, the metatarsal lyriform organ has been intensively studied for its role in vibration sensing (Barth, 2002a). In addition to sensing externally generated vibrations, spiders use slit sensilla to sense internally generated vibrations, known as proprioception (Seyfarth, 1978).

Morphological and behavioral traits in spiders are extremely variable and the mechanisms causing this variation and the implications for information transfer, whether natural or engineered, have been largely unexplored. Web-building spiders are an excellent model species to study this due to the impressive control mechanisms that can influence vibration transmission both within the environment and body, which in theory allow them to mitigate physical constraints. For spiders, morphological traits include the structures and materials of both the body of the spider and the extended phenotype of the web, and behavioral traits act to influence morphology during vibration transmission (**Table 1**). This is exemplified by orb-weaving spiders that generate aerial orb webs that act as a snare for prey capture and a vibration transmission platform (Mortimer, 2019). Through modifying the extended phenotype of the web, these spiders are thought to influence vibration transmission outside the body (Mortimer et al., 2016). Most literature on orb weaver vibration transmission is on prey capture, although the web is also used to transmit vibrations during courtship (Wignall and Heberstein, 2013).

An aim of this review is to combine perspectives from across biology and the physical sciences to explore the mechanisms governing vibration transmission. One concept that we will come back to in the Discussion is the idea of morphological computation, which is a term more typically applied to artificial systems (such as robots). Morphological computation is where the body, rather than central nervous system (CNS), is used to control actions or information, thus reducing the total level of processing required by the CNS. Recently, there has been increasing recognition of its role in biological systems—for example, animals rely heavily on the material properties of their leg muscles and tendons to walk/run, rather than these leg movements being closely controlled by the CNS (Mo et al., 2020). In sensory systems, morphological computation could be used to pre-process information before sensory transduction occurs, which in our context would be during the vibration transmission stage. This is thought to occur in the insect eye,

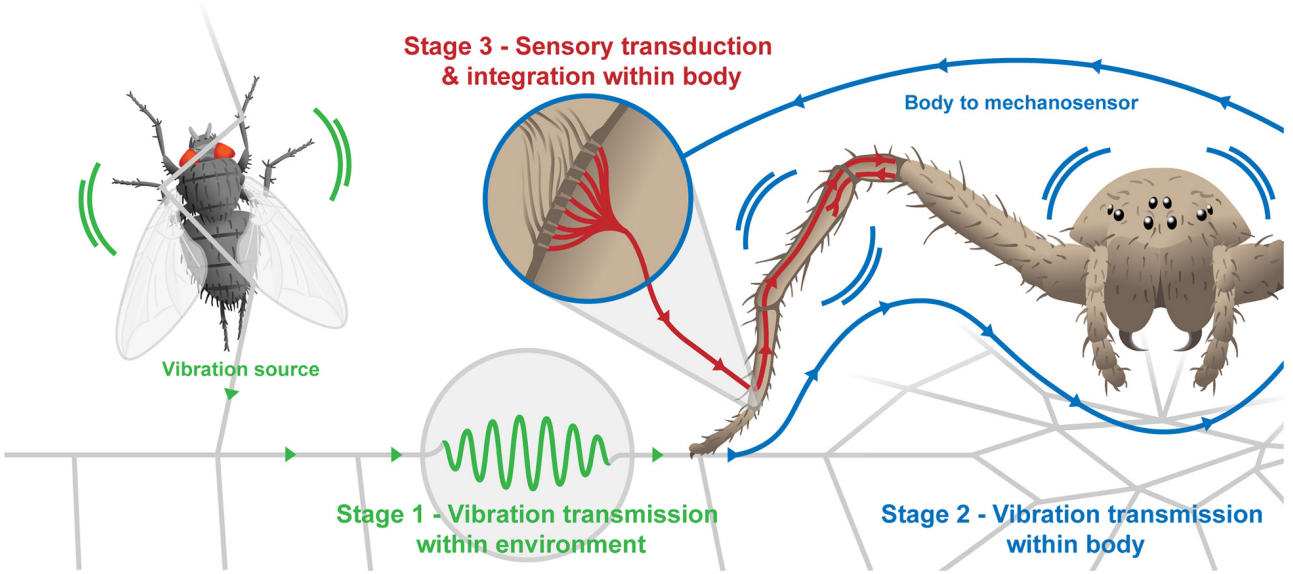

FIGURE 1 | diagrammatic representation of the stages in vibration sensing detailed in **Table 1**. Stage 1—transmission from source of vibration through environmental substrates (in this case the web) to the body. Stage 2—body transformation of input wave before it reaches the mechanosensor (lyriform organ shown in insert). Stage 3—sensory transduction of vibrational information, which feeds back to determine behavior, in turn influencing Stages 1 and 2.

where the arrangement of light-sensitive cells allows insects to accurately gauge distance without additional processing in the CNS (Franceschini et al., 1992). During vibration information transfer in terrestrial arthropods, since mechanosensors are embedded into the cuticle or leg (Yack, 2004), vibratory waves must travel through the body of the animal before they are sensed. This means vibration information transfer is more heavily influenced by morphology than other senses (whether acoustic vibration, olfaction or visual), and morphological computation is likely to play a role in this context.

Using orb weaving spiders as a case study, this review will overview the known and theoretical contributions of morphological and behavioral traits to the biomechanics of vibration transmission (sections "Influence of Morphological Traits" and "Influence of Behavioral Traits," respectively). We deconstruct the effect that these traits have on three key parameters that can be used to model the system—mass/density, springs/dampers (energy storage/loss), and geometry. We explore the interactions and trade-offs between these three parameters, morphological and behavioral traits in the Discussion. Using this approach, we show that orb-weaving spiders possess a high level of control over morphological and behavioral traits during vibration transmission over multiple timescales. We follow with a discussion of how variation in these traits (within an individual, between individuals of the same species, and between species) can be explained by the action of control mechanisms and constraints (Discussion). We suggest that within a species, morphology is controlled flexibly via behavior, which acts to mitigate against various constraints in variable contexts. We conclude that morphology likely plays a key role in transforming information before the vibrational wave reaches a mechanosensor, and hypothesize a role for morphological computation (section "Discussion"). The review provides insights into the relative roles of natural selection, evolutionary and physical constraints in shaping vibration sensing in terrestrial arthropods, but also reveals the mechanisms that can be applied to bioinspired design of devices that use material-bound vibrations for information.

INFLUENCE OF MORPHOLOGICAL TRAITS

From a biomechanical perspective, morphological traits can be seen as systems with a series of masses, springs, and dampers arranged in a specific geometry that influences its motion over time, whether in the environment (here the web) or within/through the body (here the spider's body). These three parameters are not mutually exclusive and they interact with each other to influence vibration propagation. Yet thinking of vibrating systems, including animals, as these constituent parameters provides a framework to help us to understand the underlying biomechanics governing its motion and compare between biological systems that vary to different degrees to gain insights into its evolution. Within this section ("Influence of Morphological Traits"), each component will be discussed separately, with the aim that the importance of each component to vibration transmission can be outlined.

Mass is distributed both within the substrate and within the body and is particularly important due to physical constraints acting to dampen (or dissipate energy), filter, and distort vibrational information (i.e., influence the relationship between frequency components). Density is a measure of distributed mass,

TABLE 1 | The focus of this review is vibration transmission, which is in the physical realm and involves mechanical vibrations propagating through the environment (Stage 1) and body (Stage 2).

Stages in vibration information transfer	Traits under selection to promote vibration information transfer	Relevant physical constraints
1. Vibration transmission within environment.	If applicable, production and mechanics of own substrate (e.g., web); Choice of substrate; Sensing behaviors (e.g., positioning and freezing)	Damping, filtering and distortion; Vary with substrate and transmission distance
2. Vibration transmission within/along body.	Mechanics of body materials and joints (masses, springs, dampers); Body plan and geometry; Sensor placement and integration; Sensing behaviors (e.g., posture control)	Damping, filtering and distortion; Vary with coupling, body mechanics and sensor position
3. Sensory transduction and integration within body.	Mechanotransduction mechanism (e.g., ion channel type, number and position); Sensory thresholds (e.g., frequency, amplitude); Neuronal anatomy and sensory integration (e.g., positive/negative feedback); Sensorimotor feedback	Physiological constraints act; Vary with all aspects of neurophysiology

Vibration sensing, or sensory transduction, is in the physiological realm and involves mechanotransduction and integration of sensory information within cells (Stage 3). Both morphological and behavioural traits that are under selection to promote vibration transmission are given. Physical constraints vary to different extents within each stage.

so is discussed within the section on Masses (section "Masses"). Whereas springs govern energy storage in the system when the springs are compressed or stretched, dampers govern the energy lost from the system, usually due to friction. In our orb-weaver spider context, springs/dampers are the silk threads, the cuticle that forms the exoskeleton of the spider and the spider's joints. Finally, these parameters come together into a specific geometrical arrangement (web shape, body plan, and mechanosensor structure) to govern vibration transmission and shape the information that can be extracted by the animal due to sensor placement. Here we discuss the known and theoretical contributions of masses, springs, dampers, and geometry to vibration transmission, using this interdisciplinary framework in the context of orb-weaving spiders, where the behaviors that influence these factors are reviewed in section "Influence of Behavioral Traits."

Masses

Vibration transmission through a material is influenced by mass distribution, including changes in density of a material through which a wave propagates. In our spider context, we will focus on masses in the web, as the spider's engineered substrate, and in the body, through which vibrations need to propagate before reaching embedded mechanosensors (section "Web and Body Transmission"). Mass and density (in combination with material properties and geometry) govern the speed of vibration transmission, how it loses energy over distance and time, and how the wave is filtered (section "Mechanical Impedance and Resonance") (Mortimer, 2017). Masses also interact with the other biomechanical parameters of springs and dampers (section "Springs and Dampers"), according to their geometry (section "Geometry").

Web and Body Transmission

The influence that masses have on vibration transmission differs markedly between the web and the body. The mass of the silk threads comprising the web is negligible compared with the spider's body and prey items in the web—therefore, the dynamics of vibration transmission are largely dependent on the masses suspended by the silk threads, rather than the web itself. All spider body plans consist of a fused head and body segment (cephalothorax) joined to an abdomen segment via the waist-like pedicel, and eight legs, all of which vary in their mass and geometry (Foelix, 1979). In particular, these masses modulate how vibrations are transmitted through the legs at different joint angles, where higher masses lead to greater forces exerted around the leg joints (via gravity), influencing their springs and dampers (see section "Springs and Dampers").

The mass distribution of a spider and its web is stochastic, with the mass of objects caught in the web (such as prey and debris), as well as the mass of the spider's body, varying over time. The mass of the spider's cephalothorax will fluctuate due to factors such as starvation and dehydration, which will reduce the volume of internal fluid (haemolymph) in the abdomen, as well as the production of eggs by female spiders (Foelix, 1979). Similarly, the amount of silk protein stored in the silk glands will deplete when the spider spins a new web and will then gradually refill (Vollrath, 1999). This is variation that spiders have some control over, but largely cannot avoid, and thus mass fluctuation imposes a physical constraint. Mhatre et al. (2018) simulated the vibrational response of a female black widow spider to vibration, which showed that abdomen mass could vary from 20% to 150% of its *in vivo* value without a measurable effect on the vibrational response to stimuli between 5 and 200 Hz. Spiders likely possess mechanisms that enable them to account for wave transformation due to their own mass fluctuation, which may be "known" by the spider—this is a topic requiring further research.

Mechanical Impedance and Resonance

As a vibratory wave propagates through a system, the masses it encounters will resist changes in motion that will act to transform the vibratory wave. This tendency for a mass to resist changes in velocity as it is oscillated is known as mechanical impedance, which leads to energy loss and/or reflection of vibrations as a wave passes from a material of one density to another (e.g., from silk to cuticle) (Main, 1993). From a biological perspective, it may therefore be desirable to minimize changes in density and material properties (such as stiffness) throughout transmission, as energy loss will lead to reduction in signal amplitude and therefore make vibrations more difficult to detect. This is especially important in two contexts where waves are transmitted between disparate materials: within a heterogenous substrate

(or multi-material substrate) (Elias and Mason, 2014); and also at the coupling points between the substrate and the body.

Starting with the former, damping in heterogenous materials may be reduced through morphological adaptations such as impedance matching (O'Connell-Rodwell, 2007), where materials with similar properties are used to minimize changes in mechanical impedance. The degree of sclerotization (cross-linking) in a spider's exoskeleton varies across different parts of the body and legs (Blickhan and Barth, 1985), therefore altering the material properties through which vibrational waves propagate. Gradients in material properties of the cuticle are often found [e.g., elastic modulus varying from 8.3 (±1.1) to 2.8 (±1.3) GPa across the cuticular pad] (Young et al., 2014; Erko et al., 2015), which would reduce mechanical impedance changes and so reduce damping and filtering of the vibration. Impedance matching mechanisms via gradients in material properties in terrestrial arthropods require further research.

Moving on to the effect of coupling, the energy loss caused by the coupling between silk and the spider claw (tarsus) is expected to differ between wave types, which is the type of vibrational wave propagating through the material. In the spider web, wave types vary in the direction of oscillation, where longitudinal waves oscillate within the axis of the fiber and transverse waves oscillate perpendicular to the fiber axis. Whilst only longitudinal wave transmission to the tarsus has been measured, the fact that transmission of longitudinal waves through the web shows less damping compared transverse (c. −2 dB on the same silk thread compared with c. −16 at 20 Hz) (Masters and Markl, 1981; Masters, 1984a), means that we can estimate the results for transmission to the tarsus. These differences are expected to become more pronounced above 100 Hz, after which transverse wave damping increases (down to c. −50 dB on the same silk thread) (Masters, 1984a; Landolfa and Barth, 1996). Since longitudinal vibrations are particularly important for initiating predatory behavior in orb weavers (Klärner and Barth, 1982), these observations support the idea that coupling functions to preserve longitudinal wave transmission between the silk and tarsus.

Changes in mechanical impedance also affect vibration transmission when objects sit in the web, such as the spider, prey or debris. Evidence from modeling suggests that the presence of the spider itself at the web centre (hub) leads to significant damping compared with empty webs—c. −16/−36 dB for transverse/longitudinal waves, respectively (Mortimer et al., 2018). Often the presence of these objects cannot be avoided and so present a constraint on vibration transmission. However, some spiders may be able to use the mechanical impedance of their body mass functionally in vibration information transfer. This is because a mass at the hub alters both the speeds and amplitudes of vibrations in the web (Mortimer et al., 2018), which provide information on the location of a vibration source (Mortimer et al., 2019). Mechanical impedance of prey items may also be used by orb weavers as a prey localization cue (Mortimer et al., 2019).

Vibrations often contain multiple frequencies at once. Resonance is where the frequency of an input vibration matches the natural frequency of the system through which it travels, resulting in these frequencies being amplified. The natural frequency of the system is governed by its masses and geometry (Balachandran and Magrab, 2008)—in this case it includes objects in the web and the silk threads comprising the web. At low frequencies (<30 Hz), in theory resonance may occur in the body of the spider itself, or other large objects (such as prey bundles) in the web (Frohlich and Buskirk, 1982). Frequencies in this range contain noise from environmental factors (such as wind) (Masters, 1984b; Wu and Elias, 2014), so resonance of objects in the web is therefore unlikely to be used functionally by the spider. However, spiders may be able to use resonance of silk threads [predicted to be in the hundreds to thousands of Hertz range (Frohlich and Buskirk, 1982)] to locate small objects, by "plucking" silk threads and detecting vibrational cues specific to the thread on which the object sits (Klärner and Barth, 1982). A hypothesized mechanism for this object localization method is the use of wave reflection off the object due to mechanical impedance changes that result in high frequency vibrations on these specific threads (Mortimer, 2019).

Springs and Dampers

The springs and dampers in a vibrating system governs the energy stored and lost during vibration transmission. This is determined by the material and structural properties of the material through which a vibrational wave travels. Most biological tissues contain biopolymers, meaning that they behave as viscoelastic materials (Vincent, 1990). Many biological viscoelastic materials behave like springs at low deformations, meaning they have a linear relationship between force and displacement up to a certain displacement. In this range, they can store and return energy like a spring. Outside this deformation range, they no longer behave as springs, but the material deforms to dissipate energy from the system, which is viscous behavior. This energy dissipation, or damping, depends on the time that the material has to deform (e.g., it can extend more over longer timescales) and the temperature (e.g., it can extend more at higher temperatures), as both influence the energy introduced to the biopolymer, which alters its structure beyond a transition point (the glass transition temperature of the biopolymer) (Guan et al., 2012). This makes energy dissipation time-, frequency-, and temperature-dependent. Viscoelastic materials therefore act as both springs and dampers, where this behavior changes with both deformation range and frequency (Vincent, 1990).

In context, the most important viscoelastic materials for spiders are the silk threads that make up the web, and the spider's body tissues—especially the cuticle of the exoskeleton, in which the slit sensilla vibration sensors are embedded (Barth, 2019). The springs/dampers within the materials interact with mass to influence vibration transmission: the stiffness and density of these materials are linked to wavespeed and damping, which in turn are involved in frequency filtering and hence determine the shape of the vibrational wave that arrives at the sensor (Mortimer, 2017). These materials also make up structures such as joints that have their own springs and dampers as they move. The properties of the materials and structures therefore have a profound effect on the transmission of vibrational waves.

Vibration Transmission Within Web Environment

The spring-like behavior of silk at low deformations governs the transmission of vibrations through the web. A key property of a spring is its stiffness, which is the gradient of the relationship between force and extension, which governs the tension or force on the silk when a displacement is applied (Vollrath, 2000). Spider dragline silk is unique in the range of stiffness available, due to an unusual property called supercontraction, where high humidity [>70% relative humidity (RH)] dramatically alters the silk structure and reduces its stiffness (Liu et al., 2008). Here, we will briefly review the effects of silk tension, stiffness, and supercontraction on vibration transmission in webs, whilst the behaviors that are able to control these features are reviewed later (section "Influence of Behavior").

Silk tension both controls web geometry and is determined by it. This feedback system is vital for maintaining the mechanical integrity of the web, with mooring threads that attach to the environment being under the greatest tension (Wirth and Barth, 1992). Silk tension is particularly important for the transmission of transverse waves as it directly affects wavespeed and amplitude when interacting with mass per unit length and geometry (Mortimer et al., 2014, 2016). Conversely, longitudinal wave transmission is influenced by stiffness (Frohlich and Buskirk, 1982), which is of particular importance for predatory behavior due to its low damping in the web (almost no damping, > -2 dB, from prey-capture region to hub along same silk thread in the biologically relevant range of 1–10,000 Hz) (Masters and Markl, 1981). Stiffness is influenced by supercontraction, which decreases the order of the protein structure and reduces stiffness (Liu et al., 2008; Guan et al., 2011). The degree of supercontraction is altered by the proportion of proline amino acid in the silk proteins—silks from different species of spider contain varying amounts of proline (ranging from as little as 0.6 to 14.3 mole%), and thus experience different degrees of supercontraction (Liu et al., 2008).

Vibration Transmission Within/Along Body

As slit sensilla are embedded sensors, the material properties of the cuticle directly influence vibrations before they reach the sensors, and also govern how the slit sensilla deform in response to vibrational waves. Since the cuticle behaves like a spring at low deformations, the slit sensilla are able to deform when extended and then return to their original state (Hössl et al., 2006). The exoskeleton covering the legs, where the largest concentrations of slit sensilla are found, is composed of stiff exocuticle. The opisthosoma (posterior part of the abdomen), on the other hand, is covered by less stiff mesocuticle, and therefore the slit sensilla found here will undergo greater deformation in response to a vibrational wave compared with those surrounded by a stiffer material (Barth, 2002a).

A cuticular pad situated distally (toward the claw) to the metatarsal lyriform organ is involved in transmitting and filtering vibrations as they propagate from the tarsus to the metatarsus leg segments (McConney et al., 2007; Young et al., 2014; Erko et al., 2015). The viscoelasticity of the pad allows it to act as a high-pass filter, meaning it filters out low-frequency background noise (e.g., vibrations of c. < 10 Hz). At low frequencies, the pad behaves in a viscous manner, producing maximum damping properties. This means that large tarsal deflections (angular displacements c. 10° and above) are required to activate the slit sensilla, which also acts to prevent damage to the organ at high deflections (Young et al., 2014; Erko et al., 2015). At higher frequencies (>10 Hz), the pad transitions to a spring-like state, becoming stiffer and transmitting vibrations much more effectively. These properties are temperature-dependent, with the pad damping vibrations at higher temperatures (>21°C at 10 Hz) (McConney et al., 2007; Young et al., 2014).

In addition to the cuticle, the joints between leg segments themselves act as springs and dampers. For some joint motions (loading along the leg axis), joints behave as springs (Blickhan, 1986). For other joint motions (dorsoventral and lateral deflections), increased joint deflection stretches the articular membrane, which is thought to increase the alignment within the material, increasing stiffness (Blickhan, 1986). This results in energy being lost as the joint is loaded and unloaded, so the joint acts as a damper under certain conditions (Blickhan, 1986). As the lyriform organs are typically embedded near the leg joints, their mechanical sensitivity (here taken as the ratio of input force to cuticle deformation, i.e., the strain) is influenced by the strains and changes in stiffness in the cuticle that is generated by joint movement. When the legs are loaded axially, the deformation in the cuticle is negligible around 170° (between c. −0.1 and 0.1 $\mu\varepsilon$/mN), but the deformation, and presumably lyriform organ sensitivity, increases above/below this joint angle as the cuticle is put under tension/compression, respectively (Blickhan and Barth, 1985). There are some exceptions to this (e.g., VS-5 lyriform organ), where organs are compressed (and therefore stimulated) during leg extension (Blickhan and Barth, 1985).

Geometry

The vibrational motion of a system made of masses, springs, and dampers is determined by its overall geometrical structure. Diversity in web shapes and body plans influences vibration transmission through these materials. Variation in the geometry of morphological traits between species can be explained by the evolution of divergent hunting strategies, shaped in part by various constraints. In addition, mechanosensor geometry and placement varies within an individual, both spatially over different areas of the body, and temporally due to development and regeneration.

Vibration Transmission Within Web and Body

Web geometry is highly variable both within and between individuals. Individual variation in web geometry usually arises due to external and internal factors that change over time. For example, orb webs built at 20% relative humidity have c. 80% the capture area of webs built at 70% relative humidity (Vollrath et al., 1997). Starvation has also been shown to affect the variability of web geometry—when sufficiently fed, spiders use web modifications (such as increased capture area) to reduce variability in web geometry, which is thought to produce a more optimal phenotype for prey capture effectiveness (Vollrath and Samu, 1997). Whilst this variation has been well quantified, the trade-offs between mechanical functions vs.

vibration transmission when geometry is modified have largely not been investigated, and future experiments should aim to quantify both mechanical and vibration transmission properties as web geometry changes and their implications for prey capture.

Web geometry due to the spider's resting location on the orb web alters vibration transmission, where some individuals sit in a retreat at the edge of their web rather than being positioned at the hub (center). Some spider species are more likely to employ this strategy than others, such as *Zygiella x-notata*, which senses web vibrations using a signal thread that joins the retreat to the hub, passing through a sector devoid of crossing threads (Klärner and Barth, 1982; Mortimer et al., 2018). Thus, they initially forgo the ability to localize prey on the web in favor of being less conspicuous at the retreat to avoid predation (Pasquet et al., 2007). After sensing prey struggling in the web, *Z. x-notata* moves along the signal thread to the hub, where it is able to orient toward the source vibration, making prey capture take longer (Klärner and Barth, 1982). The change in resting position does not impose any biologically relevant time cost due to vibration propagation through the web, but signals arriving at the retreat will be more damped than those at the hub (-1.37 ± 0.91 dB on the signal thread relative to the hub) (Mortimer et al., 2018).

There is high interspecific variation in web geometry (Foelix, 1979), and different web types are characteristic of separate taxonomic groups—these include orb webs, funnel webs, tubular webs, sheet webs, and tangle webs. Whilst most studies have focused on orb webs as their regular structure makes them easier to model, other web geometries are a promising area for future research, as each has their own unique transmission properties. For example, whilst the transmission efficiencies of transverse and longitudinal waves through orb webs differ greatly (especially with masses in the web as described previously) (Masters, 1984a; Landolfa and Barth, 1996; Mortimer et al., 2016), in other web geometries, such as sheet and tangle webs, they may propagate equally well (Naftilan, 1999; Vibert et al., 2016). In these web types, there are a large number of points where threads come into contact with each other, and unlike in orb webs, the angles between threads tend to be quite variable (Vibert et al., 2016). This irregular web architecture means that there is a lot of variation in what frequencies are transmitted best within and between webs, suggesting that frequency may not be the most important factor for discriminating between vibratory sources for these spiders. Modeling suggests that resonance of the silk threads may be useful in enhancing the amplitude of prey generated vibrations in funnel/sheet webs, with amplification occurring within some of the frequency range that prey struggles at (2–200 Hz) (Naftilan, 1999).

The influence that the overall body plan of the spider has on vibration transmission is a largely unexplored area, yet is likely to prove vital in our understanding of how evolution and constraints have shaped vibration information transfer. A useful approach will be to model diverse spider body plans as mass/spring/dampers systems to investigate the physical relationships underlying vibration transmission. From a mass/spring/damper perspective, overall body plan matters because it determines the dynamics of the vibrating system (including torques and resonances). We can consider two extremes of spider morphology and compare the body plan of the Pholcidae (cellar spiders) with the Theraphosidae (tarantulas) (**Figure 2**; Foelix, 1979). In Pholcidae, a pendulous abdomen is connected to a small cephalothorax, which is in turn supported by very long, thin legs. In Theraphosidae, the legs are much shorter and thicker, comprising a larger proportion of the spider's overall mass. These families have evolved disparate hunting strategies, with the Pholcidae employing a web to trap prey, and Theraphosidae being ambush predators. Therefore their difference in morphology is also correlated with a difference in substrate through which they sense vibrations (ground/tree vs. the web), and a difference in the vibrations of prey. The leg and body shapes can be assumed to be adaptive for their respective hunting strategies, shaped by evolutionary and physical constraints including trade-offs; for example the strong, muscular legs in Theraphosidae have to be able to overpower prey as well as sense vibrations. Whether the shapes of the legs and body are adaptive in vibration transmission remains to be seen, but in theory these differences will influence vibration transmission.

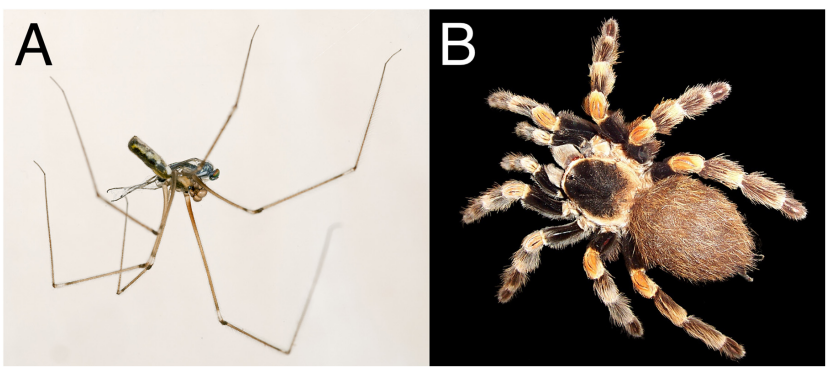

FIGURE 2 | Comparison of the body geometries of *Pholcus phalangioides* **(A)** and *Brachypelma smithi* **(B)**. Open source images: A—Filename "Pholcus phalangiodes MHNT male. jpg," courtesy of Didier Descouens, License CC BY-SA 4.0, B—Filename "Brachypelma edit. jpg," courtesy of user "Fir0002," License CC BY-SA 3.0.

Mechanosensor Geometry and Placement

Sensor geometry and placement is key to how mechanosensors respond to deformations in the exoskeleton, from the position of individual slits, through to the geometry and position of the sensors on the body. The spatial and temporal variation in slit sensilla geometry across individual spiders has been extensively studied in a single species—*Cupiennius salei* (Barth, 2019). Understanding interspecific variation in sensor geometry is currently limited, but further research would reveal how spiders in different contexts solve the problems of sensor placement to promote vibrational information transfer. More research is needed on the distributions of slit sensilla across all parts of the spiders' bodies on a range of spider species other than the well-studied *Cupiennius salei*.

We can first consider the shape and structure of individual slits. Variation in slit length (5–200 μm) affects the sensitivity of the organ, as a larger slit will deform more than a smaller slit. This induces a greater deflection in the covering membrane that spans the slit and which sensory cells are attached to, which in turn makes vibrations easier to sense and decode (Barth, 1972; Barth and Pickelmann, 1975). The base curvature of the covering membrane also varies, with some forming a deeper trough within the slit than others—again, this affects sensitivity as greater curvature will mean that the membrane deflects more as the slit deforms (Barth and Bohnenberger, 1978). This is linked to the position of the neurons that innervate the membrane, with most dendrites connecting near the middle, where the greatest deformation occurs (Molina et al., 2009).

The arrangement of slits within the lyriform organs also has implications for vibration sensing. Slit arrangements and overall organ shape differ between lyriform organs located on different areas of the leg, which give each organ subtly different sensing capabilities (**Figure 3A**; Barth et al., 1984; Hössl et al., 2007, 2009). Heart-shaped arrangements found on the trochanter (leg segment), for example, are particularly sensitive to load direction (Barth et al., 1984), whilst slightly asymmetrical triangular arrangements can be used for measuring different response ranges (Hössl et al., 2009). The orientation of these slits relative to the leg axis is also important for vibration sensing, as the greatest deformation will be induced when the long axis is perpendicular to the direction of the input force. Because of this, most slit sensilla, including those in the lyriform organs, are oriented parallel to the long axis of the legs—which is the direction of vibrational wave transmission. However, not all of them are, and the reasons for this variation are currently unclear. This could be investigated by comparing slit orientation in different species and correlating this with morphological/life strategy differences.

Lyriform organ geometry changes over development, where the lyriform organs and metatarsal pads grow at different rates (**Figure 3B**; Morley et al., 2016). The pad shows isometric scaling (proportional) relative to metatarsal length, as the pad grows at the same rate as the rest of the leg. However, the lyriform organ starts out relatively large and grows slowly using hypoallometric scaling in order to preserve function (Morley et al., 2016). This results in these two components being mismatched during early molt stages due to developmental constraints and trade-offs, with the small pad meaning that juveniles are less able to filter low-frequency environmental noise (<40 Hz) (Morley et al., 2016). Whether the effect of this mismatch is compensated for by the nervous system, handled by morphology or mitigated through behavior, remains an open question.

Both slit length and lyriform organ geometry are also affected by limb regeneration. Some spiders lose limbs and regrow them during successive molts. However, it can take several molts before the limb regains normal morphology, where the regenerated leg from the first molt may be only half the length of the others, yet appears to function normally (Vollrath, 1995). Crucially, the shape of the lyriform organs on regenerated legs differs from normal legs—the triangular organ, for example, has a lower apex whilst legs are regenerating (Speck-Hergenröder and Barth, 1988). Further research is needed to determine whether differences in the shape of the lyriform organ are adaptive, whether due to regeneration, development, and interspecific differences. Using modeling, evidence could be provided that this variation is adaptive by demonstrating that changes in lyriform organ morphology produce a compensatory transformation in the input wave.

Finally, the location of the slit sensilla on the body determines how they respond to forces, and may be linked to their function (Barth and Stagl, 1976). Slit sensilla are distributed throughout the spider's body and are extremely numerous, with around 3,500 in *Cupiennius salei* (Barth, 2002b). In general, the highest concentrations of slits (both single slits, loose groups, and lyriform organs) are found in the proximal segments of the legs (closer to body), which is correlated with increased musculature in this region. Lyriform organs are typically located at or near the leg joints (Barth, 2019), where the forces through the cuticle are likely to be highest. Single slit sensilla, on the other hand, are often found well away from the joints in the middle of the leg segments. They are, however, frequently located near where the muscle attaches to the cuticle, which supports the idea that these organs function in proprioception, through the sensing of internally generated strains from muscular contraction (Seyfarth et al., 1985). Other organs are located toward the distal ends of the legs, such as the metatarsal lyriform organ and the tarsal slits (Barth, 2002a). These organs respond to external vibrations (Barth and Bohnenberger, 1978; Speck and Barth, 1982), which makes sense given they are located closer to the coupling point with the substrate. However, vibrations will continue to propagate up through the leg and body after reaching the metatarsal lyriform organ and hence more proximally located organs are likely to be involved as well, which is supported by evidence that spiders still orient toward vibration sources (albeit with reduced accuracy) when the metatarsal lyriform organs are ablated (Seyfarth and Barth, 1972; Seyfarth et al., 1982; Bleckmann and Barth, 1984).

INFLUENCE OF BEHAVIORAL TRAITS

Behavior is a key mechanism influencing vibration transmission as it acts to influence morphology in different biological contexts. This includes altering mass distribution, the parameters of springs and dampers through stiffness and tension, and changing

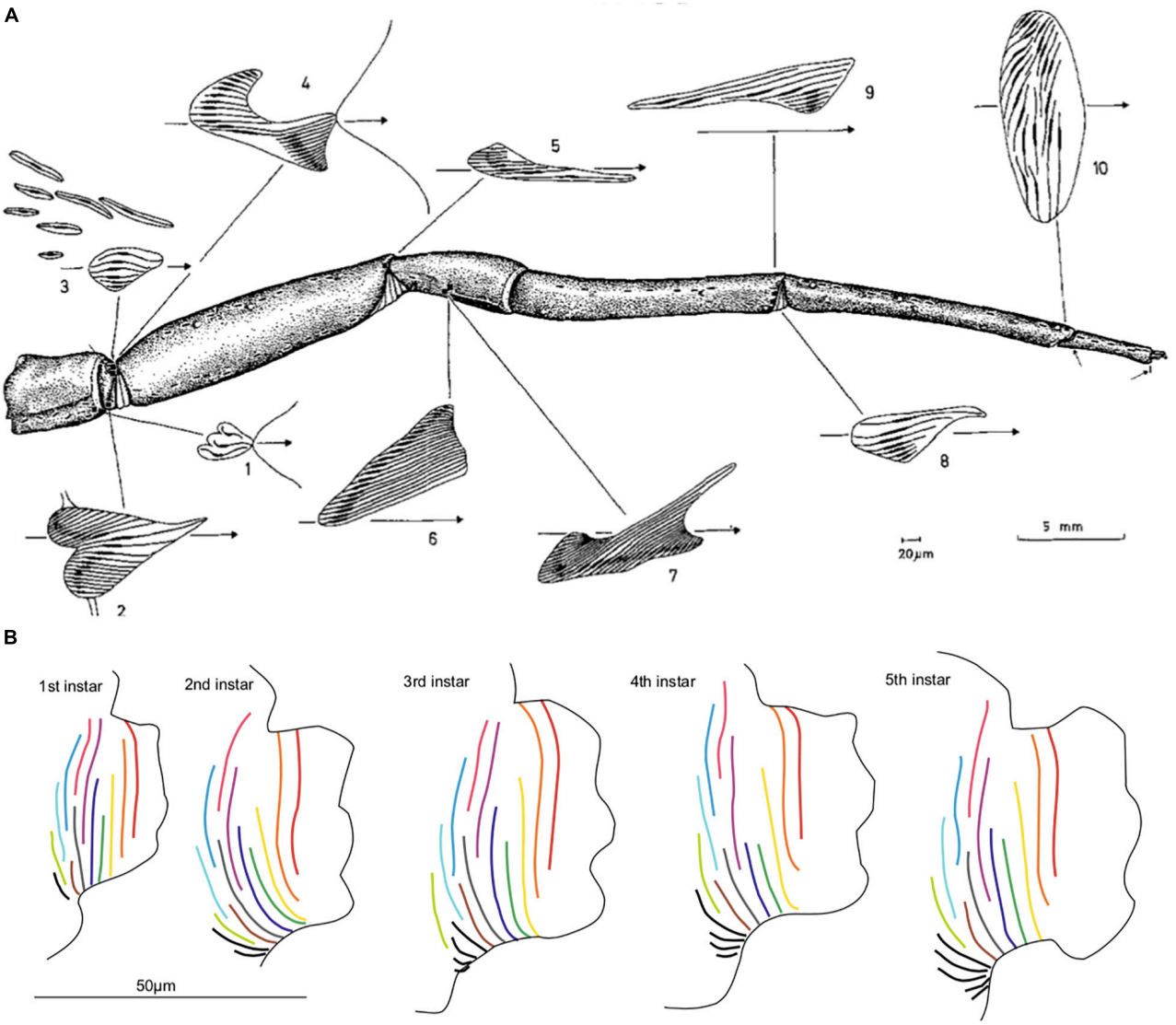

FIGURE 3 | A—spatial variation in lyriform organ geometry on the posterior of the first leg of *Cupiennius salei*, including heart-shaped, triangular, oval and irregular arrangements of slits. **B**—temporal variation in the HS10 lyriform organ on the first leg over the first 5 juvenile instars of *Latrodectus hesperus*, demonstrating changes in shape as well as increase in overall number of slits through growth. **(A)** Reprinted by permission from Springer Nature Copyright Clearance Centre: Springer Nature, Zoomorphologie (Slit sense-organs of arachnids - Comparative-study of their topography on walking legs (*Chelicerata. Arachnida*), Barth, F. G. and Stagl, J.) [Copyright], (1976). **(B)** Reprinted by permission from Springer Nature Copyright Clearance Centre: Springer Nature, Zoomorphology (Developmental morphology of a lyriform organ in the Western black widow (*Latrodectus hesperus*), Morley, E. L., et al.) [Copyright], (2016).

overall geometry—both in the extended phenotype of the web and in the spider's body.

Vibration Transmission Within Web

The extended phenotype of the spider web is an excellent example of using behavior to control an individual's vibratory environment. Different elements of the web can be modified to alter different aspects of vibration transmission, whilst maintaining other functions such as prey capture ability. We have already discussed how masses, springs, dampers, and geometry within the web may influence vibration transmission, and here we discuss the degree of behavioral control spiders have over these factors, and link these with the biological contexts that drive spiders to make these changes.

Starting with the effect of masses, some spiders will actively remove foreign objects from their web (Cloudsley-Thompson, 1995; Pasquet et al., 2007). Whilst these tidying behaviors may be driven by a number of different selection pressures [e.g., avoiding damage to the web, hygienic reasons (Straus and Avilés, 2018)], they will also affect vibration transmission by modifying mechanical impedance on the web. Orb weavers have been shown to locate these objects using a "plucking" behavior (Klärner and Barth, 1982). The presence of some masses in the web (such as stored prey items) is, however, unavoidable, despite their

impact on vibration transmission. Behaviors such as caching prey (storing food e.g., in *Nephila*) are used to cope with a variable food supply (de Crespigny et al., 2001) and also attract more insects into the web (Henaut et al., 2010). However, caching prey only within a small area may mitigate the impact of stored food on vibration transmission as their effect on vibration transmission would be location specific.

Orb weaving spiders have behavioral mechanisms to control the springs and dampers of the web, via changing tension, stiffness, and supercontraction. Spiders may adjust silk tension in order to deliberately modulate the transmission of vibrations through the web in response to a change in conditions (Watanabe, 2000). As well as controlling silk tension, spiders are also able to modulate silk stiffness. Orb weavers can adjust silk stiffness during web construction [by spinning silk more quickly (Vollrath et al., 2001)] and after the web is complete (by applying a force to a thread). Both of these processes result in increased ordering of the protein structure and hence increase stiffness (Guan et al., 2012). Spiders can modulate supercontraction to a certain degree by changing silk tension, as threads will not supercontract at high tensions (>140 MPa) (Boutry and Blackledge, 2010; Guan et al., 2011). Therefore, leaving threads slack will lead to increased supercontraction. Threads may also end up being unintentionally slack due to web damage, where supercontraction could act to increase thread tension in these areas. Spiders can also stretch out supercontracted threads to increase stiffness following supercontraction (Guinea et al., 2005). Spiders may therefore modify stiffness using these mechanisms to modulate trade-offs, for example increasing silk stiffness to prioritize mechanical function at the expense of prey sensing ability, as increased stiffness reduces longitudinal wave amplitude (Mortimer et al., 2016). Although these are mechanisms in theory, whether orb weavers actually apply their ability to modulate stiffness to control web properties and vibration transmission is currently unclear. Experiments would be needed to link sensory cues with spider control mechanisms and the resulting mechanical and vibrational properties of the web would need to be quantified.

Individual spiders will modify the geometry of their webs to influence trade-offs in response to environmental variables (Vollrath et al., 1997). When it is windy, garden cross spiders will produce a smaller, rounder orb; with fewer and more widely spaced capture spiral threads (40,686 mm^2 total area and 14,545 mm spiral length in still conditions vs. 35,235 mm^2 total area and 13,039 mm spiral length in windy conditions) (Vollrath and Samu, 1997)—this geometry is less likely to catch the wind like a sail and get damaged (Zaera et al., 2014). We hypothesize that in these situations, spiders prioritize the mechanical integrity of their web over vibration transmission, as fewer capture spirals reduce transverse wave amplitude (Mortimer et al., 2016). However, there are other relevant trade-offs at play here—these same modifications are also likely to reduce the prey-capture effectiveness of the web.

Orb weavers can also alter the transmission properties of their web in response to their energetic state (Watanabe, 2000). Starved spiders are more likely to target smaller prey items that would usually be ignored when satiated (Herberstein et al., 1998). Some orb weavers (e.g., *Octonoba sybotides*) modify web geometry when starved by forming webs with spiral rather than linear stabilimenta (web decorations). This is thought to increase radial thread tension (Eberhard, 1972), which makes low amplitude transverse vibrations produced by small prey easier to detect (Mortimer et al., 2016). In turn, this allows starved spiders to respond to small prey more quickly (Watanabe, 2000).

Using all of these mechanisms, orb-weaving spiders in theory are able to adjust the vibration transmission properties of their web at multiple temporal levels, possibly even specifically targeting certain wave types (longitudinal or transverse waves). This high level of control enables them to effectively respond to changes in environment, web damage, fluctuations in body mass, and climatic factors—variation which might otherwise impair vibration information transfer.

Vibration Transmission Within/Along Body

The dynamics of vibration transmission in a spider's body is strongly influenced by posture, which spiders actively control. We have already discussed how leg angle influences the springs and dampers of the joints, as well as the sensitivity of the lyriform organs, and here we examine how spiders use behavior to control this.

Spiders in theory can use postural changes to alter mass distribution, springs/dampers and geometry in response to different behavioral contexts. For example, the crouching posture tends to be adopted more frequently when a spider is starved (Mhatre et al., 2018). Empirical evidence for the effect of posture is currently limited to the role of springs/dampers at individual leg joints (Blickhan, 1986). Whilst evidence from modeling indicates that full-body geometry and mass distribution is also important for vibration dynamics (Mhatre et al., 2018), this is an area that is yet to be fully investigated. We do know that different postures are correlated with the sensitivity of the lyriform organs, as different joint angles influence the extension and stiffness of the cuticle surrounding the joint (Blickhan, 1986). Spiders adopt different resting postures when sitting at the hub or retreat of their webs, such that their legs are in contact with the relevant silk threads for vibration sensing (Klärner and Barth, 1982). Resting positions are typically used when the spider is sensing externally generated vibrations—in this configuration, the tibia-metatarsus angle is small (<120°), and the sensitivity of the organs is maximized. When the spider is walking, the legs are extended through much of the stepping cycle, reducing the sensitivity of the lyriform organs (Blickhan and Barth, 1985). Therefore, the spider maximizes lyriform organ sensitivity in contexts where they are most likely to show predatory behavior in response to vibrations. During locomotion, other functions are prioritized, where lyriform organs may serve more of a proprioceptive function (Barth, 2002a).

DISCUSSION

So far, this review has covered the mechanisms governing vibration transmission using orb-weaving spiders as examples of animals that influence transmission both within the environment and the body. We have demonstrated that a wide range

of morphological and behavioral traits influence vibration transmission via their effects on masses, springs, dampers, and geometry, which interact together to govern vibrational motion. We have also shown that these traits have considerable variation at different levels. How can we understand this variation to uncover the constraints acting on this sensory mode and the evolutionary drivers? Here we discuss the roles that constraints and control have in shaping this trait variation across different time scales and taxonomic levels, and the implications for vibration sensing. Through this discussion, we hypothesize a role for morphological computation in shaping vibration transmission, which can be tested for in future studies and applied within bioinspired engineering.

Firstly, we see that constraints of different types are acting on the morphological traits. The first type is physical constraints—the action of damping, filtering or distortion caused by the physics underlying the dynamics of vibration transmission. Physical constraints are governed by the geometry and properties of masses, springs, and dampers, and act to filter and dampen vibrational information, exemplified by the effects of the spider's body properties and geometry on vibration transmission both in the web and through the body. These physical constraints ultimately influence propagation distance, the transmission quality of different frequencies, and the ability of the spider to filter information from noise (Mortimer, 2017). The second type is evolutionary trade-offs, given that the web and spider bodies are multifunctional, their morphologies are important not only for vibration transmission, but also other biological functions. For example, web geometry, tension, stiffness, and supercontraction affect both the function of the orb web to capture prey, as well as vibration transmission (Mortimer et al., 2016). Also, the spring and damper properties of spider leg joints are important for locomotion, as well as vibration sensing (Blickhan and Barth, 1985). The third type of constraint is developmental constraints, where the development of the spider's morphological traits determines their geometry and properties, such as the effects of spider age or leg regeneration. For example, vibration transmission through the body and sensory transduction are affected over the course of development by the different growing rates of the metatarsal lyriform organs and associated metatarsal pads (Morley et al., 2016). These constraints do not act equally on morphological traits; some are more prone to one type of constraint than another.

The constraints do not solely act to limit or fix variation in morphological traits; we found examples of the constraints leading to non-functional variation and even constraints being harnessed for functional uses. For example, some morphological features such as leg length affect vibration transmission, but are relatively fixed within an individual due to development constraints (except over development and regeneration). In terms of non-functional variation, some morphological features will vary, but their variation may not be functionally useful for vibration sensing, such as masses in the web. As we have seen, masses have significant effects on vibration transmission due to the strong influence of mechanical impedance, a physical constraint. There are also a few examples of where apparent physical constraints cause variation that may be harnessed for functional uses in the web. For example, although the spider body mass affects vibration transmission via mechanical impedance, this accentuates orientation cues in the web, which spiders may use to locate prey (Mortimer et al., 2019). As a final thought, few of the morphological traits we have reviewed appear to be free from any form of constraint, with the possible exception of mechanosensor placement. The sheer number of sensors and their wide distribution across spider bodies suggests that evolution was able to come up with this solution repeatedly for functional uses, resulting in apparent redundancy in the system. Investigation of sensor placement differences and functionality across different species that differ in their ecology will be able to solve this mystery.

Even where constraints may be acting on morphology, there are different control mechanisms that can mitigate constraints to maintain functionality for vibration sensing. We can infer a control mechanism is in place when a trait shows variation that is functionally useful, but species comparisons and behavioral studies would provide direct evidence. Control mechanisms can be at the individual level due to plasticity that acts at small time scales to shape trait variation. However, they can also be due to evolution acting over longer timescales driving species differences through niche adaptation. We see that individual spiders are unable to directly control leg and body morphology to mitigate constraints, as these are generally fixed following development. This means that an individual's vibration transmission system must be robust to variation, which may explain the number of sensors and redundancy in the system. However, spiders can directly control web morphological traits as they have silks with an impressive range of properties, which is functionally useful to the spider. Silk property variation can control the spring and dampers of their extended phenotype at short timescales in response to changing conditions (Mortimer et al., 2014). We also see evidence for control of constraints via niche adaptation in different spider species as certain morphological traits correlate with particular hunting strategies. However, data on the functionality of specific differences between species in body morphology for vibration sensing is lacking. How different species morphologies influence inherent trade-offs, as well as physical and developmental constraints, requires further study. An interdisciplinary approach utilizing modeling (informed through experimental determination of relevant biomechanical parameters) and behavioral trials would be a promising line of research. In combination, we note that spider morphology does not vary as much between species as other terrestrial arthropods, where morphological diversity is more common in silk-use than overall body plan (Vollrath, 1999).

Behavior is an important mechanism controlling morphology. The constraints and control mechanisms above do not apply in the same way to behavioral traits, which in its nature can be more variable, with plasticity at the individual level and niche adaptation at the species level. At the individual level, we see examples of using behavior to mitigate physical constraints by removing masses on the web (Cloudsley-Thompson, 1995),

controlling web properties and geometry (Watanabe, 2000; Mortimer et al., 2016), as well as dictating spider position and posture on the web (Mhatre et al., 2018; Mortimer et al., 2018). We also see evidence of using behavior to shape trade-offs between different functions, for example adjusting web geometry in response to changing environmental conditions to shape mechanical verses sensory functions (Vollrath et al., 1997). These behavioral control mechanisms are extremely useful as they effectively harness the multifunctionality of morphological traits that underlie the trade-off, to enable the spider to deal with variable conditions by changing a multifunctional trait (Vollrath, 1999). Whether behavior can be used to mitigate developmental constraints (such as lyriform organ/pad mismatch) is a current research gap, but could be explored by investigating behaviors that juvenile spiders might use to avoid the problem of filtering low-frequency environmental noise.

The high level of involvement that morphology has in vibration transmission suggests a role for morphological computation, where morphology acts to perform useful tasks within a system (Muller and Hoffmann, 2017). Morphological computation is uniquely applicable to our spider context as vibrational information propagates through both the web and body morphology before reaching embedded mechanosensors. In this context, you can see how morphology, or behavior via its action on morphology, could act on vibrational information during propagation to filter and transform vibrational waves, which could be used in theory to promote information transfer. Using morphological computation, the filtering of information from noise is not handled solely by the nervous system, but by the masses, springs/dampers, and geometry of the web/body morphology, with each of these parameters potentially being tuned to transform the input wave differently under varying conditions. We might expect morphological computation to be selected for in terrestrial arthropods as it is in theory an efficient way, in terms of computational cost and potentially energetic cost, to promote information transfer. This is because complex computational tasks that would usually be performed by the CNS are replaced with relatively simple and potentially passive responses that are governed by a system's morphology (Muller and Hoffmann, 2017). This reduces computational cost as the animal, in theory, can compute more quickly, as CNS processing takes time, but it also reduces the complexity and number of connections required in the nervous system (Pfeifer and Bongard, 2006). It can potentially increase energetic efficiency as the computational tasks could involve passive responses of morphology rather than active metabolic processes in the CNS (Muller and Hoffmann, 2017).

Since morphological computation would be influenced by both morphological and behavioral traits, control of these traits can be selected for in vibration-sensing animals to influence vibration transmission via filtering input waves. Individual plasticity in these traits is likely to influence vibration information transfer through morphological computation. Whether morphology is controlled by an individual to functionally influence vibration transmission through the body warrants further research, but we can make predictions for how control via morphological computation would work in natural contexts. Possible examples of plasticity in spider morphological traits include the shape of the lyriform organs throughout development and after regeneration. If morphological variation here is functional for vibration information transfer, we would predict lyriform organ shape to transform input waves to compensate for leg length changes before sensory transduction occurs, thus avoiding the need for extra processing in the CNS. In this example, both leg length and lyriform organ shape are morphological traits influencing vibration transmission, as these traits alter the mass and material property distribution of the spider's leg (aspects of mass, springs/dampers, and geometry). Spider behavioral traits potentially involved in morphological computation include posture changes and modifying web geometry, which could be used to functionally influence vibration transmission. If the web uses morphological computation, we would predict that spiders use behavior to modify web properties in response to environmental factors to alter the vibrational information that arrives at the spider, thus avoiding the need for extra processing in the CNS as environment changes. In this example, spider web building is a behavioral trait influencing web geometry as a morphological trait. The geometry of the web in turn influences vibration transmission, as this governs mass and material property distribution of the spider's web (aspects of mass, springs/dampers, and geometry). Web-building spiders are special in this case; since they control their vibratory environment, they can mitigate the physical constraints faced by many other organisms that are limited by the transmission properties of the substrate upon which they live. This again highlights how web-building spiders are an ideal model organism for studying vibration information transfer, since they have control over morphology in both their vibration transmission platform and their body.

Using spiders as inspiration, these control mechanisms could have direct applications for developing new, bioinspired technologies that use material-bound vibrations for information (Lipson, 2014; Barth, 2019)—for example soft robots for search-and-rescue applications (Hawkes et al., 2017). Designing robots to cope with unknown environments in real-time is a challenge for engineers, as environmental variability can pose problems for artificial systems (Hauser et al., 2011). Sensing here is key, where material-bound vibrations can be used to monitor the internal and external environment to respond appropriately in real-time to environmental variability to maintain functionality. Morphological computation is an efficient way to solve these problems for artificial systems as computational tasks can be outsourced to the system's morphology, which reduces power requirements. If the insights of how animals, such as spiders, achieve this can be applied to robotic design, it can open up new applications for robots that are adaptable to changing environments. A spider-inspired soft robot would use morphological computation to increase not only efficiency and adaptability, but also damage tolerance, for example using "smart" structures to detect and compensate for damage to part of its body (Hauser et al., 2014). To reach this stage, it is necessary to fill the current

research gap in our fundamental understanding of how an eight-legged spider-shaped morphology transmits vibrations. What are the most important biomechanical parameters influencing wave transmission? Is there any evidence for morphological computation significantly reducing processing cost in the CNS? Is the system robust enough for morphological computation to still be effective in the face of variation in morphology over time, and could an individual spider exploit this? How does this process vary in different species, with different life strategies and different morphologies? Mathematical modeling of the whole-body system, combined with experimental manipulations of the animal systems, may provide the answer.

Further research is likely to show that morphological computation in nature is far more widespread than has previously been recognized. We suggest that evolution has produced an array of sensory solutions to problems faced by engineers, and that continued research into understanding the mechanisms that natural systems use to promote information transfer will lead to new types of technologies for varied applications.

AUTHOR CONTRIBUTIONS

TM wrote the first draft and prepared the figures. BM and TM edited the manuscript. BM provided supervision for TM. Both authors contributed to the article and approved the submitted version.

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The Subgenual Organ Complex in Stick Insects: Functional Morphology and Mechanical Coupling of a Complex Mechanosensory Organ

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Leg chordotonal organs in insects show different adaptations to detect body movements, substrate vibrations, or airborne sound. In the proximal tibia of stick insects occur two chordotonal organs: the subgenual organ, a highly sensitive vibration receptor organ, and the distal organ, of which the function is yet unknown. The distal organ consists of a linear set of scolopidial sensilla extending in the tibia in distal direction toward the tarsus. Similar organs occur in the elaborate hearing organs in crickets and bushcrickets, where the auditory sensilla are closely associated with thin tympanal membranes and auditory trachea in the leg. Here, we document the position and attachment points for the distal organ in three species of stick insects without auditory adaptations (*Ramulus artemis*, *Sipyloidea sipylus*, and *Carausius morosus*). The distal organ is located in the dorsal hemolymph channel and attaches at the proximal end to the dorsal and posterior leg cuticle by tissue strands. The central part of the distal organ is placed closer to the dorsal cuticle and is suspended by fine tissue strands. The anterior part is clearly separated from the tracheae, while the distal part of the organ is placed over the anterior trachea. The distal organ is not connected to a tendon or muscle, which would indicate a proprioceptive function. The sensilla in the distal organ have dendrites oriented in distal direction in the leg. This morphology does not reveal obvious auditory adaptations as in tympanal organs, while the position in the hemolymph channel and the direction of dendrites indicate responses to forces in longitudinal direction of the leg, likely vibrational stimuli transmitted in the leg's hemolymph. The evolutionary convergence of complex chordotonal organs with linear sensilla sets between tympanal hearing organs and atympanate organs in stick insects is emphasized by the different functional morphologies and sensory specializations.

Keywords: mechanoreception, chordotonal organ, stick insect, neuroanatomy, vibration, hearing, sensory evolution

INTRODUCTION

Structural differentiation in sensory organs commonly correlates to functional specializations (Steinbrecht, 1999; Ridgel et al., 2001; Homberg and Paech, 2002; Land and Nilsson, 2012; Scherberich et al., 2017; Strauß, 2017; Zhao and McBride, 2020). This is also found for chordotonal organs, which are internal mechanoreceptors occurring over the insect body plan (Howse, 1968; Field and Matheson, 1998; Kavlie and Albert, 2013). With scolopidial sensilla as sensory units, the chordotonal organs are versatile for adaptations to different mechanical stimuli acting on the sensilla by stretching or tilting of the dendrite. In one organ like the antennal Johnston's organ, sensilla can be functionally specialized in different subgroups which are not anatomically separated (eg., Kamikouchi et al., 2009; Matsuo and Kamikouchi, 2013). In other cases, sub-groups of sensilla or several organs occur in close proximity (Field and Matheson, 1998). For the latter, the subgenual organ complex in orthopteroid insects provides an example of 2–4 chordotonal organs located in the leg (Strauß et al., 2014). This is a notable expansion of sensory structures, as the subgenual organ in several insect lineages is the sole chordotonal organ in the proximal tibia and may consist of only few sensilla which are sufficient for detection of vibration signals (Michel et al., 1982; Nishino et al., 2016; Čokl et al., 2019). The subgenual organ is the most sensitive receptor organ for substrate vibrations and it occurs in most insects (Čokl and Virant-Doberlet, 2009; Lakes-Harlan and Strauß, 2014).

Distinct types of chordotonal organs are recognized based on their functional morphologies, including connective chordotonal organs, or tympanal (auditory) organs. The different attachments to surrounding structures like joints, cuticle, or trachea, couple the sensory organs to the sites of stimulus transfer on the insect body. Depending on the coupling/attachment, chordotonal organs can thus adaptively function as proprioceptors or exteroceptors. The mechanical coupling structurally allows to transfer mechanical force to the sensory organ as a group of sensilla, and ultimately to the dendritic membrane of the sensilla (French, 1988).

Connective chordotonal organs are attached to a strand of connective tissue to a tendon or body parts (Howse, 1968; Wright, 1976; Field and Matheson, 1998). They can function as proprioceptors, responding to body movements if they are located at or linked to joints by a receptor apodeme (Bässler, 1965, 1977; Field and Pflüger, 1989), tendons (Godden, 1972), or occur at pleural membranes (Hustert, 1974). Here, they can respond to movements like leg extension and flexion (Bässler, 1993; Tuthill and Azim, 2018) and motion of abdominal segments (Hustert, 1974). Other chordotonal organs can function as exteroceptors, detecting environmental stimuli. In these cases, they are usually coupled e.g., to structures resonating to airborne sound, like tympanal membranes and associated tracheal spaces in hearing organs. In these cases, both the tympana of very thin cuticle, as well as enlarged trachea, can provide entrance for sound energy (Stumpner and Nowotny, 2014; Montealegre-Z and Robert, 2015; Römer and Schmidt, 2016; Windmill and Jackson, 2016). Another modality detected by chordotonal organs are substrate-borne vibrations transmitted over the legs and the body. Receptor organs adapted to substrate vibrations are often located in the legs, like the subgenual organ. The subgenual organ spans the hemolymph channel in the proximal tibia and is excited by substrate vibrations transferred over the leg and the hemolymph system (Lakes-Harlan and Strauß, 2014). However, vibration stimuli are also detected by connective chordotonal organs like the femoral chordotonal organ, which usually has the highest sensitivity at relatively low frequencies (locust: Field and Pflüger, 1989; green lacewing: Devetak and Amon, 1997; stick insect: Stein and Sauer, 1999; stink bug: Čokl et al., 2006; cerambycid beetle: Takanashi et al., 2016).

For insect chordotonal organs, which repeatedly evolved a complex structure or a high number of sensory neurons (Field and Matheson, 1998; Yack, 2004), the functional morphology can indicate their physiological role by identifying how sensilla are stimulated by mechanical energy from their coupling to different structures like cuticle or tracheae. Elaborate chordotonal organs in the legs with linearly arranged sensilla occur in Ensifera, the long-horned grasshoppers (Nishino and Field, 2003; Strauß and Lakes-Harlan, 2009; Strauß et al., 2017). Especially the tympanal hearing organs in crickets and tettigoniids are well studied, with the linear sensilla forming the basis for frequency analysis (Stumpner and Nowotny, 2014; Montealegre-Z and Robert, 2015; Hummel et al., 2017; Nishino et al., 2019). Notably, these hearing organs in Ensifera are located next to other chordotonal organs which are sensitive to substrate vibrations. The differentiation of distinct organs can allow functional specialization by divergent tuning of individual organs, or by multimodal stimulus detection (Lin et al., 1993; Kalmring et al., 1994). A sensory organ similar in neuroanatomy to these tympanal organs is the distal organ (DO) of stick insects (Phasmatodea), located also in the proximal tibia, which is not associated with tympanal membranes (Strauß and Lakes-Harlan, 2013; Strauß, 2020a). This neuroanatomical organization evolved in convergence in these two taxa, and in stick insects independent to the evolution of tympanal membranes (Strauß and Lakes-Harlan, 2013). The DO in stick insects contains ∼20 sensilla in a linear array with decreasing cell sizes from proximal to distal (Strauß and Lakes-Harlan, 2013; Strauß, 2020b). This neuroanatomical complexity strongly suggests an important physiological role of the stick insect DO, as well as a mechanosensory adaptation different to the subgenual organ. The physiological function of the DO has not been specifically studied, but it is possibly vibrosensitive (Strauß and Lakes-Harlan, 2017). A more detailed understanding of the DO functional anatomy and its attachments will also show the similarities and differences to the auditory organs of Ensifera, and likely give insights into the different adaptations in diverse sensory organs.

Here, we investigate the functional morphology and neuroanatomy of the DO in stick insects. Previously, the attachments of the organ in the tibia were not studied in detail (Strauß and Lakes-Harlan, 2013). We investigate the DO in three species of stick insects to provide information for the functional morphology and their similarity in different genera. We included *Carausius morosus* (Lonchodinae) as model species for neurophysiology (eg., Bässler, 1983; Bässler and Büschges, 1998; Mantziaris et al., 2020), *Sipyloidea sipylus* (Necrosciinae) as this

species has been studied for the neuroanatomy and physiology of the subgenual organ complex (Strauß and Lakes-Harlan, 2013, 2017), and *Ramulus artemis* (Clitumninae) to sample a further species with leg sizes accessible for vital stainings. The aim here is to document the structure of the DO within the tibia (the hemoplymph channel), to identify the points of suspension or attachment for the DO, and thereby to indicate the possible mechanical coupling of the DO to other leg structures. For this, the organ is investigated for possible connections to tendons, connective tissue, trachea, and the leg's cuticle. The connections to these surrounding structures can indicate possible mechanical input pathways and the sensory activation (eg., Shaw, 1994b; Strauß et al., 2017; Stritih-Peljhan et al., 2019). Understanding the functional morphology of the DO of stick insects in more detail will also give insights into the extent of evolutionary convergence in the DO to the auditory sensilla in crickets and tettigoniids.

MATERIALS AND METHODS

Insects

This study investigated adult female *Ramulus artemis* (Westwood, 1859), *Carausius morosus* (Sinéty, 1901), and *Sipyloidea sipylus* (Westwood, 1859) for their sensory organs in the proximal tibia. *R. artemis* was included in the study to gain data on the subgenual organ complex for a previously unstudied species of stick insects [see Strauß and Lakes-Harlan (2013)]. The larger body size made preparations of the legs for vital stainings more feasible (see below). For all species, parthenogenetically reproducing females were reared in a laboratory culture at the Institute for Animal Physiology, Justus-Liebig-Universität Gießen. They were reared at 21–23°C, and under a 12:12 h light-dark cycle. The insects were provided with leaves of Rosaceae *ad libitum* and sprayed daily with water.

The experiments documented here comply with the principles of animal care of the Justus-Liebig-Universität Gießen, Germany, and with the current law of the Federal Republic of Germany.

External Leg Morphology

The external leg cuticle was documented for isolated legs with a Leica 9Si dissection microscope and an in-build digital camera (1,024 × 768 pixels) *via* the Leica Application Suite version 4.12 (Leica Microsystems CMS GmbH, Wetzlar, Germany). The tibia was photographed from the anterior and the posterior side. Series of photographs of each leg were combined using the freeware program CombineZP[1].

Neuroanatomy and Axonal Tracing

For neuroanatomical experiments, all insects were checked for intact legs and tarsi to avoid possible influences of regeneration after leg autotomy in postembryogenic development. The sensory organs of the subgenual organ complex and their neuronal innervation were stained intracellularly by axonal tracing using cobalt solution (5% $CoCl_2 \times 6\ H_2O$; Merck, Darmstadt, Germany, dissolved in *Aqua dest.*). The procedure for the dissection and tracing of the nervus cruris followed Strauß (2020a): legs were fixed with insect pins in a glass dish that was covered with Sylgard (Sylgard 184, Suter Kunststoffe AG, Fraubrunnen, Switzerland) with the ventral side facing upward. The ventral cuticle was removed with a piece of a blade (Feather FA-10, 0.1 mm, Feather, Osaka, Japan). The nerve dissection took place while covering the legs with *Carausius* saline [see Bässler (1977); 177.96 mmol NaC1, 17.4 mmol KC1, 25.1 mmol $MgC1_2 \times 6\ H_2O$, Roth, Karlsruhe, Germany; 7.48 mmol $CaC1_2 \times 2\ H_2O$, Merck, Darmstadt, Germany; 1.98 mmol Tris, from Sigma-Aldrich, St. Louis, MO, United States; dissolved in *Aqua dest.*, adjusted to pH = 7.4]. The nervus cruris was cut with iridectomy scissors, and the ending of the nerve was placed in a glass capillary filled with a 5% cobalt solution. The preparations were incubated at 4°C for 48 hr. The neuronal staining was achieved by precipitating the cobalt with ammonium sulfide (Alpha Aesar, Karlsruhe, Germany) in a 1% solution in *Carausius* saline. Prior to the incubation, the tarsi and distal tibia was cut off. Legs were placed in the ammonium sulfide solution for 15 min, rinsed in *Carausius* saline, and fixed in paraformaldehyde (4%; Sigma-Aldrich, St. Louis, MO, United States) for 60 min. Following dehydration in a graded ethanol series (Carl Roth, Karlsruhe, Germany), the legs were cleared and stored in methyl salicylate (Merck, Darmstadt, Germany).

Light Microscopy and Documentation

Before microscopy of the tracing preparations, the posterior cuticle in the tibia of tracing preparations was removed with a piece of a blade. The presence of the posterior subgenual organ and its neuronal innervation (see section "Results") were checked to ensure that the complete sensory organs were present after opening the leg. The preparations were mounted on a microscopy slide and were in most cases viewed from the posterior side. Occasionally, legs were also documented from dorsal direction.

The legs were viewed on a microscopy slide under methyl salicylate (Merck, Darmstadt, Germany) with an Olympus BH-2 microscope (Olympus, Shinjuku, Japan). Digital photographs were acquired with a Leica DFC 7000 T camera (1,920 × 1,440 pixel) attached to the microscope *via* the Leica Application Software V4.9 (Leica Microsystems CMS GmbH, Wetzlar, Germany). Series of photographs were combined using CombineZP. Photographs were assembled into panels, adjusted for contrast and brightness, and labeled using CorelDraw 11 (Corel, Ottawa, Canada).

The innervation pattern of the tibia was drawn using a Leitz microscope combined with a drawing attachment (Leitz, Wetzlar, Germany), and digitally redrawn and labeled using CorelDraw 11.

μCT Analysis of Sensory Organs

For the comparative micro-computed tomography (μCT) analysis, at least one tibia each of the foreleg and midleg of *R. artemis*, *C. morosus*, and *S. sipylus* were fixed for 24 h in Bouin's solution and then stored in 70% ethanol. To increase soft tissue contrast in the μCT scans, all samples were subsequently stained in a solution of 0.3% phosphotungstic acid (PTA; Sigma-Aldrich,

[1]http://www.hadleyweb.pwp.blueyonder.co.uk/

St. Louis, MO, United States) in 70% ethanol (Metscher, 2009) for 21 days, and subsequently washed and scanned in 70% ethanol. The tomography imaging was performed with a commercial μCT desktop system (Skyscan 1272, Bruker microCT, Kontich, Belgium) at the Zoological Research Museum A. Koenig (ZFMK). Scan settings are summarized in **Table 1**.

Thermal drift correction and digital section reconstruction was performed in NRecon 1.7 (Bruker microCT). The resulting image stacks were analyzed in DataViewer 1.5 (Bruker microCT).

Gray level-based three-dimensional volume renderings of the relevant organs were created in Drishti (Limaye, 2012) making use of the "crop" function and various "Clip" planes.

The cropped but otherwise unchanged μCT-scans of the tibiae are available as **Supplementary Material** at Zenodo[2].

Vital Staining of Sensory Organs

Vital staining of the subgenual organ complex was achieved with Janus Green B solution [Sigma-Aldrich, St. Louis, Missouri; dissolved at a concentration of 0.02% in *Carausius* saline; see Yack (1993)]. Isolated legs were fixed with insect pins in a glass dish with the leg's dorsal side facing upward. The cuticle was cut open dorsally with a piece of a blade and covered with *Carausius* saline. After removal of the saline, Janus Green B solution was applied for up to 60 s, and the legs then rinsed repeatedly with *Carausius* saline. The sensory organs were viewed with a dissection microscope (Leica), and digital photographs were taken with a Leica DFC 7000 T camera (1,920 × 1,440 pixel) mounted on the dissection microscope.

Terminology of Nerves and Nerve Branches

The tibia is innervated from the main leg of the nerve, termed nervus cruris. The terminology of nerve branches from the nervus cruris in the tibia follows that established for the more proximal leg segments (Bässler, 1983) by numbering the branches consecutively from proximal to distal. The smaller nerve branches originating from these first-order branches were numbered accordingly [see Strauß (2020a) for the subgenual organ complex in *S. sipylus*]. The terminology for the sections of the tibia along the different leg axis follows Ball and Field (1981).

Statistical Analysis

Statistical analyses were performed in GraphPad Prism 4 (GraphPad, San Diego, CA, United States) to test for differences in the number of sensilla among the legs pairs in *Ramulus artemis* with ANOVA, omnibus normality test, and Tukey's Multiple Comparison Test.

RESULTS

Neuroanatomy of the Subgenual Organ Complex

Ramulus artemis is an apterous stick insect with the body form characteristic for many Phasmatodea with thin legs (**Figure 1**).

TABLE 1 | μCT scan settings of all samples.

Species	Leg	Tube voltage (kV)	Tube current (μA)	Total sample rotation (°)	Angular step size (°)	Exposure time (ms)	Binning	Filter	Averaging	Random movement (px)	Voxel size (μm)
R. artemis	[T1], [T2]	30	200	360	0.20	1,980	1 × 1	No	8	15	1.80
C. morosus	[T1], [T2]	29	200	360	0.20	1,900	1 × 1	No	5	15	1.00
S. sipylus	[T1], [T2]	29	200	360	0.20	1,900	1 × 1	No	7	15	1.80

[T1], tibia of foreleg; [T2], tibia of midleg.

[2]https://doi.org/10.5281/zenodo.3856675

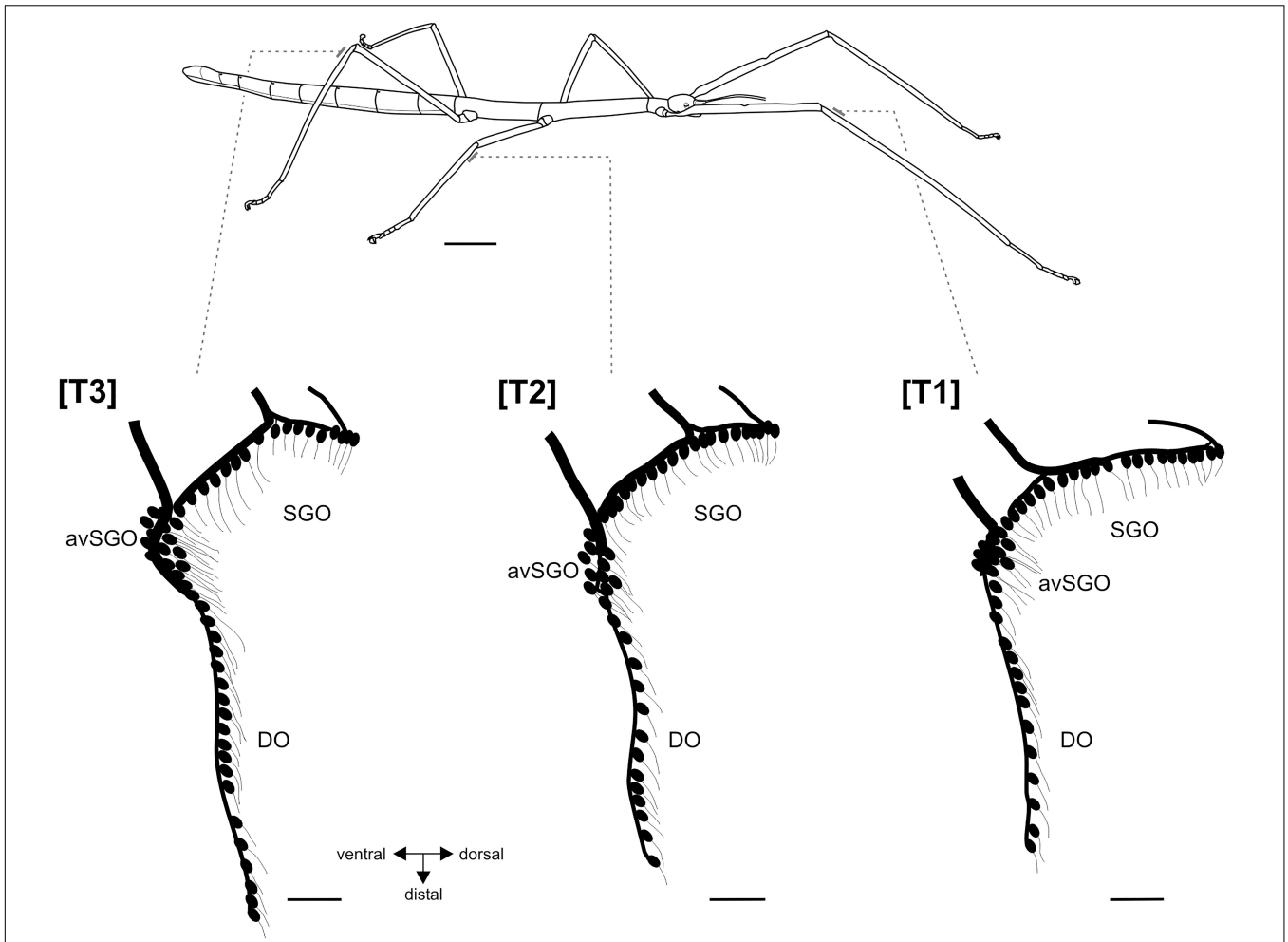

FIGURE 1 | Habitus of female *R. artemis* (top) and the serial organization of the subgenual organ complex (below). Bars at the legs indicate the position of the sensory organs in the proximal tibia. Schematics of the sensory neurons in the subgenual organ complex, redrawn from tracing preparations. Scales: habitus = 1 cm, sensory organs = 100 μm. avSGO, anterior-ventral subgenual organ; DO, distal organs; SGO, subgenual organ; [T1], foreleg; [T2], midleg; [T3], hindleg.

All legs are slender and elongated, with shorter middle legs (**Figure 1** and **Supplementary Figure 1**). The leg's cuticle at the position of the subgenual organ complex next to the femur-tibia joint was solid and showed no thinner cuticle compared to adjacent lateral leg areas. There were no differences between the anterior and posterior sides (**Supplementary Figure 1**).

The subgenual organ complex in *R. artemis* consisted of the subgenual organ (SGO) and the distal organ (DO) recognized by distinct anatomies and innervation (**Figures 1**, **2a**). The sensory organs were present in all leg pairs with no obvious differences in the overall organization (**Figure 1**). Axonal tracing showed that the SGO was oriented perpendicularly to the leg's main axis with dendrites pointing distally (**Figures 2a,b**). The most anterior sensilla of the SGO formed a dense group, termed the anterior-ventral subgenual organ (avSGO; Strauß and Lakes-Harlan, 2013) with ∼15 sensilla in all leg pairs (**Figures 2a,e**). The avSGO sensilla were placed next to the remaining subgenual sensilla (**Figure 2a**), and their dendrites were oriented in dorso-distal direction. The DO had a linear organization of sensilla (**Figure 2c**), while gaps in the line of cell bodies were occasionally seen (**Figure 2d**; in five out of 30 leg preparations). The DO dendrites were oriented in distal direction (**Figures 2c,d**). The overall neuroanatomy of the subgenual organ complex in *R. artemis* resembled that of the other stick insect species (*S. sipylus*, *C. morosus*) included in this study.

The intracellular staining allowed reconstructing the innervation pattern for the chordotonal organs and campaniform sensilla from the nervus cruris (**Figure 2e**). In general, the sensory neurons at the anterior and posterior side of the tibia were innervated by separate nerve branches on the anterior and posterior side: the sensilla of the subgenual organ were innervated by separate nerves on the anterior side (innervated by nerve branch T12) and posterior side (pSGO, innervated by nerve branch T22; see **Figures 2a,b,e**). The sensilla of the SGO occur continuous without gaps between the sensilla with different innervating nerve branches [**Figure 2e**; also Strauß (2020a) for *S. sipylus*]. The sensilla of the DO were innervated jointly with the anterior-ventral

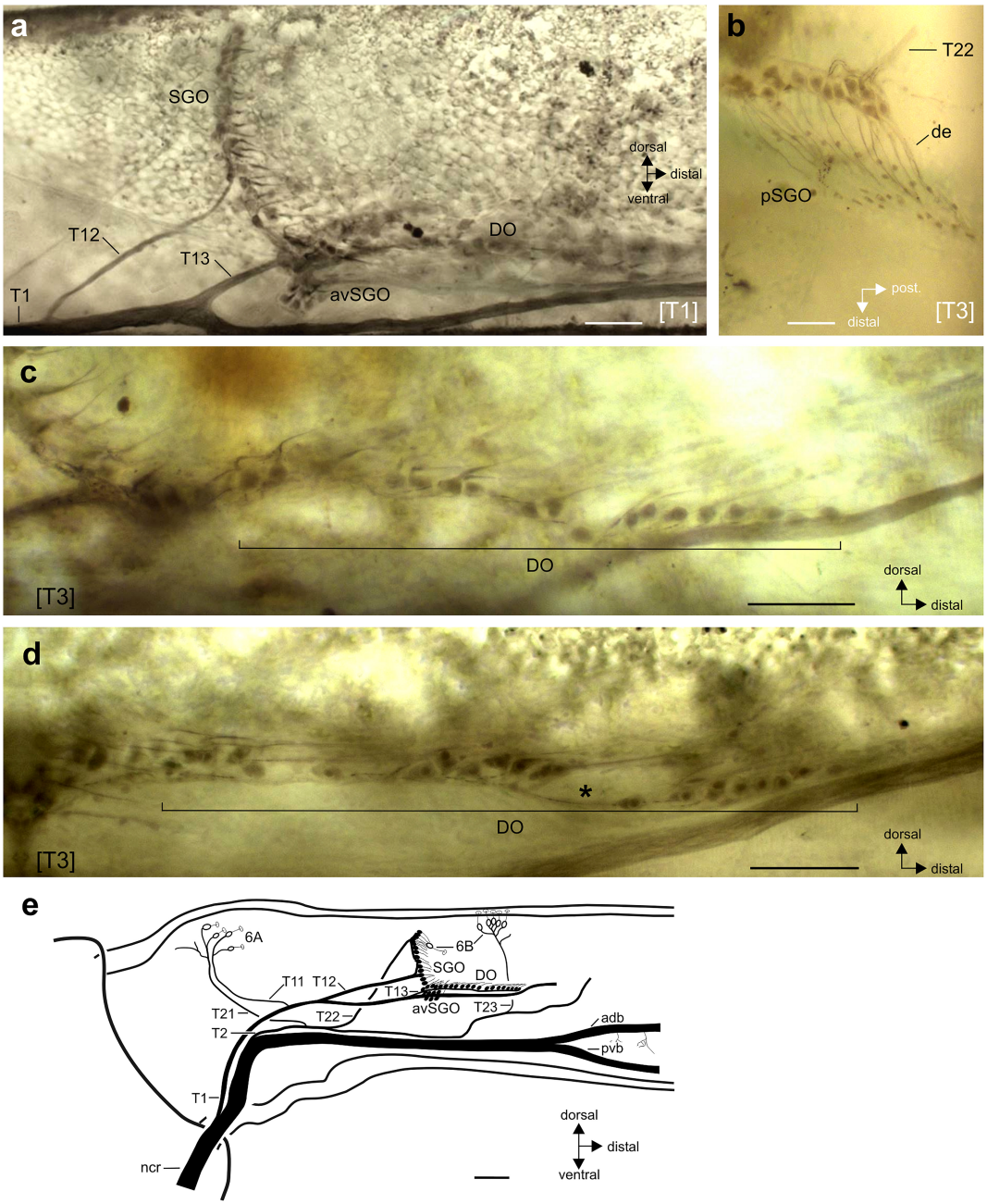

FIGURE 2 | Neuroanatomy of the subgenual organ complex in *R. artemis*. Perspective is from lateral unless stated otherwise. **(a)** Wholemount staining of the subgenual organ complex with the subgenual organ (SGO), anterior-ventral subgenual organ (avSGO) and distal organ (DO). **(b)** The sensilla in the posterior subgenual organ (pSGO) are innervated by a distinct nerve branch, T22. The sensilla's dendrites (de) point in posterior-distal direction. Viewed from dorsal. **(c,d)** Sensilla in the distal organ are arranged linearly in proximo-distal direction. They are usually continuously organized **(c)** but can show gaps between the somata (d; indicated by asterisk). **(e)** Schematic of the innervation pattern of the subgenual organ complex and campaniform sensilla in *R. artemis*, viewed in lateral perspective from the anterior side. The cell bodies of scolopidial sensilla are shown in black, the cell bodies of campaniform sensilla (groups 6A, 6B) in white. Scales: **(a,c,d)** = 100 μm; **(b)** = 50 μm; **(e)** = 200 μm. adb, anterior dorsal branch of nervus cruris; avSGO, anterior-ventral subgenual organ; de, dendrite; DO, distal organ; ncr, nervus cruris; pvb, posterior-ventral branch of nervus cruris; SGO, subgenual organ; [T1], foreleg; [T3], hindleg.

subgenual organ by nerve branch T13 (**Figures 2a,e**). The campaniform sensilla in the proximal tibia (group 6A) on either side were innervated by two nerve branches (anterior: T11, posterior: T12, **Figure 2e**).

The subgenual organ contained 37 ± 5 sensilla in the foreleg, 36 ± 4 sensilla in the midleg, and 39 ± 3 sensilla in the hindleg (n = 10 for each leg). The differences in the numbers of subgenual organ sensilla among the leg pairs were not statistically significant

(ANOVA: $p = 0.3912$; $F = 0.9720$; df = 29; D'Agostino and Pearson omnibus normality test: $p = 0.1745–0.6189$). The distal organ contained 17 ± 2 sensilla in the foreleg, 16 ± 1 sensilla in the midleg, and 21 ± 3 sensilla in the hindleg ($n = 10$ for each leg). These differences in the distal organ sensilla among leg pairs were statistically significant (ANOVA: $p < 0.0001$; $F = 17.54$; df = 29; D'Agostino and Pearson omnibus normality test: $p = 0.3336–0.4518$), with sensillum numbers significantly higher in the distal organ of the hindleg compared to the fore- and midlegs (Tukey's Multiple Comparison Test: $p < 0.001$).

Functional Morphology of the Subgenual Organ Complex

The two sensory organs of the subgenual organ complex and associated tissues were stained *in situ* with Janus Green B for *R. artemis* (**Figure 3**), and analyzed by μCT for

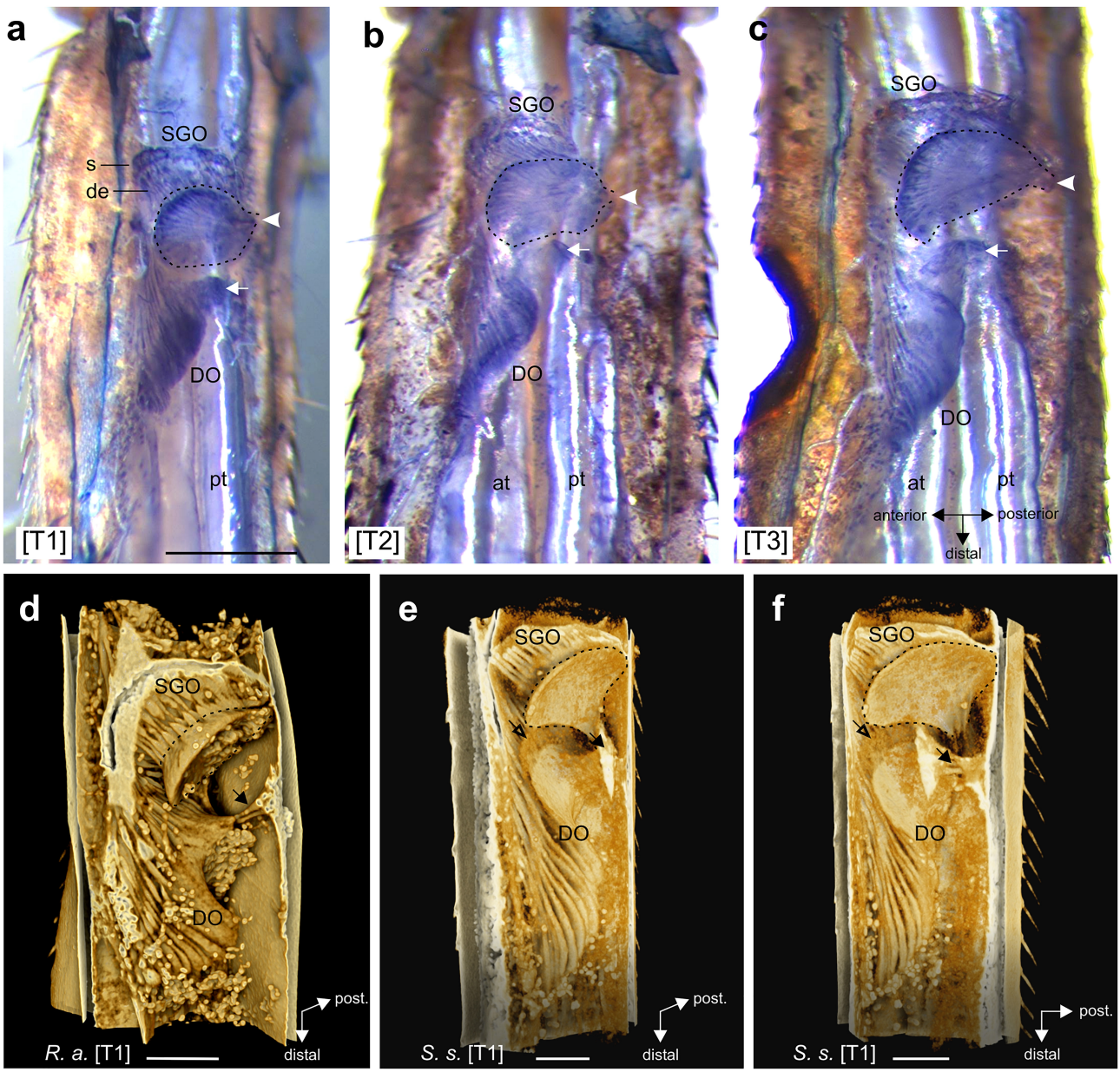

FIGURE 3 | The subgenual organ complex in the legs. **(a–c)** Vital staining in *R. artemis* with Janus Green B in **(a)** foreleg, **(b)** midleg, and **(c)** hindleg. In the subgenual organ (SGO), the dendrites (de) point distally from the somata (s) into the SGO tissue (outlined by hatched line) that attaches at the dorsal cuticle (arrowhead). The distal organ (DO) has a triangular form and attaches at the dorsal cuticle (arrow). **(d–f)** 3D renderings of the subgenual organ complex from μCT scans in **(d)** *R. artemis* and **(e,f)** *S. sipylus* with the SGO and DO. The SGO tissue (hatched line) and the proximal DO attach at the dorsal cuticle (solid arrow). Empty arrow indicates the position of the membrane between SGO and DO. Note the globular fat at the sensory organs. Scales: **(a)** = 500 μm; **(d–f)** = 200 μm. at, anterior trachea; de, dendrites; DO, distal organ; pt, posterior trachea; s, somata; SGO, subgenual organ; [T1], foreleg; [T2], midleg; [T3], hindleg.

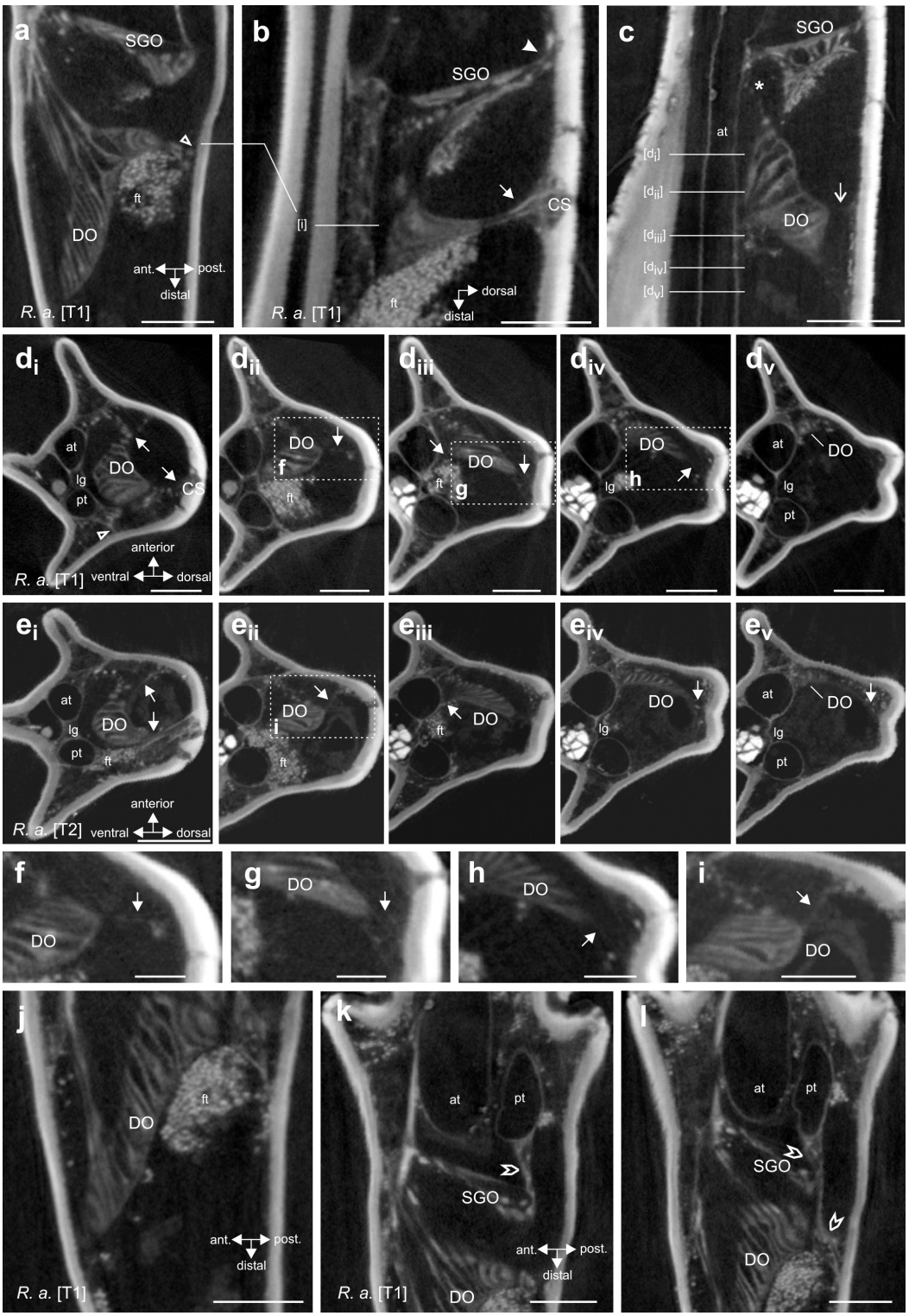

FIGURE 4 | Morphology and attachment structures of the distal organ (DO) in *R. artemis*. **(a)** The DO is placed at the anterior cuticle of the tibia, with an extension to the posterior side (empty arrowhead) (foreleg, horizontal longitudinal section). **(b)** At the dorsal tibia, a strand of tissue extends from the DO to the cuticle (solid arrow) (foreleg, vertical longitudinal section). The subgenual organ (SGO) is located more proximally in the tibia with a separate connection at the dorsal cuticle (solid arrowhead). **(c)** The main part of the DO extends to the dorsal cuticle by a fine strand (open arrow), with diffuse tissue in the space between the DO and the dorsal cuticle (foreleg, vertical longitudinal section). A strand from the DOl extends to the ventral SGO (asterisk). **(d)** Transversal sections of the foreleg show the

(Continued)

FIGURE 4 | Continued
DO suspended by the two larger tissue strands [**(d_i)**, solid arrows] and thin tissue strands [**(d_{ii}–d_{iv})**, solid arrows] from the cuticle. Section levels are indicated in **(c)**. The tissue strand to the posterior side originates from the posterior trachea (empty arrowhead). Note the distance of the DO to the anterior trachea in proximal sections. **(e_{i-v})** Transversal sections of the midleg DO at corresponding levels with identical strands. **(f–i)** Details of the boxed areas in **(d_{ii-iv},e_{ii})** with thin strands and tissue indicated by arrows. **(j)** The distal end of the DO is close to the anterior cuticle (foreleg, horizontal longitudinal section). **(k,l)** Connective tissue (pointed empty arrowheads) between the posterior trachea (pt) and the subgenual organ (SGO), located proximally to the DO. The section shown in **(k)** is located 27 μm dorsally to the section in panel **(l)**. Note that in panel **(l)**, the connective strand extends distally to the level of the DO but runs to the posterior cuticle and not the DO placed more anteriorly (empty arrowhead). Scales: **(a,b,d,e,j–l)** = 200 μm, **(c)** = 300 μm, **(f–i)** = 100 μm. at, anterior trachea; CS, campaniform sensilla; DO, distal organ; ft, fat tissue; lg, ligament between tracheae; pt, posterior trachea; SGO, subgenual organ.

R. artemis (**Figures 3**, **4**, **6**), *C. morosus* and *S. sipylus* (**Figures 5**, **6**). The anatomy was highly similar for *R. artemis* (**Figure 4**), *C. morosus* (**Figures 5d–f,h**) and *S. sipylus* (**Figures 5a–c,g**).

The subgenual organ complex was located dorsally to the two tibial tracheae (**Figures 3a–c**). The SGO spanned the proximal tibia, and was suspended in the hemolymph channel with attachment to the dorsal and

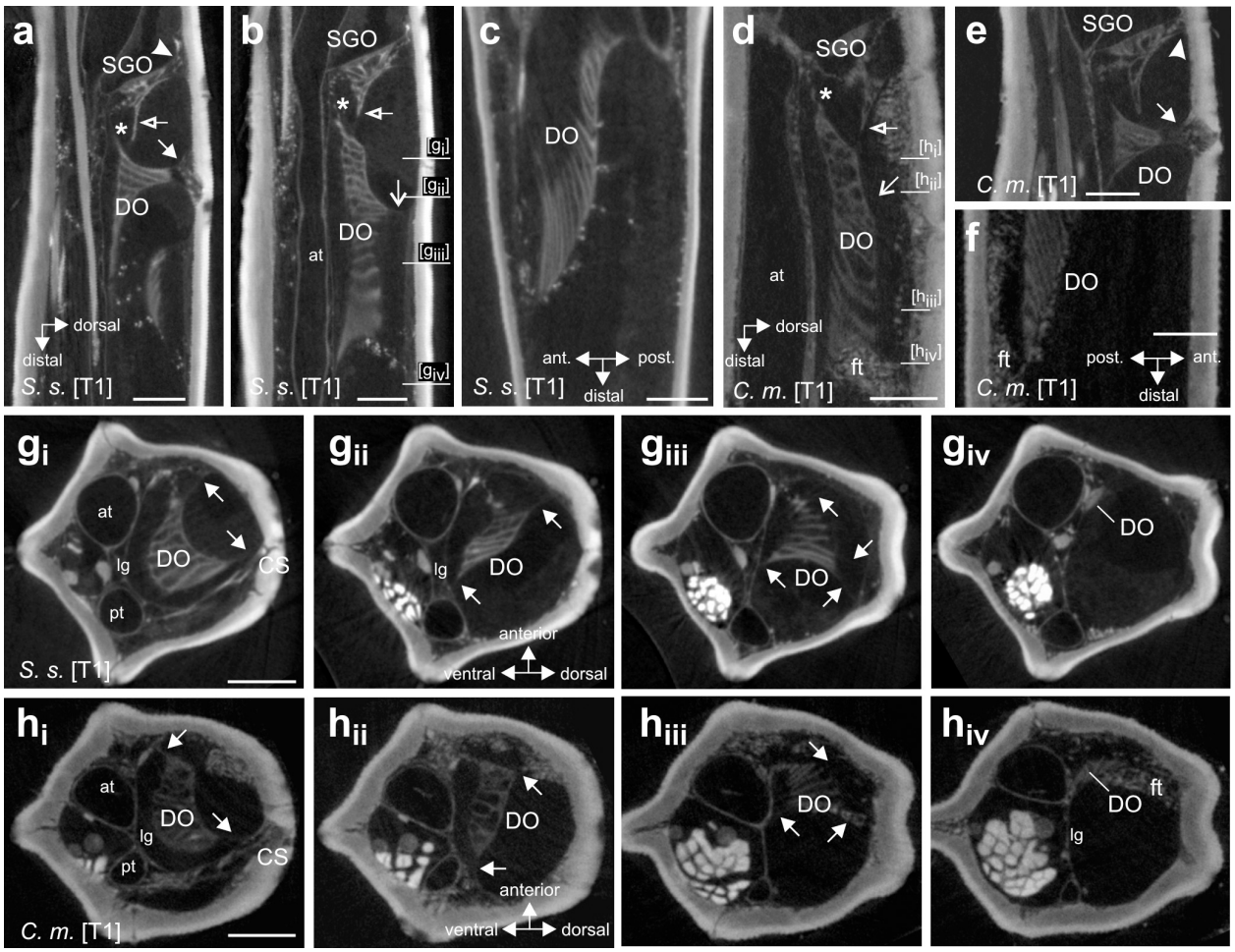

FIGURE 5 | Morphology and attachment structures of the distal organ in *S. sipylus* and *C. morosus* forelegs. In **(a)** *S. sipylus* and **(e)** *C. morosus*, the distal organ (DO) shows a proximal strand of connective tissue to the dorsal cuticle (solid arrow) (vertical longitudinal section). From the DO extends a strand to the ventral SGO (asterisk). The subgenual organ (SGO) is linked to the dorsal cuticle by a separate tissue strand (solid arrowhead). A thin membrane (empty arrow) connects the SGO and DO in panel **(b)** *S. sipylus* and **(d)** *C. morosus* (vertical longitudinal sections). More distally, the main body of the DO shows a dorsal connection to the cuticle (open arrow) in panel **(b)** *S. sipylus* and **(d)** *C. morosus*. The distal DO is located near the anterior leg cuticle but is not directly inserted or linked to it in **(c)** *S. sipylus* and **(f)** *C. morosus* (horizontal longitudinal sections). Transversal sections of the foreleg tibia in **(g)** *S. sipylus* and **(h)** *C. morosus* show the DO and strands to the cuticle (arrows); the levels of the sections are indicated in **(b,d)**. The most distal part of the DO is thinner in diameter and is located just dorsally to the anterior trachea. Scales: **(a,b)** = 200 μm, **(c,f)** = 100 μm, and **(d,e,g,h)** = 150 μm. at, anterior trachea; *C. m., Carausius morosus*; CS, campaniform sensilla; DO, distal organ; ft, fat tissue; lg, ligament; pt, posterior trachea; SGO, subgenual organ; *S. s.*, *Sipyloidea sipylus*.

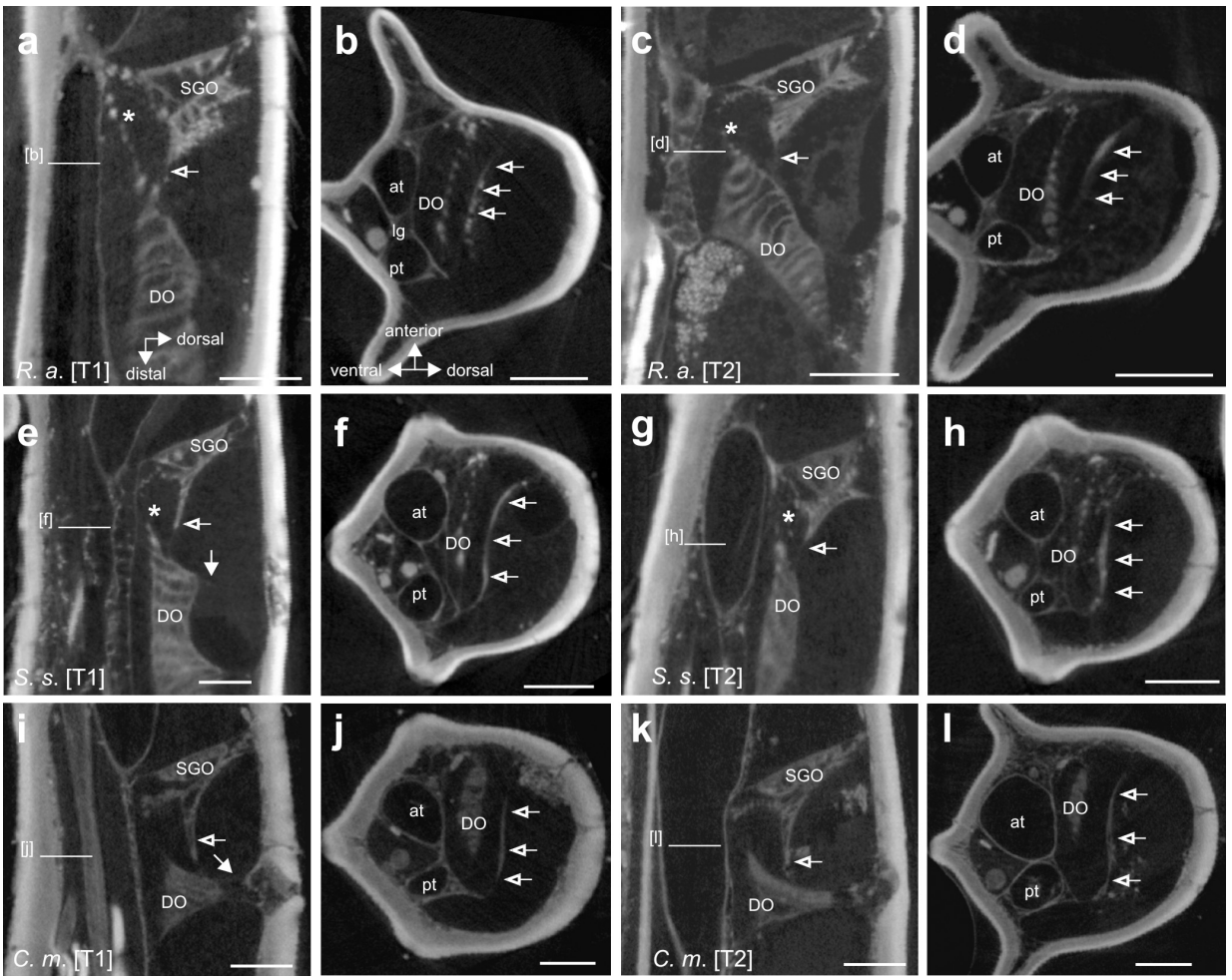

FIGURE 6 | The membranous connection between the subgenual organ (SGO) and distal organ (DO) in **(a–d)** *R. artemis*, **(e–h)** *S. sipylus*, and **(i–l)** *C. morosus*, indicated by empty arrows. The tissue connection is present in forelegs **(a,e,i)** and midlegs **(c,g,k)** (vertical longitudinal sections). The tissue spans the tibia in transversal sections between the anterior and posterior cuticle in **(b,f,j)** forelegs and **(d,h,l)** midlegs (empty arrows). Ventral to this, a strand from the DO extends to the SGO. Scales: **(a–d)** = 200 μm, **(e)** = 100 μm, and **(f,h-l)** = 150 μm. at, anterior trachea; DO, distal organ; pt, posterior trachea; SGO, subgenual organ; [T1], foreleg; [T2], midleg.

anterior cuticle (**Figures 3**, **4a,b**, **5b,e**). The SGO tissue attached most strongly at the posterior side of the tibia (**Figures 3a–c**). The dendrites of the SGO sensilla with their distal orientation inserted into the rounded SGO tissue located almost perpendicularly to the leg's main axis (**Figure 3**).

The DO was located at the anterior side of the tibia (**Figures 2**, **4a,j**, **5c**). The dendrites, cap and accessory cells of the DO formed a triangular, continuous structure at the anterior side of the tibia that became narrower at the distal end (**Figures 3**, **4a,j**, **5c**). The accessory cells of the DO are long and give a lamellar structure (**Figures 3d–f**). The DO extended posteriorly in the proximal part (*R. artemis*; **Figures 3d**, **4a,d$_i$,e$_i$**; *S. sipylus*: **Figures 3e,f**; **Supplementary Videos 1,2**) but the sensilla did not directly contact the leg's cuticle (**Figures 4a,d$_i$** and **Supplementary Figure 2**). The proximal DO was attached to the dorsal and anterior cuticle by separate tissue strands (**Figures 3**, **4b,d$_i$,e$_i$**, **5a,e**). This organization was identical in all leg pairs (**Figures 3a–c**, **4d,e** and **Supplementary Figures 3A,B, 5, 6**). The attachment to the dorsal cuticle also supplies the dorsal campaniform sensilla (dorsal group 6B) located at the level of the SGO (**Figures 4b,d$_i$**). In cleared tracing preparations of the tibia, the stained axons from the campaniform sensilla can be seen (not shown). These structures from the DO and campaniform sensilla can also be more separated (**Figures 5g$_i$,h$_i$**). During preparations, no attachment of the DO to a tendon or a receptor apodeme was noted.

In the central part, the DO extended toward the dorsal cuticle (**Figures 4c,d$_{ii,iii}$**, **5b,d,g$_{ii,iii}$,h$_{ii,iii}$**). At the extension of the DO in dorsal direction toward the cuticle, the organ is suspended by fine strands (**Figures 4c**, **5b**, **6a**). Similar thin strands occurred more distally between the DO and the cuticle (**Figures 4d–f,h,i**, **5b,g,h**). The space between the DO and the dorsal leg cuticle was filled with a diffuse tissue (**Figures 4c,d$_{ii,iii}$,g**, **5d,g$_{ii,iii}$,h$_{ii,iii}$**).

While this could represent hemolymph, especially at the distal DO it appeared more consistent than hemolymph seen in the remaining hemolymph channel (**Figures 4d**$_{iv,v}$, **5g**$_{iv}$,**h**$_{iv}$). At the distal end, the DO was located in the hemolymph channel close to the anterior cuticle (**Figures 4j**, **5c,f** and **Supplementary Figures 3–7**). The DO was placed dorsally to the anterior trachea (**Figures 4d**$_{v}$,**e**$_{v}$, **5g**$_{iv}$,**h**$_{iv}$). The elongated accessory cells run in parallel (**Figures 4j**, **5c** and **Supplementary Figures 3C, 4C, 5D**). In some preparations, fat depositions occurred in the legs adjacent to the SGO and the DO, for the latter at the proximal and distal end (**Figures 4a,b,j**, **5d** and **Supplementary Figures 3C, 4C, 5D,E**). At the distal end, fat cells were in some legs placed between the DO and the anterior cuticle (**Figures 5c,f** and **Supplementary Figures 3C, 6C**), while fat was largely absent in other legs (**Figure 4j** and **Supplementary Figure 5D**). At the distal end, no attachment of the DO to a ligament or an apodeme was noted.

The proximal DO extends a fine tissue strand to the ventral SGO (**Figures 4c**, **5a,b,d**, **6a**). Dorsally to this strand runs a fine membrane, which connects the SGO and the DO in the middle of the tibia (**Figure 6**). In transverse sections, this membrane is located dorsally to the DO in anterior-posterior direction in all legs from all species studied here (**Figure 6**). Due to the low contrast of the membrane compared to surrounding tissue in the μCT scans, the membrane is not visible in the 3D-rendering of *R. artemis* (**Figure 3d**) and only represented as a grainy structure in *S. sipylus* (open arrows, **Figures 3e,f**). Both the SGO and the DO were located dorsally of two tracheae, which run in the ventral tibia (**Figures 3**, **4**, **5**). The proximal and middle DO was placed in the hemolymph channel with a clear gap to the underlying anterior trachea (**Figures 4d**$_{i-iii}$,**e**$_{i-iii}$, **5g**$_{i-iii}$,**h**$_{i-iii}$). The middle DO formed a strand to the anterior trachea (**Figures 4d**$_{iii}$,**e**$_{iii}$). At the distal section, the DO was placed at the dorsal side of the anterior trachea (**Figures 4c,d**$_{iv,v}$,**e**$_{iv,v}$, **5d,g**$_{iv}$,**h**$_{iv}$). Notably, the anterior trachea expanded slightly in diameter between the proximal and distal end of the DO (**Figures 4c,d**, **5b,d,g,h**). This expansion did not affect the position of the DO, which was located more dorsally with a gap to the trachea (**Figure 5d**). The DO showed a coupling to the anterior trachea by a strand in the middle part of the DO, while the SGO was linked by a stronger tissue strand to the posterior trachea (**Figures 4k,l**). The leg tracheae did not show obvious tracheal vesicles at the level of the DO.

DISCUSSION

The subgenual organ complex in stick insects consists of the subgenual organ (SGO) and the distal organ (DO), and the latter has a notable linear organization of sensilla (Strauß and Lakes-Harlan, 2013). Here, we show a unique functional morphology for the DO by different types of attachments. This structural complexity raises the question for its physiological function, and for the adaptations driving the evolutionary convergence of linear sets of sensilla which occur in the stick insect DO as well as the tympanal hearing organs in Orthoptera (Strauß and Lakes-Harlan, 2013).

The Subgenual Organ Complex in Stick Insects

The overall neuroanatomy of the subgenual organ complex in *R. artemis* is identical to two other stick insect species investigated previously (*S. sipylus*, *C. morosus*; Strauß and Lakes-Harlan, 2013). Few minor differences occur, such as the relatively long nerve branches of T1 and T2 splitting off in the femur-tibia-joint in *R. artemis*, as in *S. sipylus* the nerve branches split off more distally in the proximal tibia (Strauß, 2020a). The number of SGO sensilla (averages between 36–39) is slightly lower than in the two other species studied previously which have on average 40–44 sensilla, while the number of DO sensilla is similar to *C. morosus* but slightly lower than in *S. sipylus* in the fore- and midleg (Strauß and Lakes-Harlan, 2013). The presence of the elaborate DO in three different groups of stick insects (Clitumninae, Lonchodinae, and Necrosciinae) supports the common physiological relevance of this mechanosensory organ. The three species investigated here belong to Oriophasmata, the Old World Phasmatodea (Simon et al., 2019). The DO likely also occurs in further groups of stick insects, but the distribution including the New World Phasmatodea (Occidophasmata) and the earliest branching Euphasmatodea requires a broader taxonomic study, also relating to differences in ecology, body size, and anatomy. The current data show that the elaborate DO anatomy occurs in species with cryptic body shape and elongated, slender legs, and appears to be independent of wings and flight capacity or secondary chemical defense [see Carlberg (1984, 1985) and Bradler and Buckley (2018)].

Functional Morphology of the Sensory Organs and Possible Sensory Adaptations

For chordotonal organs, the functional morphology and the attachment of the sensilla's dendrites determine the detectable stimuli and their parameters like frequency and amplitude (eg., Shaw, 1994b; Barth, 2019), since both substrate-borne vibrations and airborne sound mechanically interact with parts of the insect body (Cocroft et al., 2000; Römer and Schmidt, 2016; Stritih Peljhan and Strauß, 2018). In several cases, chordotonal organs can respond to both airborne sound and substrate vibrations if they attach to soft membranes or trachea (Shaw, 1994a; Jeram et al., 1995; Stumpner, 1996; Pflüger and Field, 1999). Other chordotonal organs can respond to proprioceptive stimuli, cuticular strain, as well as low-frequency substrate vibrations. In such cases, the functional differentiation of distinct groups of sensilla depends on different mechanical couplings of these groups, which are connected to a receptor apodeme and the inside of the cuticle. Such differentiation is described for the femoral chordotonal organ in locusts, crickets, weta, and stick insects (Field and Pflüger, 1989; Matheson and Field, 1990; Kittmann and Schmitz, 1992; Stein and Sauer, 1999; Nishino, 2000, 2003).

The SGO neuroanatomy in stick insects is similar to that in other orthopteroid insects (Lakes-Harlan and Strauß, 2014). The DO in stick insects shows different types of attachments, but there is no connection with a tendon or a tissue strand toward muscles or joints to support a proprioceptive function,

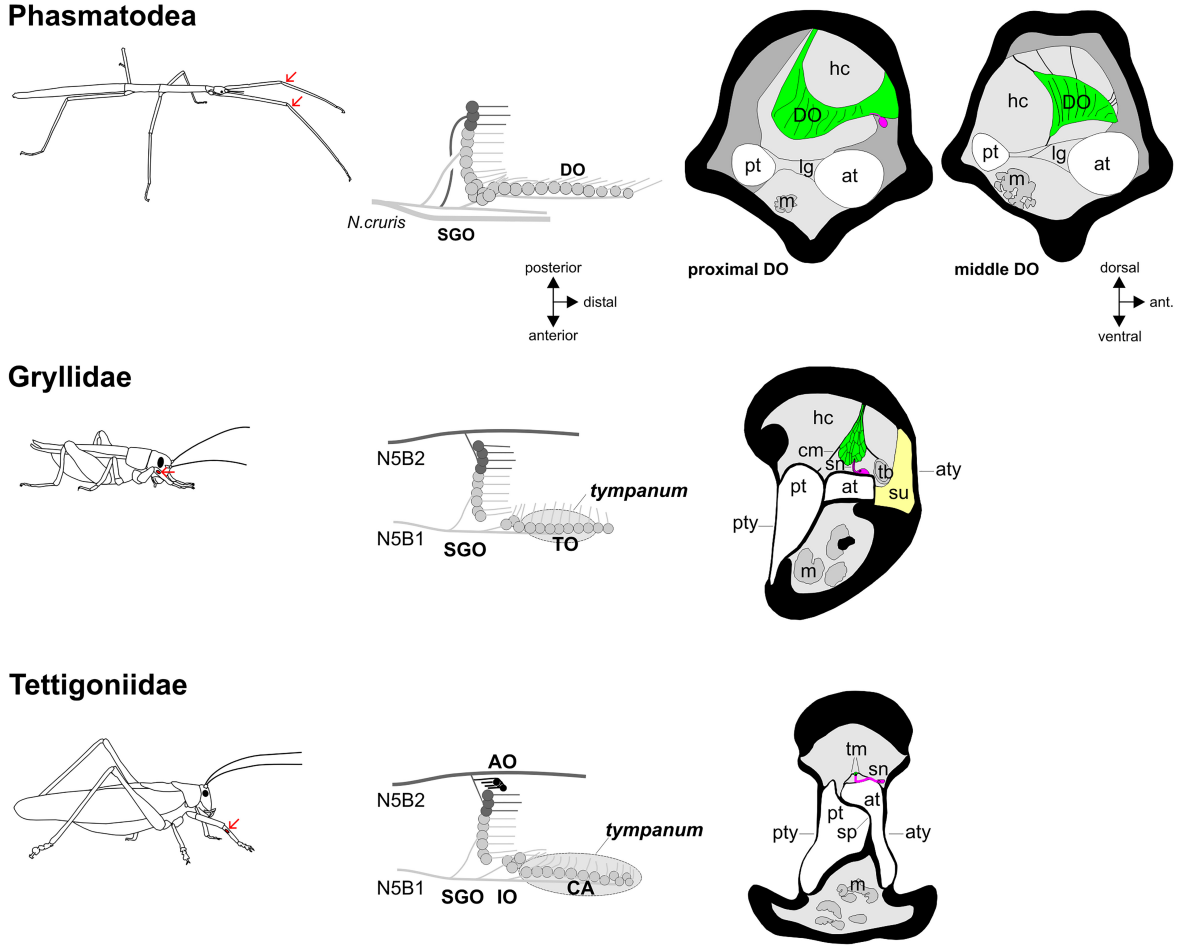

FIGURE 7 | Functional morphology of the stick insect distal organ (DO) and of orthopteran hearing organs in the foreleg. The position of the subgenual organ complex in forelegs is indicated by arrows (the subgenual organ complex is present in all leg pairs). Schematics of the subgenual organ complex show the sensory organs and innervating nerves. In the schematics, only the anterior tympanal membrane is shown. Transversal sections of the distal organ and auditory organs show the leg tracheae, sensory neurons (magenta), accessory cells (green) and cuticle/tympanal membranes, with hemolymph channels (light grey) and tissue in the leg (dark grey). AO, accessory organ; at, anterior trachea; aty, anterior tympanum; CA, *crista acustica*; cm, covering membrane; DO, distal organ; hc, hemolymph channel; IO, intermediate organ; lg, ligament; m, muscle; pt, posterior trachea; pty, posterior tympanum; SGO, subgenual organ; sn, sensory neuron; sp, septum; su, suspensorium; tb, tracheal body; tm, tectorial membrane; TO, tympanal organ. Cross-section of cricket ear reprinted by permission from Springer Nature: Springer, Zeitschrift für Morphologie der Tiere, Das Tympanalorgan von *Gryllus bimaculatus* Degeer (Saltatoria, Gryllidae), K. Michel, copyright 1974, https://link.springer.com/article/10.1007/BF00298805). Cross-section of tettigoniid ear reprinted by permission from *J Comp Neurol*, J. Hummel, M. Kössl, and M. Nowotny, Morphological basis for a tonotopic design of an insect ear, pp. 2443–2455, copyright 2017, with permission from John Wiley and Sons.

as it would be typical for a connective chordotonal organ. The linear organization of sensilla in the DO neuroanatomically resembles the auditory sensilla in tympanal organs in crickets and tettigoniids, where it forms the anatomical basis for frequency analysis (Michel, 1974; Imaizumi and Pollack, 1999; Stumpner and Nowotny, 2014; Montealegre-Z and Robert, 2015). In crickets and tettigoniids, the auditory sensilla are closely associated with the trachea in the leg (**Figure 7**; Michel, 1974; Schumacher, 1975; Oldfield et al., 1986; Lin et al., 1994; Hummel et al., 2017; Schneider et al., 2017; Nishino et al., 2019). In tettigoniids, the hemolymph channel in certain species is adapted as an acoustic vesicle which is filled with fluid and plays an important role for frequency analysis (Montealegre-Z and Robert, 2015). In the stick insect species studied here, the ventral side of the DO at the proximal end floats in the hemolymph, while the middle DO connects to the leg cuticle as well as the anterior trachea by fine strands, and the distal DO locates dorsally of the anterior trachea (**Figures 4d,e,f**, **5g,h**). No auditory adaptations are evident from the morphological structures. In the hearing organs of Orthoptera, sound energy can reach the auditory sensilla *via* tympanal membranes or through enlarged auditory trachea and spiracles. In comparison to auditory systems in Orthoptera, the stick insect DO shows only a weak coupling of sensilla to the tracheal system (**Figure 7**). In the stick insects, the anterior tibial trachea is larger in diameter than the posterior trachea (**Figures 4–6**), but not notably enlarged either at the DO, or at the thoracic spiracle as a potential pathway for sound input (J. Strauß, in preparation). However,

an auditory detection of far-field sound can also occur without the elaboration of tympanal membranes (Shaw, 1994a; van Staaden and Römer, 1998). Based on the functional anatomy, any possible physiological and behavioral roles of such sound stimuli require further experiments. With respect to acoustic behaviors, sound production is known in few stick insects in defense to predators (Bedford, 1978; Carlberg, 1989). Signaling behavior using substrate vibrations is so far not described for species of stick insects, while many insects use the detection of vibrations in predator avoidance (Cocroft and Rodriguez, 2005; Hill, 2008).

The lack of obvious auditory specializations leaves the question for the DO adaptations still open. The strongest connection of the DO to other structures occurs at the proximal end by tissue strands to the dorsal and anterior cuticle in the three species studied (**Figures 4d,e**, **5g,h**). The middle part of the DO is closer to the dorsal cuticle than the proximal part of the DO, and it is suspended by fine tissue strands to the dorsal and anterior cuticle, and also the anterior trachea (**Figures 4d,e**, **5g,h**). The biomechanical properties of these strands or their biochemical components are not known. These would affect the possible movements of the DO, by determining if the organ is merely suspended from the cuticle, or if the DO could be displaced by hemolymph movements in the tibia. These strands are notably very thin, and can be assumed to have some flexibility. The DO in heelwalkers is also attached by stronger strands to the epidermis and posterior trachea (Eberhard et al., 2010). The ventral side of the DO is at the proximal end without contact sites to other tissues, and it is at the distal end placed dorsally of the anterior trachea. We did not note the connection of DO sensilla to a membrane covering the organ which was described previously for *C. morosus* (Friedrich, 1929). Given the elongated structure of the DO, this attachment would allow for more than one possible mechanical coupling. The strand connections to the cuticle could mediate the detection of vibrations transmitted over the leg cuticle. The relatively large diameter of the proximal DO, located in the dorsal hemolymph channel, could imply the detection of hemolymph vibrations caused by substrate-borne vibrations, similar to the tibial SGO [see Kilpinen and Storm (1997), on the bee SGO]. This is also supported by the direction of the DO dendrites in distal direction in the tibia, making them well suited to respond to forces like vibrations transferred in longitudinal direction of the tibia. While the bee SGO is best studied for the activation of this organ, the similarity may be restricted by the differences in the leg's size, diameter, and also the attachment points of the SGO (Kilpinen and Storm, 1997). For bees, the role of mechanical signal detection in intraspecific communication and the leg mechanoreceptors are analyzed at different levels (Kilpinen and Storm, 1997; Rohrseitz and Kilpinen, 1997; Michelsen, 2014), while the behavioral roles of mechanical signal detection in stick insects is not documented in detail.

Other chordotonal organs located next to the SGO respond to substrate-borne vibrations with a tuning shifted to other frequencies than those detected by the SGO in tettigoniids and cave crickets (Ebendt et al., 1994; Kalmring et al., 1994; Čokl et al., 1995). This tuning shift has been shown for the intermediate organ (IO), a chordotonal organ located just distally to the SGO in some Ensifera. In these groups, the IO is located dorsally of the trachea, and is also attached to the inner cuticle which allows for multimodal stimulation (Lin et al., 1994; Jeram et al., 1995). The DO in stick insects could possibly complement the SGO by such a sensory specialization in vibrational frequency tuning. Contact to the leg cuticle is established by the fine tissue strands from the DO and the diffuse tissue in the space between DO and cuticle, though the latter seems to provide only a weak coupling to the cuticle. A mechanical interaction between the SGO and the proximal DO could be possible from the structural connections between both organs (**Figure 6**).

Another functional aspect of the DO in orthopteroid insects is that the organ could detect changes in the hemolymph pressure which occur during leg movements. This sensory function was discussed for the DO in cockroaches, which did not respond to substrate-borne vibrations (Schnorbus, 1971). So far, this remains a tentative function for chordotonal organs. In conclusion, two features may support the detection of substrate vibrations, apart from the attachment to the cuticle: with the relatively large diameter of the DO in the hemolymph channel and with DO dendrites oriented distally in the tibia, it is most likely that the sensilla respond to forces acting in the longitudinal axis of the leg. The position in the hemolymph channel would make it difficult to isolate the DO from hemolymph movements caused by external substrate vibrations, and restrict it to detecting only pressure changes occurring during locomotion. The enlarged size of the DO by the long, lamellar accessory cells (**Figure 3**) also supports the exposition to stimuli from the hemolymph.

Comparative Morphology and Evolutionary Convergence in Tibial Sensory Organs

The DO is homologous to a chordotonal organ identified distally of the SGO in some Orthoptera, the intermediate organ (IO) (Lin et al., 1995; Strauß and Lakes-Harlan, 2013). The IO in Ensifera is studied eg., in tettigoniids, cave crickets, or weta, where it contains 12–20 sensilla (Lin et al., 1994; Jeram et al., 1995; Nishino and Field, 2003; Strauß and Stritih, 2017). In tettigoniids and cave crickets, the IO responds to substrate vibration but also airborne sound (Kalmring et al., 1994; Čokl et al., 1995; Stölting and Stumpner, 1998). In orthopteroid insects, the IO and DO can occur in diverse neuroanatomical organizations, which are as a compact group of sensilla (cockroach: Schnorbus, 1971), to extending dorsally in the leg (raspy crickets: Strauß and Lakes-Harlan, 2008; weta: Strauß et al., 2017), and linear sensilla in part of the organ (cave cricket: Jeram et al., 1995) or the complete organ (stick insects: Strauß, 2020b). The organization of linear sensilla in Ensifera and stick insects, independent of tympanal membranes, is an evolutionary convergence in these two taxa (Strauß and Lakes-Harlan, 2013). The specific organization of the DO in the common ancestor of Orthoptera and Phasmatodea is not known, as species from more basal taxa of Polyneoptera (Wipfler et al., 2019) from Plecoptera (Wittig, 1955) and Dermaptera (Friedrich, 1929) lack a DO and have only the SGO. However, all other orthopteroid taxa studied so far have more compact sensilla in the DO/IO, which can differ in their attachment structures (Schnorbus, 1971; Lin et al., 1995;

Eberhard et al., 2010). The DO in locusts (Lin et al., 1995) and especially the IO in cave crickets (Jeram et al., 1995) as early branching Ensifera (Song et al., 2020), are simpler in their neuroanatomy. This indicates that the elaboration in Ensifera was absent in the ground pattern of this group (Strauß and Lakes-Harlan, 2009).

The DO/IO position in the hemolymph channel distal to the SGO could result in the extension in distal direction, when the number of sensilla is increased (Jeram et al., 1995; Strauß and Lakes-Harlan, 2013). In stick insects, the overall organization of tibial trachea and muscle/hemolymph channels and their proportions are similar to those seen in cave crickets, which also have long and very slender legs (Jeram et al., 1995; Stritih-Peljhan et al., 2019). The structure of the sensory organs seems not to be primarily affected by the leg elongation in stick insects, as their SGO morphology is similar to other orthopteroid insects (Lakes-Harlan and Strauß, 2014), and the distally located IO in cave crickets takes even more space in the anterior hemolymph channel (Jeram et al., 1995). Therefore, the stick insect DO would not be forced to extend only in the distal direction by forming a linear organ.

A linear organization is also found in atympanate sensory organs in some lineages of Orthoptera, suggested to contribute to the detection of vibrational signals from conspecifics (Strauß and Lakes-Harlan, 2008; Strauß et al., 2017). The organs distally of the SGO in Orthoptera are usually placed closely to the leg trachea (Jeram et al., 1995; Lin et al., 1995). This likely makes them sensitive to both vibrational stimuli and airborne sound (Kalmring et al., 1994; Čokl et al., 1995). In crickets, the auditory sensilla and accessory cells connect to the dorsal cuticle by a covering membrane (Michel, 1974; Oldfield et al., 1986; Nishino et al., 2019). In tettigoniids, the cap cells over the auditory sensilla's dendrites are covered by the tectorial membrane and held by supporting bands (Schumacher, 1975; Lakes and Schikorski, 1990; Lin et al., 1994). The stick insects DO is broadest in the proximal part, where the organ attaches to the inner cuticle in the hemolymph channel, with a notable distance to the anterior trachea. Only the distal part, which is smaller in diameter, is close to the anterior trachea. Thus, the DO lacks the clear morphological adaptations seen in the elaborate tympanal hearing organs (Ball et al., 1989; Lakes and Schikorski, 1990; Lin et al., 1994; Hummel et al., 2017; Nishino et al., 2019). The stick insect DO therefore shows a unique organization among orthopteroid insects with the linearly arranged sensilla with distally oriented dendrites and long accessory cells. The neuronal convergence between the tympanal organs and the DO in this insect group is striking, while the functional anatomy of the stick insect DO indicates a sensory specialization other than detecting air-borne sound, and it likely detects vibrational stimuli.

DATA AVAILABILITY STATEMENT

The cropped but otherwise unchanged μCT-scans presented in this study can be accessed at Zenodo: doi.org/10.5281/zenodo.3856676.

AUTHOR CONTRIBUTIONS

JS, LM, and PTR: carried out the experiments and edited the manuscript. JS: carried out the preparations, assembled figures, and drafted the manuscript. JS and PTR: analyzed the data. PTR: provided reconstructions and videos. All authors read the manuscript and agreed with the publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.632493/full#supplementary-material

Supplementary Figure 1 | The tibia in the thoracic legs of *R. artemis*.

Supplementary Figure 2 | Posterior structures of the proximal distal organ in *Ramulus artemis* (foreleg).

Supplementary Figure 3 | The distal structure of the distal organ in *Ramulus artemis* (midleg).

Supplementary Figure 4 | The distal structure of the distal organ in *Sipyloidea sipylus* (foreleg).

Supplementary Figure 5 | The distal structure of the distal organ in *Sipyloidea sipylus* (midleg).

Supplementary Figure 6 | Attachment of the distal organ in *S. sipylus* (midleg).

Supplementary Figure 7 | The distal organ in *Carausius morosus* (midleg).

Supplementary Table 1 | *Ramulus artemis* SGOC sensilla.

Supplementary Video 1 | 3D-Reconstruction of the subgenual organ complex in *Ramulus artemis*.

Supplementary Video 2 | 3D-Reconstruction of the subgenual organ complex in *Sipyloidea sipylus*.

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REVIEW
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Survival Sounds in Insects: Diversity, Function, and Evolution

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Insect defense sounds have been reported for centuries. Yet, aside from the well-studied anti-bat sounds of tiger moths, little is understood about the occurrence, function, and evolution of these sounds. We define a defense sound as an acoustic signal (air- or solid-borne vibration) produced in response to attack or threat of attack by a predator or parasitoid and that promotes survival. Defense sounds have been described in 12 insect orders, across different developmental stages, and between sexes. The mechanisms of defensive sound production include stridulation, percussion, tymbalation, tremulation, and forced air. Signal characteristics vary between species, and we discuss how morphology, the intended receiver, and specific functions of the sounds could explain this variation. Sounds can be directed at predators or non-predators, and proposed functions include startle, aposematism, jamming, and alarm, although experimental evidence for these hypotheses remains scant for many insects. The evolutionary origins of defense sounds in insects have not been rigorously investigated using phylogenetic methodology, but in most cases it is hypothesized that they evolved from incidental sounds associated with non-signaling behaviors such as flight or ventilatory movements. Compared to our understanding of visual defenses in insects, sonic defenses are poorly understood. We recommend that future investigations focus on testing hypotheses explaining the functions and evolution of these survival sounds using predator-prey experiments and comparative phylogenetics.

Keywords: defense, acoustic, mechanism, signal variation, communication, predator, prey, disturbance

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INTRODUCTION

When threatened or attacked by a predator, many insects produce sounds. Familiar examples are hisses of the Death's-head hawkmoth, *Acherontia atropos* (Sphingidae) (Brehm et al., 2015), and the Madagascar hissing cockroach, *Gromphadorhina portentosa* (Blaberidae) (Nelson, 1979), as well as the tymbal clicks of tiger moths (Erebidae: Arctiinae) (e.g., Corcoran et al., 2010). However, insect defense sounds are widespread and diverse, including those produced by small stridulating bark beetles (Lewis and Cane, 1990) and large whistling caterpillars (Bura et al., 2011). Despite centuries of research reporting that insects use sounds for defense (e.g., Lesser, 1738; Sanborn, 1869; Darwin, 1889), we know very little about the roles these sounds play in insect survival (Conner, 2014). How do sounds stop an attack by a predator? Do different sound characteristics serve different functions? Why do only some insects produce defense sounds? To address these questions, we need a review of insect defensive sound production that highlights research to date and identifies what remains to be investigated. Previous reviews of insect defense sounds focused on potential functions (e.g., Conner, 2014), a single mechanism (e.g., stridulation, Masters, 1980), or a specific taxon (e.g., termite alarm

signals, Hager et al., 2019; tiger moth anti-bat clicks, Waters, 2003; Conner and Corcoran, 2012). Our review takes a broader approach by exploring the diversity, function, and evolution of defense sounds across the Class Insecta. Specifically, we document which taxa, sexes, and developmental stages are reported to produce defense sounds, the mechanisms by which they produce them, and the diversity of their signal characteristics. We review hypotheses explaining the functions and evolutionary origins of defensive sound production, as well as the adaptive significance of signal variation. While we focus specifically on defense sounds, we recognize that some insects use other forms of acoustic anti-predator strategies such as adaptive silence and acoustic crypsis, and we refer readers to Conner (2014) for further discussion of these strategies.

What Is a Defense Sound?

What do we mean by "defense sound"? Variously called "distress signals" (Ossiannilsson, 1949), "protest sounds" (Alexander, 1956), "disturbance sounds" (Masters, 1979), and "defense sounds" (Bura et al., 2016), the general consensus is that these acoustic signals are produced in response to disturbance and used to repel predators or warn conspecifics (cf. Alexander, 1967). In social insects such as termites and bees, the sounds protect not only the individual, but the colony. We exclude from this review sounds associated with territorial and agonistic behaviors (e.g., those occurring in male-male interactions) as such sounds are generally aimed at competitors rather than predators. We also exclude cases of "incidental" sounds, meaning sounds associated with another defense like the chemical ejections of bombardier beetles (Eisner et al., 2001), unless the sounds are noted by the authors as being part of the defensive display. We recommend the following definition: "A defense sound is an acoustic signal produced in response to attack or threat of attack by a predator (or parasitoid) and that promotes survival." Here we refer to an acoustic signal in the broadest sense, meaning a vibration that is airborne, waterborne (both commonly referred to as sounds), or solid-borne (commonly referred to as vibrations). For simplicity, hereafter we refer to all of these acoustic signals as "sounds" [see Hill (2014) and Yack (2016) for further discussion of acoustic signal nomenclature].

WHICH INSECTS PRODUCE DEFENSE SOUNDS?

Understanding which individuals possess a trait and how it varies is important for testing hypotheses on the function and evolution of that trait. Until now, a review of the distribution of insect defense sounds across taxa, sexes, and developmental stages had not been conducted. Using our definition of defense sounds, we reviewed the literature to identify which insect orders and families include species reported or proposed to produce sonic defenses. Among these examples, we also noted the sex and life stages of the species investigated. Our goal was not to identify every reported occurrence, but to obtain a general overview of the variation between taxa, sexes, and developmental stages to identify gaps in the literature and trends that will inform future studies.

Defensive sound production has been reported in at least 12 of the 28 currently recognized insect orders (**Table 1** and **Figure 1**). However, the number of reports varies between orders, ranging from multiple species within 15 families of Lepidoptera to a single species in Odonata. Coleoptera and Hemiptera have the next most abundant number of reports at 12 and 10 families, respectively. Defense sounds are also commonly described in Blattodea (8 families) and Orthoptera (7 families). The remaining orders have reports from 6 or fewer families, though some of these families contain numerous well-known sound producers [e.g., buzzing in Apidae (Hymenoptera), Kirchner and Röschard, 1999]. Importantly, we cannot conclude based on a single sound-producing species that all members of a given family, or even a given genus, produce defense sounds. For example, not all Phasmatidae (Phasmatodea) stridulate when disturbed (Bedford, 1978), and in Sphingidae (Lepidoptera), both sound- and non-sound-producing larvae occur in the genus *Manduca* (Bura et al., 2016). While defensive sound production appears to be widespread in insects, it is certainly not an even distribution. We discuss why defense sounds might not occur in all species in the next subsection.

Within species that produce defense sounds, the sex that produces these sounds can vary. It is important to note, however, that many studies do not indicate the sex of the specimens tested. Indeed, of the 404 species identified as sound producers in this review, sex was identified in fewer than half the species (194 of 404). In the 152 species for which both sexes were tested, both males and females produce sounds in the majority of cases (126 of 152). Examples include hissing cockroaches (e.g., Nelson and Fraser, 1980) and king crickets (e.g., Field, 1993). Sexual dimorphism of sound features was noted in 21 of these 126 species, and the differences are attributed to morphological traits such as body size (e.g., Coelho, 1998; Hill, 2007), or because the male and female use different mechanisms of sound production (e.g., Kowalski et al., 2014). In the remaining 26 (of 152) species where both sexes were tested, only one sex produces defense sounds. In 20 of these, it's the male sex, and examples include cicadas (e.g., Smith and Langley, 1978) and katydids (e.g., Kowalski and Lakes-Harlan, 2011). In contrast, there are only 6 species where defense sounds are produced solely by the female, including *Heteropteryx dilatata* stick insects (Heteropterygidae) (Carlberg, 1989) and *Ips pini* beetles (Curculionidae) (Dobai et al., 2018). Based on the species investigated to date, when defensive sound production occurs in a species, it is typically produced by both sexes. When only one sex produces sounds, it is usually the male. This male bias could be attributable to males experiencing higher predation risk as they are more mobile when searching for a mate (e.g., Burk, 1982). We recommend that future studies aim to test both sexes wherever possible.

The majority of reports of defensive sound production come from adult insects, but many juvenile life stages, including larvae, nymphs, and pupae, also produce these sounds (**Table 1** and **Figure 1**). We did not, however, find any accounts of insect eggs producing sounds in response to a disturbance. Of the

TABLE 1 | Distribution of insect defense sounds across orders, families, and developmental stages including information on mechanisms and proposed functions. A, adult; N, nymph; L, larva; P, pupa. "**Figure 3** #" refers to the corresponding label on **Figure 3**. Asterisks denote cases where the sound was tested with a predator/receiver in the selected reference.

ORDER Family	Life stage	Sound type	Mechanism	Figure 3 #	Proposed function	Selected references
ODONATA						
Epiophlebiidae	N	Stridulation	Abdomino-femoral	1		Haskell, 1961
ORTHOPTERA						
Acrididae	A	Stridulation	Mandibles	2	Not specified*	Blondheim and Frankenberg, 1983*
Anostostomatidae	A, N	Stridulation	Abdomino-femoral	4		Field, 1993
	A	Stridulation	Mandibular tusks	2		Field, 1993
	A	Stridulation	Tergo-tergal	5		Field, 1993
Gryllacrididae	A	Stridulation	Abdomino-femoral	4	Startle	Field and Bailey, 1997
Gryllidae	A	Stridulation	Tegmino-tegminal	6	Aposematism	Desutter-Grandcolas, 1998
Prophalangopsidae	A	Stridulation	Abdomino-thoracic	7		Gwynne, 2001
Stenopelmatidae	A	Stridulation	Abdomino-femoral	4		Weissman, 2001
Tettigoniidae	A, N	Forced air	Mouthparts	3		Bailey and Sandow, 1983
	A	Stridulation	Tegmino-tegminal	6	Startle	Heller, 1995; Kowalski and Lakes-Harlan, 2011; Kowalski et al., 2014
	A	Stridulation	Legs		Startle	Belwood, 1990
	A	Stridulation	Femoro-alary	8		Rentz, 1993
	A	Stridulation	Abdomino-alary	9, 10	Startle*	Sandow and Bailey, 1978*; Bailey and Sandow, 1983; Heller, 1995
	A	Stridulation	Labrum-mandible	2		Lloyd and Gurney, 1975
PHASMATODEA						
Diapheromeridae	A	Stridulation	Wings		Startle Aposematism	Bedford, 1978 Edmunds, 1974
Heteropterygidae	A	Stridulation	Wings		Mimicry	Carlberg, 1989
Phasmatidae	A	Stridulation	Wings		Startle	Bedford, 1978
Phylliidae	A, N	Stridulation	Antenno-antennal	11		Henry, 1922
MANTODEA						
Empusidae	A	Stridulation	Tegmino-femoral	12	Startle*	Carpenter, 1921*
Hymenopodidae	A	Stridulation	Wings		Startle	Shelford, 1903
	A	Stridulation	Abdomino-alary	13	Startle	Edmunds, 1972
Mantidae	A	Stridulation	Abdomino-alary	13	Startle*	Maldonado, 1970*; Hill, 2007
	A	Stridulation	Pronoto-femoral	14	Startle	Robinson, 1969
BLATTODEA						
Archotermopsidae	A	Percussion	Head		Alarm*	Kirchner et al., 1994*
Blaberidae	A, N	Forced Air	Spiracles	15		Nelson, 1979; Hunsinger et al., 2018
	A	Stridulation	Tergo-tergal	16		Roth and Hartman, 1967
	A	Stridulation	Abdomino-alary			Roth and Hartman, 1967
	A	Stridulation	Pronoto-tegminal	17	Startle* Aposematism	Guthrie, 1966* Roth and Hartman, 1967
Cryptocercidae	A, N	Percussion	Head		Alarm*	Seelinger and Seelinger, 1983*
Ectobiidae	N	Stridulation	Sterno-sternal	18	Aposematism	Schal et al., 1982
Hodotermitidae	A	Percussion	Head		Alarm	Bugnion, 1913
Mastotermitidae	A	Percussion	Abdomen		Alarm*	Delattre et al., 2015*
	A	Tremulation			Alarm*	Delattre et al., 2015*
Rhinotermitidae	A	Percussion	Head		Alarm	Hertel et al., 2011
Termitidae	A	Percussion	Head		Alarm*	Connétable et al., 1999*; Hager and Kirchner, 2013*
	A	Tremulation				Röhrig et al., 1999
HEMIPTERA						
Aphididae	A, N	Stridulation	Abdomino-tibial	19		Broughton and Harris, 1971
Aphrophoridae	A	Tymbalation	Tymbals	20		Ossiannilsson, 1949
Cicadellidae	A	Tymbalation	Tymbals	20		Ossiannilsson, 1949

(Continued)

TABLE 1 | Continued

ORDER Family	Life stage	Sound type	Mechanism	Figure 3 #	Proposed function	Selected references
Cicadidae	A	Tymbalation	Tymbals	20	Startle*	Smith and Langley, 1978*
Cydnidae	A	Stridulation	Abdomino-alary	21		Dupuis, 1953; Gogala, 1970
	A	Tremulation	Abdomen		Not specified*	Nakahira and Kudo, 2008*
Membracidae	A, N				Recruitment*	Cocroft, 1996*; Morales et al., 2008*
Reduviidae	A, N	Stridulation	Prosterno-rostral	22	Startle	Yinon et al., 1972; Schilman et al., 2001
Scutelleridae	A, N	Stridulation	Abdomino-tibial	19		Leston, 1957
Tessaratomidae	A					Leston, 1954
Veliidae		Stridulation				Miyamoto, 1953
HYMENOPTERA						
Apidae	A	Tremulation	Flight muscles		Aposematism* Alarm* Mimicry	Kirchner and Röschard, 1999* Sen Sarma et al., 2002* Seeley et al., 1982
Crabronidae	A	Tremulation	Flight muscles			Coelho, 1998
Formicidae	A	Percussion	Mandibles		Alarm*	Fuchs, 1976*
	A	Percussion	Abdomen		Alarm*	Fuchs, 1976*
	A	Stridulation	Tergo-tergal	23	Alarm Aposematism	Pavan et al., 1997 Ware, 1994
Mutillidae	A	Stridulation	Tergo-tergal	23	Aposematism*	Masters, 1979*; Polidori et al., 2013
Tenthredinidae	L	Stridulation	Abdomen tip	24	Aposematism	Boevé, 2015
Vespidae	A	Percussion	Abdomen tip		Alarm*	Jeanne and Keeping, 1995*
COLEOPTERA						
Carabidae	A	Stridulation	Abdomino-elytral	25, 26	Aposematism*	Wheeler et al., 1970*; Bauer, 1976*
Carabidae: Cicindelinae	A	Stridulation	Alary-elytral	27	Müllerian mimicry	Yager and Spangler, 1997
	A	Stridulation	Elytro-femoral	29		Serrano et al., 2003
Cerambycidae	A	Stridulation	Mesonoto-pronotal	28	Müllerian mimicry Alarm*	Miller, 1971; Schmitt and Traue, 1990 Li et al., 2013*
Chrysomelidae	A	Stridulation	Abdomino-elytral	25		Schmitt and Traue, 1990
	L	Tremulation			Aggregation	Greenfield, 2002
Curculionidae	A	Stridulation	Elytro-femoral	29		Gaiger and Vanin, 2006
	A	Stridulation	Elytro-abdominal	25	Startle	Wilson et al., 1993; Fleming et al., 2013
	A	Stridulation	Vertex-pronotal	30	Startle*	Lewis and Cane, 1990*; Dobai et al., 2018
Dytiscidae	A	Stridulation				Aiken, 1985
	L	Forced Air	Spiracles	31	Startle	Mukerji, 1929
Geotrupidae	A	Stridulation	Elytro-thoracic	32		Palestrini et al., 1988
	A	Stridulation	Coxo-abdominal	33		Palestrini et al., 1988; Carisio et al., 2004
	L	Stridulation	Legs			Pavan et al., 1990
Hydrophilidae	A	Stridulation	Elytro-abdominal	34	Aposematism*	Ryker, 1976; Masters, 1979*; Aiken, 1985
	L					Aiken, 1985
Hygrobiidae	A	Stridulation	Elytro-abdominal	25		Aiken, 1985
Passalidae	A	Stridulation	Abdomino-alary	35	Startle*	Dumortier, 1963b; Buchler et al., 1981*
Scarabaeidae	A	Stridulation	Alary-abdominal	36		Kasper and Hirschberger, 2005
	A	Stridulation	Abdomino-elytral	37		Mini and Prabhu, 1990
	A	Stridulation	Elytro-abdominal	38		Palestrini et al., 1990
	A	Stridulation	Coxo-abdominal	33		Joseph, 1991
	P	Stridulation	Gin traps	39		Dumortier, 1963b
Silphidae	A	Stridulation	Abdomino-elytral	40	Aposematism Müllerian mimicry	Rothschild and Haskell, 1966 Lane and Rothschild, 1965
Tenebrionidae	A	Stridulation	Abdomino-elytral	41	Aposematism	Eisner et al., 1974
TRICHOPTERA						
Hydropsychidae	L	Stridulation	Head-femoral	42	Not specified*	Johnstone, 1964; Jansson and Vuoristo, 1979*

(Continued)

TABLE 1 | Continued

ORDER Family	Life stage	Sound type	Mechanism	Figure 3 #	Proposed function	Selected references
LEPIDOPTERA						
Brahmaeidae	L	Forced air	Alimentary canal	3	Startle	Low, unpublished
Drepanidae	L	Stridulation	Mandibles	2	Not specified*	Guedes et al., 2012*
Erebidae: Arctiinae	A	Tymbalation	Tymbals	44	Aposematism* Batesian mimicry* Müllerian mimicry* Startle* Sonar jamming*	Hristov and Conner, 2005*; Dowdy and Conner, 2016* Barber and Conner, 2007* Barber and Conner, 2007* Bates and Fenton, 1990* Corcoran et al., 2009*, 2011*
Geometridae	A	Tymbalation	Tymbals	45	Batesian mimicry	Corcoran and Hristov, 2014
Heliozelidae	L		Abdomen tip			Low, 2008
Hesperiidae	P	Stridulation	Probosco-abdominal	46		Hinton, 1948
	P	Percussion		n/a		Hinton, 1948
Lycaenidae	P	Stridulation	Tergo-tergal	43		Hinton, 1948; Downey, 1966
	P	Percussion	Anterior end			Hinton, 1948; Downey, 1966
	L	Stridulation			Recruitment*	Pierce et al., 1987*
Nolidae	P	Stridulation	Abdomen tip	47	Startle	Dodd, 1916; Hinton, 1948
Notodontidae	L	Stridulation	Mandibles	2		Federley, 1905
Nymphalidae	A	Tymbalation	Wing buckling	48	Startle*	Møhl and Miller, 1976*; Vallin et al., 2005*; Olofsson et al., 2012*
	A	Stridulation	Wings	49		Dumortier, 1963a
	P	Stridulation	Tergo-tergal	50, 51	Startle	Dolle et al., 2018
Papilionidae	A	Stridulation	Alary-tibial			Jobling, 1936
	P	Stridulation	Sterno-sternal	52	Startle	Dolle et al., 2018
Riodinidae	P	Stridulation	Tergo-tergal	53		Downey, 1966
	L	Stridulation	Vibratory papillae	54	Recruitment*	DeVries, 1991*
Saturniidae	L	Stridulation	Mandibles	2	Aposematism*	Brown et al., 2007*; Bura et al., 2016
	L	Forced air	Spiracles	55	Startle	Bura et al., 2016
	L	Forced air	Alimentary canal	3	Startle	Low, unpublished
Sphingidae	A	Stridulation	Genitalia	56	Sonar jamming*	Kawahara and Barber, 2015*
	A	Forced air	Pharyngeal	3	Startle	Zagorinsky et al., 2012; Brehm et al., 2015
	P	Stridulation	Tergo-tergal	50		Hinton, 1948
	L	Stridulation	Mandibles	2	Aposematism	Bura et al., 2012, 2016
	L	Forced air	Spiracles	55	Startle*	Dookie et al., 2017*; Sugiura and Takanashi, 2018*
	L	Forced air	Alimentary canal	3	Startle	Rosi-Denadai et al., 2018
Yponomeutidae	A	Tymbalation	Wing buckling	57	Müllerian mimicry	O'Reilly et al., 2019
DIPTERA						
Syrphidae	A	Tremulation	Flight muscles		Mimicry*	Brower and Brower, 1965*; Rashed et al., 2009
MECOPTERA						
Meropeidae	A	Stridulation	Jugum-metanotal	58		Sanborne, 1982

References have been selected to include those that best represent the diversity of taxa and developmental stages reported to produce defense sounds. However, it should be noted that this is not a comprehensive list.

69 families identified in our study in which defense sounds occur (**Table 1**), 30 families include reports from juveniles. These examples primarily occur in Lepidoptera (12 families), but also Coleoptera (5), Hemiptera (4), and Blattodea (3). Juvenile sound producers include nymphs in Ectobiidae (Blattodea) (Schal et al., 1982), larvae in Bombycoidea (Lepidoptera) (Bura et al., 2016), and pupae in Lycaenidae and Riodinidae (Lepidoptera) (Hinton, 1948; Downey, 1966). There is no obvious relationship between juvenile and adult defensive sound production within a species. In hemipterans such as Reduviidae (e.g., Yinon et al., 1972) and Membracidae (e.g., Cocroft, 1996), both juveniles and adults can produce defense sounds. However, in Lepidoptera and other holometabolous orders, where a juvenile produces defense sounds, the adult may not. For instance, of the 33 Bombycoidea caterpillar species known to produce defense sounds (Low, unpublished), only two species are reported to produce sounds as adults (e.g., Brehm et al., 2015) and the mechanisms used by the caterpillars and adults differ. Yack (2016) proposed that juvenile insects are under-represented in the literature on acoustic communication, and we argue that this pertains to defensive sound production as well. Juvenile insects are attacked by many predators and parasitoids, and

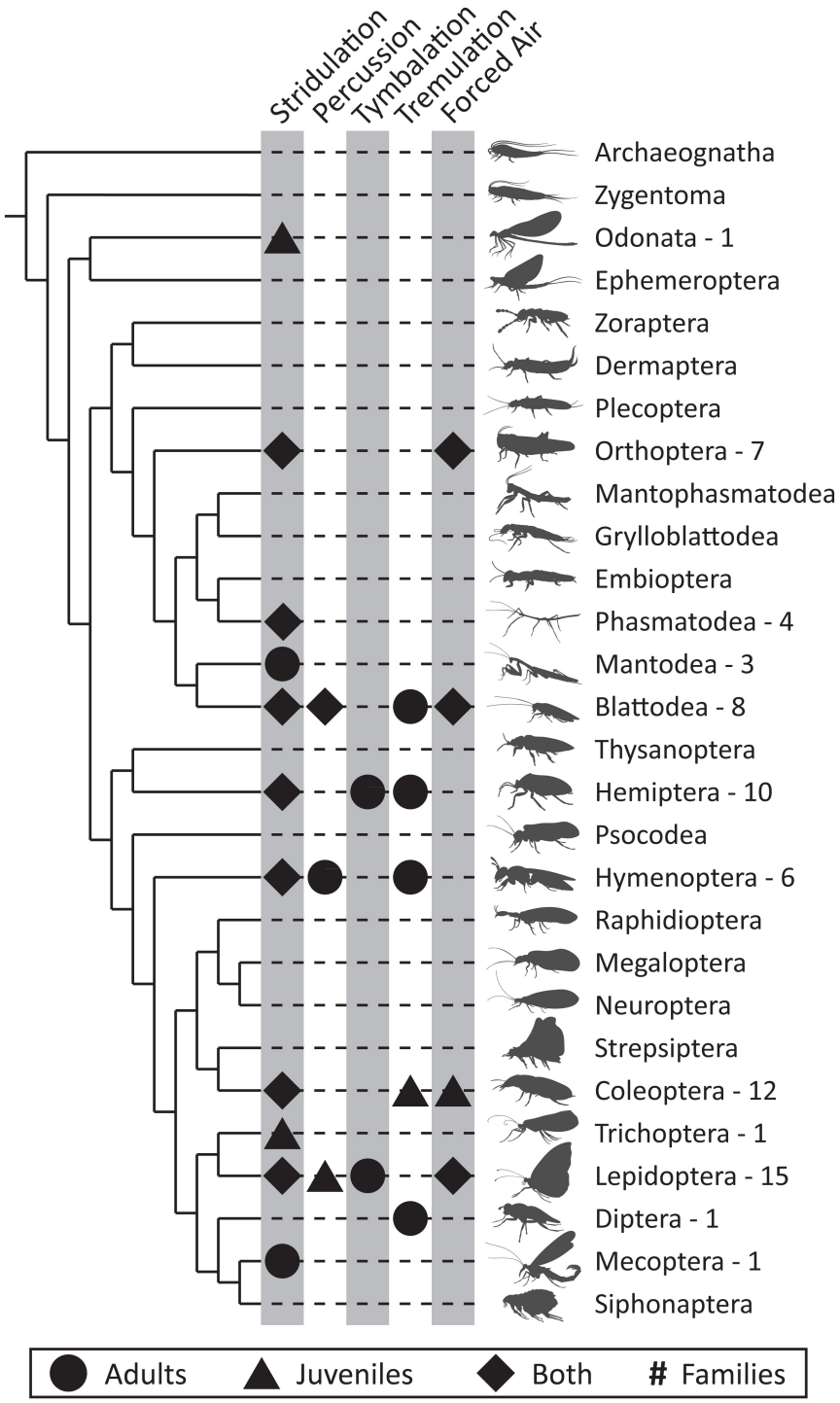

FIGURE 1 | Distribution of defense sounds across orders and life stages of the Class Insecta. The five different mechanisms used to produce defense sounds (stridulation, percussion, tymbalation, tremulation, and forced air) are indicated within their respective columns. Shapes represent the life stage at which defensive sound production occurs, with pupae included under "Juveniles." Numbers represent the number of families within that order where defensive sound production has been noted. Cladogram adapted from Misof et al. (2014).

as such there should be selective pressures to produce defense sounds. Comparative analyses among juvenile sonic defenses would be ideal for testing hypotheses on the functions and evolution of these signals, as in juveniles, defensive sound production is not confounded by sounds used in sexual selection. Future research should focus on identifying the

distribution and diversity of defensive sound production in juvenile insects including eggs.

Why Don't All Insects Produce Defense Sounds?

Why is there such diversity with respect to who does and does not produce defense sounds? There are several, non-mutually exclusive explanations. First, morphological or phenotypic traits may enhance or constrain a species' ability to evolve sound production. For example, smaller insects may not produce airborne defense sounds because such sounds could be too quiet for a predator to hear (Bennet-Clark, 1998), and indeed, we did not find any accounts of defensive sound production in orders with tiny insects (e.g., Psocodea, Thysanoptera, and Siphonaptera, **Figure 1**). Sclerotization, or hardness, of an insect's cuticle may restrict sound production to insects with hard exoskeletons (though this would vary by mechanism), and we did find the most defense sound producers in Coleoptera adults and Lepidoptera pupae (**Table 1**). Additionally, some insects may have co-opted a pre-existing mechanism used in a different signaling context, such as cricket and katydid defensive sound production that is thought to have evolved from sexual signaling (Alexander, 1960) (see section "Evolutionary Origins" below). Second, the assemblage of predators may influence who does or does not produce defense sounds. For instance, tymbal click production varies seasonally in tiger moths of south-eastern Canada whereby species that emerge during peak bat foraging season are more likely to click than species that emerge earlier in the year (Ratcliffe and Nydam, 2008). Third, there are costs to producing defense sounds that may preclude some insects from using them due to trade-offs with other defenses. Corcoran and Woods (2015) found that the metabolic rate of the tiger moth *Bertholdia trigona* (Arctiinae) is 66% higher when producing sonar jamming sounds than when at rest, but is negligible when compared to the 277% higher rate of flight. If defense sounds are costly to produce, perhaps insects that have already invested in other costly defenses (e.g., chemical defenses, Zvereva and Kozlov, 2015; Knapp et al., 2020) will not produce them. Fourth, the insect's habitat may not be conducive to transmitting sounds effectively due to interference with sound propagation from absorption, scattering, or masking by background noise (Römer, 2020). For example, insects may be living in wood or soil where the distance that sounds can travel is heavily influenced by both the source amplitude and the properties of the substrate that can absorb or interfere with the sound wave. Hager and Kirchner (2013) found termite vibrational signals produced by an individual have a range of only 0.4 m due to attenuation properties of the soil that dampen the signal amplitude. Despite this limited range, the signals can be propagated through the rest of the nest by receiving termites that relay the signal onward. Finally, sampling biases may explain differences in reports of defensive sound production between taxa, sexes, or developmental stages. For instance, Lepidoptera, Coleoptera, and Hemiptera are not only speciose orders, but also generally well-studied, thereby increasing the likelihood of researchers encountering species that produce defense sounds. Also, some signals may go undetected without the use of specialized recording equipment, such as laser vibrometers that are required to record solid-borne defense sounds. Similarly, in many instances, defense sounds are intended for close range communication with a predator and do not travel long distances (e.g., Bura et al., 2016). In these cases, unless a researcher is explicitly attacking an insect and recording sounds, the signals may not be noticed. For example, Walters et al. (2001) investigated the antipredator defenses of *Manduca sexta* caterpillars (Sphingidae), but overlooked their defensive clicking [subsequently reported by Bura et al. (2012)] by not recording sounds during their trials. Hypotheses to explain the evolution and variation of traits such as communication signals are being increasingly tested using phylogenetic comparative methods (e.g., acoustic communication in Orthoptera, Song et al., 2020). We believe that using phylogenetic comparative analyses that incorporate morphological traits, predator types, habitats, and other defenses will be particularly insightful for testing and developing hypotheses explaining why some species produce defense sounds while others do not.

DIVERSITY OF MECHANISMS

Insects are highly acoustic animals and have evolved numerous mechanisms of sound production that occur on all different body parts. The hardened exoskeleton of insects has enabled the evolution of this range of mechanisms, although even soft-bodied insects such as caterpillars can produce sounds using forced air or limited sclerotized body parts (e.g., Bura et al., 2016). Sound-producing mechanisms in insects have been reviewed by several authors, including Haskell (1961), Dumortier (1963b), Ewing (1989), and Hill (2008). These authors use a variety of terms to describe different mechanisms, including stridulation, percussion, vibration, click mechanisms, air expulsion, frictional mechanisms, and vibrating membranes. Even though sound production in insects has been reviewed, sounds produced specifically in a defensive context have not. Here, we describe the various mechanisms used for producing defense sounds as well as the locations on the body where these mechanisms occur.

We recognize five categories of sound-producing mechanisms derived from terms used by Ewing (1989) and Hill (2014): stridulation, percussion, tymbalation, tremulation, and forced air (**Figure 2**). Stridulation involves the rubbing of two body parts together or one body part on the substrate to produce sounds. The body parts may be unspecialized, such as mandibles rubbing against one another (e.g., Brown et al., 2007), or specialized, such as a file and scraper (e.g., Dobai et al., 2018, **Figure 2A**). Coleoptera exhibit the greatest diversity of stridulatory mechanisms (**Figure 3**). Percussion, which involves "tapping" or "drumming" the substrate, is common among ants (e.g., Fuchs, 1976) and termites (e.g., Hager and Kirchner, 2013, **Figure 2B**) that use these sounds as alarm signals. Tymbalation occurs via the buckling action of specialized body parts, often called tymbals, and is found in Lepidoptera and Hemiptera. This mechanism is well-known in moths that produce anti-bat

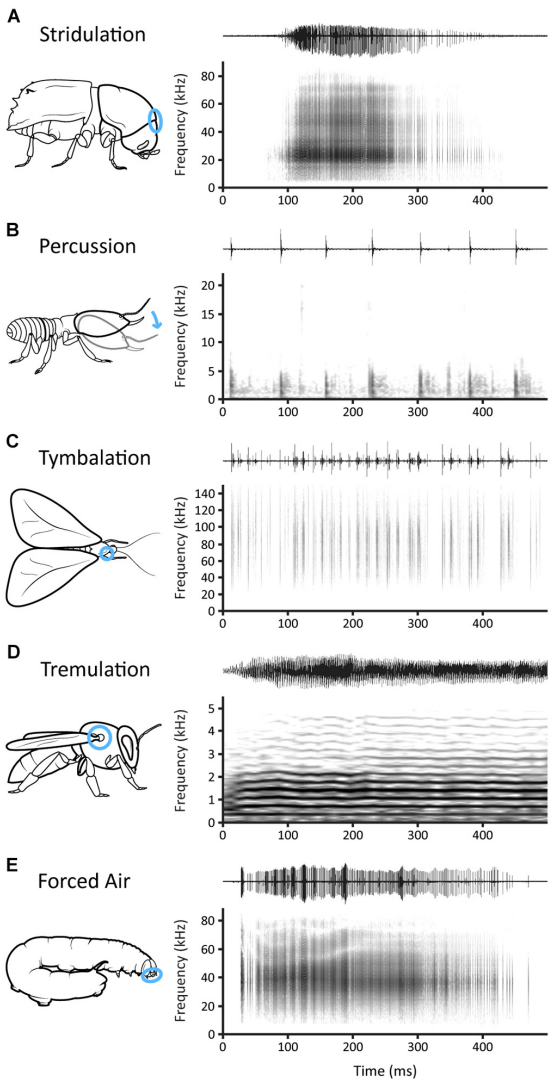

FIGURE 2 | The five types of mechanisms that insects use to produce defense sounds, illustrated by examples from five different insects. Blue circles correspond to the location of the mechanism in the chosen example. Waveforms and corresponding spectrograms show 500 ms of sound from each species. **(A)** Stridulation: a single chirp produced by an *Ips pini* bark beetle. Chirps are produced using a file and scraper mechanism located between the head and pronotum. **(B)** Percussion: a series of drumming signals by a *Macrotermes natalensis* termite. The signals are produced by tapping the substrate with its head. **(C)** Tymbalation: a series of clicks produced by a *Eubaphe unicolor* moth. Clicks are produced via a pair of tymbals located one on each side of the prothorax. **(D)** Tremulation: a group piping signal produced by *Apis florea* bees. Piping sounds are produced through vibrations of the flight muscles. **(E)** Forced Air: a single vocalization produced by an *Amphion floridensis* caterpillar. Vocalizations are made by airflow from the alimentary canal. Sound files were contributed by the Yack Lab (*I. pini* and *A. floridensis*), F. Hager (*M. natalensis*), A. Corcoran (*E. unicolor*), and S. Fuchs (*A. florea*). See text and **Table 1** for further details on these examples.

clicks (e.g., Conner and Corcoran, 2012; Corcoran and Hristov, 2014, **Figure 2C**), and in cicada anti-predator squawks (e.g., Smith and Langley, 1978). Tremulation involves bodily motions that are transmitted as vibrations through the substrate and is usually produced by bobbing movements (Virant-Doberlet and Čokl, 2004). We broaden this definition of tremulation to include the vibration of flight muscles of both Diptera (e.g., Rashed et al., 2009) and Hymenoptera (e.g., Sen Sarma et al., 2002, **Figure 2D**). Forced air mechanisms are produced via airflow from an orifice such as spiracles (e.g., Bura et al., 2011) or the alimentary canal (e.g., Brehm et al., 2015; Rosi-Denadai et al., 2018, **Figure 2E**). All five of these broad categories are used by different insects for defense sounds, but the locations where each is found varies.

Mechanisms used to produce defense sounds have evolved on almost every part of the body, as illustrated in **Figure 3**. We excluded percussion and tremulation from the figure as these usually involve large undifferentiated regions of the body, such as the head of termites and the flight muscles of flies (**Table 1**). Also excluded from the figure are mechanisms such as "rustling of the wings" where no specific location is mentioned. Our results show that mechanisms of defensive sound production occur on numerous different body regions in both adults (**Figure 3A**) and juveniles (**Figures 3B–D**). Of the 58 mechanisms illustrated, 49 are stridulatory. Stridulatory mechanisms predominantly occur either between neighboring segments (e.g., #7, **Figure 3A**; #52, **Figure 3C**), or on a section of the body that can be rubbed by appendages such as the hind legs (e.g., #19, **Figures 3A,B**) or, in adults only, the wings (e.g., #26, **Figure 3A**). Tymbals used to produce defense sounds are only noted in adults. These are located on the prothorax (#45, **Figure 3A**) or metathorax (#44, **Figure 3A**) in Lepidoptera, and on the first abdominal segment in Hemiptera (#20, **Figure 3A**). However, some Nymphalidae butterflies use a portion of their forewing membrane as a tymbal-like structure that buckles to produce clicks (#48, **Figure 3A**) (Møhl and Miller, 1976), and some Yponomeutidae moths use a part of their hind wing in a similar manner (#57, **Figure 3A**) (O'Reilly et al., 2019). Forced air mechanisms are found in both adults and juveniles. Spiracles are used for air expulsion in larvae of Coleoptera (#31, **Figure 3D**) and Lepidoptera (#55, **Figure 3D**), as well as both adults and nymphs in Blattodea (#15, **Figures 3A,B**). Airflow via the alimentary canal is found in both adults and juveniles of Orthoptera (#3, **Figures 3A,B**) and Lepidoptera (#3, **Figures 3A,D**).

Why might a mechanism evolve where it does on a given insect? For stridulation, the mechanisms are found where body parts can be easily rubbed together (Dumortier, 1963b) and the neuromuscular control is likely already available. In hard-bodied insect adults, the site possibilities are vast as demonstrated by the diversity within Coleoptera. In soft-bodied insect larvae, stridulatory mechanisms are restricted to sclerotized areas such as the head and legs (**Figure 3D**). Tymbalation requires a flexible region of the exoskeleton as well as a method of buckling this region. Tymbals located on the main body occur on or near the thorax where locomotory muscles can be used to pull the tymbal in and out (Wessel et al., 2014). On the other hand, tymbal-like mechanisms on wings will require either an opposing structure to press against (e.g., Møhl and Miller, 1976), or rely on movement of the wings themselves to buckle the flexible wing membrane (e.g., O'Reilly et al., 2019). For forced air, which

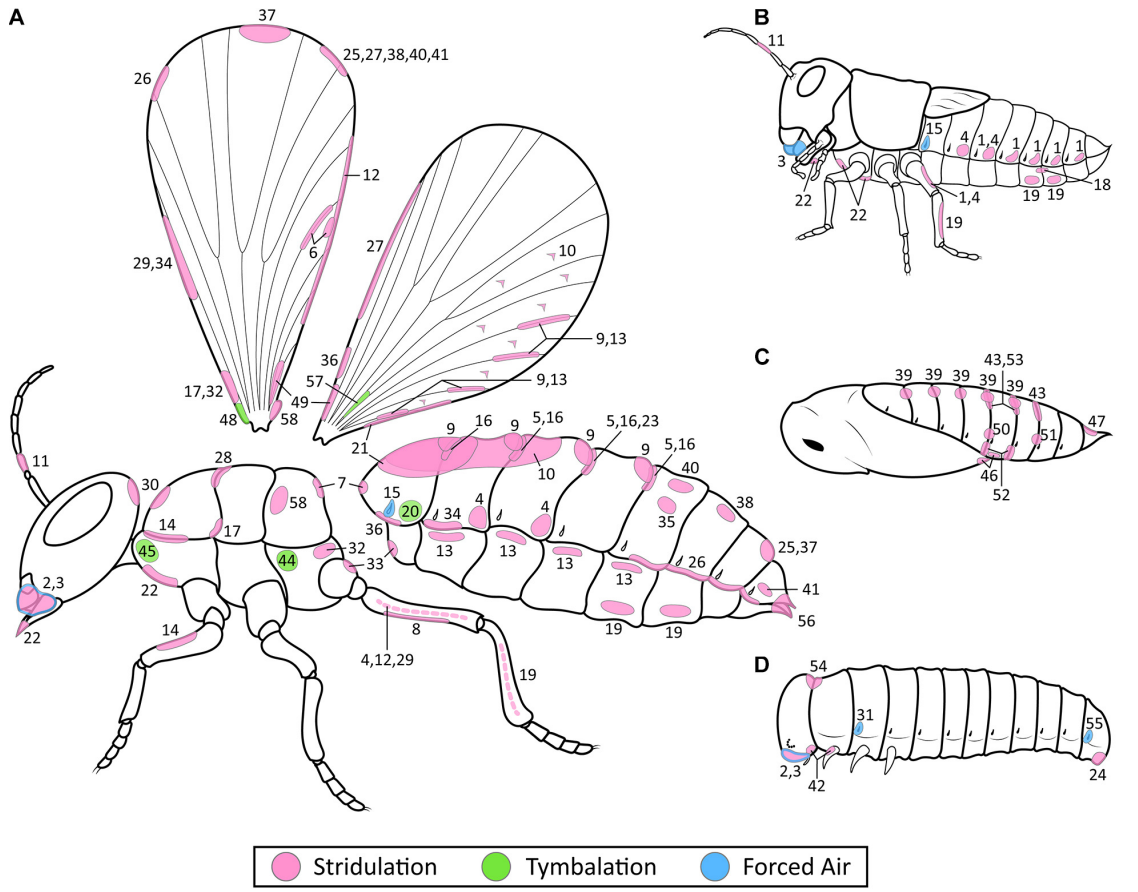

FIGURE 3 | Location of stridulation, tymbalation, and forced air mechanisms used for producing defense sounds in adult and juvenile insects. Percussion and tremulation were excluded as they generally employ undifferentiated body parts. Each number represents a position where a sound-producing mechanism has been recorded in the literature. **(A)** Generic adult insect. **(B)** Generic nymph. **(C)** Generic pupa. **(D)** Generic larva. The numbers correspond to their order of appearance in **Table 1**, which includes information on the orders, families, and literature citations. Mechanisms by insect order are as follows: 1: Odonata. 2–3: Orthoptera and Lepidoptera. 4–10: Orthoptera. 11: Phasmatodea. 12–14: Mantodea. 15–18: Blattodea. 19–22: Hemiptera. 23–24: Hymenoptera. 25–41: Coleoptera. 42: Trichoptera. 43–57: Lepidoptera. 58: Mecoptera. Tymbal mechanisms (numbers 20, 44, 45, 48, and 57) are in green. Forced air mechanisms (numbers 3, 15, 31, and 55) are in blue. Stridulatory mechanisms (all other numbers) are in pink. In stridulatory mechanisms that involve two rubbing parts, both parts are given the same number. A dotted line represents a mechanism located on the inner surface of that appendage. Note that numbers 8 and 35 do not have a corresponding location marked on the wings as the entire wing is used in these mechanisms.

pair of spiracles evolved for sound production likely depends on the underlying control of airflow within each insect's tracheal system. Directional airflow is controlled by the coordinated action of the spiracles and ventilatory movements (Heinrich et al., 2013). A spiracle used for sound production likely evolved from one originally used in air output, as demonstrated in hissing cockroaches (Nelson, 1979). The alimentary canal mechanism of forced air may originate from a sucking-pump as proposed for *Acherontia atropos* moths (Brehm et al., 2015), or from regurgitation as proposed in *Amphion floridensis* caterpillars (Sphingidae) (Rosi-Denadai et al., 2018). Percussion mechanisms use body parts already equipped with the flexibility to drum on a surface, while for tremulation the only described mechanisms used for defense sounds are flight muscles that can be decoupled from the wings to prevent flight. The morphological variation among and between these mechanisms creates a diversity of signal characteristics, and we explore this relationship in the next section.

HOW AND WHY DEFENSE SOUNDS VARY

Insect defense sounds demonstrate wide variation in their physical characteristics. Sound unit durations (unit being an individual sound as perceived by the human ear, Broughton, 1976) range from 250 μs (tiger beetle clicking, Yager and Spangler, 1997) to periods of over a minute (bee hissing and their mimics, Sen Sarma et al., 2002). Dominant frequencies range from 152 Hz (wasp buzzing, Coelho, 1998) to 90 kHz (hawkmoth stridulation, Kawahara and Barber, 2015). The loudest sound level reported is 110 dB SPL at 8–10 cm (butterfly clicks,

Møhl and Miller, 1976) while the quietest reported is 49.8 dB SPL at 2 cm (caterpillar whistling, Sugiura and Takanashi, 2018). This wide variation in signal characteristics disagrees with past proposals that defense sounds tend to share similar characteristics (e.g., Masters, 1980; Schmitt and Traue, 1990; Field, 2001). So why do defense sounds vary? While there are many factors that could explain this variation, we consider the following: morphological influences, the sensory system of the receiver, and the information content of the signal.

An insect's morphological features can impact their signal characteristics. First, the physical structure of the mechanism itself directly influences the traits of the sound produced (Dumortier, 1963c). For example, the number of ridges on a tiger moth's tymbal correlates to the maximum click rate of their sounds (Dowdy and Conner, 2019). Second, sound production and propagation are highly affected by body size. Smaller insects not only have less muscle power available to produce sounds, but also have smaller resonating structures that are more efficient at transmitting high frequency than low frequency airborne sounds (Bennet-Clark, 1998). Third, the form of the resonating structures influences the frequencies that are transmitted. For instance, the unspecialized gaster of ants provides a resonating structure that transmits broadband alarm signals (Masters et al., 1983). Crickets on the other hand use a specialized portion of their forewing called the "harp," and the frequency and bandwidth of the sounds produced depend on how well the harp is coupled to the sound-producing mechanism (Montealegre-Z et al., 2011). However, the latter example has only been studied in a mating context. Fourth, properties of the insect's exoskeleton will influence the sounds produced. For example, a certain degree of sclerotization is necessary for both percussion and stridulation. Perhaps this is why soft-bodied larvae use fewer of these mechanisms, and why the mechanisms are isolated to sclerotized body parts (see **Figure 3D**). Researchers should take into account morphological factors when testing hypotheses to explain why defense sounds differ in their physical characteristics.

Just as the hearing of prey insects is shaped by the sounds of their predators (see Yack et al., 2020), the defense sounds of prey should be shaped by their receivers. A receiver may be a predator (vertebrate or invertebrate), parasitoid, or non-predator (conspecific or heterospecific). If the receiver is very specific, then we might expect these signals to be specialized to that receiver. Tiger moth tymbal clicks are an example of targeted defense sounds. The dominant frequency of these clicks is usually ultrasonic, ranging from 28 to 82 kHz (Corcoran et al., 2010), and matches the ultrasonic hearing of insectivorous bats (Sales and Pye, 1974). Similarly, vibratory signals in termites that function to warn colony members of a threat contain most energy between 1 and 5 kHz, matching the frequencies that initiate conspecific behavioral responses (Hager and Kirchner, 2013). In contrast, a defense sound may target a wide range of predators each with different acoustic sensitivities. In such cases, we might expect the sounds to be of broader bandwidth and simpler temporal structure to reflect a range in sensory processing and cognitive skills (Masters, 1980). For instance, stridulation of *Dasymutilla* sp. wasps (Mutillidae) is broadband, simple, and shown to inhibit attacks by both mice and spiders (Masters, 1979, 1980). Additionally, *Antheraea polyphemus* caterpillars (Saturniidae) are attacked by a range of invertebrate and vertebrate predators, and their stridulatory defense sounds are broadband and simple in temporal structure (Brown et al., 2007).

Comparing characteristics of sounds produced within a species but in different contexts can lend insights into the selective pressures on defense sounds enacted by receivers. **Figure 4** illustrates a few examples of insects that produce sound in both defensive and reproductive contexts. *Dendroctonus valens* and *I. pini* bark beetles (Curculionidae) as well as *Cyphoderris monstrosa* orthopterans (Prophalangopsidae) produce defense sounds that are simpler in temporal structure (e.g., lower pulse rates and shorter durations) than their songs aimed at potential mates (Mason, 1991; Lindeman and Yack, 2015; Dobai et al., 2018; Mason, pers. comm.). One possible explanation for defense sounds being simpler in these species is that they target a broad predator audience, whereas songs directed at potential mates advertise individual characteristics (e.g., skill, health, size, and energy reserves) that are communicated by signal traits. For example, *D. valens* courtship sounds consist of interrupted chirps proposed to advertise male body size and condition to females, and females prefer more complex interrupted chirps than simpler ones (**Figure 4**) (Lindeman and Yack, 2015). In *Manulea japonica* tiger moths (Arctiinae), the opposite is true, as courtship songs produced by males are simpler in temporal structure than defense sounds (Nakano et al., 2013). Females must distinguish between courtship songs and bat sounds as the latter induces a defensive response in females including sound production and/or escape. Perhaps courtship songs evolved to be simpler in temporal structure to prevent the female from responding with an evasive response. At present, there are few studies in insects that compare signal traits of sounds produced in different behavioral contexts (e.g., defense, reproduction, and aggression). In addition to the above-mentioned examples, see also Kowalski and Lakes-Harlan (2010) on ground crickets, and Stölting et al. (2004) on cicadas. We encourage more such studies to better understand receiver selective pressures on defense sound characteristics.

Defense sound characteristics are also proposed to vary according to the message being conveyed (Bura et al., 2016). However, only two authors have tested this hypothesis in insects. Corcoran et al. (2010) found that the signal characteristics of tiger moth anti-bat clicks cluster into two groups: sound units with low duty cycle (proportion of time occupied by sound) are proposed to function as warning sounds, and high duty cycle units as sonar jamming sounds. In Bombycoidea caterpillars, short duration sounds are associated more with chemical defense than longer sounds, which are instead proposed to function in deimatic displays (Bura et al., 2016). The functional implications of different signal characteristics in conveying different messages to receivers are discussed further in the next section.

PROPOSED FUNCTIONS OF DEFENSE SOUNDS

A defense sound can be directed at either the attacker or a conspecific. Proposed functions of sounds directed at the attacker

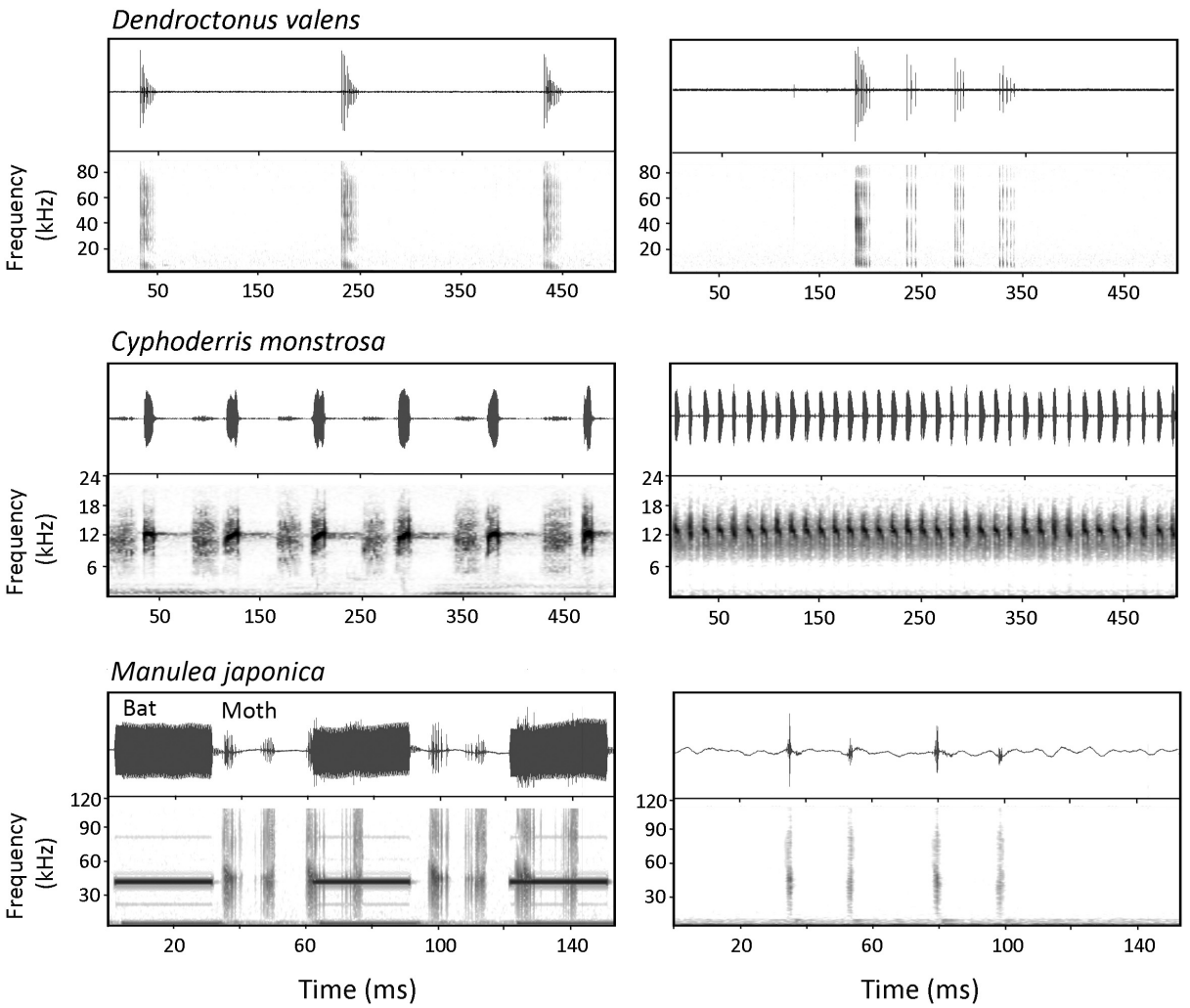

FIGURE 4 | Sounds produced by insects in both defense and reproduction to illustrate contextual differences in signal characteristics. In a defensive context, the male bark beetle *Dendroctonus valens* produces 'simple' chirps that are temporally simple compared to the more complex 'interrupted' chirps that are predominant during courtship displays. The male orthopteran *Cyphoderris monstrosa* produces a series of disyllabic chirps as defense sounds while the calling song is a continuous series of rapid trills. The male *Manulea japonica* tiger moth produces a burst of tymbal clicks in response to simulated bat calls (simulated bat calls in the spectrogram are shown as longer duration calls at ∼40 kHz that precede the moth clicks), while the courtship tymbal clicks are simpler in structure. Sound files were contributed by the Yack Lab (*D. valens*), A. Mason (*C. monstrosa*), and R. Nakano (*M. japonica*). See text and **Table 1** for further details on these examples.

include deimatism, aposematism, mimicry, or interference, while those aimed at conspecifics (and sometimes heterospecifics) are usually alarm signals. We define each of these proposed functions based on the broader defense literature (Ruxton et al., 2018) and provide examples if available. We also summarize the predicted receiver responses and defense sound characteristics for each functional category in **Figure 5**.

Signals Directed at Predators

Deimatic Displays

Deimatic displays have been defined as signals that act on the innate startle response of a predator (Skelhorn et al., 2016; Umbers et al., 2017). Although there has been debate on what comprises a deimatic display and how a predator will react (see Umbers et al., 2015; Skelhorn et al., 2016; Umbers and Mappes, 2016), predicted predator responses typically include the predator fleeing from the prey or hesitating long enough for the prey to escape, as well as habituation to the signal with repeated exposure, provided the prey is not otherwise defended (**Figure 5**) (Skelhorn et al., 2016; Umbers et al., 2017). In the visual realm for example, eyespots in butterflies and caterpillars are often recognized as a form of deimatic display that can cause predators to retreat (e.g., Vallin et al., 2005; Hossie and Sherratt, 2013) and habituate with repeated exposure (e.g., Blest, 1957). In the acoustic realm, there are few

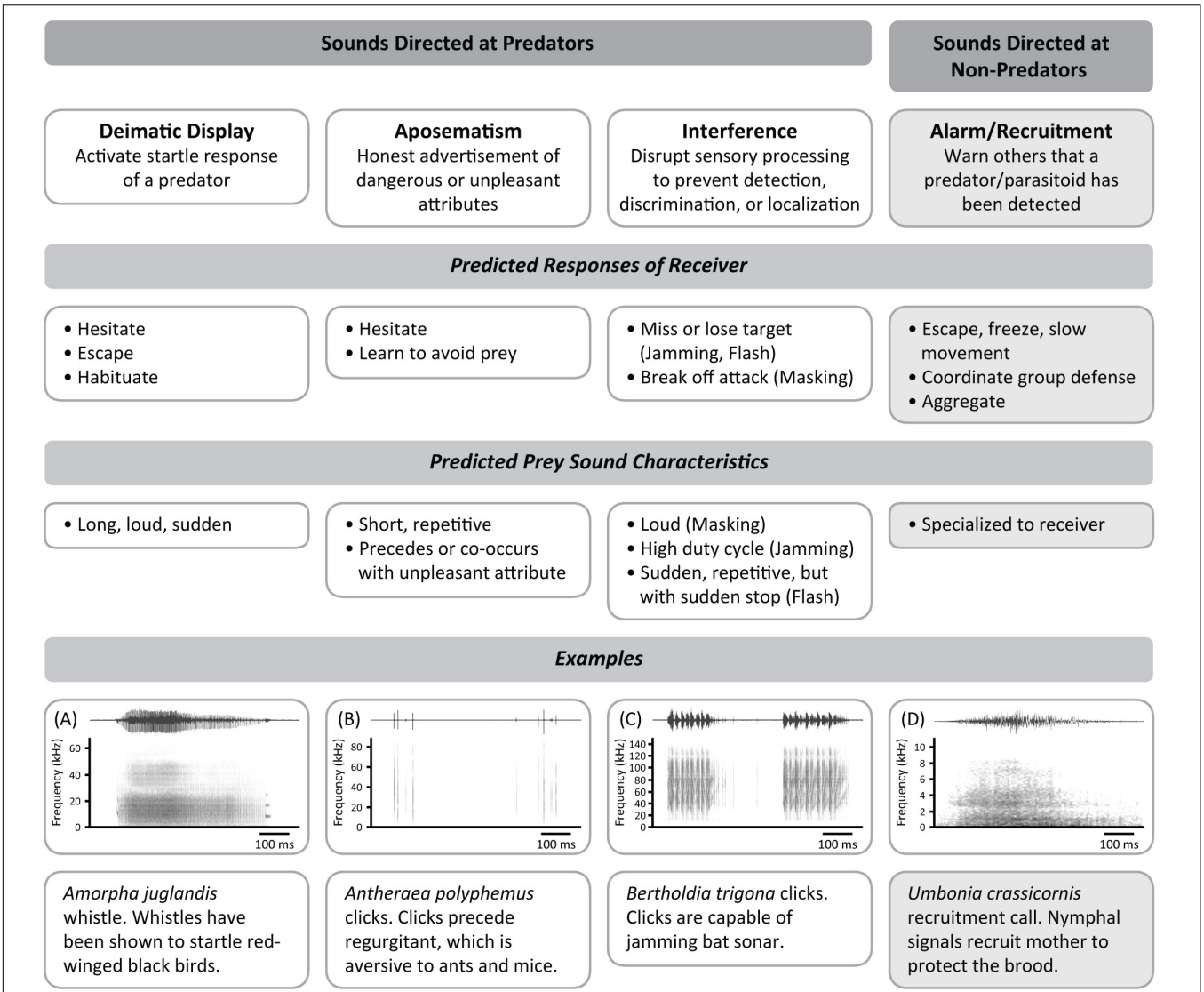

FIGURE 5 | Hypotheses explaining the function of insect defense sounds with corresponding predictions of both prey characteristics and receiver responses. Sound files **(A–D)**, shown as examples, were contributed by the Yack Lab (*A. juglandis* and *A. polyphemus*), A. Corcoran (*B. trigona*), and R. Cocroft (*Umbonia crassicornis*). See text for further discussion of these functions and corresponding examples.

experimentally tested examples of deimatic sounds involving predator responses (**Table 1**). Stridulation in *Henschoutedenia epilamproides* cockroaches (Blaberidae) startles rats and voles, causing them to withdraw (Guthrie, 1966). Clicking in *Aglais io* butterflies (Nymphalidae) causes bats to scream, jump, and retreat (Møhl and Miller, 1976), and causes mice to run away without attacking (Olofsson et al., 2012). Whistling in *Amorpha juglandis* caterpillars (Sphingidae) startles birds, causing them to dive and fly away (Bura et al., 2011; Dookie et al., 2017, **Figure 5A**), though birds habituate with repeated exposure to the sounds (Dookie et al., 2017). Deimatic sounds in insects have been characterized as being long, loud, and sudden – physical characteristics that are proposed to activate the predator's startle response (e.g., Hill, 2007; Kowalski et al., 2014). For instance, *A. juglandis* whistles are 440 ± 272 ms long, 69 to 82 dB SPL at 5 cm, and elicited upon contact (Bura et al., 2011). While insect defense sounds are sometimes proposed to be deimatic based on their signal characteristics alone (e.g., Kowalski et al., 2014; Bura et al., 2016), it is key when assessing any defensive function that the predator's responses be recorded (Skelhorn et al., 2016). Also, some insects combine deimatic sounds with visual displays, and the components of such multimodal defenses may operate synergistically, or individually to target different predators (Rowe and Halpin, 2013). For example, while clicks alone in *A. io* butterflies startle bats and mice, it is the hidden eyespots alone (which are revealed simultaneously with the sounds) that have a startling effect on birds (Vallin et al., 2005). This example highlights the necessity of studying responses to defense sounds by different predators. Future research should involve experiments with predators (and not

focus only on signal characteristics of the prey) as well as investigate potential synergistic effects of the insect's entire defensive display.

Aposematism

Aposematism is the honest advertisement of dangerous or unpleasant attributes (Edmunds, 1974; Ruxton et al., 2018; Caro and Ruxton, 2019). These attributes include not only distastefulness or toxicity, but also stinging spines, ability to fight back, and a vicious bite. Naïve predators are predicted to hesitate prior to sampling novel aposematic prey, while experienced predators will have learned to associate the signal with the unpleasant attribute and reject such prey based on the signal alone (**Figure 5**) (Speed, 2000). For example, red is a common aposematic color that can cause naïve predators to hesitate (Exnerová et al., 2007) and also facilitates learning more so than other colors (Rönkä et al., 2018). *A. polyphemus* caterpillars (Saturniidae) provide strong evidence for their sounds being aposematic. After simulated attacks or attacks from chicks, caterpillars produce clicking sounds followed by regurgitation. The regurgitant is aversive to ants and mice, and chicks stop their attacks following the caterpillar's regurgitation (Brown et al., 2007, **Figure 5B**). In another caterpillar, *Saturnia pyri* (Saturniidae), short chirping sounds are induced by simulated predator attacks and these sounds precede or accompany the secretion of defensive phenolic chemicals from scoli (bristle-bearing outgrowths) (Bura et al., 2009). These chemicals were shown in previous studies to be aversive to birds and ants (Deml and Dettner, 1993, 1995). Learned avoidance to an aposematic sound has been demonstrated in bats. Bats learn to associate tiger moth tymbal clicks with distastefulness in as few as two exposures to clicking moths, and will thereafter avoid the moths in a lab setting (Hristov and Conner, 2005). Similar to acoustic deimatism, some defended insects may combine sounds with warning coloration or odors, and such multimodal displays may increase the efficacy of an aposematic display or improve predator's learning of defended prey (Rowe and Halpin, 2013).

While it has been hypothesized that some sound characteristics may be more effective as aposematic signals than others (e.g., Bura et al., 2016), this has not yet been experimentally validated. However, we can draw on visual aposematic characteristics to make predictions. To improve prey discrimination and enhance learning, visual aposematic signals are predicted to be simple, symmetrically patterned, and conspicuous (Forsman and Merilaita, 1999; Ruxton et al., 2018). Simple visual signals such as bold patterns and few colors may be more reliably recognized than complex patterns (Cott, 1940), while symmetric patterns are easier to detect, learn, and remember than asymmetric patterns (Forsman and Merilaita, 1999). We predict the equivalent acoustic traits to be short (simple) and repetitive (symmetrical) sound units that are distinct from the background noise (conspicuous). For example, *A. polyphemus* aposematic clicks are short (24.7 $\pm$ 17.2 ms in duration), repetitive (52.7 $\pm$ 82.2 clicks in a train of sound), and distinct (ranging from 58.1 to 78.8 dB SPL at 10 cm) (Brown et al., 2007). Additionally, Masters (1980) noted a short and repetitive trend among disturbance stridulations of beetles, true bugs, and velvet ants, and Bura et al. (2016) found that short sounds correspond to the presence of chemical defenses (regurgitation or secretions from scoli) in caterpillars. These traits may aid in learning and memory retention as reported for clicker training with vertebrates (Smith and Davis, 2008). It has been reported that some moths produce ultrasonic aposematic sounds continually during flight (O'Reilly et al., 2019). Such proposed warning sounds that are always turned 'on,' analogous to warning coloration, would be interesting to explore further with live predators. We recommend that future studies focus on identifying effective aposematic sound characteristics and their impacts on a predator's psychology.

Mimicry

Defensive mimicry occurs when one individual (the mimic) copies the appearance or behavior of another (the model) to gain a selective advantage (Jamie, 2017). There are several ways that mimicry may be used for protective measures, but how a predator is predicted to respond depends primarily on its experience with the model. We address three forms of acoustic mimicry. First, in Müllerian mimicry both the mimic and model are defended, and the predator is predicted to respond as it would to an aposematic signal (**Figure 5**). Müllerian mimicry may also facilitate a predator's learning and memory retention of aposematic signals (Bradbury and Vehrencamp, 2011; Ruxton et al., 2018). Acoustic Müllerian mimicry is proposed to occur among defended clicking tiger moth species (Barber and Conner, 2007) and between defended clicking tiger moths and tiger beetles (Yager and Spangler, 1997). Second, in Batesian mimicry an undefended mimic copies an aposematic model. The predicted response of a predator that has had previous experience with the model is to avoid the mimic, whereas if the mimic is encountered prior to the model, then we would not expect a naïve predator to learn to avoid the signal. For example, Barber and Conner (2007) reported that after having sampled *Cycnia tenera*, a noxious clicking tiger moth (Arctiinae), red and big brown bats refuse palatable, clicking *Euchaetes egle* tiger moths. Another proposed acoustic Batesian mimic is *Eubaphe unicolor* (Geometridae), a palatable moth that produces tymbal clicks very similar to its sympatric, unpalatable tiger moth species (Corcoran and Hristov, 2014). Third, it has been proposed that insects can mimic the alarm calls of their predators. In vertebrates, such calls signal danger to conspecifics who react by increasing their vigilance or seeking cover (Fallow et al., 2013). An example of acoustic mimicry in insects is whistling in *A. juglandis* (Sphingidae) that is proposed to mimic the alarm calls of the caterpillar's avian predator (Dookie et al., 2017). Further studies on acoustic mimicry in insects should include comparisons of acoustic characteristics between models and mimics to determine their degree of similarity, playback studies to monitor predator responses, and palatability trials using different predators.

Interference Signals

Another way that insects may thwart attack is to interfere with a predator's sensory processing capabilities by impeding detection, discrimination, or localization of prey. For example,

flash coloration (a form of flash behavior) can cause a predator to lose track of its original target when a flash of color is revealed while fleeing, then hidden when stopped (Loeffler-Henry et al., 2018; Caro et al., 2020). Predicted predator responses to interference signals vary based on how the signals affect their cognitive processing. To date, three types of acoustic interference have been described in insects: sonar jamming, flash noise, and masking (**Figure 5**). Sonar jamming prevents prey localization by disrupting the returning echolocation signals of bats and causing the bats to miss their target (Corcoran et al., 2011). Jamming sounds are predicted to have a high duty cycle (proportion of time occupied by sound) to effectively disrupt echoes (Corcoran et al., 2009), and indeed, *B. trigona* tiger moths (Arctiinae) produce tymbal sounds with a 44% duty cycle (Corcoran et al., 2011, **Figure 5C**). However, after reporting that hawkmoths produce jamming sounds via genital stridulation with duty cycles as low as 18%, Kawahara and Barber (2015) proposed that a duty cycle threshold of about 20% or higher is necessary to jam bat sonar. The second type of acoustic interference is flash noise. Similar to flash coloration, flash noise occurs when the prey produces a sound that a predator can track acoustically (Edmunds, 1974). The predator is predicted to lose track of the prey when the signaler falls silent. For instance, a disturbed grasshopper (species unspecified) may be tracked via its wing-beat sounds, but when it lands and is again silent, the predator is unable to pinpoint its location (Edmunds, 1974). We believe that this would be especially true if the grasshopper stops flapping before it hits the ground, leaving a gap between where the sound was last heard and where the insect lands. However, the function of flash noises in hindering predators remains speculative. Masking, the third type of acoustic interference, occurs when one sound makes other sounds difficult to hear (Carterette and Friedman, 1978). Smith and Langley (1978) proposed that cicada disturbance squawks decrease the auditory acuity of a mouse by being loud, possibly preventing the mouse from hearing its own predators while handling the cicada. This could thereby make the cicada unprofitable to pursue, and the mouse would be predicted to break off the attack.

Signals Directed at Non-predators

Alarm Signals

Defense sounds directed at non-predators are referred to as alarm signals and are used to warn conspecifics, and sometimes heterospecifics, that a predator or parasitoid has been detected (Bradbury and Vehrencamp, 2011). Thus, alarm signals are usually found in group-living animals. Acoustic alarm signals in insects are predominantly transmitted and perceived through the substrate as vibrations, though some may have an airborne component (Hunt and Richard, 2013). Generally, receivers of an alarm signal respond by escaping, slowing their movement, coordinating a group defense, or approaching the signaler to provide aid (**Figure 5**). For example, drumming in *Camponotus* spp. carpenter ants (Formicidae) induces a context-dependent response where workers that were mostly still prior to the signal will freeze altogether, but those that were already in motion begin moving faster (Fuchs, 1976). Piping by returning foragers of *Apis florea* honeybees (Apidae) alerts the colony to a nearby threat and initiates coordinated hissing among the whole colony (Sen Sarma et al., 2002). The hissing in turn is thought to be an aposematic signal to approaching predators. Coordinated group responses to alarm signals also include tapping within nests by *Parachartergus colobopterus* (Vespidae) that calls the wasps outside to spray venom at intruders (Jeanne and Keeping, 1995). Alarm signals can also coordinate group formation, as observed in disturbed *Stolas* sp. beetle larvae (Chrysomelidae) where vibrational signals recruit other larvae to form a circle (Greenfield, 2002). This coordinated response may offer a survival advantage through safety in numbers (Dury et al., 2014). A rather unique type of alarm signal is the "stop" signal of honeybees (Apidae). When *Apis cerana* bees encounter danger at a foraging site, they prevent further recruitment to the site by using vibratory signals to stop any waggle dance that would otherwise recruit bees to that site (Dong et al., 2019). Insects that use alarm signals to recruit help to the signaler include *Umbonia* sp. treehopper nymphs (Membracidae) that produce vibrations when disturbed by a predator (Cocroft, 1996; Ramaswamy and Cocroft, 2009, **Figure 5D**). As the vibrational signals are transmitted across the brood, the mother responds by approaching the disturbance and often succeeds in repelling the attacker. Recruitment calls can also be directed toward heterospecifics, as seen in Lycaenidae and Riodinidae caterpillars that use solid-borne vibrations to induce ants to attend to them (Pierce et al., 2002). Tending by ants offers protection from predators and parasitoids, with untended caterpillars facing more predation by wasps than tended ones (Pierce et al., 1987; DeVries, 1991). Despite the examples that demonstrate the benefits of acoustic alarm signals to a colony, these signals have mostly been studied in the eusocial Hymenoptera and Blattodea. However, as many other substrate-bound insects (e.g., caterpillars and sawfly larvae) form groups (see Costa, 2006), the role of vibratory communication in mediating group defensive responses should be further explored in these insects.

EVOLUTIONARY ORIGINS

How did defense sounds evolve in the first place? There are two possibilities discussed in the literature: (1) they evolved from non-signaling behaviors; or (2) they were co-opted from acoustic signals that evolved for another function. Ideally, testing hypotheses on the evolutionary origins of a trait are conducted using a phylogenetic comparative approach which requires examples of homologous traits at various stages of evolution as well as a robust phylogeny (Petak, 2019). However, to the best of our knowledge this approach has not been used to test hypotheses explaining the origins of insect defense sounds. Nonetheless, we can speculate on the evolutionary origins of sonic defenses given the limited evidence available.

A communication signal can evolve from a non-signaling behavior through the process of ritualization (Scott et al., 2010; Petak, 2019). Non-signaling behaviors such as walking, flying, or using a spray apparatus can produce incidental sounds

which act as cues, meaning traits that have not evolved to alter a recipient's behavior (Yack et al., 2020). Cues can evolve into signals through ritualization whereby they become more conspicuous and stereotyped (Petak, 2019). Ritualization seems the most likely explanation for how sonic defenses evolved, especially when a defense sound is the only form of acoustic communication in a species. Several such hypotheses have been proposed for insects. Alexander (1956) suggested that defense sounds evolved from incidental sounds produced during a struggle with a predator. Nelson and Fraser (1980) proposed that hissing in cockroaches evolved from a spray apparatus since some insects produce defensive froths from their spiracles or other body openings, and can even produce incidental sounds while doing so (e.g., Carpenter, 1938). Tiger moth tymbals and the clicks they produce are proposed to have evolved through modification of the thoracic pleura which buckle when the moth is walking or flying, and so may have been associated with escape behaviors (Fullard, 1992).

A communication signal can also evolve secondarily from a pre-existing signal. For example, ultrasonic courtship signals were co-opted from anti-bat clicks in several tiger moth species (Simmons and Conner, 1996; Weller et al., 1999). Though evolving from a pre-existing signal has been less commonly proposed for defense sounds, there are a few examples. Defense sounds in crickets and katydids are proposed to have evolved from sexual signals due to the ubiquity of acoustic sexual signals in this group and the relative lack of defensive sound production (Alexander, 1960). Cases where only one sex produces both sexual and defense sounds may also indicate that the sonic defenses evolved from a pre-existing sexual signal. For instance, many species of bark beetles show sexual dimorphism in sound production whereby only one sex produces sounds in both sexual and defensive contexts (Bedoya et al., 2019). In cicadas, Smith and Langley (1978) proposed that disturbance squawks evolved secondarily to courtship sounds as both sound types, when produced via tymbals, are found only in males. If signals evolved initially for defense, it is difficult to explain the lack of tymbal sound production in the opposite sex (Smith and Langley, 1978). Territorial signals may provide another origin for some defense sounds, possibly in caddisfly larvae (Hydropsychidae) that produce sounds both when poked with forceps and when other larvae attempt to enter their shelter (Johnstone, 1964). To the best of our knowledge, co-opting a pre-existing acoustic communication signal for use in defensive sound production has yet to be formally investigated.

Future research on the evolutionary origins of defense sounds must involve phylogenetic comparative studies. Such studies require documentation of a series of variable, but homologous, morphological and associated behavioral traits within extant species, as well as a robust phylogeny to assess which traits are ancestral or derived. Behavioral traits are more challenging to document than morphological traits as they must be studied in living specimens. Consequently, there are currently few phylogenetic comparative analyses on the evolution of insect defensive displays (but see Vidal-García et al., 2020). This review has identified several insect groups that are promising for studying evolutionary origins of defense sounds in that the groups display variable sonic defenses and recent phylogenies are available. These insect groups include praying mantids (e.g., Vidal-García et al., 2020), tiger moths (e.g., Zaspel et al., 2014; Dowdy and Conner, 2019), Lepidoptera pupae (e.g., Dolle et al., 2018; Espeland et al., 2018), and Bombycoidea caterpillars (e.g., Bura et al., 2016). These model systems will allow us not only to test hypotheses on evolutionary origins of defense sounds, but also test hypotheses on why some insects evolve sonic defenses and others do not.

CONCLUSION

Defense sounds occur widely in insects. Yet, compared to our understanding of visual defenses, there are major gaps in our understanding of which insects produce defense sounds, how these sounds promote survival, and how they evolved. Here, we recommend some key, non-mutually exclusive lines of investigation and questions to focus on in future research. First, further testing is necessary to better document the extent of defensive sound production throughout the Class Insecta. Because defense sounds often occur only during close encounters with predators and thus may be "quiet," they may go unnoticed unless "attacked" by the experimenter. Also, defense sounds that are ultrasonic, or transmitted as solid-borne vibrations, can easily be overlooked by scientists not using specialized equipment. We recommend conducting experiments on those orders that are not well-represented in the literature, being careful to document any differences in the sex of the species being assessed. We also recommend further investigations on juvenile stages such as larvae, nymphs, pupae, and eggs. Second, what are the key selective pressures that led to the evolution of defense sounds? Variables including the types of predators, the presence of other defenses, body size, and life history traits should be documented and analyzed using phylogenetic comparative methods, meta-analyses, and developmental studies to address this question. Third, what explains the wide variation in signal characteristics? How much of this variation is related to factors such as the type of mechanism, the type of message being conveyed, and the hearing of the receiver(s)? We encourage experimental investigations to test the hypothesis that different acoustic characteristics are more efficient at conveying different messages, such as aposematism or deimatism. Fourth, how did defensive sound production evolve originally? Did it evolve from escape or fighting behaviors, or from pre-existing signals used in another context? To understand the evolutionary origins of defense sounds, phylogenetic comparative analyses are necessary. Fifth, and perhaps most important, testing hypotheses on the survival benefits of these sounds requires experiments with natural predators. Most studies have been conducted by humans pinching insects and reporting the sounds. While these studies are important, experiments with a diversity of natural predators are necessary to record the effectiveness of sonic defenses. Such studies should monitor the initial predator responses as well as the effects over time, such as habituation and learning

rates. Additionally, the behavior of the prey should be closely monitored to determine how long the display lasts, if the prey tries to escape, and if there are other behaviors associated with sound production. While predator-prey experiments can be challenging to conduct, they are essential to furthering our understanding of the efficacy of survival sounds in insects. Defense sounds clearly play an important role in the survival strategies of many insects, and further research on acoustic communication is critical for establishing a comprehensive understanding of insect predator-prey interactions.

AUTHOR CONTRIBUTIONS

ML led the project and wrote the first draft. All authors contributed to data collection, editing the manuscript, and figure and table preparation. All authors approved the final version of the manuscript.

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Tenors Not Sopranos: Bio-Mechanical Constraints on Calling Song Frequencies in the Mediterranean Field-Cricket

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Male crickets and their close relatives bush-crickets (Gryllidae and Tettigoniidae, respectively; Orthoptera and Ensifera) attract distant females by producing loud calling songs. In both families, sound is produced by stridulation, the rubbing together of their forewings, whereby the plectrum of one wing is rapidly passed over a serrated file on the opposite wing. The resulting oscillations are amplified by resonating wing regions. A striking difference between Gryllids and Tettigoniids lies in wing morphology and composition of song frequency: Crickets produce mostly low-frequency (2–8 kHz), pure tone signals with highly bilaterally symmetric wings, while bush-crickets use asymmetric wings for high-frequency (10–150 kHz) calls. The evolutionary reasons for this acoustic divergence are unknown. Here, we study the wings of actively stridulating male field-crickets (*Gryllus bimaculatus*) and present vibro-acoustic data suggesting a biophysical restriction to low-frequency song. Using laser Doppler vibrometry (LDV) and brain-injections of the neuroactivator eserine to elicit singing, we recorded the topography of wing vibrations during active sound production. In freely vibrating wings, each wing region resonated differently. When wings coupled during stridulation, these differences vanished and all wing regions resonated at an identical frequency, that of the narrow-band song (∼5 kHz). However, imperfections in wing-coupling caused phase shifts between both resonators, introducing destructive interference with increasing phase differences. The effect of destructive interference (amplitude reduction) was observed to be minimal at the typical low frequency calls of crickets, and by maintaining the vibration phase difference below 80°. We show that, with the imperfect coupling observed, cricket song production with two symmetric resonators becomes acoustically inefficient above ∼8 kHz. This evidence reveals a bio-mechanical constraint on the production of high-frequency song whilst using two coupled resonators and provides an explanation as to why crickets, unlike bush-crickets, have not evolved to exploit ultrasonic calling songs.

Keywords: bioacoustics, insect communication, Ensifera, biomechanics, coupled resonators

INTRODUCTION

Male crickets (Ensifera, Gryllidae) produce loud musical songs to attract conspecific females by rubbing their raised forewings together, a process known as stridulation. During stridulation, the plectrum – a sharp sclerotised region at the anal edge of the left wing (LW) – engages with the file, a row of teeth on a modified, serrated vein on the underside of the right wing (RW) in a clockwork-like manner (Elliott and Koch, 1985; Prestwich et al., 2000). In Gryllidae, the RW usually sits on top of the LW, and during stridulation, both wings open and close in a rhythmic cycle, with sound being generated during the closing phase only (Koch et al., 1988; Bennet-Clark, 1999). The dorsal field of each bilaterally symmetric wing displays a number of clearly delineated wing cells involved in sound radiation. These are the harp, mirror, chord, and the hardened, non-membranous anal surface (Montealegre-Z et al., 2011) (**Figure 1**).

The male is under strong sexual selection to sing at a high amplitude in order to effectively attract and provide phonotactic information for distant females (Forrest and Green, 1991; Römer, 1998). In most cricket species, acoustic energy is concentrated within a narrow-band, pure-tone signal centred on a single low-frequency carrier (∼5 kHz in the case of the field-cricket *Gryllus bimaculatus* De Geer) which is amplified and radiated by wing regions functioning as natural resonators (Bennet-Clark, 1999, 2003). A loud, pure-tone calling song extends the signal range, aiding the females in determining the direction of the sound source through the enhancement of binaural hearing (Kostarakos et al., 2008; Michelsen and Larsen, 2008) and makes it possible to obtain a large signal-to-noise ratio for transmission across the environment (Michelsen, 1998; Warren et al., 2006; Wiley, 2006). For optimal power transfer from sound source to the surrounding medium, a resonator like the cricket wing should have a radius of at least 1/6 of the sound wavelength λ (λ = ∼7 cm at 5 kHz; assuming a monopole radiator; the radius increases to 1/4–1/3λ for dipoles) (Fletcher, 1992; Bennet-Clark, 1998). Small, sound-producing insects like crickets with wings about 0.5–1 cm in size are therefore under strong selection to optimize power output in order to maximize signal range. Crickets approach this optimization problem by using both symmetric forewings together as sound radiators during stridulation to increase the sound radiating surface for low-frequency songs (Bennet-Clark, 1999, 2003; Montealegre-Z et al., 2011). In contrast, their close relatives bush-crickets (Tettigoniidae) have evolved high-frequency singing using asymmetric wings as a derived trait where the overlying LW bears the file and is usually mechanically dampened, while the plectrum-bearing RW is highly adapted for efficient sound radiation (e.g., Montealegre-Z and Postles, 2010; Sarria-S et al., 2016; Song et al., 2020). The drivers for the evolution of this asymmetry are unknown but it has been hypothesised to be linked to ultrasonic sound production and signal purity (Montealegre-Z, 2005; Gu et al., 2012).

Signal transmission is facilitated by resonance – an inexpensive way of enhancing sound output while conserving metabolic energy – whereby the call's carrier frequency (f_c) is determined by the resonance frequency f_0 of the wings, which implies that both wings in a symmetric system should resonate at similar f_0. Reliance on two coupled resonant structures requires that crickets have to achieve and maintain a high degree of phase locking between the two wings in order to add vibrations constructively (Prestwich et al., 2000). Only when the two resonators are vibrating at similar f_0 with minimal phase differences (ϕ) is constructive wave superposition providing the desired effect of increasing the amplitude of radiated sound energy. When optimal (ϕ = 0), this constructive interference

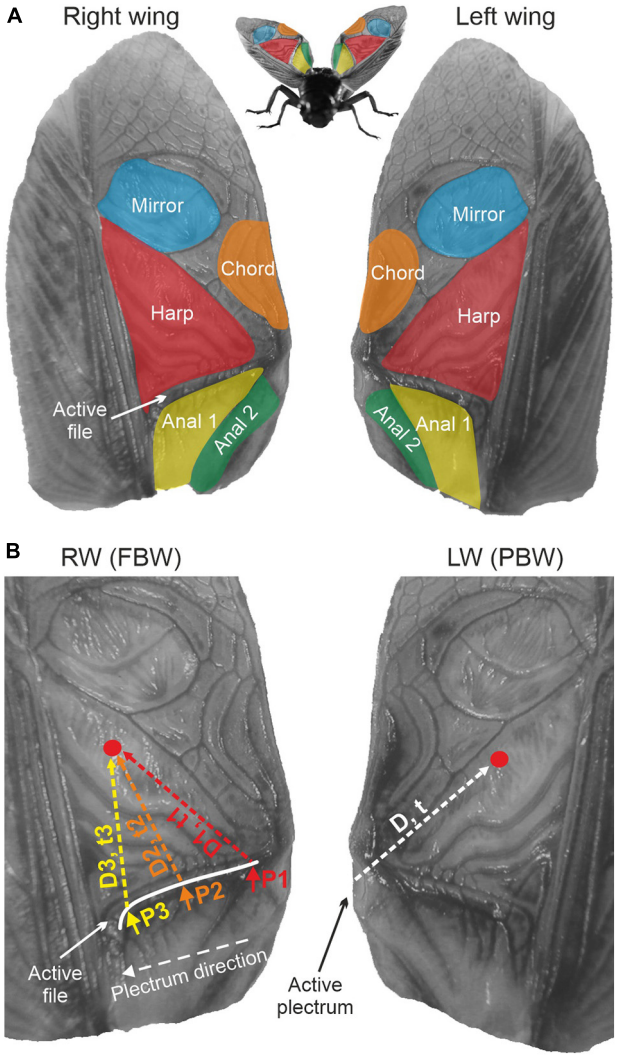

FIGURE 1 | Extended tegmina of *Gryllus bimaculatus*. **(A)** The main regions involved in sound production are highlighted. Nomenclature of wing regions follows Montealegre-Z et al. (2011). **(B)** The problem of phase interference during tegmino-tegminal stridulation. For the left, plectrum-bearing wing (PBW), energy from tooth impacts will travel a constant distance (D) from the plectrum region to a specific region of the same wing (e.g., the red dot; arbitrarily chosen). Conversely, for the file-bearing right wing (FBW), the point of energy input will change as the scraper moves over the file. Energy will travel different distances (D1–D3), reaching the red dot at variable times *t*, resulting in varying phases of vibration as the scraper moves.

results in a doubling of the amplitude of the combined output (Rossing, 1990). How can this behaviour, defined here as in-phase, take place?

The in-phase resonance between wings is facilitated by an escapement mechanism that allows both wings to vibrate together and radiate sound efficiently (Koch et al., 1988). However, prior mechanical analyses of cricket stridulation showed that the mechanism of sound production is asymmetrical (Bennet-Clark, 2003; Montealegre-Z, 2005; Montealegre-Z et al., 2011): While the RW receives its energy input along the file's ca. 200 teeth distributed over a distance of some 4 mm, the underlying LW receives energy only through the small region of the plectrum (0.1 mm^2, **Figure 1B**). **Figure 1B** shows that as the plectrum is dragged on the file from left to right, it generates mechanical impacts at different locations along the file. The input of mechanical energy therefore varies in time and location, potentially resulting in a complicated dispersion of substrate-borne waves across the surface area of the RW (**Figure 1B**, left). On the other hand, the LW has only one input, the plectrum, and vibrations will travel constantly to the various LW regions from that input (**Figure 1B**, right). Therefore, the LW should vibrate with constant phase, independently of the plectrum's position on the RW. In contrast, the RW should be more vulnerable to phase changes as the moving plectrum delivers energy impulses along the file. If these assumptions hold true, the constant phase generator (LW) and the variable phase generator (RW) are expected to interact and generate beats in their summed acoustic output, in particular at locations where LW and RW vibrations cancel each other out (Sismondo, 1993). Yet, the natural song of the male does not exhibit such beats; instead, song pulses have sustained and regular amplitude and phase profiles.

In addition, it is also implied that the wings' resonances are perfectly in tune with the input stimulus, each wings' f_0 is equal to the song carrier frequency f_c. However, previous studies revealed that the left and RWs exhibit different f_0, above and below the output f_c (<5 kHz>) (Nocke, 1971; Bennet-Clark, 2003; Montealegre-Z et al., 2011). Non-contact laser Doppler vibrometry (LDV) measurements showed that the left and RWs of field-crickets are mechanically different, with resonant frequencies differing by as much as 2 kHz ($f_{0\,\text{left}} < f_{0\,\text{right}}$; Montealegre-Z et al., 2011). It remains unclear how the seemingly imperfect and differently tuned resonators can generate the high quality pure-tones observed in crickets.

Using LDV, focal microinjection of the neuropharmacological neuroactivator eserine, and specialised acoustic equipment, we measured wing vibrations in actively stridulating Mediterranean field-crickets (*G. bimaculatus*). From physical acoustics, we hypothesise that efficient, high gain, pure-tone radiation results from the in-phase oscillation of both wings when coupled during the stridulation process. We furthermore formulate and test a second hypothesis: different wing regions vibrate in phase, despite differential tuning and inputs, and thereby generate the coherent acoustic radiation typical of field-cricket songs. As a consequence, any imperfections in the coupling of the wings that lead to temporal and phase shifts between the resonators should result in sub-optimal amplitude of the output signal and ultimately impose constraints on signal frequency.

MATERIALS AND METHODS

Animals

Adult male crickets (*G. bimaculatus*) obtained from a breeding colony maintained at the University of Bristol were used. Animals were kept at room temperature (20–22 °C) under a 12 h:12 h light:dark cycle and were fed with oats, dry dog food and water *ad libitum*. Adult males were randomly taken from the colony, their wings inspected for damage and kept individually in cages prior to the experiments. After isolation, 18 males that sang for prolonged periods of time were chosen for the experiments, as these animals usually responded better to pharmacological stimulation. All males recorded were singing with the usual wing overlap (RW over LW).

Neuropharmacological Stimulation

To elicit persistent stridulation in tethered crickets, we followed methods established and described in detail in earlier studies (Hedwig and Becher, 1998; Wenzel et al., 1998; Wenzel and Hedwig, 1999; Montealegre-Z et al., 2011). In short, we used borosilicate glass microcapillaries (1B120F-3; ID = 0.68 mm; World Precision Instruments, Inc., Sarasota, FL, United States) pulled with a Sutter microelectrode puller (Sutter Instrument Company, Novato, CA, United States) to produce ca. 10 µm wide tips. These microcapillaries were then filled with eserine/ringer solution (10^{-2} mol l^{-1}; Sigma-Aldrich Company Ltd., Dorset, United Kingdom) and connected to a picospritzer (Picospritzer II, Parker Hannifin, Pneutronics Division (formerly General Valve, NJ, United States). Small quantities of eserine (an acetylcholinesterase inhibitor) were injected into a brain neuropil, located between the pedunculus and the α-lobe of the mushroom bodies. Successful procedures elicited sustained stridulation in the typical calling song pattern (see **Supplementary Video 1**). Crickets were removed from the study if we recorded no singing activity within 1 h after the first injection.

Crickets exhibit frequency modulation (FM) in their calls, and the envelope of this modulation has been shown to be a fingerprint of each individual (Montealegre-Z et al., 2011). The quality of the pharmacologically elicited calls was examined by correlating their FM pattern with that of the natural calls obtained by zero-crossing analysis. Calls were judged of sufficient quality when the correlation was higher than 0.85 (see Montealegre-Z et al., 2011, for more experimental details).

Recordings of Wing Vibrations in Stridulating Animals (Wings Engaged)

Vibrations from the tegminal surface were successfully quantified from 11 of the 14 stridulating animals using two coupled laser Doppler vibrometers (Polytec PSV-300-F, and a PSV-400; Polytec GmBH, Waldbronn, Germany) and corresponding scanning heads (OFV-056) fitted with close-up attachments. The velocity output of the PSV-300-F served as an input channel for the PSV-400 vibrometer, thus allowing for synchronization of the recordings. Sound signals were recorded using a 1/8″ condenser microphone Brüel & Kjær Type 4138, connected to a Brüel &

Kjær 2633 preamplifier and a Type 5935L amplifier (Brüel & Kjær, Nærum, Denmark), which was in turn connected to the PSV-400 acquisition system. Measurements were performed in single-shot mode (one recording per chosen spot on the wing, no averaging) in the temporal domain (1,024 samples at 512 kHz sampling rate, leading to recordings with 2 ms duration and a temporal resolution of ∼1.95 μs). Acoustic and vibrational measurements were recorded with Polytec Scanning Vibrometer software (PSVSoft, Version 8, Polytec GmbH, Waldbronn, Germany). The microphone was positioned posterior to the specimen, 3–4 cm away from the wings as to not interfere with the laser beams. Simultaneously, wing vibrations were recorded with the laser beams focused on the anal regions, harps, chords, and mirrors (**Figure 1** and see **Supplementary Video 1** showing a singing male after pharmacological stimulation). Through the video feed of the two LDVs, we were able to visually place the laser points with some acuity within the regions in question, ensuring that the recordings from left and RW came from equivalent locations. Results for the chord regions are shown in the **Supplementary Material** but are not included in the main results as we were able to obtain chord recordings in only 7 out of the 11 animals used (the left chord regions are usually covered by the RW during stridulation and thus not easily accessible). The laser spot position and signal strength (the amount of laser light reflected from the target) was monitored and controlled via the live video feeds to the controlling computers of both laser systems. Using earlier LDV systems, signal strength often had to be increased by applying minute reflecting beads or powder to the wing surfaces. This was not the case here as the focussed laser light ($\lambda \sim$ 630 nm) was well reflected by the wing cuticle, which allowed us to perform contactless vibration measurements without further manipulation of the wings.

The microphone signal was used as a measurement trigger, so only wing vibrations involved in sound production were recorded. Data acquisition was programmed to last for 2 ms during the maximum amplitude event of a song pulse. This duration was chosen to minimise the movement of the wings during recording (∼ 8–10 teeth) while still gathering sufficient data for analysis (see also Montealegre-Z et al., 2011).

Individual Resonances of Unengaged Fixed Wings (Free Vibration)

After the previous experiment, each of the wings of each live specimen (n = 14) were extended and separated from each other by fixing the axillary sclerites with a bee's wax (Fisher Scientific United Kingdom, Limited, Leicestershire; product code W/0200/50), and Colophony (Sigma-Aldrich Co. St. Louis, MO, United States; Product No. 60895-250G) mixture (1:1). The wings were extended to not be in contact with the pronotal lateral and posterior edges. A loudspeaker (ESS AMT-1; ESS Laboratory Inc., Sacramento, CA, United States) was used to broadcast periodic chirps in the range 1–20 kHz, with a flat (55 dB SPL ± 1.5 dB) spectrum. The microphone was placed dorsally in the middle of both extended wings (**Figure 2**). The laser system was set to record in the scan mode. A complete scan of the extended wings in response to the periodic chirps was performed with the PSV-400 LDV, using 250–300 scanning points per wing with 10 measurements averaged per point. Fast Fourier transforms (FFT) with a rectangular window and a sampling rate of 512 kHz, 128 ms sampling time, and a frequency resolution of 7.81 Hz were generated for each point.

Data Analysis

Experimental data was either analysed directly with the PSV software or with custom written scripts in MATLAB (R2019a; The MathWorks Inc., Natick, MA, United States). Instantaneous phase in the time domain was obtained with Hilbert transform using custom MATLAB code (Hartmann, 1997). We tested whether the frequency differed between left and RWs, and between areas (mirror, harp, chord, anal) using linear mixed effects models run in R 4.0.0 (R Core Team, 2020). Models were run separately for free and engaged wings, with male ID included as a random effect. Models were run using lme4 (Bates et al., 2015) and lmerTest (Kuznetsova et al., 2017), with *post hoc* testing carried out using emmeans (Lenth, 2020). We also tested the difference in the normalised amplitude of the mechanical response (μm/Pa), between left and RWs using a paired t-test.

RESULTS

Using focal microinjection of the neuroactivator eserine into the cricket's brain (Wenzel et al., 1998; Hedwig, 2000), long-lasting and stable stridulation was elicited in 14 restrained males (**Supplementary Video 1**). Using two synchronised micro-scanning LDVs, we successfully measured the spatially resolved vibration of both wings simultaneously during the "engaged" phase of the stridulation process in 11 of the 14 actively singing males, following a previously established protocol (Montealegre-Z et al., 2011). After the cessation of singing, the wings of each specimen were extended and fixed basally and stimulated with sweeps of broadband sound to reveal their natural resonances f_0 and relative magnitudes of vibration. The surface area of these "unengaged" wings was scanned in its entirety, providing a detailed map of vibrational patterns (**Figures 2**, **3** and **Supplementary Video 2**, showing wing vibrations of one male at resonance of 4.6 kHz).

Natural Frequencies of Wing Vibrations

Full wing scan recordings of unengaged (extended and fixed) wings show that the RW f_0 is significantly higher than the LW (RW = 5.168 ± 0.434 kHz, SE 0.116; LW = 4.827 ± 0.396 kHz, SE 0.106; LMM: $F_{1,152.60} = 15.93$, $p < 0.001$). However, when comparing vibration amplitudes at the average f_0 of both wings, no difference between left and RWs was found. This was true for both average vibration amplitudes per wing and maximum vibration amplitudes of the harp areas alone (RW_{harp} = 0.32 ± 0.24 μm/Pa; LW_{harp} = 0.40 ± 0.35 μm/Pa; $t = 0.988$, $df = 13$, $p = 0.34$). When each wing is stimulated at its average f_0, one always exhibits a higher vibration amplitude (on average by a factor of ∼1.7; **Figures 2B,C**), but this dominant wing can be either LW or RW (cf. **Supplementary Video 2**, where the animal's LW vibrates with higher amplitude). In a previous

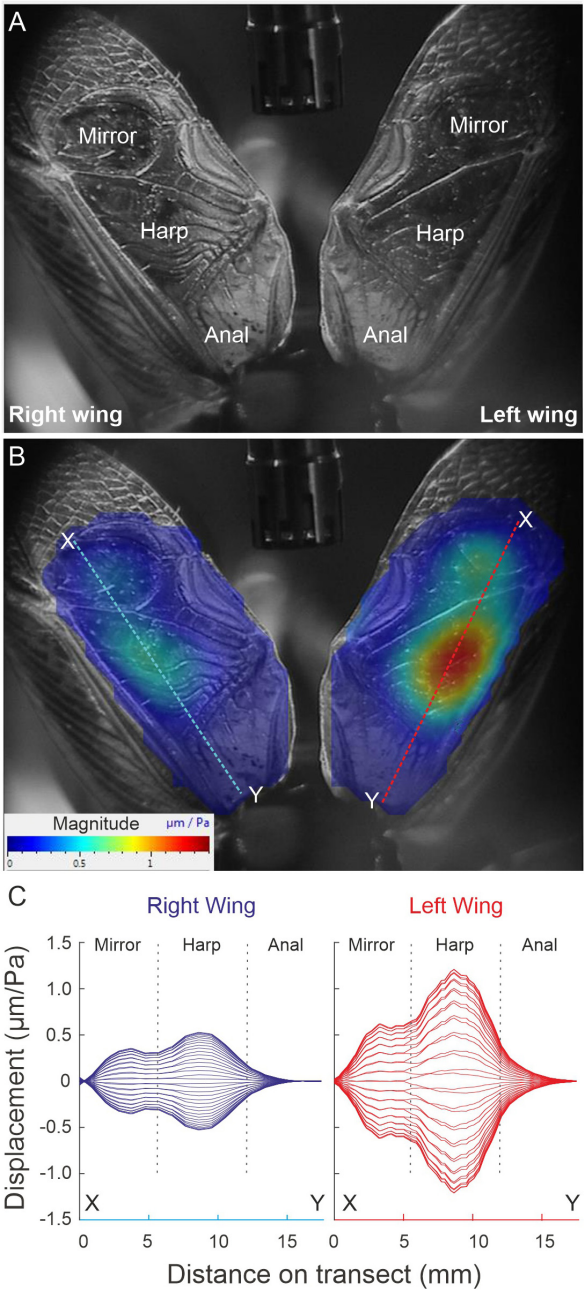

FIGURE 2 | Amplitude response of extended wings to sympathetic acoustic stimulation. **(A)** Orientation image relating tegmen topography to the position of the scanning lattice. **(B)** Scanned area and deflection shapes of the tegmen dorsal surface (harp and mirror). Dashed lines illustrate the sections through which the deflection envelopes in panel **(C)** were built. **(C)** Envelope of mechanical deflections along transects shown in B for a series of phases (in steps of 10°) in the full oscillation cycle. For this specimen: RW f_0 = 4.71 kHz, LW f_0 = 4.62 kHz).

study, we reported a trend of LW dominance which we could not identify here, which is most likely due to our low sample size (n = 44 in Montealegre-Z et al., 2011).

Examining wing vibrations in more detail, LDV measurements reveal that each wing region exhibits its own resonance spectra with varying peak frequencies (**Figure 3A**); there were significant differences in the f_0 between areas (LMM: $F_{4,152.56}$ = 72.55, p < 0.001). Post hoc testing revealed that the mirror of each wing consistently showed higher f_0 than the average wing f_0 (LW_{mirror} = 6.858 ± 0.540 kHz, SE 0.127 kHz; LW average f_0 of other areas = 4.827 ± 0.396 kHz, SE 0.106; RW_{mirror} = 7.007 ± 0.865 kHz, SE 0.204 kHz; RW average f_0 of other areas = 5.168 ± 0.434 kHz, SE 0.116; n = 18) (**Figure 3A**), with other areas of the wing not significantly different from each other.

Wing Vibrations in Stridulating Animals

Wing vibrations were recorded during active stridulation using two LDVs in single shot mode, enabling vibration measurement at defined locations and times (see **Supplementary Video 2**). Remarkably, vibrations of engaged wings during stridulation (**Figure 3B**) differ from sound-evoked vibrations in unengaged wings (**Figure 3A**). When the wings are engaged, all regions exhibit near identical, narrow vibrational frequency spectra with maximum power concentrated at the carrier frequency f_c of the calling song (here 5.125 kHz; LMM: $F_{3,66.29}$ = 1.56, p = 0.208; **Figure 3B**). There is also no difference between the left or RW (LMM: $F_{1,65.20}$ = 0.77, p = 0.383). The convergence of all resonators toward one very narrow frequency band of oscillation is reminiscent of entrainment, a process similar to synchronization between Huygens' clocks (Peña Ramirez et al., 2016).

Apart from identical oscillation frequency, an additional key feature of synchronised resonators is their phase relationship. Time-resolved LDV data were obtained by recording vibrations from different regions of both wings at synchronised points during stridulation (see methods). Results across 11 specimens show that the wings are not perfectly in phase during sound production, but that phase lags ϕ exist over a wide range between left and RWs (**Figure 4**). In some individuals, ϕ is small and relatively constant between wings (both over time and between regions, **Figure 4A**), while others show larger differences in phase (**Figure 4B** and **Supplementary Figure 1**). Within an individual, average phase lags across wing regions seem to be relatively consistent, although considerable variation exists (see **Supplementary Figure 1**).

Time domain recordings of single point measurements at the harps, anal regions and mirrors also show that LW vibration amplitudes are mostly higher than RW amplitudes (red and blue lines in **Figure 4A**, respectively) but there is also considerable variation in amplitudes across animals and wing regions (**Figure 4B**). The high variation in vibration amplitude can be explained by the limitations of the experimental set up. As two lasers had to be manually aligned on the stridulating animals, space restrictions and changes in the way the animals held their wings during stridulation often prevented a perfect orthogonal alignment of the laser beams to the vibrating surfaces, resulting in absolute displacement amplitudes that are hard to compare between wings. Relative phase relationships between the wings, however, can be measured with high accuracy, as they

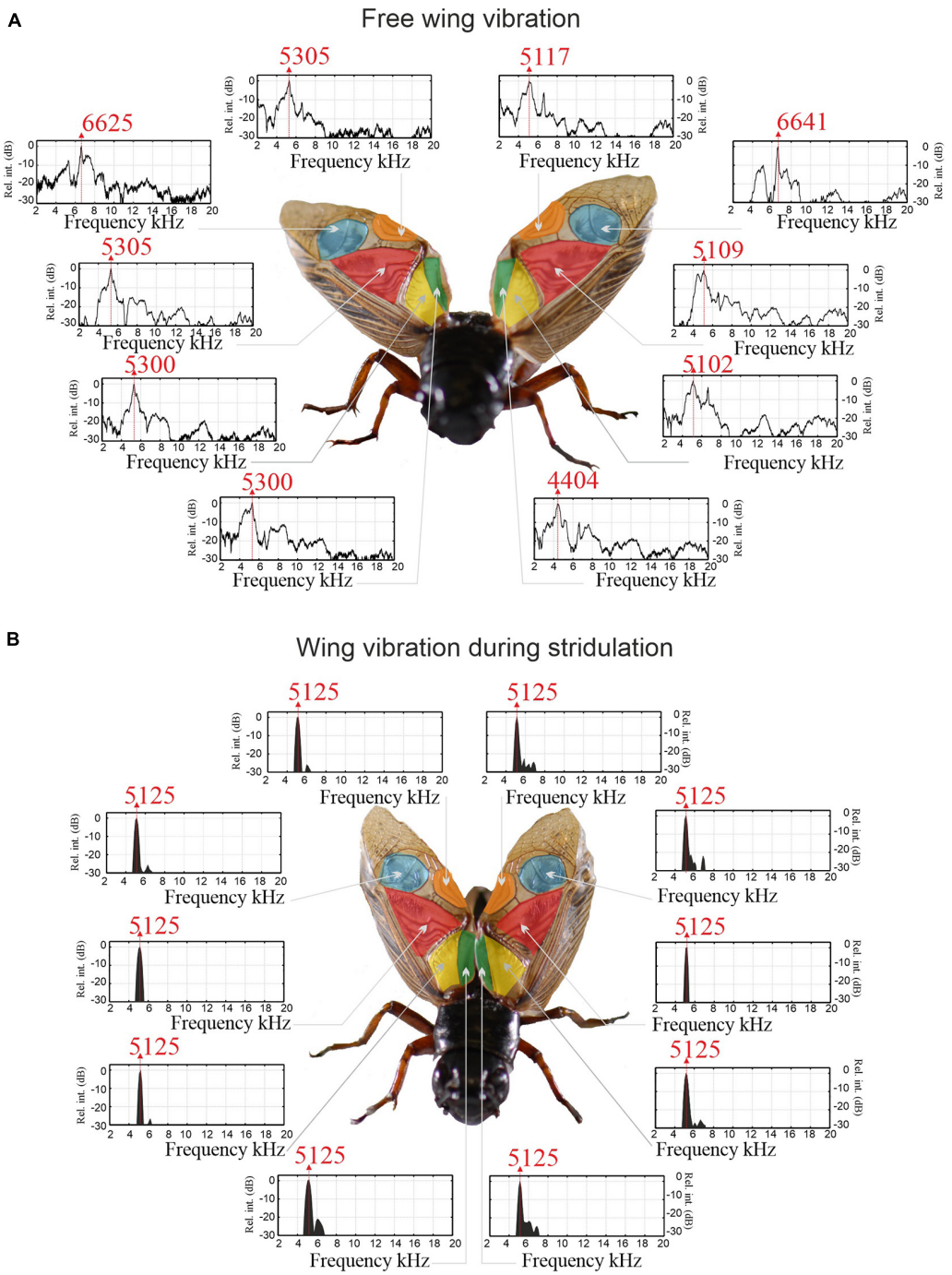

FIGURE 3 | Wing region resonances of unengaged and engaged wings of a male *Gryllus bimaculatus*. **(A)** Natural resonances of wing regions measured with LDV in unengaged wings. **(B)** Wing resonances measured in the same individual during stridulation (engaged). Vibration amplitudes have been normalised to a relative dB scale.

are not affected by laser beam–target orthogonality. In theory, mathematical superposition of LW and RW vibrations allows estimating the resulting combined output vibration. For example, the net vibratory response at a given place and time caused by the two harps is the sum of the responses which would have been caused by each harp individually (**Figures 5A,B**). These calculations show that the greater the phase lag ϕ (and thus time lag Δt for a given frequency; cf. **Supplementary Figures 1**, **2**) between LW and RW, the lower the amplitude of the resulting vibration and therefore the gain as compared to using only one wing (**Figures 5A–C**). Without exact amplitude information for engaged wings, we can nevertheless show the effect of phase

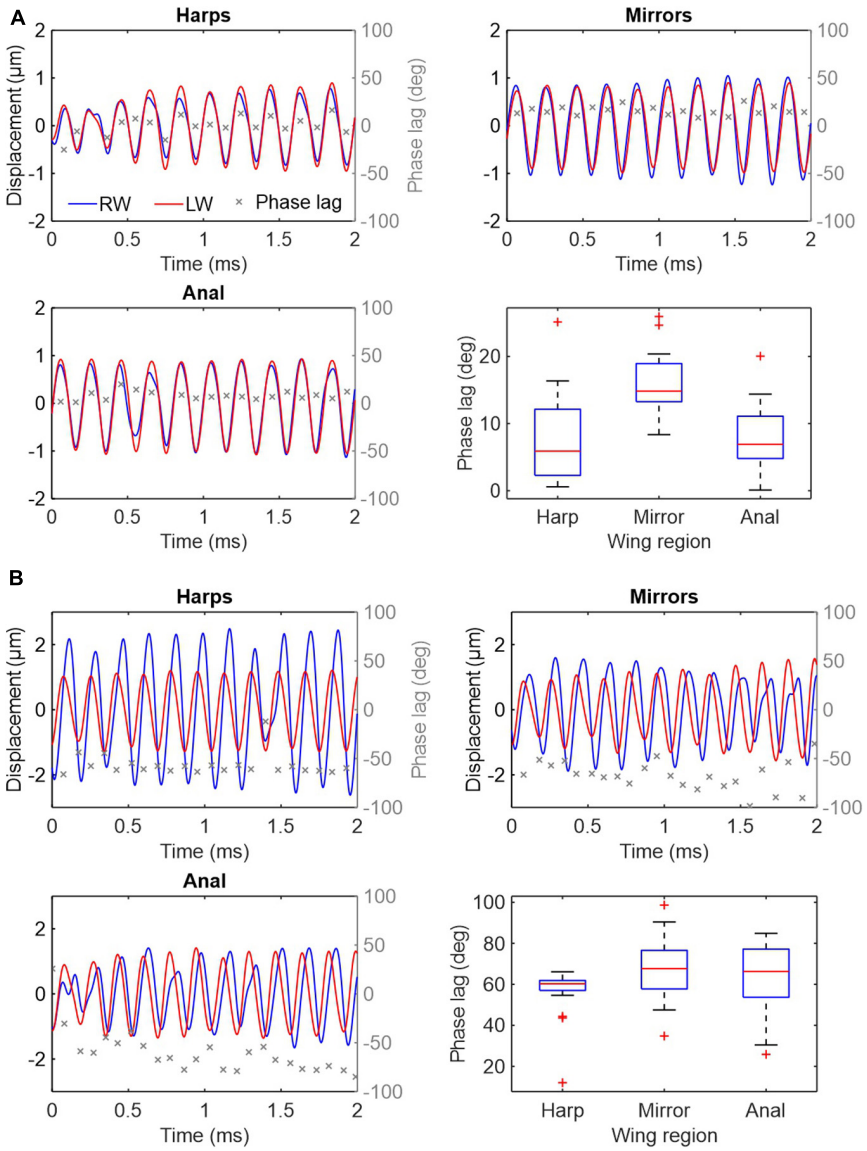

FIGURE 4 | Vibration displacements and phase relation in three major wing regions during stridulation in two *Gryllus bimaculatus* males. Wing vibration measurements were obtained simultaneously from two homologous wing regions using two LDVs. **(A)** An individual with nearly perfect phasing of the wings (median ϕ between 6° and 15°). **(B)** An individual with more prominent phase differences and variation between the wings (median ϕ between 60° and 68°). Each panel represents an independent recording showing RW in blue, LW in red and phase lag ϕ in grey. ϕ is measured as the difference in phase between LW and RW at the LW local maxima and minima. Boxplots show the median (red line), 25th, 75th percentiles (box) and 1 IQR whiskers for all ϕ per wing region. Outliers are marked as red +.

shifts between wings on the overall output amplitude assuming that vibration amplitudes are equal for both wings (as shown in **Figure 5A**). Thus, **Figure 5C** shows normalised RMS (root mean square) gain as a function of phase lag ϕ of three different wing regions using normalised vibration velocity amplitudes. In ideal conditions, where both wings exhibit equal vibration amplitudes at equal frequencies, perfect phase locking ($\phi = 0°$) produces a gain of 2, while a phase lag of 120° (Δt = 67 µs at 5 kHz) would produce a gain of 1 or no amplification of the resulting output as compared to using only one resonating wing. For example, the phase lags recorded from left and right harps (median values ranging from 6° to 79° across all specimens; this equates to Δt values between 3 and 43 µs; see **Supplementary Figures 1**, **2**) produce relative amplitude gains ranging from 1.97 to 1.34 (**Figure 5C**, blue stars). Other wing regions (mirror and anal regions, red circles and yellow squares, respectively), exhibit similar values.

Figure 5D illustrates the effect imperfect coupling of the wings has on the overall combined output amplitudes at different song carrier frequencies (assuming both wings vibrate with the same frequency and amplitude). While animals producing pure-tones at 5 kHz can afford to have relatively uncoupled wings with

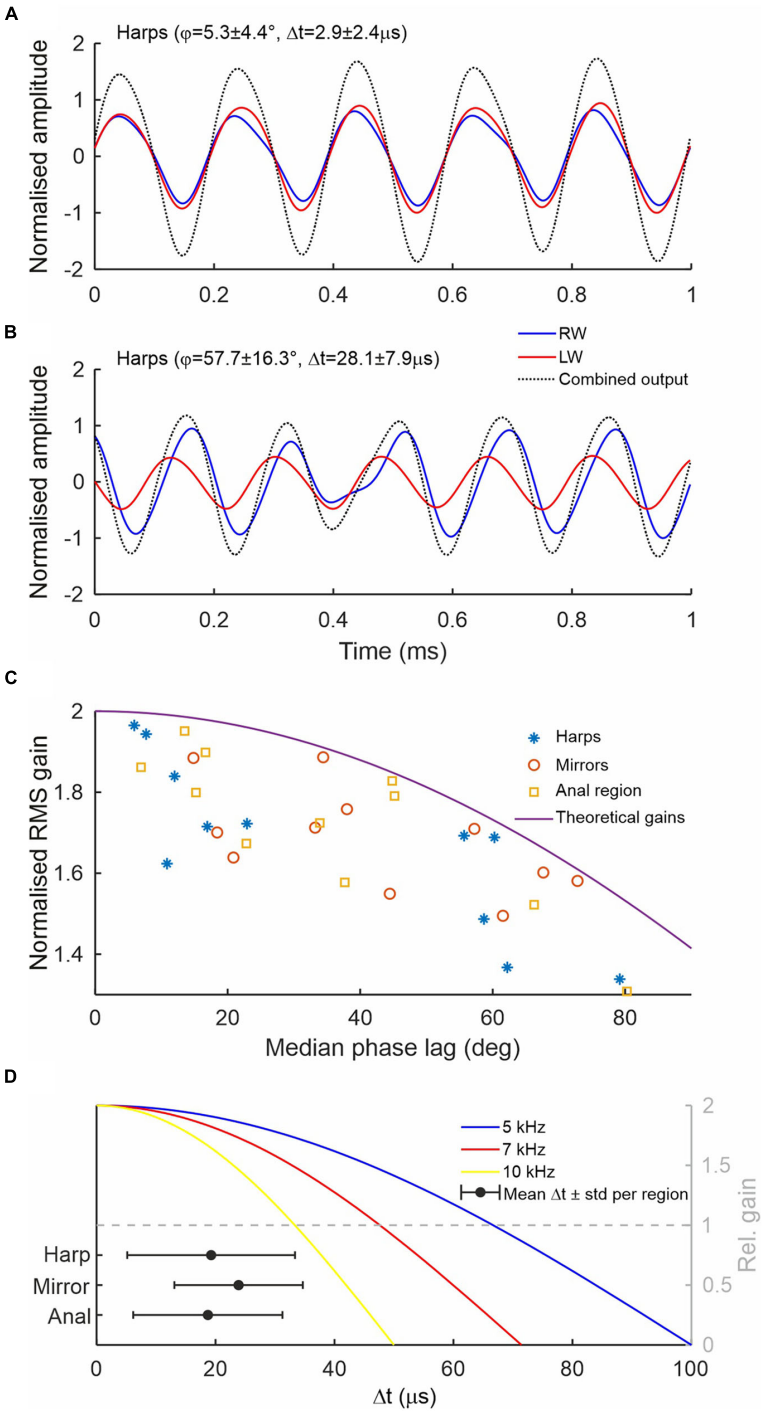

FIGURE 5 | Sound wave superposition to illustrate amplitude gains. **(A)** Theoretical harp output calculated from a *Gryllus bimaculatus* showing small phase differences between both harps ($\phi \sim 5°$; $\Delta t \sim 3$ μs; $f_c = 5.07$ kHz). **(B)** Harp output from an individual with large phase differences ($\phi \sim 58°$; $\Delta t \sim 28$ μs; $f_c = 5.7$ kHz). Note that in spite of large phase differences, the output (black dotted line) shows a gain, which is larger in A. In both cases, tracks have been normalised to the highest amplitude. **(C)** Comparison of median absolute phase lag per specimen and RMS gain of three major wing regions. Vibrations were obtained simultaneously from the paired respective regions (harps, mirrors, and anal) of LW and RW. RMS gains were calculated from the superposition of normalised LW and RW displacement responses measured with each laser. Each data point per region represents one individual; $n = 11$. The solid line shows theoretical gains with increasing ϕ assuming equal vibration amplitudes and frequencies. **(D)** Mean absolute time lags Δt (black circles) and standard deviation between LW and RW for three major wing regions and 11 animals. Coloured solid lines show the theoretical amplitude gains (right y-axis in grey; equal amplitudes and frequencies) as function of Δt for three different carrier frequencies (blue, red, and yellow for 5, 7, and 10 kHz, respectively). Values below 1 (dashed grey line) signify lower combined output amplitudes compared to using only one resonator.

time lags up to ∼67 μs before destructive interference occurs (**Figure 5D**, intersection of blue and grey dashed lines), Δt at which destructive interference starts is reduced to ∼48 and 34 μs when singing at 7 or 10 kHz, respectively (red and yellow lines). The inset in **Figure 5D** showing the average time differences and standard deviations between wings for the 11 specimens recorded shows that the span of Δt values (like ϕ) is generally small enough to ensure amplitude gains well over 1.5 when singing with a 5 kHz carrier frequency.

DISCUSSION

Here, we have revealed the presence of an elegant additional mechanism at work in crickets that contributes to generating high amplitude, pure tone signals using distinct yet coupled sound generators: the two forewings and their individual wing cells. Although the wings appear to be mirror images of each other (**Figure 1**), they are asymmetrical in their mechanical properties and structure (**Figure 2A**), as previously reported (Simmons and Ritchie, 1996; Bennet-Clark, 2003; Montealegre-Z et al., 2011). For *G. bimaculatus*, it is known that the RW on top is slightly larger in surface area and exhibits a higher f_0 than the LW (Montealegre-Z et al., 2011).

In addition, differences in resonant properties between both wings and among single wing regions are characterised in some detail. The biomechanical data demonstrate that, within a single wing, different regions have variable resonance peaks close to that of the harp f_0 value (apart from the mirror, which generally resonates several kHz higher) and overall resonance curves also differ in their spectral composition (**Figure 3**). Interestingly, the observed differences between both the individual wing regions and between the wings themselves (**Figure 3A**), vanish when the wings engage in active stridulation (**Figure 3B**). These results confirm for the first time that all regions of both wings actively radiate sound at the carrier frequency during stridulation and that the resonance properties of the LW dominate the frequency output. This suggests that, during stridulation, the LW harp vibrations, generated through plectrum-teeth impacts, drive the vibrations of all other wing regions, including those of the RW, so that the engaged wings vibrate together at the LW f_0.

In order to produce the best possible signal output from both coupled resonators, we hypothesised that both wings and the wing regions therein should not only oscillate at one common frequency, but also, ideally, in-phase ($\phi = 0°$), thereby creating maximal constructive interference (and thus a two-fold amplitude gain as compared to using only one wing). Whilst the whole system is indeed driven and oscillating at one specific frequency, we find considerable incoherence in the phase relationships between LW and RW and their respective regions. **Figures 4**, **5** clearly show that individual wing regions are not phase-locked to each other but exhibit average phase differences ϕ ranging from ca. 6° to 79°, equating to temporal differences Δt between the wings of 3–43 μs at the carrier frequency (f_c = 5.125 kHz). **Figure 4** and **Supplementary Figure 1** also show that individuals exhibit roughly similar phase differences within their wing regions but phase shifts between individuals are quite variable. This leads us to suggest that the ability to tightly control the wing movements and the coupling of the resonators is an individual trait depending on either wing morphology or neuro-muscular control of the stridulation process or a combination thereof. As a consequence, the phase differences ϕ and corresponding time lags Δt seen across the recorded individuals would approach the distribution of this trait over the population.

Figures 5A,B depicts the consequences of these phase shifts in two male crickets on the opposite sides of the range of observed ϕ. While the lower ϕ of Male 1 ($\phi = 5.3°$, $\Delta t = 2.9$ μs, **Figure 5A**) results in a considerable output gain in comparison to the individual harp amplitudes (ca. 1.85 times the highest LW amplitude), the higher phase differences of Male 2 ($\phi = 58°$, $\Delta t = 28$ μs, **Figure 5B**) result in only a moderate gain (ca. 1.3). For this animal, a further increase in ϕ and consequently Δt would result in destructive interference, whereby the combined output of both wings would be less than the output of one wing alone, negating the advantage of using coupled resonators. This is shown in more detail for three major wing regions over all animals in **Figure 5C**. It is noteworthy that no instance of destructive interference was observed in the specimens studied.

Figure 5D shows the effect frequency has on the overall gain of this imperfect coupling in the temporal domain. While a cricket singing at 5 kHz will experience an increase in combined output amplitude (gain $>$ 1, above dashed grey line, **Figure 5D**) for temporal differences between the wings of up to 67 μs (corresponding to a 120° phase shift and assuming equal vibration amplitudes), crickets singing at higher frequencies will encounter this threshold much earlier (at 48 μs and 33 μs for 7 kHz and 10 kHz, respectively). Consequently, the animals' observed inability to tightly synchronise the wing movements in time will act as an acoustic constraint for crickets to exploit higher song frequencies using two (imperfectly) coupled resonators. In addition, **Figures 5C,D** demonstrate that the observed imperfections in wing coupling in *G. bimaculatus* are still sufficiently low to ensure theoretical amplitude gains well above 1.5 times in comparison to the output of one wing alone. It is unknown, however, if ϕ and Δt are, for example, dependent on temperature. Due to the clockwork escapement mechanism involved in stridulation (and different from wing motion dynamics; Prestwich and Walker, 1981) tooth strike rates and f_c are largely independent of temperature in many Gryllidae, as are the resonant properties of the wings (Elliott and Koch, 1985; Bennet-Clark and Bailey, 2002). However, some species can show slight changes in f_c with temperature. Furthermore, the temporal song patterns, including syllable duration, are often affected by changes in ambient temperature (Walker, 1962; Pires and Hoy, 1992; Walker and Cade, 2003). It would therefore be conceivable that ϕ is also temperature dependent, potentially increasing with temperature and changes in singing behaviour. Further experiments including other cricket species and varying recording temperatures are planned to address inter-species variability and temperature dependence of the animals' wing coupling abilities.

If the higher values of Δt we observe in *G. bimaculatus* (**Figure 5D** for averages and SD; see **Supplementary Figure 2** for

a depiction of the range of observed values across all animals) are an indicator for the minimal amount of temporal control crickets in general are able to exert during stridulation, then one can attempt to calculate a cut-off frequency above which the sound production with two symmetrical and coupled wings becomes inefficient. The highest median value for ϕ we measured for the three wing regions were between 72° and 80°, equating to Δt values between 38 and 43 μs at f_c = 5.125 kHz. Using simple trigonometric relationships between phase, amplitude, Δt and frequency of waves and under the simplified assumption that both waves have the same frequency and amplitude, one can calculate the frequency $f_{\max}$ at which the gain of the combined output of the superimposed waves becomes 1:

$$f_{\max} = \arccos\left(\frac{Gain}{2}\right)/(\pi * \triangle t) \quad (1)$$

Using Eq. (1) and the range of Δt stated above, theoretical $f_{\max}$ values range from 7.8 to 8.8 kHz (for 43 and 38 μs, respectively), denoting frequencies above which stridulation using the mechanism described above becomes inefficient for some animals in the population. Taking the mean and standard deviation values for Δt shown in **Figure 5D** as rough population measure (harp: 19.3 ± 14.1 μs; mirror: 23.9 ± 10.8 μs; anal region: 18.7 ± 12.5 μs; see also **Supplementary Figures 2**, **3**), one could state that ∼16% of males would not be able to produce song above ∼10 kHz with an amplitude gain above 1 when using both wings as active resonators.

These cut-off frequencies correspond very well with maximal carrier frequencies observed in the majority of Gryllidae, which lie between 2 and 8 kHz (Bennet-Clark, 1989; Robillard et al., 2015). A notable exception are members of the subfamily Eneopterinae, which produce calling songs with frequencies of up to 26 kHz (Robillard et al., 2013). Interestingly, in this subfamily, there is a clear gap between species singing at low frequencies and species singing at high frequencies. This gap is located between 7.9 and 12.2 kHz and members of the high-singing species form a distinct clade within the Eneopterinae (the Lebinthini) (Desutter-Grandcolas and Robillard, 2004). Additionally, Robillard et al. (2013) found that these species exhibit resonance patterns and stridulation mechanisms quite different to the ones employed by other Gryllids and other Eneopterinae. Here, the resonances in the LW and RW are clearly asymmetrical, only partly (or not at all) overlapping the carrier frequencies and they generally show lower vibration magnitudes when compared to, e.g., the wings of *G. bimaculatus*. Furthermore, instead of employing constant tooth strike rates (like *G. bimaculatus* and most other Gryllids), some Lebinthini employ a stridulation mechanism (resembling those commonly observed in bush-crickets) whereby the wing stops during the closing phase to build up elastic energy which is then quickly released to produce highly increased tooth strike rates and therefore higher frequency calls (Robillard et al., 2013). These adaptations for high-frequency song production are similar to those encountered in bush-crickets. In bush-crickets, the wings are generally highly asymmetric as well, both morphologically and acoustically: The LW (lying on top of the RW and bearing the active stridulatory file) is often thicker, usually shows no clear stridulatory fields and is highly damped, therefore playing only a minor role in sound radiation (Montealegre-Z and Postles, 2010; Baker et al., 2017). The RW on the other side (which receives its mechanical input vie the plectrum) often exhibits extremely thin to translucent stridulatory fields with clear resonance properties, thus constituting the acoustically active wing (e.g., Sarria-S et al., 2014; Baker et al., 2017). Thus, the sound production system in Tettigoniidae only contains one resonator, reducing the surface for sound radiation, whilst eliminating the problems inherent to two imperfectly coupled resonators as described here for crickets. This allows for a shift to higher song frequencies (and shorter wavelengths) without destructive interference from a second resonator, and simultaneously ensures that the size of the remaining resonator is still (closer to) optimal for pure tone sound radiation.

In conclusion, the results presented here suggest a mechano-acoustical constraint on the bilateral near-symmetrical, dual resonator sound production mechanism common to most Gryllidae which prevents the exploitation of higher song frequencies above ∼8–9 kHz whilst still being able to produce loud and pure-tone calling songs to effectively attract mates. This could have been an important constraint for the majority of Gryllidae (restricting them to the role of tenors) which the Tettigoniidae (the sopranos within the Ensifera) seem to have overcome by evolving a highly asymmetric singing mechanism (Montealegre-Z et al., 2017; Song et al., 2020) which allows them to produce high-frequency songs without the drawback of undesirable destructive interference reducing song amplitude.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

FM-Z, TJ, and DR designed the research and wrote the manuscript. FM-Z and TJ performed the experiments. CS conducted the statistics. FM-Z, TJ, and CS analysed the data. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.647786/full#supplementary-material

Supplementary Video 1 | A male *Gryllus bimaculatus* producing calling song in the experimental setup after pharmacological injection of Eserine (10^{-2} mol/l) into the brain. The cricket is mounted and fixed on a holder in front of the LDV. The LDV's laser dot is visible on the harp area of the right wing.

Supplementary Video 2 | Animation of the vibration map of unengaged left and right wing of a male *Gryllus bimaculatus* as derived from LDV recordings. The wings are elevated upward from the animal's body at a similar angle to the natural singing position, spaced apart and imaged from the front; the reference microphone is visible between and slightly behind the wings. The overlaid vibration map shows the colour-coded relative displacement (μm/Pa; red = max. positive displacement; blue = max. negative displacement) of the wing surface as a response to acoustic stimulation at the wings' overall resonance frequency (4.62 kHz). Here, the LW displacement amplitude is higher than the RW's.

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Convergent Evolution of Wingbeat-Powered Anti-Bat Ultrasound in the Microlepidoptera

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Bats and moths provide a textbook example of predator-prey evolutionary arms races, demonstrating adaptations, and counter adaptations on both sides. The evolutionary responses of moths to the biosonar-led hunting strategies of insectivorous bats include convergently evolved hearing structures tuned to detect bat echolocation frequencies. These allow many moths to detect hunting bats and manoeuvre to safety, or in the case of some taxa, respond by emitting sounds which startle bats, jam their biosonar, and/or warn them of distastefulness. Until now, research has focused on the larger macrolepidoptera, but the recent discovery of wingbeat-powered anti-bat sounds in a genus of deaf microlepidoptera (*Yponomeuta*), suggests that the speciose but understudied microlepidoptera possess further and more widespread anti-bat defences. Here we demonstrate that wingbeat-powered ultrasound production, likely providing an anti-bat function, appears to indeed be spread widely in the microlepidoptera; showing that acoustically active structures (aeroelastic tymbals, ATs) have evolved in at least three, and likely four different regions of the wing. Two of these tymbals are found in multiple microlepidopteran superfamilies, and remarkably, three were found in a single subfamily. We document and characterise sound production from four microlepidopteran taxa previously considered silent. Our findings demonstrate that the microlepidoptera contribute their own unwritten chapters to the textbook bat-moth coevolutionary arms race.

Keywords: bat-moth arms race, acoustic mimicry, micromoths, Tineidae, Oecophoridae, Depressariidae, *Yponomeuta*

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INTRODUCTION

Roeder's seminal discovery of anti-bat hearing (Roeder and Treat, 1957) sparked research into the defences of nocturnal moths against echolocating bats in the bat-moth acoustic evolutionary arms race. Many nocturnal insects, including moths, have evolved hearing structures to detect bats (e.g., Miller and Surlykke, 2001) and the Arctiinae (tiger moths) are well known for their defensive sounds that function through startling their predators, acoustic aposematism (warning

sounds), and/or echolocation jamming (e.g., Corcoran et al., 2010). However, a recent surge of new discoveries has arisen in this arms race: taxa other than the Arctiinae have been shown to produce anti-bat sounds (Barber and Kawahara, 2013; Corcoran and Hristov, 2014; O'Reilly et al., 2019), the hindwing "tails" of some moths have been discovered to act as acoustic decoys (Barber et al., 2015; Lee and Moss, 2016), and the acoustic absorptive power of moth scales as acoustic metamaterials has emerged as a fascinating and complex new area of research (Zeng et al., 2011; Ntelezos et al., 2017; Shen et al., 2018; Neil et al., 2020a,b). This spate of recent discoveries suggests the true extent of moth anti-bat adaptations might substantially exceed current knowledge.

Lepidoptera have been crudely divided by their size into two suborders: the smaller micro- and the larger macrolepidoptera. Most research into the anti-bat defences of moths has focussed on the macrolepidoptera, yet preferred prey size varies both within (Waters et al., 1995) and between bat species. Some species such as *Myotis septentrionalis*, rely heavily on microlepidoptera as dietary constituents (e.g., Dodd et al., 2012). Microlepidoptera are therefore also under significant predation pressure from bats.

It would seem highly likely that such pressure on the microlepidoptera would lead to the evolution of anti-bat defences analogous to those found in macrolepidoptera. However, research into such defences has seemingly just recently begun, with only two studies, other than those investigating the well-known pyralid hearing (e.g., Skals and Surlykke, 2000), addressing the subject. Firstly, Kovalev (2016) suggested that the feather-like wing plumes of *Alucita hexadactyla* (Alucitidae) may have evolved to reduce its echo intensity, and secondly O'Reilly et al. (2019) discovered that the hyaline (transparent) hindwing patches of the microlepidopteran genus *Yponomeuta* (Yponomeutidae) are wingbeat-powered aeroelastic tymbals (ATs) that render these deaf moths acoustic Müllerian mimics of aposematic Arctiinae.

Like the sound-producing tymbals located on the thorax of the macrolepidopteran Arctiinae, *Yponomeuta* ATs produce two bursts (one longer and one shorter burst) of ultrasonic clicks through buckling of a series of striations. However, unlike arctiine sound production, AT clicks are not produced upon detection of an approaching bat. Instead, they occur during every wingbeat, one burst per wing stroke. As these moths are deaf and unable to detect and respond to hunting bats, these structures allow them to bypass predator detection by constantly producing warning sounds. *Yponomeuta* provide the first example of constitutive acoustic aposematism in the bat-moth arms race (O'Reilly et al., 2019). This elegant defence solution for unpalatable, deaf microlepidoptera is unlikely to be exclusive to the Yponomeutidae and here we specifically investigate whether other microlepidopteran taxa possess yet undocumented defences based on ATs.

As the AT of *Yponomeuta* reveals itself as a hyaline patch in the wing, hyaline patches in other microlepidopteran taxa might suggest similar acoustic functionality. The presence of hyaline wing patches is indeed not exclusive to *Yponomeuta*. For example, *Monopis*, *Crypsithyrodes*, and *Crypsithyris* (Tineidae) species are characterised by a subhyaline patch in the discal cell of the forewing (Robinson, 1980; Xiao and Li, 2005; Lee et al., 2016), and members of the *Tinea pellionella* species complex (Tineidae) possess hyaline/subhyaline patches at the base of the forewing, just below the *subcosta* (Robinson, 1979). Generally, hyaline wing patches, such as the above examples, are only documented in the literature if they serve as identification features.

Given that microlepidoptera are under significant predation pressure from echolocating bats, and that ATs provide an elegant method of passive acoustic protection, we anticipated that these structures would be taxonomically widespread. Thus, through a comprehensive morphological assay as well as acoustic characterisation, we investigated our prediction that ATs have convergently evolved throughout the microlepidoptera.

MATERIALS AND METHODS

Phylogenetic Spread of Candidate ATs

Image Analysis

For each of our two phylogenetic analyses we assessed taxa for the presence of known and candidate ATs. This was primarily achieved by examining online image databases of microlepidoptera. The majority of photographs were assessed from the Barcode of Life Database (BOLD) (Ratnasingham and Hebert, 2007), but microscopic assessment of specimens from the Bristol Museum and Art Gallery and the Natural History Museum, London was also used.

A *known AT* was defined as a hyaline patch in the same position on the wing as related taxa known to produce wingbeat-powered sound, e.g., a hindwing hyaline patch in an *Yponomeuta* species or relative. A *candidate AT* was defined as a hyaline patch on the wing with no obvious other function. If possible, for every species suspected of possessing an AT, multiple specimens were assessed to confirm the presence of the structure. This aided in preventing false positives due to symmetrical specimen damage.

Candidate ATs in the Microlepidoptera

This comprehensive assessment of the presence of ATs includes all microlepidopteran taxa from the 11 superfamilies in a recent molecular phylogeny of the Lepidoptera (Regier et al., 2013), from Nepticuloidea up to and including Gelechioidea. Despite the Pyraloidea being considered microlepidoptera, they were excluded from this analysis, because they have ultrasound sensitive ears, which constant sound production by ATs would excite and habituate, rendering the combination of ears and AT counterproductive. Furthermore, any transparent areas of the wings of the Sesiidae were not considered as potential ATs as they more likely function as part of their visual mimicry of Hymenoptera.

We used a simplified phylogenetic tree based on Figure S1 from Regier et al. (2013) for this study, to identify likely points of independent evolution of ATs within the microlepidoptera. The genus *Monopis* (Tineidae) and the Scaeosophinae (Cosmopterigidae) were not included in the original phylogeny (Regier et al., 2013) but were found to possess candidate ATs, so they were includes in our analysis.

Candidate ATs in the Tineidae

Photographs of 751 species (148 genera) of Tineidae were assessed. Between one and 20 photographs (individuals) were assessed per species for the presence of hyaline patches on either the forewing or hindwing. In the case of the genus *Chrypsithyris* (four species) images and species descriptions were used from Xiao and Li (2005). Images for *Niditinea sabroskyi* were used from (Metz et al., 2018).

Phylogenetic Analysis

As far as we are aware, a detailed molecular phylogeny of the Tineidae does not exist; thus, using publicly available data, a phylogenetic tree was inferred using the Cytochrome Oxidase Subunit 1 (COI) amino acid sequence from 90 species from 19 genera of the Tineidae family. *Dolophilodes distinctus* (Philopotamidae: Trichoptera) was used as an outgroup. Sequences were downloaded from BOLD Systems (Ratnasingham and Hebert, 2007), aligned using MAFFT version 7 (Katoh and Standley, 2013), and the alignments were trimmed using BMGE 4.0, using a BLOSUM62 matrix to remove poorly aligned positions (Criscuolo and Gribaldo, 2010). The phylogenetic tree was inferred using IQ-Tree (Nguyen et al., 2015), and the best-fitting substitution model (LG+C60+G) was selected by Bayesian Information Criterion (Le and Gascuel, 2008). Moreover, empirical profile mixture models were used to improve model fit (Quang et al., 2008). The bootstrap supports were estimated using UFBoot2 (Minh et al., 2013). Trees were visualised and edited in ITOL (Letunic and Bork, 2016). For links to software used see **Table 1**.

Sound Production by Candidate ATs

Species

We gained access to live specimens of five relevant species to test in laboratory conditions: two Tineinae (Tineidae) species possessing candidate ATs (*Monopis crocicapitella*, Clemens, 1859 $n = 2$; and *T. pellionella*, Linnaeus, 1758 $n = 7$), one Tineinae species lacking candidate ATs (*Tineola bisselliella*, Hummel, 1823 $n = 6$), one Oecophoridae species possessing candidate ATs (*Endrosis sarcitrella*, Linnaeus, 1758 $n = 4$) and one lacking them (*Hofmannophila pseudospretella*, Stainton, 1849 $n = 2$), which were all tested for sound production. All *T. pellionella* specimens were wild caught from three houses in Bristol, United Kingdom, all *T. bisselliella* specimens were taken from a wicker basket found in Bristol, United Kingdom, both *M. crocicapitella* specimens were caught at one location in Weston-Super-Mare, United Kingdom using a mercury vapour moth trap in a suburban garden, and all *E. sarcitrella* and *H. pseudospretella* were caught from two houses within Bristol, United Kingdom. Moths were either acoustically assessed immediately or kept in a refrigerator between 4 and 6°C for up to 24 h before beginning assessment. If refrigerated, moths were kept at room temperature for at least 2 h prior to testing.

TABLE 1 | Resources used in the creation of the Tineidae phylogeny.

Resource	Source	Web link
Data	NCBI	https://www.ncbi.nlm.nih.gov/protein/
Data	BOLD Systems	http://www.boldsystems.org/
MAFFT	Katoh and Standley, 2013	https://mafft.cbrc.jp/alignment/software/
BMGE	Criscuolo and Gribaldo, 2010	http://gensoft.pasteur.fr/docs/BMGE/1.0/BMGE_doc.pdf
IQ-Tree	Nguyen et al., 2015	http://www.iqtree.org/
LG Model	Le and Gascuel, 2008	http://www.atgc-montpellier.fr/models/index.php?model=lg
Mixture Models	Quang et al., 2008	https://academic.oup.com/mbe/article/25/7/1307/1041491
ITOL	Letunic and Bork, 2016	http://itol.embl.de/

Tethering Method

Moths were first recorded in free flight, if they did not produce sound, they were not studied further, if they did then they were subsequently tethered. Due to their small size, we tethered them following O'Reilly et al. (2019): a 0.14 mm diameter insect pin was inserted into the dorsal meso/prothorax until the tip just punctured the ventral side. Like the moths tested in O'Reilly et al. (2019), all test specimens flew for prolonged periods post tethering.

The head of the tether (insect pin) was inserted into modelling clay attached to a flexible arm (Manfrotto + Co. Spa, Cassola, Italy), which allowed flexible positioning of the moth. We positioned the moth upside down, which elicited more prolonged flight compared to normal orientation. This stronger flight is probably due to the unusual gravitational pull on the insect causing it to try and return itself to its natural flight orientation.

Audio Recordings

All audio recordings of *M. crocicapitella*, *T. pellionella*, and *E. sarcitrella* (16bit, sampling rate 500 kHz) were made using USG Omnidirectional Electret Ultrasound Knowles FG-O microphones connected to an UltraSoundGate $1216H^{200}$ recorder, run through Avisoft Recorder USGH software (all Avisoft Bioacoustics, Berlin, Germany). Recordings were made in a semi-anechoic chamber (Industrial Acoustics Company Ltd., Winchester, United Kingdom).

Individual moths were initially placed in a $24'' \times 24'' \times 24''$ BugDorm-1 Insect Rearing Cage (Megaview Science Co., Ltd., Taichung City, Taiwan) with one microphone positioned through a central sleeved hole on one side of the cage. Flight was initiated through flicking or tapping the cage where the insect was at rest. These free-flight recordings were initially analysed for the presence of any acoustic signal. If sound production was discovered, tethered recordings were subsequently made. For tethered recordings, the insect was positioned 30–50 mm from a microphone oriented perpendicular to the centre of the lateral axis of the moth. To reliably initiate flight, tethered moths were first given a small (∼5 mm diameter) ball of paper or foam to hold, this was removed when flight was required.

Additionally, one *Monopis cf monachella* (Hübner, 1796, collected in Germany) was assessed for free-flight sound production using an ultrasound bat detector, and one *Ethmia bicolorella* (Guenée, 1879, Depressariidae, collected in Kenya) specimen was recorded in free-flight using a USB ultrasonic microphone (Ultramic250K, Dodotronic, Italy) in 16 bit using a 250 kHz sampling rate. This recording was made in the field, as such it was only analysed for the presence/absence of ultrasonic click production. Four other *Ethmia* (*E. sabiella, E. oculigera, E. cascineutis,* and *E. livida*; Felder et al., 1875; Möschler, 1883; Meyrick, 1913; Zeller, 1852, respectively) *species* were collected and recorded in South Africa using the same methods. Recordings of these species were assessed for the presence or absence of ultrasonic clicks.

Ablation Experiments

Ablation of the candidate ATs was attempted on all individuals of *M. crocicapitella, T. pellionella*, and *E. sarcitrella*, however, due to the small size of the moths and their hyaline patches, this proved difficult. In all but one individual of each of the two Tineinae species the ablation attempt resulted in enough damage to the wings to render them unable to fly. Therefore, ablation results were only taken from one individual of each Tineinae species. *E. sarcitrella* patches were more fragile than those of the Tineinae, therefore a cruder method of ablation (removal of the hindwings) was initially used to confirm the general location of the sound producer (n = 1). More specific ablation attempts, similar to those used on Tineinae, were unsuccessful in the three remaining *E. sarcitrella* individuals. For the successful ablations of *M. crocicapitella* and *T. pellionella*, recordings were made from two treatments for each moth, firstly the right hyaline patch was ablated, and secondly the left hyaline patch was ablated.

Tineinae ablation was achieved using a size 0.14 mm diameter insect pin under a 50× magnification dissection microscope (Leica EZ5 Stereo Microscope, Leica Microsystems, Wetzlar, Germany). Moths were anaesthetised using CO_2 and secured to foam by placing insect pins in a cross over (not penetrating) both the abdomen and head of the insect, as well as individual pins over the fore and hindwings to hold them extended from the body, thus exposing the hyaline patches. The patch was then punctured with an insect pin and the membrane removed using fine forceps and microdissection scissors. All pins were removed, and the insect was positioned within the recording set-up, holding a small piece of paper or foam. It was left for between 15 and 120 min to recover and checked every 15 min for pre-ablation flight behaviour, and post-ablation recordings were subsequently made.

Acoustic Analysis

We analysed all acoustic recordings using Avisoft SASLab Pro (version 5.2.07, Avisoft Bioacoustics, Berlin, Germany), measuring the following acoustic characteristics for each species: source level, peak frequency, high and low frequency (bandwidth), click detection distance, shorter click burst click duration, longer click burst click duration, duty cycle, number of clicks per burst, and number of clicks per wingbeat. We analysed 10 consecutive wingbeats from a steady flight period with consistently high amplitude click bursts for each individual.

Acoustic characteristics were determined using the following methods. For each individual, click bursts from ten consecutive wingbeats were analysed. There are two click bursts per wingbeat cycle, defined here as longer and shorter as we did not confirm which burst occurred during the up- and down-stroke, respectively. We counted all clicks and further analysed the loudest click from each longer click burst. Click number was determined by totalling the number of clicks discernible in waveform for each of the two click bursts per wingbeat. The duration of each individual click was measured from the waveform. Click amplitude was determined following O'Reilly et al. (2019), by initially calculating the peak-to-peak sound pressure and converting it to dB peSPL, using a calibration tone from a signal generator which produced a constant tone of known amplitude at 40 kHz (Avisoft Bioacoustics, Berlin, Germany) and the following formula:

$$\mathrm{CA} + 20{*}\log_{10}\left(\frac{\mathrm{TS}}{\mathrm{CS}}\right)$$

$$\mathrm{CA} = \text{Calibration Tone Level (dB)}$$

$$\mathrm{TS} = \text{Test Signal Amplitude (mPa)}$$

$$\mathrm{CS} = \text{Calibration Signal Amplitude (mPa)}$$

Clicks were extracted from the waveform individually for spectral analysis including 0.05 ms of margin noise, with silent margins added (zero padding) and linearly ramped into the noise. Peak frequency and bandwidth (high and low frequencies) were measured from power spectra (Hamming window size 1024), high and low frequencies were determined as the frequencies −15 dB either side of the peak frequency.

Monopis crocicapitella, *T. pellionella*, and *E. sarcitrella* click detection distances by bats were calculated from the loudest click from each of the longer bursts from ten successive wingbeats. Following O'Reilly et al. (2019) click peak frequency and amplitude (dB peSPL) were used to calculate the distance at which these sounds could be detected by bats, using a 10 dB SPL bat hearing threshold.

$$\mathrm{CSL} - 20{*}\log_{10}\left(\frac{\delta - \delta\mathrm{ref}}{\delta\mathrm{ref}}\right) - \mathrm{FDA}{*}\left(\delta - \delta\mathrm{ref}\right) = \mathrm{HT}$$

$$\mathrm{HT} = \text{hearing threshold} = 10 \text{ dB SPL}$$

$$\mathrm{CSL} = \text{ClickSource Level}\left(\mathrm{dBpeSPLatref}\right)$$

$$\delta = \text{Distance (m)}$$

$$\delta\mathrm{ref} = \text{Reference Distance} = 0.1\mathrm{m}$$

$$\mathrm{FDA} = \text{Frequency Dependent Attenuation}\left(\mathrm{dBm}^{-1}\right)$$

Additionally, video footage was taken using a smartphone at 120 fps of *T. pellionella*, *M. crocicapitella*, and *E. sarcitrella* in order to determine their wingbeat frequency, so it could be linked to the frequency of click burst production. The quality of these videos was sufficient to determine wingbeat frequency, but did not provide clarity or frame rates capable of creating useful synchronised high-speed video and audio recordings.

Hearing Tests

Prior to free-flight recordings, every moth in the flight cage was exposed to an ultrasonic stimulus known to elicit the anti-bat behaviours of moths with hearing capabilities (St. Juliana et al., 2007), at a distance of around one metre from the centre of the cage. Moths were exposed to the stimulus both at rest and during flight, and their behaviours observed. The observer was not blind to the treatment, as personnel availability and time constraints with limited numbers of live animals made this impractical. A Dazer II Ultrasonic Dog Deterrent (Dazer International, London, United Kingdom) was used as the stimulus; it produces a 25 kHz tone at 118.1 dB SPL (at 0.1 m). Reactions were defined as a sudden cessation of flight, or any other typical anti-bat escape/avoidance manoeuvre (Miller and Surlykke, 2001), or twitching, commencement of flight, or dropping from its perch if the moth was at rest.

If multiple individuals of the same species were caught on the same day, they were placed in the BugDorm-1 together and their behaviour in response to flight, and therefore sound production, of other individuals was observed. This was possible once each for *M. crocicapitella* (two individuals), *T. pellionella* (two individuals), and *E. sarcitrella* (two individuals).

RESULTS

Phylogenetics

Phylogenetic Spread of ATs in the Microlepidoptera

Photographs or museum specimens of 19,596 species (2,440 genera, 50 families, 11 superfamilies) were morphologically examined for the presence of candidate ATs in the form of hyaline patches. The results were plotted on a simplified version of Regier et al.'s (2013) lepidopteran phylogeny (**Figure 1**). Candidate ATs were found throughout the microlepidoptera in nine of the eleven superfamilies assessed (**Figure 1**).

We identified ATs in four different locations on microlepidopteran wings (**Figure 2**) and named them as follows: (1) Forewing Subcostal Tymbal (FST) at the forewing base between the subcostal and radial veins in the cell directly above the discal cell (blue); (2) Forewing Discal Tymbal (FDT) directly within the apex of the discal cell itself (orange); (3) Forewing Cubital Tymbal (FCT) in the cell directly below the first cubital veins (red); and (4) Hindwing Cubital Tymbal (HCT) at the base of the hindwing in the cell directly below first cubital veins (green).

Phylogenetic Spread of ATs in the Tineidae

Analysis of 751 species from 148 genera in 14 subfamilies (as assigned by BOLD systems) of the Tineidae revealed that hyaline patches, likely to be ATs, were present in at least 46 species in eight genera, within the family, seven of which were in the subfamily Tineinae. The Tineidae contain examples of ATs in all four wing locations.

Forewing Subcostal Tymbals are present in 11 of 38 species of *Tinea* as well as one of two *Praeacedes* and four of five *Niditinea* species examined, including the newly discovered species *N. sabroskyi* (Metz et al., 2018). FSTs in the Tineinae vary in relative size, with the more conspicuous examples being found in *Tinea steueri* (Robinson, 1979), whereas, species such as *Tinea dubiella* possess much smaller structures.

Forewing Discal Tymbals are present in all *Monopis* analysed (26 species) as well as the genera *Crypsithyrodes* (one species) and *Crypsithyris* (four species), *Tinea unomaculella* possesses a light spot in the same area but we believe this is colouration not a tymbal. FDTs can vary in their size, shape (relatively round to elongated), and their location on the wing in terms of their position along the wing tip to base axis. Nevertheless, the structures always appear to be situated within the discal cell of the forewing and their position is likely due to differences in the length of this cell.

Forewing Cubital Tymbals are small (∼1 mm in length) hyaline patches near the base of the forewing, likely between veins Cu_{1b} and Cu_2 (**Figures 2**, **3**), and are limited to the genus *Trichophaga*, present in at least three of the six species analysed. Additionally, the previous analysis using the Regier et al. (2013) phylogeny revealed HCTs in the Erechthiinae genus *Erechthias*. This genus was not, however, included in our phylogenetic analysis of the Tineidae.

The maximum likelihood tree (**Figure 3**), groups all *Monopis* as a single clade, with the three *Trichophaga* species forming a sister clade. The FST-possessing species do not form a single clade but are instead split into two main clades with one species, *T. trinotella*, placed away from these two groups. The first of these two clades exclusively contains *Niditinea* and *Praeacedes* species, and the second exclusively *Tinea* species. The second, with the addition of *T. columbariella* and *T. niveocapitella*, consists of the already established *T. pellionella* species complex (Robinson, 1979). *Tineola bisselliella* (no hyaline patch) is placed as the only non-*Tinea* species in a clade containing mostly FST-possessing species.

Acoustics

Microlepidopteran Sound Production

Live specimens from three Tineinae and two Oecophoridae species were available for acoustic testing. All three species possessing hyaline patches, *M. crocicapitella* (forewing patch), *T. pellionella* (forewing patch), and *E. sarcitrella* (*Yponomeuta*-like hindwing patch), produced two bursts of broadband ultrasonic clicks with every wingbeat (**Figure 4**). These clicks are acoustically similar to the in-flight clicks produced by the ATs of *Yponomeuta* species (Yponomeutidae) (O'Reilly et al., 2019) in

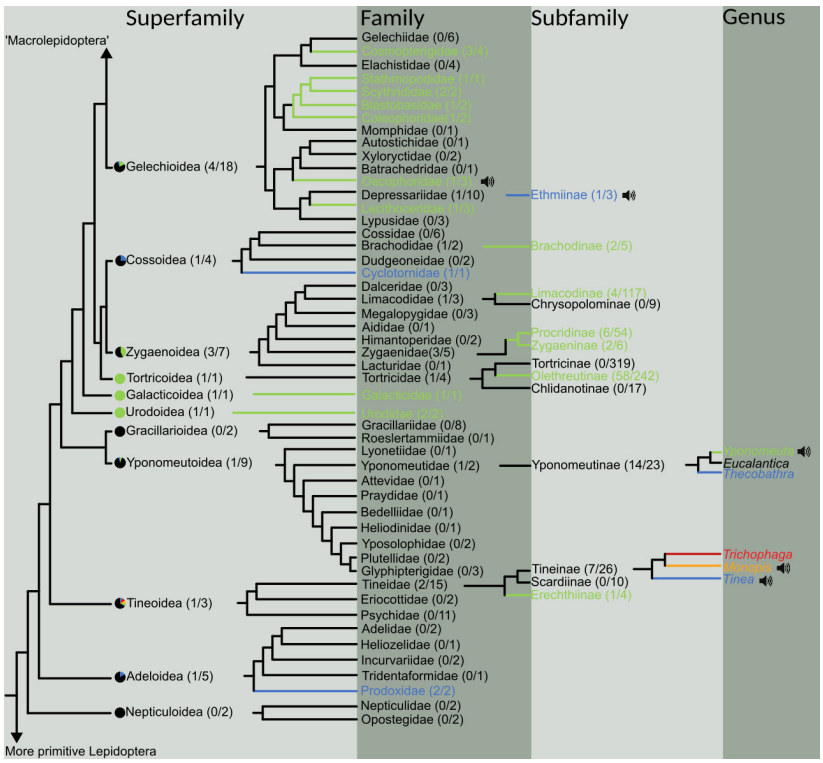

FIGURE 1 | Phylogeny of the "microlepidoptera" (here defined as taxa below and including the superfamily Gelechioidea, and above and including Nepticuloidea) adapted from Regier et al. (2013). The spread of aeroelastic tymbals (ATs) is represented at various taxonomic levels, beginning with superfamilies and ending in genera. For each taxonomic level above genus, the fraction of subtaxa possessing ATs is given in parentheses. Following superfamily, if ATs are present, all families are presented, and then only relevant subfamilies (i.e., possessing ATs or showing evolutionary relationships). In subfamilies with multiple types of AT (see **Figure 2**), a genus tree is presented to show evolutionary relationships. Pie charts before the superfamilies represent the ratio of families containing ATs. Slice colours correspond to the location of the AT on the wing and match **Figure 2**, and black represents no obviously detectable structure. Taxa names and their branches are coloured if they contain examples of ATs, and colours again correspond to the locations in **Figure 2**. Black speaker icons indicate that those AT-possessing taxa have been recorded producing sound in flight (see **Figure 4**).

that they show a bimodal regularity (likely two different bursts per wingbeat, one on the up- and one on the down-stroke), they exclusively occur during flight, and the two bursts differ in duration (**Figure 4**).

Both species lacking hyaline patches, *T. bisselliella* and *H. pseudospretella*, did not produce any acoustic emissions during flight. Males of *T. bisselliella* are known to produce low frequency substrate-borne sounds (Takács et al., 2003), yet we were not attempting to record such substrate-borne vibrations.

Successful ablation of the hyaline patches of both *M. crocicapitella* and *T. pellionella* (n = 1) eliminated sound production, whilst ablation of one hyaline patch, leaving the other intact, effectively halved the number of clicks produced per wingbeat, 22.95 ± 3.4 and 3.8 ± 0.9 pre ablation, and 12.9 ± 1.1 and 2.2 ± 0.4 post ablation (mean ± SD) for *M. crocicapitella* and *T. pellionella*, respectively. Removal of both *E. sarcitrella* hindwings eliminated sound production.

One *Monopis cf monachella* individual was found to produce ultrasonic emissions during flight using an ultrasonic bat detector, and recordings from one *E. bicolorella* in free flight in Kenya confirm alternating bursts of ultrasonic clicks characteristic of AT sound production (**Figure 4D**). Additionally, all four South African *Ethmia* species recorded in free flight produces similar bursts of clicks (data not shown).

Acoustic Characterisation of AT Sounds

We analysed 20 bursts and 10 individual clicks from *M. crocicapitella*, *T. pellionella*, and *E. sarcitrella* for amplitude, spectral, temporal, and duration information. In addition, we calculated the distance at which bats could detect these clicks (**Table 2**). All three species produce relatively loud (64.6, 56.9, and 54.0 dB peSPL at 0.1 m, respectively) ultrasonic, broadband clicks (41.2–111.7, 54.3–125.1, and 45.8–128.9 kHz, respectively) with high peak frequencies (88.1, 92.1, and 100.0 kHz, respectively) (**Table 2**).

The sounds of all three moth species fall within the known frequency range of anti-bat sounds of the Arctiinae, Sphingidae, Geometridae, and Yponomeutinae (Corcoran et al., 2010; Barber and Kawahara, 2013; Corcoran and Hristov, 2014; O'Reilly et al., 2019). Their low duty cycles (**Table 2**) are also similar to some of the aposematic signalling Arctiinae such as *Cosmosoma stibasticta* and *Amplicincia* near *mixta* (Corcoran et al., 2010). Example ultrasonic click burst recrodings of *Endrosis sarcitrella,*

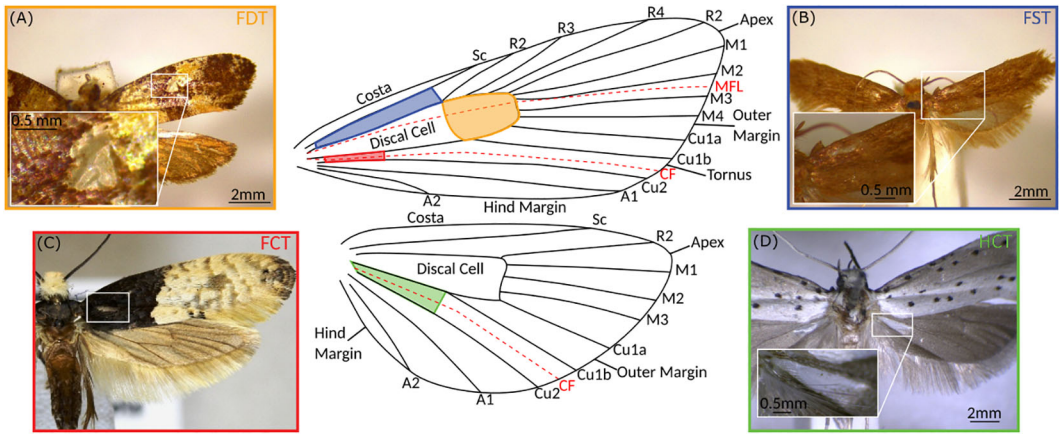

FIGURE 2 | Aeroelastic tymbals of the microlepidoptera. Typical examples of macrolepidopteran aeroelastic tymbals for each of the four locations in which they are found on the wings. **(A)** Forewing Discal Tymbal (FDT) represented by *Monopis crateroxantha* (Meyrick, 1927; Tineidae), **(B)** Forewing Subcostal Tymbal (FST) represented by *Tinea pellionella* (Linnaeus, 1758; Tineidae), **(C)** Forewing Cubital Tymbal (FCT) represented by *Trichophaga tapetzella* (Linnaeus, 1758; Tineidae), and **(D)** Hindwing Cubital Tymbal (HCT) represented by *Yponomeuta cagnagella* (Hübner, 1813; Yponomeutidae). The colours of the photograph borders correspond to the location of the tymbal on the generalised Lepidopteran wing in the centre (modified from Watson and Dallwitz, 2003 onward), as well as the colours used in **Figures 1**, **3**. Shaded areas show locations of tymbals, and dashed red lines represent flexion lines in the wing, the median flexion line (MFL) or the "fold" and the claval furrow (CF). Vein labelling: Sc (Subcosta), R (Radial), M (Medial), Cu (Cubital), and A (Anal), followed by vein number. Photograph **(C)** was taken from BOLD Systems (Ratnasingham and Hebert, 2007), the photographer was Marko and the image has a CC0 licence.

Ethmia bicolorella, Monopis crocicapitella, and Tinea pellionella can be found in **Supplementary Audio 1–4** respectively.

Hearing Tests

All live individuals (excluding *E. bicolorella*) were exposed to a sound source known to elicit the anti-bat behaviours in insects with hearing capabilities (St. Juliana et al., 2007). No individual of any species showed any reaction, such as cessation or initiation of flight, sudden movement, or any change in flight direction. Additionally, when the insects were obtained as groups of two or more individuals, they were housed together and no individual was observed reacting to flight, and therefore sound production, of the other ($n = 2$ for all tested species).

DISCUSSION

Distribution of ATs in the Microlepidoptera

Morphological analysis of 11 superfamilies highlighted that ATs are widely distributed in microlepidoptera (**Figure 1**). However, when we analysed the presence and absence of ATs on a subfamily and genus level it was apparent that ATs were a lineage-specific innovation, and that ATs can vary in both location and size. The exact number of evolutionary events is unclear; however, given we showed four different ATs in four locations on the wing (**Figures 1**, **2**). When mapped onto a phylogenetic tree, the distribution of ATs either suggests multiple evolutionary events, or significant lineage specific losses of this organ, with the former being the most parsimonious explanation for the evolutionary history. We could confirm sound production in five independent ATs (Yponomeutinae, *T. pellionella* and *E. bicolorella* FSTs, *Monopis* FDTs, and *E. sarcitrella* HCTs). These ATs were found in three different wing regions, two of which had not been documented as sound producing structures before. Our analysis suggests a remarkable example for multiple convergent evolution. The fact that all candidate ATs we were able to test with live specimens indeed produced sound, inspires confidence in the validity of our approach to identify ATs using hyaline wing patches. This does not confirm that a lack of scales is a prerequisite for an AT though.

Aeroelastic Tymbal Morphology and Function

The positioning of ATs on the wing may provide some insight into how they function. Interestingly, all four AT locations place them near flexion lines (**Figure 2**). Flexion lines are lines along which an insect wing shows flexibility (folding) during the wingbeat. The claval furrow is a flexion line found in most insect wings, and the median flexion line (sometimes referred to as the "fold") is found in the forewing (and occasionally hindwing) of many insect taxa, and usually runs between the medial and radial veins (Dudley, 2000).

The hindwing claval furrow appears to play a role in *Yponomeuta* HCT actuation (O'Reilly et al., 2019), and thus it is reasonable to assume it has similar importance in other taxa with HCTs. FCTs are located analogously to *Yponomeuta* ATs but in the fore- not hindwing, and thus, if these are sound producers, the claval furrow is again likely to play a role in actuation.

The median flexion line is not always present in insect wings, and its position on the wing when present can vary between taxa (Wootton, 1979); however, its normal location transects the discal cell and therefore FDTs. Additionally, FSTs are near the median

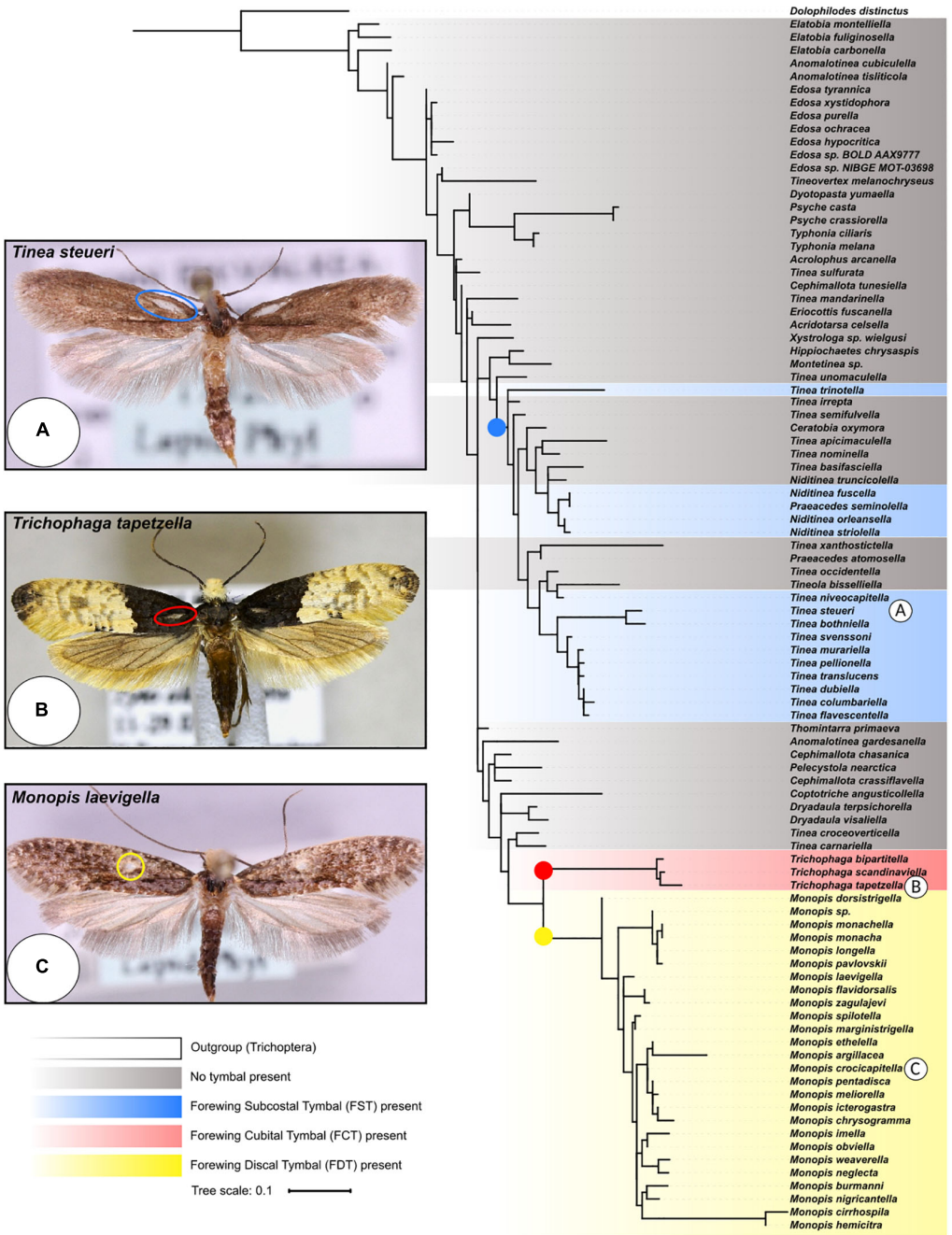

FIGURE 3 | Rooted phylogenetic tree of Cytochrome Oxidase Subunit 1 (COI) gene from the Tineidae family. The maximum likelihood tree was inferred in IQ-tree (Nguyen et al., 2015) from an amino acid alignment of the COI gene from 90 species of moth and one caddis fly - *Dolophilodes distinctus* (Philopotamidae: Trichoptera) was used as an outgroup to root the tree. Bayesian Inference Criterion was used to select the best-fitting substitution model (LG + C60 + G), and bootstrap supports from branches were calculated using UFBoot2 (Minh et al., 2013). The three Tineinae ATs (FSTs, FCTs, and FDTs) are labelled on an example species of each (**A–C** are *Tinea steueri*, Petersen, 1966; *Trichophaga tapetzella*, Linnaeus, 1758; and *Monopis laevigella*, Denis and Schiffermüller, 1775 respectively). Coloured nodes indicate likely origins of the three ATs. Colours correspond to the AT position on the wing detailed in **Figure 2**. Photographs taken from BOLD Systems (Ratnasingham and Hebert, 2007), all are CC0 licence and taken by Marko Mutanen.

flexion line if it passes through the discal cell, but there is also the possibility that in these taxa it may be situated even closer to the tymbal. It is therefore reasonable to predict that FDT and FST actuation is facilitated by this flexion line.

Aeroelastic tymbal location seems to be associated with flexion lines, but strong supporting structures appear important too, as *Monopis*, *Crypsithyrodes*, and *Crypsithyris* discal cells have thickened veins surrounding their ATs (Robinson, 1980;

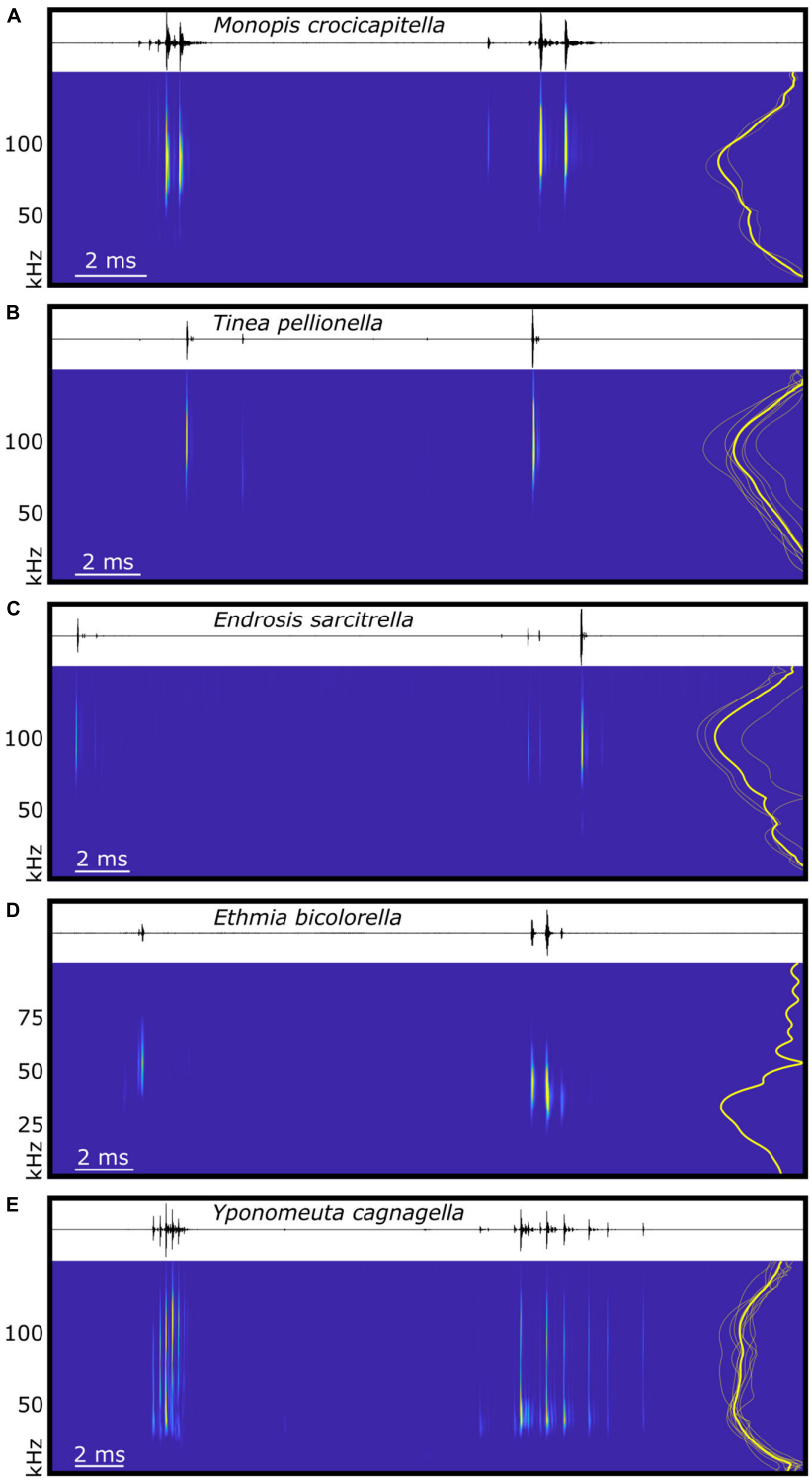

FIGURE 4 | Spectral and temporal characteristics of microlepidoptera sounds. The waveform and spectrogram (derived from continuous wavelet transformation using the Morse wavelet) of typical examples of in-flight acoustic emissions of five species of microlepidoptera, **(A)** *Monopis crocicapitella* (Tineidae), **(B)** *Tinea pellionella* (Tineidae), **(C)** *Endrosis sarcitrella* (Oecophoridae), **(D)** *Ethmia bicolorella* (Depressariidae), and **(E)** *Yponomeuta cagnagella* (Hübner, 1813; Yponomeutidae). Each panel represents one full wingbeat showing the two bursts of clicks produced with each wingbeat cycle, beginning with the first click of the burst with the shortest inter-click interval, and ending immediately prior to the first click of the next equivalent burst. To the right of each spectrogram is a power spectrum showing the normalised click amplitude for the species mean (thick yellow line) and individuals (thin translucent yellow lines, for each species *n* see section "Materials and Methods"). Time scales vary between plots, **(D)** uses a different frequency scale, and spectrograms are not calibrated for amplitude.

TABLE 2 | Acoustic properties of three microlepidoptera species.

Sp.	F	N	SL	PF	LF	HF	DD	SCD	LCD	DC	NHMC	NMC
Tinea pellionella	Tineidae	7	56.9 ± 0.7 (*n* = 70)	92.1 ± 3.7 (*n* = 70)	54.3 ± 3.1 (*n* = 70)	125.1 ± 4.8 (*n* = 70)	4.1 ± 0.2 (*n* = 70)	13.9 ± 1.0 (*n* = 70)	13.9 ± 1.3 (*n* = 70)	0.4 ± 0.2 (*n* = 7)	3.8 ± 0.9 (*n* = 140)	7.6 ± 1.0 (*n* = 70)
Monopis crocicapitella	Tineidae	2	64.6 ± 2.0 (*n* = 20)	88.1 ± 3.1 (*n* = 20)	41.2 ± 8.0 (*n* = 20)	111.7 ± 4.3 (*n* = 20)	5.9 ± 0.4 (*n* = 20)	14.5 ± 1.5 (*n* = 20)	13.8 ± 0.8 (*n* = 20)	0.8 ± 0.04 (*n* = 2)	11.2 ± 2.0 (*n* = 40)	22.95 ± 3.4 (*n* = 20)
Endrosis sarcitrella	Oecophoridae	4	54.0 ± 1.1 (*n* = 40)	100.0 ± 1.5 (*n* = 40)	45.8 ± 2.3 (*n* = 40)	128.9 ± 3.6 (*n* = 40)	3.4 ± 0.1 (*n* = 40)	9.7 ± 1.4 (*n* = 40)	13.6 ± 0.9 (*n* = 40)	0.2 ± 0.1 (*n* = 4)	4.5 ± 2.5 (*n* = 80)	9.1 ± 1.9 (*n* = 40)

Acoustic properties (mean ± SD; n = clicks) of the clicks. Sp = Species, F = Family, N = Number of individuals, SL = Source Level (dB peSPL 0.1 m), PF = Peak Frequency (kHz), LF = Low Frequency (kHz), HF = High Frequency (kHz), DD = Click Detection Distance (m), SCD = Shorter Burst Click Duration (μs), LCD = Longer Burst Click Duration (μs), DC = Duty Cycle (%), NHMC = Number of Clicks per Half Modulation Cycle (Burst), NMC = Number of Clicks per Modulation Cycle (Wingbeat).

Huang et al., 2011). This apparent importance of flexion lines and strong supporting structures in AT location, and probably in actuation, provides important indicators for mechanical modelling of these novel sound-producing systems.

Other than the known tymbals of *Amyna natalis* (Noctuidae), which are used for sexual communication and are not perpetually active during wing movement (Heller and Achmann, 1993), there are no obvious AT candidates in the macrolepidoptera. This exclusivity and convergence of ATs within the microlepidoptera suggests that a property of their wings gives them a propensity to evolve into sound producers. Therefore, differences between macro- and microlepidoptera in wing elastodynamics and structure of wing and wing membrane would be another important area of investigation. The most obvious difference between macro- and microlepidoptera is their size and we believe this is the most likely morphological factor facilitating sound production. Wing cell size may be of particular importance, as the smaller spaces between wing veins could allow for the formation of appropriately sized tymbals in micro- but not macrolepidoptera. Tymbal size is likely to be a factor in determining acoustic characteristics such as frequency; thus, the cell sizes in macrolepidopteran wings may not allow for ATs that produce clicks with frequencies appropriate for their function, e.g., anti-bat sound production.

Acoustics

All species possessing candidate ATs we were able to assess produced ultrasonic clicks linked to their wingbeat. The oecophorid species *E. sarcitrella* produces its sounds using its hindwings and has a hyaline patch at the same position as the known *Yponomeuta* HCT. *T. pellionella* and *M. crocicapitella* (Tineinae; Tineidae) have hyaline/subhyaline patches on their forewings that produce sound during wingbeats, most likely functioning similar to the hindwing HCTs of *Yponomeuta* and its relatives (O'Reilly et al., 2019). The *Ethmia* species assessed (Depressariidae) also produce sounds during flight and possess a subhyaline patch in a similar position to *T. pellionella*, but a lack of ablation tests prevented confirmation that this is the sound producer.

Several further lines of evidence corroborate that hyaline/subhyaline patches of *T. pellionella*, *M. crocicapitella*, and *E. sarcitrella* are functioning as ATs: firstly, only the species possessing candidate structures produced sounds, the two species lacking structures were silent. Secondly, like *Yponomeuta* sounds, the clicks of all these moths occur in two bursts every wingbeat, with one burst likely occurring during the upstroke and the other during the downstroke. Thirdly, for both Tineinae species, ablation of both hyaline patches eliminated sound production, and ablation of one of the two patches did not result in a change in the periodicity of the click bursts, instead halving the total number of clicks per wingbeat. This demonstrates that each tymbal is producing half the total number of clicks per wingbeat, that each tymbal contributes to both click bursts, and that the body of the moth does not prevent clicks from one wing reaching the opposite side. Although specific ablation of the hyaline patches of *E. sarcitrella* was unsuccessful, the removal of the hindwings eliminated sound production, and there is no other obvious candidate structure on these wings. Additional support comes from the location of the *E. sarcitrella* hyaline patch being indistinguishable from that of *Yponomeuta* HCTs.

We believe that, like other tymbals, ATs produce sound through bimodal buckling, and that the two click bursts each moth produces per full wingbeat are the two stages of its ATs buckling and then returning to their resting state. The exact biomechanical mechanism by which these tymbals are actuated was beyond the scope of this study and requires complex modelling, but we propose that, similarly to *Yponomeuta* HCTs (O'Reilly et al., 2019), twisting and folding of the wing (likely along flexion lines, e.g., claval furrow or median flexion line) during flight are important, as are strong supporting structures such as thickened veins.

Structurally, all three tymbals resemble *Yponomeuta* HCTs; they consist of similarly sized hyaline patches with few or no scales between two often strong veins. However, unlike *Yponomeuta* HCTs, they do not possess obvious microtymbals. Microtymbals are striations running the length of a tymbal, each functioning to produce an individual click in sequence following tymbal actuation, resulting in the production of bursts (trains) of clicks. Following initial tymbal buckling each microtymbal buckles in sequence producing a train of individual clicks, and then upon the return of the tymbal to its resting state the same process occurs in reverse order, producing a second click train.

The low click number in *T. pellionella* and *E. sarcitrella* click bursts is consistent with a lack of microtymbals; however, the higher click number in *M. crocicapitella* bursts suggests that this species may possess an alternative mechanism. Raised "bumps" are visible on the FDTs of some *Monopis* species, which may be analogues of microtymbals (**Figure 2A**).

Function of Sounds

The acoustic emissions of all recorded species most likely function as anti-bat sounds. The ultrasonic, broadband nature of the clicks is similar to the known anti-bat sounds of other moths (Corcoran et al., 2010; Barber and Kawahara, 2013; Corcoran and Hristov, 2014; O'Reilly et al., 2019) and they are loud enough to be detected by bats.

The maximum distances over which these sounds will be audible to bats is lower than *Yponomeuta* clicks (5.9, 4.1, and 3.4 m for *M. crocicapitella*, *T. pellionella*, and *E. sarcitrella*, respectively compared to 10.5 m for *Yponomeuta cagnagella*; O'Reilly et al., 2019). This is due to increased atmospheric attenuation of the sounds due to much higher peak frequencies, and for *T. pellionella* and *E. sarcitrella* sounds, lower source levels.

The different number of clicks per wingbeat might not necessarily have substantial biological relevance. *T. pellionella* and *E. sarcitrella* produce much fewer, lower amplitude clicks per burst (normally one or two, but occasionally more, see **Figure 4**) than *M. crocicapitella.* However, producing fewer clicks does not mean that these sounds are less likely to function as a bat defence. Within the tymbal-possessing Arctiinae, many species produce click bursts but others, including the sympatric *Arctia caja*, do not possess microtymbals and thus produce one defensive click per tymbal buckling event (Fenton and Roeder, 1974; Surlykke and Miller, 1985).

Additional support for these sounds having an anti-bat function is the lack of any reaction from the moths to ultrasonic stimuli, whether generated artificially or by another individual. Although tympana have been reported in the Tineidae, this is a defining feature of the subfamily Harmacloninae (Davis, 1998), and there is no evidence they are present in the Tineinae. Similarly, there is no evidence in the literature that *E. sarcitrella* possesses hearing capabilities. Therefore, these moths cannot be communicating with conspecifics using airborne sounds. Constantly producing ultrasonic clicks detectable by bats that serve no communication purpose, seems counterintuitive, unless the sounds act as acoustic defence.

The precise defensive mechanism of these sounds for each species remains unclear, with relevant unknowns including moth toxicity and their propensity to spend time on the wing producing sound. It is clear though that the low duty cycles of all their sounds cannot jam bat echolocation (**Table 2**), as this requires a duty cycle of at least 20% (Corcoran et al., 2010; Conner and Corcoran, 2012). It is also unlikely that bats will be startled by these sounds as such a defence tends to be ephemeral and only effective against naïve bats (Bates and Fenton, 1990; Hristov and Conner, 2005). This suggests that the sounds function as aposematic signals, as either Batesian (imposter) or Müllerian (true) mimics of acoustically aposematic moths such as the Arctiinae and *Yponomeuta*.

Phylogenetic Spread of ATs Within the Tineinae

There are two lines of support for the convergent evolution of anti-bat sound production by ATs within the Tineinae subfamily. Firstly, the structures are morphologically similar in many aspects, but are sufficiently different in shape and position on the wing to suggest multiple evolutionary origins. Secondly, based on their phylogeny, the distinctly separate *Monopis, Trichophaga,* and *Tinea*-like clades suggests three points of evolutionary origin for Tineinae ATs (**Figure 3**). The *Tinea*-like clade is particularly interesting as FSTs appear in three distinct lineages. The ancestor of both the *Tricophaga* and *Monopis* lineages probably had no AT, suggesting that the FCT and FDT structures found in these lineages evolved independently. Moreover, the structures are found on different locations on the wing, providing further evidence they are not homologous, and thus, the result of convergent evolution. Additionally, the distribution FSTs found in the *Tinea* and *Niditinea* species suggests either the common ancestor of all these species possessed an FST and there has been lineage specific loss, or that there has been a minimum of three independent evolutionary events of this structure, and convergent evolution has occurred. The phylogeny from which we draw our conclusions was constructed with a single marker gene, leading to species level branches only having weak bootstrap support. Thus, the conclusions on a species level must be re-assessed when more data become available and more robust phylogenies can be constructed. The distribution of FST, FCT, and FTD possessing species on the phylogenetic tree clearly indicates three independent evolutionary events of sound producing structures, highlighting remarkable convergent evolution on a subfamily level.

Anti-bat sound production in the Tineinae is exceptionally diverse as it has evolved convergently several times within one subfamily. Convergent evolution of bat defences in the Lepidoptera is common, and has occurred in terms of hearing, sound production, and hindwing decoys (Corcoran and Hristov, 2014; Barber et al., 2015; ter Hofstede and Ratcliffe, 2016), but it rarely, occurs between such closely related taxa as within one subfamily. Similar levels of convergence appear to have occurred within Saturniidae (silkmoth) subfamilies with regards to acoustic wing decoys (Rubin et al., 2018). These two examples of subfamily level converge in moths reiterate how important the bat-moth coevolutionary arms race is as a case study for evolutionary principals.

Thoughts on the Evolution of ATs in Cave-Dwelling Taxa

Records of troglophilic (cave-dwelling) invertebrates from various cave systems globally indicate that Tineidae are widely present, particularly in tropical and subtropical regions of the Americas as well as the Balkan states and Australia (Barr and Reddell, 1967; Hamilton-Smith, 1967; Peck, 1975, 1974; Robinson, 1980; Trajano, 2000; Humphreys and Eberhard, 2001; Cokendolpher and Polyak, 2004, 1996; László, 2004; Wynne and Pleytez, 2005; Wynne et al., 2005; Polak et al., 2012; Byun et al., 2014; Eberhard et al., 2014; Pape, 2014; Silva and Ferreira, 2015; Turbanov et al., 2016; Jakšić, 2017). The Tineidae is a cosmopolitan lepidopteran family (Slootmaekers, 2013), and so it is highly likely that tineids are present in cave systems globally, but records are lacking.

Larvae of the subfamily Tineinae feed on animal detritus including bat guano, resulting in independence from green plants. This independence allows these moths to permanently inhabit environments such as caves. Indeed, at least 11 Tineidae species (including species of the Tineinae genera *Monopis*, *Crypsithyrodes*, *Crypsithyris*, *Tinea*, *Niditinea*, and *Praeacedes*) are known to spend their entire lives within caves feeding as larvae on bat guano or the fungi that grow on it (Robinson, 1980). In addition, *E. sarcitrella* is also found in caves and bat roosts (Mosconi, 2011; Centelles Bascuas, 2015). *E. sarcitrella* is a pest of stored grain but is known to be able to subsist on guano and other organic matter (Carter, 1984).

All these troglophilic moth species thus exist alongside bats, feeding on the faeces of their potential predators, which puts them at a perpetual risk of predation. It seems counterintuitive for moths to have initially adapted to live on the faeces of their predators; indeed, guanophagy in cave-dwelling microlepidoptera may have originated before bats, with moths perhaps feeding on bird guano.

Guanophagous moths will indiscriminately feed on bird or bat guano, and sometimes other animal products, including bird feathers (Robinson, 1980). With birds having evolved considerably earlier than bats (Kumar and Hedges, 1998), it is plausible that the ancestral cave-dwelling, guanophagous tineid shared its abode with cave-roosting birds, like extant swiftlets or oilbirds. A cave can provide a geographic mating barrier to populations, and as multiple Tineinae species can spend their entire lives living in caves (Robinson, 1980), ancestral moth populations could have become isolated in caves, leading to speciation. Then, following the evolution of echolocating bats and their colonisation of caves, this strong predation pressure, the geographic isolation, and an apparent propensity for wings to be sound producers could have resulted in the convergent evolution of ATs in the Tineinae.

Cave-Dwelling Microlepidopteran Acoustics

We already established that the sounds produced by the species we recorded most likely function as acoustic aposematic signals, but whether these moths are Batesian or Müllerian mimics of other aposematic moths depends on their toxicity which is unknown. Toxicity in Lepidoptera is derived from sequestering secondary metabolites from food and/or synthesising compounds (Rothschild et al., 1970). Both faeces and the fungi that grow on it could conceivably provide noxious compounds to sequester for the acoustically active Tineinae and *E. sarcitrella*, or equally they could synthesise such compounds.

Bats will learn over time to ignore acoustic Batesian signals (Barber and Conner, 2007), so if these cave-dwelling moths are palatable, they then risk becoming acoustically conspicuous targets. Therefore, the persistence and convergent evolution of sound production within this group of moths suggests that they are truly aposematic. An interesting thought is that naïve juvenile bats might first learn to avoid clicking moths from within their roosts.

Alternatively, reducing the exposure of bats to these acoustic signals could allow Batesian mimicry to persist. If these moths preferentially avoid flight, and instead crawl atop the guano, they will avoid sound production. This would prevent saturating the bats with a potentially Batesian signal and therefore reducing the effectiveness of sound production as a defence. The lower detection range of the clicks we recorded compared to non-cavernicolous *Yponomeuta* and macrolepidoptera (e.g., Corcoran et al., 2010; O'Reilly et al., 2019) may be beneficial in this respect; limiting their detectability to a distance at which bats are close enough to pose a threat.

A second, not necessarily separate, scenario in which Batesian mimicry could persist may arise if the ratio of Müllerian to Batesian mimics bats encounter is so high it is not worth risking attacking any clicking target. If the amount of sound producing moths in the bats' hunting environment is above a certain threshold, then they will be regularly exposed to true aposematic signallers when foraging, reinforcing the effectiveness of acoustic aposematism. Within the roost, palatable cave-dwelling microlepidoptera could then "piggyback" on the protection afforded by ultrasound production, and reduced acoustic conspicuousness and/or flight could maintain the effectiveness of their signals.

Everything considered, based on the similarities of their sounds with those of aposematic moths, their lack of both hearing and, therefore, intraspecific communication, as well their unusual feeding ecology in close proximity to bats, we conclude that *M. crocicapitella*, *T. pellionella*, their tymbal-possessing relatives (**Figure 3**), and *E. sarcitrella* are mimics of acoustically aposematic moths. We cannot, however, state with confidence whether they are Batesian or Müllerian mimics.

CONCLUSION

The bat-moth evolutionary arms race is an area of much research interest for both sensory ecologists and evolutionary biologists, and yet a huge number of taxa remain underrepresented in the current literature. Microlepidoptera are largely ignored in terms of this topic, and our findings highlight that this suborder is greatly understudied. The remarkable level of convergence in anti-bat sound producing structures is further evidence in support of microlepidoptera being under significant selection pressure from bat predation. As a result of this pressure, the array of acoustic defences these moths possess are probably just as complex and diverse as their larger cousins, and they undoubtedly deserve increased research attention. Here, we begin a new chapter in the bat-moth coevolutionary arms race; the acoustic anti-bat defences of the microlepidoptera.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

LO conceived the study, performed all images, and museum specimen analysis, all laboratory-based acoustic recordings and analysis, and wrote the original manuscript draft. DA made acoustic recordings of *Ethmia* species in Kenya and South Africa, provided live Tineidae specimens, and provided advice on microlepidoptera. BH performed the phylogenetic analysis of the Tineidae and created the cladogram in **Figure 3**, as well as providing advice on the other phylogenetic aspects of the project. MH provided funding for LO, research methods and equipment, confirmed sound production in *Monopis cf monachella,* offered advice on acoustic analysis and contributed to later drafts of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.648223/full#supplementary-material

Supplementary Audio 1–4 | Recordings of in-flight microlepidopteran sounds. Audio 1–4 contain multiple ultrasonic click bursts from *Endrosis sarcitrella, Ethmia bicolorella, Monopis crocicapitella*, and *Tinea pellionella* respectively.

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ORIGINAL RESEARCH
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How Loud Can you go? Physical and Physiological Constraints to Producing High Sound Pressures in Animal Vocalizations

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Sound is vital for communication and navigation across the animal kingdom and sound communication is unrivaled in accuracy and information richness over long distances both in air and water. The source level (SL) of the sound is a key factor in determining the range at which animals can communicate and the range at which echolocators can operate their biosonar. Here we compile, standardize and compare measurements of the loudest animals both in air and water. In air we find a remarkable similarity in the highest SLs produced across the different taxa. Within all taxa we find species that produce sound above 100 dB_{peak} re 20 μPa at 1 m, and a few bird and mammal species have SLs as high as 125 dB_{peak} re 20 μPa at 1 m. We next used pulsating sphere and piston models to estimate the maximum sound pressures generated in the radiated sound field. These data suggest that the loudest species within all taxa converge upon maximum pressures of 140–150 dB_{peak} re 20 μPa in air. In water, the toothed whales produce by far the loudest SLs up to 240 dB_{peak} re 1 μPa at 1 m. We discuss possible physical limitations to the production, radiation and propagation of high sound pressures. Furthermore, we discuss physiological limitations to the wide variety of sound generating mechanisms that have evolved in air and water of which many are still not well-understood or even unknown. We propose that in air, non-linear sound propagation forms a limit to producing louder sounds. While non-linear sound propagation may play a role in water as well, both sperm whale and pistol shrimp reach another physical limit of sound production, the cavitation limit in water. Taken together, our data suggests that both in air and water, animals evolved that produce sound so loud that they are pushing against physical rather than physiological limits of sound production, radiation and propagation.

Keywords: bioacoustics, source level, sound propagation, sound production, vocal communication

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INTRODUCTION

Sound is the medium through which animals, including humans, can communicate complicated and unambiguous signals: from laughter when we are happy, to terrified screaming when we fear for our lives. From a baby babbling whilst practicing speech, to Feynman presenting his famous "Lectures on physics." Humans, especially, are capable of combining vocal utterances

into languages able to convey our most complicated concepts (Fitch, 2005, 2012).

Sound production is critical to the social communication and survival for many arthropods and the majority of vertebrates. Almost 10,000 bird species, 7,000 frog species, 6,000 mammal species, and an unknown number of fish and arthropod species, have evolved the ability to produce sounds, many with highly specialized organs (Bradbury and Vehrencamp, 2011), driven by complex motor patterns, and executed by exceptional muscles (Elemans et al., 2008, 2011; Mead et al., 2017). Sound plays a pivoting role in many behaviors, including courtship and territorial display signals in insects, fish, frogs, birds and mammals, and orientation and prey capture in echolocating animals. No other communication modality combines the accuracy, speed, and richness of communication over long distances as does sound, both in air and in water (Bradbury and Vehrencamp, 2011).

One critical acoustic parameter for communication is sound pressure amplitude or source level (SL) of the animal vocalizations. SL affects the range of vocal communication in a network or the range of object detection and interpretation in echolocation, because with increasing SL animals can detect sound signals in ambient noise at longer ranges. Even though many animals may not benefit from producing loud sounds, some avian and mammalian species produce particularly high SLs. The term loud here refers to high sound pressures, which is different from, and should not be confused with *loudness*, a term reserved in psychoacoustics for the *perceived* level of a sound (Troscianko, 1982). Interestingly, in air, the highest reported SL values do not seem to exceed 120 dB_{peak} re 20 μPa at 1 m (Surlykke and Kalko, 2008; Podos and Cohn-Haft, 2019), which suggests that there are certain limitations to produce high sound pressures. However, direct numerical comparison of published SL amplitudes is complicated by the different standards and methods used to compute them. We therefore currently lack a direct comparison of the highest SLs, which is critical for investigating potential limitations to producing loud sounds.

Here we compiled SLs of the loudest animals known both in air and in water and converted all reported values into standardized measures that are directly comparable. Furthermore, we use acoustic models to estimate the highest acoustic pressures generated in the entire acoustic field. We discuss what physical and physiological mechanisms could constrain the production, radiation and propagation of high sound pressures and if such boundaries are met by animals.

RESULTS

How to Compare Source Levels?

The SL of a sound source is defined as the sound pressure at a reference distance along its acoustic axis (**Figure 1**). Traditionally, the methodology of reporting SL values differs significantly between animal groups in bioacoustics research. However, comparing SLs can be done easily when considering five issues:

First, the SI unit for pressure is the Pascal, but this physical property is often reported on the decibel (dB) scale, which first scales the data to a reference value and then applies a log transform. Because the reference value is typically 20 μPa in air and 1 μPa in water, the same absolute pressure in Pascal is represented by a numerical value 26 dB higher in water than in air when represented on the dB scale. To avoid confusion, we consistently report sound pressures both in Pascal and on the relevant dB scale (Also compare the two central pressure scales in **Figure 1C**).

Second, because it is not possible to measure the pressure at the location of the source, the SL is defined at some distance from the source. The reference distance varies between scientific fields but is one meter by convention in most biological and engineering applications. Many animals do not provide a convenient way to place a microphone or hydrophone at this reference position. In such cases, if the distance to the animal is known, the SL of the animal is estimated by accounting for the transmission-loss of the pressure magnitude over the distance traveled (Urick, 1983; Madsen and Wahlberg, 2007; Wahlberg and Larsen, 2017). Often simple spherical spreading loss models are used to estimate transmission loss, but these can be imprecise especially at longer distances to the source, when acoustical properties of the environment play an important role (Wahlberg and Larsen, 2017).

Third, because sound sources are directional at high frequencies relative to the size of the sound source, it is important to record the sound on-axis or to reconstruct the radiation directionality pattern and report the on-axis SL (**Figure 1A**). Sound pressure is highest along the acoustic axis and attenuates continuous with increasing off-axis angles. For highly directional sounds produced by bats and toothed whales the direction of the acoustic axis and position of the animal can be determined by using microphone or hydrophone arrays (Madsen and Wahlberg, 2007; Jakobsen and Surlykke, 2010).

Fourth, there are several ways to quantify the amplitude of a time-varying pressure wave. Amplitude measurements are traditionally either taken peak-to-peak (ptp), zero-to-peak (peak), or root-mean-square (rms) and it is important to note the differences when comparing studies using different amplitude measures (**Figure 1B**). For a sine wave, the peak-to-peak value is 6 dB higher than the peak and 9 dB higher than the rms value. For most real-world signals these relationships are different. Especially the rms amplitude will differ and the difference between peak-to-peak and rms can be greater than 9 dB depending on the time window used for computing the rms. Sound level meters are also used for bioacoustics measurements and common measures are given as either L_{peak} or $L_{eq.}$ L_{peak} equals the peak amplitude measurement with no time averaging applied and is used widely in bioacoustics and human audiology research. L_{eq} is the equivalent continuous level and the same as the rms measure.

Fifth, the frequency response and sensitivity of the recording chain needs to be specified. For example, most sound level meters have different filters that can be selected, e.g., A, C, and Z weighing, where A and C relate to human loudness perception at different intensity levels, and Z has a constant reference pressure

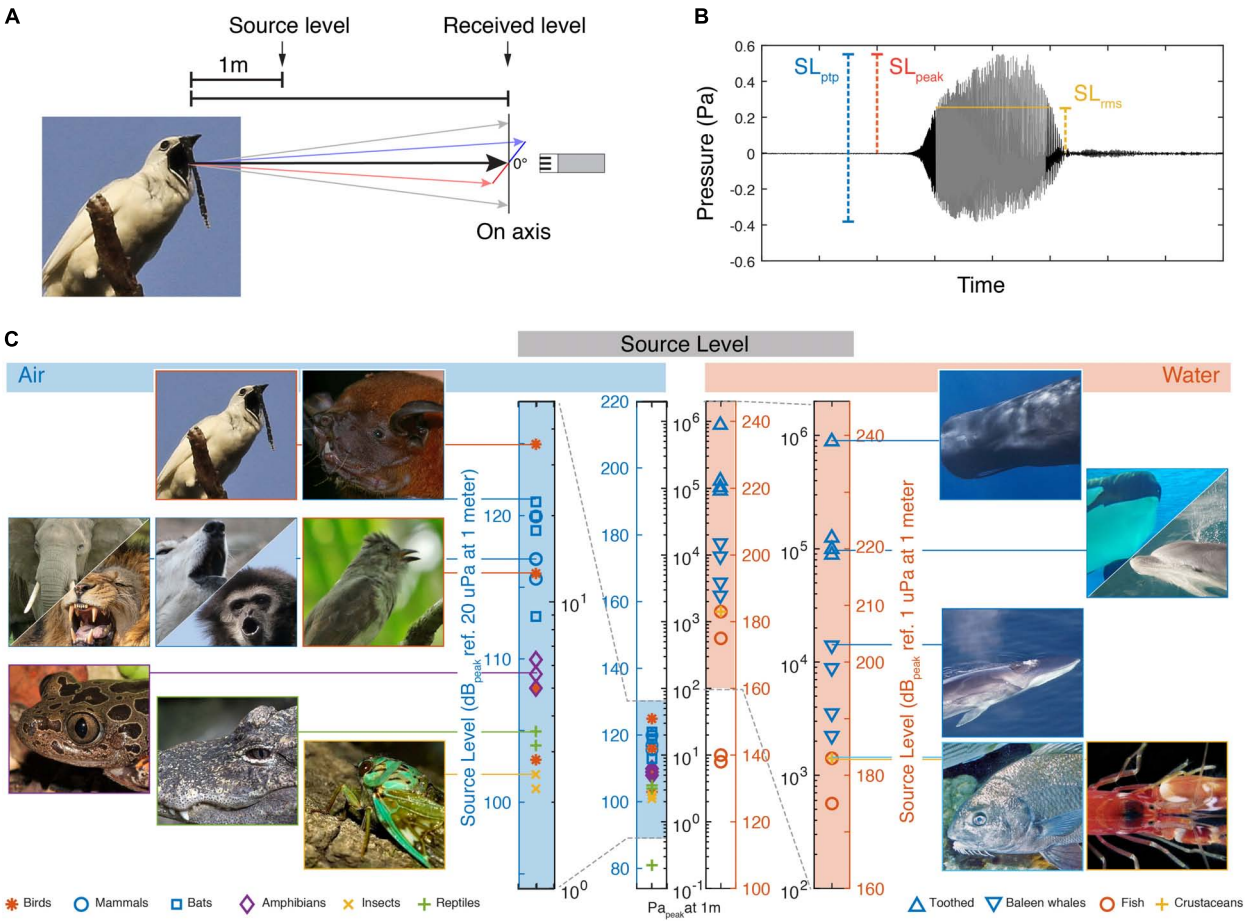

FIGURE 1 | Source levels of the loudest animals in air and water. **(A)** Source level is defined as the on-axis radiated sound pressure at 1 m distance from the source. **(B)** Three commonly used measures of pressure amplitude; SL_{peak} is the highest absolute magnitude of the signal. SL_{ptp} is the difference between highest and lowest amplitude. SL_{rms} is here shown as the rms amplitude over the duration set by using a 95% energy threshold criterion [see Madsen and Wahlberg (2007) for detail]. **(C)** SLs of the loudest reported animals in air and water (For data points and references see **Table 1**). The two vertical bars of pressure in the middle are on the same absolute pressure scale to allow direct comparison of the different dB scales in air and water.

of 20 μPa across frequencies (i.e., unweighted) (International Standard IEC61672-1, 2002). Thus, A and C weighing can be used to make conclusions about human perception. Because hearing sensitivity varies significantly across species this type of weighing should be avoided in bioacoustics research and will especially affect low- and high-frequency sounds. Lastly, sound level meters come in two classes, 1 and 2 that have difference tolerance limits for precision. Both perform almost equal between 20 Hz and 10 kHz, but class 2 has lower precision tolerance outside this frequency range. Therefore class 1 sound level meters are recommended for measurements at frequencies below 20 Hz and frequencies above 10 kHz.

Which Animals Produce the Highest Source Levels?

To identify the loudest species, i.e., the species that produce the highest SLs, within and between all clades of vocal animals in air and water, we compiled SLs of animal vocalization per taxon (see section "Materials and Methods," **Figure 1C** and **Table 1**). To prevent overrepresentation of species with lower SLs, we included only the four loudest species within each taxon. We included bats and toothed whale as separate groups because echolocation likely imposes a different evolutionary demand on the sound production system than does communication. The variable measuring conditions of acoustic fields in laboratory and field, makes comparing dB values with precision below 1 dB not very meaningful. In combination with the different methodologies used to measure peaks or average maxima, we should consider the maximal values reported here indicative within 2–3 dB of what the animals produce. Our efforts in trying to compile these data emphasized to us how infrequent SLs are reported in bioacoustics papers. Given the importance of SL for the biology of species, we thus would like to urge people to measure and report SL in their work.

In air, the loudest reported animals are birds and mammals. The White Bellbird (*Procnias albus*) is the loudest at 125 dB_{peak} re. 20 μPa at 1 m (i.e., 36 Pa_{peak} at 1 m) (Podos and Cohn-Haft, 2019). Elephants and bats are runners up at 120 dB_{peak} re.

TABLE 1 | Source level and maximum radiated pressures for animals in air and water.

Species name	Source level (peak dB re 20 μPa at 1 m)	Maximum pressure (peak dB re 20 μPa)	SL reference	Size references	Location	Weighing
Air						
Canis lupus lupus	117	139	Suter et al., 2016	Andersone and Ozolins, 2000	Field	dBA
Hylobates lar	116	146	Terleph et al., 2016	Groves, 1971	Field	None
Loxodonta africana	120	127	Poole et al., 1988	https://www.skullsunlimited.com/products/replica-african-elephant-skull?variant=3001994543128	Field	None
Panthera leo	117	134	Larom et al., 1997	Saber and Gummow, 2015	Field	?
Eptesicus bottae	113	143	Holderied et al., 2005	Hulgard et al., 2016	Field	None
Eptesicus fuscus	121	149	Hulgard et al., 2016	Hulgard et al., 2016	Field	None
Noctilio albiventris	120	137	Surlykke and Kalko, 2008	Hulgard et al., 2016; Thiagavel et al., 2017	Field	None
Noctilio leporinus	119	135	Surlykke and Kalko, 2008	Hulgard et al., 2016; Thiagavel et al., 2017	Field	None
Gallus domesticus	108	145	Brackenbury, 1979	Verdiglione and Rizzi, 2017	Field	?
Lipaugus vociferans	116	155	Podos and Cohn-Haft, 2019	Adjusted relative to blackbird measure	Field	dBA/C
Procnias albus	125	161	Podos and Cohn-Haft, 2019	Adjusted relative to blackbird measure	Field	dBA/C
Turdus philomelos	103	143	Brackenbury, 1979	https://skullsite.com/skullpage/turdus-merula-blackbird/	Field	?
Bufo gutturalis	109	142	Passmore, 1981	Passmore, 1981	Field	?
Kassina maculata	110	149	Passmore, 1981	Ahn et al., 2004	Field	?
Rana areolata	110	145	Gerhardt, 1975	Redmer, 2000	Field	?
Rana virgatipes	108	145	Gerhardt, 1975	Given, 1987	Field	?
Alligator Mississippiensis	104	120	Todd, 2007	O'Brien et al., 2019	Enclosure	?
Alligator sinensis	105	129	Wang et al., 2007	O'Brien et al., 2019	Enclosure	?
Gekko gecko	81	115	Brumm and Zollinger, 2017	Laver et al., 2020	Tank	None
Brevisana brevis	102	149	Villet, 1987	Villet, 1988; Young, 1990	Field	?
Diceroprocta apache	102	149	Sanborn and Phillips, 1995	Equal to *Brevisana brevis*	Lab	?
Oxypleura lenihani	101	146	Villet, 1987	Villet, 1988; Young, 1990	Tank	?
Pycna semiclara	102	146	Villet, 1987	Villet, 1988; Young, 1990	Tank	?
Water	**(peak dB re 1 μPa at 1 m)**	**(peak dB re 1 μPa)**				
Orcinus orca	220	220	Eskesen et al., 2011	Finneran et al., 2016	Field	None
Physeter macrocephalus	239	227	Mohl et al., 2003	Finneran et al., 2016	Field	None
Pseudorca crassidens	219	221	Madsen et al., 2004	Finneran et al., 2016	Field	None
Tursiops truncatus	222	228	Wahlberg et al., 2011	Finneran et al., 2016	Field	None
Balaenoptera acutorostrata	191	197	Wang et al., 2016	Omura and Sakiura, 1956	Field	None
Balaenoptera borealis	187	188	Wang et al., 2016	Matthews, 1938	Field	None
Balaenoptera musculus	199	195	Sirovic et al., 2007	Mackintosh and Wheeler, 1929	Field	None
Balaenoptera physalus	203	199	Wang et al., 2016	Goldbogen et al., 2007	Field	None
Argyrosomus japonicus	175	194	Parsons et al., 2012	Fisheries resources in NSW 2008/9	Field	None
Bairdiella chrysoura	138	178	Sprague and Luczkovich, 2004	https://www.fishbase.se/summary/1165	Field	None
Glaucosoma hebraicum	140	162	Parsons et al., 2013	Hesp et al., 2002	Field	None
Pogonias cromis	183	205	Locascio and Mann, 2011	Jones and Wells, 1998	Field	None
Synalpheus parneomeris	183	232	Au and Banks, 1998	Versluis et al., 2000	Tank	None

20 μPa at 1 m (i.e., 20 Pa_{peak} at 1 m) (Poole et al., 1988; Surlykke and Kalko, 2008; Hulgard et al., 2016). The loudest reported amphibian species call at 110 dB_{peak} re. 20 μPa at 1 m (i.e., 6.3 Pa_{peak} at 1 m) (Gerhardt, 1975; Passmore, 1981). The loudest reported reptile species are the alligators at around 105 dB_{peak} re. 20 μPa at 1 m (i.e., 3.6 Pa_{peak} at 1 m) (Todd, 2007; Wang et al., 2007). The loudest reported insects are several species of cicadas at 102 dB_{peak} re. 20 μPa at 1 m (i.e., 2.5 Pa_{rms} at 1 m)

(Villet, 1987; Sanborn and Phillips, 1995). These SLs represent the highest values at species level. For the bat, bird, insect and toothed whale species included here, the SL values reported represent their reported loudest vocalizations. However, for the other species we do not know if the reported SLs encompass the maximal capabilities in the species-specific vocal repertoire, and we cannot exclude they can emit higher SLs.

Also within species, SL variability can be expected. Humans deserve special attention because it is the only species where we have some information on the loudest individuals within a species. The human shouted voice is about 105 dB_{rms} re. 20 μPa at 1 m (Lagier et al., 2017). However, The Guinness Book of World Records lists the loudest voice from a schoolteacher saying "Silence" at 122 dB re. 20 μPa at 1 m and the loudest non-speech scream to be 129 dB re. 20 μPa at 1 m, which would rank humans up with the loudest mammal and birds. However, we have not been able to confirm the recording methodology of these records with Guinness, including what amplitude measure was used, and therefore do not include them here. Taken together, in air, the loudest animals all emit surprisingly similar maximum SLs around 120 dB_{peak} re. 20 μPa at 1 m, which equals 20 Pa_{peak} at 1 m.

In water, maximum SLs are much higher than in air. Toothed whales are by far the loudest group of animals in water; the sperm whale (*Physeter macrocephalus*), emits echolocation clicks with SLs up to 239 dB_{peak} re. 1 μPa at 1 m (i.e., 900,000 Pa_{peak} at 1 m) (Mohl et al., 2003). In comparison, the loudest baleen whale is the fin whale (*Balaenoptera physalus*) at 203 dB_{peak} re. 1 μPa at 1 m (i.e., 14,000 Pa_{peak} at 1 m) (Wang et al., 2016). The loudest teleost fish, the black drum (*Pogonias cromis*) (Locascio and Mann, 2011), is almost three orders of magnitude of pressure below the sperm whale at 183 dB_{peak} re. 1 μPa at 1 m (i.e., 1,400 Pa_{peak} at 1 m), as is the pistol shrimp (*Synalpheus parneomeris*) at 183 dB_{peak} re. 1 μPa at 1 m (Au and Banks, 1998). Please note that the dB values in water are 26 dB higher than in air due to the difference reference pressure of 1 μPa alone (see central, black labeled pressure scale in **Figure 1C**). In water, we thus do not observe that different animal clades converge upon a maximum SL.

Loudest Animals Are Independent of Size and Frequency in Air, but Not in Water

How much would a sound source need to move to achieve a SL of 125 dB_{peak} re. 20 μPa in air or 240 dB_{peak} re. 1 μPa in water? To approximate this, we considered the output of two simple sound sources: (1) a pulsating sphere and (2) a piston of equal diameter (see section "Materials and Methods," **Figure 2**). These models show that the velocity needed to achieve a certain fixed SL decreases with the radiated frequency and physical size in air and water (**Figures 2A,B**). We also considered the product of the wavenumber ($k = 2\pi f$) and size (a), the ka product. This dimensionless parameter represents the acoustic size of an emitter i.e., the size relative to the wavelength it is emitting since $ka = 2\pi a/\lambda$. At a fixed SL, the velocity also decreases with ka for both air and water (**Figure 2C**). While the piston model shows a power relationship (linear on the double logarithmic axes), for the pulsating sphere the velocity required becomes constant at higher frequency, size and ka. This is because the source becomes large compared to the wavelength and tends to locally radiate a plane wave, for which the ratio of sound pressure to particle velocity is the characteristic impedance of the propagation medium, ρc [see also Equation (1) in section "Materials and Methods"]. By fixing other parameters, such as rms volume velocity of the source (see section "Materials and Methods"), the SL increases with frequency, size and ka product (**Figures 2D–F**). Again, for the pulsating sphere, the SL does not increase with frequency, size and ka for a fixed velocity over a certain frequency for the reason mentioned above.

These simple models illustrate three acoustic considerations important for generating sound. First, to produce higher frequencies at the same SL, the source needs to move less. Second, reversely, with the same source velocity, a higher SL can be achieved at higher frequencies or larger size. Third, due to the impedance difference between air and water, the same source motion results in water in a three orders of magnitude higher sound pressure than in air. It is thus much easier to generate a high pressure in water.

The ka product determines how much of the power used to produce the sound is converted into acoustic power that radiates from the source, i.e., the efficiency of the source. For a pulsating sphere the maximum efficiency is at $ka \geq 2$. Below $ka = 2$ efficiency drops by 100 for every order of magnitude of ka (Michelsen, 1992; Larsen and Wahlberg, 2017). While there is no increase in source efficiency at $ka > 2$, most sound sources will exhibit a substantial increase in SL because the sound source becomes increasingly directional with increasing ka, i.e., pressure is highest along the acoustic axis and progressively decreases at greater off-axis angles. Thus, a directional source radiating the same acoustic power as an omni-directional source will emit a higher SL on the acoustic axis. However, a pulsating sphere does not become directional at high ka.

Because these simple acoustical models predict a clear dependency on frequency, size and ka product, we compiled SL of the loudest animals as a function of their peak frequency body mass, acoustic radius and ka product (**Figure 3** and **Supplementary Table 1**, see section "Materials and Methods"). We consider that applying descriptive statistics is not meaningful given the sparse nature of the data, but a few patterns do emerge. Although within a clade body size may be a good predictor of SL (Villet, 1987), for the loudest aerial species we observe no increase of highest SLs with radiated sound peak frequency over four orders of magnitude (**Figure 3A**), no increase with body mass across nearly five orders of magnitude (**Figure 3B**) and no increase with increasing ka over two orders of magnitude (**Figure 3D**). All loud insects, frogs, reptiles, birds and terrestrial mammals have ka between 0.1 and 1, which makes them omnidirectional sound emitters. The bats have $ka > 2$, which makes them efficient and more directional sound emitters. Thus, in contrast to simple linear acoustic models that show increase of SL with increasing frequency, radius and ka product, the maximal SL of around 120 dB re. 20 μPa at 1 m in air seems independent of weight, radius, frequency and ka product (**Figure 3** and **Supplementary Table 1**).

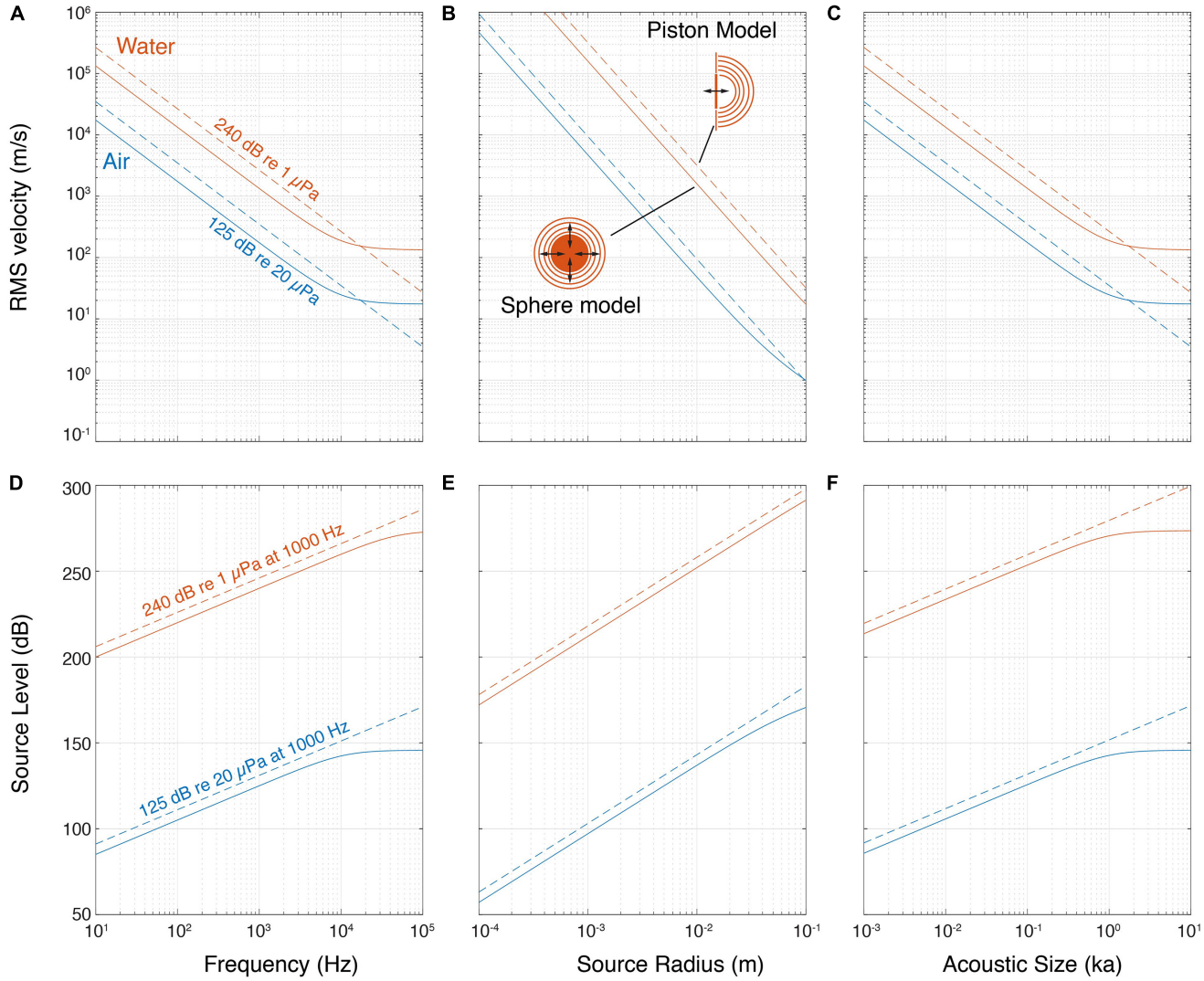

FIGURE 2 | Pulsating sphere and piston models predict that source level depends on frequency and size. **(A)** Isolines of a 240 dB re. 1 μPa and 125 dB re. 20 μPa source show that producing sound requires less movement with higher frequency, **(B)** size and **(C)** ka product. **(D)** SL increases with frequency, **(E)** size and **(F)** ka product for both sphere and piston models. The lines shown here are at a volume velocity that makes the source of 10 mm diameter produce 240 dB re. 1 μPa and 125 dB re. 20 μPa at 1,000 Hz, in water and air, respectively (see section "Materials and Methods").

For aquatic animals, the sparse observations fit the simple acoustic models that highest SL increases with frequency (**Figure 3C**), body size (**Figure 3D**) and ka product (**Figure 3E**). However, due to the sparseness of the data, we should be cautious interpreting this data. For loud crustaceans, fish and baleen whales, the ka product is between 0.01 and 0.2, which makes them omnidirectional, but not such efficient sound emitters. For tooth whales the ka product is larger than 10, which makes them efficient and highly directional sound emitters. As a consequence, while toothed whale SLs are substantially higher than the baleen whales, the high directionality means that the difference in radiated acoustic power, i.e., the combined sound radiation in all directions, is much smaller. This is because when emitting sound directionally, sound pressure is concentrated in the frontal direction and much lower pressures are radiated off-axis whereas for omni-directional sources, sound pressure radiation is roughly equal in all directions.

Physical Upper Limits to Sound Pressure Generation and Radiation

The SL of bat echolocation calls has been suggested to be close to the physical limit of maximal pressure generation in air (Madsen and Surlykke, 2014). Are animals indeed so loud they are hitting certain physical limits to sound production?

In air, pressure fluctuates around atmospheric pressure of about 100 kPa and the negative crest is limited at 0 Pa. Sound waves that are symmetric around atmospheric pressure can therefore reach an amplitude of maximally 200 kPa peak-to-peak (194 dB_{peak} re. 20 μPa). However, there is no theoretical

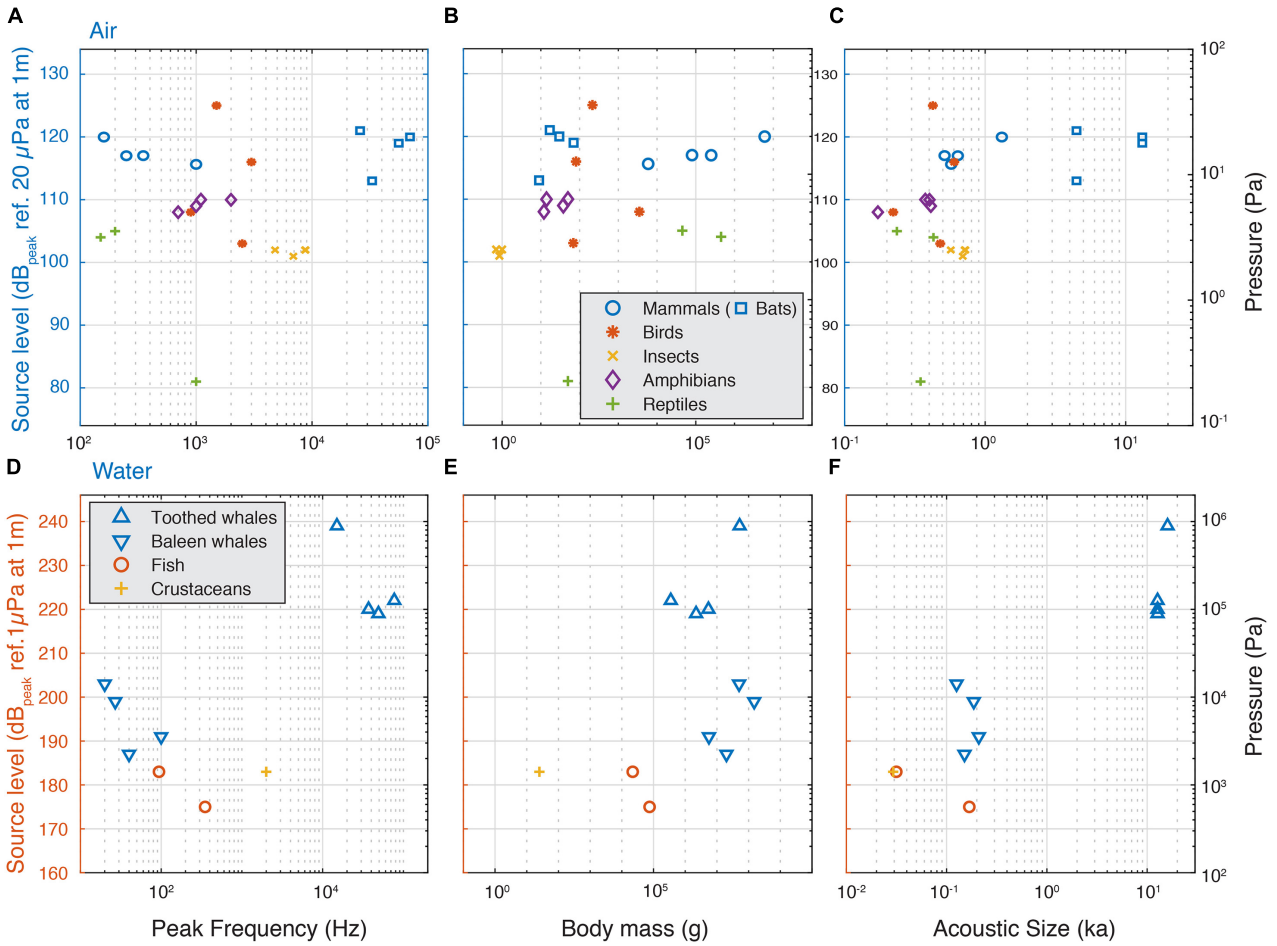

FIGURE 3 | Source levels of the loudest animals do not increase with frequency, weight and acoustic size in air, but do underwater. **(A)** The highest SLs do not increase with frequency, **(B)** body mass or **(C)** ka for animals vocalizing in air and is maximally 125 dB re. 20 μPa at 1 m. **(D)** In aquatic animals, we observe a trend that the highest SLs increase with frequency, **(D)** in aquatic animals, we observe a trend that the highest SLs increase with frequency, **(E)**, body mass and **(F)** ka, but the sparsity of the data prevents statistical interpretability.

physical upper limit to pressure, and extreme explosions can indeed surpass the 100 kPa positive crest. The supposed loudest explosion in recent human history was the 1883 Krakatoa volcano eruption with an estimated SL of about 270 dB$_{peak}$ re. 20 μPa at 1 m (Winchester, 2003). Besides many issues with approximating this particular SL, it is clear that in air, making sounds by exploding is not a viable option for animals, and vocalizations do not reach such enormous pressures.

In water, the minimal sound pressure is limited by the formation of vapor-filled cavities, i.e., cavitation, at 0 Pa. Because the ambient water pressure depends on depth in the water column, the difference between ambient pressure and cavitation also depends on diving depth. Thus, a sound wave at the water surface and symmetrical around atmospheric pressure can therefore also reach an amplitude of maximally 200 kPa peak-to-peak (220 dB$_{peak}$ re. 1 μPa). Again, there is no theoretical upper limit to pressure, but because the cavitation boundary poses a design constraint in human-made sonar systems (Woollett, 1962) it is reasonable to assume that this also is the case for biological systems. A sperm whale click of 239 dB$_{peak}$ re. 1 μPa would thus actually surpass the minimal crest limit when produced at shallower depths than 80 m.

The above physical limitations apply to acoustic pressure magnitudes irrespective of where they occur in the sound field of a source. However, what are the maximal sound pressures animals produce in the entire sound field that they radiate? Whereas SL is defined at the reference distance of 1 m, the highest pressures mostly occur much closer to most animals. To estimate the maximal acoustic pressures the loudest animals generate, we approximate them as two types of sound sources; a pulsating sphere and a piston in an infinite baffle (**Figures 4A,B**). In the far field sound pressure decreases with 6 dB per doubling of distance due to the spreading of the acoustic power over a larger area (Jacobsen and Juhl, 2013). A pulsating sphere only has a far field and the highest pressure produced is obtained at the surface of the sphere (**Figure 4A**, see section "Materials and Methods"). However, pistons and more complex sound sources also have a near-field where the pressure strongly depends on local conditions. For a piston in an infinite baffle the transition from near to far field boundary can be conservatively

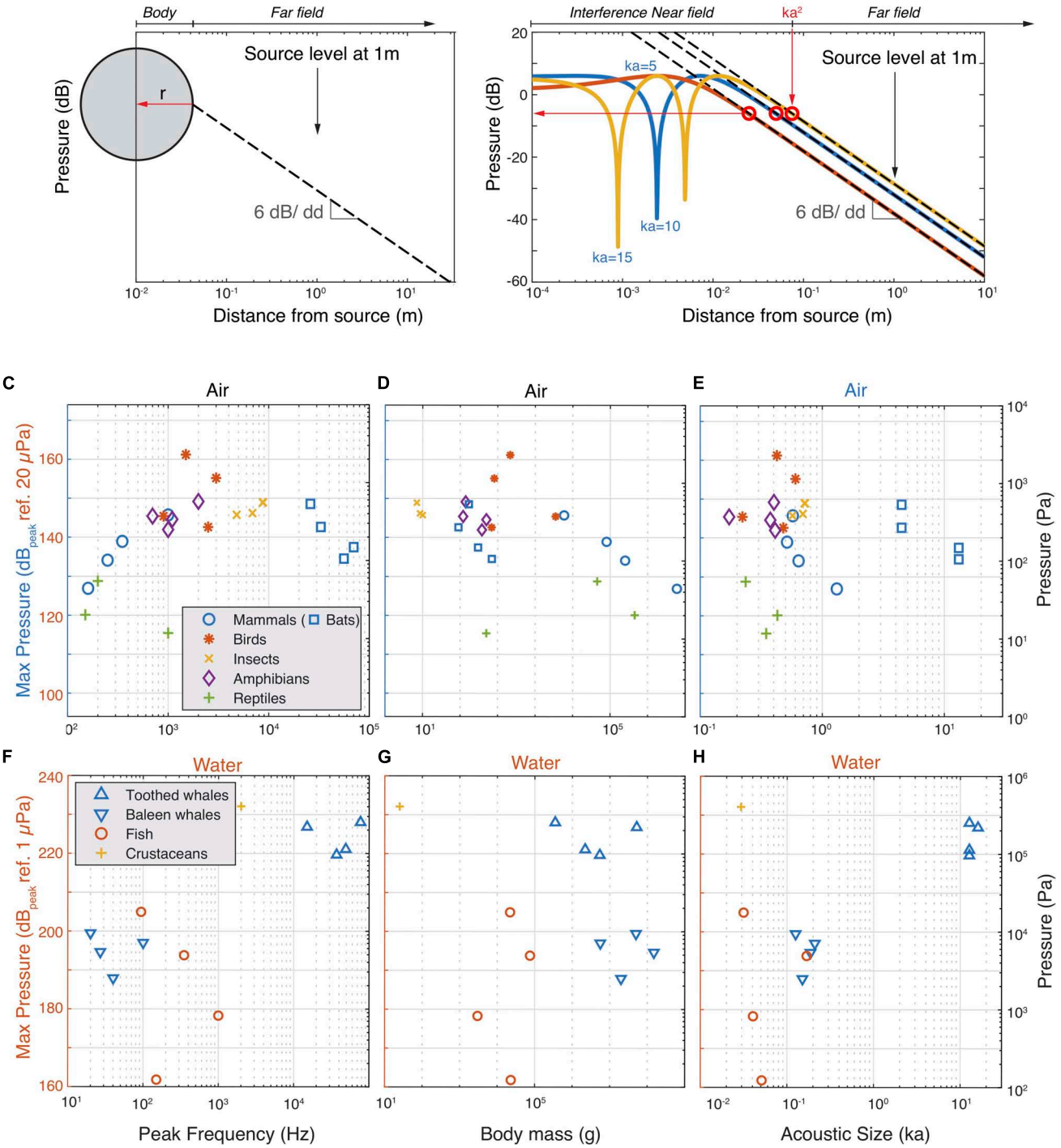

FIGURE 4 | The estimated highest occurring sound pressures in air and water. **(A)** For animals that are omni-directional sound radiators we used the monopole model to estimate the highest occurring pressure (red horizontal arrow). Because a monopole does not have a near field, we assumed the radius of the monopole to be the body wall (see section "Materials and Methods"). In far field conditions, the sound pressure decreases with 6 dB per doubling of distance. **(B)** For highly directional sound radiators (bats and cetaceans), we used the piston model to estimate the highest occurring pressure. We use the conservative estimate that the highest occurring pressure (red horizontal arrow) occurs at the border of the interference near field and far field (see section "Materials and Methods"). **(C–E)** Estimated highest produced sound pressures increase with frequency but plateau at about 150 dB ref. 20 μPa by animals vocalizing in air. **(F–H)** Estimated highest produced sound pressures seem to increase with frequency and size for animals vocalizing in water.

approximated by: $D_{piston} = k \times a^2$, where k is the wavenumber ($k = 2\pi/\lambda$) and a the radius of the emitter (**Figure 4B**; Foote, 2014). In the interference near field of a piston, pressure can be up to 12 dB higher than are the near/far field border we use for our approximation and strong dips occur that are highly sensitive to local conditions and ka-values (**Figure 4B**). Given the near field conditions are very specific for each animal, we consider it safer to use the more conservative maximum pressure at the boundary between the geometric nearfield and the far field.

Using these two models, we estimated the maximum pressures the loudest animals generate (see section "Materials and Methods"). In air, below 2 kHz the estimated maximum sound pressure increases with frequency (**Figure 4C**). However, at 2 kHz, the pressure seems to reach a plateau at 150 dB_{peak} re. 20 μPa with the exception of the Bellbird that reaches 160 dB_{peak} re. 20 μPa. This maximum pressure plateau is also maintained for animals under 10 kg but decreases with body mass over 10 kg (**Figure 4D**) and radius over 5 cm (**Supplementary Table 1**). When estimating the maximum pressure produced, the frogs and cicada's move up and interestingly, all loudest mammals, birds, cicada's and frogs converge upon 140–150 dB_{peak} re. 20 μPa. In water there is a trend that maximum pressure increases with frequency with no indication of a plateau as seen in air (**Figure 4F**). However, body mass, and ka product do not show clear relationships with the maximal pressure (**Figures 4E,F**). Both the pistol shrimp and the toothed whales produce estimated maximal pressures as high as 230 dB re. 1 μPa and reach cavitation limit pressures at depths less than 30 m.

Taken together, we observe that animals vocalizing in water roughly follow the source relations predicted by sphere or piston models. The loudest animals in water come close or reach a physical limit (cavitation) when producing loud sounds at shallow depths. The loudest animals vocalizing in air are efficient sound producers, but do not get close to the maximal amplitude for a symmetrical wave. Our data thus suggests that they are limited to amplitudes of 140–150 dB_{peak} re. 20 μPa.

Physical Upper Limits to Sound Propagation

The next physical limitation of sound production is the phenomenon that at high acoustic pressures sound propagation becomes non-linear and efficacy decreases. The non-linearities occur since the speed of sound is temperature dependent and pressure fluctuations are accompanied by temperature fluctuations. As a result, the positive pressure crest travels faster than the negative pressure crest. This effect accumulates over distance and eventually (depending on loss mechanisms) shockwaves may form, even from a waveform that is initially a sinusoid (Pierce, 1981). This distance from the source at which the shock wave is formed is called the shock formation distance. The relevant propagation (e.g., communication or prey detection) distance is thus a key factor to include when estimating shock formation distance. The creation of shockwaves is frequency and level dependent and the radiated waveshape at the source also plays a major role. The sound producing process itself might lead to a waveform that is close to that of a shockwave, thereby reducing the shock formation distance. Because of these propagation non-linearities, very loud sounds attenuate much more rapidly with distance than dictated by simple spherical spreading loss and atmospheric attenuation. The introduction of propagation non-linearity can (depending on level, frequency, and range) even give rise to a saturation effect for sound propagation in air and water, because increased SL beyond this level is not associated with an equivalent increase in signal range (Pierce, 1981).

However, the effects of spherical spreading and absorption counteract the formation and propagation of shockwaves. Since absorption in both air and water increase with frequency, the higher harmonics caused by the transition into a shockwave are attenuated more than the fundamental frequency leading to a sinusoidal waveform at large distances (the so-called old-age region) (Pierce, 1981). The strength of this counteracting effect depends on amplitude, frequencies and propagation distances. This effect along with the saturation effect is in particular relevant for animals communicating over long distances.

Shock wave formation can thus be considered a realistic but "soft" limit to sound production in air and water, because it is frequency, level, waveshape and distance dependent. Due to the complicated non-linear acoustics involved, analytical models of the attenuation of shock waves are limited to approximate cases such as plane wave propagation of an initially sinusoidal waveform. As a rule of thumb and at moderate distances, sound pressure can reach 150 dB ref. 20 μPa in air and 240–250 dB ref. 1 μPa in water before physical non-linearity and additional losses significantly reduce amplitude (Larsen and Wahlberg, 2017). Thus at least in air, the loudest birds, mammals, frogs and insects create sound pressure levels that approach the level at which non-linear propagation losses become significant and further increase would be inefficient as a mean to increase communicative distance. Thus, radiation non-linearities may provide a realistic physical limitation to making louder sounds. The resulting skewed sound waveforms are at least consistent with the bellbird calls and mammalian screams.

Definitively answering the question if propagation non-linearities are physically limiting sound production requires non-linear modeling and precise measurements. The acoustic nearfield and spherical spreading have to be taken into account and can only be solved numerically. Measurements of shock waves and thereby high-order harmonics from animals producing high-frequency vocalizations should be definitive, but also impose high demands to the equipment in terms of sampling frequency and transducer response. The conditions are so different for each species that the question must be solved on a case-by-case basis, which is beyond the scope of this paper.

Physiological Limitations to the Production of Loud Sounds

All extant vocalizing species have undergone millions of years of evolution and sound production is only one of a multitude of trade-offs individuals face in their survival. Many factors could thus play an important role in explaining why most species do not produce loud vocalizations. First of all, making high

acoustic pressures is also conspicuous and thus not necessarily an advantage. Another major factor is the energetics and efficiency of vocal production in relation to the ecology and behavior of a species. In frogs, birds, and bats it has been shown that high SLs come with a substantial increase in energy expenditure (Currie et al., 2020). Obviously, the duty cycle of calling plays a major factor in this; some frog species call at high duty cycle for several hours, but other species may only produce a few vocalizations per day. However, if power plays a major role, we would hypothesize that large animals would be louder as they could afford more energy, but our data does not support this. Additionally, loud sounds can become too loud and may temporarily deafen the receiver (Finneran, 2015). These are just a few reasons why an animal may not invest in making high sound pressures. However, can we identify more principal constraints in the physiology that pose a limitation to producing high sound pressures?

To answer this question, we need to look at the different mechanisms animals use to generate sounds. Sound production mechanisms differ widely and pose phylogenetic and evolutionary constraints. In some case they are not well-understood or even unknown. Most air-breathing tetrapods produce vocalizations by converting respiratory flow to modulated flow by self-sustained oscillation of laryngeal vocal folds or syringeal analogous structures. The resulting air pressure disturbances constitute the acoustic excitation of the system (Titze, 2000). This framework is called the myo-elastic aerodynamic theory of sound production or MEAD. The theory of sound production using MEAD is best studied in humans, but also found applicable to non-human mammals (Herbst et al., 2012) and birds (Elemans et al., 2015; Jiang et al., 2020). Amphibians and the few vocal reptiles probably also use MEAD (Rand and Dudley, 1993; Reber et al., 2015).

We identified at least four MEAD features that potentially pose limits to producing high SLs. A first limit is the efficiency by which aerodynamic energy is converted into acoustic energy. This efficiency is referred to as the glottal efficiency in laryngeal sound producers including humans (van den Berg, 1956; Bouhuys et al., 1968; Schutte, 1980) or vocal/mechanical efficiency (ME) (Titze et al., 2010; Zhang et al., 2019) and is defined as the ratio of radiated acoustic power over driven aerodynamic power of the subglottal/subsyringeal air. Acoustic power is typically determined by combining the measured sound pressure, impedance and an approximation of the area over which the energy is radiated. Aerodynamic power is calculated as the product of measured mean tracheal/bronchial airflow and pressure. When measured *in vivo*, ME captures both (i) the transformation of aerodynamical power into acoustic flow within the vocal tract, (ii) transmission efficiency through the airways, and (iii) the transformation of sound from the surface (mouth/beak/air sacs) to the environment (Titze and Palaparthi, 2018). ME varies greatly with bronchial pressure (Herbst, 2014), frequency (Zhang et al., 2019), vocal fold position, geometry and pathologies and also in between species (e.g., Brackenbury, 1979; Titze et al., 2010; Herbst, 2014; Maxwell et al., 2021) and values are reported between 10^{-4} to 2% (e.g., a factor of −60 to −20 dB).

Many animals have evolved anatomical or behavioral adaptations that aid in radiating the sound energy from their vocal organs to the radiated sound field. Indeed, the ME of excised vocal organs is typically lower because there is no upper vocal tract (Titze, 2006). Anatomical adaptations to increase sound radiation efficiency, such as air sacs in frogs (Rand and Dudley, 1993), birds (Riede et al., 2004), and mammals (Riede et al., 2008), or enlarged larynges in howler monkeys (Dunn et al., 2015) and hammerhead bats (Schneider et al., 1967). Additionally, behavioral adaptations can be found such as posture modifications to increase mouth/beak opening when emitting high SLs, as seen in the bell bird and, howler monkeys. Models suggest that for mammals and birds, adjustments of head size, mouth opening, and beam direction can make the power transformation efficiency from vocal tract to radiated sound as high as 100% in the 1–50 kHz range (Titze and Palaparthi, 2018). Some animals even change their environment by constructing horns or baffles that aid in radiating the sound (Mhatre et al., 2017).

A second limitation is the amount of aerodynamic energy an animal can produce. *in vivo* and excised larynx and syrinx work has shown that SL increases with mean bronchial pressure (Schutte, 1980; Zhang et al., 2019). The increasing pressure leads to higher VF displacement, sharper flow starts and stops and therefore a higher SL. The maximal expiratory pressure is limited by the maximal effort of respiratory muscles and in humans ranges from 5 to 7 kPa during crying in infants and up to 10–15 kPa in adults during shouting (Wilson et al., 1984; Dimitriou et al., 2000; Lagier et al., 2017). Without vocalizing, higher expiratory pressures over 20 kPa can be achieved by both normal and brass instrument playing adults (Fiz et al., 1993).

However, before the maximal respiratory pressure or flow is achieved, a third limit is typically reached. As bronchial pressure and flow increases, at specific values the dynamics of VF vibration behavior bifurcates from regular to chaotic regimes. This point is called the phonation instability pressure or flow (Jiang and Titze, 1993; Hoffman et al., 2012). As pressures exceed the phonation instability pressure (PIP) the SL does not increase further in the few species studied (Jiang and Titze, 1993; Zhang et al., 2007; Hoffman et al., 2012), probably because the vocal efficiency decreases. Although using pressure above the PIP is unfavorable from an energetics point of view, irregular or chaotic vocal fold regimes are common in mammalian vocalizations (Wilden et al., 1998; Fitch et al., 2002) and their signaling function in communication thus likely outweighs the loss of energy efficiency.

Fourth, with increasing amplitude the collision force of vocal folds, or impact stress, increases. Although short peak impacts may not be a limiting factor *per se*, accumulative vocal fold damage due to a large amount of high impacts, aka the vibration doses, may be limiting. Through intense voice use, damage can accumulate over time and tissue stress is suggested as the tradeoff for peak performance (Titze and Hunter, 2015). Impact stress is also the main traumatizing mechanism in human voice production, and the main cause of vocal fold nodules (Horacek et al., 2009). In humans, many impact related VF pathologies are known, but to our knowledge there is no reports on VF pathologies in animals.

Taken together, for animals using MEAD to produce vocalizations, at least the above four physiological constraints could pose limits to SL. However, we suggest that these

constraints are not hard limits, but should be more seen as trade-offs in energy expenditure or vocal fold damage. Furthermore, our current dataset does not allow investigation of allometric scaling with anatomical and physiological parameters (e.g., Charlton and Reby, 2016), because we did not systematically sample across a range of SLs and taxa that use MEAD. Instead we specially mined the literature for the highest SLs. It would be interesting to see if within phylogenetically related taxa of animals using MEAD allometric relationships can be found, as between SL and size within the cicada's (Villet, 1987).

The loudest insects, the cicadas, use a fundamentally different mechanism to produce sound. Cicada's buckle ribs on their tympanum that results in clicks, which provides a resonant source that drives the abdominal resonator, from which sound is radiated *via* the tympana (Young and Bennet-Clark, 1995). The limit to produce clicks is unknown, but most likely related to mechanical failure of the tympanic ribs.

Animals producing loud sounds in water do so by at least three mechanisms. The unique mechanism by which pistol shrimp produce sound using their large snapper claw is well-understood. Muscle co-contraction builds up tension that is released by contraction of another muscle. The rapid closure of the claw pushes a plunger into a socket, and creates an outward water jet at such velocity that a cavitation bubble forms. It is the implosion of this cavitation bubble that creates the loud snapping sound (Versluis et al., 2000).

Bony fishes have evolved perhaps the largest diversity of sound generating organs among vertebrates (Fine and Parmentier, 2015; Ladich and Winkler, 2017). For the few species studied, the most common mechanisms are muscle driven vibration of a gas-filled bladder, and stridulation mechanisms of pectoral girdle or fin (Ladich and Winkler, 2017). The loudest teleost fish reported here most likely produce sound by swim bladder vibration (Locascio and Mann, 2011). Because all vertebrate muscles trade-off muscle power and speed, the fastest muscles can move at rates of 270 Hz (Mead et al., 2017). These extreme contraction rates still produce low frequencies for sound. Given the size of the fish, these result in ka $<$ 1, which makes them poor pressure radiators. However, many fish are mostly sensitive to particle motion, not pressure, and thus pressure may not be the most relevant cue for communication (Radford et al., 2012).

In cetaceans sound production has received much attention, however, we have no convincing direct evidence of how the sounds are produced. Cetaceans have shared ancestry with the artiodactyla and sound production is thought to be driven by air flow. In mysticetes, the hypothesis that sound is produced by laryngeal tissue vibration is based on anatomy (Damien et al., 2019) and we still lack direct experimental observation to test outstanding hypotheses. Their relative low ka values make them suboptimal sound radiators, but the low-frequency emission may be favorable because of low absorption and thus allow long-range communication. The odontocetes produce the highest sound pressures of all animals (Mohl et al., 2003). Several lines of evidence suggest that sound production occurs at the phonic lips in the upper nasal passages, either by a muscle-driven catch-release mechanism or an air-flow driven MEAD system. The sound radiates from the melon is highly directional. In the sperm whale, the produced sound is collimated inside the enormous nasal complex, resulting in the most directional sound source known where most energy is concentrated in a beam of only a few degree (Mohl et al., 2003). However, given the fact that odontocetes are producing the highest sound pressures of any animal on the planet especially warrants further investigation to understand how they manage to produce 1 MPa sounds.

CONCLUSION

Across the animal kingdom we find that the loudest animals span several orders of magnitude of size and frequency and can be found in all phylogenetic groups and habitats. To investigate what potential mechanism could limit the generation of loud sounds, we compiled SL data for animals vocalizing in air and water. In air we see that SLs are limited to 125 dB_{peak} re. 20 μPa at 1 m after correcting for scaling conventions. The maximum actual pressure generated are 140—150 dB_{peak} re. 20 μPa, typically much closer to the source than one meter. Several physiological processes could be limiting but given the many tradeoffs the different animals face during evolutionary history it is hard to point to a single constraint that explains the maximally observed values. Two physical constraints are of a magnitude to pose serious limitations. First the acoustical size (ka) constraints the efficiency of sound radiation. The loudest animals in air all seem to be good radiators, maybe except for the elephant, with ka close to or above 1. Second, non-linear propagation makes it inefficient, but not impossible, to make louder sounds. Thus, in air, physical limitations and particularly non-linear propagation could play a major role in how loud animals can maximally get.

In water, pistol shrimp and odontocetes produce extreme acoustic pressure close to the zero pressure (cavitation) limit. The loudest fish reach a physiological limit that muscle-powered swim bladder motion is limited to generating frequencies of 300 Hz. The mechanisms of sound production in both baleen and tooth whales are not well understood. How these animals achieve these incredible SLs is not well known.

Being loud is one of many strategies of the surprising tapestry of animal vocalizations. The loudest animals produce sound pressures where several physical processes become highly non-linear. To solve which process poses a limitation to producing higher SLs requires the development and detailed testing of numerical models on a case-by-case basis. Although for the majority of animals, being loud has not been an evolution strategy, we see that both in air and in water, species have evolved that are pushing against the physical limits of sound production.

MATERIALS AND METHODS

Source Level Comparison and Compilation

We determined SLs by making the following two conversions to the literature data if relevant: First, we use sound pressure level (peak) as the proxy for sound amplitude (**Figure 1A**). For the

particular purpose of this study, peak is a better measure than RMS because it represents the maximum pressure the animals are producing while RMS averages the pressure over the duration of the sound. We did this conversion using the relationship between peak, peak-to-peak and RMS for a simple sinusoid, i.e., by adding 3 dB to RMS values or subtracting 6 dB from peak-to-peak values. For RMS values this underestimates the peak value for non-sinusoid signals, which makes our SL_{peak} values conservative estimates. Second, we calculate SL to the standard reference distance of 1 m using spherical spreading attenuation. While atmospheric attenuation becomes substantial in air at frequencies >20 kHz, it is negligible over the short distances we encounter here and very likely less than the overall uncertainty involved in the reported measures. All our values are based on the highest reported values in each study.

Pulsating Sphere and Piston Model

To relate sound pressure measurements at one position to another we must adopt a model of the sound source and the propagation medium. For the medium we assume lossless free space and discuss air/water-attenuation at ranges where these effects are relevant. For the sound source we employ two models: the pulsating sphere and the piston in a baffle, which despite being simple approximations are quite often used in bioacoustics.

For a pulsating sphere the relation between pressure amplitude and surface velocity is (Jacobsen and Juhl, 2013):

$$\left|p\left(r\right)\right| = \frac{k}{\sqrt{1+\left(ka\right)^2}} \frac{\rho c\left(4\pi a^2 U\right)}{4\pi r} \quad (1)$$

where ρ is the density of medium, c is the speed of sound in medium, wavenumber $k = 2\pi$ f/c,

a is the radius of the sphere, U is the velocity of the sphere surface and r is the distance to center of sphere. If the velocity is given as an RMS value, the resulting sound pressure is an RMS as well and so forth for peak or peak-to-peak values. The quantity ($4\pi a^2$ U) is the volume velocity of the sphere, which is often used to characterize source strength in acoustics.

For a piston in a baffle, we limit the discussion to the on-axis pressure, the amplitude of which can be calculated by, (Jacobsen and Juhl, 2013)

$$\left|p\left(x\right)\right| = 2\rho cU\left|\sin[0.5k(\sqrt{x^2+a^2}-x)]\right|, \quad (2)$$

where x is the distance to the center of the piston. For high frequencies and close distances strong interference can occur (**Figure 4B**), whereas an approximate expression can be found for long distances (compared to both radius and wavelength):

$$\left|p\left(x\right)\right| = 2k\frac{\rho c\left(\pi a^2 U\right)}{4\pi x}, \quad (3)$$

Note that the volume velocity of the piston, (πa^2 U), is one-fourth of that of the sphere.

For a given radius and volume velocity, the frequency response of the sphere is increasing by 6 dB/octave at low frequencies before reaching a limit at ka = 1 (3 dB corner frequency). For the piston in a baffle, there is no such limit in the far-field, but evidently the near-field extends further with increasing frequency.

Estimation of Maximal Acoustic Pressure

For sources that can be considered equivalent to oscillating pistons, we used the theoretical boundary between the interference near field and far-field as the distance to the source where the highest sound pressure occurs. According to Foote (2014), this can be approximated conservatively as:

$$\text{Distance} = \frac{2 \times \pi \times a^2}{\lambda}$$

Where a is the radius of the piston and λ is the wavelength of the sound. We use this approximation for the toothed whales and bats who's highly directional sound emission patterns have been shown earlier to fit well with piston model predictions (see e.g., Mohl et al., 2003; Jakobsen and Surlykke, 2010). For bats we used the piston-fit to the measured directionality of *E. fuscus* as reported in Hulgard et al. (2016). We assumed that *E. bottae* emits similar directionality to *E. fuscus* and computed a using emitted frequency as reported by Holderied et al. (2005). For The two Noctillio, we assume higher directionality based on the much higher emission frequency relative to body size, we therefore adjust the size by the difference in estimated maximum gape size as reported by Thiagavel et al. (2017). For Toothed whales, the end of the near field of *T. truncatus* is ca 0.5 m Finneran et al. (2016). Given that *P. crassidens* emits the same directionality as *T. truncatus* and assuming that *O. orca* does so as well, we estimated a from the known nearfield of *T. truncatus* and the emitted frequencies of each species. Directionality is higher for *P. macrocephalus* and we accounted for this by multiplying the assumed a at equal directionality to *T. truncatus* by the difference in directivity index (2 dB = 1.25) [see Jensen et al. (2018) for directivity measures].

For sources that can be considered monopoles, the limitation is essentially the size of the animal as there is no interference nearfield. We approximate animals that emit sound with no apparent directionality as monopoles, i.e., a ka product < 1 (see **Figure 4**), which included all animals other than bats and toothed whales. Acoustic size estimates are not commonly given in the literature, so we used approximations based on available morphological measures. For frogs we estimated the size of the vocal sac as half the length of the animal (snout-vent length) and assume that the vocal sac is equal to the size of the monopole. For the cicada we estimated the width of the body from the commonly given hemelytra length using the known relationship between hemelytra length and body width reported for *Cyclochila australasiae* (Young, 1990). For the pistol shrimp, we used the size of the cavitation bubble reported by Versluis et al. (2000). For the fish, we computed the radius of a cylinder based on reported lengths and weights assuming the same density as water. For all other animals we used the halfwidth of the skull as the monopole radius. All values are given in **Table 1** and **Supplementary Table 1**.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

LJ, JC-D, PJ, and CE: conceptualization, formal analysis, and writing – review editing. LJ, PJ, and CE: methodology. LJ and CE: writing – original draft. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.657254/full#supplementary-material

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Cues for Directional Hearing in the Fly *Ormia ochracea*

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Insects are often small relative to the wavelengths of sounds they need to localize, which presents a fundamental biophysical problem. Understanding novel solutions to this limitation can provide insights for biomimetic technologies. Such an approach has been successful using the fly *Ormia ochracea* (Diptera: Tachinidae) as a model. *O. ochracea* is a parasitoid species whose larvae develop as internal parasites within crickets (Gryllidae). In nature, female flies find singing male crickets by phonotaxis, despite severe constraints on directional hearing due to their small size. A physical coupling between the two tympanal membranes allows the flies to obtain information about sound source direction with high accuracy because it generates interaural time-differences (ITD) and interaural level differences (ILD) in tympanal vibrations that are exaggerated relative to the small arrival-time difference at the two ears, that is the only cue available in the sound stimulus. In this study, I demonstrate that pure time-differences in the neural responses to sound stimuli are sufficient for auditory directionality in *O. ochracea*.

Keywords: directional hearing, eardrum, insect, phonotaxis, interaural difference, coupled ears, *Ormia*

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INTRODUCTION

The fly *Ormia ochracea* (Diptera:Tachinidae) possesses an auditory system that performs analagous functions to those of vertebrate hearing (detection, recognition, segregation, and localization or sources), albeit for a restricted range of stimuli (Gray et al., 2007). The flies are parasitoids and females must locate a cricket host in order to reproduce (Wineriter and Walker, 1990). They accomplish this by localizing the calls of singing male crickets using an auditory system dedicated to this task (Cade, 1975). Tympanal hearing is unusual for flies. All known examples are species that are parasitoids of acoustic insects (Allen, 1995; Robert et al., 1999; Lehmann, 2003), and these include two families (Tachinidae and Sarcophagidae) in which tympanal hearing has evolved independently through convergent adaptation of the same precursor organ (Edgecomb et al., 1995; Robert et al., 1996a; Lakes-Harlan et al., 1999).

Due to the small size of the flies (ears are < 0.5 mm apart) relative to the wavelength of cricket sound ($\sim$7 cm), acoustic directional cues are severely restricted (Kuhn, 1987). Sound waves impinging on the fly auditory system generate no interaural level difference (ILD) and interaural time differences (ITDs) are very small (maximum 1.5 μs for a sound source at 90° relative to the midline axis). Nevertheless, flies can localize a cricket sound source with exceptional accuracy ($<$2° azimuth, Mason et al., 2001).

Abbreviations: BMAA, biomimetic antenna array; ICE, internally coupled ears; ILD/ITD, interaural level/time difference; MEMS, micro-electromechanical system; nITD/nILD, neural interaural level/time difference; tITD/tILD, tympanal interaural level/time difference.

Directional hearing in *Ormia* is derived from a specialized mechanical coupling between the two tympanal membranes (Robert et al., 1998). Mechanical coupling of the two eardrums amplifies the small direction-dependent ITDs in the sound field, and generates ILDs in the tympanal vibration responses, so that both cues are present in the tympanal (Robert et al., 1996b) and neural responses (Mason et al., 2001; Oshinsky and Hoy, 2002). Modeling of *Ormia* auditory mechanics (Miles et al., 1995) demonstrated that intertympanal coupling results in a system with two resonant modes of vibration in response to acoustic stimulation (**Figure 1**): a symmetric mode, in which the two tympani vibrate with equal amplitude and phase; and an antisymmetric mode, in which the two tympani vibrate with equal amplitude but opposite phase. Under normal acoustic conditions, a sound source located directly ahead of the fly (0° azimuth) generates vibration in the symmetric mode (each tympanum driven by identical sound pressure waves). Sound impinging from any other direction, however, will stimulate a combination of both modes of vibration with the result that the two tympani will respond with different (direction-dependent) amplitudes and phases of vibration, with maximum interaural differences of ∼12 dB in amplitude and ∼50 μs delay (Robert et al., 1996b).

Analyses of the mechanical properties of *Ormia* tympanal membranes (Miles et al., 1995; Robert et al., 1996b; Akçakaya and Nehorai, 2008) have demonstrated that the mechanical coupling between the two eardrums enhances the system's sensitivity to the minute direction-dependent differences in arrival time of sound at the two ears. The nature of this effect is two-fold. (1) The arrival-time difference is amplified to result in a larger ipsilateral-leading phase difference between vibrations of the two tympani, creating a tympanal interaural time difference (tITD). (2) The amplitude of contralateral tympanal vibration is reduced relative to ipsilateral, creating a tympanal interaural level difference (tILD).

The majority of auditory receptors associated with each ear respond with tonic bursts at the onset of sound pulses (Oshinsky and Hoy, 2002) with response latencies that are dependent on tympanal vibration level, such that tILDs result in direction-dependent interaural latency differences in receptor responses. These neural interaural time differences (nITD) scale with the azimuth of the sound source location (Mason et al., 2001; **Figure 2**). Receptor thresholds vary, however, and another effect of tILDs is (direction dependent) differential recruitment of receptors in the two ears, such that directional sound sources will also generate interaural differences in the amplitude of summed neural responses – neural interaural level differences (nILD).

The directional mechanism of interaural coupling in ormiine ears was at first considered to be a unique evolutionary innovation. However, *Ormia* directional hearing is now considered to be a specialized example of a taxonomically widespread phenomenon by which acoustic directional cues (mainly ITDs) are amplified via interactions between the two ears to generate larger ITDs and ILDs in tympanal vibration which can then be used to encode directional information in neural responses. Internally coupled ears (ICE) include the majority of vertebrate auditory systems (van Hemmen et al., 2016), and general models of ICE have been derived (Vedurmudi et al., 2016a).

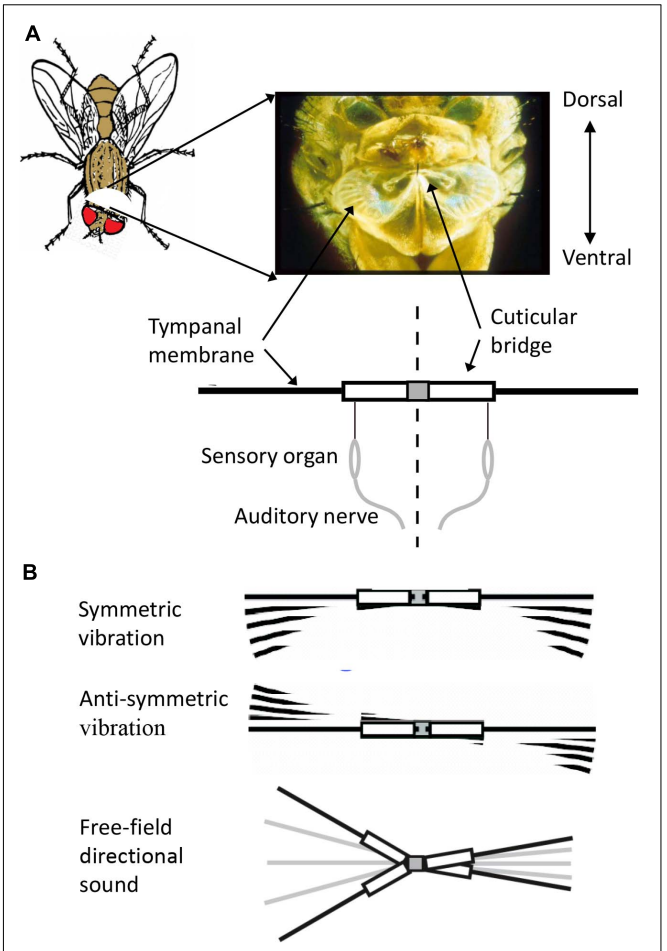

FIGURE 1 | (A) *Ormia* auditory system. Ears are located behind the head – less than 0.5 mm apart. Mechanical coupling between eardrums, via the cuticular bridge, allows resolution of miniscule interaural time differences – the only acoustic directional cue available. Auditory receptors in each ear are attached to a single point at the end of the cuticular bridge. **(B)** Symmetric and antisymmetric modes of vibration. Symmetric mode (upper) corresponds to non-directional sound source (0° azimuth). Equal, in-phase sound pressure drives equal-amplitude, in-phase vibration of the two tympani, bending at the midline. In antisymmetric mode vibration (middle) the tympani vibrate with equal amplitudes and opposite phase – rocking like a teeter-totter. Sound stimulation from any direction other than 0° would elicit tympanal vibration representing a combination of these two modes resulting in unequal vibration of the two tympani (lower).

The principle of directionality via coupled hearing has been the basis of multiple auditory adaptations (Römer and Schmidt, 2016), just as insect ears in general show a striking diversity of independent evolutionary origins (Yack and Dawson, 2008). *Ormia* hearing in particular has been a subject of considerable interest as a model for evolutionary arms races between this eavesdropping parasitoid and its acoustically communicating hosts (e.g., Zuk et al., 1995; Wagner and Basolo, 2007; Sakaguchi and Gray, 2011).

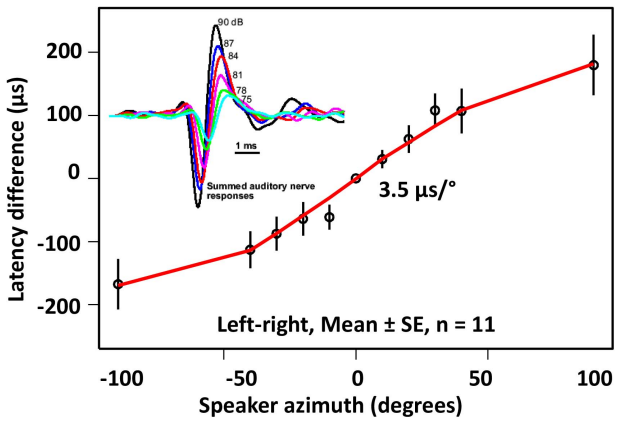

FIGURE 2 | Interaural latency differences as a function of sound source azimuth [redrawn from Mason et al. (2001)]. Inset: Summed auditory nerve responses recorded for different sound levels. Using sound level as a proxy for directional variation in relative tympanal vibration amplitude, these data show that in addition to the nITDs represented in the main plot, contralateral neural responses are weaker for a directional sound source.

Ormia auditory directionality has also emerged as an adaptable model for novel technology, and the past couple of decades have seen considerable interest in biomimetic applications of the flies' intertympanal coupling principle to engineering problems related to source localization for waveform signals, with two main areas of research. Efforts to design biomimetic directional microphones (BDMs, with application, for example, in hearing aids) have sought to mimic the mechanical properties of the fly eardrums in micro-electro-mechanical-system (MEMS) devices (Ishfaque and Kim, 2018; Zhang et al., 2018). Efforts to design biomimetic antenna arrays (BMAAs) have applied the principle of coupled detectors to electromagnetic signal localization (Grüner et al., 2019). Much of this work has focused on quantifying the contribution of sensor coupling to directional resolution (Akçakaya and Nehorai, 2008; Grüner et al., 2019), tuning the effective bandwidth of the system (Rahaman and Kim, 2020), extending the mechanism to more than one axis (Lisiewski et al., 2011), and optimizing noise-robustness and sensitivity in the readout of ILDs and ITDs (Miles et al., 2009).

Previous work has shown that directional information is represented in the fly auditory system by amplified ITDs in the responses of auditory receptors (Mason et al., 2001). Other work (Oshinsky and Hoy, 2002) suggests interaural differences in the amplitude of neural responses based on differential receptor recruitment may also play a role in directional hearing. There is also evidence that small time-differences between competing auditory sources may play a role in source segregation in *Ormia* (Lee et al., 2009), and that noise sources may introduce a systematic bias in directional sensing that is a direct consequence of the coupling mechanism (Lee and Mason, 2017). Thus, as in vertebrate hearing, ITDs and ILDs may both contribute to directional hearing in *Ormia ochracea*, although the way these cues are combined in fly directional hearing is not fully resolved.

This study examines in more detail the relationship between ITDs and ILDs at the tympanal and neural levels, and the contribution of these cues to directional hearing using a series of experiments that measure behavioral, tympanal and neural responses to stimuli with manipulated phase and amplitude to generate ITDs in isolation. The aim is to answer a fairly simple and specific question – can the flies make use of pure time differences in auditory responses to generate directional responses to acoustic stimuli? The results show that interaural time differences do mediate directional responses in the absence of interaural level differences. I consider implications of these data for how ITD and ILD mechanisms may represent alternative solutions to differing physical and evolutionary constraints.

MATERIALS AND METHODS

Animals

Experiments were conducted on lab-reared gravid female *Ormia ochracea* derived from specimens originally collected in Gainesville FL. Flies were maintained at 25°C and 75% humidity on a 12-h:12-h light:dark regime and fed nectar solution (The Birding Company, Yarmouth, MA, United States) *ad libitum*.

Acoustic Stimuli

Single tone pulses (5 kHz, 10 ms duration, 0.1 or 0.5 ms rise/fall time) or synthetic cricket chirps (10 pulses at 50/s) were delivered from two speakers at 84 dB SPL (unless otherwise specified). Acoustic stimuli were synthesized using Tucker-Davis Technologies (TDT) hardware (System 3) and custom scripts written in C or Matlab. The stimuli were amplified (NAD S300), passed through a programmable attenuator (TDT model PA5) and broadcast from piezoelectric horn tweeters (Radio Shack Realistic, Taiwan). Stimulus amplitude and timing were controlled by computer and calibrated with a probe microphone (B&K Type 4182, Denmark). The relative phase and amplitude of simultaneous stimuli were adjusted to manipulate auditory ITDs and ILDs independently (see below).

Experimental Measurements

Behavior

Phonotactic responses were recorded with flies mounted on a spherical treadmill which transduced walking movements for recording by computer (Mason et al., 2001). This open-loop setup allowed stimulus conditions to be held constant throughout the duration of presentation. For comparisons of flies walking direction under different stimulus conditions I measured the angle of the fly's trajectory at the halfway point of each (virtual) walking path.

Tympanal Vibration

Following behavioral experiments, flies' heads were removed and tympanal vibration measured under identical acoustic conditions, using a laser Doppler vibrometer (LDV) (Polytec OFV 3001 controller, OFV 511 sensor head).

Auditory Nerve Recording

For some stimulus conditions, I recorded summed auditory nerve responses simultaneously from both ears, under stimulus conditions similar to behavioral and tympanal measurements, using tungsten wire electrodes (AM Systems, 0.25 mm). Amplified (AM Systems Model 1800) neural responses were averaged (50 sweeps) and recorded by computer (TDT AD1, 100 kHz sampling rate).

Behavioral, physiological, and mechanical measurements were all carried out in the same setup, with behavioral and mechanical measurements made on the same individuals. Physiological recordings were made on separate cohort of specimens under identical conditions.

I first repeated the measurement of eardrum responses to directional stimuli using the same setup as the other experiments and confirmed comparable results to those in the literature. I then conducted a set of experiments (1–3) aimed a manipulating nITDs and nILDs separately, to address the question of how much each of these response parameters contributes to the coding of auditory directionality.

RESULTS

Auditory Cues for Sound Localization

Figure 2 shows variation in the timing and amplitude of summed auditory nerve responses over a 15 dB range of stimulus levels comparable to the range of tILDs. Previous studies (Oshinsky and Hoy, 2002) have suggested that these nILDs could contribute to the coding of sound source direction, with some data suggesting that nILDs provide more accurate directional information than nITDs (Pollack and Mason, 2014).

I conducted a set of experiments aimed a manipulating nITDs and nILDs separately, to address the question of how each of these response parameters contributes to the coding of auditory directionality.

Experiment 1 – Standing Wave

This experiment was designed to exploit the antisymmetric mode of tympanal vibration by placing a fly at the node of an acoustic standing wave.

Flies were tethered in place atop the spherical treadmill (Mason et al., 2001), between two speakers positioned at ± 90°. Stimuli were calibrated with a probe microphone just above the midline of the tympanal membranes, and the amplitude and timing of identical, but opposite-phase, acoustic stimuli from each speaker were adjusted to create a null at the fly's midline. With a node positioned at the midline of the fly (i.e., the junction of the two tympani), sound pressure acting on the two tympana is equal-amplitude and opposite phase, and the tympanal membranes should rock in the antisymmetric mode of vibration (**Figure 1**). Reversing the phase of the standing wave would reverse the relative phases of tympanal vibration so that if this phase difference provided a directional cue, flies directional response should also change.

By broadcasting the same stimuli in phase from both speakers, I could also generate a summed waveform at the midline of the fly.

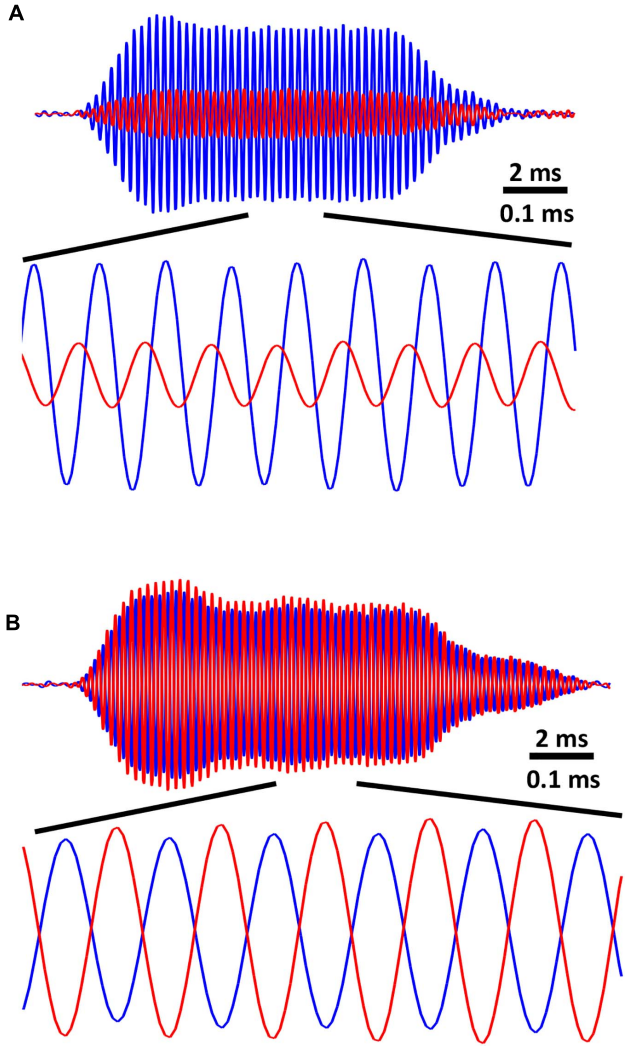

FIGURE 3 | (A) Recorded tympanal vibration (velocity, 6 sweeps averaged) for a sound source located 90° relative to midline. Ipsilateral (blue) tympanum vibrates with 12dB higher amplitude than contralateral (red), and contralateral tympanal vibration is delayed by 70 μs relative to ipsilateral. Note that traces show the response of the same tympanum with ipsilateral and contralateral referring to the location of the sound source. **(B)** Tympanal vibration (velocity) for two sound sources located ± 90° relative to midline. Sources broadcast identical, but opposite-phase stimuli, adjusted to create a standing wave with a node located at the midline of the two tympani. Tympanal vibrations are equal in amplitude and opposite phase, such that there is a 100 μs delay between corresponding wave peaks.

This condition should elicit symmetric mode tympanal vibration (mimicking a phantom source at 0° azimuth, Lee et al., 2009).

Flies' behavioral responses (phonotaxis toward synthetic cricket chirps) were recorded (n = 6) for: (i) directional signals from each speaker individually; (ii) the standing wave condition (signals canceling at the fly's midline), recorded for both relative phases of tympanal vibration (i.e., left-leading and right-leading); and (iii) the summing signals condition. After behavioral recordings I measured tympanal vibration under

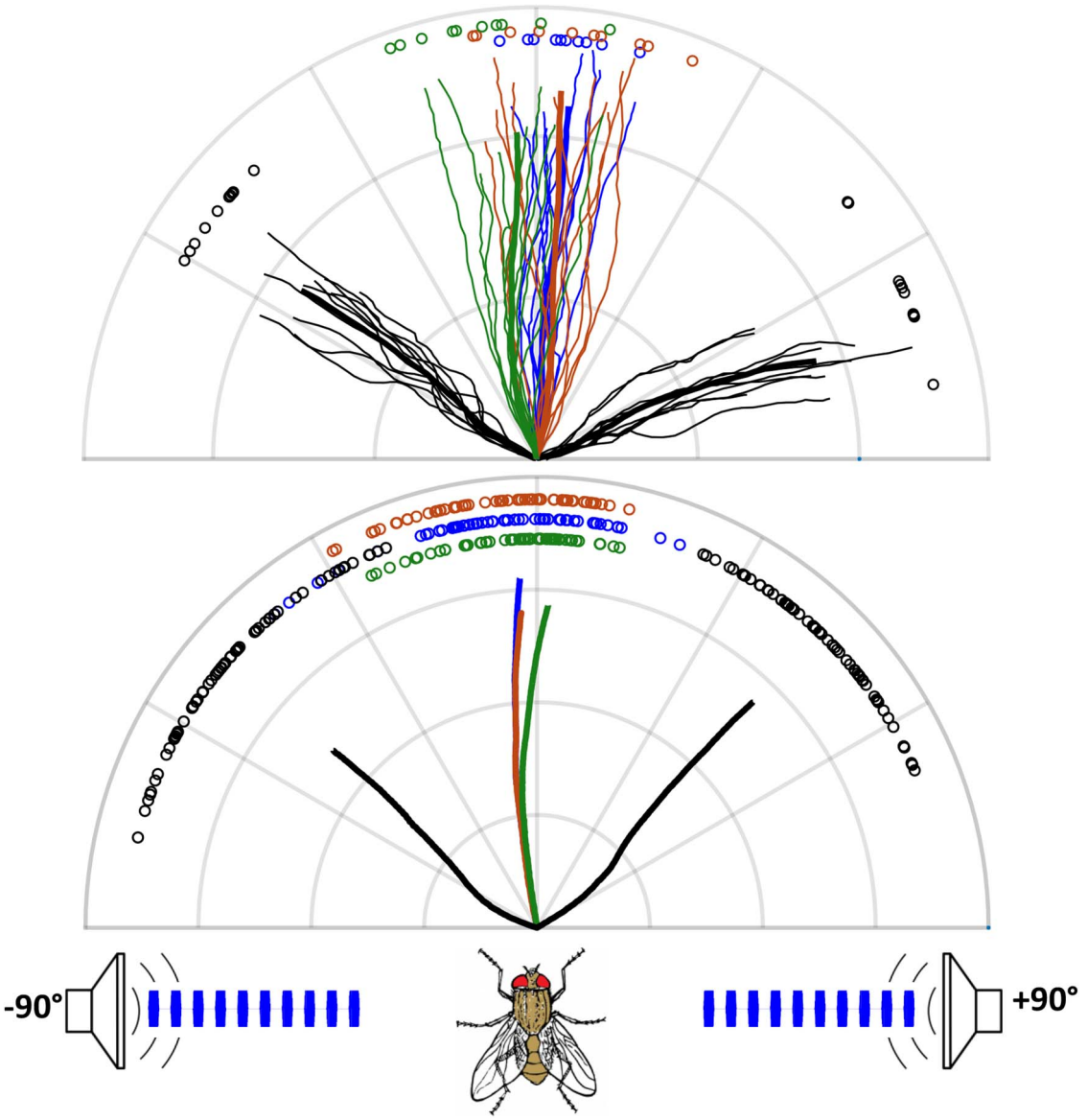

FIGURE 4 | Phonotactic walking responses for standing wave stimuli. The upper panel shows data for a single fly (grid = 5 cm). Thick lines represent averages (10 runs per trace); fine lines show the corresponding individual responses. Symbols (here and in subsequent figures) show the angles of the individual responses measured at the halfway point of each walking path, and statistical comparisons were based on these angles. The lower panel shows pooled responses for six flies (10 runs per fly in each trace, grid = 2 cm). Black traces are responses to stimuli from single speakers on the corresponding side of the fly. The green traces show responses to both speakers broadcasting in-phase stimuli (symmetric tympanal vibration simulates a single source at 0°). The blue and red traces show responses to both speakers broadcasting opposite-phase stimuli adjusted to create a standing wave (anti-symmetric vibration, for equal-amplitude but out-of-phase tympanal vibration), with blue and red traces representing opposite-phase standing waves. The flies' responses were not affected by the phase of the standing wave (single fly – Watson's $U = 0.093$, $p > 0.1$; pooled data – Watson's $U = 0.0416$, $p > 0.1$, $n = 6$). Responses to these conditions are similar to a forward source and show no directional response to cycle-by-cycle phase differences in the stimulus waveform as a cue for directionality (single fly – Rao's homogeneity test for vector direction = 1.55916, $p > 0.4$; pooled data – Friedman chi-squared = 3.0333, df = 2, $p > 0.2$).

identical conditions using the LDV to validate the stimulus conditions. I show the tympanal vibration data first.

Tympanal Responses

First, I verified the behavior of the system for conventional free-field auditory stimulation. Tympanal vibration in response to a single source located at 90° was exactly as predicted by the original analyses of the mechanics of the system (Miles et al., 1995; Robert et al., 1996b). Tympanal vibration in responses to an ipsilateral (90° azimuth) sound source was greater in amplitude by 12 dB and leading by 70 μs relative to the source-contralateral tympanum (**Figure 3A**).

In the standing wave condition, ipsi- and contralateral tympanal vibrations are equal-amplitude and 180° out of

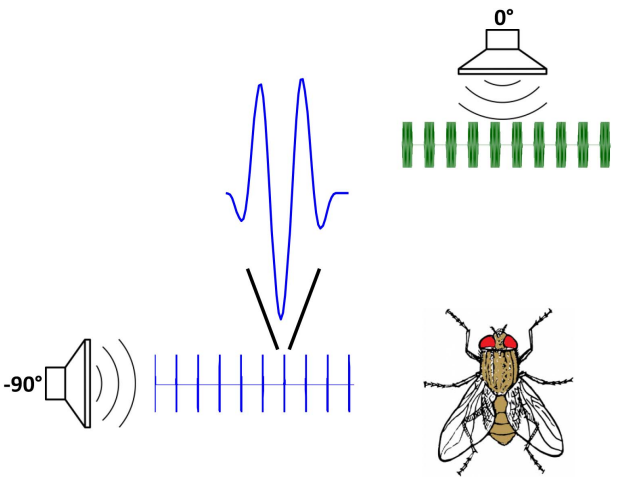

FIGURE 5 | Stimulus setup for onset-cancelation (Experiment 2). One speaker broadcasts an attractive stimulus at 0° (which should elicit phonotaxis in the forward direction). A second speaker, at 90°, broadcasts a series of impulses adjusted in amplitude and phase to cancel the initial portion of the attractive stimulus waveform. Due to the directional properties of the tympanal membranes, and the lateral location of the impulse source, cancelation will be greater for the tympanum ipsilateral to the impulse source (contralateral tympanal vibration for a 90° source will be attenuated relative to ipsilateral by 12 dB in these measurements). This condition should elicit equal-amplitude, in-phase tympanal vibration (consistent with a forward source location) but with a delay in the onset of the stimulus envelope on one side (ipsilateral to the impulse source).

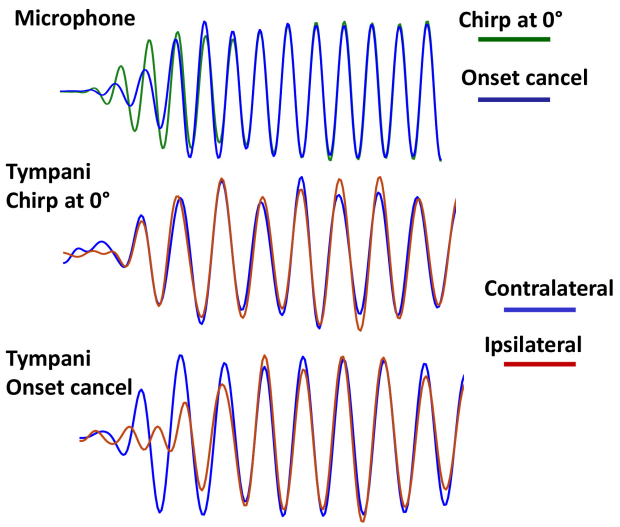

FIGURE 6 | Onset-cancelation tympanal responses. Upper traces show the onset of the stimulus waveforms recorded at the midline position of the fly (green – forward chirp alone; blue – chirp with impulses canceling initial onset). The rise-time of the stimulus waveform is delayed in the cancelation condition. Middle traces show the corresponding vibration responses for the two tympani for the forward chirp alone (simultaneous responses). Lower traces show tympanal vibration with onset cancelation. The impulse-ipsilateral tympanal response is delayed.

phase, equivalent to a ± 100 μs tITD (depending on the phase of the standing wave, **Figure 3B**). In the summed stimulus condition, the tympani showed equal-amplitude, in-phase vibration (symmetric mode) similar to a sound source directly ahead (0° azimuth, data not shown).

Behavioral Responses

Flies' sound localization behavior was highly consistent within each stimulus condition (**Figure 4**). In response to stimuli broadcast from either speaker alone, flies showed appropriately oriented phonotaxis. There was no difference in the orientation of phonotaxis between the standing wave and summed stimulus conditions and no effect of a phase reversal in the standing wave. In each condition flies walked directly ahead (0° azimuth). Pure asymmetric mode tympanal vibration did not generate directional cues, despite a 100 μ s tITD.

Previous work has demonstrated, however, that small differences in the timing of stimulus onset can affect fly responses and mediate selective attention to one among multiple simultaneous sources, via a precedence effect (Lee et al., 2009). The next experiment examined whether time differences in the stimulus amplitude envelope can mediate auditory directionality in response to a single source.

Experiment 2 – Onset Cancelation

In this experiment, conditions were similar to experiment 1, except that one speaker was placed at 0° azimuth (directly forward of the fly), while a second speaker was placed at 90° (lateral to the fly). The forward speaker broadcast a synthetic cricket chirp, which should elicit phonotaxis in the forward direction. The second speaker broadcast a train of brief impulses, approximating a half-cycle of the 5 kHz chirp waveform, timed to coincide with the initial onset of the individual pulses of the synthetic chirp, and phase-adjusted to cancel the initial cycle of each chirp pulse (**Figure 5**). Due to the directional properties of the tympanal membranes, and because the impulse source is located lateral to the fly (90° azimuth), its effect will be greater on the ipsilateral tympanum than the contralateral by 12 dB (see above, **Figure 3**).

The overall result of this stimulus arrangement is that the onset cancelation has a greater effect on the ipsilateral side (relative to the impulse source) than the contralateral, resulting in a delay in the rise-time of the amplitude envelope of the chirp pulses at the ipsilateral tympanum (**Figure 6**). This has no effect on the overall amplitude of the stimulus at either tympanum but results in a delay in onset timing that is also measurable in summed auditory nerve responses (**Figure 7**). The additional apparatus required for nerve recordings made it more difficult to calibrate the stimuli in these experiments. Interaural delays measured in auditory nerve responses were variable, with a mean ± s.d. nITD in the cancelation condition of 48.6 ± 125.5 μs ($n = 7$).

Behavioral Responses

Fly behavior ($n = 5$) clearly indicated that interaural differences in the timing of stimulus onset constituted a directional cue in the absence of an amplitude difference (**Figure 8**). Phonotactic

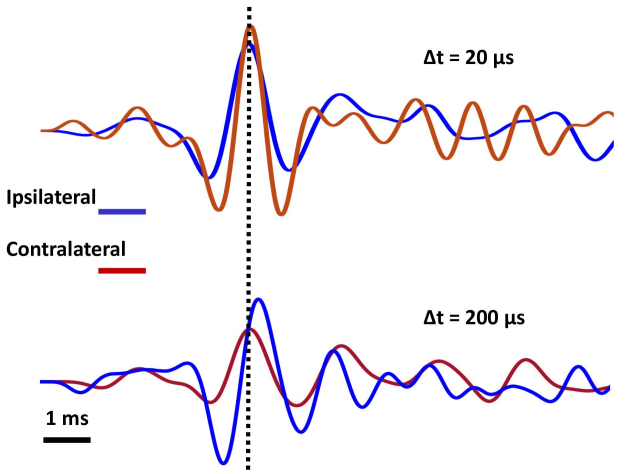

FIGURE 7 | Paired auditory nerve recordings for a stimulus source at 0° alone (upper traces) and in the onset-cancelation condition (lower traces). Auditory responses are nearly simultaneous for a forward source alone. In the onset-cancelation condition, ipsilateral responses (relative to the cancelation source) are delayed.

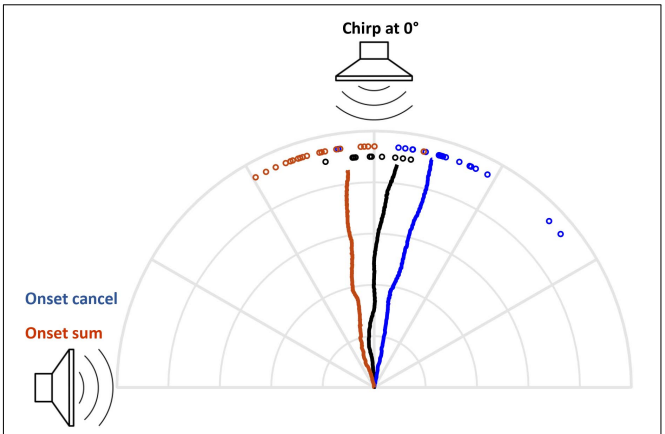

FIGURE 8 | Onset-cancelation behavioral responses for a single fly. Lines represent average (n = 10) walking paths (grid = 0.5 cm); symbols show the individual response angles (as in **Figure 4**). Flies walk forward, toward an attractive acoustic stimulus located on midline (0° azimuth, black trace). When impulses broadcast from a lateral location (± 90° azimuth) are timed to cancel the onset of the attractive stimulus pulses, flies' responses are in the direction contralateral to the impulse source (blue line, 0° alone vs. 0° + cancel: Watson's U = 0.6626, $p < 0.001$). Reversing the phase of the impulses (so that they sum, rather than cancel the attractive pulse onsets) causes the flies' responses to be oriented toward (ipsilateral to) the impulse source (red line, 0° alone vs. 0° + sum: Watson's U = 0.5016, $p < 0.001$).

walking paths were diverted contralateral to the impulse source (toward the side with leading chirp pulse onsets). Reversing the phase of the impulse waveforms (summing rather than canceling ipsilateral pulse onsets) reversed the effect, and phonotaxis was diverted ipsilateral to the impulse source.

For comparison with free-field auditory directionality, I shifted the position of the attractive sound source to determine what source azimuth elicited responses with similar directionality to the onset-cancelation condition. The directional effect of onset cancelation was equivalent to a source azimuth of 2° (**Figure 9**).

DISCUSSION

Although flies are not sensitive to cycle-by-cycle phase differences in the stimulus waveform, they do show a directional response for stimuli that differ only in the timing of the amplitude envelope, as shown by experiment, and which generate only latency differences in the responses of auditory receptors. However, the magnitude of these directional responses (i.e., the perceived source direction as indicated by the direction of the flies' walking path) is somewhat smaller than would be predicted by measurements of the nITD induced by the stimuli, although still within the range of nITDs elicited by directional sound sources in free field stimulation. Measurements of nITDs in response to variation in sound source azimuth (Mason et al., 2001) showed a slope of 3.5 μs/°. The mean value in these measurements was approximately 50 μs, which would correspond with an angle of incidence of ∼15 degrees. While these results clearly demonstrated that interaural time differences alone can mediate auditory directionality, they clearly do not rule out a contribution from interaural level differences. Experiments involving dichotic stimulation in orthopteran insects (grasshoppers, katydids, and crickets) have demonstrated a separate contribution of ITD and ILD cues in directional hearing (Rheinlaender and Mörchen, 1979; Kleindienst et al., 1981; von Helversen and Rheinlaender, 1988; Rheinlaender et al., 2006).

Temporal cues play an integral role in auditory processing (beyond the obvious importance in temporal pattern recognition for the pulsatile acoustic signals of their cricket hosts). Flies' auditory receptors respond almost exclusively to pulse onsets (Oshinsky and Hoy, 2002) and the strength of the response (the fly's perceived stimulus level) is determined by the amplitude increment relative to the noise-floor (effective amplitude, Lee and Mason, 2017). Small time-differences between competing sources mediate selective responses (Lee et al., 2009). While small interaural time-differences (for a single source) mediate directionality.

In a number of functional characteristics, *Ormia* hearing is convergent with more familiar (i.e., vertebrate) auditory mechanisms, and these may be seen as common principles arising from adaptation to the physics of sound. There are also clear differences, however, which could be consequences of the specific implementation of directionality in *Ormia* ears. For example, noise can disrupt directional acuity in *Ormia* and this is not alleviated by spatial separation of noise and signal (flies show no spatial release from masking, Blauert, 1997). Instead, under some circumstances, separation of signal and noise sources increases directional masking in *Ormia* (Lee and Mason, 2017).

What we know about the flies' hearing suggests that they accomplish as much as possible via peripheral filtering, with their auditory system functioning as a high-resolution, rapidly responding, symmetry detector that makes discrete measurements corresponding to the onset of each pulse in the

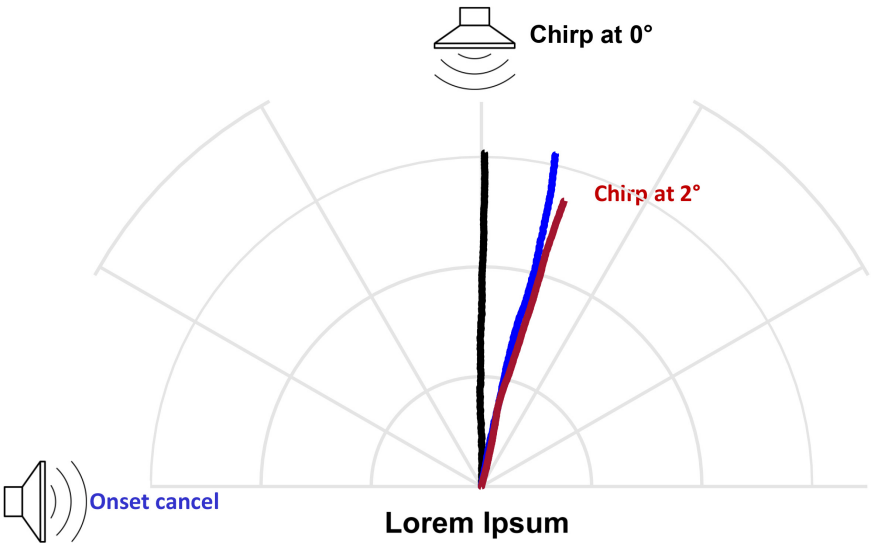

FIGURE 9 | Comparison with free-field auditory directionality. The directional effect of onset cancelation corresponded to a source azimuth of 2°. Chirp at 0° vs. onset cancel: $n = 5$ flies, 10 runs/fly/angle, Watson's $U = 0.4607$, $p < 0.001$. Grid = 1 cm.

signal. Flies simply orient to the direction that balances auditory input in time and amplitude. The lack of spatial release from masking and biased response to noise is a consequence of this (Lee and Mason, 2017) and even the precedence effect that mediates source segregation is based on the refractoriness of peripheral receptors (Oshinsky and Hoy, 2002; Lee et al., 2009). This simplified set of information (relative to vertebrate hearing systems) allows for the major functions of hearing: segregation, recognition, and localization of sources, albeit for a specific pre-determined set of stimuli (host communication signals).

The major evolutionary innovation for *Ormia* hearing is that tympanal coupling relieves them from size limitation in auditory directionality. Comparative and phylogenetic studies (Edgecomb et al., 1995, Robert et al., 1996a) have identified the suite of morphological adaptations that constitute the tympanal ear of this group, and the homologous structures in atympanate flies. Hearing arises as a single-origin evolutionary innovation shared by members of the subfamily Ormiini. The sensory organ itself is derived from a proprioceptive chordotonal organ present, but of uncertain function, in atympanate flies. Surprisingly, tympanal hearing based on the same homologous precursor organ, but independently evolved, was also identified in one species belonging to a second family of flies (Sarcophagidea, Lakes-Harlan et al., 1999). This species shows a number of convergent characteristics with *Ormia*, including a parasitoid life cycle with an acoustic insect (cicada) as host and directional hearing via coupled eardrums (Robert et al., 1999), although the details of tympanal mechanics are distinct in this species.

Despite the fact that auditory directionality via ICE is now known to be a rather widespread phenomenon (van Hemmen et al., 2016), *Ormia* should still be considered a highly specialized example, with a number of striking adaptations that appear to optimize their directional acuity despite the relative simplicity (and small scale) of their auditory processing apparatus (both mechanical and neural). An interesting contrast between *Ormia* and other (vertebrate) examples of ICE is that in most systems, there is a segregation in the frequency domain of the directional cues derived from the coupling mechanism, with ITDs at lower frequencies (relative to the fundamental frequency of the tympanum) and ILDs at higher frequencies (Vedurmudi et al., 2016b), whereas these cues are combined in *Ormia* (Robert et al., 1996b). On the other hand, the auditory system in *Ormia* is adapted to exploit the specific temporal and spectral structure of the host cricket acoustic signals; working in a relatively narrow frequency band and detecting the onset timing of individual sound pulses in the trill like call of the host. Expanding the usable bandwidth of systems designed to mimic *Ormia* hearing has been a major focus for biomimetic engineering efforts inspired by *Ormia* (Zhang et al., 2018). The subfamily Ormiini, though not a large group, includes nearly 70 species (Lehmann, 2003), with different species exploiting hosts with diverse acoustic signals in terms of both frequency and temporal characteristics. Comparative studies examining how auditory directional mechanisms are adapted to this diversity of signal parameters should be of great interest.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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frontiers in Ecology and Evolution

REVIEW
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Evolutionary and Biomechanical Basis of Drumming Behavior in Woodpeckers

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Understanding how and why behavioral traits diversify during the course of evolution is a longstanding goal of organismal biologists. Historically, this topic is examined from an ecological perspective, where behavioral evolution is thought to occur in response to selection pressures that arise through different social and environmental factors. Yet organismal physiology and biomechanics also play a role in this process by defining the types of behavioral traits that are more or less likely to arise. Our paper explores the interplay between ecological, physiological, and mechanical factors that shape the evolution of an elaborate display in woodpeckers called the drum. Individuals produce this behavior by rapidly hammering their bill on trees in their habitat, and it serves as an aggressive signal during territorial encounters. We describe how different components of the display—namely, speed (bill strikes/beats sec^{-1}), length (total number of beats), and rhythm—differentially evolve likely in response to sexual selection by male-male competition, whereas other components of the display appear more evolutionarily static, possibly due to morphological or physiological constraints. We synthesize research related to principles of avian muscle physiology and ecology to guide inferences about the biomechanical basis of woodpecker drumming. Our aim is to introduce the woodpecker as an ideal study system to study the physiological basis of behavioral evolution and how it relates to selection born through different ecological factors.

Keywords: display behavior, muscle physiology, sexual selection, spring mass system, behavioral evolution

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INTRODUCTION

Understanding how behavioral traits evolve is a longstanding goal of organismal biology. Indeed, most research within the field of behavioral ecology that addresses this objective explores the ecological factors that influence changes to a species' behavioral program over time (Westneat and Fox, 2010). Such work has resulted in an extremely rich knowledge of environmental factors that create selection pressures, which in turn modify the way that individuals interact with their social and physical surroundings to better survive and reproduce. Yet, at the same time, we must remember that behavior itself is often a manifestation of complex neurobiological and physiological processes. In these cases, complex behaviors occur through concomitant changes to the nervous and/or musculoskeletal systems that determine how individuals express behavior (Bauwens et al., 1995;

Clifton et al., 2015; Ding et al., 2016; Fuxjager et al., 2016; Barkan et al., 2018). Our understanding of behavioral evolution from this mechanistic standpoint is murkier than it is from the ecological standpoint—yet, both perspectives are necessary to fully uncover the complex processes by which behavioral changes can (or cannot) occur.

Here, we explore the evolutionary interplay among behavioral evolution and organismal ecology and physiology. We do this by focusing on the evolution of woodpecker "beak behavior," or the actions of drilling for foraging and nest creation as well as drumming for social signaling. We start by discussing the woodpecker clade and its diversity. We then review how these birds use their bill for important naturally selected and sexually selected behavioral traits. A deeper exploration of the evolution of drumming displays (a territorial signal that is produced by rapidly hitting their bill against a resonant substrate) allows us to assess how selection promotes behavioral diversity, particularly in the face of morphological constraint. We then move the discussion to the physiological and biomechanical basis of woodpecker drilling and drumming. We do this by reviewing the relatively limited literature on the topic and then developing a model for how drumming may be controlled. Our aim is to begin to merge our understanding of the ecological factors associated with the diversification of "beak behavior" and the physiological and mechanical factors that shape this behavior.

WOODPECKERS: A FAMILY-WIDE MODEL FOR STUDIES OF INTEGRATIVE EVOLUTION

Woodpeckers are an intriguing group of birds. They are contained within the clade Coraciimorphae, which includes a large assemblage of cavity nesting species such as trogons (order: Trogoniformes), hornbills and hoopoes (order: Bucerotiformes), and rollers and kingfishers (order: Coraciiformes) (Jarvis et al., 2014). Woodpeckers themselves are part of the family Picidae, which together with puffbirds (family: Bucconidae), Jacamars (family: Galbulidae), and a variety of toucan and barbets (infraorder: Ramphastides), form the order Piciformes (Jarvis et al., 2014).

Woodpeckers are also highly diverse. For example, they occupy nearly all terrestrial habitats across the globe (except for Australasia and Antarctica). This includes rich temperate and tropical forests, arid plains and savannas, swamps and marshes, and deserts (Bent, 1939; de Kiriline Lawrence, 1967; Short, 1970, 1971). Likewise, woodpecker behavior is equally variable. Some taxa adopt cooperative lifestyles, in which large family groups live together to feed and shelter (Koenig, 1981; Lennartz et al., 1987), whereas other species adopt isolated, nomadic lifestyles (Collins, 2017b; Nickley and Bulluck, 2020). Woodpeckers have also evolved an array of diets and foraging tactics; some species feed generally, while others forage on highly specialized items, such as sap or ants (Spring, 1965; Tate, 1973; Leite et al., 2013). From a morphological perspective, woodpecker body sizes span a wide range. For example, certain piculets are roughly 10 g in mass and no more than 10 cm in body length, whereas *Dryocopus* woodpeckers weigh around 300 g and are almost 50 cm in body length (Koenig, 1996; Miles et al., 2018). Plumage characteristics of woodpeckers are equally diverse—many species exhibit their own unique color ornaments, which often consist of bright red and yellow on the head (Stradi et al., 1998; Wiebe and Vitousek, 2015; Lammertink et al., 2016; Miller et al., 2019). Some species even have brilliant crests that exaggerate the aesthetic of their head.

Phenotypic differences are not the only reason why woodpeckers are a compelling family for studies of evolution. Similarities among these birds provide an opportunity to explore how different forces of constraint and/or preservation of adaptive traits influence phenotypic evolution in species that have otherwise undergone significant diversification over time. For most woodpeckers, the primary "phenotypic tie" that binds the clade together is their uncanny propensity to use their bill as a hammer or drill on wood in their environment. Here, we refer to this as "beak behavior," and we roughly categorize it into one of three different functional groups: (*i*) foraging, (*ii*) nest building, and (*iii*) displaying (**Figure 1**). We hypothesize that this behavior is built from a conserved physiological scaffolding that supports the extensive use of the head and body as a hammer. Presumably, features of this scaffolding arose deep within the Coraciimorphae lineage, where nest excavation first emerged. Yet something must have happened in the woodpecker's history to modify the mechanisms of pecking, which allows the species to use this

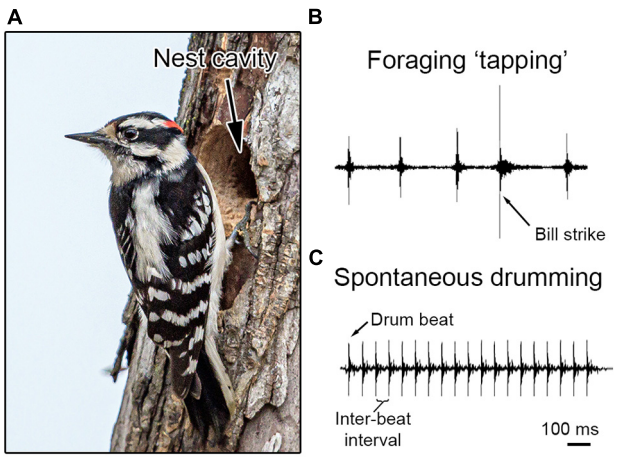

FIGURE 1 | Bill behaviors in the downy woodpecker (*Dyrobates pubescens*). **(A)** During the breeding season, downy woodpeckers excavate nest cavities within trees using their bill. These nest cavities serve as a place to raise offspring during the breeding season. Photo credit: Shiva Sheney (CC BY 2.0, no changes or modification were made to the photograph). **(B)** Throughout the year, woodpeckers will also use their bill to forage on trees for food, including probing for insects (see *main text*). **(C)** Most woodpeckers communicate by hammering their bill repeatedly against a tree, which produces an acoustic signal used in courtship and competition. This loud atonal signal is called a "drum." Each time the bill makes contact with the tree it produces a "drum beat," with the timing between two beats called the inter-beat interval. Both recordings are at the same temporal scale for comparison, with a scale bar at the bottom right.

behavior more broadly in contexts of feeding, reproduction, and communication (**Figure 1**). A major goal of our research is to elucidate the physiological scaffolding that underlies "beak behavior," and assess how it influences the trajectory of phenotypic evolution.

WOODPECKER "BEAK BEHAVIOR": NEST EXCAVATION AND FORAGING

As discussed above, nest excavation is likely the ancestral form of "beak behavior" in woodpeckers. Woodpeckers and other primary cavity nesting species have the incredible task of excavating a portion of a tree that will serve as a safe place for nesting and egg laying during the breeding season and for roosting year-round (**Figure 1A**) (Kilham, 1958; Short, 1979; Rudolph et al., 1990). Acquiring and defending territories that have multiple sites suitable for these uses is integral to the bird's survival and reproductive success. Most of the Picids construct nest cavities each breeding season, with the trees that particular species choose for cavity construction ranging from already-rotting specimens in wetlands to fully mature live trees in densely forested areas (Short, 1979). In either case, nest construction often requires woodpeckers to expend a significant amount of time to use their bill as a drill, chipping away bits of wood to create a relatively large hole in which the bird and its clutch can fit. This process often lasts multiple weeks, but in the end the bird creates a site that provides suitable protection for future offspring (Kilham, 1958; Short, 1979). Although the strenuous physicality of this task requires numerous physiological and morphological adaptations, recent work suggests that nest excavation into multiple layers of dense wood is sometimes facilitated by fungi that soften the wood (Farris et al., 2004; Jusino et al., 2016). Indeed, red-cockaded woodpeckers (*Dryobates borealis*) transmit fungal spores that hasten the wood-decay of pine trees from their beak into the fresh live wood, as individuals build their nest (Jusino et al., 2016). One might consider this an unusual form of tool use on the part of the woodpecker, which may be adaptive considering that it speeds up the nest excavation processes and likely buffers the sheer physical challenge associated with this part of the reproductive process.

Woodpeckers also forage using different styles of bill-hammering. Some species chip away or excavate tree bark to extract food items from these sites, whereas other birds look for food in holes that are already present in a tree or shrub (Short, 1971; Conner, 1981; Askins, 1983). The latter behavior is probably the more iconic mode of woodpecker feeding, as it is often performed vigorously. In such cases, birds scrape and chip away large pieces of bark to gain access to insect larvae hiding underneath, creating an aesthetic of a jackhammer drilling on a substrate (Askins, 1983). Hairy woodpeckers (*Leuconotopicus villosus*) and downy woodpeckers (*Picoides pubescens*), two common North American species, are well known for exhibiting such behavior in parks, greenways, and forests. As downy woodpeckers forage, for example, they often slow pecking motions (**Figure 1B**) on bark to excavate small insects (Lima, 1983, 1984; Peters and Grubb, 1983). At the same time, other species use their bill to make caches in which they store food items. Acorn woodpeckers (*Melanerpes formicivorus*) create incredible acorn caches, whereby entire trees are littered with holes that can be used to keep acorns (Koenig et al., 2008).

Tropical woodpeckers differ slightly in their foraging behavior than temperate ones. For example, feeding via excavation is rarer, and tropic woodpeckers are even less likely to cache food for later (Askins, 1983). Instead, woodpeckers of the tropics often raid arboreal ant nests and termite mounds, pecking through the dense structures these invertebrates have constructed to shield themselves from predators (Askins, 1983). Other species, like the Kaempfer's Woodpecker (*Celeus obrieni*), drill through the internodes of bamboo stems and feed on the ants that shelter inside (Leite et al., 2013). Although these strategies are quite different from their temperate relatives, it is abundantly clear that tropic woodpeckers still leverage bill-hammering to get access to food.

WOODPECKER DRUMMING

Woodpeckers also use "beak behavior" for social communication. The most common example is the drum, or the loud staccato sound that penetrates the environment when an individual rapidly hammers its bill against a tree (**Figure 1C**). Ornithologists have long known that drumming is a territorial signal produced by resident birds as they settle and defend their territory during the breeding season. Early documentation of drumming comes from researchers like Brewster (1876), who described the drums of yellow-bellied sapsuckers (*Sphyrapicus varius*) and noted that they were often produced when an individual's breeding territory was being invaded by another (Brewster, 1876). This phenomenon-increased drumming behavior during territorial interactions—has been noted many times since, often in North American woodpeckers (Bent, 1939; Kilham, 1959, 1960, 1969, 1974, 1977; de Kiriline Lawrence, 1967; Ligon, 1970; Winkler and Short, 1978).

One of the interesting aspects of drumming as a social signal is that it may function over both short and long spatial scales. During agonistic encounters, for example, individuals will perform drums when opponents invade their territories. At the same time, residents often broadcast drums at specific times in the day (e.g., dawn) (Kilham, 1958, 1974; de Kiriline Lawrence, 1967), much like a resident songbird sings at the morning's first light to broadly advertise to neighbors that they still occupy the area (Burt and Vehrencamp, 2005).

Several studies used field experiments to better understand the function of drumming behavior. This work often employs simulated territorial intrusions, or STIs, in populations of free-living birds. The idea is to test how residents respond to encounters in which they hear a drum display on their territory, as though it is being invaded by an interloper. STI methodology is compellingly simple: an experimenter broadcasts a putative aggressive signal (in this case a drum) in a resident's territory and then observes the resident's behavioral response (Searcy et al., 2006). Studies across multiple woodpecker species demonstrate that playback of drums reliably elicits aggressive behavior from

resident individuals, including both males and females (Winkler and Short, 1978; Dodenhoff et al., 2001; Schuppe et al., 2016; Figarski, 2017; Schuppe and Fuxjager, 2018). For example, when downy woodpeckers are presented with drumming playback, both sexes engage in a wide range of behaviors to defend their territories, including calls and attack flights, as well as drums (Schuppe et al., 2016; Schuppe and Fuxjager, 2018). Additional work in red-bellied woodpeckers demonstrates a significant level of dynamism in these responses (Miles and Fuxjager, 2019). For instance, males that encounter an unfamiliar intruder (via STI) on their territory for the first time begin their agonistic defensive routines largely through flight displays, and not drums. However, if resident males experience additional territorial intrusions on subsequent days, they flip the order in which they produce these behaviors (they start encounters off with drums, and then segue into flights). This latter context also results in the production of significantly more drumming across the board. Interestingly, these territorial strategies change if a resident male's female partner (who does not partake in territorial defense) is present during the STI—in such cases, males dramatically reduce the number of drums they produce, no matter how many STIs they have accrued. Instead, they rely mostly on aggressive calling behavior and attack flights.

Altogether, these observational and experimental studies imply that drumming is an aggressive signal. In fact, drumming meets well-established criteria that distinguish agonistic display behavior: (*i*) drum production increases in aggressive contexts, (*ii*) receivers respond to drums by also producing this behavior, and (*iii*) drum signals predict robust aggressive responses from territorial residents (Searcy and Beecher, 2009). It is also important to recognize, however, that drumming may have other functions related to social signaling. For instance, some authors propose that drumming mediates elements of mate choice (Kilham, 1974, 1979). Tests of this idea, or any other that explores the functional significance of drumming outside the context of territoriality, are rarely (if ever) addressed in a rigorous experimental fashion.

Drum Displays: Speed, Length, and Rhythm

Establishing a connection between drumming and territoriality is only the first step toward understanding how this display works. Like many studies in animal communication, one can attempt to "decode" drumming by figuring out the way that the signal's components underlie its functionality. Drumming is ideal for such work, because the display's acoustics are relatively simple—each beat is an atonal burst of broadband sound, much like a handclap (**Figures 1C**, **2**). As such, there are only a handful of ways to regulate this display. Individuals, for example, might modify the drum's speed (number of beats produced over time) or length (total number of beats) (**Figure 2**). Likewise, individuals can adjust elements of the signal's rhythm by altering elements of cadence (description of how speed changes over time) or acceleration (description of the direction of speed change over time) (see **Figure 2**; Miles et al., 2018, 2020; Schuppe and Fuxjager, 2018). Of course, individuals may also modify amplitude (volume) or dominant frequencies within the broadband spectra that define beat acoustics.

Recent experimental work focuses on the effects of drum speed, length, and rhythm during territorial contests. Hints that these variables are functionally important arise through research showing notable variation in parameters of speed and length within populations and among species (Stark et al., 1998; Schuppe et al., 2016; Miles et al., 2018; Schuppe and Fuxjager, 2018). For example, studies in red-bellied woodpeckers show that individuals actively engaging in territorial interactions exhibit drums that are longer than drums produced spontaneously in the morning (Wilkins and Ritchison, 1999). This finding provides further support for the notion that individuals adjust how they drum to regulate the effectiveness or potency of the signal in a context-dependent manner.

Other research looks specifically at the role of drum speed in territorial competition. In downy woodpeckers, for example, the average drum speed is about 16 beats s^{-1}, with 23 beats s^{-1} as the absolutely fastest drum we have ever recorded (Schuppe and Fuxjager, 2018). If resident birds are presented (via STI) with engineered drums in which the time interval between subsequent beats is decreased by 8 ms, they become significantly more aggressive and produce more agonistic behavior in response (see **Figure 3A**; Schuppe and Fuxjager, 2018). This high performance "rapid drum" falls within the natural distribution of downy woodpecker drum speeds, which means that resident birds are responding to a display that they might normally encounter. It is therefore thought that residents increase their aggressive response to this display because they perceive it as a more potent threat from an intruder. Consistent with this notion is additional work showing that resident birds presented with the "rapid drum" stimulus attempt to match its speed by producing drums with inter-beat intervals that are roughly 4 ms faster than residents presented with control drums (slower, low performance drums; **Figure 3B**). If this finding did in fact reflect a resident's attempt to match the social threat with that of an intruder, it is notable that the resident falls short of fully doing so (**Figure 3C**). This is likely because drum speed may be bound by an upper physiological limit (more on this topic below). Regardless, these data support the idea that speed is a critical component of drum effectiveness during aggressive disputes.

Similar types of studies have also looked at drum length. For example, downy woodpeckers produce drums that include roughly 16 beats. When residents are subjected to STIs that broadcast longer drums with 19 beats, they again become more aggressive and produce more agonistic behavior (Schuppe et al., 2016). This study did not assess whether residents attempt to match drum length with that of the intruder; however, this work showed that resident males and females appear to coordinate their aggressive response to intruders when they hear engineered "long drums." Again, these findings suggest that residents perceive longer drums as more threatening agonistic signals, and thus adjust their territorial response accordingly.

We suspect that sexual selection by male-male competition drives the evolutionary elaboration of drum speed and length, at least in downy woodpeckers. This idea is based on recent studies that suggest that elaborate displays produced through

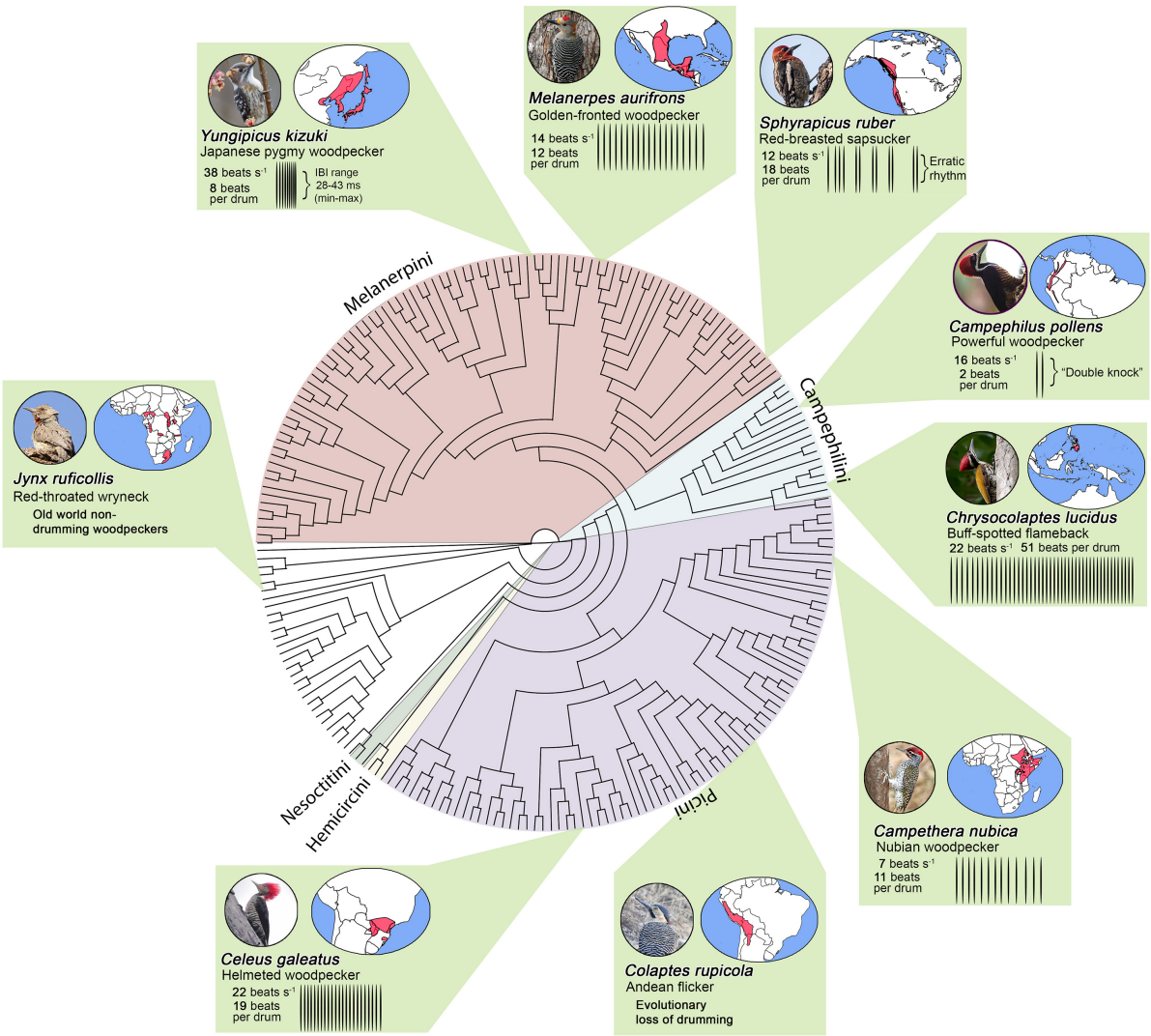

FIGURE 2 | Cladogram of the woodpeckers (Picidae) from Shakya et al. (2017). Colors within the phylogenetic tree illustrate the five main woodpecker tribes and non-drumming old world woodpeckers (e.g., wrynecks). Green boxes illustrate how drums vary across the woodpecker phylogeny. Woodpecker drums differ in terms of speed (beats/sec), length (total number of beats), and rhythm (Miles et al., 2018, 2020). The Japanese pygmy woodpecker (*Yungipicus kizuki*) exhibits the fastest drums. This species is able to strike its bill at rates that can exceed 38 beats s^{-1} (or a strike every 28–43 ms). The buff-spotted woodpecker (*Chrysocolaptes lucidus*), a species found throughout eastern Asia, exhibits one of the longest drums (~51 beats per drum). Some birds also exhibit drums with a-typical patterns. For instance, sapsuckers exhibit erratic drum rhythms, and the Powerful woodpecker produces "double-knocks" rather than the longer drums seen in most woodpeckers. Boxes also illustrate that woodpeckers occupy diverse habitats throughout the world. Photo credits: Japanese pygmy woodpecker (Ik T, CC BY 2.0); golden-fronted woodpecker (Roberto González, CC BY 2.0); red-breasted sapsucker (Beck Matsubara, CC BY 2.0); powerful woodpecker (Alan Harper, CC BY 2.0); buff-spotted flameback (Tareq Ahmed, CC BY 2.0); nubian woodpecker (Brad Schram, CC BY 2.0); Andean flicker (Vil Sandi, CC BY 2.0); helmeted woodpecker (Hector Bottai, CC BY 3.0); and red-throated wryneck (Derek Keats, CC BY 2.0). No photographs have been altered.

body and/or appendage movement reflect honest information regarding the health or condition of the signaler (Nowicki et al., 2002; Müller et al., 2010). The physiological link between these two variables—motor control of gesture and condition—are still being worked out, but there is a growing body of literature that suggests even the performance of routine locomotory tasks quickly becomes more challenging when they are performed at higher speeds (Wynn et al., 2015; Amir Abdul Nasir et al., 2017). In this way, competitive drumming may push woodpeckers to their performance limit, such that low quality individuals are incapable of drumming as fast or as long. Research in other taxa is fully consistent with this view, as signal length appears to influence the outcome of social interactions across a broad range of animals. In territorial disputes, for example, individuals capable of producing longer signals (e.g., more repetitive elements) are often perceived as superior competitors (Behr et al., 2006; Rivera-Gutierrez et al., 2010; Mager et al., 2012). Future work will be needed to test these ideas using multiple approaches. One starting point is to determine whether individuals with faster drums exhibit better health or body condition. Another more

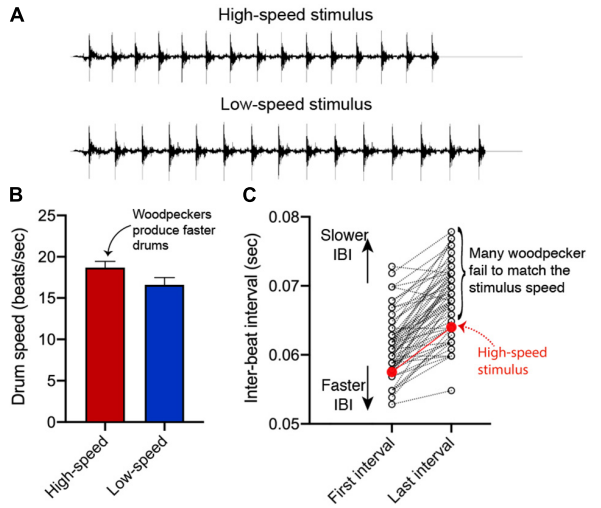

FIGURE 3 | Residents modulate the speed of their own drum displays to resemble that of the high-speed stimulus. **(A)** Representative waveform diagrams of high-speed and low-speed drum stimuli that were used during stimulated territorial intrusions (STIs). **(B)** In response to STIs with the high-speed stimulus, territorial residents produce significantly faster drums compared to those that heard the low-speed drum. Bars represent mean ± standard error **(C)**. Yet, many residents were not able to produce drums that were as fast as the high-speed drum stimulus. Residents on average produced drums that were approximately 4 ms slower than the actual high-speed stimulus. All data are reanalyzed from Schuppe and Fuxjager (2018).

direct way includes determining whether individuals with faster and longer drums exhibit greater reproductive success.

Several studies also look at the functional effect of drum rhythm. Downy and red-bellied woodpeckers, for example, live in sympatry across much of eastern North America, and the two species frequently share overlapping territories. Although both taxa produce drums that are similar in average speed and length, rhythm is the one key difference between their drums—downy woodpeckers produce a drum with a cadence that slows down at a linear rate, whereas red-bellied woodpeckers produce a drum that speeds up at an exponential rate (Schuppe and Fuxjager, 2018). If a typical downy woodpecker drum is engineered so that its rhythm resembles that of a red-bellied woodpecker, then downy woodpeckers stop responding to it during an STI. Likewise, if a red-bellied woodpecker drum is engineered to resemble the rhythm of a downy woodpecker, then red-bellied woodpeckers stop responding to it (Schuppe and Fuxjager, 2018). Among other North American species, resident woodpeckers also exhibit subdued behavioral responses to STIs of either sympatric or allopatric species that exhibit markedly different drum speeds, cadences, or acceleration patterns (Dodenhoff et al., 2001). However, when species encounter (via STI) heterospecific intruders that produce drums of similar length and speed then their response is comparable to that of a conspecific intruder (Dodenhoff et al., 2001). These results are consistent with the idea that rhythm encodes species identity, a concept that extends across much of the woodpecker family. Large-scale comparative analyses indicate that sympatric sister taxa are more likely to have different cadence patterns to their drum (and to a lesser extent different acceleration patterns), compared to allopatric sister pairs.

Evolution of Drumming Behavior

Because drumming behavior is shared among most of the woodpecker lineage, we can also begin to study this signal at a macroevolutionary level. This approach can highlight potential principles that guide the evolutionary "construction" of drum displays. Such work is challenging when applied to many other types of signals. Birdsong, for example, is so complex and variable among species that it becomes difficult to track how homologous elements of the display might change through time, without reducing these elements into variables (e.g., principle components) that poorly track the display's complexity (Goodale and Podos, 2010; Weir and Price, 2019). But drumming behavior differs because of the limited number of ways by which it varies from species to species; thus, we can more easily track how specific components of the signal likely change over time.

Ancestral state reconstruction of drumming behavior reveals that the signal likely first arose at the base of Picinae. This observation suggests that the signal was then retained through time, such that variation in the signal that deviates from this ancestral state evolved by way of either selection or neutral processes. Machinery that underlies a bird's ability to drum therefore presumably evolved early in the species' history and was similarly likely retained as the taxa within this clade diversified. Only a few woodpeckers (≈5) have completely lost drumming behavior, and many of these birds inhabit environments without trees (Miles et al., 2018). For example, ground woodpeckers (*Geocolaptes olivaceus*) live in holes in the ground throughout the treeless grasslands of southern Africa; individuals of this species have evolved wing displays in lieu of drumming (Short, 1971). Andean flickers (*Colaptes rupicola*) have also lost drumming from their behavioral repertoire, as they occupy the grasslands high up in the Andean mountains (Short, 1970, 1971). Interestingly, there are published anecdotal observations of Andean flickers producing drum-like displays in populations that have re-colonized habitats containing trees (Fjeldsa, 1991). Such findings speak to the notion that woodpeckers have a largely conserved neurobiological program for drumming, even in species that have lost the behavior altogether.

How exactly do drum displays differ across the woodpecker family? The answer centers on the three main components of the drum described above: speed, length, and rhythm (**Figures 2**, **3**). For example, Japanese pygmy woodpeckers (*Yungipicus kizuki*) from the deciduous forests of northeastern Asia drum at ≈40 beats s^{-1}, whereas Nubian woodpeckers (*Campethera nubica*) from Central and Eastern Africa drum at ≈8 beats s^{-1}. Length variation is similarly extreme, with many *Campephilus* woodpeckers producing short "double knock" (2 beat) drums, while greater flamebacks (*Chrysocolaptes lucidus*) of the Indian subcontinent produce ≈50 beats per drum. Similarly, species like the South American helmeted woodpecker (*Celeus galeatus*) show a striking linear deceleration in speed, whereas other species like the North American golden-fronted

woodpecker (*Melanerpes aurifrons*) show non-linear change in speed characterized by acceleration and then deceleration. Outside of these patterns, species within the *Sphyrapicus* genus exhibit unusual drum rhythms that are characterized by erratic changes in speed.

Our past work tests whether phenomenological signatures of strong sexual selection mark species differences in any of these display parameters. Specifically, we use sexual size dimorphism (SSD), which is one of the best predictors of the strength of sexual selection in birds [males are generally larger than females in taxa that evolve in response to relatively strong intrasexual selection; SSD (Dale et al., 2007; Székely et al., 2007)]. Analyses indicate that SSD positively predicts variation in drum length, but not drum speed (Miles et al., 2018). This finding suggests that these two components of the display are modular, in that they can change over time somewhat independently of each other. Morphological constraint provides insight into why length may be more modular and become elaborated by sexual selection. Although we found no evidence of a relationship between body size and drum length, this morphological variable is associated with speed (Miles et al., 2018). These two variables form a triangular distribution, in which the hypotenuse reflects a statistically significant negative relationship between body size and species' drum speed. In short, larger woodpeckers exhibit a notable tradeoff between size and speed, but length is unconstrained in this manner. Thus, these findings suggest that sexual selection drives the elaboration of drum elements that are less constrained. Another insight from our work that is consistent with this idea is that drum length shows a greater evolutionary rate than drum speed.

In a separate study, we were interested in how a component of this signal, rhythm, that is in part used for species recognition is also influenced by sexual selection. We find that greater SSD values positively predict whether complex cadences and acceleration patterns are present in a particular species (Miles et al., 2020). Woodpecker species with larger males are therefore more likely to produce a drum that either increases or decreases in speed (or both) at a linear (e.g., consistent slowdown in inter-beat interval) or non-linear cadence (e.g., exponential increase in inter-beat interval speed). This suggests that sexual selection by male-male competition potentially influences how drum rhythm changes over time, alongside effects of selection through conspecific recognition (see above). Importantly, we also find that body size does not predict facets of rhythm, suggesting that morphology itself does not constrain innovation in this feature of the drum like it does for speed.

The evolutionary interplay among speed, length, and rhythm is also likely complex, as these components of the drum can influence how the others evolve. Rhythm, for example, can have potent effects on the way that drum speed and length change over time (Miles et al., 2020). When a drum takes on a complex cadence or acceleration pattern (anything not constant), then evolutionary rates of both speed and length are depressed. Likewise, this also means that drums with a constant rhythm—no change in speed over the course of a single drum—potentiate the evolution of display speed and length parameters. Either way, these findings provide clear evidence that one component of the display can dramatically alter how other components evolve, which of course influences the phenotypic "options" for sexual selection.

However, our studies also suggest that rhythm unequally constrains length and speed evolution. Rates of length evolution, for instance, are lower in species that drum with linear cadences, compared to those that drum with non-linear cadences (Miles et al., 2020). This difference is not observed with respect to rates of speed evolution, whereby constraint severity is likely similar for non-linear cadences and linear cadences alike. Thus, these data again point to drum length as a less constrained element of the drum signal, creating a potential "path of least resistance" for length display elaboration to occur.

Physiology and Biomechanics of Drumming and Drilling

Like any motor activity, beak behavior in woodpeckers must operate within the constraints of physical principles, as well as physiological "rules" that are common to muscle-driven systems. Below we review how physical and physiological demands may shape the evolution of beak behavior. Because there are few empirical studies of mechanics in drumming or drilling, we use inference from other forms of movement or communication signals to explore how mechanical demands may shape the evolution of these behaviors.

Drumming Is a Fast Activity

An obvious feature of drumming is that it is a high-speed activity. By plotting average drumming rates for different species, organized by genus, one can easily see that (*i*) drumming speeds are variable both within and across genera; and (*ii*) many species drum at rates exceeding 20 beats per second (**Figure 4**). Speed matters, because fast motions place demands on the neuromuscular system, and thus the evolution of fast drumming may have been driven by its usefulness as an honest signal of

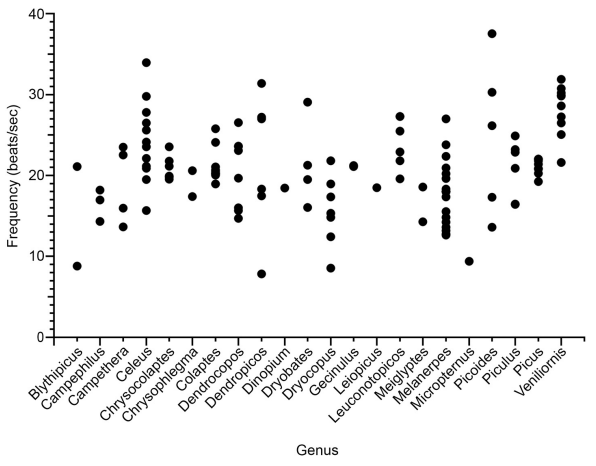

FIGURE 4 | Variability in drumming frequency (beats s^{-1}) within and between 22 woodpecker genera. Each point represents the mean ($n \geq 2$) frequency of a single species extracted from audio recordings accessed through the Macaulay Library at the Cornell Lab of Ornithology and Xeno-canto (data from Miles et al., 2018).

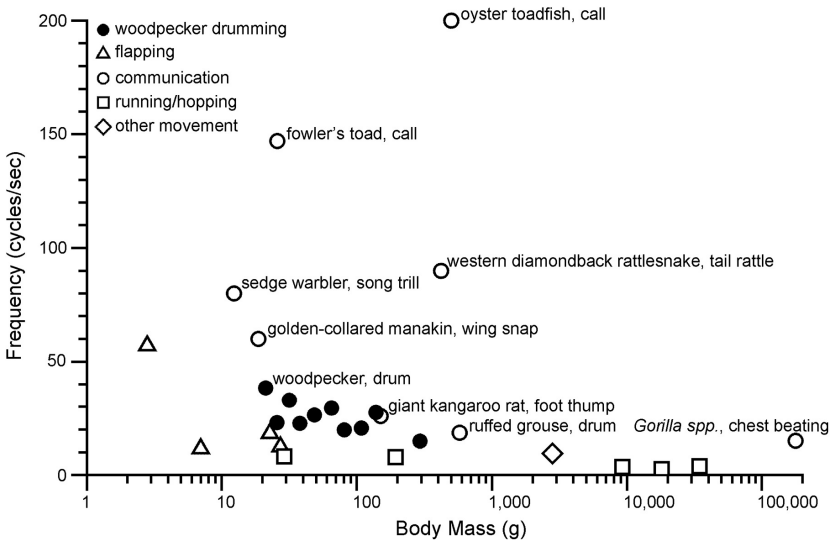

FIGURE 5 | Woodpecker drumming relative to other fast frequency (cycles s^{-1}) movements plotted by body mass (g) on a logarithmic scale. Woodpecker drumming (solid circles) includes from left to right: Japanese pygmy woodpecker, Downy woodpecker, Ladder-backed woodpecker, Nuttall's woodpecker, Red-cockaded woodpecker, Hairy woodpecker, Acorn woodpecker, Lewis's woodpecker, Northern flicker, and Pileated woodpecker. To limit woodpeckers to a subset of species that drum, the species with the fastest known drum (Japanese pygmy woodpecker) and North American species were selected. Communication (open circles) organisms are labeled on the graph. Flapping (open triangles) includes from left to right: Ruby-throated hummingbird, little broad-nosed bat, common chaffinch, and downy woodpecker. Running/hopping (open squares) includes from left to right: white mouse, ground squirrel, domestic dog, red kangaroo, and cheetah. Other movement (open diamond) includes domestic cat paw shake. Data sourced from: Zweifel (1968), Dawson and Taylor (1973), Walton and Anderson (1988), Heglund and Taylor (1988), Griffiths (1991), Rome et al. (1996), Schaeffer et al. (1996), Tobalske (1996), Goller and Suthers (1996), Randall (1997), Langefors et al. (1998), Pennycuick (2001), Bullen and McKenzie (2002), Stein and Uy (2006), Fine et al. (2009), Zihlman et al. (2011), Garcia et al. (2012), Hudson et al. (2012), Mahalingam and Welch (2013), Fusani et al. (2014), Miles et al. (2018), Schuppe and Fuxjager (2018), Déaux et al. (2020). See electronic **Supplementary Material** for more detail.

condition. Below we present benchmarks to put drumming in context of other movements, and we speculate how fast speed may translate to physiological challenges.

Is drumming *unusually* fast? **Figure 5** compares cyclic motion frequency across a range of vertebrate activities, from locomotory movements to motions involved in communication signals and some non-locomotor movements (e.g., cat paw-shake). The goal in compiling these data is not to produce an exhaustive summary, but rather to visualize broadly how motion frequencies compare for different kinds of activities. Perhaps the most obvious insight from **Figure 5** is that communication signals can occur at much higher frequencies than locomotor movements. This is arguably consistent with the idea that sexual selection pushes performance to physiological limits, while also undoubtedly related to the differing mechanical demands of communication vs. locomotion. Drilling also involves rapid motions, but drilling frequencies are generally lower than drumming frequencies (Miles et al., 2018), and so selection for the fastest speeds has most likely been associated with drumming.

Given the uncertainties of our broad analysis of motion frequency (discussed below), conclusions drawn from this comparison must be made with caution. However, we feel that a roughly quantitative assessment leads to a few insights. The observation that the frequency of woodpecker drumming falls above the frequency of locomotor movements for similarly sized vertebrates supports the idea that drumming is a fast activity that presents a physiological challenge. At the same time, woodpecker drumming frequencies fall below the very fast communication signals of a diverse range of vertebrates. For some of the fastest motions in **Figure 5**, "superfast" muscles have been identified as a key specialization for generating high frequencies (Rome et al., 1996; Schaeffer et al., 1996; Rome and Lindstedt, 1998; Elemans et al., 2004; Fuxjager et al., 2016; Zweifel, 2017). These muscles allow for very fast activation and deactivation, but at the expense of maximum force production (peak tetanic isometric force). Woodpecker drumming frequencies fall below the frequencies of movements in which superfast muscles have been identified, but contractile property measurements will be required to evaluate whether such specializations occur in woodpeckers.

While at first glance woodpecker drumming may appear to occur at a relatively low frequency when compared with the calls of toads and toadfish, it is important to acknowledge that frequency is only one component of what makes a movement mechanically demanding. All of the very high frequency motions included on **Figure 5** involve muscles driving the motion of relatively light loads. The muscles that drive a warbler's trill, for example, are moving only air and relatively light structures of the syrinx, as well as possibly the mass of some respiratory muscles (Hartley, 1990; Wild et al., 1998; Suthers et al., 1999). Woodpecker drumming involves motions of the head and there appear to be specializations of neck muscles for this motion (Jenni, 1981; Schuppe et al., 2018), but many models of drumming suggest that motion of the body is important,

driven by muscles of the hindlimbs (Vincent et al., 2007). This is significant, because the load determines the force and power that muscles must produce, thus the combination of relatively high frequency and high load of woodpecker drumming may be quite demanding.

Muscular Demands of Drumming and Drilling

Studies of woodpecker drumming mechanics are quite limited, but several lines of evidence support the idea that drumming places significant demands on the mechanical performance of skeletal muscles. When presented with a high frequency drum in an STI, woodpeckers appear to have a limited ability to increase their own drum speed—they can only boost their speed a few milliseconds faster than their typical, "unchallenged" speed. As mentioned above, this finding supports the idea that drum speeds occur at or near an individual's physiological limit (Schuppe et al., 2018; **Figure 3**). Such high frequency motions can challenge muscles in several ways. Foremost, to cycle at high frequencies, muscles must turn on (activate) and off (deactivate) rapidly. Activation of muscles is governed by voltage-gated Ca^{2+} channels in the sarcoplasmic reticulum. This action is passive (does not require energy) and can be quite rapid. Muscle deactivation requires the use of ATP for active pumping of Ca^{2+} from the myoplasm to the sarcoplasmic reticulum, against a concentration gradient, and thus muscle relaxation is generally slower than activation. Muscles that cycle rapidly, as measured typically by a short duration of twitch force, require specializations such as a high density of activation machinery (e.g., sarcoplasmic reticulum, t-tubules) and a high mitochondrial density to fuel the high metabolic demand of Ca^{2+} pumps (Rome and Lindstedt, 1998; Rome, 2006). This takes space that might otherwise be occupied by contractile machinery, and the energy demands of calcium pumps incur a metabolic cost in every muscle contraction. In muscles specialized for very fastest cycling, superfast muscles, a well-developed calcium buffering mechanism involving parvalbumin also appears to be essential (Rome et al., 1996; Rome and Lindstedt, 1998; Nelson et al., 2018).

Anatomical and molecular specializations provide some clues regarding potential modifications for rapid calcium cycling in woodpeckers. The longus colli ventralis muscle of the neck is enlarged and studies have identified physiological adaptations associated with quick relaxation in this muscle (Jenni, 1981; Schuppe et al., 2018). Elevated expression of two protein encoding genes [*parvalbumin* and *sarcoplasmic reticulum* Ca^{2+} *ATPase 1* (SERCA1)] that promote rapid Ca^{2+} transients was observed in the *longus colli ventralis* muscle in both downy and red-bellied woodpeckers. The protein products of these genes assist in moving and retaining myoplasmic Ca^{2+} back into the sarcoplasmic reticulum, leading to muscle relaxation. This increase was not seen in a woodpecker muscle with no role in drumming or in a non-woodpecker species that exhibits slower drum-like movements during foraging (Schuppe et al., 2018). Given this evidence, the fivefold increase in gene expression of parvalbumin and SERCA1 in a drumming muscle appears to be a specialization that supports the drumming behavior.

In addition to the challenges of muscle activation/deactivation, high-frequency movement can involve a high speed of muscle shortening, and this presents challenges that are different and somewhat independent from those of turning muscle on and off. The simplest measure of intrinsic muscle speed of shortening is V_{max}, the theoretical unloaded maximal speed of shortening, which can be measured via a series of contractions at different speeds in an isolated, maximally activated muscle (Hill, 1938). Muscles with a high V_{max} are metabolically costly, as faster shortening speeds involve higher activities of the ATPase involved in cross-bridge cycling (Bárány, 1967). Further, the force-velocity relationship of muscle dictates a trade-off between speed and force that can impact fast motions, because fast motions often require high forces.

The mechanical power required for drumming is likely also considerable. Power is the product of force and velocity. It has been established that drumming involves high speeds of movement, and the accelerations required with each reversal of direction of body motion will also involve high forces. Liu et al. (2017) used a piezoelectric film mounted under a wood block to measure impact forces during beak behavior (likely drilling) in a great spotted woodpecker (*Dendrocopos major*) and found values as high as 19.6 Newtons, a force corresponding to 200–300X body weight for this species. High-speed film estimates of acorn woodpeckers measured decelerations on impact that were 500–1500 g (May et al., 1979). Further, whether drums are produced for territorial defense or for attracting mates, drum volume (acoustic energy) is likely important. The production of acoustic energy requires muscular work, and, while the total acoustic energy produced in a drum is unknown, it is clear from other examples of sound production in nature that acoustic signals can be mechanically demanding.

While drilling motions are generally slower than drumming motions, they are likely to be mechanically demanding for reasons beyond speed. The drilling motions associated with excavation require mechanical work to break down the substrate. The relative proportion of drilling power that is associated with motions of the body vs. substrate breakdown is unknown and likely to be variable depending on substrate qualities and behavior (e.g., nest excavating vs. foraging).

Sources of Power for Drumming and Drilling

It seems reasonable to assume that the evolution of rapid drumming involved selection for morphological and physiological features that allow for sustained power production to maintain the drumming motion and produce sound. There are few empirical studies of drumming or drilling kinematics or kinetics, and so our understanding of where and how power is produced is quite limited. Theoretical models of the drumming motion are in many cases based on minimal empirical data, and assumptions vary. Some models place power production within the neck (Liu et al., 2015), whereas others assume hindlimb muscles play a role (Vincent et al., 2007).

A theoretical, schematic analysis of the flow of power during drumming highlights the many possible energy sources and sinks,

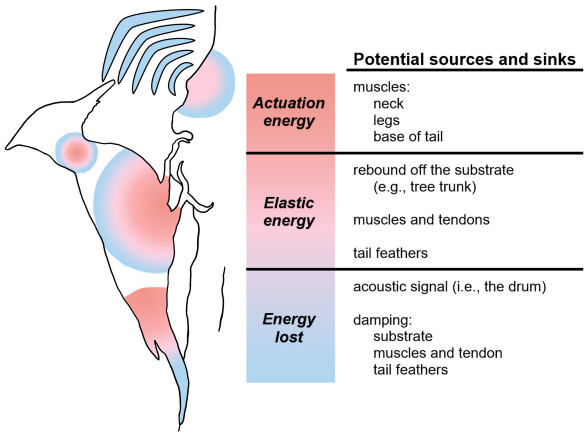

FIGURE 6 | Schematic demonstrating potential energy sources and sinks in the woodpecker drumming system. Red represents the actuation energy to begin and maintain drumming; pink, the elastic energy that aids in the continuation of the behavior after actuation; and blue, the energy lost from the system.

as well as a number of possible sites for elastic mechanisms that might recycle energy (**Figure 6**). Ultimately, muscle actuation must drive the motion, and muscles in the limbs, neck, and even potentially the base of the tail may act as a source of energy for movement. The back-and-forth motion of drumming involves as much deceleration as acceleration, and so the muscles that power, for example, the acceleration of the head towards the substrate might also sink (dissipate) energy as they reverse the motion of the head backwards in preparation for the next strike. Elastic mechanisms can store and recover energy, for example, kinetic energy lost as the body decelerates might be stored in springy tendons, and the recoil of these tendons could reaccelerate the body in the other direction.

One known sink of energy is the acoustic energy of the drumming sound. Mechanical work done by muscles is necessary to produce this energy. Does the acoustic energy in the drum signal represent a significant energy sink? We are not aware of existing measures of acoustic power of drumming. Measurements of sound power in other animal signals tell us that acoustic energy content is generally low. For example, Brackenbury (1979) measured acoustic power output for 17 species of songbirds and found values ranging from 10 to 870 mW kg^{-1} body weight. Even the loudest bird in the study, the song thrush (*Turdus philomelos*), produced a call with a sound energy of 0.87 W kg^{-1}, which, for reference, is nearly two orders of magnitude lower than the 40–60 W kg^{-1} of mechanical power measured for steady flight in similarly sized budgerigars (*Melopsittacus undulatus*). It is difficult, however, to dismiss the mechanical demands of acoustic energy production as negligible because the efficiency of conversion of mechanical work to acoustic energy is generally quite low (Fletcher, 2007).

Measures of metabolic efficiency of sound production (metabolic power/sound power) in several insects and anurans provide at least an upper limit of the direct cost of producing sound energy. Efficiency values of biological sound production range from 0.2 to 6.4% (Prestwich, 1994). The metabolic cost of birdsong has received considerable attention. Results vary but generally find a relatively low cost of singing, with an elevation in energy consumption during song that is generally below 2-fold, and in some cases negligible (Oberweger and Goller, 2001; Ward et al., 2003; Zollinger and Brumm, 2015). Though birdsong is metabolically inexpensive, other acoustic signals, such as echolocation in bats, can be costly, with rates as high as 9.5 RMR in stationary bats (Speakman and Racey, 1991). The mechanical efficiency of sound production depends on a number of physical characteristics, including the size of the radiator, the wavelength of the sound, and the relative impedance of the radiator and sound-conducting medium (Prestwich, 1994). Given the distinctive nature of woodpecker drumming, it is difficult to draw conclusions about whether the acoustic energy content of a drum represents a significant portion of the mechanical energy budget of the drumming motion. Measurements of the energy content of the drum sound would improve our understanding.

Given the potentially high cost of rapid cyclic motions of the body and head during drumming, we hypothesize that the evolution of drumming as a signal hinges on mechanistic innovations that increase the efficiency of the drumming motion. The storage and recovery of energy by elastic mechanisms has the potential to significantly decrease the work that must be done by muscle contraction. Energy stored as elastic strain energy can be subsequently released, and work recovered from elastic sources is work muscles do not have to perform. For example, when the head decelerates from its backward motion, kinetic energy can be converted to elastic strain energy, and the release of this energy can power the acceleration of the head toward the substrate. **Figure 6** identifies several hypothesized sites of elastic energy storage and recovery. Muscles and tendons of the limbs and neck may store and recover energy cyclically. Such mechanisms have been assumed in some mathematical models of drumming mechanics (Vincent et al., 2007; Liu et al., 2015), and woodpecker drumming has been modeled as a forced harmonic oscillator (Collins, 2017a). Tail feathers often brace the body during drumming. They can be observed to bend with the drumming motion, and may serve a spring-like function, as has sometimes been assumed (Vincent et al., 2007). Unknown is the extent to which elastic rebound on impact with the substrate contributes to motion. Spring-like behavior of wood has been considered in some mathematical models of drumming (Vincent et al., 2007), but whether such rebound is a significant or insignificant contributor remains to be determined. The material properties of the drumming substrate will be an important determinant of the elastic behavior. Woodpeckers choose a variety of drumming substrates, typically dead wood sites but also flexible metal substrates (e.g., gutters, chimney flashing). Studies of whether birds choose drumming sites with favorable elastic properties are underway.

Playback experiments suggest that woodpeckers operate near a physiological limit when drumming, but it is not clear what mechanical and/or neurological tasks may set this performance limit. At the muscle level, rapid drumming requires both rapid processes of activation/deactivation (Schuppe et al., 2018), as well as possibly high speeds of muscle shortening. Peak power output

of muscle is a potential constraint, and elastic mechanisms may also set limits that are difficult to escape. And, physiologically demanding movement tasks are also demanding of motor control mechanisms (Barske et al., 2011; Clifton et al., 2015; Fuxjager et al., 2016), thus fidelity of motor control is also a possible limiting factor for performance.

The potential sources of power for drilling are the same as those for drumming. The need to break down substrate during drilling is likely to mean more energy is lost in each cycle, thus drilling may be associated with higher demands for net positive muscle power. We speculate that for this reason elastic mechanisms may be less important during drilling than during drumming, but this hypothesis remains to be tested.

Impact Risk of Beak Behavior

Many, possibly most, studies of the mechanics of drumming focus on the question of how woodpeckers can repeatedly strike their heads against a relatively stiff substrate without suffering brain injury. These studies are often motivated by a desire to reduce the chance of human brain injury, for example by improving helmet design through bio-inspiration (May et al., 1979; Mao et al., 2014; Liu et al., 2017). The production of sound with a high speed impact is unusual as an acoustic signal, and casual observation suggests that when impacts of similar speed and stiffness occur elsewhere in nature, the goal is often to cause or at least threaten damage [e.g., the impact of high-speed mantis shrimp claw "clubs" with mollusk shells or the horn collisions of big-horn sheep (Kitchener, 1988; Crane et al., 2018)]. Drumming itself is assumed to be derived from the drilling behavior that is meant to be destructive to wood as birds forage and excavate nests. Thus, it seems reasonable to hypothesize that anatomical or physiological specializations may have been required to reduce the chance of injury in the high-impact behavior of drumming.

Studies of mechanisms that might reduce the chance of injury during the impact phase of drilling or drumming have used mathematical models, anatomical observations, and some materials testing to probe for possible adaptations. Early analysis acknowledged that small size provides some protective effect (May et al., 1979). For a given acceleration the force on the brain will be proportional to mass while the cross-sectional area will scale with the 2/3 power of mass, thus stress (force/area) should decline with decreasing size for a given acceleration. Gibson (2006) observed further that differences in the orientation of the braincase between birds and humans increase the relative cross-sectional area of the brain in the direction of acceleration. Using measures of head deceleration taken for acorn woodpeckers (May et al., 1979) and a concussion tolerance curve for humans (Ono et al., 1980; Gibson, 2006) concluded that the accelerations woodpeckers experience on impact (≈600 to 1500 g) are well below that expected to cause injury (≈4,600 to 6,000 g). This analysis requires a number of assumptions, including that injury leading to concussion occurs at the same stress in human and woodpecker brains. It remains unclear if such a calculation, which puts woodpecker drumming and drilling impacts under a threshold for concussion in a single blow, means that mechanisms are not needed to reduce possible damage from repeated high impact accelerations.

Several anatomical features have been proposed to act as protective mechanisms against brain injury in woodpeckers. An idea central to many studies is that anatomical structures act as a damper, dissipating the energy of impact and thus reducing the energy left to accelerate the brain, much like a crumple zone in a car protects passengers. Features that have been proposed as dampers include the microanatomy of skull spongy bone (Wang et al., 2011), the micro and nano-structure of the ramphotheca of the beak (Lee et al., 2014; Liu et al., 2017), and the hyoid and associated muscles (Wang et al., 2011; Jung et al., 2016). Implicit in these functional interpretations is the idea that selection has favored these energy dissipating mechanisms. A challenge to this reasoning, acknowledged in some cases (Shaw, 2002; Liu et al., 2017), is that energy dissipated by anatomical structures is energy lost to its intended purpose, that is, the breakdown of wood in the case of drilling or the production of acoustic energy in the case of drumming. Other protective mechanisms that have been proposed include a tight packing of the brain within the brain case, which reduces the "sloshing" of the brain that may be associated with injury (Shaw, 2002) and a minimization of rotational accelerations that may also increase the risk of injury (May et al., 1979; Shaw, 2002). The putative mechanical risks associated with drumming need further investigation, as they may help explain why this signal evolved in the first place.

SUMMARY

Here, we review the relatively small body of literature that explores woodpeckers drumming. We emphasis how ecological and mechanical factors likely interact to shape display design, painting an integrative picture of behavioral evolution. We highlight many avenues for future work that further expand our understanding of this process. In this way, research on woodpecker drumming serves as an example of how classic organismal biology can elucidate broader principles that underlie life and its diversity. Our manuscript is therefore as much of a starting point for additional research as it is a snapshot of completed work.

AUTHOR CONTRIBUTIONS

ES and AR contributed to the writing of the manuscript and the synthesis of its ideas. TR and MF contributed to the writing of the manuscript, establishing the manuscript main points and ideas, and provided funding to support the research and effort. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.649146/full#supplementary-material

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REVIEW
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Bridging the Gap Between Mammal and Insect Ears – A Comparative and Evolutionary View of Sound-Reception

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Insects must wonder why mammals have ears only in their head and why they evolved only one common principle of ear design—the cochlea. Ears independently evolved at least 19 times in different insect groups and therefore can be found in completely different body parts. The morphologies and functional characteristics of insect ears are as wildly diverse as the ecological niches they exploit. In both, insects and mammals, hearing organs are constrained by the same biophysical principles and their respective molecular processes for mechanotransduction are thought to share a common evolutionary origin. Due to this, comparative knowledge of hearing across animal phyla provides crucial insight into fundamental processes of auditory transduction, especially at the biomechanical and molecular level. This review will start by comparing hearing between insects and mammals in an evolutionary context. It will then discuss current findings about sound reception will help to bridge the gap between both research fields.

Keywords: auditory evolution, insect hearing, mammal hearing, auditory transduction, hearing biomechanics

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INTRODUCTION

Detection of air-borne sounds can enable animals to perceive key information about conspecifics, predators and prey over long distances and with a high directional precision. Both, insects and mammals have evolved unique and fascinating solutions—often with common principles of operation—to identical problems of sensitive sound detection, frequency discrimination and sound localization. In this review we compare and contrast evolution and present day function of ears in insects and mammals. We discuss this in the context of evolutionary drivers and constraints that sculpted them through ∼600 million years of evolution since they separated. We start with *Early aquatic evolution of primary mechanosensitive receptors* which accounts for nearly a third of the evolutionary time since the last common ancestor of insects and mammals. Once animals ventured onto land ∼400 million years ago (MYA) it is informative to list and appreciate the *Evolutionary drivers and constraints of mammal and insect ears* that act on the auditory organs, including predator detection, conspecific communication and prey detection. We address the evolutionary innovations of ear physiology, through the constrains of both their evolutionary history, i.e., natural selection can only work on the range of phenotypes a species has, and the physical properties of sound propagation and detection by biological systems. We then review the function of today's insect and mammalian ears in the sections *Convergent evolution: sculpting similar biomechanical function of ears* and *Convergent evolution: mechanisms of sound amplification*.

It is a testament to the strict laws of physics and the persistent, harsh and relentless selection pressures of hearing that the same optimal solutions are found both in insects and mammals. Finally, we delve into the *Mode of transduction—closing the gap on the identity of the transduction ion channel in mammals and insects*. Insect and mammalian ears are exquisitely tuned for their respective detection of sound. Although they use homologous development genes to control ear development and uncannily similar molecular mechanisms, this is achieved through a combination of similar and different protein components. Identifying and confirming the identity of the transduction channel—different between insects and mammals—has proved especially challenging in both animals and as each research field closes in on the transducer identity it is an especially exciting time to review the progress.

EARLY AQUATIC EVOLUTION OF PRIMARY MECHANOSENSITIVE RECEPTORS

The earliest life existed some ∼3.7 billion years ago in a hot, oxygen-poor primordial broth (Garcia et al., 2017) of simple single-celled prokaryotic organisms. A tapestry of membrane-bound receptor proteins enabled interactions with their environment. Some two billion years later eukaryotes evolved out of an endosymbiotic amalgamation of prokaryotic components (Cooper, 2000; Knoll, 2004). One key difference that evolved in eukaryotes, was a microtubule cytoskeleton. This linear repeating chain of tubulin proteins would later push finger-like protrusions out into the environment: flagella/cilia (Mitchell, 2004; Jékely and Arendt, 2006; Satir et al., 2008). Their ensuing rhythmic bending created water currents necessary to filter and ingest food but also endowed cell motility so that, together with the adaptability of this new eukaryotic form, single celled life could move to exploit new environmental niches. The basic cytoskeleton of cilia—their nine doublet microtubules that form an elongated internal ring (**Figure 1**)—are ubiquitous in all branches of eukaryotes and evolved before the last eukaryotic common ancestor (Doolittle et al., 1996; Douzery et al., 2004; Berney and Pawlowski, 2006; Mitchell, 2007). This microtubule flagellum is hypothesized to have been such a competitive advantage that it was the only eukaryote whose descendants survive to this day (Mitchell, 2007). In addition, cilia acted as sensory antennae where receptor proteins congregate. This innovation proved key to the formation of all specialized sensory organs of today's eukaryotes—insect and mammalian (Moran et al., 2014).

From single-celled organisms sprouted multicellular life, nearly two billion years after first single-celled life (Knoll, 2004). Using minimum evolution criterion of the molecular clock, which aligns with the geological record, recent analyses put the last common ancestor of vertebrates and invertebrates as late as 573 MYA (Peterson et al., 2004) and as early as 634 MYA (Peterson and Butterfield, 2005). This is well before the Cambrian explosion of complex life and ecosystems (541 MYA) and well before animals appear in the fossil record (Knoll, 2004). It is speculated that the last common ancestor of invertebrates and vertebrates—termed Urbilaterian (**Figure 1**)—had a well-defined body-axis (de Robertis and Sasai, 1996) bearing a head with dedicated sensory systems including photoreceptors (Arendt and Wittbrodt, 2001) with probably nine opsins (Ramirez et al., 2016), a gut (Hejnol and Martindale, 2008) and touch-sensitive appendages, equipped with sensory cilia linked to a nervous system (Carroll et al., 2001). The common origin of the sensory systems of invertebrates and vertebrates in this Urbilateria is strikingly evidenced by a suite of homologous genes in today's insect and mammals. For example, both modern-day flies and mammals have different but homologous pro-developmental genes for ear development (Math1 and Atonal, **Figure 1**) and eye development (Pax6) that can be functionally swapped between them (Xu et al., 1999; Wang et al., 2002; Weinberger et al., 2017). Such genetic conservation is not limited to sensory systems and it appears that the basic genetic architecture for a bilateral body design already existed before invertebrate and vertebrates became two distinct animal groups. In this respect, we could think of the widespread genetic homology in animals not in terms of the relatively short evolutionary time that they shared in this animal form, but the evolution of a genetic and developmental program that was ready at an optimal moment; at the start of the Cambrian explosion and flourished during it when all of today's animal lineages were established—an enduring unchallenged bilateral monopoly on nearly all animal life on earth (Knoll, 2004).

The vertebrate multiciliate receptor cells, called hair cells, are thought to have evolved even before a centralized dedicated mechanoreceptive system in aquatic vertebrates [similar to modern day ascidians or filter feeders (Burighel et al., 2003)]. The integration of hair cells into a dedicated head-based mechanosensory organ was the first of two major milestones in the evolution of vertebrate hearing that took place in a purely aquatic environment. Evolution would sculpt this prototypical mechanoreceptive organ to fulfill the selective pressures of vertebrates to hear as they diversified into terrestrial niches. The earliest fossil evidence of an internalized mechanoreceptor is from an early (pre-Devonian age > 416 MYA) vertebrate armored "fish" creature—the Ostracoderm *Protopteraspis micra* (**Figure 1**; Stensiö, 1927). Embedded in the skull are bilateral labyrinths, resembling basic acceleration or balance organs. Ostracoderms, with their bilateral balance organs, were so prosperous that they gave rise to most vertebrates alive today over their 100-million-year reign (Forey and Janvier, 1994; Janvier, 2008). The second milestone, evolution of an articulating jaw, is first evidenced in another type of armored fish, placoderms, with the earliest fossil 419 Million years old (Zhu et al., 2013). Evolution of the jaw was a key innovation that allowed jawed vertebrates (Gnathostomes) to outcompete their jawless competition, probably first for buccal (mouth)-based respiration (Mallatt, 1996), then for biting and chewing their prey; few jawless chordates, such as the lamprey, exist today. The evolution of an articulating jaw transmitting sound-induced bone vibration to the ear was essential for the evolution of hearing of all vertebrates over the next 300 million years of terrestrial evolution.

Invertebrate evolution, like vertebrates, was constrained to an aquatic environment. The first terrestrial fossil tracks come

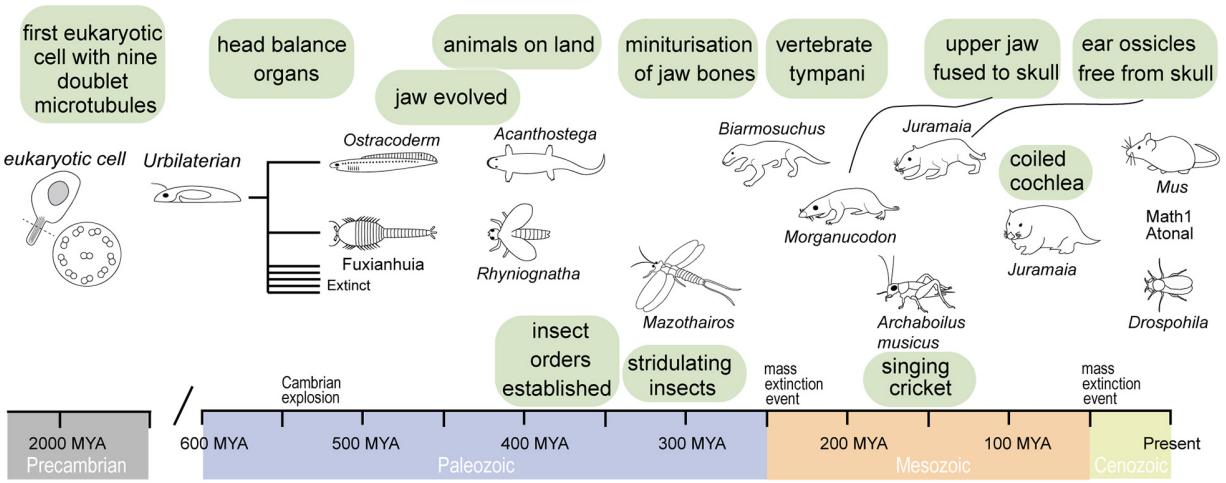

FIGURE 1 | Timeline of evolution of important ear structures in vertebrates and invertebrates that have a common ancestor in the Urbilaterian. This bilateral organism probably had already mechano-sensitive cells.

from invertebrates, dated around 480 MYA (MacNaughton et al., 2002). Phylogenetic analysis puts the appearance of insects around the same time (Misof et al., 2014). Such tracks are thought to represent transient incursions on land of marine-based invertebrates but provide also evidence for the ease at which invertebrates can exploit terrestrial niches. Their hard exoskeleton could support their body weight and prevent desiccation out of water—unique advantages over their vertebrate counterparts. In contrast to vertebrates, aquatic invertebrates did not evolve a central mechanosensitive organ, from which all insect ears evolved. What proved crucial for insect ear evolution was their widespread proprioceptors present in all their articulating joints necessary for sensing all their movements. These proprioceptors formed the basis of all insect sound detecting organs.

EVOLUTIONARY DRIVERS AND CONSTRAINTS OF MAMMAL AND INSECT EARS

Mammals

From water to land, the first vertebrate ears are speculated to have taken their first evolutionary steps in semi-aquatic tetrapods that straddled the aquatic-terrestrial shoreline. One of the earliest terrestrial tetrapods, such as *Parmastega aelidae* and *Acanthostega* (**Figure 1**) gives us a glimpse of these aquatic-terrestrial transitional forms of vertebrates. *P. aelidae* obtained oxygen from both water—as assumed from nostrils positioned under the water line—as well as from the air—through a spiracle opening at the back of the head above the water line (Beznosov et al., 2019). As these early tetrapods evolved, the nostrils became larger and migrated above the water line to permit oxygen to be obtained solely from the air. This fossil record not only evidences their advancement onto land but sets the stage for airborne detection of sound that requires head-based air conducting channels to funnel sound to the balance organs of their aquatic ancestors—that would (much) later evolve into organs dedicated to detect airborne sound—ears. At this time ∼400 MYA, however, the bilateral balance organs of gnathostomes had already evolved over ∼150 million years in aquatic environments to detect low-frequency head-based accelerations necessary for the control of swimming (van Bergeijk, 1967; Baird, 1974). Such low-frequency detecting organs were quite unsuited for detecting higher frequency airborne sound. The frequency range of their balance organs was only one of two more substantial barriers to the detection of airborne sound. The first was a lack of structures [such as tympani (ear drums)] to capture sound energy, in the form of sound pressure differences. The second was a lack of structures (a middle ear) to transmit any airborne sound energy to their internal balance organs—so called impedance transformers because air-borne sound must be converted into vibrations of the high impendence saline that bathes the sensory receptors. Our interpretation goes against the "standard view" (Lombard and Bolt, 1979) that aerial hearing evolved soon after tetrapods moved onto land. In defense of our interpretation, there was a complete lack of middle ear specializations for hearing in early tetrapod animals that represent the five major amniote lineages (Clack and Allin, 2000) and no tympani were even thought to exist for early aquatic tetrapoda (Allin and Hopson, 1992; Clack and Allin, 2000). Their balance organs were perfectly sufficient to detect the footfall of predators or competing conspecifics through surface-borne ground vibrations, picked up by conduction of sound through the leg and then jaw. It is hence debatable if these first tetrapoda "heard" anything that we would call sound, induced by pressure changes in air. Modern amphibians lack middle ear cavities, but still sensitivity detect the vibration of predators. Therefore, vibration detection through limbs, which are in contact with the ground proves an effective strategy and, hence, why vertebrates were in no hurry to evolve solely aerial hearing (Hildebrand and Goslow, 1995). In

support of this, there is a secondary loss of specialized air-borne receivers in favor of surface-borne vibrations in amphibians like salamanders (Wever, 1978; Hetherington, 1992). As such, the first evolutionary steps to the solely aerial hearing system of today's mammals were probably in the form of better coupling of ground-borne vibrations to their balance organs.

Middle ear evolution began with a bone later to become the stapes, which in present day mammals feeds sound vibrations into the cochlea. About 400 MYA ago the stapes had an obvious structural role to stabilize articulation of the jaw for breathing and chewing, but crucially it bridged between the otic (ear) bone and the upper jaw (Clack, 1992). Early fossils of aquatic tetrapoda, *Acanthostega* and *Ichthyostega* (Clack, 1992; Clack et al., 2003), suggest that, in addition to its main structural role, the stapes already served to transmit ground-borne vibrations to the inner ear in early semi-aquatic tetrapods (Clack, 1983). From an extensive fossil record of vertebrates, we know that two former lower jaw bones underwent a change of function and became also part of the mammalian middle ear (Reichert, 1837). It is hypothesized that miniaturization of the jaw was the primary driver for the transformation of the jaw joint (Lautenschlager et al., 2018) and that the transformation of the jaw elements into cranial ear bones occurred several times in the mammal evolution. Allotheria, an extinct group of early mammals, evolved a unique palinal joint as a feeding adaption, whereas other Mammaliaformes have hinge joints (Han et al., 2017). However, the incus and malleus decoupled in different stages from the lower jaw and only function in sound transmission in modern mammals (Lopatin, 2019). Due to the poor preservation, the origin of the tympanum in the different vertebrate taxa is less understood. It is believed that tympana also evolved independently several times in vertebrates (Grothe and Pecka, 2014) and is based on thinning of the skin of the lateral head. Two main hypotheses are discussed for the origin of the tympanum holding bone elements; one origin is considered by a postquadrate tympanum and another by a postdentary tympanum (Gaetano and Abdala, 2015).

Based on the fossil record of small rodent-like mammals like Sinoconodon and Morganucodon (**Figure 1**), the cochlea ∼200 MYA was little more than a thumb-like stump (Graybeal et al., 1989; Luo and Ketten, 1991) less than 2 mm long (Zhexi et al., 1995). By the end of the Jurassic period, the cochlea of Dryolestoida, a basal relative to marsupial and placental mammals, had coiled by about three quarters. As the length of cochlea correlates with the frequency range of hearing, this fossil record suggests an evolutionary trajectory in mammals toward ever higher frequency hearing (West, 1985). The selection pressures that drove our Mesozoic mammalian ancestors to specialize sensitive, high frequency hearing are threefold (Meng and Wyss, 1995). During the Jurassic mammals exploited nocturnal niches in the face of larger predatory dinosaurs (Liu et al., 2018). Due to this there was large selection pressure for sensitive hearing as visual information was more limited at night. Secondly, an effective sound shadow to allow small mammals to detect the direction of sound—through comparative differences in sound amplitude at their ears—is only effective for high frequencies (Schnupp and Carr, 2009). Finally, vocalizing at higher frequencies would have made their larger low-frequency hearing predators less likely to hear and locate them, giving them a further selective advantage.

Insects

About 40 Million years after the colonialization of land by plants (Early Silurian, about 480 Ma), and probably before vertebrates established themselves on land, ectognathous insects such as *Rhyniognatha* (**Figure 1**, including Orthoptera, what we would recognize today as crickets, bush crickets and locusts) were present. Their descendants went onto evolve acoustic communication but the earliest proof of insects producing sound is from a Permian insect, *Permostridulus brongniarti* that existed ∼260 MYA (Béthoux et al., 2003). This is based on its specialized grooved veins under the wing—modern day crickets rub this vein along its other wing to stridulate and produce mating calls. The ability to produce sound does not necessarily imply the ability to hear. However, tympanal membranes on forelegs are found in Triassic and Jurassic fossils (Zeuner, 1939; Plotnick and Smith, 2012) and most modern-day Orthoptera that stridulate have ears (Jost and Shaw, 2006). Later stridulating insects such as *Archaboilus musica* ∼165 MYA (Gu et al., 2012), in the Jurassic, and Tertiary *Pseudotettigonia amoena* ∼55 MYA (Rust et al., 1999) shows that Orthoptera maintained the ability to produce sound through stridulation. It is likely that ears evolved to hear this stridulating sound, due to its high reproductive advantage for conspecific localization. During the Jurassic, as *Archaboilus musica* was chirping, other acoustic groups such as Diptera and Lepidoptera diverged alongside the radiation of flowering plants (Doyle, 2012).

There is further reason to believe that insect ears evolved early in their terrestrial occupation and this is the apparent evolutionary ease of acquiring a sound sensitive organ for insects. Whereas all vertebrate ears evolved from specialized head-based acceleration organs, insects' ears, by contrast, evolved from proprioceptors littered throughout their body. While the evolutionary barriers for vertebrates were formidable—evolution of middle ears (for impedance transformation) thinning of bone to form a tympanum (to capture sound pressure)—insects already possessed three components to form ears—in abundance. Their stretch- and vibration-sensitive proprioceptors, which are widely dispersed throughout their body, evolved into the auditory receptor cells, the tympani were a "simple" evolutionary thinning of their exoskeleton and the impedance of airborne sound could be simply matched by the backing the tympanum with their ample tracheal network filled with air. This evolutionary ease is evidenced by the independent evolution of several tympani—found in at least 10 different body parts, across several insect taxa (Fullard and Yack, 1993) and the high diversity of insect tympanal ears.

If we dive deeper into the morphology of insects and compare insects either with or without a tympanum (atympanate) in closely related taxa, here proprioceptors that monitor body motions in atympanate insects, acquire an auditory response with little change in their morphology (Yack and Fullard, 1990, 1993). An intuitive example is the proprioceptors in the "knee" joint of ancient insects that, was anchored at either end, and

suspended between, the tibia femur joint. As the tibia-femur joint flexed the stretch-sensitive neurons were stretched and activated. These proprioceptors were also ideally placed to detect substrate borne vibration; much like early tetrapods that detected vibration by conduction through their legs (and jaw) to their head-based mechanosensitive organs. In extant crickets this single proprioceptor has evolved into three distinct sensory organs each with their own afferent nerve bundle (see review Strauß and Lakes-Harlan, 2014): the subgenual organ, intermediate organ and the crista acustica. The archetypal composition of the receptor cell complex, known as a chordotonal organ (Kavlie and Albert, 2013), is similar in all of these three organs, only the connection to the structure that picks-up the signal differs: Sensory cells of the subgenual organ are connected to the cuticle of the leg, to detect vibration (Kühne, 1982), the cells of the crista acustica are found on top of an inner air-filled trachea in the leg to detect high frequency sound that travels along the trachea (Hedwig, 2014). Evolution of dedicated sound receivers in many other extant insect groups involved the thinning of the cuticle onto which the receptor cells were anchored as found for example in locusts, cicada and lepidoptera (for review see Yack, 2004). We can even "capture" the apparent evolutionary transition of proprioceptors into dedicated auditory receptors in the act. Cockroaches possess a dual responsiveness to substrate and air-born sounds. This provides convincing evidence that auditory organs of crickets evolved from an ancestral subgenual organ (Shaw, 1994).

Although insects lack a distinct middle ear, they have none-the-less exploited biomechanical first-order levers to enhance sound detection. Antennal receivers that exploit first-order lever mechanics—like that of the mosquito or fruit flies'—stand above all other auditory receptors in their sensitivity to angular displacement (Göpfert and Robert, 2001). Here, the end of the lever directly stretches the ciliated end of the auditory receptors. This is different to the end of the stapes in mammals that pushes, through the oval window, the fluid in the cochlea; the fluid then moves the hair bundles on top of the hair cells. Bush crickets, also developed a middle ear (Bangert et al., 1998) with characteristics similar to a 1st order lever through phase shifted motion along their tympani, which induce a motion of the fluid around the receptor cells (Montealegre-Z et al., 2012; Montealegre-Z and Robert, 2015). However, there are bush cricket species with tympanal ears that function like a 2nd order levers also without a phase difference along the tympanum motion (Nowotny et al., 2010). In the taxon ensifera, which includes crickets and bush crickets, there are even basal groups (Gryllacrididae) with ears without a tympanum (middle ear) that show functional crista acustica homologs (Strauß and Lakes-Harlan, 2008).

The physical properties of sound, such as its relatively fast speed and wide diffraction, impose constrains on evolutionary solutions of auditory systems to detect and extract directional information. Given insects small size, these constraints are particularly severe. Unlike vertebrates, insects produce a poor sound shadow thus sound approaching from one side has a similar amplitude at both ears. To locate the source of a sound, many insects like crickets, bush crickets and flies exploit and amplify subtle phase differences either through biomechanical levers made of cuticle (Robert et al., 1996) or passing sound through a tracheal network. In such a network, sound reaches the tympanum, not only from the direct external route, but through a longer internal route. The extra distance traveled by the sound shifts the phase such that for each half-period of sound the tympanum is not only pulled by low pressure on the outside, it is additionally pushed by high pressure inside (*visa versa* for the next half-phase of sound), creating a pressure difference receiver where tympanal motion is amplified on the side nearest the sound (Michelsen et al., 1994).

CONVERGENT EVOLUTION: SCULPTING SIMILAR BIOMECHANICAL FUNCTION OF EARS

The fine-tuned ability of animal ears to discriminate frequency and maximize sensitivity relies on microscopic biomechanical specializations that couple sound-induced motion to the auditory receptors. Frequency discriminating traveling waves are a pertinent example of convergent evolution between the mammalian and Orthopteran ears (Montealegre-Z et al., 2012; Udayashankar et al., 2012). In mammals, the basilar membrane shows a gradient of mass and stiffness along its length. This leads to a filter bank of damped resonators, spatial separation and gradient of auditory receptors, called tonotopy (von Békésy, 1960). The basilar membrane, the elastic membrane on which the sensory epithelium (organ of Corti) sits on, is driven by sound-induced fluid waves inside the cochlea. This creates traveling waves of the auditory epithelium, which were first observed in human cadavers (von Békésy, 1960). Traveling waves in both mammal and insect hearing organs, are much slower than air-carried sound waves. They reach velocities of about 5–25 m/s (Udayashankar et al., 2012), more than ten times slower than in air. A consequence of the mechanical based filter bank is that tonotopic motion of the epithelium always starts in the high-frequency region, independent of the frequency of sound or where it enters (this is also the reason why bone-conduction hearing aids and headphones work).

Half a century after von Békésy's pioneering work on human-based cochlea traveling waves (von Békésy, 1960), similar waves were recorded in the simple—ear drum like—tympanum of the locust (Windmill et al., 2005) and cicada (Sueur et al., 2006). The oval-shaped tympanic membrane is mainly composed of cuticle and the traveling wave results from the passive and non-homogeneous anatomical properties (thickness and tension) of the tympanum (Malkin et al., 2013). Here, a thin and light part of the tympanum vibrates best at high frequencies and a thicker and more massive thick tympanum vibrates best at lower frequencies (Michelsen, 1971; Römer, 1976). These inhomogeneous properties result in a wave that travels from high frequency to low frequency (Windmill et al., 2005)—just like the basilar membrane. Three discrete attachments of auditory neurons from Müller's organ are attached onto the inside of the tympanum and are stimulated best by the respective frequencies of the tympanum, permitting frequency discrimination (Jacobs et al., 1999). The elongated and linear

arranged auditory epithelium of the bush cricket, crista acustica, resembles the biophysical properties of an uncoiled mammalian cochlea (Udayashankar et al., 2012, 2014). Like the basilar membrane of the mammalian cochlea, the crista acustica has graded changes in its stiffness and mass to make it tonotopic (Hummel et al., 2017; Olson and Nowotny, 2019). A specially evolved adaptation in hearing organs is an overrepresentation of key ecologically important frequencies along the length of epithelium, called an auditory fovea found in some mammals (Müller et al., 1992; Neuweiler and Schmidt, 1993; Kössl, 1997), birds (Köppl et al., 1993; Corfield et al., 2011) and insects (Scherberich et al., 2016, 2017).

CONVERGENT EVOLUTION: MECHANISMS OF SOUND AMPLIFICATION

Auditory transduction is localized to evolutionary-ancient membrane protrusions that have specialized to actin-based villi or microtubule based cilia in mammals and insects respectively. Mechanosensitve ion channels are located on these projections and are opened in response to sound-induced forces. Transduction channels of mammals and insects not only convert sound-induced displacements into electrical potentials but also amplify quiet sound to enhance the hearing capacity, and therefore the survival, of its owner. This final section reviews recent experiments on electrical tuning and amplification in insects and mammals and address two key areas where we expect parallel breakthroughs in our understanding of auditory transduction for mammals and insects: visualization of amplification in the insect auditory neurons and the identity of the transduction channel.

Electrical Amplification in Early Vertebrates and Insects

Electrical tuning, found in turtles, frogs and chicks (Crawford and Fettiplace, 1981; Ashmore, 1983; Lewis and Hudspeth, 1983; Fuchs et al., 1988), relies on the sinusoidal influx of cations in response to sound which then triggers the opening of other voltage gated ion channels at the base of the hair cell (**Figure 2A**). Voltage-dependent calcium channels in the base of the hair cell are opened in response to a transduction potential flowing through the transduction channels. Calcium flows in depolarizing the cell but calcium ions also bind and open calcium-gated potassium channels which consequently leads to potassium exiting the cell and depolarizing it. The interplay of the inflow and outflow of cations leads to an oscillation, sometimes also observed as spontaneous oscillations (Crawford and Fettiplace, 1980) and when the frequency of this oscillation matches that at which the hair cell is driven by sound it amplifies the electrical potential (Crawford and Fettiplace, 1981). Electrical amplification in hair cells is considered an evolutionary old solution restricted to non-mammalian vertebrates (Popper and Fay, 1997) and has an upper limit of about 1 kHz, which is perhaps why high-frequency hearing mammals have not exploited this mechanism. Recent work by Warren in the locust's Müller's organ has shown a lack of electrical oscillations in auditory neurons (**Figure 3**). The sharpness of tuning of individual auditory neurons appears the same both upon entry of cations through the transduction channels, at the apical end of the neuron, through to the spike encoding axon at the opposite end of the auditory receptor (**Figure 3**). These first experiments to test for electrical tuning in insect auditory neurons suggest no electrically-based mechanism is involved to sharpen or amplify acoustic signals, at least in locusts. This finding agrees with the working theory (Field and Matheson, 1998) that frequency tuning in insects is accomplished solely by the mechanical properties (mass and stiffness) of their hearing organs. Antennal hearing organs, with a set mass and stiffness, discriminate frequencies (Kamikouchi et al., 2009), so there may be more of a precedent for electrical tuning here however.

Mechanical Amplification and Receptor Movements

In mammalian and vertebrate hair cells mechanical movements that power mechanical amplification have been conventionally imaged—in outer hair cells—or movements below the optical diffraction limit ingeniously measured using a glass probe attached the stereocilia and pairs of photodiodes to localize hair bundle movements to a couple of nanometers. Based on these intricate measurements of displacements and forces directly from the receptor cilia of hair cells, and their morphology we have a good understanding of how stereocilia pivot at their base and are coupled along their axis to transduction channels to amplify quiet sound (Martin et al., 2000).

For insects, most notably two-winged insects, measurements of forces from an assemble of auditory receptors can be indirectly measured and properties of the transduction channel calculated by measuring displacements of the antennae to which they attach (Albert et al., 2007; Su et al., 2018). Despite the advancements in auditory transduction this has afforded us, the tiny and inaccessible nature of insect hearing organs and the auditory receptors themselves means we are in the dark as to how—at the auditory receptor level—these movements are generated. There is general agreement that active movements that power mechanical amplification are localized to the cilium of the auditory neurons but at this stage we can not rule out other parts of the auditory neuron or other cells that compose the mechanosensory (scolopidial) unit contributing—as is the case for Prestin-based mechanical amplification in mammals, which are separate from the sensory inner hair cells.

Prestin-Based Amplification

Discovery of outer hair cell based mechanical amplification can be traced back about 40 years ago. Dallos and Harris (1978) selectively destroyed outer hair cells and found severely impaired auditory response in chinchillas. They hypothesized that outer hair cells, somehow, sensitized the sensory inner hairs cell. Then, labs reported on mechanical responses in isolated outer hair cells from the mammalian cochlea (Brownell et al., 1985; Zenner, 1986; Ashmore, 1987). In response to sound-induced

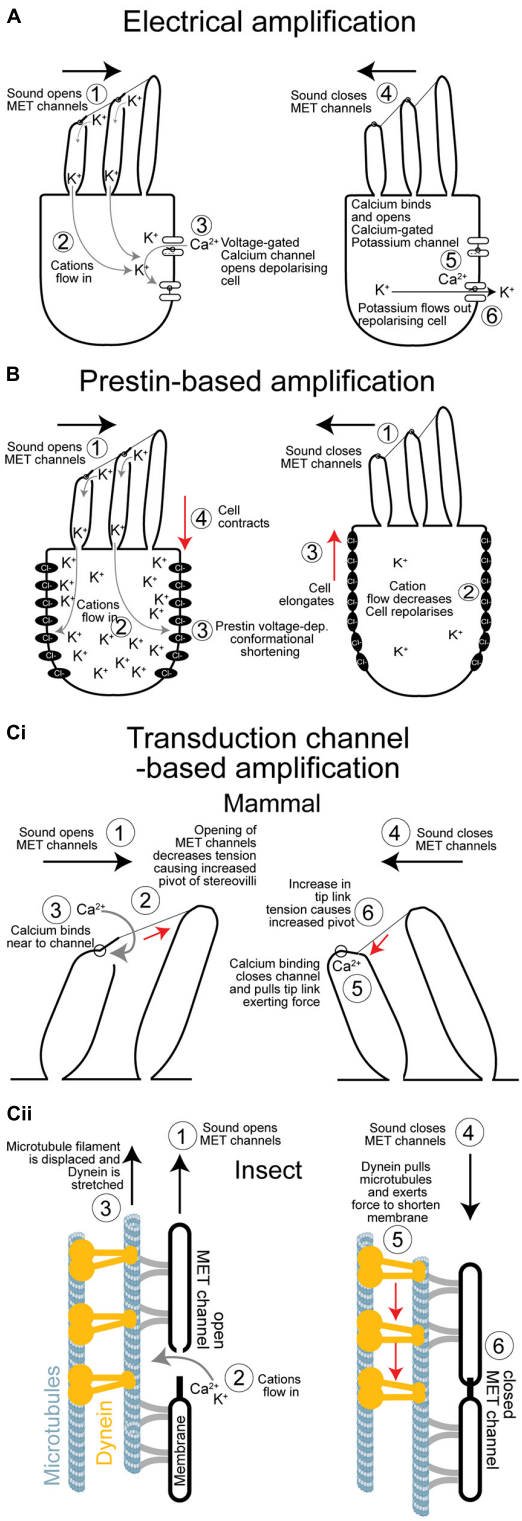

FIGURE 2 | Comparison of the three cellular mechanisms to amplify sound. **(A)** Electrical amplification in the frog's sacculus is based on reciprocal feedback loop created by voltage- and calcium-gated ion channels that sets up an electrical resonance which amplifies quiet sound of relatively low frequencies. **(B)** Prestin-based amplification of sound exclusive to mammals. *(Continued)*

FIGURE 2 | Continued
Elongation and contraction of the cell body depends on the voltage-dependant—and transduction induced—changes of the conformational state of a repurposed anion co-transporter. There is no evidence that sensory cells in insect ears undergo voltage-dependant changes in their body shape. **(Ci)** Simplified mechanism of hair bundle based amplification. Amplification is based on a transient reduction in stiffness as channels open causing the hair bundle to pivot further. As the transduction channels close, due to a calcium-dependant feedback, they pull the hair bundle through the tip links in the opposite direction augmenting its movement. **(Cii)** Proposed mechanism of amplification in insect auditory neuron cilia. Sound forces open transduction channels and causes a transient decrease in stiffness that increases movement in the direction of the force. Dynein responds to an increase in tension by contracting pulling the microtubule filament, and the membrane housing the transduction channel, to mediate its closure. Dynein-based force production amplifies sound when in phase with it. MET, mechano-electrical transduction.

sinusoidal voltage changes, caused by the transduction potential, these cells elongate and contract to mediate rapid changes of hair cell length (**Figure 2B**) that push and amplify movements of a sound-induced traveling wave. This outer hair cell electromotion is facilitated by a motor protein called Prestin (Zheng et al., 2000). The motor function of this protein (protein family: SLC26) was a mammalian-specific evolutionary repurposing of an anion transporter, which existed either in the last mammalian common ancestor ∼200 MYA or later in the last common ancestor of therians ∼130 MYA (Manley, 2000). In insects, homologs of Prestin are found (Weber et al., 2003) but without any motor function (Kavlie et al., 2015). In mammals, Prestin is essential for sensitive responses to low sound pressure levels (Liberman et al., 2002; Cheatham et al., 2004) but also for compression at high sound pressure levels (Robles and Ruggero, 2001). A complex interaction of basilar membrane motion and Prestin-induced outer hair cell motion optimize cochlea tuning (Cooper et al., 2018). During evolution, the new piezo-electric motility (Dong et al., 2002) by Prestin enabled mammals to hear ultrasonic frequencies. Here, mammals have made and exploited their own channel of communication, like some insect taxa.

Hair bundle motility amplifies and tunes their responses (**Figure 2Ci**). This probably existed in the primitive balance organs of the earliest vertebrates ∼500 MYA (Manley, 2000). Active hair bundle motility is caused by the coordinated opening and closing (adaptation) of transduction ion channels that pull to exert forces on the hair bundle, through filamentous tip links, to amplify their movements (Howard and Hudspeth, 1988). A rapid reduction in stiffness is the result of the mass opening of transduction channels pulled by filamentous tip links. The reduction in stiffness can be so severe as to become negative—it provides a force in the same direction of sound-induced forcing—thus amplifying movements (Martin et al., 2000). Rapid channel closure—known as fast adaptation—can then pull the hair bundle, through the tip links, in the opposite direction exerting a recoil force (Kennedy et al., 2003). If these channel-based forces coincide with sound-induced movement of the hair bundle it results in mechanical amplification of the hair cell displacement and therefore an amplified receptor potential. This mechanism can drive spontaneous bundle oscillations, which

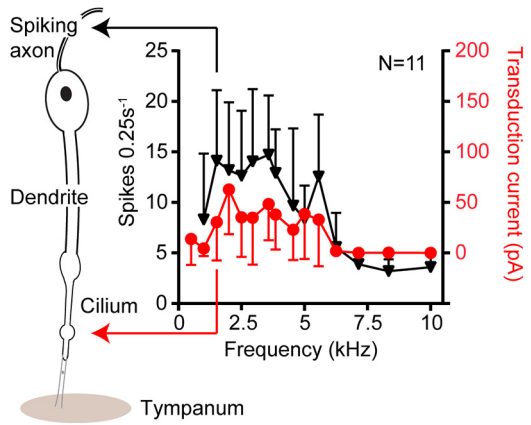

FIGURE 3 | Isolevel auditory tuning curves in response to 80 dB SPL from single auditory receptors of the locust ear. The black triangles are tuning curves derived from sound-induced axonal spikes at the neuron's basal end. The red circles donate tuning curves based on the transduction current at the ciliated apical end. No change in the shape of the tuning curves suggesting no electrophysiological processes, like that of reptiles to, sharpen tuning in this insect model.

were measured in the frog sacculus for example (Martin and Hudspeth, 1999). In mammals the importance of this mechanism is still discussed (Nin et al., 2012). Hair bundle-based motility based on the properties of transduction channel opening and closing is elegantly explained by a mathematical "gating spring" model first used to quantitatively describe bullfrog saccular hair bundle dynamics (Howard and Hudspeth, 1988).

Active hearing in insects was first shown in mosquitos and *Drosophila* and was later shown to have remarkable parallels with hair bundle motility. Sound-receiving antennal receivers were discovered to oscillate spontaneously, but under CO_2-indcued hypoxia these active oscillations disappeared (Göpfert and Robert, 2001, 2002). Later, the first insect with tympanal ears was shown to exhibit spontaneous oscillations that disappeared upon death (Mhatre and Robert, 2013). An interaction of mechanotransduction channels and motor proteins in the sensory cells are hypothesized to be the basis of these oscillations (Nadrowski et al., 2008). Most striking of all is that, despite separate ~ 600 million year evolutionary trajectories, the "gating-spring" model, first used to describe frog hair bundle movements also quantitatively explains the mechanics of antennal sound-receivers—ears—of fruit flies and mosquitoes (Albert et al., 2007; Su et al., 2018). This is a spectacular illustration of convergent evolution of two hearing systems, with very different architecture, that have combined the gating of transduction channels with mechanical amplification of sound. Key in the gating spring model is adaptation and rapid closure of the channel after opening. In insects a dynein motor is hypothesized to rapidly close the channels after opening (Karak et al., 2015). However, for hair cell receptors Ca^{2+} binding close to the channel complex is thought to conformationally close it (**Figure 2Ci**; Peng et al., 2013; Corns et al., 2014) and this might also be the case for insect auditory cilia. The energy for this process presumably comes from the very steep electrical gradient of ~130 mV (von Békésy, 1952; Russell and Sellick, 1978) across the hair cells. A steep electrochemical gradient is shown to be the case for some insect mechanoreceptors (Thurm and Küppers, 1980) and assumed to be so for auditory neurons of insects (Kavlie and Albert, 2013; Warren and Matheson, 2018). In lower vertebrates, such as turtles and bullfrogs, slow adaptation of the channel maintains an optimal tension and open probability of the channel and is powered by myosin motors anchored to the actin cytoskeleton (Gillespie and Cyr, 2004; Stauffer et al., 2005). As insect auditory cilia have a microtubule cytoskeleton bearing dynein arms (Karak et al., 2015), channel closing in insects is thought to be powered by dynein (see for review Göpfert and Robert, 2008; Göpfert and Hennig, 2016; **Figure 2Cii**). Further supporting dynein's role in transduction, are measurements of the temperature dependence of spontaneous oscillations in mosquitoes (Warren et al., 2010), and distortion-product otoacoustic emissions in locusts (Möckel et al., 2012) both hallmarks of the transduction process. The temperature dependence of biological processes gives information about the chemical reactions that produce them. In this case the activation energy measured for both spontaneous oscillations and distortion-product otoacoustic emissions matched that of the enzyme dynein ATPase, which is hypothesized to provide the energy for mechanical amplification.

MODE OF TRANSDUCTION – CLOSING THE GAP ON THE IDENTITY OF THE TRANSDUCTION ION CHANNEL IN MAMMALS AND INSECTS

Over the past 40 years a fascinating and persistent search for the mammalian transduction channel has captured the imagination of sensory biologists. This dramatic roller-coaster of discovery and dismissal of various promising hair cell transduction channel candidates has led to today's more tentative approach to claims that *the* channel has been found. One realization is that the channel works in a complex with other membrane proteins, which exist in different isoforms—quelling the idea of *the* transduction channel and setting out a longer more gradual journey to discover of all the interacting components. About 20 years ago insects jumped into search for its own auditory transduction channel, powered by the genetic versatility of the fruit fly. As it will become clear in the next paragraphs, both research fields have made remarkable progress, but it is those working on the mammalian hair cell transduction channel that appear to be cautiously closing in on the channel identity.

In mammals, tip links, composed of cadherin 23 at their apical end and protocadherin 15 at their lower end (Kazmierczak et al., 2007), orientate in one direction and connect adjacent stereovilli in hair bundles (**Figure 4A**; Hudspeth, 1985). In zebrafish protocadherin 15 connects to a candidate protein of the hair cell transduction channel, a transmembrane channel-like channel (TMCs) (Maeda et al., 2014). In contrast, sensory cells of insect ears have only one ciliate hair and tip links are not present (**Figure 4B**; Kavlie and Albert, 2013). Therefore,

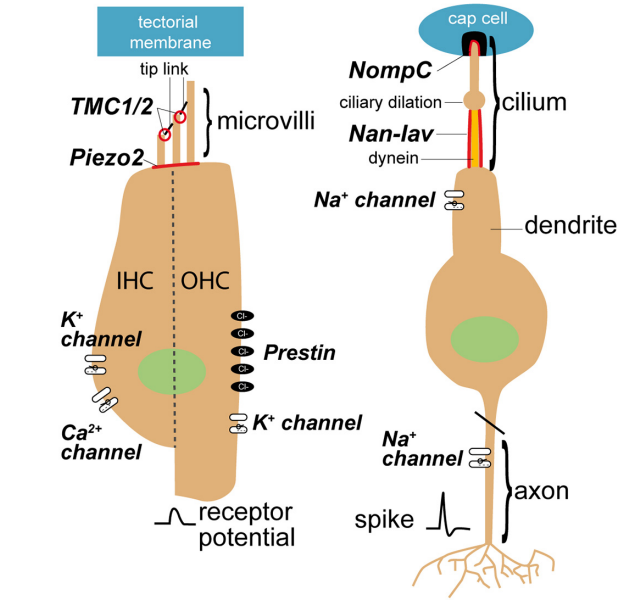

FIGURE 4 | Sensory cells in mammals **(A)** and insects **(B)** and mechanosensitive channels that are involved in the transduction process. Mechanosensitive channels in mammals belong to the transmembrane channel-like proteins (TMCs), whereas mechanosensitive channels in the insect ears are members of the transient receptor potential (TRP) family (see for review Jin et al., 2020). TMC are believed to evolved from TRP channels (Saier, 2016). Nan-lav, nanchung-inactive; NompC, no mechanoreceptor potential C.

the question arises how in an insect ear the transduction channels are opened without the connection by tip links. In insects, the protein structure of the mechanosensitive channel protein NompC (Jin et al., 2017) is well known. The other contender for the auditory transduction channel, Nanchung-Inactive, not. The interplay and role of these channels is still under discussion (Albert and Göpfert, 2015; Hummel et al., 2016). However, in mammals, the position and function of the transduction channel is well characterized (Beurg et al., 2009; Peng and Ricci, 2011; Ó Maoiléidigh and Ricci, 2019), but the involved proteins and their interplay are being established (Qiu and Müller, 2018).

The first candidate for the hair cell transduction channel were epithelial sodium channels (ENaC) because they were localized to the stereovilli tips (Hackney et al., 1992), had isoforms in the chick cochlea (Killick and Richardson, 1997) and homologous MEC genes in *C. elegans*, when knocked out, had mechanosensory defects (O'Hagan et al., 2005). Despite such early promise the selectivity of ENaC's to Na^+ and Ca^{2+} were too high and low respectively compared to that measured in hair cells (Kellenberger and Schild, 2002) so were the first to be ruled out. At the turn of the century, a decade after the first transient receptor potential (TRP) channels, required for vision, were discovered in *Drosophila* (Hardie and Minke, 1992), mechanically sensitive TRP channels were being discovered and characterized across the animal kingdom from flies and zebrafish to frogs and worms (Walker et al., 2000; Sidi et al., 2003; Shin et al., 2005; Li et al., 2006). As two TRP channels had been identified essential for hearing in *Drosophila* (Walker et al., 2000), at this stage, it was thought possible that, for both insects and mammals, TRP channels were the transduction channel. The accumulating widespread sensory functions of TRPs across animals (Madrid and Bacigalupo, 2015) made them all the more convincing and, for mammals, there was one outstanding candidate, TRPA1 (Corey et al., 2004). Transduction currents were severely affected, sometimes absent, in mice with TRPA1 knockdowns, TRPA1 localized to the hair bundle tips, where the channels are, and the start of expression coincides with hearing. Disappointingly, it was later shown that hair cells with knockout of TRPA1 have normal transduction currents (Kwan et al., 2006), they were dismissed and the search continued.

Adding to an already complex system, a distinct mechanically-elicited electrophysiological current (reverse-polarity current) was discovered that manifested in hair cells even when tip links are severed (Marcotti et al., 2014). This current was proposed to be a candidate for the transduction channel (Beurg and Fettiplace, 2017) but later electrophysiological characterization strongly suggested they were different channels (Marcotti et al., 2014). This reverse polarity current was discovered to be carried by Piezo channels (Wu et al., 2017) already identified to have diverse roles in touch sensation (Gottlieb, 2017). Thus, Piezo could be the penultimate candidate for the hair cell transduction channel, expressed in the cochlea (Wu et al., 2017), if the latest candidate—transmembrane channel-like protein (TMC) channels—withstand the battery of tests it is currently undertaking to win the race (Pan et al., 2018). Two transmembrane channels isoforms (TMC1 and TMC2) and associated proteins are the leading contenders for core components for the transduction channel (Corey et al., 2019; **Figure 4A**). As well as localization to the site of the channel, TMC2 expression coinciding with onset of mechanotransduction (Kawashima et al., 2011) and multiple pore mutations predicted to alter the channel's ion selectivity and binding with the blocker dihydrostreptomycin do so (Pan et al., 2013, 2018; Corns et al., 2016). TMC orthologs are functionally conserved in *Drosophila* larvae for touch sensation but the scattering of TMC expression in auditory neurons suggests that TMCs are not the insect auditory transduction channel (Guo et al., 2016).

Two outstanding candidates for the insect transduction channel were discovered with forward genetic screens (Kernan et al., 1994; Kim et al., 2003; Gong et al., 2004). These transduction channels are: NompC and Nanchung-Inactive (**Figure 4B**). Both, NompC and Nanchung-Inactive, belong to the TRPN and TRPV sub families of the TRP superfamily of sensory ion channels and localize to the tip and proximal part of the cilium respectively (Gong et al., 2004; Liang et al., 2010). Although NompC forms a bone fide mechanotransduction channel, even when expressed in heterologous cells or ectopically (Gong et al., 2013; Zhang et al., 2015), intracellular recordings of the transduction current support Nanchung-Inactive as the transduction channel (Lehnert et al., 2013; Warren and Matheson, 2018).

NompC connects the membrane to the microtubule cytoskeleton (Howard and Bechstedt, 2004; Jin et al., 2017) where the cilia are attached to the cap, most clearly shown for cuticle strain-sensitive campaniform receptors (Zhang et al., 2015). This is because the 29 ankyrin repeats of each NompC ion channel form a helical spring (Michaely et al., 2002; Jin et al., 2020), are compliant structures and absent when NompC is knocked out (Liang et al., 2013). Connection of the cap to the ciliary tip membrane is through a membrane-embedded filamentous extracellular matrix protein, including NompA (Chung et al., 2001). NompA could also directly gate the transduction channels of insect auditory neurons, like the vertebrate tip link, because the leading candidate mechanotransduction ion channel NompC also localizes to ciliary tip (Lee et al., 2010).

Two competing models exists to explain the respective functions of NompC and Nanchung-Inactive (Göpfert et al., 2006; Lehnert et al., 2013; Albert and Göpfert, 2015). The NompC hypothesis assumes that NompC, at the tip of the cilium, is the primary transduction ion channel and Nanchung-Inactive, propagates the transduction potential down the cilium, like an action potential (Göpfert et al., 2006). NompC produces forces, by channel gating, that move the antennae. The origin of these forces are thought to be due to the mechanical forces produced by dynein (Shingyoji et al., 1998; Karak et al., 2015) or due to a conformation change of the channels gate powered by the steep electrochemical gradient across the channel (Mhatre, 2015). In this model NompC produced force is regulated by Nanchung-Inactive. NompC is a clear frontrunner for the channel. It forms a mechanotransduction ion channel in heterologous cells, the permeability of which can be altered through pore mutations (Yan et al., 2013). It functions as a mechanotransducer when expressed ectopically in non-mechanically sensitive neurons (Yan et al., 2013) and is a bone fide mechanotransduction ion channel in other mechanoreceptors (Gong et al., 2013). When Nanchung or Inactive are knocked out, spontaneous active motility of the antennae increases 10-fold, explained by the model's lack of feedback regulation by Nanchung-Inactive. Whereas mutations in NompC lead to a reduction in the compound potential recorded from the fly Johnston's organ and total loss of mechanical amplification (Göpfert et al., 2006). When NompC is knocked out there remains a small sound-evoked compound potential from Johnston's organ. The NompC model accounts for this as being due to gravity dedicated neurons that weakly respond to sound (Kamikouchi et al., 2009). Because mechanical amplification of the auditory neurons depends on channel gating—as predicted by the gating spring model—a lack of amplification would be predicted when the mechanotransduction channel is mutated, which supports NompC.

Evidence against NompC stems from intracellular recordings of the sound-evoked current flowing though the mechanotransduction ion channel. Here no potentials are detected in NompC mutants or when Nanchung-Inactive are pharmacologically impaired (Lehnert et al., 2013; Warren and Matheson, 2018). As such, the Nanchung-Inactive hypothesis (Lehnert et al., 2013) states that NompC regulates the tension delivered to the true mechanotransduction channel Nanchung-Inactive. The only direct intracellular voltage-controlled recordings in locust auditory neurons failed to show any voltage activation of Nanchung-Inactive (Warren and Matheson, 2018), casting doubts on the electrical propagation role predicted by the NompC model, at least in morphologically similar orthopteran auditory receptors. Other recent work on age-related hearing decline in *Drosophila* also adds to our understanding of the respective roles of NompC and Nanchung-Inactive. When the transcription factor, Onecut, involved in sensory organ development and maintenance, is knocked down auditory transduction is nearly completely lost—including the antennae's ability to mechanically amplify quiet sound (Keder et al., 2020). In Onecut knockdown, both, Nanchung and Inactive expression levels are decreased, but NompC expression levels are unchanged, suggesting that Nanchung and Inactive are more critical for transduction than NompC (Keder et al., 2020). NompC is also expressed and essential for the function of two other non-auditory mechanoreceptor types—bristle and campaniform—and when genetically knocked out results in a decrease of mechanotransduction (Kernan et al., 1994; Liang et al., 2010). However, transient knockdown of mechanotransduction channel candidates in cockroach, using RNAi, resulted in reduction of the bristle receptor response only for Nanchung and Inactive but not for NompC (Hennenfent et al., 2020). Thus, it appears that Nanchung-Inactive are, at least, drawing level with NompC as contenders for the auditory transduction channel in insects. NompC, no doubt, has a critical role in auditory transduction, especially to coupling forces to the cilium, but previous work on NompC has relied of germline genetic mutations of NompC, which makes it hard to discern between a developmental phenotype and a functional phenotype. The crucial experiment that will break the two contender deadlock are direct recordings of the transduction current (Warren and Matheson, 2018) with pore mutations of the channel candidates.

In insects, channel gating is determined by the relative stretch of the cellular membrane and microtubule cytoskeleton. However, the effective stimulus to open transduction channels in the cilium is largely speculative and based on the morphology of the cilium; is it pull along the ciliary axis, bending or tilting for instance? The two contenders for the insect auditory transduction channel, NompC and Nanchung-Inactive are positioned along opposing sides of a dilation in the sensory dendrite (**Figure 4B**). NompC is located at the ciliary tip above the dilation (Liang et al., 2011) and Nanchung-Inactive located at the proximal dendrite below the dilation (Kim et al., 2003). Although dynein is only located below the dilation it could be coupled to either prospective channel; longitudinally through the microtubule cytoskeleton, that passes through a ciliary dilation, for NompC or through a possible direct connection for Nanchung-Inactive (Field and Matheson, 1998) through structures termed microtubule integrated cones (Thurm et al., 1983). In either scheme stretch-activation of dynein is necessary for any dynein-based force production in cilia. It was suggested that pull along the axis of the cilium is the effective stimulus of all chordotonal organs (Field and Matheson, 1998; Todi et al., 2004). This is based on (i) the rigid channel or receptor lymph space maintained by the scolopale cell and its actin cytoskeleton,

(ii) connections of the cilium are commonly in line with the ciliary axis, and (iii) the cilium is long and slender. Recent work on bush crickets by Hummel et al. (2016), however, shows that tilt of the ciliary tip that is the most effective way to stimulate the chordotonal sensory cell. Here the phase delay of the traveling wave, where the tilt of the ciliary tip is maximal, leads to the largest neural response.

Nanometer displacement of hair cell stereocilia has been resolved through an ingenious projection of the shadow of a glass probe, attached to the stereocilia tips, onto a pair of photodiodes (Howard and Hudspeth, 1988). The nanometer displacement of the glass probe, due to stereocilia movement, can be calculated from the proportion of photons blocked on each adjacent photodiode. In insects it may be possible to optically interpret ciliary movements by projecting the cilium directly onto adjacent photosensors and measure their proportional activation. Such an approach seems impossible in flies, mosquitoes and one species of tree cricket where mechanical amplification has been proven (Göpfert and Robert, 2001, 2002; Mhatre and Robert, 2013) due to the inaccessible nature of their hearing organs. However, such an approach would be feasible in the locust's Müller's organ and perhaps other non-model insects. It could be argued, only cilia in insects known to provide mechanical amplification would be motile but the inability to detect mechanical amplification should not rule out the absence of ciliary movements. Dynein is present in the cilia of all chordotonal organ neurons so far examined. Thus, it has a role, active or otherwise, in chordotonal organ transduction. Until measured directly ciliary movements are purely speculative but we predict that the cilium would twist. This is because the dynein-tubulin connections follow a ring formation and forces could only be generated through relative movement between adjacent microtubule doublets. Other cilia with $9 \times 2 + 0$ arrangement rotate their free apical end clockwise (Nonaka et al., 1998). Imaging the auditory receptors themselves has provided a deeper and powerful understanding of mechanotransduction in hair cells that has accelerated understanding. We predict that such a breakthrough will have similar repercussions for insect auditory transduction.

As the insect labs push forward to find the channel the search for the mammalian transduction channel has gifted some key lessons. For instance, as many mammalian channel candidates fell to the road side, we must be open to the possibility that neither NompC nor Nancung-Inactive is the hearing channel for insects. For mammals, it is a channel complex as opposed to a single protein that is required for auditory transduction and there may well be different isoforms to account for different conductivities along the cochlea (Beurg et al., 2018). Further complicating matters is the redundancy of TMC channels; knockout of TMC1 results in TMC2 taking up its function (Asai et al., 2018). Thus, even if a knockout of a single gene has no effect it may still be the channel. Our forlorn hope is that the insect hearing channel is a simple one channel solution and that it is either NompC or Nanchung-Inactive but our sneaking suspicion is that it will not be so simple.

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Both authors developed the concept of the publication and wrote the publication.

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OPINION
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Strange Seal Sounds: Claps, Slaps, and Multimodal Pinniped Rhythms

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BEYOND VOCAL COMMUNICATION

Within mammalian bioacoustics, vocal communication has received much attention. Efforts to understand sound production often focus on sounds generated *via* apparatuses that specifically evolved to phonate, such as the larynx. However, while mammals mostly perceive sounds *via* one organ, the ear, they can produce sounds *via* limbs, tails, flippers, tools, and several other mechanisms which, at first, may not seem to have primarily evolved for sonation (Tyack and Miller, 2002; Frankel, 2009; Clark, 2016). For example, kangaroo rats drum their foot to communicate (Randall, 1984), while non-human primates drum using artificial tools (Remedios et al., 2009), resonant surfaces (Ravignani et al., 2013), and their hands (e.g., Dufour et al., 2015). Also aquatic mammals can produce a variety of non-vocal sounds (such as whistles, snorts, and others; Tyack and Miller, 2002). These sound production modes may enable communication even when laryngeal phonation is ineffective or impaired (Munoz and Blumstein, 2012; Partan, 2017). Research on sound production *beyond phonation* is key to properly characterise the richness of animal communication.

Recent exploratory work (Hocking et al., 2020) provides an example of non-vocal sound production in a pinniped: grey seals (*Halichoerus grypus*) clapping their fore flippers underwater, a signalling behaviour previously attributed to vocalising. While Hocking et al.'s observation is limited to few events, it is reminiscent of previous, seemingly unrelated work reporting water-slapping behaviour in other species, including a close relative, the harbour seal (*Phoca vitulina*) (Venables and Venables, 1957; Newby, 1973; Hanlan, 1998; Hayes et al., 2004; see also humpback whales: Dunlop et al., 2010). The preliminary data reported by Hocking et al. naturally invite a host of questions, whose answers rely on a characterisation of when, how often, and under what circumstances these claps occur. Are they a frequent or seasonal phenomenon? Are they modulated by social context? Furthermore, the mechanism of knock production should be considered: while clapping the fore flippers can generate loud knock-like sounds, other mechanisms have also been proposed (e.g., in walruses: teeth clacking, tongue movement, or suction; Sjare and Stirling, 1981; Sjare et al., 2003; Reichmuth et al., 2009; Larsen and Reichmuth, 2012). This is a good starting place for future research, though, clearly, more observations are required to answer these questions. In particular, the field should perform more empirical, foundational work. This should (1) provide robust observations and descriptions in addition to anecdotes, (2) evaluate context, timing and seasonality in the production of percussive sounds, (3) determine which sex produces these signals and potential sexually dimorphic characteristics, and (4) design rigorous experiments that test potential function of percussive sounds. Once these absolutely necessary foundations are established, we suggest exploring more complex topics related to bioenergetics, signal evolution, multimodality, and rhythm production/perception. In this Opinion piece, we discuss these more hypothetical research directions, which however can only be performed after more thorough biological descriptions of the basic phenomena.

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MECHANISMS FOR (DIS)HONEST SIGNALLING?

If replicated, this research may encourage to re-evaluate previous evidence. Could previously recorded "vocalisations [which] sounded like a loud piercing clap" (pg. 61, McCulloch, 2000) and "knocks" (pg. 2213, Asselin et al., 1993) in grey seals have been actual underwater claps? A hypothetical reassessment of the production mechanism underlying a sound would entail several implications. Awareness of the sound source could be helpful to test potential sound-body allometric links.

One testable hypothesis is that claps may be a partly dishonest signal as they give away limited information about body size while their source level is surprisingly high, especially compared to captive individuals (Wahlberg et al., 2002). This hypothesis dovetails with some empirical evidence of water claps functioning as aggressive and territorial behaviours in harbour seals (Hayes et al., 2004), since claps have been so far observed mostly in males and in presence of other seals (for example in Weddell seals: Russell et al., 2016). Furthermore, limited underwater visibility, as reported by Hocking et al. (2020), would promote a signalling strategy concealing body size. To test whether clapping is a dishonest signal, field studies should investigate the conditions under which underwater claps take place (e.g., water visibility, social context).

Conversely, claps could be honest signals, since the strength of the animal or the size of its flipper may determine the intensity of the clap and the perceived loudness. This second hypothesis would comply with allometric scaling (e.g. Garcia et al., 2017): if sound-producing structures scale with body size, honest signalling ensues (Garcia and Ravignani, 2020). Larger individuals should also be able to produce stronger or (visually) larger claps (Partan, 2013). Anatomical observations in, and tests of allometry across, individuals producing underwater claps might contribute to disentangling these contrasting hypotheses.

EXAPTATION AND REPURPOSING OF BIOMECHANICAL PROCESSES

This research underlines the process of evolutionary exaptations for communicative purposes (Gould and Lewontin, 1979; Buss et al., 1998). Seal clapping "may be a ritualised version of a swimming stroke" (pg. 1, Hocking et al., 2020). In other words, a movement evolved for essential in-water displacement (Fish, 2000; Kuhn and Frey, 2012) may have been repurposed for acoustic communication (see also Clark, 2016). As a parallel, the tree drumming of woodpeckers is now a purely communicative signal, probably repurposed from what originally was a simpler foraging behaviour (Dodenhoff et al., 2001; Miles et al., 2018, 2020; Garcia et al., 2020). Both seals and woodpeckers may provide fascinating examples of the evolution and repurposing of biomechanical processes. At what point in pinniped phylogeny may swimming strokes have been recruited for communication, and did this happen in multiple pinniped species? Communicative non-vocal sounds have been observed in several pinnipeds (Schusterman and Van Parijs, 2003; Russell et al., 2016[1]); more specifically, claps have been reported, to our knowledge, in grey seals (e.g., Hocking et al., 2020), harbour seals (Venables and Venables, 1957; Newby, 1973; Hanlan, 1998; Hayes et al., 2004), and walruses (e.g., Reichmuth et al., 2009). Underwater recordings of similar non-vocal sounds in other pinnipeds might contribute to answering this question. With limited evidence it is difficult and unwise to generalise to all pinnipeds. However, one may hypothesise that the swimming style of phocids, propelled by their hindflippers, may free up their foreflippers for communicative purposes, while the swimming style of otariids, using their foreflippers to "fly" underwater, may have hindered their exaptation for communication (Kuhn and Frey, 2012).

MULTIMODALITY AND ENERGETICS

Most work mentioned above advocates multimodal approaches to communication. Multimodality is sometimes neglected, with some research programs only focusing on one production and one perception channel (Slocombe et al., 2011). Grey seals exhibit a communicative behaviour that is motorically produced (bypassing specialised laryngeal neurons) and might be perceived acoustically at long ranges and visually at short ranges (Wahlberg et al., 2002; Ravignani et al., 2016; Hocking et al., 2020). As the larynx is hidden from sight, the act of mammalian vocalisation is invisible to the receiver (cf. Fitch and Reby, 2001; Higham and Hebets, 2013; Nowak, 2020). A clap, instead, could potentially reach the receiver visually, acoustically, or haptically, also allowing for multisensory integration.

Multimodality also entails energetic considerations. While laryngeal phonation is relatively cheap, other modes of controlled sound production may be more energy-expensive. Indeed, research on the energetic costs of communication generally assumes higher expenditure for multi-modal as compared to uni-modal interactions (Partan, 2013). For example, in sympatric wolf spiders, multi-modal displays (as in *Schizocosa ocreata*) require higher energy levels than unimodal displays (as in *S. Rovneri*) (Cady et al., 2011). Rather than being disadvantageous, such a costly display might serve as an honest signal indicating a male's good condition (Zahavi, 1975; Byers et al., 2010; Mitoyen et al., 2019). Yet, questions concerning the relative costs and benefits of pinniped sound production *via* clapping and slapping (Beier and Wartzok, 1979; Wahlberg et al., 2002; Gillooly and Ophir, 2010) remain open, as well as their function (e.g., territorial, reproductive, etc.; Russell et al., 2016). If they indeed relate to mating, one may expect a variation in clapping/slapping abilities due to ontogeny (Rado et al., 1991), an increase at puberty onset, a cyclical variation entrained with mating seasonality or a decline due to senescence (Soulsbury and

[1]We are here referring to jaw claps, which the authors originally grouped as vocalisations for methodological reasons.

Halsey, 2018). The reliable evidence of such seasonal and developmental trends in the vocal displays of seals (e.g., Van Parijs et al., 1999; Galimberti et al., 2008; Reichmuth and Schusterman, 2009) may invite comparisons with non-vocal displays and facilitate the understanding of their function. By combining quantitative techniques (e.g., Gillooly and Ophir, 2010), allometric considerations (Garcia and Ravignani, 2020), and field observations (Hocking et al., 2020), some of these questions may be addressed.

COMMUNICATIVE RHYTHMS IN THE MILLISECOND-SECOND RANGE

More observations on the clapping behaviour of grey seals, their characteristics, and context of use are needed: Are these sounds produced occasionally or routinely? Do they contain rhythmic components? The presence of rhythmic features in claps would allow to link Hocking et al.'s (2020) finding to research on communicative rhythms and could spur a subfield of ecologically-relevant percussive rhythms in mammals. Recently, cross-species evidence has shown rhythmic capacities, sometimes employed for communication, in pinnipeds (Cook et al., 2013; Rouse et al., 2016; Mathevon et al., 2017; Ravignani, 2019). "Rhythm" is not meant here in its circadian sense, studied for instance in ecology, but instead as "temporal structure" at short timescales (de Reus et al., 2020). When little information is encoded in the frequency domain, as in seals' claps and slaps (Wahlberg et al., 2002; Hocking et al., 2020), this temporal structure could emerge in sound signals and serve to encode information. Within animal cognition and behaviour, evidence for rhythm in pinnipeds is particularly interesting (Ravignani et al., 2016; Wilson and Cook, 2016). In fact, pinnipeds constitute a key taxon to test a cross-species hypothesis which links rhythm and vocal learning capacities (Patel, 2006). Still, work on rhythm in mammals is relatively limited, especially when compared to the richness of rhythm production research, for instance, in insects and frogs (Greenfield, 1994; Hartbauer and Römer, 2016). While these species can produce extremely fast rhythms, the rate and complexity of non-vocal rhythms in mammals may be hampered by the physical limitations occurring when moving a limb (but see, for example, Randall, 1984 on the foot drumming behaviour of kangaroo rats). Mammalogists and comparative psychologists may still be inspired and benefit from decades of work on rhythm and percussive behaviour in arthropods and anurans (Ravignani et al., 2014). In particular, since the 1960's, entomologists and then herpetologists have been measuring the communicative rhythms of their species with almost millisecond accuracy (e.g., Buck and Buck, 1968). Avian researchers followed, while mammalogists and primatologists are slightly lagging behind (de Reus et al., 2020). Applying concepts such as phase resetting and period correction, for instance, to communicative rhythms in apes could also inform the evolution of rhythmic capacities in our own species (cf. Bittman, 2020).

CONCLUSIONS AND FUTURE WORK

Hocking et al.'s (2020) finding, albeit preliminary, can inspire at least six hypothetical strands of future work. First, to establish a base from psychophysics, *propagation* experiments could test how far the sound of grey seals clapping carries underwater (Wahlberg et al., 2002); this strand of research would help disentangle the role of claps as either honest or dishonest signals, as the latter may be more relevant at short distances (e.g., Tyack and Miller, 2002). Second, *biomechanics* and metabolic work could pinpoint the energetic costs and evolutionary benefits of clapping (Fish, 2000; Kuhn and Frey, 2012); this research should consider the context in which a signalling behaviour occurs (e.g., occasional vs. prolonged use; environmental and social conditions). Third, a larger dataset (McCulloch, 2000) would allow onset-to-onset temporal measurements to investigate whether claps may feature putative *rhythmic structures*, linking either claps within a series (e.g., based on their inter-onset intervals) or repeated series performed in succession. After that, to test for homologies and analogies, comparative analyses could be attempted with water slaps in harbour seals and other pinniped percussive behaviour (Wahlberg et al., 2002). Fourth, it would be important to test how nearby conspecifics perceive claps. Therefore, connecting to recent work on pinniped timing (Heinrich et al., 2016, 2020), one could explore how grey seals *perceive temporal information* in sequences of claps. Fifth, one could target the proximate and ultimate *function of clapping*, and its potential role in sexual or natural selection. Sixth, methodological advances in neuroimaging techniques (e.g., Cook et al., 2021) may be employed to inform on the neural underpinnings of cross-modality and their interface with the physiological and physical constraints imposed on flippers by their original function (i.e., swimming). All this work, we stress, can only come once more fundamental research is performed to tackle basic biological questions. For the time being, we will keep looking for more percussive performances by these fascinating mammals.

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AR and LV developed the arguments and co-wrote the paper. All authors contributed to the article and approved the submitted version.

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Functional Analyses of Peripheral Auditory System Adaptations for Echolocation in Air vs. Water

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The similarity of acoustic tasks performed by odontocete (toothed whale) and microchiropteran (insectivorous bat) biosonar suggests they may have common ultrasonic signal reception and processing mechanisms. However, there are also significant media and prey dependent differences, notably speed of sound and wavelengths in air vs. water, that may be reflected in adaptations in their auditory systems and peak spectra of out-going signals for similarly sized prey. We examined the anatomy of the peripheral auditory system of two species of FM bat (big brown bat *Eptesicus fuscus;* Japanese house bat *Pipistrellus abramus*) and two toothed whales (harbor porpoise *Phocoena phocoena;* bottlenose dolphin *Tursiops truncatus*) using ultra high resolution (11–100 micron) isotropic voxel computed tomography (helical and microCT). Significant differences were found for oval and round window location, cochlear length, basilar membrane gradients, neural distributions, cochlear spiral morphometry and curvature, and basilar membrane suspension distributions. Length correlates with body mass, not hearing ranges. High and low frequency hearing range cut-offs correlate with basilar membrane thickness/width ratios and the cochlear radius of curvature. These features are predictive of high and low frequency hearing limits in all ears examined. The ears of the harbor porpoise, the highest frequency echolocator in the study, had significantly greater stiffness, higher basal basilar membrane ratios, and bilateral bony support for 60% of the basilar membrane length. The porpoise's basilar membrane includes a "foveal" region with "stretched" frequency representation and relatively constant membrane thickness/width ratio values similar to those reported for some bat species. Both species of bats and the harbor porpoise displayed unusual stapedial input locations and low ratios of cochlear radii, specializations that may enhance higher ultrasonic frequency signal resolution and deter low frequency cochlear propagation.

Keywords: biosonar, cochlea, basilar membrane, stapes, inner ear, echolocation, bat, dolphin

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INTRODUCTION

The adaptive importance of detecting sound cues is underscored by the universality of "hearing." There are lightless habitats on earth with naturally blind animals, but no terrestrial habitat is without sound, and no known vertebrate is naturally profoundly deaf. Mechanistically, hearing is conceptually a relatively simple chain of events: sound energy is received and converted by biomechanical transducers (middle and/or inner ear) into electrical signals (neural impulses) that provide a central processor (brain) with acoustic data. The complexity of these structures varies considerably by taxa, from relatively simple acoustic pressure detectors to the typical mammalian ear which packs over 75,000 mechanical and electrochemical components into an average volume of 1 cm^3. The focus of this paper is on comparisons of ears of two mammalian groups, microchiropteran bats and odontocete cetaceans, both of which are echolocators.

Inner ear anatomy is similar across all mammals. There is a tri-chambered spiral cochlear labyrinth with a major partition, the basilar membrane, which functions as a tonotopic resonator and that supports the organ of Corti. Hair cells and supporting cells in the organ of Corti are the primary transducers of acoustic energy into neural impulses and which also control intracochlear afferent and efferent responses. Variations in the structure and number of these ear components account for most of the differences in hearing capacity among mammals (Echteler et al., 1994; Ekdale, 2016). In particular, basilar membrane dimensions, membrane support structures, cochlear spiral configurations, and neural densities and distributions have been proposed as critical determinants of hearing range and sensitivity (von Békésy, 1960; West, 1985; Greenwood, 1990; Heffner and Heffner, 1992). Further analyses of these variations also led to the designations of "generalist" and "specialist" ears (Fay, 1988; Echteler et al., 1994), the latter referring primarily to differences in the structure of the basilar membrane that affect stiffness and mass and therefore frequency encoding.

During the explosive period of mammalian radiation, two orders, Chiroptera (bats) and Cetacea (whales and dolphins), emerged with a wide range of highly evolved adaptations for arboreal and aquatic habitats, respectively, including hearing in radically different media. Two subdivisions of these orders, the suborder Microchiroptera (largely insectivorous microbats) and parvorder Odontoceti (toothed whales, dolphins, and porpoises), further evolved into echolocators with sophisticated biosonar systems for the production and analysis of ultrasonic signals and the returning echoes. For an echolocator, the key element is not simply the ability to hear and discriminate ultrasonic signals but rather the ability to produce an explicit signal that is tied to the objects of interest, either prey or obstacles, and to analyze returning echoes to decipher the presence, direction, and speed of targets of interest.

While we can find in some fossil specimens anatomical indicators of inner ears that were tuned to ultrasonic signals, we cannot be certain at what point in time the ability to echolocate occurred in any mammal. These changes in the skulls of bats and odontocetes occurred gradually and on different timelines. Bats were fully arboreal in the Eocene (56-34 MYA), whereas cetacean fossil skulls do not display clear evidence of telescoping until the Miocene (23-5 MYA) (Barnes et al., 1985). The emergence of exaggerated, complex pinnae and narial specializations such as nose leaves in bats and cranial alterations in dolphins are features in bat and dolphin evolution consistent with the onset of echolocation. For laryngeal echolocating microbats, a distinguishing characteristic is the unusual placement of the stylohyal bone connecting to the tympanic ring (Veselka et al., 2010). In toothed whales, there was a dramatic remodeling of the skull, termed "telescoping," referring to changes that relate to both life in water and the production and reception of underwater signals for echolocation. These include the migration of the narial bones dorsally to produce a "blowhole" for respiration, displacement of the frontal bones posteriorly, and elongation of the maxillae and mandibles, providing a hollow or scooped platform accommodating, in modern odontocetes, the fatty "melon" through which odontocetes emit outgoing echolocation signals.

For both groups, one driving force for biosonar may have been the absence of light. Microchiropteran bats are largely nocturnal, insectivorous predators. Odontocete cetaceans prey on fish, invertebrates, and aquatic mammals. They typically forage in daylight hours but hunt in deep or murky waters and therefore operate in essentially crepuscular conditions at best. Some species, such as the beaked whales, are capable of foraging as deep as 2,000 m with dives lasting over 2 h in lightless regions of the ocean (Tyack et al., 2006; Baird et al., 2008). Thus, while the primary target prey are quite different in size and behavior for bats and dolphins, and they operate in radically different habitats, they do share some environmental pressures that may have resulted in parallel evolution of echolocation, resulting in sophisticated biosonar systems and evident similarities in their ability to produce, detect, and analyze ultrasonic signals.

Because of the similarity of tasks and information that odontocetes and microchiropteran bats obtain acoustically from their environments, we expect that there are some commonalities in their auditory reception and processing mechanisms as well as differences related to alternative echolocation strategies and especially to media dependent elements reflected in the structure of their ears. These differences are manifested in differences in the structure and peak spectra of their echolocation signals, which in turn likely reflect wavelength and speed of sound in each medium, habitat and prey parameters, and spectral features of prime targets, all of which evolutionarily shaped hearing abilities. Further, there are niche and task dependent signal elements (Siemers and Schnitzler, 2004) and anatomical variations common to frequency-modulated (FM, short FM sweeps) and constant-frequency (CF-FM, long duration constant frequency tones followed by short FM sweeps) bats and mid vs. ultrahigh ultrasonic frequency odontocete ears (Pye, 1966; Ketten and Wartzok, 1990; Wartzok and Ketten, 1999; Fenton et al., 2012; Southall et al., 2019) that dictate critical feature extraction of echoes in air vs. water. Although there has been extensive research on the comparative anatomy of

mammalian ears, we still lack a precise understanding of how multiple anatomical variations observed across species affect hearing abilities.

The objective of the present study is to understand the similarities and differences of dolphin and bat inner ear morphometry related to the issues detailed above. Preliminary results from a smaller data set were published previously as an extended abstract in conference proceedings (Ketten et al., 2012). This paper provides the data for the first major stage in a research project focusing on similarities and differences of cochlear architecture and the implications for ultrasonic encoding and acuity amongst these groups. The primary goal is to put that data into a functional and comparative context. The key issues addressed are: (1) how do bat and dolphin ears differ from other terrestrial ears; (2) how do these differences correlate with air vs. underwater sound perception; and (3) what do the findings imply about the parallel evolution of adaptations for biosonar.

SOUND IN AIR VS. WATER

In analyzing air vs. water borne sound adapted hearing, it is important to consider how the physical aspects of sound in each medium relates to acoustic cues. The following section summarizes key variables and their effect on measures of sound in air and water. For a comprehensive discussion see Urick (1983) and Rossing and Fletcher (2004).

In elastic media like air and water, "sound" is a disturbance that takes the form of acoustic waves. Basic measures of sound are speed, frequency, wavelength, and intensity. Because water is denser than air, sound in water travels faster and with less attenuation than sound in air. Sound speed in moist ambient surface air is approximately 340 m/s. Sound speed in sea water averages 1,530 m/s but will vary with any factor affecting density, such as salinity, temperature, and pressure. For each 1% increase in salinity, speed increases 1.5 m/s, for each 1°C decrease in temperature, 4 m/s, and for each 100 m depth, 1.8 m/s (Ingmanson and Wallace, 1973). Because these factors act synergistically, any marine, estuarine, or freshwater habitat has a variable sound profile that may change seasonally and with depth. For practical purposes, given in water sound speed is 4.5 times faster, and because frequency, measured in cycles/s or Hertz (Hz), is defined as the speed of sound (m/s) divided by the wavelength (m/cycle), the wavelength for any given frequency is 4.5 times greater than in air.

Concerning measures of hearing, intensity is a key feature, and its measures are dependent upon sound speed and arbitrary sound reference pressure. Sound intensity (I) is the acoustic power (P) impinging on a surface perpendicular to the direction of sound propagation, or power/unit area (I = P/a). In general terms, power is force times velocity (P = Fv). Pressure is force/unit area (p = F/a). Therefore, intensity can be rewritten as the product of sound pressure (p) and vibration velocity (v):

$$I = P/a = Fv/a = pv \quad (1)$$

For a traveling spherical wave, the velocity component becomes particle velocity (u), which can be defined in terms of effective sound pressure (p), the speed of sound in that medium (c), and the density of the medium (ρ):

$$u(x, t) = p/\rho c \quad (2)$$

We can then redefine intensity (2) for an instantaneous sound pressure for an outward traveling plane wave in terms of pressure, sound speed, and density (3):

$$I = pv = p(p/\rho c) = p^2/\rho c \quad (3)$$

The product ρc is the characteristic impedance of the medium. For air c = 340 m/s and for sea water c = 1,530 m/s. For air, ρ = 1.29 kg/m^3 = 0.0013 g/cm^3; for sea water, density varies with temperature, salinity, and depth but on average, ρ = 1,032 kg/m^3 = 1.03 g/cm^3. The following calculations show how these physical property differences for air vs. water influence intensity and sound pressure values:

$$I_{air} = p^2/(0.442\ g - m/s - cm^3) \quad (4)$$

$$I_{water} = p^2/(1575.9\ g - m/s - cm^3) \quad (5)$$

For a mammal to have an equivalent threshold in air and water requires the same acoustic power/unit area ($I_{air} = I_{water}$):

$$I_{air} = p_{air}{}^2/(0.442\ g - m/s - cm^3) = p_{water}{}^2/(1575.9\ g - m/s - cm^3) = I_{water}$$

$$p_{air}{}^2(3565.4) = p_{water}{}^2$$

$$p_{air}(59.7) = p_{water}$$

Therefore, the sound pressure in water must be ~60 times that required in air to produce the same threshold response at the ear.

Because intensity (W/m^2) is difficult to measure, most studies of hearing thresholds rely on measures of sound pressure level (SPL) (see Au, 1993 for discussion). Sound pressure levels are expressed in decibels (dB) and are defined as:

$$dB\ SPL = 10 \log (p_m{}^2/p_r{}^2) = 20 \log (p_m/p_r) \quad (6)$$

where p_m is the pressure measured and p_r is an arbitrary reference pressure. However, there are different standardized reference pressures for SPL in air and water. For air-borne sound measures, the reference pressure is re 20 μPa. For underwater sound measures, the reference pressure is 1 μPa.

Consequently, for an ear with the same sound intensity threshold in air and water, the underwater sound pressure level would need to be 35.5 dB + 20 (log 20) dB greater than the airborne value. That is, a sound level measured as 61.5 dB re 1 μPa in water is equivalent to a sound measured as being 0 dB re 20 μPa in air.

These equations describe idealized and controlled measures of air and water borne sound. In comparing behavioral data from different species, particularly in comparing airborne and marine sound for mammalian hearing data, differences in experimental conditions are extremely important. We have no underwater equivalent of anechoic chambers, thus there are unavoidable ambient noise effects even in captive aquatic test conditions. In

addition, data for marine mammals are often available from very few individuals for which there may be no life history or prior hearing data and under test conditions that are highly variable particularly for studies on wild stranded animals. By combining research results from behavioral studies with biomechanical and anatomical studies, we obtain a more comprehensive picture of what and how each species hears and particularly how they hear in their respective habitats.

MATERIALS AND METHODS

The ears from four species, two FM bats [the big brown bat *Eptesicus fuscus* (n = 6) and the Japanese house bat *Pipistrellus abramus* (n = 1)] and two odontocetes [the harbor porpoise *Phocoena phocoena* (n = 6) and the bottlenose dolphin *Tursiops truncatus* (n = 10)] were analyzed for this study (**Table 1**). Ears were examined using submillimeter imaging with two radiographic techniques, conventional helical computed tomography (CT) and microCT scanning using two analytical, fixed anode, rotating specimen scanners. All scans were performed on post mortem specimens of intact heads or extracted temporal bones.

Specimens

The dolphin and porpoise heads and ears were obtained postmortem from male and female adult stranded animals under letters of authorization and USFW/NMFS permits (932-1489-08, 493-1848-00, 493-1848-02, 130062, and 130062-1) issued to DK. The specimens selected for study were relatively fresh material (postmortem condition designation Code 1 or 2) collected 1–24 h post mortem and with no evident auditory system pathology, such as intracochlear blood, evidence of torn or absent inner ear membranes or other cochlear partitions, necrotic middle ear mucosa, disarticulations of the ossicles, degenerate or absent auditory nerve, based on gross anatomical and CT examinations. The tissues were held chilled at 4°C until scanning. In the case of whole head specimens, post scanning, one or both temporal bones were extracted from each specimen, fixed in formalin by immersion and low pressure injection of formalin through the internal auditory canal and/or round window, and rescanned after 2 weeks or more to visualize any alterations in fixed compared to fresh tissue. Whole ears collected at the stranding site were held chilled and scanned the day of extraction, then processed as described above. Selected ears from these specimens were decalcified in EDTA and processed for transmission electron microscopy (TEM) or embedded in celloidin, stained with hematoxylin and eosin (H&E) or osmium tetroxide, and sectioned at 20 microns (for processing protocols see Schuknecht, 1953, 1993).

Adult big brown bats (four females, two males) were captured from attics and barns under permits issued by the State of Rhode Island, United States to JS. Because these animals were wild caught, the ages are unknown. The bats were housed in groups in the Brown University laboratory. All bats were in good health and echolocated normally during exercise and training. They were euthanized by intraperitoneal injection of Beuthanasia solution (0.03 ml). Heads were fixed in 4% paraformaldehyde and scanned in this solution. One additional bat was perfused with 0.9% saline followed by 4% paraformaldehyde. The head was placed in a decalcifying solution, embedded in paraffin, sectioned in the coronal plane at 5 μm thickness on a cryostat, and stained with trichrome. Use of animals was approved by the Brown University Institutional Animal Care and Use Committee and are consistent with United States federal regulations.

Japanese house bats were captured from a large colony living on bridge girders in Kyotanabe, Japan. They were brought to the laboratory and euthanized by intraperitoneal injection of sodium pentobarbital (65 mg/kg). Bat heads and extracted ears were fixed in 4% paraformaldehyde and scanned in this solution. Capture and use of these bats were approved by the Doshisha University Animal Experiment Committee and are consistent with Japanese law.

Head and Ear Imaging

Heads and ears of all specimens were examined first using a Siemens Volume Zoom at the Woods Hole Oceanographic Institution Computerized Scanning and Imaging Facility[1]. The specimens were scanned using an imaging protocol of 0.5 mm acquisitions, 0.5 mm table speed. KV and effective mAS varied according to the mass of tissue being imaged. Data were acquired with an ultra-high resolution (U90 and U95 head) kernel, 200 FOV for whole heads. All helical CT images were produced with isotropic 100 micron voxels. Bone and soft tissue windows at standard and extended scales (see section "Middle Ear") were used for image reconstructions. All data and images were archived as both raw acquisition data and DICOM formatted image data files. Primary images were formatted at 0.1 mm slice thickness in the transaxial plane. Raw acquisition data were employed for imaging at smaller fields of view and for multiplanar reconstructions in sagittal and coronal planes and to digitally realign the slice plane to match a mid-modiolar cochlear axis.

[1]http://csi.whoi.edu

TABLE 1 | Study specimens.

Species	Common name	Ear specimens	Weight range (kg)	Average cochlear length (mm)	Standard deviation	Peak echolocation frequency (kHz)
Phocoena phocoena	Harbor porpoise	6	55–78	25.6	1.42	100–110
Tursiops truncatus	Bottlenose dolphin	10	150–250	37.3	2.78	40–70
Eptesicus fuscus	Big brown bat	6	0.014–0.021	8.7	0.48	35–45
Pipistrellus abramus	Japanese house bat	1	0.005	6.8	–	43–52

For extracted ears, the same parameters were employed with images acquired at a 50 FOV. Each ear was scanned in a position approximating an *in situ* prone, anterior first position for the axial; i.e., short axis, cross-sectional slice images. This orientation typically gives the best initial approximation of a mid-modiolar cochlear projection.

MicroCT studies were performed on bat heads and extracted dolphin ears. Data were obtained first on an X-Tek MicroCT at the Harvard University Center for Nano Systems. For these studies, depending upon the dimensions and mass of the tissues, a Molybdenum or Tungsten anode was used with varying parameters for voltage and exposure times. The X-Tek uses a fixed head with a rotating specimen plate. For each study, 2,000–4,000 radial projection data were obtained and reformatted using VGStudio Max 2.0 into DICOM format into transaxial contiguous sections with an isotropic voxel of 11–40 microns. Additional data were obtained for bat specimens using a Zeiss Xradia Versa 520 at the Micro-CT and X-ray Microscopy Imaging Facility of Boston University. These data were acquired at 7–100 micron isotropic voxel resolutions and formatted by Zeiss platform software as DICOM images.

All image sets were further processed and reconstructed into 3D still and video images using Siemens proprietary VRT software, Amira 5.4, VG Studio Max 3.4, RadiAnt version 2020.1.1, software programs on 64-bit PC and Mac platforms.

Cochlear Morphometrics

Cochlear canal midpoints and basilar membrane paths were identified based on membrane visualizations or, in their absence, on laminar positions from CT images for both the odontocete and microchiropteran ears to obtain Cartesian triplets (X, Y, Z) for three-dimensional (3-D) mapping, measurement, and reconstruction of the cochlear canal and basilar membrane path. Up to 30 mid-canal or membrane midline triplets, from the hook region (a recurved section at the most basal portion of the cochlear canal) to the helicotrema (the U-shaped section at the apex of the cochlear canal that connects scala media and scala tympani), were used to map each cochlea and measure spiral parameters (modiolar height and radii at each turn). For the odontocete specimens, measurements of the radii and of basilar membrane dimensions were obtained from mid-modiolar histology sections and by reslicing digitally 3-D reconstructions of the cochlea to produce radial slices along the spiral path. Parallel measures were made of the spiral from registered histology sets for the two odontocete species. These measurements were used to calculate cochlear and basilar membrane lengths with calculations based on the spiral parameters using the procedures and formulae described in detail in Ketten et al. (1998). These results were compared with cochlear length values obtained by measurement tools in the Amira software program. Basilar membrane thickness and width were obtained from specimens processed for transmission electron microscopy (TEM) (n = 1 *T. truncatus* ear) and from histology sections stained with hematoxylin and eosin (H&E) (n = 3 *P. phocoena*, 5 *T. truncatus* ears), with osmium tetroxide (n = 1 *P. phocoena* ear), and with trichrome (n = 1 *E. fuscus* ear). Ganglion cell counts and mapping were obtained from specimens in this study and from published data in prior studies as indicated in **Table 2**.

RESULTS

Because of the stringent criteria for collection, postmortem condition, and checks on quality of tissues, particularly in the case of odontocete specimens, processing and analyses from the specimens in this study were completed over more than a decade. Some data on a few specimens have therefore been published previously, specifically those listed for ganglion cell counts (**Table 2**) and basilar membrane thickness and width (**Table 3** non-echolocating species). New data presented in this paper are found in **Table 1** for cochlear length averages and in **Table 3** for membrane and cochlear ratios in the species in bold. Additional new, important findings reported here are on variations in stapedial input and cochlear radii ratios and their functional significance.

Auditory Bullae

While the tympanic and periotic bullae of the microchiropteran specimens analyzed are large in comparison to the total skull volume (see **Figure 1** and **Supplementary Video 1**), there are few differences in the actual bony structure, placement, and orientation compared to most mammals. The tympanic and periotic bullae in the bat are bulbous and are fused to the cranium. The periotic is positioned such that the apex of the cochlea points anteriorly with a slight ventral rotation (**Figures 1A,B**). This is a common orientation for land mammal inner ears. There is

TABLE 2 | Auditory and vestibular nerve densities.

Species	Common name	Membrane length (mm)	Auditory ganglion cells	Density (cells/mm cochlea)	Vestibular ganglion cells
Phocoena phocoena	**Harbor porpoise**	**25.93**	**70,137**	**3117.20**	**3,200**
Tursiops truncatus	**Bottlenose dolphin**	**40.65**	**96,716**	**2486.27**	**3,489**
Rhinolophus ferrumequinum	Horseshoe bat	16.1	15,953	991/1,750*	
Pteronotus parnellii	Mustached bat	14.0	12,800	900/1,900*	
Homo sapiens	Human	32.1	30,500	950	15,590

*Ganglion cell count data were compiled from this study (species in bold) and from previously published data by Bruns and Schmieszek (1980), Nadol (1988), Echteler et al. (1994), Gao and Zhou (1995), and Kössl and Vater (1995). *Densities at auditory fovea as described by Bruns and Schmieszek (1980). Ganglion cell counts for Phocoena and Tursiops are from histologies of the same specimens listed in **Table 3**.*

TABLE 3 | Cochlear morphometry of high and low frequency adapted cetacean and terrestrial mammals.

Species	Common name	Total frequency range (kHz)	Turns	Basilar membrane length (mm)	Basal T/W (μm)	Apical T/W (μm)	Basal ratio (t/w)	Apical ratio (t/w)	Radii ratios
Phocoena phocoena	**Harbor porpoise**	**0.35–180**	**1.5**	**25.93**	**25/30**	**5/290**	**0.833**	**0.0172**	**3.62**
Tursiops truncatus	**Bottlenose dolphin**	**0.2–160**	**2.25**	**40.65**	**25/35**	**5/380**	**0.714**	**0.0132**	**4.39**
Balaenoptera acutorostrata	Minke whale	0.02–30	2.25	50.6	11/130	3/920	0.085	0.00326	7.17
Balaenoptera musculus	Blue whale	0.01–18	2.25	71.0	7/120	<2/2,200	0.058	0.0009	10.45
Elephas maximus	Asian Elephant	<0.20–5.7	2.25	60.0	–/–	–/–	–	–	8.7
Felis domesticus	Cat	0.125–60	3.0	25.8	12/80	5/420	0.150	0.0119	5.71
Mus musculus	Mouse	5–60	2.0	6.8	15/40	1/160	0.363	0.0063	4.0
Rattus norvegicus	Rat	1–59	2.2	10.7	18/80	2/250	0.300	0.0106	4.3
Rhinolophus ferrumequinum	Horseshoe bat	7–90	3.25	16.1	35/80	2/150	0.438	0.0133	–
Eptesicus fuscus*	**Big brown bat**	**10–100**	**2.25**	**8.7**	**21/100**	**4/147**	**0.21**	**0.0272**	**3.4**
Pipistrellus abramus	**Japanese house bat**	**4–80**	**2.5**	**6.8**					**3.1**

Data in this table were obtained from specimens in this study (in bold) and from data published previously by Bruns and Schmieszek (1980), West (1985), Ketten and Wartzok (1990), Echteler et al. (1994), and Ketten (2000). Values for turns, radii ratios, and basilar membrane lengths were obtained from 3D reconstructions from CT scans and histology. Thickness and width of the basilar membrane (T/W) were measured by light microscopy from cochlear H&E histology sections for one bottlenose dolphin, one harbor porpoise, and one bat. Therefore, membrane lengths differ from average lengths in* ***Table 1****. Hearing ranges are based on audiometric or electrophysiological data (Wartzok and Ketten, 1999; Surlykke and Moss, 2000; Boku et al., 2015; Southall et al., 2019) where available. Frequency ranges for the blue whale are based on vocalization data and for the minke whale, on vocalizations and FEM and cochlear frequency map models (Ketten and Mountain, 2011; Tubelli et al., 2012).*

**Data for basilar membrane dimensions for P. phocoena and T. truncatus specimens in this study were taken from radial sections located at 5–7% of cochlear length for the basal values and 98–100% for the apical values. These locations are consistent with locations for the remaining species except E. fuscus. E. fuscus data were taken from a paramodiolar section with basal values at a point approximately 20% of and 80% of length for the apex. The E. fuscus data are preliminary pending a full cochlear membrane morphometry map.*

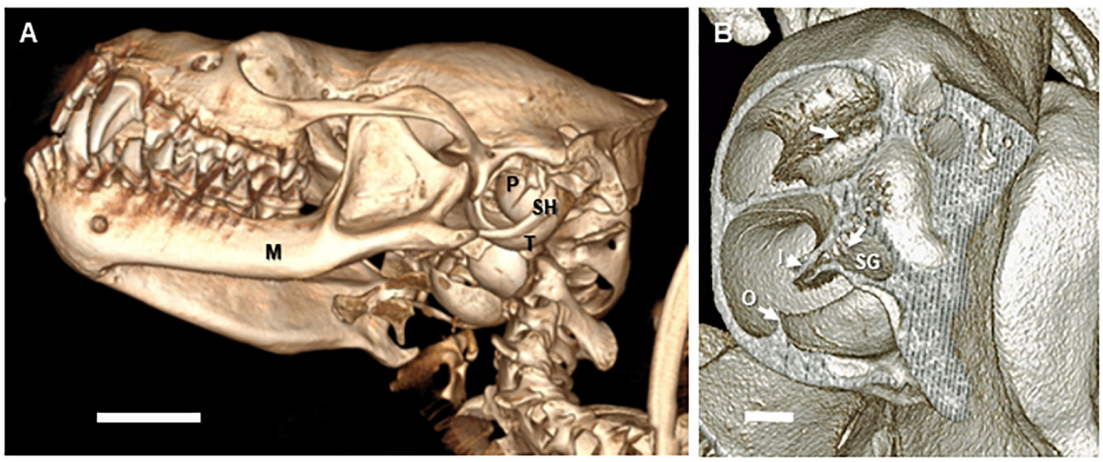

FIGURE 1 | Three-dimensional reconstructions of microCT images of the skull and auditory bullae of *Eptesicus fuscus* (big brown bat). The images are reconstructed from microCT data obtained at 11–17 micron voxel resolutions. **(A)** Left lateral view of skull of an adult female *E. fuscus* head. The manubrium of the malleus is visible inside the tympanic ring. M, mandible; P, left periotic bulla; SH, stylo-hyoid; T, tympanic bulla. Scale bar = 2.5 mm. **(B)** Dorso-lateral view of left cochlea in the same specimen. The wall of the periotic was removed digitally to reveal the mid to upper basal turns and laminae. Note the regular distribution of the foraminae of the habenula perforata (arrows) through which the afferent and efferent auditory (VIIIth) nerve fibers traverse the basilar membrane. I, inner osseous lamina; O, outer osseous laminae; SG, spiral ganglion (Rosenthal's canal). Scale bar = 0.1 mm. Images copyright 2020 DK, all rights reserved.

also in *E. fuscus* a well-developed, bony stylohyal flange that connects directly to the latero-posterior wall of the tympanic bulla (**Figure 1A**), consistent with bats that generate echolocation signals via the larynx (Veselka et al., 2010).

By contrast, the tympanic and periotic in the odontocetes in this study differ from the bat anatomy in location, orientation, and degree of attachment to the skull. The odontocete tympanic and periotic are connected to each other, forming a tympano-periotic complex, but are not fused to the skull (**Figures 2A,B**). The periotic is attached at its posterior margin to the tympanic (**Figures 2B,C**). The periotic which houses the cochlea and vestibular system is composed of exceptionally dense compact bone. The tympanic is hollow and distinctly cone shaped with a broad, thickened posterior and thin, friable body. This tympano-periotic complex is extra-cranial, suspended by ligaments in the peribullar fossa, ventral and posterior to the extended flange of the squamosal bone and just medial to the posterior edge of the mandibular ramus. The stylohyal bone

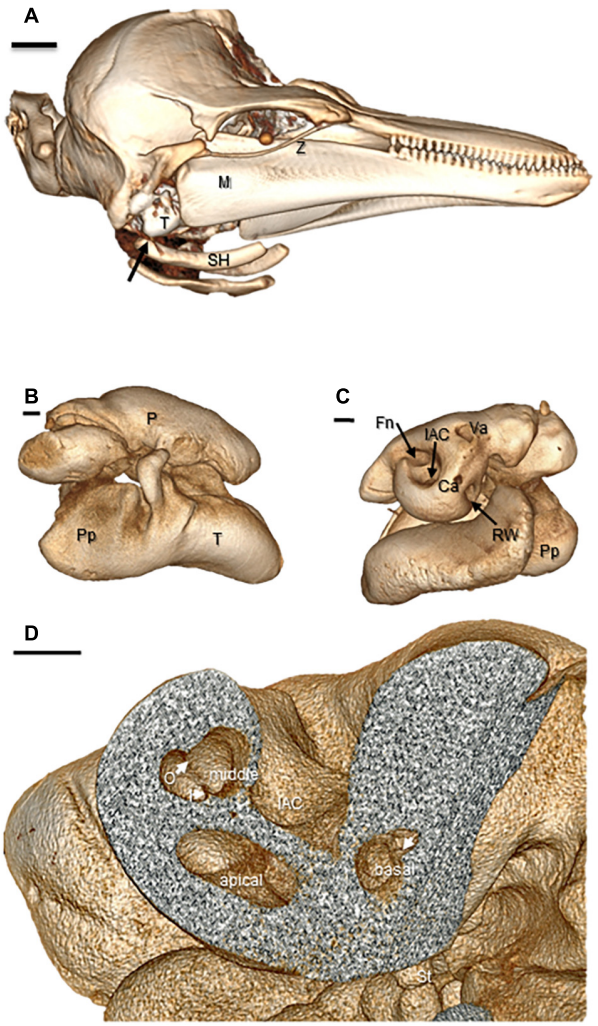

FIGURE 2 | Three-dimensional reconstructions of CT images of the entire head and auditory bullae of an adult male *Phocoena phocoena (harbor porpoise*). Ca, cochlear aqueduct; Fn, facial (VIIth) nerve canal; IAC, internal auditory canal of the acousto-vestibular (VIIIth) nerve; I, inner osseous lamina; M, mandible; O, outer osseous laminae; Pp, tympanic posterior prominence; P, periotic bulla; RW, round window; SH, stylohyal bone; St, stapes head; T, tympanic bulla; Va, vestibular aqueduct; Z, zygomatic (Images copyright© 2020 DK, all rights reserved). **(A)** Whole head image reconstructed from helical CT scan data with 1 mm slice acquisitions. Right lateral view of head shows the tympano-periotic complex in the peribullar fossa posterior to the mandible and associated stylohyal structures. Scale bar = 2.0 cm. **(B)** Lateral view of right tympano-periotic bullae images obtained from microCT with 500–1,500 projections and 33 micron voxel resolution. Scale bar = 2.0 mm. **(C)** Medial views of right ear tympano-periotic complex. Scale bar = 2.0 mm. **(D)** Cut-away of the periotic showing cochlear canal cross sections of the basal, middle, and apical turns with differences in spiral laminae separations (arrows) that reflect basilar membrane widths at each level. Scale bar = 2.0 mm.

(also referred to as stylo-hyoid) of odontocetes is well-developed but is connected to the tympanic typically by only a small ligament which attaches to a cartilaginous cap on the outer posterior prominence of the tympanic bone (**Figure 2A**). This suggests there is little or no transmission of laryngeal sound via the stylohyal bone in toothed whales and is consistent with ultrasonic signals generated via narial passages with "phonic lips" and nasal sacs; which are not found in baleen whales (Reidenberg and Laitman, 2018).

The whole complex is rotated medially 15–20°. The cochlear spiral within the periotic is oriented with the apex directed ventrally (**Figure 2C**). The acousto-vestibular (VIIIth) nerve projects inward from medial surface of the periotic, crossing the retro-peribullar space, to enter the temporal bone of the skulls; i.e., it is not enclosed in a bony internal auditory canal although it is encased in a heavy fibrous sheath. Species-specific variations in some of these features among odontocetes and particularly in comparison to the bullar and cochlear anatomies of mysticete (baleen) whales have been described in prior studies (see Reysenbach de Haan, 1956; Norris, 1969; Oelschlager, 1986; Ketten, 1992; Echteler et al., 1994; Nummela, 1995; Fordyce and de Muizon, 2001; Yamato et al., 2012).

Middle Ear

Microchiropteran bats and odontocetes have similar features in their middle ears that enhance stiffness, including dense calcified middle ear ligaments, struts, and stiffer annular ligaments than most mammals. A new, notable feature of middle ears in both bat and odontocete specimens found in this study is that microchiropteran and odontocete ossicles, despite radical differences in size, have similar, exceptionally high Hounsfield values (HU) ranging 1,500–4,800. HU, named after the primary inventor of computed tomography, are dimensionless units that represent the summated relative attenuations at each detector for the multiple radiation beams transmitted in each transit of the radiation source. HU's are a representation of the measured attenuation coefficients of tissues or objects detected normalized to the density of air (−1,000) and water (0). The HU upper bound depends upon the scanning protocol and machine software. Standard clinical ranges are −1,000 to +3,071, and most animal tissues do not exceed +2,000 HU. Some systems are able to use "extended scales" developed primarily for imaging metallic implants, which provide HU values up to +44,000.

The ossicles and periotic capsules of the ears examined in this study commonly ranged over 3,000 HU compared to maxima of 1,000–1,200 for these structures in humans and most other mammals. HU are not a direct measure of density but they are interrelated, and these high HU values are consistent with exceptionally dense, stiff ossicular bones. HU values also indicate the tensor tympani is partially calcified, which was confirmed on histology. The stapedial muscle is disproportionately large compared to humans and cats, and the tympanic membrane and annular ligament are thick and relatively stiff; i.e., resisting manual movement of the stapes. This is consistent with nanoindentation studies (Miller et al., 2006) that showed *T. truncatus* and one bat species, *Rhinolophus ferrumequinum*, the horseshoe bat, to have acoustic stiffness values of $\sim 10^{17}$ Pa/m^3, which was two orders of magnitude greater than the majority of all other species in their study. Further, in both bat species in this study, there is a well-developed band of fibrous tissue, analogous to the stylo-hyoid ligaments

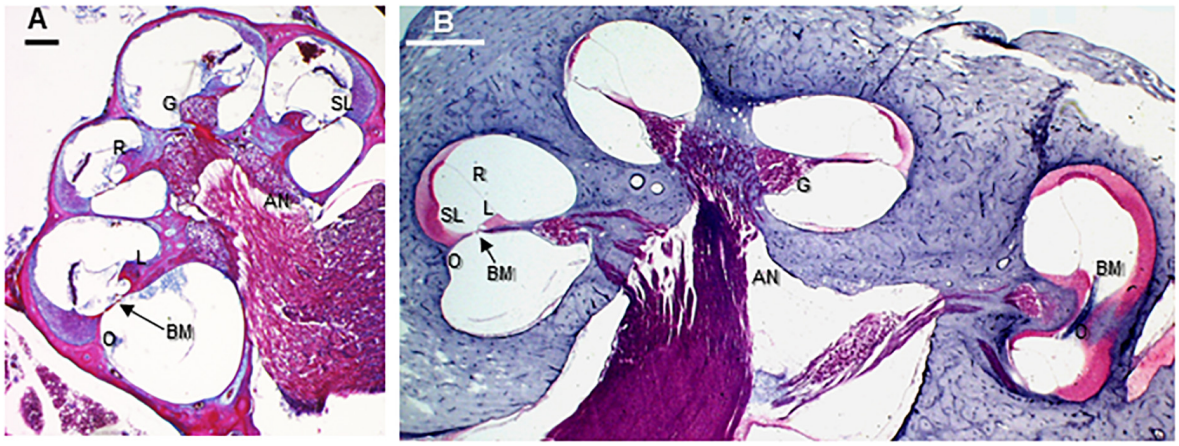

FIGURE 3 | Paramodiolar sections showing basal, middle, and apical turns in the big brown bat (*Eptesicus fuscus*) and harbor porpoise (*Phocoena phocoena*). AN, auditory nerve; BM, basilar membrane; G, ganglion cells; L, spiral limbus; O, outer spiral lamina; R, Reissner's membrane; SL, spiral ligament. Scale bars = 0.1 mm. **(A)** Big brown bat (*E. fuscus*) trichrome stained paramodiolar cross-section. **(B)** Harbor porpoise (*P. phocoena*) H&E stained midmodiolar section. The location of this section approximates the position of the microCT cross-section in **Figure 2D**. The basilar membrane is shown in an ascending longitudinal position in the hook region. The cochlea is inverted from the *in vivo* position to match conventional cochlear section image orientations. Images copyright© 2020 AS and DK, all rights reserved.

in other mammals and has been reported for other bat species (Veselka et al., 2010). This band joins the posterolateral edge of the bulla to the posterior margin of the mandible and stylo-basihyoid complex. As discussed in Veselka et al. (2010), these fibrous tissues may be important for coordinating vocalizations with auditory attention and receptivity.

Cochlear Cytoarchitecture and Morphometry

Odontocete and microchiropteran cochleae have the prototypic mammalian divisions: scala media (cochlear duct), scala tympani, and scala vestibuli. The membranous labyrinth of the scalae form a spiral inside the bony labyrinth of the periotic, curving around a core, the modiolus, containing the auditory branch of the VIIIth nerve (**Figure 3**). Three anatomical features of the inner ear which influence resonance characteristics and frequency perception are addressed in detail here: basilar membrane construction and support specializations, spiral ganglion cell distributions, and cochlear spiral morphometry.

In all species examined in this study, the organ of Corti anatomy has the same basic cellular cohort as non-echolocating mammals but there are differences in the number, packing, cellular substructure of many features. Some structures of the scala media are hypertrophied, such as enlarged support cells, thickening of the basal basilar membrane primarily through increased collagen fiber density (**Figure 4**), and increased cellular density of the stria vascularis and spiral ligament (**Figures 3–5**). Similar features have been discussed in detail by a number of authors for some species of both dolphins (Wever et al., 1971a,b, 1972) and CF-FM bats (Vater, 2004).

Outer laminae in conjunction with the spiral ligament in most mammals buttress the basilar membrane, particularly those with high frequency hearing. The presence and extent of the outer laminae that hold the basilar membrane rigidly both laterally and medially varies by species. The specimens we examined had substantial outer osseous laminae running 20–60% of the basilar membrane length, varying by species. The thickness of the inner laminae varies inversely with distance from the stapes. The outer lamina in the basal end is as much as 40 μ in depth in *P. phocoena* (**Figures 4**, **5A**) and is heavily calcified (see **Figures 3–5** and **Supplementary Video 3**). MicroCT scans of *E. fuscus* (**Figure 1B**) indicate that similarly deep-layered laminae are present in that species as well. Further measurements of laminar thickness and percentage of cochlear length from histology for the bat specimens are in progress.

In mammals, basilar membrane thickness and width vary inversely from base to apex (von Békésy, 1960; West, 1985). Highest frequencies are encoded in the narrow, basal region; toward the apex, as the membrane broadens and thins, the membrane responds preferentially to progressively lower frequencies. Width and thickness change at different rates according to species in both land and marine animals (Ketten, 1992, 2000; Echteler et al., 1994). **Table 3** provides recent basilar membrane data for the specimens in this study from both CT and histology and compares findings in other mammals. Basal thickness and width ratios are similar in both the air and water echolocators, and are significantly different than in species with better lower frequency hearing. In the case of the porpoise, the basalmost membrane region was virtually a square cross-section as discussed below in more detail. The greatest differences across species in the membrane ratios were found in the apical regions. Estimations of basilar membrane width and thickness can be made from microCT, but require histologic preparations for accurate measurement. Data for basilar membrane dimensions for *P. phocoena* and *T, truncatus* specimens were taken from radial sections located at 5–7% of cochlear length in each specimen for basal values and 98–100%

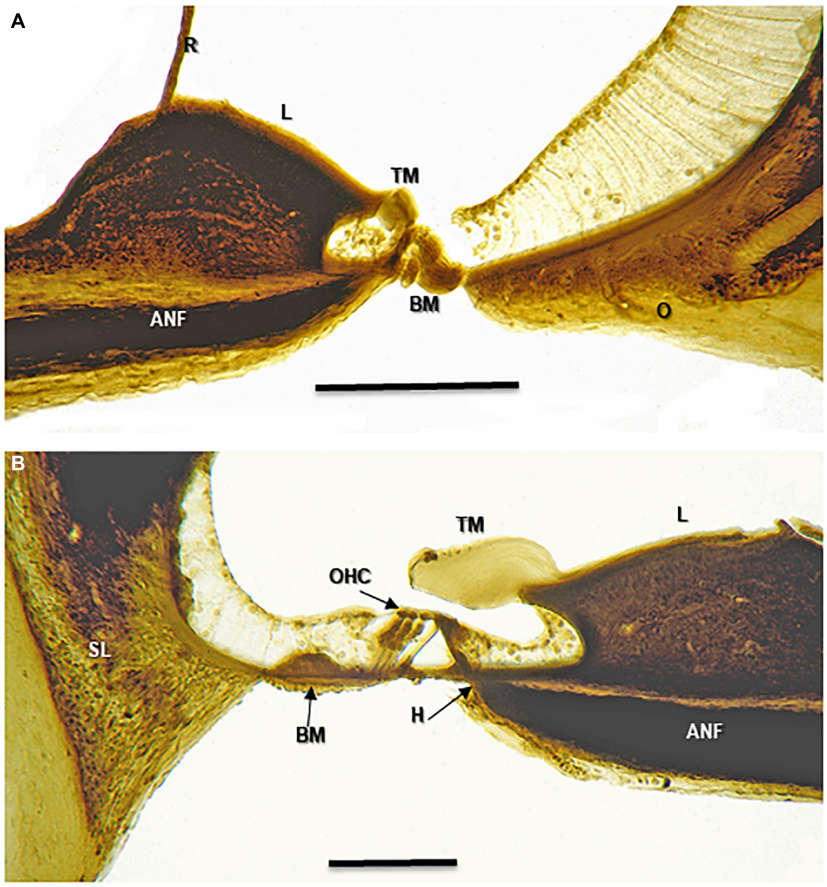

FIGURE 4 | Osmium tetroxide stained 25 micron sections of the of a harbor porpoise (*P. phocoena*) cochlea. These images should be compared with the TEM and schematic images in **Figure 5**. ANF, auditory nerve fiber; BM, basilar membrane; H, habenula; L, spiral limbus; O, outer spiral lamina; OHC, Outer hair cells; R, Reissner's membrane; SL, spiral ligament; TM, tectorial membrane. Scale bars = 0.1 mm. **(A)** Lower basal turn. **(B)** Mid apical turn. Images copyright© 2020 DK, all rights reserved.

for apical values, which are consistent with locations for the data for the other species except *E. fuscus*. The *E. fuscus* data were taken from a paramodiolar section with basal values at a point approximately 20% of length from the base and 80% length for the apex values. They are therefore not directly comparable to the other data in the table and are preliminary pending a full cochlear membrane morphometry map.

Total ganglion cell counts and ganglion cell densities measured from histologies of the odontocete specimens are given in **Table 2**. Average ganglion cell densities for the two odontocetes are more than twice those counted in the CF-FM horseshoe and mustached bats (Bruns and Schmieszek, 1980) and in humans (Nadol, 1988). They are also 30–50% greater than the highest densities reported in the basal, foveal regions in the two species of bats. Ganglion cell counts and distribution data are not yet available for bat species in this data.

Three-Dimensional Anatomical Features

Reconstructions from microCT images coupled with the detailed histology of middle ear and cochlear features provided unexpected insights into peripheral auditory system architectures. **Figures 1**, **2** show images of the bullae; **Figures 3–5**, the cochlear duct; and **Figure 6**, the ossicles, the cochlear capsule, basilar membrane paths, and cochlear spiral variations. Videos revealing the exterior and interior cochlear topography and the relationship of the basilar membrane to stapedial locations in the sampled species are available in the **Supplementary Material**.

These reconstructions revealed unusual fenestral placements for the stapedial input to the cochlea compared to most mammals. The *Tursiops* specimens have a typical mammalian inner ear spiral configuration with the stapes located near the vestibule toward the base of hook region. However, in the other three species, the position of the stapedial input differs from this expected placement. In *E. fuscus* (**Figure 6A**) and *P. abramus* (**Figure 6B**), the oval window/stapedial footplate is located well above the vestibule and descending portion of the hook. This unusual placement was earlier observed in one *E. fuscus* ear (Ketten et al., 2012). We have now confirmed this placement in the ears of five additional big brown bats, both males and females. The *P. phocoena* cochlea (**Figures 6C,D**) exhibited the most extreme modification with the oval window located at the

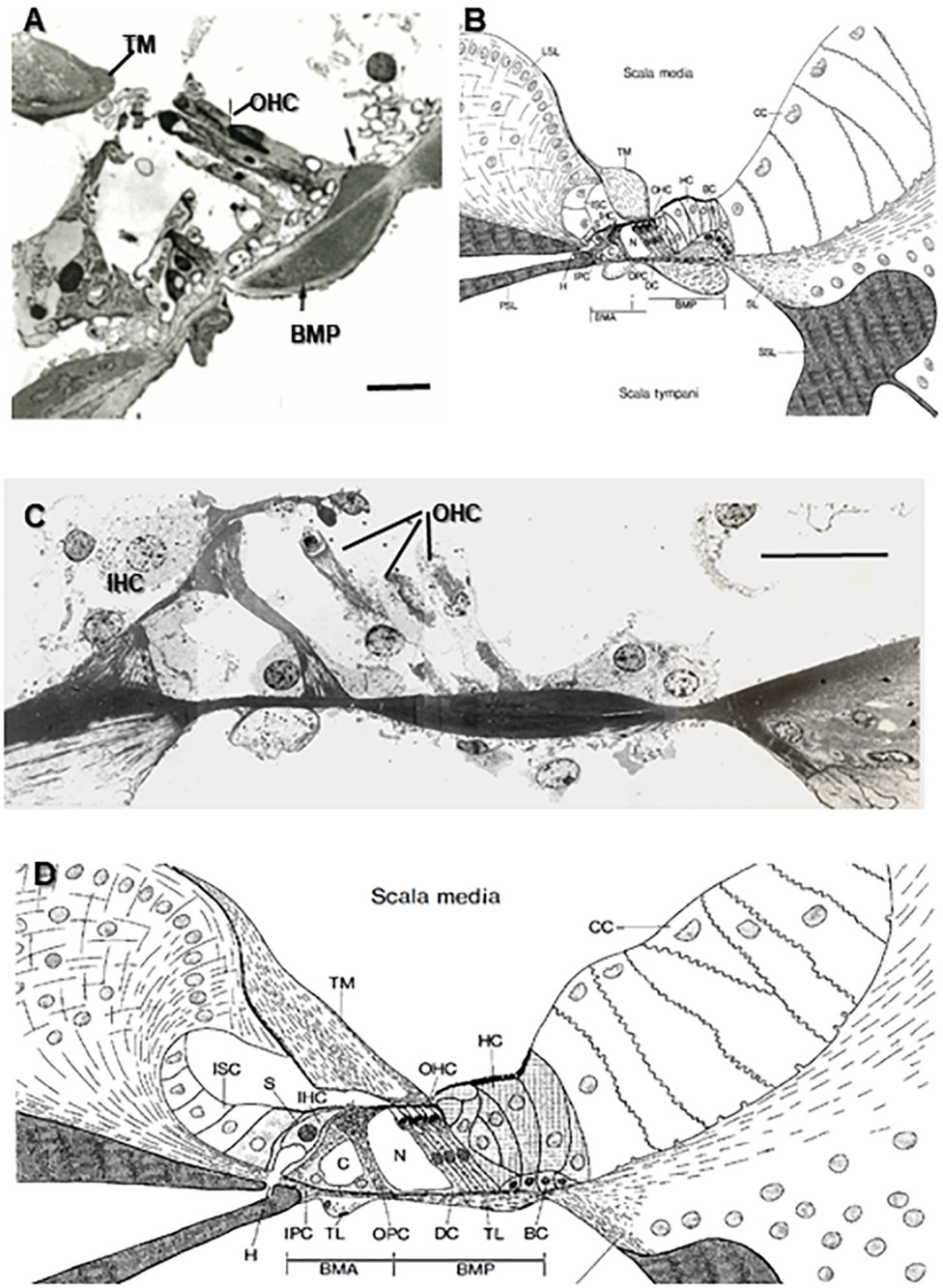

FIGURE 5 | Transmission electron microscopy (TEM) images of odontocete basilar membranes and organ of Corti compared with schematics of the cochlear sections from the horseshoe bat (*Rhinolophus ferrumequinum*). The specialized basal regions of the porpoise and bat have similar thickened regions of collagen fibers (arrows) attached to the basilar membrane that run longitudinally (scala media side) and transverse/radially (scala tympani side) that are hypothesized to act as stiffening agents. In both species, the outer hair cells (OHC) sit atop the bundle of longitudinal fibers. Specialized bundles are absent in the upper basal and second turn of the bottlenose dolphin and bat. Note: Because the OHC are actually staggered, all three may not be fully shown in the TEM images. This is not indicative of hair cell loss. BMA, Arcuate zone of the basilar membrane; BMP, Pectinate zone of the basilar membrane; C, Tunnel of Corti; CC, Claudius' cells; DC, Deiters' cells; H, Habenula; HC, Hensen's cells; IHC, Inner hair cells; IPC, Inner pillar cells; ISC, Inner sulcus cells; LSL, Limbus of the spiral lamina; N, Nuel's space; OHC, Outer hair cells; OPC, Outer pillar cells; PSL, Primary spiral lamina; S, IHC supporting cells; SL, Spiral ligament; SSL, Secondary spiral lamina; TL, Tympanic layer; TM, Tectorial membrane. Scale bars = 0.02 mm [TEM images copyright© 2021 DK, all rights reserved. Diagrams from Bruns (1980) reprinted by permission from Nature/Springer from Anatomy and Embryology, vol. 161]. **(A)** TEM image from the specially adapted lower basal half turn of a harbor porpoise (*P. phocoena*, 1200X magnification). **(B)** Schematic from Bruns (1980) of lower basal turn location in the horseshoe bat (right, *R. ferrumequinum*). **(C)** TEM image from the unspecialized region of the upper basal turn in a bottlenose dolphin (*T. truncatus* 2000X magnification). **(D)** Schematic of the basilar membrane and organ of Corti in a horseshoe bat (*R. ferrumequinum*) in the unspecialized upper second turn.

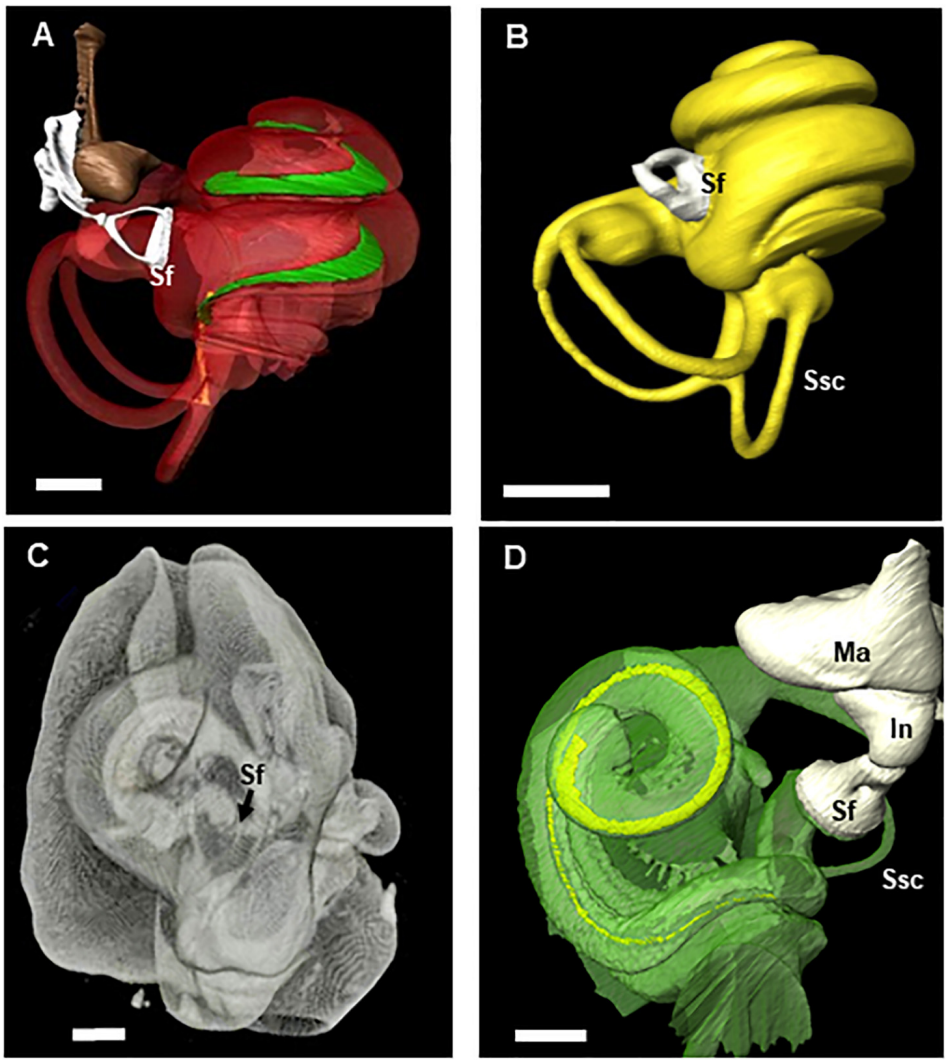

FIGURE 6 | 3D reconstruction from microCT scans of the middle ear ossicles and inner ears in two species of echolocators (big brown bat and harbor porpoise) with unusual stapes input positions. Videos (**Supplementary Videos 2, 3**) show rotations of the cochlear canals that become transparent to reveals the path and width changes of the basilar membrane from base to apex as well as the placement of the stapes and oval window in each of these species (images and multimedia copyright© 2021 DK, all rights reserved). Sf, stapes footplate; In, incus; Ma, malleus; Ssc, semi-circular canal. Scale bar = 1 mm. **(A)** *Eptesicus fuscus* (big brown bat). 3D reconstruction using Amira of a left ear obtained from 17 micron voxel X-Tek MicroCT scan data. The cochlea has 2.25 turns. The basilar membrane (green) length is 8.7 mm and has a post-hook basal turn stapedial input (Sf) (see **Supplementary Video 2** to view rotations and basilar membrane path within the cochlear capsule). **(B)** *Pipistrellus abramus* (Japanese house bat) 3D reconstruction of left ear obtained from 17 micron voxel X-Tek MicroCT scan data. The basilar membrane length is 6.8 mm with a post-hook lower basal turn stapedial input (Sf). **(C)** *Phocoena phocoena* (harbor porpoise) right ear is shown reconstructed with the periotic and cochlear walls transparent to reveal the basilar membrane (yellow) path and stapes located at end of an extended, double hook. The image was reconstructed from 100 micron voxel scans of the entire tympano-periotic complex within the head. The darkened line along the cochlear canal is the edge of the outer osseus lamina, but the basilar membrane itself cannot be fully resolved in this scan series. **(D)** This higher resolution image of a *Phocoena* cochlea was reconstructed from 18 micron voxel microCT scan data. The cochlea has 1.5 turns and basilar membrane length of 24.5 mm. In this species, a second arc rises from the first descending portion with the stapes footplate (Sf) located at its terminus (see **Supplementary Video 3** to view rotations and basilar membrane path within the cochlear capsule).

end of a second, reversed hook extending from the end of the primary descending basilar membrane hook region.

Table 3 contains radii ratios for these cochleae. The ratio of the radii of curvature is defined as the radial length from the modiolus to the outermost length of the basal turn divided by the radius at the point of the helicotrema. It is an approximation of the curvature gradient (Manoussaki et al., 2008). The lower the value, the tighter the coiling. Equiangular curves, the broad based spirals with logarithmic increases in interturn distances that are most common in nature, therefore have larger ratios than Archimedean curves which have a constant interturn distance, as seen in a flat, tightly coiled rope.

The *T. truncatus* cochlear canal is a conventional equiangular curve common to most mammalian ears and has a radii ratio of 4.9. *E. fuscus* and *P. abramus* approximate Archimedean spirals and have ratios of 3.5 and 3.1, respectively. *Phocoena* has a ratio

of 4.3 and appears to be an Archimedean spiral but is difficult to categorize with certainty because it has only 1.5 turns. These ratios are in sharp contrast to the values for low frequency adapted ears, which typically range 8–12 in both land and aquatic species (**Table 3**; see also Manoussaki et al., 2008).

DISCUSSION

Air vs. Water: Matches and Misses

How well do the ears of echolocating mammals, in air or water, mesh with the general land mammal hearing scheme and how different or similar are microchiropteran and odontocete ears in this context of substantial differences in their natural habitats but common echolocation abilities?

In echolocation, or biosonar, the auditory system serves as a real-time sonar system that performs with greater versatility than man-made systems (Simmons, 2017). Identifying the auditory mechanisms responsible for superior performance is of great technological interest. The middle and inner ears of bats and toothed whales differ substantially with regard to mechanical coupling of sound from air or water to the middle and inner ear, or more specifically to the receptor array of the organ of Corti and the critical step of transducing acoustic parameters into neural inputs to higher auditory centers. By comparing the ears of aerial and aquatic echolocators we are beginning to explore this coupling to better understand how the auditory structures, their mechanics, and their respective environments result in similarly effective strategies for echolocation. Critically, several behavioral tests of biosonar performance show that big brown bats and bottlenose dolphins have perceptual acuity for echo delay and for the phase of biosonar echoes (Simmons et al., 1990; Finneran et al., 2020). For this to occur, both bat and dolphin auditory receptors, particularly the cochlea, must capture and convey fine echo delay and phase information via afferent signals to the auditory brainstem and temporal lobes, and be responsive to efferent control of peripheral responses in return. There are major differences in microchiropteran and odontocete ears related to air vs. underwater hearing, but the point in this sequence of reception, transduction, and processing where these differences fade and the functional anatomies converge is the cochlea. The goal of this on-going study is to describe this convergence to address how biosonar "works" and at the same time how it works in two very different acoustic realms.

Sensory systems evolved to allow animals to receive and process information from their surroundings but also to avoid overload (von Uexküll, 1957 translation, Wartzok and Ketten, 1999). In that sense, they are tuned to stimuli of greatest relevance, preferentially admitting some signals and incapable of receiving or processing others. Ears in all species act as highly selective, tuned filters, selecting and attending to signals that, evolutionarily, proved to be important in the context of their local environment (Ketten, 1992). Most animals, including whales and dolphins (Ketten and Wartzok, 1990), have vocalizations linked to their peak hearing sensitivities in order to maximize conspecific communication but also hear beyond their peak range to detect acoustic cues from predators, prey, or significant environmental cues. Further, hearing evolved in the context of natural ambient noise, which varies significantly by habitat. Wenz (1962) laid the ground work for assessing marine ambient noise and showed that it is dominated by frequencies below 5 kHz. Recently, growing concern for sound impacts has led to extensive efforts globally to assess the acoustic environment of diverse habitats, both at sea and on land. These studies have shown that even relatively small contiguous areas can vary significantly based on landscape and vegetation differences (Slabbekoorn, 2004).

Both bats and whales evolved from land-dwelling ancestors during the explosive period of mammalian radiation. Bats of course continued to evolve in air, while the archeocetes moved into aquatic habitats but retained the essentials of air-adapted ears; e.g., an air-filled middle ear and spiral cochlea (see Barnes et al., 1985; Fordyce and de Muizon, 2001; Ekdale, 2016). Therefore, some similarities in land and aquatic mammal hearing anatomy mechanisms are not surprising. For microchiropterans and odontocetes, however, the most striking similarities are not the basic mammalian ear components but rather the specializations or modifications that link to ultrasonic hearing and echolocation abilities.

Land and marine ears, and specifically bat and dolphin ears, do have considerable structural differences. The majority of those differences are in the structure of the reception pathways and the locations of the ears rather than in the middle and inner ear anatomy. As marine mammal ancestors became more aquatic, air-adapted mammalian ears had to not only be coupled to water-borne sound but also adapted to an ambient sound field dominated by low frequencies for hearing to remain functional.

Ear evolution in cetaceans took place in tandem with, and in part in response to, body reconfigurations. Just as the physical demands of operating in water exacted a structural price in the locomotory and thermoregulatory systems of whales, physical differences in underwater sound required some auditory system remodeling. As the rostrum elongated, the cranial vault foreshortened, and the nares and narial passages were pulled rearward to a dorsal position behind the eyes. Many conventional land mammal auditory components, like external pinnae and air-filled external canals were lost or reduced and the middle and inner ears migrated outward (Ketten, 1992, 2000). In most odontocetes, the ears have no substantial bony association with the skull. Instead, they are extra-cranial, suspended by ligaments in a foam-filled fossa outside the skull. In addition, there are specialized fatty bundles with distinct and unique lipid profiles in all odontocetes that parallel the mandible, connecting the middle ear, that have a discrete shape resembling elongated pinnae (Ketten, 1997, 2000; Koopman et al., 2006).

Several factors related to the physical characteristics of sound in water, such as speed, frequency of echolocation signals vs. target object size, drove the specializations of the auditory system in odontocetes. The speed of sound in water drove cetacean ears to be farther apart compared to other mammals; new sound reception pathways matched to acoustic impedance characteristics of water developed, and acoustic isolation of outgoing signals from the ear was achieved by ears that are uncoupled from the skull, given the five-fold increased speed of sound in water, the almost cartoonish large cetacean heads and

extracranial ear placements provide odontocetes with interaural time difference discriminations comparable to that of bats.

Since bats evolved and remained in air, acoustic properties of the media were not so evident a factor for major retooling of the auditory periphery although there are clear anatomical specializations for flight. In one sense, they can be seen as enhancing or honing rather than reshaping their auditory systems. The complex and relatively delicate structures of the pinnae and nose leaves in some species are as striking and intriguing as telescoping and specialized fats in odontocetes. All of these features require more extensive biomechanical analyses as well as the related questions of if and how bats deal with air flow noise in flight and dolphins deal with water flow noise in dives to reduce interference with echo perception.

The most striking and functionally significant observations related to the specimens in this study, and the observations that set them apart from the majority of mammalian ears, are in fact their similarities, particularly the augmentations observed in the middle ear ossicular stiffening and control structures in the middle ear, the unusual stapedial locations for three of the studied species, the basilar membrane foveal membrane regions in one species, and the increased ganglion cell densities compared to other mammals. Our data on ganglion cell counts are preliminary at this time, and it is important to clarify whether the location of high ganglion cell densities coincide with frequency place maps for the peak spectral characteristics of echoes in each group.

The intracochlear distribution of the outer lamina expressed as a percentage of membrane or cochlear ranges from 20% in *Tursiops* to over 60% in *Phocoena*. The data from microCT images suggest that the bat distributions are similar. Extensive buttressing is consistent with higher resonant frequencies as well as less potential variability from more elastic suspension systems. Fleischer (1976) observed that osseous laminae may have material properties in the basal region comparable to solid compact bone and decreasing apically as fibrous inclusions increase, producing a potential 100-fold to 1,000-fold base to apex stability gradient. If correct, these values suggest that differences in laminar support may be a far more influential element of basilar membrane dynamics than is currently understood. They also underscore that material property measurements on a species basis should be prioritized to aid accuracy in Finite Element Models (FEM) of tissues in both the middle and inner ear (Tubelli and Ketten, 2019; Puria, 2020).

Within the inner ear of all cetaceans, one major dissimilarity from bats and in fact other mammals as well is the differences in vestibular dimensions. Not only is the vestibular system smaller in proportion to the cochlea, it is relatively poorly innervated (Gao and Zhou, 1995). Most mammals, including bats, have approximately 40–45% of the VIIIth nerve fibers distributed to the vestibular branch. In cetaceans, vestibular branch commonly has less than 7% of the total VIIIth nerve fibers. A number of features have been examined with regards to this question, including the possibility that the fusion of the cervical vertebrae affected inputs to the vestibular system, the velocity and frequency of rotations compared to land mammals, and the kinematics of cetacean swimming (Gingerich et al., 1994; Fish, 1998; Spoor et al., 2002; Kandel and Hullar, 2010). Nevertheless, the primary driver for this state remains unclear. Both bats and dolphins make fast and frequent re-orientations while seeking prey and avoiding obstacles. Therefore, they are subject to similar stresses on the vestibular system. That suggests that reduction of the vestibular system in cetaceans is not driven by their manoeuvers. This remains an open question.

It's a Material World

The basilar membrane is a frequency-dispersing array that shunts a succession of frequencies from high to low to different locations, and thus to different receptors, creating frequency tuned channels for subsequent auditory processing (Dallos, 1996). Variations in rate of change in basilar membrane dimensions are consistent with differences in the octave ranges of hearing in each species, with gradations in thickness and width a reasonable proxy of the material properties of stiffness and mass. Consistent with the data in our study, Pye (1966) reported for the basilar membrane of another FM bat, *Pipistrellus pipistrellus*, a basal width of 80 μ with a thickness of 15 μ and an apical width of 115 μ with a thickness of 5 μ or less. If *P. abramus* is similar that suggests a basal membrane ratio of approximately 0.19 and apical of 0.04, which is similar to the preliminary values for *E. fuscus*. Although the membrane data are incomplete for the microchiropteran bats examined in this study, the preliminary data from histology for *E. fuscus* and microCT for *P. abramus* suggest they have smaller gradations in both thickness and width, changing little over the full cochlear length. This implies a narrower hearing range, with much higher low frequency and lower high frequency cut-offs compared to the odontocetes. Our data for these two bat species are consistent with those in CF-FM bats (reviewed by Echteler et al., 1994; Kössl and Vater, 1995). Of the two odontocetes studied, a full length basilar membrane morphometric maps of *P. phocoena* show markedly less gradation than *T. truncatus* and more closely resembles the *R. ferrumequinum* membrane gradient in its basal regions (Ketten and Wartzok, 1990).

Based on the anatomy of the basilar membrane in *P. phocoena*, specialist ears exist in both odontocetes and microchiropterans. *P. phocoena* has a basal cochlear membrane structure consistent with a specialized basilar membrane "foveal" region in the lower basal turn, similar to that reported for the CF-FM bat *R. ferrumequinum* (Bruns, 1980). The harbor porpoise basilar membrane has a thickened region with fairly constant width and thickness over a substantial portion of the basal basilar membrane (**Figures 4**, **5**). There are also longitudinal and transverse or radial fibers present, again paralleling those reported for *R. ferrumequinum*. These areas, dubbed "acoustic fovea" regions by Bruns (1980) and Bruns and Schmieszek (1980) are singularly devoted to frequencies near the peak spectra of their echolocation signals (100–110 kHz for the porpoise, 80–86 kHz for the CF-FM bat) and thus represent a stretched frequency map that occupies much of the basal turn of the cochlea modeled by Ketten (1994) which was later confirmed behaviorally by Kastelein et al. (2002).

There are, however, additional elements evident in these ears, including the inner and outer osseous laminae, that may have a significant role in determining responsivity, particularly for the upper limits of the hearing range by increasing the stiffness of the basilar membrane along its extent. These stiffening features

are part of the reason that the basilar membrane of *P. phocoena* has a peak sensitivity of approximately 110 kHz and extends to nearly 200 kHz (Kastelein et al., 2002), despite having a cochlear length equivalent to a cat (Greenwood, 1990). Were the *P. phocoena* frequency map derived from a single parameter, such as length, the hearing range would have been substantially lower with a cut-off near 60 kHz rather than the 180–200 kHz estimated in more complex models (Ketten, 1994; Ketten et al., 1998). This is because length is correlated with body mass. Calculations of frequency ranges and cochlear maps based solely on a single parameter such as cochlear length or number of turns are less definitive than multi-parametric estimations and are generally not reliable for species operating in different media with radically different constraints on body mass. Bats and dolphins as two extreme examples of this underscore the importance of considering multiple facets of the functional anatomy of the ear in making comparisons across species.

Waves

The most common model of intracochlear acoustic propagation is that the majority of the cochlea may have some response to introduced sound stimuli, but depending upon its properties and those of each membrane region the amount of deflection and phase of the signal will vary. The progressive phase and amplitude variations have been described as a traveling wave that produces a time dependent response "envelope" of amplitudes that characterizes the signal (Dallos, 1996).

Could species variations in location of the oval windows with respect to the basilar membrane segments suggest alternative response mechanisms? Simulation experiments (A. Hubbard, pers. comm.) indicate that changing response parameters to constant tuning from 20 to 40% resulted in a standing wave. In big brown bats, the exceptional position of the oval window opens several possibilities, including bi-directional flow propagation and resultant reflection effects that may also produce a localized standing wave phenomenon.

The concept of a standing wave has been proposed previously in relation to the acoustic fovea of CF-FM bats (Kössl and Vater, 1995). These authors proposed that the relative thickening of the basilar membrane could provide a reflection zone tuned to returning echoes. This hypothesis also would function to enhance Doppler detection. In this paper, we have presented another potential mechanism for standing wave generation in *E. fuscus,* a species not known to have an acoustic fovea. Our hypothesis is not in opposition to that put forward by Kössl and Vater. Rather, it may be an alternative means to a similar end for some species, and both have yet to be proven.

Thus far, among odontocetes, only the harbor porpoise has been shown to have basilar membrane characteristics similar to acoustic foveal regions in Microchiropterans. There is also no evidence to date that dolphins or porpoises use Doppler shift compensations. Indeed, Au (1993) concluded that sound speeds in water may produce sufficient repeat echoes over a short period of time to diminish the information that Doppler shifts may provide to dolphins about target prey velocity and direction. *Tursiops*, however, does not have the structural features that were found in *Phocoena.* Were Doppler sensitivity to be explored in any odontocete, or in fact other bats, it may be important to take cochlear anatomy into account.

Spiraling Down

Radii ratios have been proposed as a correlate of low frequency hearing cut-offs (Manoussaki et al., 2008) based on the assumption that larger ratios reflect a broader curvature that would produce a "whispering gallery" effect in which energy density paths focus at the points of concavity, producing a radial pressure gradient. This is a favorable structure for low frequency energy to propagate throughout the cochlea. The compact spiral structure encountered in the FM bats in this study imply a decrease in the propagation for lower frequencies. Even more interesting is the additional reverse curve present in the harbor porpoise which suggests an alternative but potentially equally or more effective anatomical strategy for preventing low frequencies from penetrating the cochlea. This is in turn brings up the question of whether echolocators have developed structural measures to minimize exposure to spurious signals, such as low frequencies which dominate the marine environment.

Potential Protection From Echolocation Adaptations?

Dolphin ears are essentially terrestrial ears immersed in a biologically rich but in other ways a harsh environment. Anatomically, they follow the basic land mammal pattern but they have extensive adaptations that accommodate substantial parasite loads, pressure changes, and concussive forces. It remains unclear whether the relatively noisy and literally high pressure oceanic environment led to ears more stressed by multiple impacts or the development of physiologically tougher than average ears (Maison and Liberman, 2000). On the other hand, because marine mammals evolved in a high noise environment and have adaptations that prevent structural ear damage from barotrauma, it is possible that this is a feature related to echolocation *per se*, similar to what has been hypothesized for bats. Simmons et al. (2016, 2017, 2018) found that hearing sensitivity of big brown bats is not impaired by long duration, high-intensity exposures to sounds at levels that are known to induce temporary threshold shifts in other mammals. Therefore, it may be that successful echolocators have one or more ways by which they are able to sustain hearing in the presence of their own repetitive and intense signals with the secondary benefit of being less subject to environmental noise and hearing deficits.

Cochlear microphonic studies on several species of bats have demonstrated that contractions of the stapedius are coincident with the onset of the out-going signal followed by a release, thus synchronizing signal-echo sequences (Henson, 1965; Suga and Jen, 1975; Kick and Simmons, 1984). In these experiments, attenuations of the initial signal ranged from 20 to 28 dB. These levels are consistent with attenuations in humans and other species for stapedial reflexes, but the key features that differentiate this ability in bats from a simple stapedial reflex to an intense sound is the closely timed synchronization with the emitted signal, its rapidity, and the sustainability of the

sequences. Studies have indicated an ability in several odontocete species, including *Tursiops* and *Phocoena*, to "self-mitigate" effects of exposure to loud underwater sounds in captive studies (Finneran, 2018; Nachtigall et al., 2018; Kastelein et al., 2020), the precise mechanisms of which remain unexplained.

CONCLUSION

Cross-media commonalities suggest similar cochlear specializations developed in parallel in microchiropterans and odontocetes. Cochlear anatomy observed in all specimen groups are linked to peak spectra of their vocalizations, notably with expanded frequency representation in the inner ear and, in some cases, possibly with enhanced tuning hypothesized to be derived from standing wave phenomena.

Differences that are consistent with processing of aerial vs. aquatic borne sound are found primarily in the outer and middle ear elements. Other differences among species, such as peak frequency of echolocation signals, are correlated with signal type, prey, and/or habitat features.

One speculation is that the stapedial placements and uniform, robust basilar membrane structure may enhance tuning in adjacent ear segments by generating standing wave phenomena. In the FM bats, the stapedial locus may result in a bi-directional flow. In the phocoenids, the double hook may serve to attenuate low frequency penetration and thus reduce low frequency sensitivity providing more membrane space for a stretched response map. Delphinid odontocetes, represented by the bottlenose dolphin in this study, more closely resemble the terrestrial generalist ear, with a peri-vestibular input. In all species examined, the cochlear canal curvatures are consistent with those of the highest frequency terrestrial species.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Bat studies were reviewed and approved by the Brown University Institutional Animal Care and Use Committee; odontocete studies were approved by the IACUC committee of Woods Hole Oceanographic and collected under USFW/NMFS permits (932-1489-08, 493-1848-00, 493-1848-02, 130062, and 130062-1).

AUTHOR CONTRIBUTIONS

DK collected all odontocete specimens and provided the histology and analyses of all data on odontocetes, conducted and analyzed CT scans of all specimens in the study, and wrote the primary draft of the manuscript. JS, HR, and AS provided bat specimens. JS and AS provided histological data for the big brown bat. All authors contributed to the interpretation of the data and edited the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.661216/full#supplementary-material

Supplementary Video 1 | The data for the video were obtained from images reconstructed in RadiAnt version 2020.1.1 from microCT data obtained at 11–17 micron voxel resolutions on a Zeiss Xradia Versa 520. The video shows a digital dissection of a the head of a female bat (*Eptesicus fuscus*) from the exterior surface to the inner ear that progressively reveals the tympano-periotic complex, middle ear, and inner ear labyrinth. The head is shown first in anterior view. As the head rotates to the left side, the skin and soft tissues fade from view to reveal the skull of the bat. The middle ear structures, particularly the spike-like long arm of the malleus, are clearly visible inside tympanic ring, just posterior to the mandible. The video then focuses on these structures, removing the surrounding skull structures, and rotates the bulla from a lateral to anterior view, revealing the three ossicles and the semicircular canals of the vestibular system. The stapes can be seen situated at the basal turn of the cochlea as the bony cochlear capsule fades

to show the 2.25 turn spiral of the cochlea (see **Figure 1** for labeling of structures. Images and multimedia copyright© 2021 DK, all rights reserved).

Supplementary Video 2 | The data for the video were obtained from images produced in Amira from VG Studio Max 3.4 images reconstructions of 7–11 micron voxel acquisitions of microCT data obtained with an X-Tek MicroCT. The video shows the inner ear anatomy of a male bat (*Eptesicus fuscus*) first as the full periotic capsule (red) with the stapes (white), incus (white), and malleus in place. The capsule fades as the inner ear rotates, revealing the path and profile of the basilar membrane (green) within inner ear labyrinth (see **Figure 6** for dimensions and detail of the structures).

Supplementary Video 3 | The data for the video were obtained from images produced in Amira from VG Studio Max 3.4 images reconstructions of 18–25 micron voxel acquisitions of microCT data obtained with an X-Tek MicroCT. The images were processed for video using Osirix 12.0.

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