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Active auditory mechanics in female black-horned tree crickets (*Oecanthus nigricornis*)

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Compliance with ethical standards

Experiments were performed in compliance with ethical standards of the institution and national guidelines for the care and use of animals were followed.

Abstract

The acoustic signalling behaviour of many tree cricket species is easily observed and has been well described. Very little is known, however, about the receivers in these communication loops. The exception to this is a single Indian species (*O. henryi*) which employs active auditory mechanics to enhance female sensitivity to quiet sounds at male calling frequencies. In most species male calls have been described, but whether or not sender-receiver matching is present is uncertain. Here we investigate auditory mechanics in females of the North American black-horned tree cricket (*Oecanthus nigricornis*). The response of the anterior tympanal membrane is nonlinear, exhibiting a lack of tuning at high amplitudes (60 dB and above) but as stimulus amplitude decreases the membrane becomes tuned to around 4.3 kHz. The tuning of the membrane falls within the frequency range of male calls indicating sender-receiver matching at low amplitudes, which could aid localisation of the highly directional calls of males. The extent of active auditory mechanics in tympanal insects is not yet known, but this paper provides an indication that this may indeed be widespread in at least the Oecanthinae.

Introduction

Physical constraints on acoustic communication by small animals, such as insects, are well documented (Hoy et al. 1998; Robert 2001), but despite size-related limitations, insect auditory systems nevertheless are adapted to solve similar acoustic “problems” to vertebrate systems. They must detect, discriminate, and localize specific categories of acoustic signal. Insect systems have proved useful models for identifying general principles of auditory processing, independent of the specific auditory structures involved. Recent work has demonstrated that despite the vast disparity in the origins, development, and anatomy of insect and vertebrate auditory systems, there are some fundamental similarities, one of which is the presence of active amplification in some insect auditory receptors (Göpfert and Robert 2007).

Auditory receivers must respond to relevant environmental stimuli over a range of frequencies and amplitudes that can span orders of magnitude. To achieve this many animals have evolved a nonlinear auditory response, whereby the receiver shows increased sensitivity to quiet sounds and sensory cells selectively and actively tune into frequencies of interest. Auditory nonlinearities are known across all vertebrate groups (Manley 2000; Manley et al. 2008) and in insects have been found in mosquitoes (Göpfert and Robert 2001), *Drosophila* (Göpfert and Robert 2003) and the tree cricket, *O. henryi* (Mhatre and Robert 2013). The female *O. henryi* auditory system becomes selectively tuned into male call frequencies at low amplitudes, but at high stimulus amplitudes this tuning is lost. The system shows the characteristics of a critical oscillator: there is a compressive nonlinearity demonstrated by amplification of low amplitude stimuli; the tympanum shows tuned mechanical movement in the absence of external stimuli in the form of self-sustained oscillations (SOs); there is a change in frequency selectivity; and the system is physiologically vulnerable as its response becomes linear under anaesthesia or *post mortem*. These nonlinear active mechanics are required in order for the female to tune into male song because in its passive state the ear is not selectively tuned to any specific frequency. Both the function and underlying mechanism of active auditory amplification are dynamic areas of research and debate (Göpfert and Robert 2007; Lu et al. 2009). It is not yet clear how

widespread the phenomenon is among acoustic insects. There is some comparative work on the role of active mechanisms in species-specific variation in auditory function of Johnston's Organs in fruit flies (Riabina et al. 2011), but this variation between species has not been addressed in tympanal auditory systems, which are present in many insect taxa. To date, *O. henryi* is the only tympanal insect known to show the characteristics of an active auditory system and the question of how widespread this trait is in tympanal hearing insects is unknown.

Here, we probe morphology and auditory mechanics in the North American black-horned tree cricket, *O. nigricornis*; a species found in southern-central Canada and the northern United States living in shrubs predominantly of goldenrod (*Solidago* spp.), raspberry (*Rubus idaeus*) and other weedy plants (Walker 1963). In this species, multimodal communication (involving acoustic, olfactory and vibrational cues) is used by males in mate attraction (Bell 1980). Acoustic signals are important phonotactic cues for females and comprise a long-range call with a carrier frequency between 3-4.6 kHz depending on temperature (Sismondo 1979) and a courtship song used at close range which is less well described (Bell 1980). Like other tree crickets (Mhatre et al. 2009), *O. nigricornis* have tympanal ears located on the proximal tibiae of the forelegs and although calls (Brown et al. 1996), sound production (Williams 1945; Sismondo 1979; Sismondo 1993) and many features of sexual selection (see for example Brown 1999; Brown and Kuns 2000; Bussiere 2004) in this species have been described, the capabilities and morphology of the female receiver have not yet garnered attention. This paper therefore provides an analysis of the female auditory system as well as probing the extent of active auditory mechanics in the in Oecanthinae.

Using laser Doppler vibrometry and stimuli presented at a range of amplitudes between 66 – 36 dB sound pressure level (SPL, re 20 μ Pa) we examine the linearity of the anterior tympanic membrane (TM) to reveal amplitude-dependent amplification and frequency selectivity. We expect the best frequency of the female ear to fall within the range of male calls and also anticipate finding active auditory mechanics similar to those found in the Indian *O. henryi* (Mhatre and Robert 2013). We also

examine the presence of SOs in the absence of acoustic stimuli and the physiological vulnerability of the auditory mechanics in both CO₂ anaesthetised animals and *post mortem*. The morphology of the female anterior TM is also described using scanning electron microscopy.

Materials and Methods

Animals

Vibrometry and morphological measurements were carried out with both wild-caught and lab-reared adult female *O. nigricornis*. Wild-caught animals were collected from sites around Toronto, ON, Canada, between the 4th and 31st September, 2013, and maintained in communal chambers (42 x 30 x 24 cm) in the laboratory on crushed dry cat food, diced apple and water *ad libitum*. To rear animals in the lab, oviposited stems were collected in February 2014 and incubated at 26°C for 4 weeks. Once hatched, nymphs were moved into communal chambers and maintained as above.

Animals were mounted for laser vibrometry using the modified protocol from Mhatre et al. (2009; 2011). *O. nigricornis* were anaesthetised using CO₂ and mounted to a damped metal rod using liquid latex as described previously, but instead of using a balsa block or Blu-Tack (Bostik Ltd) to immobilise the femorotibial joint, wooden posts affixed to either side of the mount served to stabilise the leg and joint using dental wax. Animals were allowed to recover from anaesthesia for at least 30 minutes before any measurements were made.

Morphology

Forelegs of adult females were frozen and subsequently fixed in 2% gluteraldehyde before dehydration using an ethanol series (50%, 70%, 95%, 100%) and critical point drying (Polaron, Watford, UK). They were then sputter coated with gold (PS3, Polaron, Watford, UK) and imaged using scanning electron microscopy (Hitachi S530 SEM, Hitachi, Tokyo, Japan). Images were acquired and

digitised using Quartz PCI imaging software (Quartz PCI, Quartz Imaging Corporation, Vancouver, Canada).

Laser Doppler vibrometry

Vibration velocities of the anterior TM in adult female *O. nigricornis* were acquired using laser Doppler vibrometry. All vibration velocity measurements were carried out on a vibration isolation table (Newport VH3036W-OPT; Irvine, CA, USA) within an acoustic isolation booth (Eckel XHD-BATTEN; ON, Canada). A microscanning laser Doppler vibrometer (Polytec PSV 400; Waldbronn, Germany) with an OVF-505 sensor head fitted with a close-up attachment was used to acquire vibration velocities. Subsequent digitisation of the signal was performed by Polytec Scanning Vibrometry software (PSV 9.0; Polytec Inc., Waldbronn, Germany) via an on-board data acquisition card (NI PCI-6110, National Instruments, Texas, USA). Both microscans of multiple points across the entire anterior TM or measurement of the velocity at the single point of maximum deflection were possible.

Anterior TM frequency response

For scans and measurements of the membrane's frequency response the acoustic stimulus was generated by the PSV software (PSV 9.0) and consisted of a periodic chirp with a frequency range from 1.5-10 kHz. The signal was flattened to provide equal power output across all frequencies, amplified (US amplifier, Avisoft, Berlin, Germany) and subsequently played back through a loudspeaker (ScanSpeak, Avisoft, Berlin, Germany). The loudspeaker was positioned perpendicular to the length of the animal as illustrated in Mhatre et al. (2009). A reference microphone (4138, Brüel and Kjær, Nærum, Denmark) positioned 5 mm above the femorotibial joint was used to monitor the acoustic stimuli throughout the acquisition of anterior TM vibration velocities. The microphone signals was amplified (2610, Brüel and Kjær) and recorded using PSV software (Polytec PSV 9.0). Stimuli were played at 6 amplitudes between 66 dB sound pressure level (SPL) (40 mPa) and 30 dB SPL (0.6 mPa) at intervals of 6 dB.

Scans comprised at least 250 points sampling the area of the anterior TM and its surrounding cuticle. Longitudinal and transverse transects interpolating anterior TM deflection shapes across the membrane were produced in PSV (V 9.0). Single point measurements were made at the location of greatest anterior TM displacement to determine the membrane's frequency response. A sampling rate of 25.6 kHz and a bandwidth of 10 kHz were used in data acquisition, with a resultant resolution of 12.5 Hz. FFTs were computed using a rectangular window and averaged over 100 samples to improve the signal to noise ratio for each measurement.

After an initial set of measurements animals were enclosed in a plastic container (4 x 4 x 5.5 cm) fed by a trickle of CO₂ for 30 minutes before re-measuring anterior TM frequency response, using the same methodology as described above. Animals were then allowed to recover from CO₂ exposure for 45 minutes and, once again, anterior TM frequency response was measured. Frequency response was also measured post mortem, after *O. nigricornis* were euthanized by abdominal injection of 100% ethanol.

A transfer function between the anterior TM displacement, measured using the laser, and the stimulus sound pressure, from the reference microphone, was calculated as in (Windmill et al. 2005), along with coherence and phase between the anterior TM response and the input stimulus. Coherence between these two signals informs on measurement quality and how much of the measured displacement can be explained by the input stimulus.

Frequency response curves were fitted with a simple harmonic oscillator model (eq. 1) using the curve fitting toolbox in Matlab (2012b; Mathworks, Natick, MA, USA) to allow extraction of Q factor and resonant frequency. Only fits with an r^2 greater than 0.65 were used in analysis.

$$A = A_0 \omega_0^2 ((\omega_0^2 - \omega^2)^2 + (2 \zeta \omega_0 \omega)^2)^{1/2} \quad \text{Eq. 1}$$

Where A is the displacement of the membrane, ω is its angular frequency, A_0 is the anterior TM displacement where angular velocity is 0, ζ is the damping ratio and ω_0 the angular resonant frequency of the anterior TM.

Gain response

A 100 ms sine wave at the membrane's best frequency, as measured at 42 dB (2.5 mPa), was used to determine anterior TM gain response. The gain response was calculated using a transfer function between the input sound stimulus, monitored by the reference microphone, and the measured anterior TM displacement as described in (Windmill et al. 2005). The stimulus was generated in Matlab (2012b) and linearly increased in amplitude to a peak of 100 mPa and subsequently linearly decreased back to zero, bounded by 100 ms of silence. A sampling rate of 128 kHz and bandwidth of 50 kHz was used resulting in a resolution of 7.81 Hz. Envelopes for the time domain anterior TM vibration velocities were generated using the Hilbert transform in Matlab.

Self-sustained oscillations

The anterior TM was also measured in the absence of any stimuli to ascertain the presence of SOs. Anterior TM response was measured at the point of maximal deflection in the absence of acoustic stimuli using vibrometer settings described above for the gain response, recording for at least 12s. To induce SOs, repetitive acoustic stimulation and injection of ethanol or DMSO have previously been used (Göpfert and Robert 2001). Here, some animals were exposed to white noise with a bandwidth of 10 kHz for 30 minutes at 60 dB SPL. However, no difference in the rate of occurrence of SOs or in their amplitude was seen between animals measured with or without prior noise exposure. This may have been due to the several minutes it often took to position and focus the laser in the correct location on the anterior TM being too long to see any carried over effects. Data from animals with pre-measurement noise exposure and those measured without noise exposure were therefore pooled.

Where possible, animals were also measured immediately after injection with 100% ethanol and shortly *post mortem*.

Results

Membrane morphology and deflection

As in other tree crickets (Mhatre et al. 2009), *O. nigricornis* have 2 tympanic membranes on the tibia of each foreleg. The anterior TM is both longer and wider than the posterior TM (anterior TM length $770\ \mu\text{m} \pm 48$; width $204\ \mu\text{m} \pm 25$; $n = 13$; posterior TM length $536\ \mu\text{m} \pm 34$; width $77\ \mu\text{m} \pm 5$; $n = 5$) (Fig. 1). The anterior TM does not have a flat uniform surface. Distally, it has a groove that runs approximately 2/3 of the anterior TM length (Fig. 1a-c). This groove is met by several smaller ridges which adjoin perpendicular to the groove length. The proximal end of the anterior TM is predominantly flat, with 2 bumps located at the proximal end of the groove. Overall, it is similar but less pronounced than that described for *O. henryi* (see Mhatre et al. 2009). Above this furrow and the midpoint of the anterior TM there is a small ridge. The posterior TM shows far fewer features and in contrast is relatively flat, with indications of a slight furrow running longitudinally and ridges perpendicular to this (Fig. 1c and 1d). However, the features on the posterior TM are far smaller than those observed on the anterior TM.

Only a small portion of the anterior TM is maximally displaced by acoustic stimuli, and the magnitude of displacement is extremely low at around $140\ \text{pm} \pm 17$ ($N = 13$) at 4 kHz and 66 dB SPL (40 mPa) (Fig. 2d). Most of the membrane moves coherently with the sound stimulus, however, high coherence levels and maximal membrane deflection is restricted to a small region located on the lateral distal edge of the membrane just below the midline (Fig. 2a-c). From the coaxial camera used while performing laser vibrometry it is possible to see that maximal deflection corresponds with the area of the membrane bounded by the lateral furrow at the bottom and the small ridge at the top (Fig. 1a-c). In *O. henryi* this region is where the auditory sensillum is located (Mhatre and Robert 2013).

Active auditory mechanics: tuning

At stimulus amplitudes above 60 dB SPL (20 mPa) the displacement of the anterior TM shows a flat response across the frequency range examined (1.5 – 10 kHz) (Fig. 3a, red). The resonant frequency of the system is where displacement peaks, and correspondingly, the phase relationship between the input stimulus and displacement transitions through 0° , from a positive phase difference at frequencies lower than resonance to a negative phase difference at frequencies above resonance. The phase response of the anterior TM remains close to 0° , showing a cycle-by-cycle response with the stimulus, tailing off as frequency increases probably due to small frequency-dependent increases in the time delay for the signal reaching the reference microphone (Fig. 3b, red). There is no observed phase transition or displacement peak in the anterior TM and the system is not resonant. As stimulus amplitude is attenuated below 60 dB SPL the anterior TM becomes resonant with both a peak in displacement at $4.3 \text{ kHz} \pm 0.04$ (mean $\pm$ SD $n = 8$, $23^\circ\text{C} \pm 1^\circ$) (Fig. 3a, blue) and a phase transition emerging, becoming more sharply tuned as amplitudes decreased down to 36 dB SPL (Fig. 3b, blue). At stimulus amplitudes of 36 dB SPL the anterior TM has a Q factor of 2.8 ± 0.6 (mean $\pm$ SD $n = 8$, $23^\circ\text{C} \pm 1^\circ$) marking it as an underdamped resonant system. As well as peak displacement becoming sharper as amplitude is decreased, the phase response shows steeper transitions from a flat response at 0° at 66 dB SPL to a transition from near 55° to -55° across a bandwidth of around 1 kHz (Fig. 3b, red). This amplitude-dependent anterior TM tuning is not observed either under CO_2 anaesthesia (Fig. 3c and 3d) or *post mortem*, indicating physiological vulnerability of the tuning mechanism (Fig. 4b and 4d). However, post- CO_2 anaesthesia anterior TM responses recover to pre- CO_2 exposure levels (Fig. 4c). The anterior TM under CO_2 anaesthesia shows a modified frequency response lacking resonance at 4.3 kHz, but a small peak is present at around 3 kHz that is not observed prior to or post- anaesthesia.

Active auditory mechanics: gain and SOs

At the membrane's best frequency, the anterior TM of *O. nigricornis* displays a higher displacement gain for low stimulus amplitudes (36-42 dB) and a lower displacement gain for high stimulus

amplitudes (60-66 dB) (Fig. 5a and 5b). This gain is symmetric, i.e. both decreases in amplitude and increases in amplitude produce the same magnitude of gain. Gain is not seen *post mortem*, with anterior TM response envelopes closely following the input stimulus envelope (Fig. 5b). SOs can be seen in the absence of stimuli as a small peak in displacement ($5.17\text{pm} \pm 0.85$, $n = 5$) around the membrane's best frequency (4.3 kHz, Fig. 5c). These measurements, although small, were consistently above the noise floor as measured at 4 kHz ($2.19\text{pm} \pm 0.6$, $n = 3$). The amplitude of the SOs increased by an order of magnitude after injection with 100% ethanol, but *post mortem* no SOs were ever observed (Fig. 5d).

Discussion

The *O. nigricornis* anterior TM is displaced in response to acoustic stimuli in a localised manner. Only a small area just below the midline near the lateral distal edge of the anterior TM responds with high coherence and maximal displacement to acoustic input across the 1.5 - 10 kHz range. At amplitudes of 66 dB SPL the mean displacement of the anterior TM is merely 140 pm at 4 kHz, and above 60 dB SPL the system is non-resonant with a flat frequency response in both displacement amplitude and phase. However, at amplitudes below 60 dB SPL the system is resonant and a displacement peak at 4.3 kHz is observed in the anterior TM along with a corresponding phase transition. At low amplitudes the anterior TM displays gain, which is lost at high stimulus amplitudes. In anaesthetised animals and animals *post mortem* tympanal mechanics become linear and stimulus amplitude has no impact on tuning, which indicates a physiological input is necessary for the observed auditory nonlinearity.

Active auditory system

The nonlinear mechanics of the *O. nigricornis* anterior TM shows several of the characteristics of an active auditory system; it produces self-sustained oscillations in the absence of stimuli, there is a compressive nonlinearity whereby quiet stimuli produce an amplified response, it shows high frequency selectivity and is vulnerable to physiological changes. These characteristics are also seen in the Indian tree cricket *O. henryi* (Mhatre and Robert 2013) suggesting that these characteristics of active amplification may be widespread in auditory systems in the genus *Oecanthus*, and it remains to be seen whether other Gryllids show similar nonlinear and potentially active auditory dynamics.

Although not direct evidence in itself, the presence of SOs are compelling support that this system is active (for a review see Mhatre 2015). In *O. nigricornis* SOs show the same characteristics as SOs in other well characterised insect auditory systems such as *Drosophila* and mosquitoes (Göpfert and Robert 2001; Göpfert and Robert 2003); they are around the best frequency of the receiver, they increase in amplitude following ethanol injection and they are never seen *post mortem*. Although their amplitude is in the picometer range, they are observed above the noise floor of the measurement

system. Whether these motion-generating contributions to auditory mechanics in *Oecanthus* come from the periphery and the mechanosensory cells themselves or involve some feedback from the central nervous system is not yet known. In *Drosophila* it is the mechanosensory modules of transduction themselves that generate the auditory nonlinearities in their antennal ear (Albert et al. 2007), as well as the amplification and frequency response properties observed (Nadrowski et al. 2008). In vertebrates too, hair cells are motile and actively produce auditory nonlinearities (Manley et al. 2008). As the auditory scolopidia in *Oecanthus* lie near the position of maximal displacement on the membrane (Mhatre and Robert 2013), and as other active auditory systems employ motile mechanosensory cells that generate nonlinearities (Göpfert and Robert 2007), it is highly probable that mechanosensory cells are responsible for the motion generation in the tree cricket anterior TM. However, further experimentation is required before muscular contribution or efferent nervous mechanisms can be ruled out.

Although the anterior TM response at best frequency (4.3 kHz) is knocked out in CO₂ anaesthetised animals, there is a small peak at 3 kHz which is not present before or after CO₂ exposure. This peak may be due to incomplete anaesthesia of the animals or perhaps some other non-specific effect of the anaesthetic. As has been reported previously in *O. henryi* (Mhatre and Robert 2013), a smaller animal than *O. nigricornis*, full anaesthesia takes at least 30 minutes of CO₂ exposure. This is a long time, but may not be sufficient to completely switch off the active mechanics in all of the *O. nigricornis* measured here.

In the *O. nigricornis* anterior TM we did not see the 'off' state observed in *O. henryi* in the light phase of the light-dark cycle (Mhatre and Robert 2013). This was not, however, explicitly searched for, but auditory nonlinearities were seen in all animals measured regardless of phase of their light cycle. *O. nigricornis* males do call both during the day and the night, indeed wild animals used here were caught while displaying diurnally. Females respond to these calls both diurnally and nocturnally (personal observation) so it would appear that their auditory system should be switched on at all times.

Matching sender and receiver

The calls of male *O. nigricornis* vary in frequency between 3-4.6 kHz at temperatures of 15-32°C respectively (Sismondo 1979). The best frequency of the female ear as recorded at 42 dB SPL is 4.3 kHz at 23°C which falls within this range, matching receiver sensitivity to the senders call frequency. This however is only true at amplitudes below 60 dB SPL. Above 60 dB SPL the mechanics of the female tympana are non-resonant and show no selectivity for male calling frequencies over any other frequency measured. At these higher amplitudes the mechanics of the ear are passive, as demonstrated by their persistence when the animal is physiologically compromised (Fig. 3 and 4). In contrast, under normal physiology, low amplitude stimuli not only tune the ear into conspecific calls but do so with increasing gain, enhancing the female's ability to detect quiet sounds around a frequency of 4 kHz. The gain provided by the auditory nonlinearity at low stimulus amplitudes should allow females to detect males at a greater distance than would be possible with a purely passive auditory system. As male tree crickets produce calls from a dipole that is highly directional (Toms 1984; Forrest 1991) the amplification of quiet stimuli should also enhance the detection of males that are calling from angles projecting only minimal acoustic energy in the direction of the listening female. How the environment impacts signal detection in *O. nigricornis* and at what range males are audible to females is not yet known.

Male *O. nigricornis* call frequencies are temperature dependent varying by over 1 kHz with changes in temperature across a range of 15 °C (Sismondo 1979). This change is known to be generated by their elongate wing geometry and its physical properties (Mhatre et al. 2012). However, whether corresponding changes in the mechanics and neurophysiology of the ear maintain signal-receiver matching has yet to be established in *Oecanthus*. At amplitudes of 66 dB SPL the *O. henryi* membrane mechanics show no modification in response to a temperature range between 18 – 27 °C (Mhatre et al. 2011). At this amplitude, however, the ear is pushed into its passive regime without active contributions driving tuning or generating gain that can be detected from external measurements of membrane mechanics. In locusts only very slight increases in tympanal displacement occur as a result

of temperature changes of 6.5 °C (Eberhard et al. 2014), and cicadas showed no change in tympanal frequency response across a frequency range of 18-35 °C (Fonseca and Correia 2007). It is known however, that insects do show changes in neural spike rates, neuronal sensitivity and temporal characteristics in response to temperature (see for example Wolf 1986; Oldfield 1988; Fonseca and Correia 2007; Korsunovskaya and Zhantiev 2007; Eberhard et al. 2014). As *Oecanthus* has an active auditory system, it is possible that the tuning of the membrane will be modified by temperature especially if the mechanosensory neurons are the drivers of this nonlinear behaviour. Indeed, the mosquito *Culex quinquefasciatus*, which has an active auditory system, shows temperature-dependent tuning of self-sustained oscillations in an antennal ear (Warren et al. 2010). However, it remains to be seen whether the mechanics of the *Oecanthus* membrane modify frequency tuning in a temperature-dependent manner, thus matching signal to receiver across a range of temperatures.

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Figure legends

Figure 1. SEM images of the *O. nigricornis* tympanal membranes. **a** Anterior TM taken from the left leg. (**b** and **c**) Close up image of sections of the anterior TM. **b** Proximal area of anterior TM, **c** distal area of anterior TM. (**d** and **e**) Posterior TM. Distal lies to the right side of the image. **e** Close up of posterior TM surface. Scale bar in **a** and **d** is 200 μm , in all other images scale bar is 100 μm . In figures **a** to **c** the distal end of the tibia is located in bottom right of each image. In figures **d** and **e** the distal end of the tibia is to the right-hand side of the image.

Figure 2. **a** Coherence values in anterior TM. **b** Vibration of the anterior TM, frontal view. **c** Deflection shapes along longitudinal and transverse transects of the anterior TM. Inset shows location of transects on the membrane. **d** Mean ($\pm$ std; N = 13) displacement of membrane at the point of maximum deflection at 4 kHz and 66 dB SPL (40 mPa). Inset image of anterior TM shows location of measurements.

Figure 3. Sensitivity (top, **a** and **d**), phase (middle, **b** and **e**) and coherence (bottom, **c** and **f**) in live *O.nigricornis* (left column (**a-c**); 36 dB SPL n = 9; 66 dB SPL n = 13) and post mortem (right column (**d-f**); 36 dB SPL n = 7, 66 dB SPL n = 11). Blue 36 dB SPL, red 66 dB SPL.

Figure 4. Anterior TM sensitivity (upper panel) and phase (lower panel) responses over a range of amplitudes in a single individual (F13) before any treatment **a**, after 30 minutes bathing in CO₂ **b**, 45 minutes post-CO₂ exposure **c** and immediately after death **d**. Red line is highest amplitude (66 dB SPL, 40 mPa) and blue is lowest amplitude (30 dB SPL, 0.6 mPa).

Figure 5. Auditory gain in female *O. nigricornis*. **a** Response envelope showing anterior TM vibration (red) and stimulus (black dotted). **b** Mean of 5 responses showing auditory gain in a single individual. Measurements from live animal (red) and post mortem (black). Grey lines are individual recordings. **c** Self-sustained oscillations (SOs) in live (red) and *post mortem* (black). **d** SOs post-injection with 100% ethanol (grey) with live and dead as in **c** for scale comparison.

A **B** **C** **D**