

Active auditory mechanics in mosquitoes

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In humans and other vertebrates, hearing is improved by active contractile properties of hair cells. Comparable active auditory mechanics is now demonstrated in insects. In mosquitoes, Johnston's organ transduces sound-induced vibrations of the antennal flagellum. A non-muscular 'motor' activity enhances the sensitivity and tuning of the flagellar mechanical response in physiologically intact animals. This motor is capable of driving the flagellum autonomously, amplifying sound-induced vibrations at specific frequencies and intensities. Motor-related electrical activity of Johnston's organ strongly suggests that mosquito hearing is improved by mechanoreceptor motility.

Keywords: active sensation; bioacoustics; cell motility; insect ear; mechanotransduction; otoacoustic emissions

1. INTRODUCTION

Cochlear amplification, the active enhancement of the ear's mechanical input, is a salient feature of vertebrate audition. Through their self-sustained motion, the auditory hair cells of vertebrates actively amplify the sound-induced vibrations to which they also respond. This positive mechanical feedback sharpens the ear's passive resonant mechanics, and thus provides the basis for sensitive and frequency-selective hearing (reviewed by Dallos 1992; Ruggero 1992; Yates *et al.* 1992; Hudspeth 1997; Nobili *et al.* 1998). While a comparable active amplification process has been suggested in insects (Moran *et al.* 1977; Coro & Kössl 1998; Kössl & Boyan 1998*a,b*), direct mechanical evidence for active audition—such as autonomous vibration—was lacking. Consequently, the prevailing view was that insect hearing organs are purely passive, with the auditory response being determined by the ear's mechanical properties alone (French 1988; Field & Matheson 1998; Hoy 1998; Eberl 1999; Yager 1999).

In mosquitoes, as in vertebrates, hearing organs mediate the detection of sounds at particular frequencies and low intensities (Tischner 1953; Belton 1974; Clements 1999). Mosquitoes hear with their antennae. Serving as a sound receiver, the distal elongated flagellum of each antenna vibrates in response to particle oscillations in the sound field (figure 1) (Clements 1999; Göpfert *et al.* 1999). The flagellum is suspended by its base in the second segment of the antenna (the pedicel, figure 1). When stimulated acoustically, it vibrates as a forced damped harmonic oscillator (Göpfert *et al.* 1999). Accordingly, the flagellum is resonantly tuned and acts like a mechanical frequency filter (Göpfert *et al.* 1999; Göpfert & Robert 2000). The pedicel at the base of the antenna contains the sensory apparatus proper, Johnston's organ (figure 1). This complex chordotonal organ (Field & Matheson 1998) consists of thousands of radially arranged, multicellular mechanoreceptor units, each composed of two or three sensory neurons and two auxiliary cells (Risler & Schmidt 1967; Boo & Richards 1975*a,b*; Clements 1999). In total, about 7500 sensory neurons are contained

within the organ in females (Boo & Richards 1975*b*), and about 15 000 are found in males (Boo & Richards 1975*a*). We have previously shown that in both sexes Johnston's organs are sensitive to nanometre-range displacements of the flagellum (Göpfert & Robert 2000). *A priori*, there was no reason to expect active mechanics in mosquito hearing, but in the course of investigating its mechanical properties, this is what we found.

In vertebrate hearing, active auditory mechanics manifests itself in characteristic ways. These include: (i) a metabolic vulnerability of sensitivity and tuning, which in turn depend on the physiological condition of the animal; (ii) a mechanical nonlinearity that is also metabolically vulnerable; (iii) reversible effects of hypoxia on the mechanical response; and (iv), most compellingly, the occurrence of autonomous vibrations that can be measured in the absence of external acoustic stimulation, commonly known as spontaneous otoacoustic emissions in vertebrates (Dallos 1992; Ruggero 1992; Yates *et al.* 1992; Yates 1995; Hudspeth 1997; Nobili *et al.* 1998). We used these four key features as operational criteria to investigate active auditory mechanics in mosquitoes. In the culicid species *Toxorhynchites brevipalpis*, we examined the mechanical response of the flagellum by measuring the flagellar displacement d_f and its relation to the particle displacement d_p under various, non-invasive experimental conditions.

2. MATERIAL AND METHODS

(a) *Animals*

Animals of the culicid species *T. brevipalpis* were taken from laboratory cultures. Prior to the mechanical examination, the wings and the legs were removed, and the animals were waxed dorsum-down to a holder. The head, which protruded from the holder, was additionally waxed to the thorax to prevent head movements. All experiments were carried out on a vibration isolation table (TMC, MA, USA; type 78-442-12) at room temperature (22–25 °C).

(b) *Mechanical analyses*

The set-up and methods used to examine flagellar sound-induced vibrations in mosquitoes have been described (Göpfert *et al.* 1999; Göpfert & Robert 2000). In brief, the antennae were exposed to acoustic stimuli, which were generated by a network

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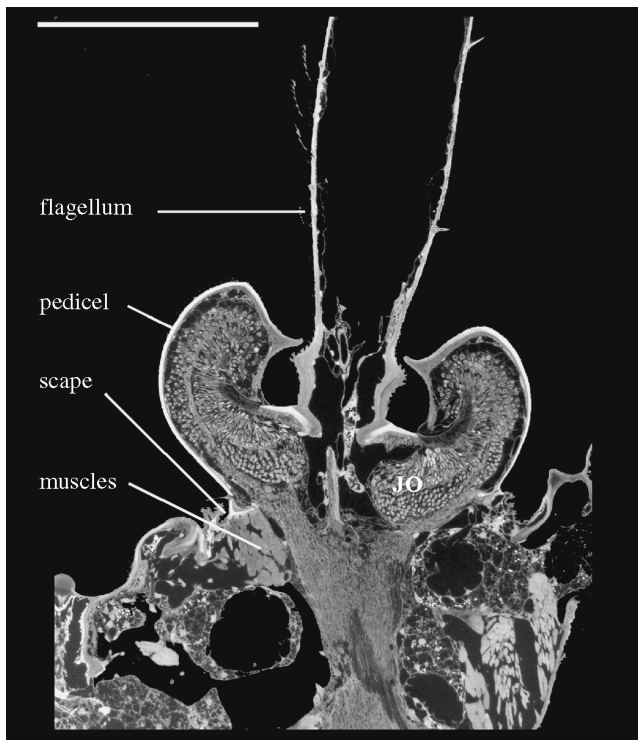


Figure 1. Auditory anatomy. Histological section through the base of a male antenna. The antennae of both sexes are divided in three main parts (scape, pedicel, flagellum), with the pedicel comprising Johnston's organ (JO). The most distal antennal muscles insert between the scape and the pedicel. Scale bar, 0.2 mm.

analyser (Stanford Research Systems, Inc., Sunnyvale, CA, USA; type SR 780), amplified, and broadcast via a loudspeaker (Uher; model UL-1302) positioned at a distance of 0.07 m from the preparation. Only one antenna was examined per animal. Sound-induced vibrations and air particle oscillations were assessed by simultaneously measuring flagellar vibration velocity and particle velocity via a Polytec PSV-200 scanning laser Doppler vibrometer (Brüel & Kjaer, Denmark) and a miniaturized Knowles NR-3158 particle velocity microphone (Knowles Electronics Co., West Sussex, UK), respectively. The latter had been calibrated against a precision B&K, type 4135 sound-pressure microphone (Polytec GmbH, Walderbronn, Germany) under acoustic far-field conditions (Göpfert *et al.* 1999). Hence, its output voltage could be directly converted to the corresponding particle velocity. During the measurements, the microphone was positioned close to the side of the antenna being measured, with its membrane orientated perpendicular to the direction of sound propagation. The responses of both the laser vibrometer and the microphone were linear in the intensity range examined (Göpfert *et al.* 1999). For offline analyses, the laser vibrometer and the microphone signals were digitized using an Analogic Fast-16 A/D board (Analogic Corporation, Peabody, MA, USA). Frequency spectra were estimated by means of fast Fourier transforms (frequency resolution 3.2 Hz; rectangle window). The flagellar vibration velocity and the particle velocity were subsequently converted to the corresponding displacements, i.e. the flagellar displacement (d_f) and particle displacement (d_p) (displacement = velocity/ $2\pi \times$ frequency). Mechanical sensitivity d_f/d_p was determined by computing transfer functions between the Fourier-transformed laser and microphone signals, calculated as the cross-power

spectrum of the laser and microphone signals divided by the auto-power spectrum of the latter. The magnitude information of these transfer functions was finally converted to the corresponding d_f/d_p values.

(c) *In vivo*–*post-mortem* comparisons and hypoxia induction

Immediately after measuring the *in vivo* response, animals were killed by microcapillary injection of ethanol (70%) in the thorax. Post-mortem measurements are problematic. Usually, the overall stiffness increases soon after the animal's death (rigor mortis). As a consequence, the flagellar resonance slowly shifts towards higher frequencies, precluding meaningful comparisons between *in vivo* and post-mortem vibration responses. To overcome this problem, we measured the post-mortem response within the short time-window between the animal's death and the onset of rigor mortis. To do so, we continuously monitored the flagellar vibration response after ethanol injection. Such online monitoring revealed that the changes in the vibration response described here occurred long before the flagellar resonance frequency started to shift. The changes, however, coincided with the appearance of autonomous vibration activity (a phenomenon described in §3). Since these vibrations clearly outlasted any other detectable movements of the animal, their cessation was used as a criterion for the animal's death. At this time, slight frequency shifts of the flagellar resonance were occasionally observed, but there was no temporal correlation between these shifts and the effects we describe.

To induce hypoxia, CO₂ was delivered to the animals through a steel tube (0.25 inch diameter). Measurements were only taken before and after exposure to avoid measuring direct mechanical effects due to the turbulent flow of CO₂. Online observation of the antenna's position via the video monitoring system of the laser vibrometer also confirmed that changes in the flagellar mechanics cannot be attributed to hypoxia-induced changes of the antenna's position; such changes, which have been reported for other insects (Kössl & Boyan 1998*b*), were not observed in *T. brevipalpis*, either in males or in females.

(d) *Electrophysiology*

Compound neural activity of Johnston's organ was recorded extracellularly via an electrolytically tapered tungsten electrode inserted directly in the organ. For grounding, an indifferent electrode was inserted in one of the compound eyes. Activity recorded this way represents the summated receptor potential of the mechanoreceptor units of Johnston's organ (Tischner 1953; Clements 1999). After preamplification, the electrode signal was digitized and converted to the frequency domain in the same manner as described for the laser vibrometer and microphone signals.

3. RESULTS AND DISCUSSION

First, physiologically vulnerable components of flagellar mechanics were identified by *in vivo* and post-mortem measurements. In comparable mammalian studies, such analysis revealed that the basilar membrane's sensitivity, tuning and nonlinearity depend on the physiological condition of the cochlea, and decreases or vanishes post-mortem (Rhode 1971; Sellick *et al.* 1982; Johnstone *et al.* 1986; Ruggero & Rich 1991; reviewed by Ruggero 1992). We examined changes in sensitivity and tuning by measuring the flagellar mechanical response to

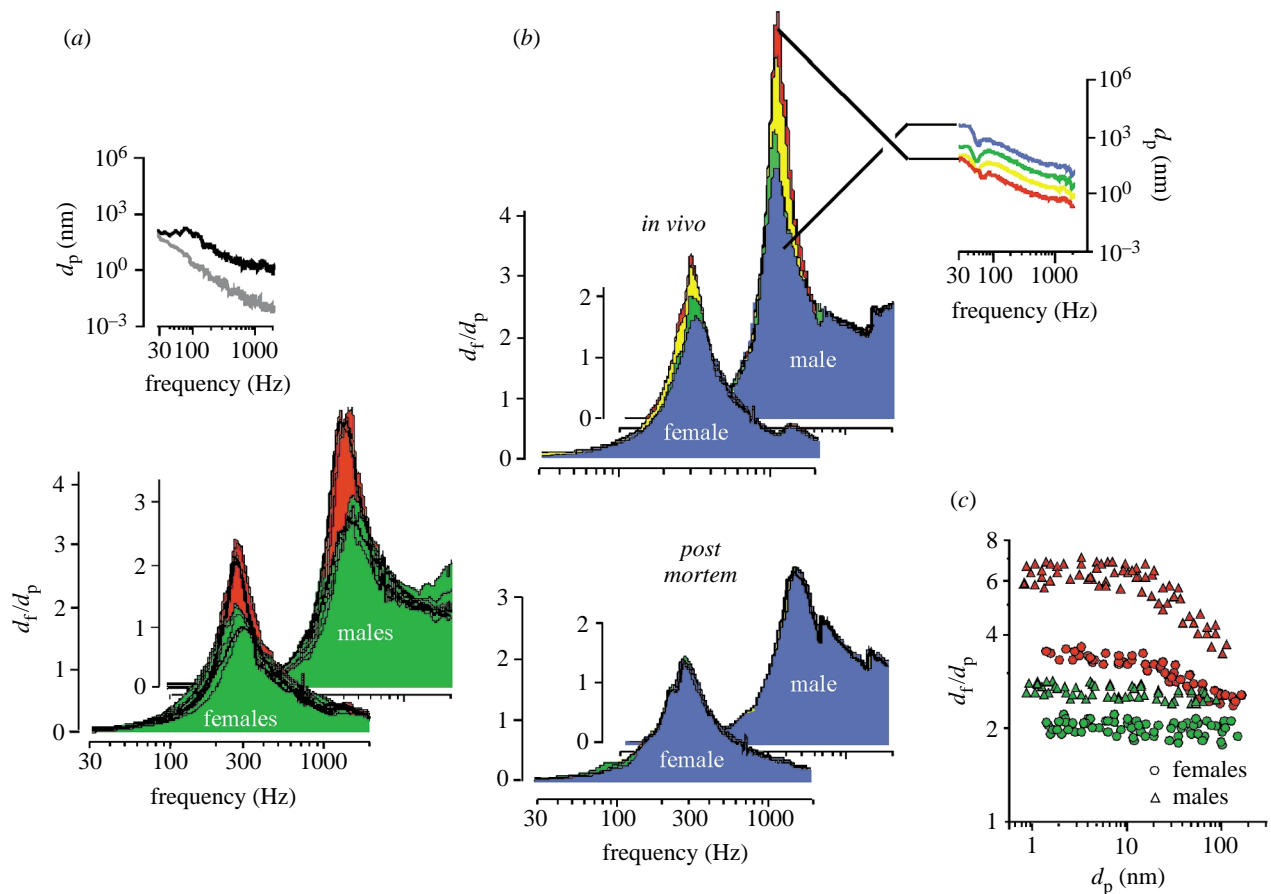


Figure 2. Metabolic vulnerability of flagellar vibrations. (a) Sensitivity and tuning. Lower panel: superimposed flagellar-tip responses to acoustic random-noise stimulation measured *in vivo* (red) and post-mortem (green) (five flagella per sex). Magnitude is computed as mechanical sensitivity d_f/d_p . Upper panel: frequency spectra of the stimulus (black) and the background noise (grey). (b) Nonlinearity. *In vivo* and post-mortem flagellar-tip responses to chirp sounds of different intensities (12 dB steps; the colour code for intensities is given in the panel on the upper right) (one flagellum per sex). (c) Sensitivity d_f/d_p at flagellar best frequency as a function of d_p (colour convention as in (a); four flagella per sex).

identical random-noise stimuli before and just after the animal's death (figure 2a). *In vivo*, the response of the flagellar tip was characterized by a sharp resonant peak, with female flagella vibrating maximally at *ca.* 250 Hz and those of males at *ca.* 430 Hz. Post-mortem, this peak consistently dropped in amplitude, whereas the response at other frequencies remained unaffected (figure 2a). Quantitatively, this reduction was similar in both sexes. Mechanical sensitivity d_f/d_p , defined as flagellar displacement (d_f) normalized by particle displacement (d_p), decreased on average by a factor of 1.5 ($d_f/d_p = 2.9 \pm 0.2$ (*in vivo*) and 1.9 ± 0.3 (post-mortem) in females and $d_f/d_p = 4.4 \pm 0.2$ (*in vivo*) and 2.9 ± 0.3 (post-mortem) in males; $n = 5$ animals per sex). In addition, tuning sharpness, measured as quality factor Q_{3dB} (Bennet-Clark 1999), consistently dropped by a factor of 1.6 ($Q_{3dB} = 2.6 \pm 0.3$ (*in vivo*) and 1.6 ± 0.3 (post-mortem) in both sexes; $n = 5$ animals per sex). Accordingly, the physiological vulnerability of sensitivity and tuning, as known for the basilar membrane of mammals (Ruggero 1992), also characterizes the vibrational response of the mosquito flagellum.

Second, active auditory amplification is characteristically nonlinear (Kemp 1978; Robles *et al.* 1991; Jaramillo *et al.* 1993). This nonlinearity results from an intensity-dependent amplification in which only minute vibrations,

induced by faint sounds, are strongly enhanced. Such nonlinear amplification was observed when the flagella were exposed to chirp sounds (frequency-modulated sweeps) of different intensities (figure 2b). Sound intensity was initially adjusted to a low level, and subsequently increased stepwise. *In vivo*, the responses were nonlinear around the frequency of highest sensitivity (figure 2b). At those frequencies, sensitivity d_f/d_p was inversely related to intensity d_p ; the lower the intensity, the larger the height of the sensitivity peak. This band-limited nonlinearity disappeared post-mortem (figure 2b). To evaluate this vulnerable effect in more detail, we increased the number of intensities tested and plotted the mechanical sensitivity d_f/d_p at flagellar best frequency against the corresponding d_p (figure 2c). For a linear system, d_f would be proportional to d_p , so the data points of d_f/d_p would be parallel to the intensity axis. This was indeed the case for the post-mortem responses of the flagella of both sexes (figure 2c). *In vivo*, however, the sensitivity of male and female flagella was higher in the entire range of intensities examined. Maximum sensitivity occurred at low d_p only, i.e. between 1 nm and 10 nm (figure 2c). Here, the vibration amplitudes of female and male flagella were 1.8- and 2.5-times higher than post-mortem, corresponding to a 5 dB and 8 dB enhancement, respectively. These results suggest that some physiological process improves the resonant

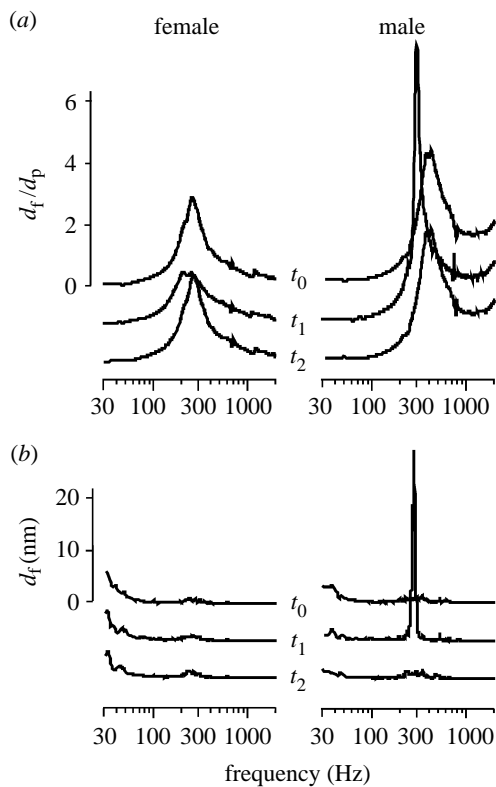


Figure 3. Sensitivity to hypoxia. (a) Superimposed flagellar-tip responses to acoustic random-noise stimulation measured before (t_0), immediately after (t_1), and 1 min after (t_2) exposure to CO_2 for 60 s (one flagellum per sex). (b) As in (a), but here d_f was measured in the absence of acoustic stimulation.

mechanics of mosquito flagella in a frequency- and intensity-dependent way.

Third, the effects of hypoxia on the antennal mechanics were studied by briefly exposing the animals to CO_2 . In female mosquitoes, hypoxia caused a drop of the sensitivity peak, similar to that observed post-mortem (figure 3a). The effect was reversible, and the sharp peak observed before exposure reappeared 1 min later (figure 3a). Comparable effects have been reported for vertebrates. In mammals, hypoxia causes a reversible decrease of the endocochlear potential (the presumed driving voltage for hair cell motility) (Rebillard & Lavigne-Rebillard 1992), and hypoxia also transiently suppresses auditory nonlinearity in other vertebrates (Manley *et al.* 1988; Köppl & Manley 1993). In male mosquitoes, the effect was surprisingly different. The sensitivity peak reversibly shifted by *ca.* 100–150 Hz towards lower frequencies, sharpened, and increased in amplitude (figure 3a). Thus, the treatment that suppresses flagellar vibrations in females facilitates them in males. The reasons for these opposite effects remain elusive. Interestingly, in geckos, salicylates have been observed to either enhance or suppress otoacoustic emissions, whereby the sign of the effect depends on the initial power of the emissions (Stewart & Hudspeth 2000). In male mosquitoes, hypoxia not only enhanced sound-induced vibrations. It even consistently induced vibrations of male flagella in the absence of acoustic stimulation. Transient, sharp flagellar vibration peaks reliably appeared after CO_2 exposure (figure 3b). Peak frequency was *ca.* 300 Hz, corresponding to the best frequency in the mechanical

response to sound (figure 3a,b). The flagellar vibrations in the absence of acoustic stimulation cannot be related to external forces. Maximum d_f reached amplitudes of 20–40 nm, whereas the background d_p was below 1 nm at the corresponding frequencies. Comparable peaks were not detected in females (figure 3b). These results mean that the hypoxia-induced vibrations of male flagella are autonomous and self-sustained, and generated by an internal motor.

Fourth, autonomous vibration (AV) was occasionally observed in untreated males (figure 4a). Moreover, it could be reliably induced in animals of both sexes by injection either of dimethylsulphoxide (DMSO; 30%, dissolved in saline) or, alternatively, of ethanol (70%). DMSO is a local analgesic (Sawada & Sato 1975) that has been reported to affect sensory transduction and spike generation in insect chordotonal organs (Theophilidis & Kravari 1994). AV, when induced by DMSO, typically lasted for 1–1.5 h (figure 4b). During this time, the vibration shifted towards lower frequencies and the amplitude changed (figure 4b,c). After an initial increase, reaching d_f of *ca.* 200 nm in females and 400 nm in males, amplitude gradually decreased, until the vibrations finally disappeared (figure 4b). Interestingly, AV could be detected on all sides of the antenna, indicating a precessing vibrational pattern. Several lines of evidence suggest that AV, as induced by DMSO injection, is related to the active amplification process revealed by the *in vivo*–post-mortem comparisons. AV only occurred within a restricted range of frequencies (figure 4c). This range was different in males and females. In each sex, it spanned those frequencies at which amplification took place (figures 4c and 2a,b). AV also exhibited signs of non-linearity that became apparent when the flagella were acoustically driven at nearby frequencies (figure 4d). AV was transiently suppressed, demonstrating a nonlinear interaction between autonomous and sound-induced vibrations. In addition, AV was affected by hypoxia (figure 4e). As expected, AV was reversibly suppressed by CO_2 exposure in females, and in males it shifted by *ca.* 100 Hz in frequency and increased in sharpness and amplitude. As a striking parallel, spontaneous otoacoustic emissions in vertebrates are also restricted to frequencies at which amplification takes place, suppressed by external tones, and sensitive to hypoxia (Probst 1990; Köppl 1995), key properties identified here for AV of mosquito flagella.

Altogether these findings indicate the presence of a mechanical motor that actively improves flagellar vibrations in mosquitoes. This raises the question of the nature and location of this motor. Mosquitoes, like most other insects, can actively move their antennae with muscles at each antenna's base (Clements 1999). Hence, a simple explanation would be that the active auditory amplification arises from muscular activity. In mosquitoes, the most distal muscles insert between the two basal segments, the scape and the pedicel (Risler 1953; Belton 1989) (figure 1). Since no muscles attach directly to the flagellum, muscle activity must result in a sympathetic movement of the flagellum and the pedicel (Risler 1953; Belton 1989; Clements 1999). We systematically measured the vibrations along the length of the antennae during AV, and show that AV occurs along the entire length of the flagellum, but does not extend to the pedicel

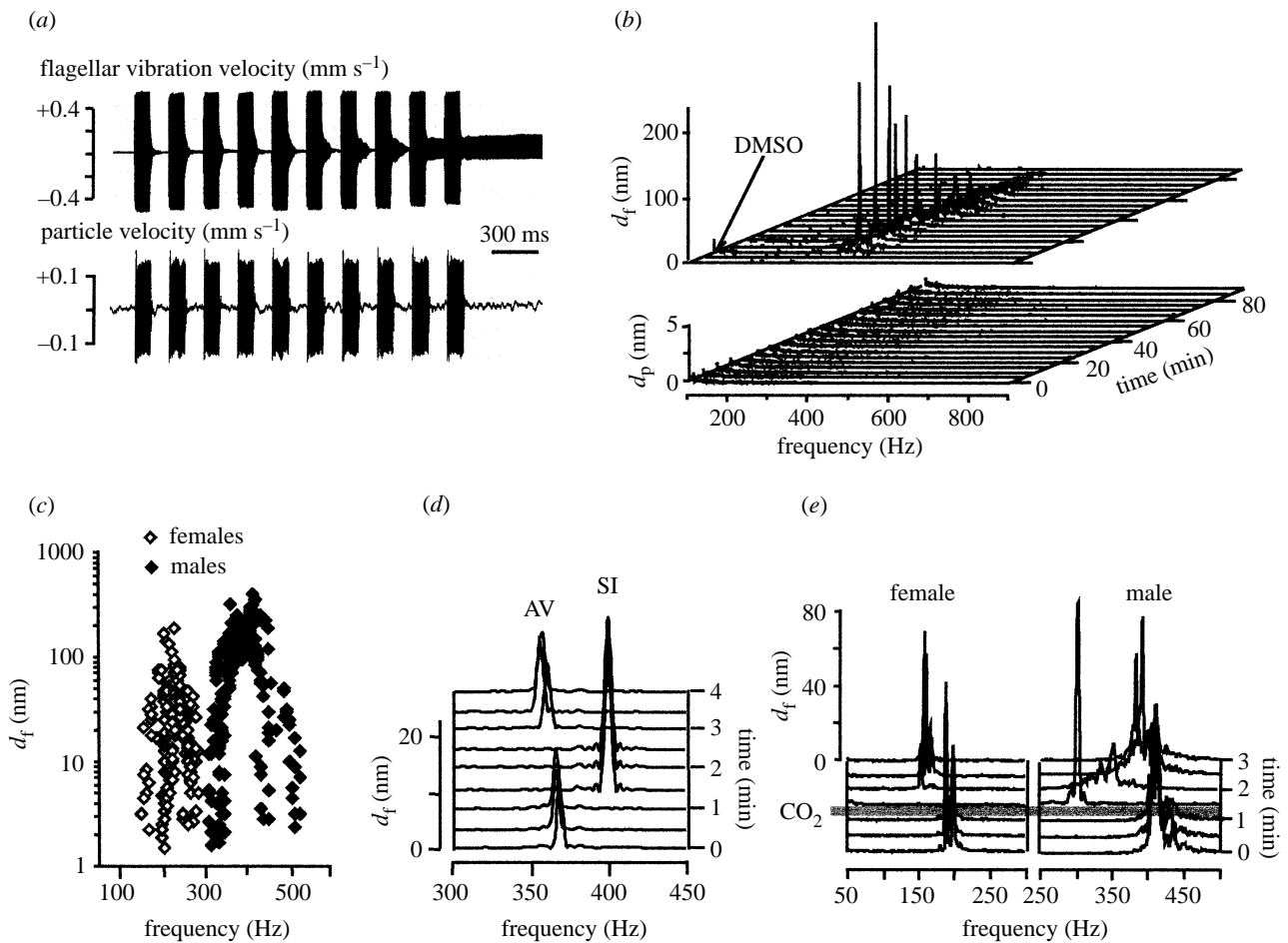


Figure 4. Autonomous vibration (AV). (a) Emergence of AV in an untreated male. Flagellar-tip vibrations (upper trace) and particle oscillations (lower trace) were recorded while the animal was exposed to a series of sound pulses at 400 Hz. AV first manifested itself by a prolonged ringing of the flagellum that outlasted the stimulus pulses. After the last pulse of the series, the flagellum vibrated autonomously at its own best frequency (430 Hz) for 2.4 min. (b) DMSO-induced AV. d_f (upper panel) and d_p (lower panel) simultaneously measured at 5-min intervals before and after DMSO was injected in a male animal. (c) Amplitude of DMSO-induced AV as a function of frequency. The amplitude and frequency of the vibration peak were determined in frequency spectra taken at 3-min intervals (four flagella per sex). (d) Transient suppression of AV (at 350–360 Hz) in a male by sound-induced (SI) vibrations at 400 Hz. (e) Effect of hypoxia on AV. Flagellar-tip displacement d_p before and after a 20 s exposure to CO₂ (one animal per sex).

(figure 5a). Moreover, AV of the flagellum could be detected even when the pedicel was immobilized to the head capsule (figure 5a). Consequently, the flagellum must move at its suspension in the pedicel when vibrating autonomously. Such a vibrational pattern rules out the possibility that AV results from activity of muscles at the antenna's base. Instead, the motor must be non-muscular and located in the pedicel.

Anatomically, the mechanoreceptor units of Johnston's organ are the only structural components that attach to the flagellar base (Risler 1953; Belton 1989; Clements 1999). Thus, they may serve as actuators driving the flagellum. The radial arrangement of these units could explain the detectability of AV from all sides of the antenna, and their number (*ca.* 3000 units with 7500 sensory neurons in females versus *ca.* 7000 units with 15 000 sensory neurons in males (Boo & Richards 1975a,b)) could account for sex differences in the strength of amplification. Simultaneous measurements of flagellar-tip vibrations and electrical activity in Johnston's organ provide evidence for an active mechanical role of these units. Such measurements show that AV persists when

sound-induced vibrations fail to elicit electrical responses (figure 5b). At this point, it is possible to separate electrical activity that is associated with the motor from electrical responses resulting from flagellar vibrations. Hence, we could identify electrical potentials in Johnston's organ that occurred at the instantaneous AV frequency (figure 5b). These potentials disappeared when the electrode was inserted more proximally, close to the antennal muscles and nerve, but reappeared when it was moved back inside the sensory organ. This correlation between AV and local electrical activity inside Johnston's organ strongly suggests that active auditory mechanics in mosquitoes is based on mechanoreceptor motility.

This study demonstrates that active auditory mechanics occurs in insects. Like vertebrates, mosquitoes actively improve hearing by enhancing sound-induced vibrations in a frequency- and intensity-dependent way. Considering the vastly different anatomies of mosquito hearing organs and vertebrate ears, such similarity in the mechanical processing of sound is surprising. At first, it seems reasonable to assume that active auditory mechanics in vertebrates and insects evolved independently and in

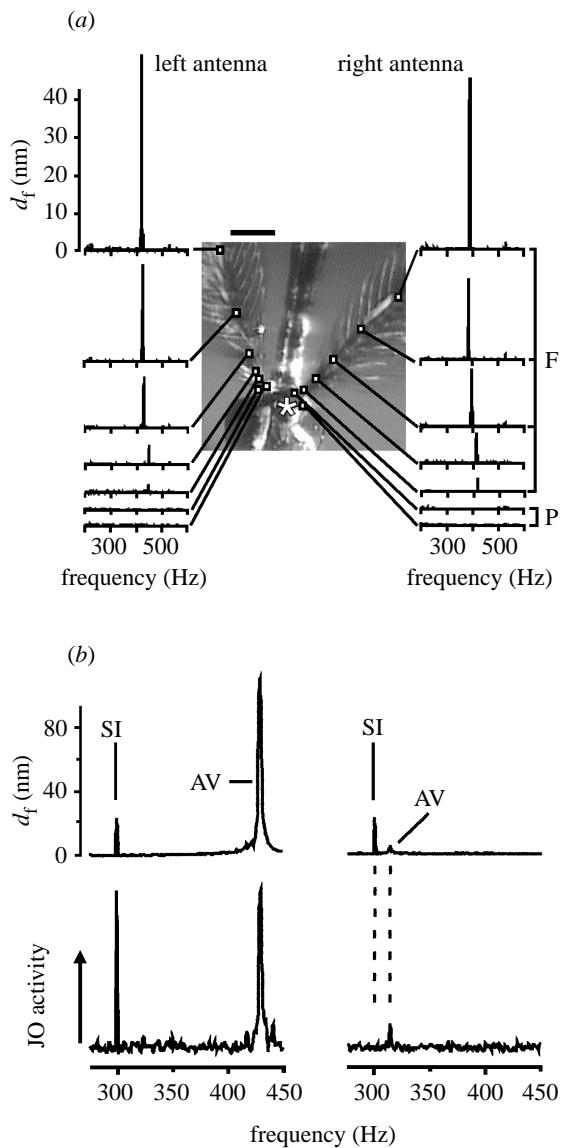


Figure 5. Autonomous vibrations (AVs) from different parts of the antenna and identification of motor-related electrical activity. (a) Vibrations in different regions of the flagella (F) and pedicels (P) during AV. Vibration was measured in a male in which the pedicel of the right antenna was waxed to the head capsule (asterisk). Vibrations are shown for 14 representative measurement points chosen from a total of 247 points examined (total time of data acquisition, 3.6 min). Scale bar, 0.5 mm. (b) d_f (upper traces) and electrical activity of Johnston's organ (lower traces) recorded during AV and acoustic stimulation (sound-induced vibration, SI). Five minutes after DMSO injection (left panel), electrical activity accompanies both AV (at ca. 430 Hz) and SI vibrations (at 300 Hz). Seventy-five minutes after injection (right panel), AV has severely decreased in amplitude and shifted to 319 Hz. At this frequency, Johnston's organ is still electrically active, although SI vibrations no longer elicit an electrical response.

parallel. The biophysics underlying acute hearing may have independently promoted the evolution of active auditory mechanics in both groups. Some recent findings, however, suggest a common evolutionary origin. Insect mechanoreceptors have been demonstrated to share the fundamental transduction characteristics of vertebrate hair cells (Walker *et al.* 2000). Increasing evidence also

suggests that, in vertebrates, auditory amplification presumably is an ancient feature of audition (Manley & Köppl 1998; Stewart & Hudspeth 2000). Moreover, the development of cochlear hair cells in mammals and Johnston's organ mechanoreceptors in insects has been shown to be controlled by homologous genes, *Math1* and *atonal* (Bermingham *et al.* 1999), indicating an ancestral link between the evolution of their auditory receptors. Considering the accessibility of their hearing organs, mosquitoes and possibly also other insects (Kössl & Boyan 1998*a,b*; Coro & Kössl 1998; Ewing 1978) constitute attractive model systems for the study of active auditory mechanics. The comparative examination of hearing, in insects and vertebrates not only promises fundamental insights into active sensation, but also into its evolution.

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