Tectorial Membrane Morphological Variation: Effects Upon Stimulus Frequency Otoacoustic Emissions

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Abstract

The tectorial membrane (TM) is widely believed to play an important role in determining the ear's ability to detect and resolve incoming acoustic information. While it is still unclear precisely what that role is, the TM has been hypothesized to help overcome viscous forces and thereby sharpen mechanical tuning of the sensory cells. Lizards present a unique opportunity to further study the role of the TM given the diverse inner ear morphological differences across species. Furthermore, stimulus–frequency otoacoustic emissions (SFOAEs), sounds emitted by the ear in response to a tone, non-invasively probe the frequency selectivity of the ear. We report estimates of auditory tuning derived from SFOAEs from twelve different species of lizards with widely varying TM morphology. Despite gross anatomical differences across the species examined herein, low–level SFOAEs were readily measurable in all ears tested, even in non–TM species whose basilar papilla contained as few as 50–60 hair cells. Our measurements generally support theoretical predictions: longer delays/sharper tuning are found in species with a TM relative to those without. However, SFOAEs from at least one non-TM species (*Anolis*) with long delays suggest there are likely additional micro–mechanical factors at play that can directly affect tuning. Additionally, in the one species examined with a continuous TM (*Aspidoscelis*) where cell–cell coupling is presumably relatively stronger, delays were intermediate. This observation appears consistent with recent reports that suggest the TM may play a more complex macro–mechanical role in the mammalian cochlea via longitudinal energy distribution (and thereby affect tuning). Although significant differences exist between reptile and mammalian auditory biophysics, understanding lizard OAE generation mechanisms yields significant insight into fundamental principles at work in all vertebrate ears.

Key words: cochlear mechanics ; lizard ; tectorial membrane ; stimulus–frequency otoacoustic emission; auditory tuning

Abbreviations: ANF, auditory nerve fiber; TM, tectorial membrane; BM, basilar membrane; OAE, otoacoustic emission; SOAE, spontaneous otoacoustic emission; SFOAE, stimulus–frequency otoacoustic emission; Q, quality factor

1 Introduction

The tectorial membrane (TM), a gelatinous ribbon sitting atop the sensory cells of the inner ear, is widely believed to play a critical role in producing the ear's remarkable sensitivity and selectivity (1). This latter feature, also referred to as sharpness of tuning, determines the ear's ability to resolve different frequency components of incoming sound. However, the precise functional role of the TM is still not well known given the fragility and complexity of the inner ear. A traditional point of view posits that the mammalian TM provides an additional mechanical resonance in the cochlea and thereby a means for sharpened tuning (2), (3), a prediction supported by direct empirical observation (4). More recent reports, both theoretical and experimental, have indicated additional possible roles the TM plays. Several theoretical studies have suggested the TM contributes toward sharper tuning at the level of the stereovillar bundle by counteracting viscous forces (5), (6). Other recent studies have examined the role the TM plays in coupling responses across groups of hair cells (7), (8), (9) and longitudinally along the mammalian cochlea (10), indicating that such coupling is likely important. Observations in a genetic knockout mouse model have suggested that mutations to the underlying fibrillar network of the mammalian TM can have large consequences for longitudinal coupling in the cochlea and thereby lead to changes in sharpness of tuning (11).

Remarkably, the ear not only responds to sound, but somehow in the process of forward transduction (i.e., converting mechanical sound stimuli to electrical signals at the level of the auditory nerve), the inner ear generates and subsequently emits sound. These sounds (known as otoacoustic emissions, or OAEs), are emitted from the ear either spontaneously or via an evoking stimulus and provide a non–invasive window into the function of the peripheral auditory system. Emissions have been demonstrated to be present in a wide variety of species (12), (13), including non-vertebrates (14). Furthermore, the development of extensive clinical OAE applications have been of great value to audiologists (15), motivating further study of the underlying generation mechanisms. Spontaneous otoacoustic emissions (SOAEs) present compelling (but not definitive) evidence for active mechanisms at work inside the inner ear that lead to an amplification of low-level stimuli (e.g., (16)). Stimulus frequency emissions (SFOAEs), evoked using a single tone and occurring at that same stimulus frequency, have been suggested as a means to noninvasively probe the sharpness of tuning (or bandwidths) of the underlying auditory filters inside the ear (17), (18). Specifically, the SFOAE phase–gradient delays, expressed non–dimensionally as

N_{SF} (see Methods), are hypothesized to reflect build-up time towards the steady–state response of the underlying filters. Theoretical models for the mammalian (19) and lizard (20) ear provide a foundation for such a correlation, indicating a proportionality between phase-gradient delays associated with low-level SFOAEs and filter bandwidths. Specifically, the larger the emission delay, the sharper the tuning.

The present study utilizes the morphological diversity of the inner ear across the Lacertilia (lizards) to non–invasively probe functional consequences of TM structure (or lack thereof). Lizards exhibit robust emissions (21), (22), (23), (24), (13) in addition to wide variations in inner ear structure (25). As put forth by Manley, the lizard inner ear represents '*a playground of evolution*' (26) and differs significantly from mammals in that lizard ears lack a traveling wave that propagates along the basilar membrane (27), (28). Lizards have been described as having two populations of different hair cell types (29): I – cells whose stereovillar bundles are uni–directionally oriented, sensitive to frequencies below ∼1 kHz (30), (31) and have a continuous overlying TM, and II – cells whose bundles are bi–directionally oriented, responsive to frequencies above 1 kHz, and have a diverse TM morphology (differing across species). The latter cell group typically comprise the majority of the basilar papilla (e.g., ∼70–90% for iguanids) and are the focus of the present study. We diverge somewhat from the convention of Wever (25) and Miller (29) with regard to defining the TM. In their framework, a TM refers to a structure that is attached at another anchoring point (i.e., the limbic lip). Here, we refer to any structure composed of a gelatinous/fibrillar matrix that resides directly atop the sensory cells as a TM, regardless of whether there is a secondary attachment point.

Lizard TM morphology comes in many different forms. A continuous TM attached to the limbic lip is present in some families over the entirety of the papilla (e.g., teiids), superficially similar to that of mammals. Several lizard families have essentially what amounts to a discretized TM (sallets), such as in skinks, geckos, and gerrhosaurids. While unconnected to the limbic lip, there are interconnecting processes coupling adjacent sallets (25), but the functional significance of these connections is unknown. One possibility is that the connections introduce a small degree of longitudinal coupling that might improve sensitivity (28) at the sacrifice of selectivity (32). Other families lack a TM altogether over the bulk of the papilla, such as iguanids and anguids. Much of this diversity in TM morphology has been proposed to stem from various selection pressures (26), (32). It is worth noting that a previous study has taken a similar approach to that here: utilizing OAEs and the diversity of TM morphology in non–mammals to infer filtering prop-

erties of the TM with respect to certain types of emissions (33). However, that work differs significantly from ours in that it was focused upon how the TM might act as a band–pass filter in producing distortion product emissions (DPOAEs), OAEs evoked by the simultaneous presentation of two tones. Another study (34) also examined the effects of TM detachment upon DPOAEs in the mammalian cochlea via a genetically–modified mouse model.

The specific goal of the present study is to make a broad comparison of OAE–derived estimates of tuning across lizards with known differences in TM morphology. Theoretical studies have hypothesized that the presence of a TM, which presumably plays a role in how the hair cells are coupled, can provide sharpened tuning to the underlying auditory filters (5), (6), (8). However, there is little direct empirical verification of such aside from comparisons of auditory nerve fiber (ANF) responses across various different studies (e.g., (35), (26)). Assuming SFOAEs provide an objective measure of peripheral auditory tuning (17), (20), we hypothesize that non–TM species would exhibit shorter SFOAE delays relative to those species with a TM. We systematically test such a prediction by examining twelve different lizard species, spanning across eight different families and four infraorders. Salient morphological features for all species are summarized in Table 1. Additionally, Figs.1–3 contain highly simplified schematics to illustrate the TM structure over the bi–directional portion of the papilla (type II hair cells) for the indicated species. For the sake of clarity, the results initially focus on three species (*Anolis carolinensis*, *Gerrhosaurus flavigularis*, and *Aspidoscelis tigris*) in order to demonstrate results from non-TM, salletal, and continuous TM papillae respectively. Data from several other species can be found in the *Supporting Material*.

2 Methods

All measurements reported in this study were obtained using the same stimulus paradigms, acquisition codes, and OAE probe for all species/individuals (13). A desktop computer housed a 24-bit soundcard (Lynx TWO-A, Lynx Studio Technology), whose synchronous root mean square (RMS) input/output was controlled using a custom data–acquisition system. A sample rate of 44.1 kHz was used to transduce signals to/from an Etymotic ER-10C probe containing a microphone and two earphones. The microphone response was amplified by 40 dB and high-pass filtered with a cut-off frequency of 0.41 kHz to minimize the effects of noise. The OAE probe was coupled to the

external ear using a short tube attached to the foam tip and sealed to the head using vaseline or silicone grease. This ensured a tight (closed) acoustic coupling and minimized low-frequency losses. The probe tip was ∼0.5–1.25 cm from the tympanic membrane. The probe earphones were calibrated *in-situ* by presenting flat-spectrum, random-phase noise. By computing the ratio of response to that of the output signal, the frequency response and associated delays could be determined. Calibrations were verified repeatedly throughout the experiment. All stimulus frequencies were quantized such that an integral number of cycles were contained within the sampling window.

To evoke the SFOAEs, a low probe level (L_{p} = 20 dB SPL) was chosen for several reasons. First and foremost, this level was large enough to evoke a detectable emission with suitable signal-to-noise ratio in all species examined and was low enough such that the SOAE activity was not suppressed by the stimulus tone (see *Supporting Material*). Second, SFOAE phase–gradient delays (defined below) are fairly insensitive to stimulus level at these lower intensities, whereas moderate and higher level stimuli exhibit significantly smaller N_{SF} values (e.g., (36), (13)), consistent with the general observation of broadened tuning in ANF responses at higher stimulus levels. Third, as previously reported (22), lizard OAEs evoked using a relatively low stimulus level are highly dependent upon the physiological state of the animal (e.g., hypoxia) while those evoked at higher levels are less sensitive to such. Thus, emissions generated via low-level stimuli are presumably more critically tied to active mechanisms at work in the inner ear. Fourth, given that there is evidence for multiple OAE generation sources in at least certain types of lizard ears (13), a low stimulus level helps minimize the potentially confounding factor of (nonlinear) source–mixing from different generation mechanisms (37). Lastly, for low stimulus levels the acoustic noise floor completely masks any system distortion that can create artifactual emissions.

The range of stimulus frequencies (f_{p}) employed was typically 0.4–8 kHz. The stimulus and emission frequency are one and the same for SFOAEs. A two–tone suppression paradigm was employed to extract the SFOAE (37), (13). The suppressor parameters were: $f_{\mathrm{s}} = f_{\mathrm{p}} + 40$ Hz, $L_{\mathrm{s}} = L_{\mathrm{p}} + 15$ dB (where f_{s} and L_{s} are the suppressor frequency and level, respectively). A total of 35 waveforms (8192 sample window) were averaged, excluding any flagged by an artifact-rejection paradigm (37). A period of 20 ms was allowed before the start of the sample window so to allow for the associated response to reach steady–state. Frequency step-size during sweeps was small enough to avoid ambiguity during the phase unwrapping. Delays associated

with the measurement system were determined and subtracted out. The noise floor was defined as the average sound-pressure level centered about (but excluding) the frequency of interest. It was quantified via averaging the magnitudes of the ± 3 bins in the fast Fourier transform (FFT) of the response.

The phase-gradient delay expressed (N_{SF}) in number of periods is the product of the derivative of the phase function with respect to frequency (τ_{OAE}) and the emission frequency (f_{p}). For linear systems, this is directly related to the group delay. By quantifying the frequency dependence of the steady–state response of a system, the phase-gradient delay provides a useful means to identify delays inherent in the dynamics of the system. It is given by

$$N_{\mathrm{SF}} = f_{\mathrm{p}}\,\tau_{\mathrm{OAE}} = -\frac{f_{\mathrm{p}}}{2\pi}\frac{\partial \phi_{\mathrm{OAE}}}{\partial f_{\mathrm{p}}} \tag{1}$$

where ϕ_{OAE} is the emission phase (in radians) and f_{p} is in hertz.

Expressing the delay in dimensionless form as N_{SF} is useful when making comparisons to other dimensionless quantities such as the filter bandwidths (Q). Delays (i.e., τ_{OAE}) were computed from individual (unwrapped) phase responses using centered differences (38). As shown in Fig.2, delay trends were computed across individuals of a given species via a locally–weighted regression (*loess*) (39) [weighting factor $\alpha \approx 0.1 - 0.2$, polynomial of degree one, robust fit]. To further reduce the effects of outliers at the end points, only N_{SF} values whose corresponding magnitude (as well as the magnitude of its neighbors) was at least 10 dB above the noise floor were included in the fits. The data in general do not appear well fit by a simple power–law (38) across all frequencies tested, and it is desirable to make comparisons without *ad hoc* statistical modeling assumptions. While *loess* fits are a first–order approximation (e.g., note deviation in Fig.2 between $4 - 5$ kHz for *Aspidoscelis*), they do provide a useful starting point for quantitatively comparing delay trends across species (38).

All experiments were performed at the University of Arizona with approval from the Institutional Animal Care and Use Committee. Experiments were performed during the months of March–August. For all species in this study, OAE data were collected from both adult males and females and from both ears in a given individual; the results as presented here do not distinguish between sex nor between data collected from left versus right ears. Species native to southern Arizona/California were wild caught while non-native species were obtained via local vendors. All lizards were housed in glass terraria with a 9–hour light cycle and fed meal worms and crickets

(occasionally dusted with calcium powder) 2–3 times a week. All lizards were healthy and active. Prior to each experiment, an animal was anesthetized via a 25–36 mg/kg Nembutal intraperitoneal injection to prevent movement. Anguids and iguanids required higher doses while the teiids and scincomorphs were given lower doses to obtain similar anesthetic states such that they do not move. These doses were effective for approximately two to five hours. The animal recovered completely within a few hours after the experiment. During the experiment, lizards were placed in a noise-attenuating chamber. Body temperature was kept constant by the use of a regulated heating blanket (Harvard Apparatus) and monitored using a calibrated thermocouple placed in the mouth (propping it open) or in the leg pit for cases where the lizard spit out the thermometer. Body temperature was kept in the range of approximately 32–33° C (verified via a quick–reading cloacal thermometer). Preliminary data indicate SFOAE phase-gradient delays appear relatively insensitive to temperature (40) or depth of anesthetic state.

3 Results

Of the 49 different ears examined during the present study, in addition to the 21 gecko ears from a previous study using the same system/paradigms (13), low–level SFOAEs were readily detectable in all ears (top traces in Fig.1). Significant phase accumulation was also apparent as the stimulus tone was swept in frequency (ϕ_{OAE} in Fig.1), indicative of delays on the order of milliseconds. Spontaneous activity, as identified via temperature dependence and suppressibility due to external tones (41), (42), was apparent in the vast majority of ears examined (see *Supporting Material*). In two instances of accidental overdose, both SOAEs and low-level SFOAEs were found to rapidly disappear upon death. These observations indicate these emissions are dependent upon a healthy physiological state for the animal, consistent with previous studies of lizard emissions (21), (22), (23).

For each species, three different individuals (chosen at random) are shown in Fig.1 to demonstrate SFOAE similarities and differences across ears. For all species examined, SFOAE magnitudes fall off into the noise floor by $f_{\mathrm{p}} \approx 7 - 8$ kHz, though typically at lower frequencies depending upon species (Fig.1, *Supporting Material*). Low–level SFOAE magnitudes extend beyond the highest frequency of SOAE activity (*Supporting Material*), though how far beyond varies from animal to animal. While SFOAE magnitude and phase trends are broadly apparent for a given species (as f_{p} was varied), significant variations across individuals are observed (Fig.1).

Each ear exhibits a distinct set of peaks and valleys in magnitude and these spectral characteristics were highly reproducible within a given recording session, provided body temperature was kept constant. In some instances, individual responses appear qualitatively different from the others within a species. For example, one individual *Gerrhosaurus* shown in Fig.1 shows reduced magnitudes above ∼3 kHz, although the rate of phase accumulation is similar to the others. Other magnitude features, such as the notch at 2–2.5 kHz for *Aspidoscelis*, are consistent across individuals within a species.

As shown in Figs.2 and 3, the delay (N_{SF}, expressed in number of stimulus cycles) was computed by numerically differentiating the phase responses shown in Fig.1. While a steady rate of phase accumulation is apparent for a given individual (Fig.1), sudden magnitude variations such as notches can lead to phase discontinuities and thus ambiguities in the phase unwrapping (e.g., negative delays). This ambiguity can make it difficult to precisely quantify the phase–gradient delay for a given ear, despite the clear trend. However, given a sufficiently large population for a particular species (≥ 4 ears), a suitable trend in N_{SF} can be determined via a locally-weighted regression (see *Methods*) (38), (36) as shown in Fig.2. Only delays whose corresponding magnitude (as well as that of its neighbors) was at least 10 dB above the noise-floor were included in further analysis.

Comparing SFOAE phase-gradient delays across species, Fig.3 shows that delays are typically larger in TM–species for f_{p} above ∼1–2 kHz. One notable exception is *Anolis*, whose emissions extend out to higher frequencies relative to other non–TM species and exhibit relatively large N_{SF} values. Of all species examined, *Gekko* exhibits the largest delays above ∼1.5 kHz. The one species with a continuous TM, *Aspidoscelis*, has intermediate N_{SF} values. In all species, with the exception of *Elgaria* and *Uta*, N_{SF} generally increases with frequency.

4 Discussion

While it is commonly accepted that the tectorial membrane plays an important role in the ear's ability to transduce sound into neural signals, there is still presently much debate as to precisely what that role is. Given the robust OAEs and diverse TM morphology across the lizard taxa, the present study systematically explores SFOAE properties across a broad array of lizard species. Specifically, we make use of the notion that SFOAEs (emissions evoked in response to a single stimulus tone) can be used to objectively determine auditory filter bandwidths (17), (18). Thus an underlying goal here

is to examine the functional role the TM potentially plays in determining the ear's ability to discriminate different frequencies.

4.1 SFOAE Delays and Tuning

A theoretical model inspired by the gecko ear (20) predicts a proportionality between SFOAE phase-gradient delay and the reciprocal of the auditory filter bandwidth (for example, as derived from ANF responses). In a nutshell, the model is a collection of coupled, tuned oscillators (manifesting a small degree of irregularity) that act as the underlying filters (43), (44). There is no direct TM coupling: longitudinal coupling comes entirely via the rigid papilla. The model assumes that the underlying auditory filters are second order, although the model predictions do not appear constrained by this assumption. More complicated filter assumptions can still lead to a prediction of proportionality between N_{SF} (phase-gradient delay) and Q (tuning bandwidth) (20). For second order filters, the model predicts that

$$N_{\mathrm{SF}} \approx 2Q/\pi = 6Q_{10\mathrm{dB}}/\pi \ , \tag{2}$$

where Q is the quality factor of the resonance of the filter and $Q_{10\mathrm{dB}}$ is derived from the bandwidth 10 dB from the peak response as is commonly reported in physiological measurements (see (1)). Although the model SFOAEs receive contributions from all the oscillators, the response (and subsequent rate of phase accumulation) is dominated by those tuned about the emission frequency. The basic intuition is that the more sharply tuned the filter is, the longer it takes to build up to steady–state and hence a sluggish response, or longer emission delay apparent via the phase–versus–frequency relationship.

As predicted by the model, bandwidth estimates derived from both ANF responses and SFOAEs have been shown to correlate well for *Gekko gecko* (20). Additionally, while the model does not directly distinguish the role of the TM, the model's prediction (i.e., $N_{\mathrm{SF}} \propto Q$) holds well in the non–TM species *Elgaria multicarinata* (20), indicating the model is applicable across a variety of species. Furthermore, the results shown in Fig.3 are strikingly similar to comparisons of tuning estimated directly from the auditory nerve for a variety of lizard species (see Fig. 4.17 in (35)). Lastly, estimates of tuning derived from SOAE suppression tuning curves (42), (24) also correlate well to low–level SFOAE estimates for geckos (20) and anoles (see *Supporting Material*).

The larger SFOAE phase-gradient delays observed in the majority of TM-species above $1.5 - 2$ kHz (Fig.3) are generally consistent with the hy-

pothesis that those species have sharper tuning. Several possible explanations could account for how the TM leads to sharper tuning. The increased mass due to loading by TM could lead to a reduction in the effective damping working against the hair cell bundle (5), (6). Longitudinal and radial coupling could also play a significant role in the mechanical response of groups of hair cells by concentrating the efforts of active hair cell bundles (e.g., (7), (8), (45)). Thus, the role of the TM in sharpening mechanical tuning could stem from both passive and active force considerations. Note that there are apparent exceptions to this rule, such as *Anolis* (non–TM, large delays) and *Aspidoscelis* (continuous TM, intermediate delays). These observations, indicate that increased coupling does not always result in sharpened tuning, as discussed further in subsequent sections.

As shown in Fig.3, it is difficult to distinguish differences in N_{SF} across species below 1–1.5 kHz where there is significant overlap. All species included in this study (except for *Eumeces*) have a TM connected back to the limbic lip that covers the low frequency portion of the papilla (25). Without further knowledge of how OAE generation mechanisms differ in the lower frequency portion of the papilla (where SOAE activity is not readily observable), one might reasonably expect N_{SF} to be similar across species at these lower frequencies. It is possible that there is source mixing (37) between generators in the low–frequency TM region (present in most species tested here) and the higher frequency region as outlined in Table 1. At sufficiently high enough frequencies, such mixing becomes negligible and a more apparent distinction can be made. It is worthwhile to note that the TM in the Scincidae family (including *Eumeces*) is unconnected to the limbic lip (25), (46). As such, for the only skink species examined here, N_{SF} sits above that for all other species at the lowest frequencies where SFOAEs were detectable.

The majority ($\sim$80%) of ears examined in this study also produced detectable SOAEs, consistent with previous studies (e.g., (24); see *Supporting Material*). However, low–level SFOAE activity was more readily produced than SOAE activity in most ears (i.e., when little or no SOAE activity was detected, SFOAEs were clearly present). This observation suggests that SFOAEs might provide a more robust probe into OAE generation mechanisms than SOAEs alone. For example, suppression tuning curves derived from SOAE peaks have been shown to correlate well to tuning curves derived from ANF responses (e.g., (42)), but SOAE peaks only manifest at certain frequencies, limiting their practicality. Tuning measures derived from SFOAEs, which appear to correlate well to those derived from SOAE suppression (see *Supporting Material*), can provide a more rapid measure with finer frequency resolution.

Within a given species, significant magnitude differences exist across individuals (Fig.1). This observation supports the hypothesis that individualized irregularity, such as manifest in bundle stiffness or variations in active force contributions, has functional consequences as observed in the OAEs (e.g., (47), (7), (20)). Such individualized differences presumably relate to the unique SOAE spectra measured in different individuals (see *Supporting Material*).

4.2 Mammalian/Non–Mammalian Differences

The functional role of the TM could be quite different between mammals and non–mammals. The demonstration that the mammalian TM is capable of propagating energy longitudinally along the length of the cochlea (10), coupled with the lack of a BM traveling wave in the lizard ear (27), (28), could point toward significant differences in the underlying mechanics between the two types of ears. For example, one proposed cochlear model (48) posits what essentially amounts to two coupled parallel transmission lines that trade energy back and forth to produce sharp and spatially–localized tuning as observed in mammalian BM responses. If such a model holds, the TM could potentially play a critical role in one of those paths (e.g., (11)). Given the absence of lizard BM traveling waves, such a model may be implausible for their ears. Thus, the TM's role towards creating a 'second resonance' (49) could be a feature specific to the mammalian cochlea.

The only species examined with a continuous TM, *Aspidoscelis*, exhibited relatively moderate delays (Fig.3). This observation, barring additional unknown morphological considerations at the micro-mechanical scale [e.g., differences in how the TM specifically attaches to the stereovillar bundles, non–uniform bundle bi–directionality (29)], suggests that stronger TM coupling does not always lead to sharper tuning. One possibility is that in the lizard, too strong of a coupling may be disadvantageous (32). Potential reasons could stem from both passive (e.g., strong coupling on papillae with steep tonotopic gradients would smear out responses) and active considerations (e.g., phase cancellations from active force contributions), similar in essence to differences between lizards and mammals discussed in the previous paragraph. Such a limiting effect could have led to the discretized nature of the TM in the salletal species, such as geckos who indeed appear to be auditory specialists amongst the lizards (32). Put another way, there may be an optimal amount of TM coupling that works to effectively balance active force contributions: too much or too little can ultimately lead to broader tuning. Such a notion would be consistent with recent observations

in the mammalian ear that indicated weaker longitudinal coupling via the TM leads to sharper tuning at the level of the BM (11).

Additionally, the morphological and mechanical properties of the TM itself might be very different in non–mammals. For example, the collagen–based fibrillar network in the mammalian TM (50), (51) that appears functionally important (11), (52) could take on a very different composition in the lizard TM. As noted by Miller, the skink TM (an individual sallet specifically) is 'thrown into complicated folds and twisted structures that are interconnected by both thick and very fine strands of material' (53).

One possible means to gain further insight into differences between mammalian and non–mammalian OAE generation mechanisms (and the subsequent role of the TM) could be a comparative study of SFOAEs across bird species. There is evidence to suggest that there is some degree of similarity in OAE generation mechanisms between chickens and humans (13), despite large morphological differences of the inner ear: chickens lack hair cell somatic motility (54), (55) and have a massive TM more firmly coupled to the apical surface of the basilar papilla (56).

4.3 Species Specifics

As shown in Fig.3, N_{SF} for the Tokay gecko (*Gekko*) sits well above the other species for frequencies $\geq$1–2 kHz. This species is the only one examined here known to extensively vocalize for territorial and mating purposes, save for *Eublepharis*, whose vocalizations are much more limited. In fact, Gekkonidae appears unique amongst lizard families in that it is the only one to possess elasticized vocal cords (57), (58), allowing them to make more spectrally–rich vocalizations than the 'hissing' observed in other lizard groups. Sharper peripheral tuning, as indicated by the larger SFOAE delays, could potentially provide significant benefits to *Gekko* for the perception of these vocalizations. Furthermore, the spectral content apparent in the SFOAE responses of Tokay geckos ((13), *Supporting Material*) correlates well with that of their vocalizations (57), where responses remain relatively flat up to $\sim$4–5 kHz and fall off sharply above. It has been proposed that geckos are similar to mammals (and birds) in that evolution has produced a dichotomy of hair cell types in the high frequency portion of their papilla (i.e., within the type II hair cell region): those that act as detectors (to send information to the brain) and those that act as amplifiers (to boost low-level stimuli) (59). It is presently unclear how such a distinction might extend across the Lacertilia, but further study of OAE properties should help determine if such a dichotomy is present in other lizard groups.

Of all species examined, emission magnitudes were smallest in *Callisaurus*. This observation is not surprising given their close relation to the *Holbrookia* and *Coposaurus* genera, also known as the 'earless lizards' due to their apparent lack of an external ear. While *Callisaurus* has a more pronounced external tympanum (similar to frogs) than *Holbrookia*, it nonetheless has reduced function relative to the external/middle ears of other species examined including other species in the *Phrynosomatidae* family. Thus the smaller magnitudes in *Callisaurus* presumably stem from poorer forward and reverse transmission via the middle and external ear (see (60)).

Relative to other non–TM species, anole (*Anolis carolinensis*) OAEs appear unique: robust SOAEs and relatively large SFOAE delays with emission magnitudes extending well out to higher frequencies. It is not presently clear why their delays are relatively larger: as Table 1 indicates, their papilla is roughly the same length and contains the same number of hair cells as the alligator lizard (*Elgaria*) whose delays are significantly shorter. Furthermore, there does not presently appear to be any outwardly obvious differences in the hair cells themselves across these two species (e.g., number of stereovilli in a given bundle (61), (62)), though *Anolis carolinensis* does have an enclosed external auditory meatus. It seems unlikely that the longer SFOAE delays in anoles are due to some other factor not associated with tuning, given that tuning estimates derived from SOAE suppression in a similar *Anolis* species are relatively large (24) and appear to correlate well to tuning estimates derived from SFOAEs (see *Supporting Material*). One possibility is that anoles (and possibly other non–TM species) have evolved such that the underlying amplification mechanisms (at the micro–mechanical level) could be enhanced relative to other species. Indeed, the *Anolis* genus is highly diversified and has served as an prime example of adaptive radiation in evolutionary biology ((63)). These observations suggest that anoles could serve as excellent models for future auditory research, given the relative simplicity of their papilla and robust OAEs, as well as potential applications stemming from the sequencing of the *Anolis carolinensis* genome (see (63)). Lastly, the anole OAE data presented here further reinforce the observation that reptile hearing, even in those with a relatively simple papilla, need not be confined to lower frequencies (i.e., below 5 kHz) (24).

Lastly, SFOAE magnitudes in *Pogona* fell off at lower frequencies (typically by ∼3 kHz) but exhibited relatively large N_{SF} values (Fig.3). While the inner ear morphology of *Pogona* is presently unknown, it may likely be similar to that of iguanids (i.e., minimal TM) (32). If this is the case, perhaps agamids are similar to *Anolis* in that they have evolved mechanisms to sharpen mechanical tuning despite the lack of a TM. Other possibilities:

Pogona has a more complex TM morphology, as is apparent in some agamids such as *Leiolepis belliana* (25) or that there is some additional source of delay present in their ears that does not contribute to tuning, as appears to be the case for the frog (13).

5 Summary

The present study demonstrates that features of SFOAEs, a non–invasive measure of auditory function, appear critically tied to local mechanical and morphological properties of the inner ear. Specifically, our results imply that the structure of the TM, in a region where the emissions are presumably being generated, likely play an important role in determining the observed OAE properties in lizards. A lack of a TM in general leads to shorter emission delays, while species with a TM (either continuous or discretized) typically exhibit longer delays. However, at least one non–TM species exhibits relatively large delays, suggesting that additional factors could be at work across species that can affect tuning (e.g., differences in an underlying active process). In light of theoretical considerations that hypothesize that TM–coupling can lead to sharper tuning and that SFOAE delays are inversely proportional to auditory filter bandwidths, our results generally support these predictions, but with the added caveat that there might be an optimal amount of TM coupling: too little or too much can lead to broadened tuning.

Given the significant differences that exist between the lizard inner ear and the mammalian cochlea, the functional role of the TM could very well be different between these two groups. Whereas in lizards the TM's primary role might be to help overcome viscous forces and thereby sharpen mechanical tuning, the TM in mammals could have the added role of coupling energy longitudinally along the length of the cochlea. Regardless, further understanding of emission generation mechanisms in non-mammals will inevitably lead to deeper insights into the function of the mammalian ear.

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Table 1. Species examined in the present study. Cited values are from [25] and [64] (the latter in parentheses). Where unknown, inferences based upon similar species are included (designated via ?). Family abbreviations as follows: Ag – *Agamidae*, Po – *Polychrotidae*, Ph – *Phrynosomatidae*, An – *Anguidae*, Sk – *Scincidae*, Gr – *Gerrhosauridae*, Gk – *Gekkonidae*, Te – *Teiidae*. Families Ag, Po and Ph all fall within infraorder Iguania. The designations non–TM, salletal, and continuous TM are meant simply to indicate the morphology of the TM over the majority of the papilla (i.e., for the bi–directional hair cells). All species except *Eumeces* have a continuous TM attached to the limbic lip overlying the portion of the papilla sensitive to frequencies below 1 kHz (see [26], [32]). For *Eumeces*, the TM over the low frequency portion of the papilla is unconnected to the limbic lip [46]. Note that for clarity, the TM morphologies listed here are a simplification; see [25], [29] for more detailed descriptions. Total hair cell counts in the last column are per ear. Data from species indicated by a * are from a previous study [13] while those with a † were animals locally native/wild-caught.

	Anatomical parameters			
Species (common name)	Family	TM type (≥ 1 kHz)	Papilla Length [mm]	# of hair cells
Anolis carolinensis (green anole)	Po	none	0.45 (0.5)	160 (182)
Aspidoscelis tigris† (whiptail lizard)	Te	continuous	0.65	370 (465)
Callisaurus draconoides† (zebra–tail lizard)	Ph	none	(0.2)	65 (73)
Elgaria multicarinata† (Southern alligator lizard)	An	none	0.4	160
*Eublepharis macularius** (leopard gecko)	Gk	sallets & continuous	1.25	970
Eumeces schneideri (Schneider's skink)	Sk	sallets	?	500?
*Gekko gecko** (Tokay gecko)	Gk	sallets & continuous	1.8	1620 (2100)
Gerrhosaurus flavigularis (yellow–throated plated lizard)	Gr	sallets	0.8?	530
Pogona vitticeps (bearded dragon)	Ag	?	?	?
Sceloporus magister† (desert spiny lizard)	Ph	none	0.35 (0.35)	80 (90)
Urosaurus ornatus† (ornate tree lizard)	Ph	none	0.29?	55
Uta stansburiana† (common side–blotched lizard)	Ph	none	0.22? (0.2)	52 (55)

FIGURE CAPTIONS

- Figure 1: Low-level SFOAE magnitude and phase (ϕ_{OAE}) for three representative individuals from each of three species chosen to highlight differences in TM morphology: **top-** non–TM (*Anolis*), **middle-** sallets (*Gerrhosaurus*), **bottom-** continuous TM (*Aspidoscelis*). A guiding schematic is included: grey shading denotes the presence of the TM, arrows illustrate bundle polarization (direction from shortest to tallest villi) for radially–clustered groups of hair cells, note the variability in orientation for *Aspidoscelis*). Different individuals are illustrated by different line shadings. SFOAE magnitude and phase (ϕ_{OAE}) were evoked using a 20 dB SPL tone. Error bars have been excluded for clarity (see *Supporting Material*). Stimulus conditions and steady-state body temperatures ($\sim$32–33° C) were identical for all curves. Dashed-dotted line indicates approximate noise floor.

- Figure 2: Low-level SFOAE phase-gradient delays, expressed in dimensionless form (N_{SF}). SFOAEs were evoked using a 20 dB SPL stimulus level with body temperature stable at $\sim$32–33° C. Only points whose magnitudes were at least 10 dB above the acoustic noise floor were included. Similar to Fig.1, different shadings represent different individuals. While significant spread is present, trends within a given species are apparent. Solid lines indicate a locally–weighted regression (*loess*) trend.

- Figure 3: Comparison of N_{SF} across 12 different species. Species lacking a TM over the majority of their papilla are denoted by dashed lines while TM–species by a solid line. Non–TM exhibit smaller N_{SF} values than those with a TM for frequencies above $\sim$1–2 kHz. All data were obtained using a 20 dB SPL stimulus level with body temperature stable at $\sim$32–33° C. For a given species, the illustrated curve was obtained via a locally–weighted regression (see Fig.2), the number of individual ears included is specified in parentheses. Only points whose magnitudes were at least 10 dB above the acoustic noise floor were included. Note that the innear ear morphology for *Pogona* is presently unknown.

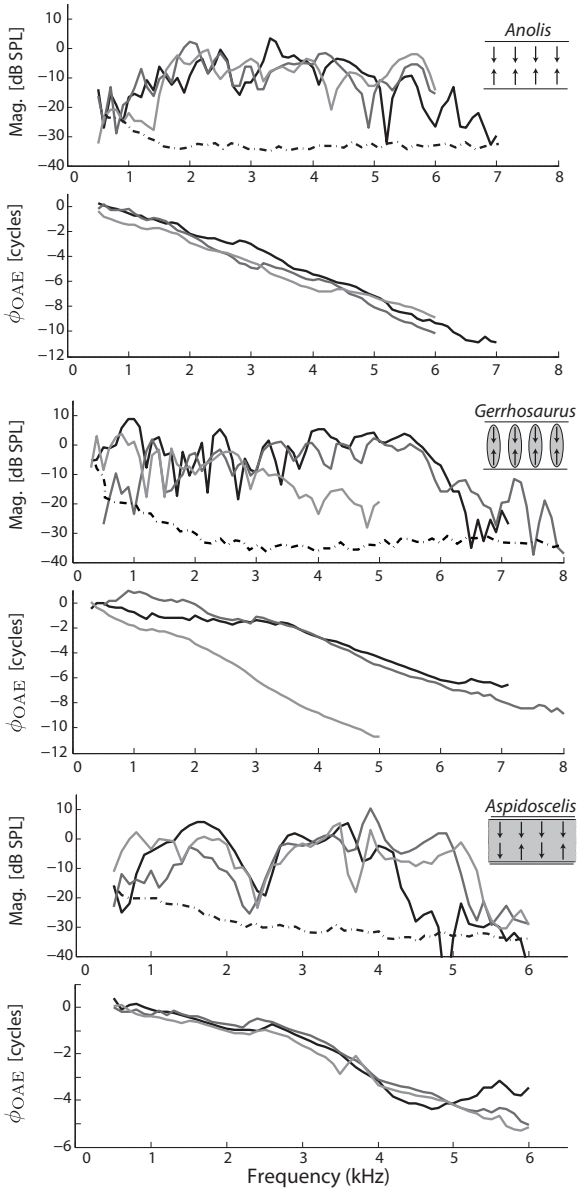
Anolis
Gerrhosaurus
Aspidoscelis
Mag. [dB SPL]
ϕ_{OAE} [cycles]
Frequency (kHz)

Figure 1:

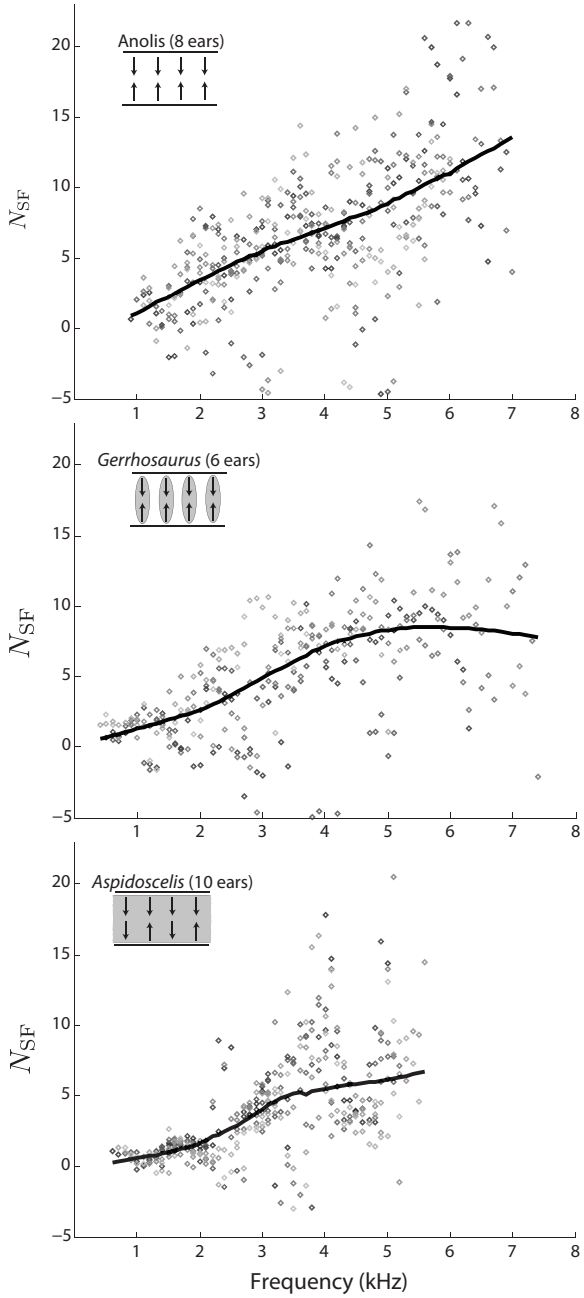
Anolis (8 ears)
Gerrhosaurus (6 ears)
Aspidoscelis (10 ears)
N_{SF}
Frequency (kHz)

Figure 2:

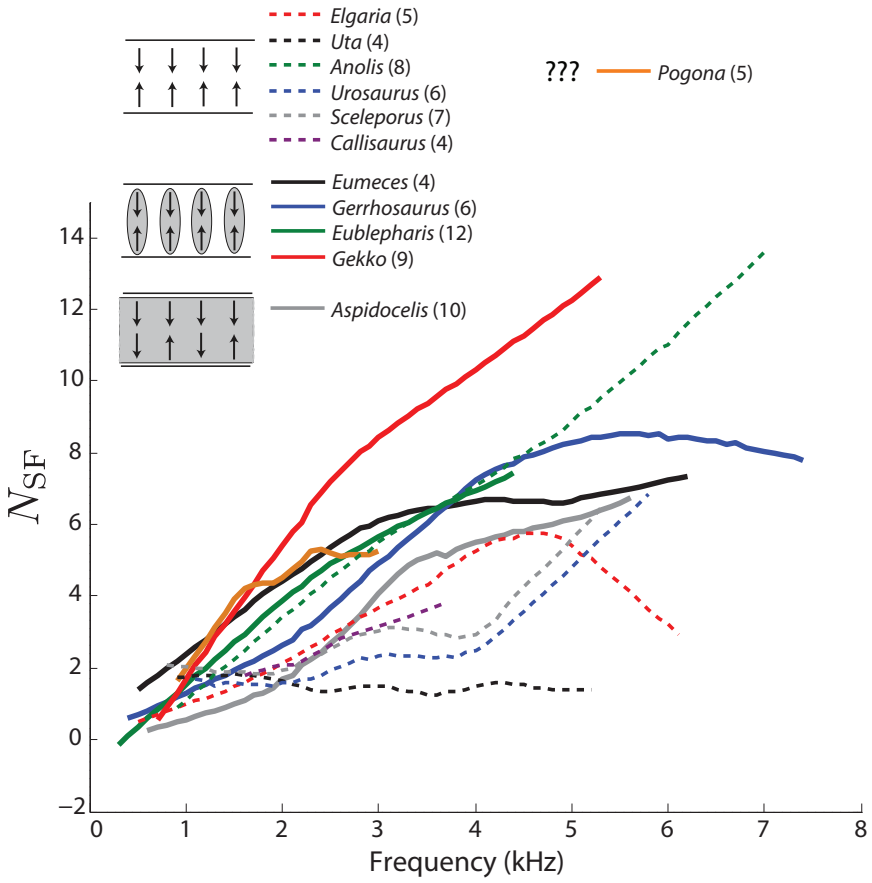
Elgaria (5)
Uta (4)
Anolis (8)
Urosaurus (6)
Sceleporus (7)
Callisaurus (4)
???
Pogona (5)
Eumeces (4)
Gerrhosaurus (6)
Eublepharis (12)
Gekko (9)
Aspidocelis (10)
N_{SF}
14
12
10
8
6
4
2
0
−2
0
1
2
3
4
5
6
7
8
Frequency (kHz)

Figure 3:

Supporting Material

NOTE: The material below supplements the article entitled *Tectorial Membrane Morphological Variation: Effects Upon Stimulus Frequency Otoacoustic Emissions* by Bergevin et al. (Biophysical Journal, 2010).

I – Spontaneous Emissions

As indicated in the main document, SOAEs were also measured during the course of these experiments. To summarize, there appears to be a clear correlation between SOAE and SFOAE activity. While further study is needed to better understand the inter–relationship between these two emission types in lizards, this correlation is presumably relevant to the primary thesis of the main document (i.e., peripheral mechanisms for tuning and the role of the TM in such).

Methods

For SOAEs, 60 waveforms (32768 sample window, SR= 44.1 kHz) were acquired and the FFT magnitudes averaged, either with or without a suppressor tone present. Despite the presence of external noise, SOAE activity could be readily distinguished in that it was both temperature–dependent as well as suppressible by a nearby external tone (41), (42).

Results

Spontaneous activity, as identified via temperature dependence and suppressibility due to external tones (41), (42), was apparent in the vast majority of ears examined (bottom black trace in Fig.4). Figure 4 also includes an SOAE spectrum with a 40 dB SPL tone present (grey trace) to demonstrate the resulting region of localized suppression due to the external tone (e.g., (41)). Spontaneous emissions commonly consist of *baseline activity* (a broad, suppressible plateau, (41), (42)) with several distinct, narrowband peaks atop it. However with the exception of the anoles, SOAE activity in the non–TM species (e.g., iguanids, anguids) comprised a suppressible baseline emission with only one or two (if any) distinct and relatively wideband peaks. Furthermore, SOAEs and low-level SFOAE magnitudes were correlated (Fig.4). A rise in SOAE activity at a given frequency correlated with an increase in SFOAE magnitude. However the converse is not always true: significant SFOAEs could be measured where no SOAE activity was detected.

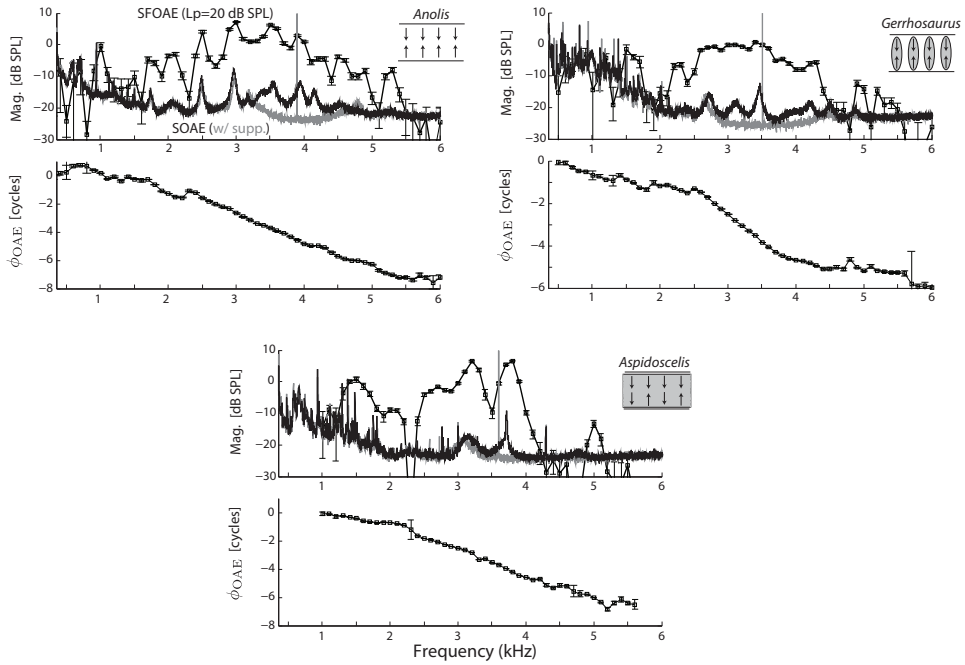

Figure 4: Correlation between SOAE and low–level SFOAEs. For each individual, SOAEs (with and without a 40 dB SPL suppressor tone, indicated by the grey and black traces respectively) are also shown with the SFOAE magnitude and phase (ϕ_{OAE}) evoked using a 20 dB SPL tone. SOAE measurements were stable before and after the SFOAE measurements. Error bars for SFOAEs denote the standard error of the mean. *Anolis* and *Gerrhosaurus* data shown here were at body temperatures ∼26–27° C (where SOAE activity in these species was observed to be more robust) while the *Aspidoscelis* data were obtained at ∼32–33° C (heating pad on). Spectral artifacts due to external acoustic noise (e.g., 4.2 kHz peak for *Aspidoscelis*) are distinguished from SOAEs by virtue of lack of temperature–dependence and suppressibility.

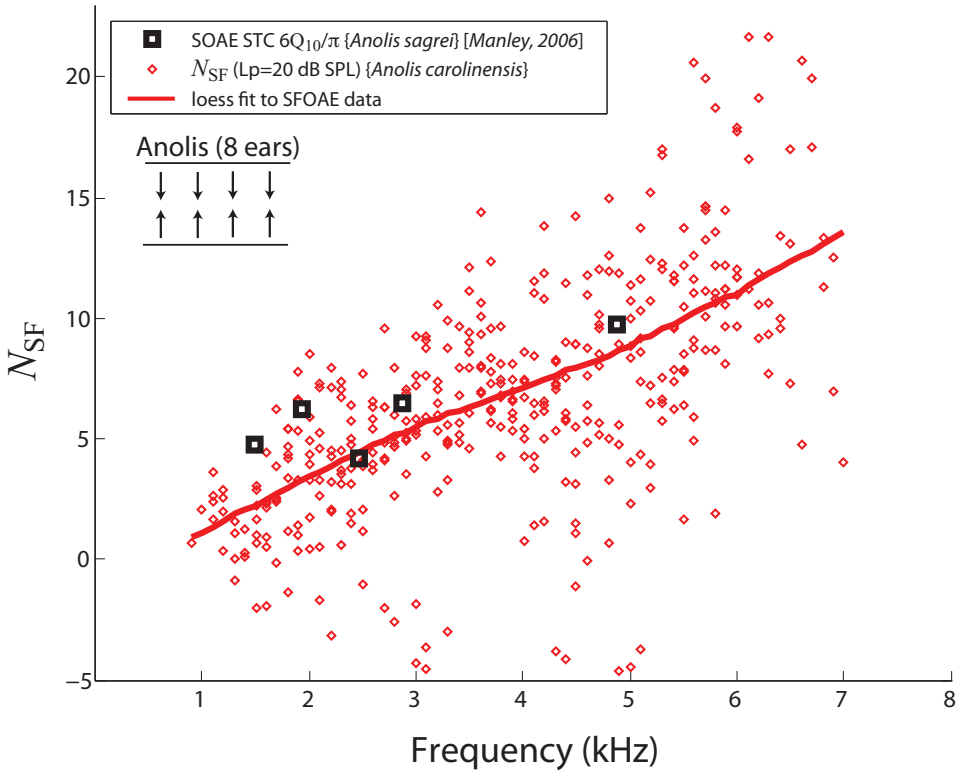

Figure 5: Correlation between tuning measures derived from SOAEs and low–level SFOAEs from two different species of *Anolis*. The SOAE data is from *Anolis sagrei* (24). From that study, which reported $Q_{10\mathrm{dB}}$ derived from SOAE suppression tuning curves, the plotted points (black squares) indicate the corresponding tuning estimate relative to that of the SFOAE via the model prediction (20) as indicated in the figure legend (Eqn.2).

Differences in SOAEs between TM and non–TM species are also apparent, consistent with previous studies (24), (65). While all species exhibit some degree of baseline activity, species with a continuous TM tend to exhibit fewer and more sharply tuned SOAE peaks while salletal species commonly exhibit more numerous and wider–band peaks. Non-TM species commonly produced one (and sometimes several) broad, smaller peaks, with anoles appearing to be an exception (as described below).

Shown in Fig.5 is two different otoacoustic estimates of tuning from a non–TM species (*Anolis*). The SOAE data comes from a different study (24), as well as from a different species (but the same genus).

Discussion

A novel feature indicated here is a degree of correlation between SOAEs and low–level SFOAEs in lizards (Fig.4), suggestive that the underlying emission generation mechanisms for the two OAE types are related (if not identical). Such a correlation between the two is remarkable in that it appears consistent with predictions from a standing model for the mammalian SOAEs (66), despite the absence of BM traveling waves in the lizard (27). Additional study is warranted of these interrelations between lizard SOAEs and SFOAEs to elucidate how generation mechanisms might be similar and different between mammals and non–mammals (e.g., (20)). Clearly the presence of a TM has some influence on SOAEs, (e.g., focusing baseline activity into distinct peaks), but little overall effect upon whether OAEs are ultimately present or not. As previously pointed out (24), this observation speaks to the robustness of the underlying generation mechanisms (e.g., hair cell bundle motility (67)).

It is worth noting that, as indicated previously (7), the external tone is probably not really suppressing per se. Clearly in the spectra there is a frequency–dependent region about the external tone where the SOAE amplitudes are reduced. However, the underlying generators are unlikely to simply stop oscillating (i.e., they are suppressed), but more likely shift their frequency to match that of the external tone (i.e., they are entrained). Thus, to some extent, SOAE *suppression* is likely a bit of a misnomer when specifically considering the dynamics of the underlying generators.

As shown in Fig.5, tuning estimates from both SOAEs (24) and SFOAEs appear to match up well via the model predictions (20). However, further study is warranted given that the comparison is made across two different species (which may manifest differences in peripheral tuning) and because of the relatively limited SOAE data shown here. Preliminarily, these data demonstrate that SOAE and SFOAE measures may reasonably be expected to yield similar estimates of tuning sharpness.

II – Additional SFOAE Data

Similar in nature to Figs.1 and 2, data from several other species is included for comparison in Fig.6.

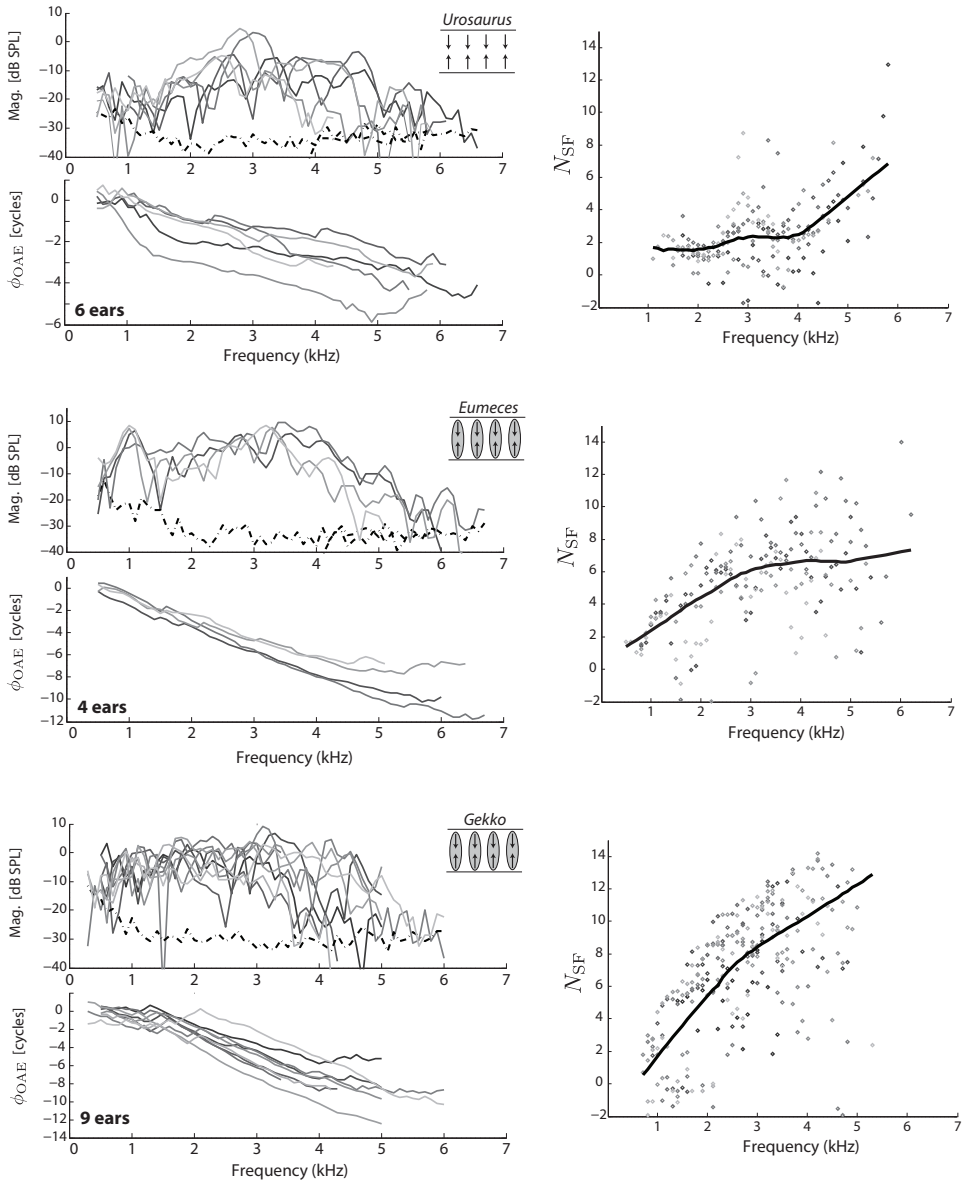

Figure 6: Comparison across individuals similar for three additional species: *Urosaurus ornatus* (top), *Eumeces schneideri* (middle), and *Gekko gecko* (bottom). Same parameters as described in the captions for Figs.1 and 2.