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1 **Auditory mechanics in a bush-cricket: direct evidence of dual sound inputs in the pressure**
2 **difference receiver.**

3

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15

16

17 Short running title: “pressure difference auditory mechanics“

18 Summary

19 The ear of the bush-cricket *Copiphora gorgonensis* consists of a system of paired eardrums
20 (tympana) on each foreleg. In these insects, the ear is backed by an air-filled tube, the acoustic
21 trachea (AT), which transfers sound from the prothoracic acoustic spiracle to the internal side of
22 the eardrums. Both surfaces of the eardrums of this auditory system are exposed to sound, making
23 it a directionally sensitive pressure-difference receiver. A key feature of the AT is its capacity to
24 reduce the velocity of sound propagation and alter the acoustic driving forces at the tympanum.
25 The mechanism responsible for reduction in sound velocity in the AT remains elusive, yet it is
26 deemed to depend on adiabatic or isothermal conditions. To investigate the biophysics of such
27 multiple input ears, we used micro-scanning laser Doppler vibrometry and micro-computed X-ray
28 tomography. We measured the velocity of sound propagation in the acoustic trachea, the
29 transmission gains across auditory frequencies, and the time-resolved mechanical dynamics of the
30 tympanal membranes in *Copiphora gorgonensis*. Tracheal sound transmission generates a gain of
31 ~15 dB SPL, and a propagation velocity of *ca.* 255 m/s, a ~25% reduction from free field
32 propagation. Modelling tracheal acoustic behaviour that accounts for thermal and viscous effects,
33 we conclude that reduction in sound velocity within the acoustic trachea can be explained, amongst
34 others, by heat exchange between the sound wave and the tracheal walls.

35

36

37 Key words: Tympanum, sound processing, katydid, bush-cricket, sound propagation, acoustic
38 trachea

39

40 1. INTRODUCTION

41

42 For the majority of animals endowed with tympanal ears, incident pressure waves act on the
43 external surface area of thin and compliant tympanal membranes. Bush-crickets (Orthoptera,
44 Ensifera, Tettigoniidae) have pairs of eardrums for each ear, located within their forelegs. Instead
45 of acting only on the external surface of the eardrums membranes, sound pressure acts on both the
46 external and internal surfaces [1-7]. The internal acoustic input is enhanced by an air-filled tube,
47 the acoustic trachea (AT), that conveys sound from an opening on the side of the thorax (the
48 acoustic spiracle) to the internal side of the eardrums [1-5, 8]. The AT is a gradually narrowing
49 pipe that extends forwards from the thorax through into the fore femoral cavity until it reaches the
50 femoro-tibial joint (the knee), whereupon it enters the tibia and divides into two branches, an
51 anterior feeding the anterior tympanal membrane (ATM) and a posterior branch connected with the
52 posterior tympanal membrane (PTM) (see figure 1 for relative position of the tympana) [7-9]. Each
53 tracheal branch leads to one tympanal membrane, and the dorsal part of the anterior branch
54 harbours the ear mechanoreceptors (known as the *crista acustica*, CA) [10-12]. Dorsal to this area,
55 between the two tympana on both sides of the tibia, lies the auditory vesicle, a fluid-filled cavity
56 that encapsulates the CA [13]. Both tracheal divisions merge again below the tympanal membranes
57 where the trachea narrows and ends right beneath the ear [9]. Each eardrum is placed against the
58 outer surfaces of these tracheal divisions, creating the only place in the system where both sides of
59 the tracheal wall are coupled to the outside air. Hence, both internal and external surfaces of the
60 tympanal membranes are readily driven by sound waves travelling through the AT and by sound
61 waves reaching the membrane externally.

62 It is broadly accepted that the AT is the main acoustic input of the ear of many Tettigoniidae
63 species [2, 7, 14-17]. However, for the subfamily Pseudophyllinae (a large group with some 1000
64 species described) the acoustic spiracle is reduced (a character used as diagnostic for this
65 subfamily) and the bulla is replaced by a small chamber [18-20] and in some species the AT forms
66 a large U-shape bend at the bulla site [6]. Although poorly understood, in Pseudophyllinae, the AT
67 is unlikely to be the main acoustic input, and some authors suggest that the tympanal slits might
68 play important role as waveguides [18, 20].

69 It is also agreed that in some species, the AT looks and functions like an exponential horn,
70 increasing the magnitude of sound pressure acting on the internal side of the tympanal membranes
71 [1, 5, 7, 14]. This gain-enhancing role is associated with the size of the spiracular opening and its
72 associated bulla [5, 21]. The enhancement of the internal pressure acting on the back surface of the
73 tympanal membranes is deemed to provide this auditory system with directional sensitivity (see
74 below) [22, 23]. Some researchers argue that this exponential horn exhibits high-pass, high-gain
75 characteristic to provide a broadband response necessary for acoustic reception [7].

76 The AT is thought to play a vital role in the formation of the pressure difference mechanism. A
77 pressure difference receiver relies on the interference of sound waves at either surface of the
78 tympanal membranes [23-25]. The internal sound pressure of sound waves travelling in the AT,
79 undergoes different degrees of attenuation or amplification and some phase shift as a result of
80 alterations in propagation velocity [23]. Phase shifts are produced because pressure waves push the
81 tympanic membranes externally and internally, but also by differences in the time of arrival of
82 sound waves on both surfaces caused by alterations in propagation velocity inside the AT. These
83 changes in the propagation velocity result from the fact that the AT seems to impose resistance to
84 sound propagation [24], effectively slowing down sound travelling through the AT compared to
85 the sound waves travelling in the surrounding air and reaching the external side of the tympanum
86 [4, 5, 26]. This time delay has been observed as a gradual change in the phase of the tympanal
87 membrane vibrations and is particularly prominent at high frequencies [5]. The internal sound
88 propagation can also be measured in the time domain when the ear is stimulated with pure-tone
89 pulses. The impulse mechanical response should therefore exhibit two distinct vibrational events,
90 which reveal the sound wave arriving twice at the tympanum, once externally and once internally.
91 The difference in time lag appears to depend on leg position with respect to the sound source [4].
92 In summary, in a pressure difference receiver, a combination of both phase shift and amplitude
93 difference is likely to take place, and to affect the ipsilateral and contralateral ears differentially [4,
94 23, 26].

95 Researchers have had differing opinions about the AT, its role in a pressure difference receiver
96 ear and its effect on sound propagation. Discrepancies on the AT/pressure difference receiver ear
97 might have arisen from different approaches, and techniques used over time (some invasive and
98 deemed less appropriate [5, 7]), and from using different species, most of which communicate with
99 broadband calls. Low propagation velocities of sound inside the AT (about 75% of sound velocity
100 in air) are well documented in two species of field crickets [4, 25]. Previous studies provided clear
101 evidence that sound propagation velocity is reduced within the AT of bush-crickets [27]. Yet, to
102 date, the biophysical mechanisms of sound propagation within the AT, its potential dimorphism, its
103 effects on spectral auditory sensitivity and on auditory mechanics remain elusive.

104 Here, we study *Copiphora gorgonensis* (Conocephalinae, Copiphorini), a neotropical bush-
105 cricket species that communicates by producing sharply-narrow-band pure-tone calls with a carrier
106 frequency centred at 23 kHz [28]. Like most Conocephalinae, males and females possess large
107 acoustic bullae and narrow tracheae [13, 29], suggesting that the acoustic trachea might function as
108 an exponential horn [1], therefore enhancing the internal pressure driving the tympanal membrane.
109 If this is the case, vibrations of the tympanal membranes of individuals placed in the acoustic free
110 field should show pressure and temporal differences produced by pressure waves acting on both
111 sides of the tympana. Our hypothesis is that the internal sound input is significantly delayed
112 compared to the sound wave following the external pathway to the tympanal membranes.

113 Following on our previous work [13], we also test the hypothesis that the ear of *C. gorgonensis*
114 works as a pressure difference receiver that is effective over a broad range of frequencies.

115 Using micro-scanning laser Doppler vibrometry (LDV), micro-computed tomography (μ -CT),
116 and an experimental platform permitting the controlled acoustic isolation of both internal and
117 external inputs, we performed rigorous measurements of tympanal vibrations to quantify acoustic
118 gain, temporal delay and spectral characteristics of sound propagation in the AT.

119

120 Our results demonstrate that the ear of *C. gorgonensis* functions as a pressure difference receiver.
121 We show that internal sound input through the acoustic trachea is significantly delayed due to a
122 25% reduction in the propagation velocity of sound. This tracheal input also contributes a nearly
123 fourfold gain as compared to the external input. The gain and propagation velocity are comparable
124 to those found in other Orthoptera. The possible mechanisms at work in acoustic trachea
125 responsible for the decrease in propagation velocity are discussed.

126

127 **2. METHODS**

128 *2.1. Experimental animals*

129 We used 21 *Copiphora gorgonensis* individuals (10 males, 11 females). This species is endemic to
130 the island of Gorgona, Colombia, located off the south-western Colombian Pacific coast. Males
131 call females in the low ultrasonic range using a pure-tone, short-duration pulse (8 ms) at 23 kHz
132 [28]. Specimens were collected as nymphs in their natural habitat and maintained in captivity in
133 cages at 25 °C, LD 11h: 23h and 70% RH, where they were fed on a mix of pollen and dry cat food
134 until they reached adulthood.

135

136 *2.2. Morphological studies of the acoustic trachea*

137 The anatomy of the bush-cricket ear and the AT was examined using X-ray μ -computed
138 tomography (μ -CT) and 3D reconstruction using standard biomedical imaging software following
139 the protocols of Montealegre-Z et al. [13]. Four specimens (two males and two females) were
140 scanned with a Bruker SkyScan 1272 (Bruker microCT, Kontich, Belgium) at 100 kV, 36 μ A and
141 with a 0.5 mm thick aluminium filter, resulting in a voxel size of 11 μ m. Reconstruction and
142 automated measurements of acoustic tracheae were carried out with Amira (v. 5.4, VSG, Berlin,
143 Germany) and results further processed in Matlab (R2014a, The MathWorks, Inc., Natick, MA,
144 USA). In addition, tracheal lengths of 20 individual (9 males and 11 females) were measured by
145 inserting a thin human hair from the spiracle to the middle of the tympanal area. The insertion of
146 the hair could be easily monitored visually through the semi-transparent leg cuticle and tympanal
147 membranes.

148

149 *2.3. Induction of tympanal vibration*

150 Tympanal vibration in response to sound was studied using two different approaches: 1) Both
151 surfaces of the tympanal membranes were exposed to sound by placing the specimen in the
152 acoustic free field, and 2) the effect of multiple sound pathways was studied by generating internal
153 and external sound inputs independent of each other.

154 1) The specimen was mounted in a bespoke holder and placed in the acoustic free field. The
155 holder consists of a movable plate (with copper wires to secure the legs) screwed on an elbowed
156 arm (for more details see Montealegre-Z et al. [13]). The forelegs were oriented forwards, in a
157 position akin to the bush-cricket standing on a leaf. The holder was solidly tethered on a vibration
158 isolation air table holding a LDV (Polytec PSV-300-F; Waldbronn, Germany). A loudspeaker
159 (ACR, FT 17H, Fostex, Tokyo, Japan; or an ESS AMT-1, ESS Laboratory, Inc., Sacramento, CA,
160 USA) was positioned 30 cm away, ipsilateral at 90° with respect to the body axis of the animal,
161 playing periodic chirps in the range of 1-50 kHz. Computer controlled correction of the acoustic
162 stimulus was used to maintain constant amplitude levels (60 ± 1.5 dB SPL, re 20 μ Pa) at the
163 tympanum across the whole frequency range. Broadband signals were generated at 512 kHz by the
164 LDV internal data acquisition board (National Instruments PCI-4451; Austin, TX, USA), amplified
165 (TAFE570; Sony, Tokyo, Japan) and passed to the loudspeaker. The velocity of the tympanal
166 membrane vibrations was measured using the LDV with an OFV-056 scanning head fitted with a
167 close-up attachment (Polytec; Waldbronn, Germany). Tympanal vibrations were analysed by
168 simultaneously recording the vibration velocity of the tympanum, and the sound stimulus
169 amplitude and frequency at the tympanum and at the spiracle entrance. Data quality was assessed
170 using coherence for each data point [30]. Data were considered of sufficient quality when
171 coherence exceeded 80%.

172 All sound pressure measurements were carried out with two 1/8" precision pressure
173 microphones (Bruel & Kjaer, 4138; Nærum, Denmark) and a preamplifier (Bruel & Kjaer, 2633).
174 The microphones were calibrated using a sound level calibrator (Bruel & Kjaer, 4231). Recordings
175 were sampled at either 512 kHz or 1 MHz.

176 In addition to broadband stimuli, the tympana were also stimulated with 4-cycle pulses at 23
177 kHz (50 Hz burst rate), produced by a function generator (Agilent 33120A, Agilent Technologies
178 UK Ltd., Edinburgh, UK) synchronized with the LDV. The microphone was carefully positioned
179 near the measured tympanum until the phase of the microphone signal and that of the tympanum
180 displacement matched. Tympanal vibrations were recorded from both anterior and posterior
181 tympanal membrane (ATM and PTM, respectively). The instantaneous phase of the stimuli and
182 responses was calculated using Hilbert transform to identify any discrepancy in phase between
183 both signals.

184 2) Specimens were mounted on a custom-built platform, which provides acoustic isolation
185 between the two main sound inputs of the bush-cricket ear (see figure 1 and supplementary
186 information for details).

187 Sound was delivered locally at the spiracle and at the tympanum [5] using a custom build probe
188 loudspeaker (figure 1b, see [13] for details). The combination of the probe loudspeaker offering
189 high acoustic impedance and the platform's front panel as an acoustic barrier was sufficient to
190 effectively attenuate high-frequency sound and allow for focal acoustic stimulation.

191 Using this setup, tympanal vibrations in response to broadband chirps (5-50 kHz) and 23 kHz
192 4-cycle tones broadcast by the probe loudspeaker at the spiracle were recorded from both ATM
193 and PTM using LDV. The spectrum of the output of the probe loudspeaker was mathematically
194 flattened using the B&K microphone as a reference placed 2 mm away from the probe tip ([13]).
195 We calculated the FFT of the transfer function between the stimulus and tympanal response to
196 obtain the phase spectrum.

197

198 *2.4. The transmission gains of the trachea*

199 We calculated AT gain (as in [5]) from broadband stimulation, and from time domain recordings
200 using 4-cycle pure-tones at specified frequencies. The response of the tympanum to both types of
201 stimuli was measured using a focal sound source [5, 24] delivering sound at the external surface of
202 the tympanum while isolating the tracheal input (figure 1b), and using a probe loudspeaker
203 delivering sound at the acoustic spiracle only (figure 1c). For broadband stimulation, we adjusted
204 the sound pressure of the output at 0.02 Pa (~60 dB SPL) as measured at 2 mm away from the
205 probe's tip. We then positioned the probe loudspeaker either at 2 mm away from ATM or PTM, or
206 at 2 mm away from the spiracle. Tracheal gain was quantified as the difference in tympanal
207 displacement (using LDV) between external and tracheal stimulation.

208

209 *2.5. Statistics and analysis*

210 We compared differences in log tympanal tuning, tracheal time delays between tympana, across
211 individuals, and between sexes using a restricted maximum likelihood linear mixed-effects model
212 (LMM) in R (v.3.2.1, [31]) using the *lmerTest* package [32]. For a detailed description, see
213 supplementary materials.

214

215 **3. RESULTS**

216 *3.1. Anatomical measurements of acoustic trachea*

217 The geometry of the tracheal system was studied using μ -CT, while also evaluating tracheal
218 length using an inserted human hair. The reconstructed 3D models of the acoustic trachea do not
219 reveal sexual dimorphism in their general appearance. However, as an effect of body size, tracheal
220 tubes are slightly, although not statistically significantly, longer in females: females ($17.239 \pm$
221 0.724 mm, $n=22$ [11 left and 11 right]), males (16.272 ± 0.7412 mm: LMM: $t=-3.07$, $d.f.=2.87$,
222 $p=0.058$). Levine test ($F=0.05$, $P=0.956$) shows that the variability in left and right measurements
223 between males and females is statistically not significantly different. There was no significant

224 difference between right and left trachea on its own ($t=0.45$, $d.f.=2.84$, $p=0.685$) or in interaction
225 with sex ($t=0.37$, $d.f.=2.84$, $p=0.735$).

226 Tracheal morphology in *C. gorgonensis* is typical for conocephaloid bush-crickets [2, 8, 29].
227 The oval spiracle opens into an ovoid tracheal atrium, the acoustic bulla (figure 2a, b, c). Past the
228 bulla, the trachea narrows quickly into a thin tube (figure 2b, c, d). Using 3D μ -CT models, we
229 measured the internal AT radii at 25 μm intervals for both left and right tracheae in 2 males and 1
230 female *C. gorgonensis*. The radius of the trachea varies along its length, progressively narrowing in
231 the first half of its length, after which it stays relatively constant until approaching the ear (figure
232 2c, e, f). The mean radii for left and right tracheae ranged from 169 μm to 185 μm (with SD
233 ranging from 72 μm to 90 μm), while the median values lay between 138 μm and 151 μm (see
234 figure 2e, f and supplementary material table S-1).

235

236 *3.2. Frequency and time domain responses of the tympanal membrane in acoustic free field*
237 *conditions*

238 Specimens were tethered in the acoustic isolation holder and the vibrational responses of
239 tympana to broadband sound chirps from the ipsilateral side were recorded with the LDV. In all
240 cases, the response frequency spectrum of both tympanal membranes was broad across the
241 measured range. However, both tympanal membranes vibrate with higher amplitudes to
242 frequencies around the frequency of the call (~ 23 kHz, figure 3a, b). Measurement quality and
243 reliability for each measurement point was high as estimated using magnitude-squared coherence
244 [30], in particular for frequencies around 23 kHz (Figure 3c, d). There was no difference between
245 frequency tuning (at maximum spectral response) between ATM and PTM across all specimens
246 (LMM: $t=-0.81$, $df=20$, $p=0.4305$). There was, however, a significant negative relationship
247 between tympanal tuning and tracheal length (LMM: $t=-2.73$, $d.f.=17$, $p=0.014$), and a trend for this
248 pattern to sex (LMM: $t=2.06$, $d.f.=17$, $P=0.055$). Similarly, there were no significant differences in
249 the tuning of the tympanal membranes between males and females (LMM: $t=-2.06$, $df=17$,
250 $p=0.055$, females $=22.92$ kHz \pm 4.18 kHz, $n=22$; males 24.41 kHz \pm 3.874 kHz, $n=20$). On average,
251 across all specimens, both tympanal membranes showed best response at 23.63 kHz \pm 4.06 kHz,
252 $n=42$, (figure 3a, b).

253 Recordings of tympanal membrane vibrations were also obtained in the time domain. For these
254 experiments, the specimens were mounted as above, and the loudspeaker placed at 30 cm
255 ipsilateral and perpendicular to the body axis delivering 23 kHz 4-cycle tones at a constant sound
256 pressure of 1 Pa. These experiments were designed to provide direct evidence of the pressure
257 difference system, evaluating the ratios of the magnitudes of the sound pressures acting on the
258 internal and external surfaces of the tympanum. Tympanal vibrations are generated by sound
259 acting from the inner and outer sides of the tympana (figure 4). LDV recordings show that the
260 tympanal vibration is composed of two parts, a segment of low amplitude, and a subsequent part

261 with high amplitude (figure 4e). The low amplitude oscillations represent the free field sound
262 waves acting directly on the external surface of the tympanal membrane (black segment, figure
263 4e), while the high amplitude oscillations are the response to sound waves travelling from the
264 acoustic spiracle via the AT and acting on the internal surface of the membrane (red segment). The
265 latter response betrays the presence of sound waves pushing the membrane in a direction opposite
266 to that exerted by sound acting externally. Hence, this mechanical response is the result of sound
267 acting externally in addition to the vibrations produced by sound acting internally. The exact
268 moment of collision between the two oscillations was identified applying Hilbert transformation to
269 the tympanal displacement to compute the phase information in the time domain [33]. Because the
270 microphone was carefully adjusted as to have the same vertical axis as the tympanal membrane of
271 interest, both the recorded stimulus and the vibration of the tympanal membrane exhibited similar
272 phase during the first sound cycles (figure 4d, e and f). Some short time after stimulus onset, a
273 clear change in phase of the mechanical response is observed (figure 4e, f, asterisk and orange
274 dashed arrow). This phase change indicates the arrival of sound at the internal side of the tympanal
275 membrane (see supplementary material, Video 1). After this moment, the phase of the
276 displacement stays constant in relation to that of the stimulus until the phase difference amounts to
277 ca. 200° (figure 4f). Because sound propagation inside the trachea is delayed, the first cycle of the
278 stimulus signal, in phase with initial tympanal vibration (black trace 1 and 2 in figure 4e), takes 62-
279 80 μ s to strike the tympanal membrane on the internal surface. Thus, the signal arrives at the
280 tympanal membranes twice, with the second arrival delayed by tens of microseconds (figure 4f,
281 orange dashed line). In specimens measured within a free sound field (without the sound isolation
282 platform), the time delay varies with distance and azimuth of the sound source in relation to the
283 spiracle and the position of the ear. Cycles of tympanal vibrations corresponding to the external
284 sound arrival are nearly 6 times (5.53 ± 1.35 , range 3.87-7.72; 14.85 dB, n=21, measured at the
285 ATM only) quieter than their respectively shifted cycles coming from the trachea (figure 4e).
286 These findings highlight the role the acoustic bulla and trachea play in sound amplification, in this
287 case enhancing gain by nearly 15 dB.

288

289 3.3. Velocity of sound propagation in the trachea calculated from the frequency and time domains

290 3.3.1 Calculations in the frequency domain

291 Periodic chirps in the range of 1-50 kHz were delivered to the spiracle using a calibrated probe
292 loudspeaker continuously monitored with a reference microphone. Acoustic phase at the spiracle
293 and subsequently at the tympanal membrane was evaluated in response to stimulation at the
294 spiracle. In general, phase changes linearly with frequency (figure 5). In the low frequency range
295 (5-10 kHz), the phase at the tympanal membrane changes slowly (by less than half a cycle $\sim 120^\circ$)
296 with respect to the phase at the spiracle. This shows that low frequency sound propagates in the
297 trachea with minor impediment. In contrast, at 23 kHz phase changes by nearly 500° (460° - 490° in

298 figure 5a, b, male and female), revealing a phase shift of nearly 1.3-1.4 cycles. At 23 kHz, such
299 shift corresponds to a time delay of approximately 60 μ s. At 40 kHz, the phase change is about
300 840°-860°, or 2.3-2.4 cycles, also corresponding to approximately 60 μ s duration. Calculations in
301 the low frequency range, such as 10 kHz, concur and show that sound propagation time is around
302 58 to 60 μ s.

303 This analysis therefore suggests that sound velocity inside the trachea remains constant across
304 the frequency range measured.

305

306 3.3.2 Calculations in the time domain

307 The velocity of sound propagation inside the trachea was established by measuring the time lag
308 between the onset of the stimulus delivered at the spiracle and its time of arrival at the tympanal
309 membrane (figure 5c, d). At 23 kHz, we measured a propagation time between 60.60 and 82.00 μ s,
310 with a mean of $66.37 \pm 4.79 \mu$ s (ATM, mean \pm SD, n=21); and 60.20-81.20 μ s, mean of $65.96 \pm$
311 4.59μ s (PTM; n=21) (figure 5c, d). There is no significant difference in the time of arrival at each
312 tympanum (LMM: $t=-0.48$, d.f.=17.99, $p=0.637$). Across specimens, the propagation time
313 calculated was not statistically different from the time lag calculated from the phase spectrum at 23
314 kHz (LMM: $t=-0.93$, d.f.=18.41, $p=0.364$). In addition, neither sex (LMM: $t=-1.17$, d.f.=22.53,
315 $p=0.254$) nor the interaction between sex and time lag were significant (LMM: $t=1.19$, d.f.=18.45,
316 $p=0.249$). At other frequencies (10, 15, 20, 30, 40 and 50 kHz), the transmission time remains
317 constant ($63.50 \pm 1.36 \mu$ s, n=21, PTM only). Altogether, these results agree with the linear
318 response between phase and frequency described in the previous section (see also figure 5a, b).

319 Using this acoustical information and tracheal dimensions, the velocity of sound propagation
320 was calculated as ~ 255 m/s and found not to differ between right and left trachea (right= 255.2
321 $\text{m/s} \pm 18.5 \text{ m/s}$, n=21; left= $255.8 \text{ m/s} \pm 14.9 \text{ m/s}$, n= 21; SE= 7.17, LMM: $t=-0.51$, d.f.=20.09,
322 $p=0.616$). The overall average velocity of sound propagation in the trachea was 255.5 m/s (n=42).
323 The velocity of sound propagation was slightly, but not significantly, higher in the female tracheae
324 ($261.3 \pm 7.9 \text{ m/s}$, n=22) than in the male tracheae ($249.1 \pm 21.1 \text{ m/s}$, n=22; LMM: $t=-0.53$,
325 d.f.=19.31, $p=0.602$).

326 Overall, these results show that sound velocity inside the trachea is reduced in relation to that in
327 free field conditions by a factor of 1.35, and confirms figures obtained from the frequency analysis
328 (section 3.5), as well as early work on other species [4, 5, 27].

329

330 3.4. Tracheal gain calculations

331 Tracheal gain functions were measured using both broadband stimulation and time domain
332 responses to 4-cycle pure-tones. The gain was determined by calculating the difference in
333 tympanal deflection between external and tracheal stimulation. The stimulus was presented using
334 equivalent sound pressures either at the external surface of the tympanum or at the acoustic

335 spiracle entrance. In response to broadband stimulation, the gain function reveals that most of the
 336 gain occurs between 15 and 35 kHz (figure 6). Within this range the gain increases from 4.2 to
 337 10.6 (i.e., 12.5 and 20.5 dB), as measured at the ATM (figure 6). This finding demonstrates that the
 338 AT performs amplification of sound pressure across a range of frequencies.

339

340 4. Discussion

341 4.1. The velocity of sound transmission in the acoustic trachea

342 Sound propagates slower inside the AT (~255 m/s), a velocity comparable to those inferred in
 343 field crickets and bush-crickets (~260 m/s) [4, 27]. In field crickets, tracheal conduction was
 344 shown to enable directional hearing by imposing resistance to sound propagation with respect to
 345 sound acting on the external surface of the tympanal membrane [24, 26, 34]. In bush-crickets,
 346 species with large thoracic spiracle and large acoustic bullae, such as *C. gorgonensis* (figure 2)
 347 [35], the tracheal signal is amplified within a specific range of frequencies, and also incurs a time
 348 lag between sound acting on the external and internal sides of the tympanum [4, 5] (figure 6).
 349 Additionally, the size of the auditory spiracle is positively correlated with hearing sensitivity [15,
 350 19, 36]. Functionally, the progressive reduction in AT radius (figure 2) has been proposed to act
 351 like an exponential horn that enhances sound pressure at its thin end [2, 7, 14]. Our data support
 352 the exponential horn model from earlier work [5], suggesting that in bush-crickets the propagation
 353 velocity of sound can be seen as largely independent of frequency and therefore non-dispersive.
 354 The mechanism responsible for the reduction of sound propagation velocity remains, however,
 355 unknown.

356 It has been long demonstrated that the velocity of sound in a solid tube is greatly reduced when
 357 the tube becomes narrow compared to the wavelength of sound, or frequency increases [37, 38].
 358 Interestingly, the empirical values reported here, although in good accord with earlier results [4,
 359 27], are clearly lower than those predicted from conventional equations for the propagation of
 360 sound velocity in narrow tubes.

361 For comparison, we used the following two different approximations of Kirchhoff's solution on
 362 the acoustical propagation velocity v developed by Benade [37] and Zwicker & Kosten [39],
 363 respectively:

$$v = c \cdot \left(1 - \frac{1}{r_v \sqrt{2}} - \frac{\gamma - 1}{r_t \sqrt{2}} \right) \quad (1)$$

364 where c is the sound velocity in free field air (343 m/s), r_v and r_t terms for the viscous and thermal
 365 boundary layers (see supplementary material, table S-2) and γ the ratio of specific heats (1.4).

366 Notably, the velocity term can be expressed as

$$v = \frac{c}{\text{Im}(\Gamma)} \quad (2)$$

367 with the propagation constant Γ as

$$\Gamma = \frac{J_0(i^{\frac{3}{2}}r_v)}{\sqrt{J_2(i^{\frac{3}{2}}r_v)}} \cdot \sqrt{\frac{\gamma}{n}} \quad (3)$$

368 where J_0 and J_2 are Bessel functions of 0-th and 2-nd order and n constitutes a term that approaches
 369 1 for $\gamma \rightarrow 1$ (see supplementary material, table S-2 for more details).

370 Using both Benade's (Eq. 1, [37]) and Zwikker & Kosten's (Eq. 2 & 3, [39]) approximations
 371 with standard values for the properties of air and a median trachea radius of 150 μm at 23 kHz, one
 372 finds propagation speeds of 318 m/s and 320 m/s respectively (supplementary material, table S-2).
 373 There could be several reasons to account for the discrepancy between the results found here and
 374 in the acoustic literature.

375 Firstly, although the equations of Benade [37] and Zwikker & Kosten [39] include the effects of
 376 viscous and thermal boundary layers (r_v and r_t , respectively) within the tube, the general
 377 assumption is that of an adiabatic system where no heat is exchanged with the surroundings.
 378 Interestingly, Fletcher [40] argues that the adiabatic assumption in narrow tubes is only valid for
 379 radial frequencies ω

$$\omega \gg \frac{\pi\kappa}{a^2} \quad (4)$$

380
 381 where κ is the thermal diffusivity ($\sim 1.9 \cdot 10^{-5} \text{ m}^2/\text{s}$) and a the tube radius and $\omega = 2\pi f$. Using 23 kHz
 382 as frequency f and 150 μm as tube radius in Eq. 4 results in ω being roughly 50 times bigger than
 383 the right hand side of Eq. 4. Although Fletcher does not state a definite cut-off value for the
 384 transition from isothermal to adiabatic, it is reasonable to assume a rather gradual transition from
 385 low-frequency isothermal to high-frequency adiabatic conditions. If so, the system described here
 386 could be viewed as isothermal, then a variation of Zwikker & Kosten's equation can be used,
 387 where $\gamma=1$ [38], which changes Eq. 3 to:

$$\Gamma = \frac{J_0(i^{\frac{3}{2}}r_v)}{\sqrt{J_2(i^{\frac{3}{2}}r_v)}} \quad (5)$$

388
 389 Substituting Eq. 5 in Eq. 2 results in a propagation velocity within the tube of 276 m/s, much closer
 390 to our experimental values (see also table S-2).

391 A second possible explanation for our relatively low propagation speed can be found in the nature
 392 of the classical equations underlying the analytical approximations used here. Although basic
 393 properties of the medium (like density, viscosity and ratio of specific heats) and dimensions of the
 394 tube are taken into account, no terms for the elasticity (like Young's modulus or bulk modulus) and
 395 thickness of the tube walls are considered. Insect tracheae are very thin and quite unlike the rigid
 396 structures assumed previously. Considering these additional material properties and others within
 397 the system (like internal pressure, changes in composition and humidity of the gas mixture, etc.)

398 may provide more accurate understanding of isothermal and adiabatic sound propagation inside
399 tracheal systems.

400

401 4.2. *The tracheal transmission gain*

402 In response to 4-cycle pure-tone stimuli, tympanal membranes undergo vibrations that are, in
403 linear terms, 4-6 times larger for internal sound pressures than for external ones, a response that
404 varies with frequency. Such response gains range from 12 to 16 dB, as calculated from time
405 domain and broadband measurements.

406 We have shown here that sound pressure amplification is a result of sound travelling inside the
407 gradually narrowing AT. The data reveal that pressure amplification originates from internal sound
408 pathways (figure 4) in *C. gorgonensis*, suggesting that each ear, working as a pressure difference
409 receiver, can independently process directional information. Although not the scope of this study,
410 the reported dependence of tympanal vibrations on the azimuth of sound incidence in other cricket
411 species [22, 24] supports the presence of a similar mechanism in *C. gorgonensis*. Operating at
412 ultrasonic frequencies, however, these ears may also exploit instantaneous phase relationships
413 between the ATM and PTM within a single ear, essentially offering the possibility that each ear is
414 directional. Ultrasonic frequencies such as that of the species calling song may generate diffractive
415 effects around the ears and the tympanal flaps and result in differences in phase of vibration
416 between the tympanal membranes of a single ear [42]. The functional morphology of such ears,
417 potentially exploiting 6 distinct acoustic inputs, remains to be studied in detail, especially
418 questioning the role of tracheal transmission and microacoustical diffraction in the biophysics of
419 auditory directionality.

420

421 5. Conclusions

422 Quantifying the acoustic transmission characteristics of the AT in *C. gorgonensis*, we present
423 direct biophysical measurements of the mechanisms at work in an auditory pressure difference
424 receiver. Both spectral and time-resolved measurements presented here allow for a deeper
425 understanding of this widespread form of auditory system, the pressure-difference receiver.

426 A pressure difference receiver relies on the interference of sound waves at both surfaces of the
427 tympanal membranes [23-25]. We demonstrate here the existence of two pressure wave fronts -the
428 differential pressure waves- their relative timing and effect of their superposition on tympanal
429 vibrations. Carried out over a broad range of frequencies, the temporal analysis of these two waves
430 demonstrates that sound travels inside the AT at a constant velocity and thus non-dispersively (as
431 found by [27]). This behaviour does not comply with the theoretical frequency dependence of
432 propagation velocities in narrow tubes. This deviation from theory reveals an interesting functional
433 characteristic of this sound transmission system, as it allows for a spectrally broadly tuned system

434 to reliably transmit finely resolved undistorted temporal and spectral informational content to the
435 ears' receptive structures. Minimal dispersion in effect serves the coherent transfer of the
436 spiracular acoustic input to the internal face of the tympanum where it interacts with the external
437 and original version of itself. Biophysically, it is presumably advantageous for this auditory system
438 to produce interference between signals void of frequency dependent delays or distortions. In this
439 sense, preserving both the spectro-temporal characteristics and temporal patterns of the species-
440 specific narrow-band song may facilitate the delicate frequency decomposition process carried out
441 by the ear of *Copiphora* [13]. It can be argued here that similar demands exist to preserve the
442 coherence of multiple acoustic inputs when they originate from the environment of other signalling
443 species.

444 It has been suggested that pressure difference receivers operate only at low frequencies and low
445 internal amplifications [43], with the consequence that for higher frequencies and amplification,
446 the system would operate more like a conventional pressure receiver, yet dominated by large
447 internal pressure input [7, 17, 44]. However, such a proposal only considers the actual
448 amplification through tracheal propagation (12-20 dB over the frequency range from 5-50 kHz in
449 this case) and does not take into account the additional level of mechanical amplification that
450 results from the lever-like energy transfer between the tympanum and the tympanal plate [13].
451 Even minuscule tympanal displacements in response to low amplitude acoustic stimulation
452 produce large deflections of the *crista acustica* surface, comparable or larger than those of the
453 tympanum (but see fig. 5 in Montealegre-Z & Robert [29]). The increased sound pressure
454 produced by tracheal amplification acting on the inner tympanal surface increases this effect and is
455 most likely dependent on wave diffraction at the position of the spiracular opening. In the same
456 way, external sound waves will be diffracted by the animal's cuticular flaps covering the ears that
457 will affect their impact on the vibration of the tympanal membranes.

458 Since pressure difference receivers are inherently directional due to the differential phase and
459 amplitude components of the two incident sound waves [24], the high amount of amplification
460 generated by the AT suggests that *Copiphora* could use these differential inputs to perform
461 accurate localization of sound sources. If this is indeed the case, the importance of diffraction at
462 both the spiracle and the cuticular ear flaps and the sensitivity of the system to directional signals is
463 currently still unknown.

464

465

466 **Authors' contributions.** F.M-Z., T.J. and D.R. conceived and designed the experiments. F.M-Z.
467 and T.J. performed the experiments. KR-B performed μ CT scans and processed X-ray images for
468 further analysis. F.M-Z, T.J. and C.S. analysed data. C.S. designed all the statistical models. T.J.,
469 D.R. and F.M-Z. wrote the manuscript. All authors reviewed the manuscript.

470

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482

483 **Figure caption**

484 **Figure 1.** Experimental setup. (a) Frontal view of the isolating platform. (b) Setup used to
485 stimulate the ear using tracheal input only. The probe loudspeaker is placed at 2 mm away from the
486 spiracle. The LDV records tympanal vibrations, while a microphone positioned at ear location
487 monitors that sound from the probe loudspeaker does not cross the isolating panel. (c) Setup used
488 to occlude tracheal input. A sound-attenuating cylinder is assembled at the posterior side of the
489 platform, enclosing the body region containing the spiracle. A microphone is inserted inside the
490 cylinder to monitor sound entering the chamber; a syringe needle allows balancing atmospheric
491 pressure inside. A probe loudspeaker is positioned near the tympanum for external sound delivery.

492

493 **Figure 2.** Anatomy of the acoustic trachea measured using μ CT. (a) Frontal view of a male *C.*
494 *gorgonensis* with head, legs and thorax in transparency showing the AT. (b) Lateral view of the
495 body in transparency showing left and right AT. (c) Close up view of the acoustic spiracle and
496 bulla.

497 (d) Internal view inside the acoustic trachea. (e) and (f) Quantitative relationship between tracheal
498 diameter and length, displayed from the acoustic spiracle to the tympanal organ area in a male and
499 a female, respectively.

500

501 **Figure 3.** Tympanal vibrations in respond to broadband stimulus in free-field conditions, shown as
502 the average spectrum ATM (a) and PTM (b), measured across 21 individuals (10 males and 11
503 females). (c) Coherence plots of ATM vibration. (d) Coherence plots of PTM vibration

504

505 **Figure 4.** ATM motion in response to free-field pure-tone stimulation. (a-b) Orientation image
506 relating ear topography to the position of the scanning lattice. (c) Vibration map of the ATM
507 response measured as displacement. Deflections are shown for different phases along the

508 oscillation cycles (numbers match the cycles shown in *d* and *e*. Note that the tympanal plate (as
 509 described in [13]) is not included in the scan. (*d*) 23 kHz 4-cycle tone played at ca. 1 Pa. (*e*)
 510 Tympanal vibrations recorded with LDV. Initial dashed line represents sound arriving at the
 511 exterior tympanum surface. The red trace shows tympanal motion with additional internal acoustic
 512 tracheal input. (*f*) Phase analysis of tympanal response. The interference between external and
 513 internal inputs results in a significant change in phase at 81 μ s. This phase shift is also apparent
 514 from the change of the otherwise sinusoidal membrane displacement (red asterisk in *e*). The
 515 oscillation marked with number 1 in the microphone trace in *d*, and in the laser trace in *e*,
 516 corresponds to the oscillation marked with 1* in *e*. (*g-i*) Average stimulus, response, and
 517 instantaneous phase (as shown in panels *d-f*) measured on the left ATM across 11 females.

518

519 **Figure 5.** Tracheal sound propagation, frequency and time domain analysis. (*a*) ATM and PTM
 520 response to broadband stimulation for a male and a female. (*b*) Phase spectrum of the response
 521 highlighting the phase lag at 23 and 40 kHz. (*c-d*) Vibration of the tympana in response to sound
 522 (23 kHz, 4-cycle tone) travelling through the AT only. (*c*) Oscillograms showing the stimulus
 523 recorded at the spiracle entrance of a male and a female. (*d*) Mechanical response of both tympanal
 524 membranes in the same individuals. The response is notably delayed in each case (shaded areas) in
 525 relation to the microphone onset as sound propagates through AT.

526

527 **Figure 6.** Gain measurements across the spectral range. (*a*) ATM response in females (n=11).
 528 (*b*) ATM response in males (n=10). Black outline shows the tympanal response to external input
 529 only. Red trace shows tympanal response when sound is delivered at the acoustic spiracle and
 530 transmitted via the AT only. Shaded areas indicate standard deviation in both cases (n=11
 531 females).

532

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