RESEARCH ARTICLE

Frequency organization of the Johnston’s organ in male mosquitoes (Diptera, Culicidae)

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ABSTRACT

The Johnston’s organs (JO) of mosquitoes are the most complex mechanosensitive organs yet found in insects. Previous findings on the behavior of mosquitoes suggest that, together with exceptional sensitivity, their auditory system can discriminate frequencies. Analysis of compound responses of the JO did not provide unambiguous evidence of such discrimination, nor did it help to find its mechanism. Using the feedback stimulation method, we measured the tuning frequencies of the JO sensory neurons. Here we present electrophysiological evidence that male mosquitoes of Culex pipiens possess at least eight groups of auditory neurons that are distinct in their frequency tuning, with individual frequencies ranging from 85 to 470 Hz. Most of the neurons are tuned to 190–270 Hz, which corresponds to the difference between male and female flight tones. Axons of the JO sensory units propagate graded amplified receptor potentials rather than all-or-none action potentials, are grouped into pairs or triplets and often respond in anti-phase to each other. Some features of the mosquito auditory system suggest an analogy to the retinal mechanisms. Together with our previous findings on frequency tuning in female mosquitoes of different species, this study presents evidence in favor of sophisticated frequency analysis of sound in mosquitoes.

KEY WORDS: Mosquito, Johnston’s organ, Frequency tuning, Primary auditory neuron, Feedback, Autoexcitation, Culex pipiens

INTRODUCTION

Sound reception plays an exceptional role in the life of mosquitoes. The males swarm in hundreds, waiting for a female to enter a swarm. As soon as the female is detected by the sound of her flight, males rush to intercept her, still guided by the sound. This behavior pattern is typical for many dipterans, including families of Culicidae (Roth, 1948; Charlwood and Jones, 1979) and Chironomidae (Rümér, 1970; Ogawa and Sato, 1993). However, it does not end with interception, as, according to recent findings (Gibson and Russell, 2006; Cator et al., 2009; Warren et al., 2009; Pennetier et al., 2010; Aldersley et al., 2016), male and female mosquitoes actively respond to the flight tones of each other by altering their own wingbeat frequencies. This mechanism of reciprocal tuning is considered to be a means of sex and species recognition, audition thus being the very focus of natural selection in mosquitoes.

First described by Christopher Johnston (1855), a sensory organ in the second segment of the antenna – Johnston’s organ (JO) – is the key part of the mosquito auditory system. Affected in response to a sound, the antenna oscillates, and the radially arranged sensory units of mechanosensory neurons of JO transduce nanometer-scale vibrations into electrical signals and pass them on to the brain via the antennal nerve (Göpfert and Robert, 2000). The mechanical sensitivity of the male mosquito’s antenna exceeds those of all other arthropod movement receivers studied so far (Göpfert et al., 1999). Moreover, its sensitivity is further improved by active auditory mechanics that amplify sound-induced vibrations at specific frequencies and intensities (Göpfert and Robert, 2001; Avitable et al., 2010; Nadrowksi et al., 2011; Simões et al., 2016). However, despite being one of the most studied among the arthropod sensory systems, the mosquito JO presents unresolved questions.

Most saliently, each mosquito JO contains a surprisingly high number of primary sensory neurons (PSN) (approximately 15,000 for a male and half that number for a female; Boo and Richards, 1975a,b), a fact which Belton (1974) called ‘a flagrant violation of Roeder’s (1963) principle of neural parsimony’.

Secondly, when the mosquito JO was studied with a tungsten electrode to record acoustically evoked field potentials, its compound response in most cases demonstrated frequency doubling compared with the fundamental tone of the stimulation. This fact could not be fully explained, despite several hypotheses being proposed (Belton, 1974; Göpfert and Robert, 2000; Jackson et al., 2009).

Thirdly, the reciprocal tuning of flight tones requires a mechanism for sound frequency analysis in both male and female mosquitoes, even if they only detect the distortion products resulting from the mixing of male and female flight tones (Warren et al., 2009; Arthur et al., 2010; Pennetier et al., 2010; Simões et al., 2016). The question of whether the resting mosquitoes are able to analyse the frequency spectrum of sounds is also unresolved (Arthur et al., 2010).

Recent methodological change, namely the use of glass microelectrodes for focal recording of responses from the confined groups of axons of the JO PSNs, partially resolved the first and second issues (Lapshin, 2010, 2011, 2012, 2013). Responses to simple acoustic stimuli as well as responses during flight simulation were recorded using this technique. Existence of narrow-tuned auditory units in the brain of Culex pipiens mosquitoes was found, which suggested that JO PSNs were heterogeneous and partly explained the high number of neurons in the mosquito JO.

The minute diameter of axons in the antennal nerve, however, did not allow for certainty in recording the responses of the individual neurons rather than their group responses. To overcome this limitation, the method of feedback stimulation was developed (Lapshin and Vorontsov, 2013) and first implemented for measurements of the frequency tuning of individual receptors in females of blood-sucking mosquitoes and later repeated in males of...
List of abbreviations

AF autoexcitation frequency
JO Johnston’s organ
PSN primary sensory neuron
SPVL sound particle velocity level

Chironomus plumosus L. (Chironomidae) (Lapshin, 2015). Experiments confirmed that: (i) feedback stimulation can be used to measure the properties of individual receptors, and (ii) the JO in both midges and female mosquitoes contains units with substantially different frequency tuning.

In this study, we used both feedback stimulation and conventional auditory threshold measurements, aiming to perform a detailed examination of a Culex male mosquito’s JO. The initial questions were: (i) whether, as in females, the JO is heterogeneous or not; and (ii) if yes, what are the auditory properties of its units? However, in addition, the male mosquito JO demonstrated several salient and totally unexpected features, which we also report here.

MATERIALS AND METHODS

The detailed procedure has been described elsewhere (Lapshin and Vorontsov, 2013). We outline it briefly below, detailing only the differences and methodological improvements.

Animal preparation

Forty-two male mosquitoes of Culex pipiens pipiens Linnaeus 1758 were captured from a natural population in the Moscow region of the Russian Federation. Experiments were conducted in laboratory conditions with air temperature 19–21°C in August and September 2011–2014 at the Kropotovo biological station (54°51′2″N, 38°20′58″E).

Microelectrode recordings

Focal recordings from the axons of the antennal nerve were made using glass microelectrodes (1B100F-4, WPI Inc.) filled with electrolyte and inserted at the scape–pedicel joint. In the preliminary series we used 3 mol l⁻¹ potassium chloride; however, the occasional breaking of the electrode tip inside the neuropile led to the immediate and irreversible cessation of responses. After that even with a new electrode it was usually impossible to record any auditory responses from the same JO, although it could be easily recorded from the contralateral (intact) JO of the same specimen. To overcome this issue in the main experimental series we filled the electrodes with 0.15 mol l⁻¹ sodium chloride or 2 mol l⁻¹ potassium acetate (to control the effect of electrode sodium ions on the axonal responses). An electrode inserted through the previously made puncture in the cuticle had the resistance of 40–80 MΩ.

Neuronal responses were amplified using a home-made DC amplifier (input resistance >30 GΩ, gain 20 dB). In this study, we recorded all neuronal responses without a 5 Hz high-pass filter which was used in our earlier study on females (Lapshin and Vorontsov, 2013) and which in that study did not give us an opportunity to compare our earlier results with the findings of Cator et al. (2009) and Arthur et al. (2010). During recordings of very low amplitude compound responses from the JO cuticle or when neuronal responses were used for feedback stimulation (see below), the output of the DC amplifier was passed through an additional AC amplifier (gain 20, 30 or 40 dB, band-pass 5–5000 Hz).

Responses and stimulation signals were digitized using E14-440 A/D board (L-Card, Russian Federation) at 20 kHz sampling rate, and LGraph2 software. Digitized recordings were examined with Sound Forge Pro 10 (Sony).

Sound stimulation

Sounds were presented to mosquitoes within a home-made acoustical chamber (Fig. 1) with two loudspeakers opposing each other and being connected via a balancing circuit. The chamber allowed cancellation of the sound pressure changes around the mosquito, leaving only the air velocity component of the sound for stimulation.

The calibration of the stimulation system has been described in detail previously (Lapshin and Vorontsov, 2013). Additionally, in this study we used the differential microphone NR-23158-000 (Knowles Electronics, USA), which was previously calibrated in the far field according to the technique described by Warren et al. (2010) using the B&K 2253 sound level meter with a B&K 4135 microphone (Bruel and Kjaer, Denmark) to control the frequency properties of the stimulation system and to record the stimulation signals.

All particle velocity data in this study are given in the logarithmic scale in dB RMS SPVL (root mean square sound particle velocity level), with a reference level of 0 dB being equal to 4.85×10⁻⁵ mm s⁻¹, which corresponds in the far field to the standard reference sound pressure of 20 µPa.

The mosquito was positioned such that the stimulation acoustic waves caused a dorsoventral deflection of the antenna flagellum. The movement of air particles during the stimulation in the dorsal direction was taken as the positive direction. The electrode was inserted at about 45 deg to the plane of movement of the stimulated antenna. The whole set-up was mounted at a vibration-isolated steel table.

The criterion of the response threshold was set at 2 dB above the average noise level in a given recording. Sequential measurements of the threshold responses were performed in the range 40 to 600 Hz, with 2, 10 or 20 Hz steps depending on the bandwidth of a given neuronal unit. The upper frequency limit was set according to our preliminary studies (Lapshin, 2012) in which we did not find any high-frequency responses of JO sensory cells, in contrast to the findings of Arthur et al. (2010) on Aedes mosquitoes.

Feedback stimulation

A feedback stimulation was used to determine tuning properties of the neuronal units. The essence of the method is a positive feedback loop established using the amplified response of a sensory unit as the signal to drive the stimulation loudspeaker. Applying such kind of stimulation to the sensory unit, we expect it to ‘sing’ at a frequency that is close to its intrinsic tuning frequency – we call this effect ‘autoexcitation’. If there are responses of several units recorded (and applied as stimulation signals) then they all must be represented in the complex signal.

From the symmetrical structure of the JO neuronal complex, one would expect that the randomly chosen unit will demonstrate either an in-phase or an anti-phase response compared with the stimulation signal. To that end, a phase inverter was added to the stimulation circuit to adapt the stimulation to the properties of a given unit. Here and below the phase inversion of the feedback complex signal means that the signal waveform that goes to the loudspeakers is flipped so that its positive wave becomes a negative one, and vice versa. The inverter also provided us with an opportunity to test each unit under conditions of both positive and negative types of feedback (see ‘Control of artefacts’ below).

During the electrode movement through the antennal nerve, the preparation was continuously stimulated with 65 ms pulses of 200–260 Hz and 60 dB SVPL at 600 ms intervals until the response
amplitude increased abruptly. After that, the stimulation equipment was switched either to the feedback mode to measure the autoexcitation frequency (AF) or left in the pulse mode to measure the audiogram. With feedback stimulation, the signal level that was sufficient to start the autoexcitation was defined as the threshold, and further tests were carried out at 10–15 dB above the threshold.

Control of artefacts

The recording electrode was held inside a mosquito head below the JO and the antennae which was itself vibrating due to the external sound stimulation as well as due to the internal mechanoreceptor motility. In addition, acoustic waves created the lateral force on the electrode micropipette, which could possibly pass the vibration to its tip. Thus the mechano-electrical artefact potentials could appear in the recording along with the PSN responses. We had to be even more cautious with the possibility of recording artefacts as our previous study on female mosquitoes (Lapshin and Vorontsov, 2013) reported the lack of true action potentials. This made these responses look quite similar to the expected mechanical artefacts. In addition, as the primary aim of this study was to measure the frequency tuning of the system consisting of the electrode, JO and the antennae which was itself vibrating due to the external sound stimulation as well as due to the internal mechanoreceptor motility. Thus the vibrations of the antenna cannot serve as a primary source of the response. This sets the upper limit for possible mechanical artefacts like the direct effect of the sound onto the electrode or the mosquito body excluding the antenna. The observed residual response could also originate from the proximal part of the antenna’s flagellum and its fibrillae, which were not fixed. However, this control does not allow us to estimate the possible mechanical artefacts mediated by the antenna vibration.

Other controls were based on the analysis of the experimental data. Below we list evidence against the artefact nature of auditory responses: (1) the resonant frequency of the antenna in male mosquitoes is about 370 Hz (Göpfert et al., 1999), which is above the range in which we recorded most of the individual tuning frequencies. Thus the vibrations of the antenna cannot serve as a primary source of resonant phenomena that we recorded from the antennal nerve; (2) abrupt changes of resonant frequency during the tiny shifts of the electrode tip can hardly be explained by the changes in the mechanical tuning of the system consisting of the electrode, JO and antenna. For example, the frequency shift from 221 to 299 Hz (as shown in Results) requires the stiffness of the system to become 1.8 times higher (proportionally to the square of the frequency ratio). This is very unlikely for the indicated shift of an electrode (40 μmol l⁻¹) within the antennal nerve; (3) simultaneous autoexcitation at two frequencies and the stepwise change of autoexcitation frequency at the moment of feedback inversion; (4) the amplitude of phasic response that followed the logarithm of the stimulus amplitude; (5) the latency of response; (6) the phase of response to sinusoidal stimulation, which varied among different recordings (compare Fig. 2B and Fig. S2B: the phase difference is about 180 deg. Note that it is better to make comparisons in a steady state of response, closer to the end of recording); (7) the poisoning of the antennal nerve with potassium chloride, which led to the irreversible cessation of auditory responses (see above).

An additional control of mechanical artefacts as well as of the damage of the PSN axons was made by recording the compound...
The significance of the non-random co-localization of the certain groups tended to appear together at the same recording site. Instead, they evidently segregated into several groups, and AFs from these groups appeared with an opposite phase of stimulation (0 or 180 deg). Two AFs were considered equal if they were within 5 Hz. We did not take into account frequencies that could be interpreted as higher harmonics or mixed harmonics. For example, we treated 420 Hz as a true AF only in the absence of 210 Hz peak under the same stimulation conditions.

**Data analysis**

When summarizing data on individual AFs of units, every single AF recorded was taken into the distribution only once regardless of how many times it appeared in a single preparation, except when this AF appeared with an opposite phase of stimulation (0 or 180 deg). Two AFs were considered equal if they were within 5 Hz. We did not take into account frequencies that could be interpreted as higher harmonics or mixed harmonics. For example, we treated 420 Hz as a true AF only in the absence of 210 Hz peak under the same stimulation conditions.

The AFs were not evenly distributed across the frequency range; instead, they evidently segregated into several groups, and AFs from certain groups tended to appear together at the same recording site. The significance of the non-random co-localization of the certain

Fig. 2. Responses to sinusoidal sound stimulation: glass microelectrode recordings. (A) Responses from axons of auditory PSNs in antennal nerve (single recording); voltage scale is 10 mV. For neuronal response, upper trace in each record. Lower traces: stimulation signals (230 Hz) of different amplitudes recorded by microphone in a stimulation chamber. Slight axial shift of electrode was applied between fragments 1 and 2, which resulted in change to presumably intracellular recording from the axon. Note (i) the 20 mV drop of potential, (ii) lack of action potentials at any amplitude of the stimulus and (iii) the amplitude of response gradually following that of the stimulus. Also note the significant change of the response waveform between responses 1 and 2: the polarity of the response and the frequency doubling in 1, but not in 2, and the following responses. Stimulation level starts from 60 dB SPVL, decreases to 40 dB SPVL and then returns. In this particular recording the response disappeared at 45 dB, but during the stimulation level increase it appeared at lower levels (41 dB); the difference is probably due to the long-term adaptation of the JO to the loud stimulation. (B) Intracellularly recorded response, on a different time scale (compare with Fig. 3B and Fig. S2B, note the different phase of response in the latter case).

In the present study the primary method to search for and measure the frequency-tuned PSNs was based on the positive feedback stimulation. However, this method did not guarantee that we observed the true resonant units. To test the individual PSN tuning in non-feedback mode in those rare recordings when only a single unit accounted for the major part of the response, the threshold curves were measured using the sinusoidal stimulation. In addition, the units initially recorded with the positive feedback stimulation were then stimulated in the negative feedback loop (phase inversion, see above). If frequency tuning in the feedback loop was determined by a resonant structure, then its optimal performance had to be observed in the range where the signal transfer is mostly effective, i.e. at the resonance. This must be equally true for the positive feedback (when we elicit autoexcitation of the system) and for the negative one (when we expect to see the frequency-specific suppression of noise, i.e. of the spontaneous broadband oscillations of membrane potential). The existence of suppression zones at the same (autoexcitation) frequencies served as an important control, which allowed us to distinguish the true autoexcitation frequencies of neuronal units from the combination harmonics of any origin. An alternative hypothesis for the origin of the noise suppression zones was also considered: these zones could appear due to hypothetical mechanisms of lateral inhibition between the units. It could be expected that the lateral inhibition, if observed, is effective not only during the feedback stimulation but also during sinusoidal stimulation with the similar amplitude of sound. In the latter case, the noise suppression zones were expected to appear in sonograms below and above the fundamental frequency of stimulation. However, this was never observed in our experiments. It should be added that active vibration of the antenna flagellum caused by the JO PSN motility (Göpfert and Robert, 2000, 2001; Warren et al., 2010) affects the reception of sounds and could result in the appearance of combination harmonics similar to the autoexcitation frequency peaks. However, such combination of spontaneous and forced oscillations of the antenna could not result in the frequency-specific noise suppression after the inversion of the feedback signal observed at many different frequencies.
Fig. 3. Response to the feedback stimulation. (A) The onset of autoexcitation during a gradual increase of positive feedback level from 50 to 70 dB SPVL (level change is not shown in the figure, threshold measured at 61 dB SPVL). Glass microelectrode focal recording from the antennal nerve, voltage scale is given for the neuronal response (upper trace). Lower trace: stimulation signal (output from the microphone), which is a neuronal response, amplified, frequency-filtered and emitted by the loudspeakers. The autoexcitation appeared with the level of the stimulation rising abruptly by 7–12 dB, AF=204 Hz. (B) Fragment of A, on an expanded time scale. Note the lack of response frequency doubling, visible in Fig. 2, response 1. (C–F) Frequency spectra of neuronal responses recorded from the antennal nerve, except D, which was recorded from the outer cuticle surface of the JO. As the phase properties of a randomly chosen unit within the antennal nerve are not predictable, the preparation was stimulated with a phase shift of either 0 or 180 deg relative to the recorded response. (C) Feedback phase 0 deg, 70 dB SPVL. Autoexcitation can be seen at 247 Hz (AF1) and 378 Hz (AF2); the suppression zone is centered at 201 Hz. The peak at 494 Hz is a second harmonic of AF1, the peak around 740–750 Hz probably consists of the third harmonic of AF1 and the second harmonic of AF2. The peak at 127–130 Hz is the mixed harmonic (AF2 minus AF1). All other spectral peaks are the higher or mixed harmonics, as in C. (F) Spectrum of spontaneous activity (no acoustic stimulation) recorded from the antennal nerve without any acoustic stimulation. Note the peak at 454 Hz.
AF groups was analysed in the following way: first, for each AF group, the expected number of its co-localizations with each other AF group was calculated according to the proportion of these groups in the summary distribution, presuming that AFs were evenly co-localized. Then, we used the exact binomial test to compare the expected numbers with the observed ones.

To measure the sharpness of frequency tuning we estimated the quality factor \( Q_{10} = F_0 / \Delta F \), where \( Q_{10} \) is the quality factor and \( \Delta F \) is the width of the threshold curve at +6 dB from its minimum at \( F_0 \) frequency. As the direct measurement of frequency band could not be reliably made at +10 dB, to facilitate the comparisons we calculated \( Q_{10} \) values from the directly measured \( Q_{10} \) using the coefficient \( Q_{10} = 0.69Q_6 \), based on the data from Bennet-Clark (1999).

**RESULTS**

**Responses of the JO sensory units to sound**

Due to the fact that the electrode tip and the average diameter of sensory axon in the antennal nerve were of comparable size (1 μm or less), we cannot claim that the recordings were made from the individual axons. However, the specifics of auditory responses we recorded, especially the numerous fast and abrupt changes of response amplitude and frequency tuning when the electrode was moved through the antennal nerve (see below) imply that at each recording site with a stable position of an electrode only a few axons contributed to the response. For the sake of simplicity, here we use the terms ‘unit’ or ‘sensory unit’ in the sense of one or several axons belonging to the PSNs of the JO, closely located within the antennal nerve and sharing similar frequency and phasic properties, thus representing a single functional unit. An example of a typical extracelluar response to a sinusoidal pulse is shown in Fig. 2A, response 1 (see also Fig. S2A). The response is a sum of fast oscillations (AC) and a slow negative wave (DC). The AC component is enriched with the second and higher harmonics; the proportion of the first harmonic (fundamental frequency) in the signal varies from −7.3 to −1.1 dB RMS (−3.5 dB RMS on average), i.e. the response shows frequency doubling. The latency of response varied from 4 to 7 ms (at 60 dB SPVL). The typical noise level was 0.09−0.15 mV outside the antennal nerve and rose higher (to ca. 0.2 mV) when the electrode was inside the nerve. In cases of better recording quality and respectively higher amplitude of auditory response the noise level was also higher (Fig. 2, compare responses 1 and 2), suggesting that at least a fraction of this ‘noise’ consisted of the axonal potentials.

The DC component usually has a fast leading edge, coinciding with onset of the AC component. In contrast, its trailing edge is usually smooth, with the potential returning to the pre-stimulus level within 70 ms. In several observations the DC component had a slow rise similar in duration to the trailing edge. Also, we observed several responses that contained the DC component, while the AC had a low amplitude or was absent. It should be noted that in this study we took into account only the AC component of the response.

In several recordings, we observed response parameters characteristic of the intracellular recording, which demonstrated higher amplitude of response while the waveform of the AC component of the response roughly followed the logarithm of the stimulus amplitude (Fig. 2A, responses 2–6). In addition, the prevalence of the second harmonic (compared with the stimulus frequency), the so-called frequency doubling which is the well-known characteristic of the JO compound response, was not present in this kind of response (Fig. 2A, compare responses 1 and 2–6). Some of the narrow-tuned units demonstrated oscillations that lasted for 100–300 ms after the end of stimulus (not shown). Remarkably, we did not see action potentials in any of the recordings made from the JO PSN axons.

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**Fig. 4. Frequency transitions during different kinds of acoustic stimulation.** Sonograms of the responses recorded from the antennal nerve to the feedback stimulation. In sonograms the red spots represent the higher amplitude of response. (A) Simultaneous autoexcitation at two close frequencies (204 and 227 Hz, recording time from 9 to 13 s) during the electrode unidirectional axial shift (feedback stimulation 86 dB SPVL, phase 0 deg). (B) Abrupt change of AF caused by the electrode axial movement across the antennal nerve. The electrode was moved back and forth (indicated by the white trace below the sonogram: up is deeper, amplitude of axial shift 40 μm). The autoexcitation switched between 221 and 299 Hz; 73 dB SPVL feedback stimulation, phase 0 deg. All unmarked frequency tracks here and in other sonograms are the higher harmonics of the response originating from its non-linear properties. (C) The autoexcitation substituted by suppression after the inversion of the phase of feedback from 0 to 180 deg. Feedback amplitude is 72 dB SPVL (0 deg) and 74 dB SPVL (180 deg). Autoexcitation threshold: 62 dB SPVL. Autoexcitation at 200 Hz at 0 deg and 260 Hz at 180 deg. Responses at 152 and 409 Hz, which appear at 180 deg, are somehow linked to each other, but we cannot be sure which of them is a fundamental AF and which is a mixed harmonic. Most probably, the AF is situated at 152 Hz as there is a corresponding suppression zone at 0 deg.
To be sure that we did not overlook the action potentials in the auditory axons by some methodological fault, we made control recordings from the brain auditory interneurons using the very same technique. The identification of the brain region that these neurons belonged to was beyond the scope of this study; however, we obtained responses to the acoustic stimulation and recorded normal action potentials (Fig. S3).

**Autoexcitation**

In all experiments, during gradual increase of the feedback power from sub-threshold levels, the autoexcitation appeared with the stimulation amplitude spontaneously rising abruptly by 7–12 dB (Fig. 3A,B). Threshold of the autoexcitation varied from 60 to 72 dB SPVL. The amplitude of fundamental harmonic, which directly excited the given unit, was −9 to −2 dB (−4.3 dB on average) from the RMS level of the signal that affected the antenna. In case of simultaneous autoexcitation of two different units, the fraction of each of the two fundamentals was respectively lower (from −11 to −2 dB, −6.3 dB on average). For stimuli below 60 dB SPVL, the possibility of eliciting the autoexcitation was substantially limited by the noise level.

Responses of sensory units during the autoexcitation usually had a line spectrum (Fig. 3C,E). Apart from one or more discrete AFs in the spectrum, there were also their derivatives, higher harmonics and mixed harmonics. To reliably separate only true AFs for further statistical analysis we had to drop all cases where unequivocal interpretation was not possible (see Materials and methods, Data analysis). In most cases (41 of 42), if there was stable autoexcitation and mixed harmonics. To reliably separate only true AFs for further statistical analysis we had to drop all cases where unequivocal interpretation was not possible (see Materials and methods, Data analysis). In most cases (41 of 42), if there was stable autoexcitation at a given recording site, it reappeared after the phase inversion but at different AFs.

**Effects of mechanical shift of the electrode**

To prove that only a few PSNs contributed to the recorded response we made gradual axial shifts of the electrode within tens of micrometers. This procedure often caused an abrupt change from one AF to another, which would not be the case if we recorded a compound response from a large pool of axons (Fig. 4A). To ensure that the change of the AF was not due to damage of the axons, several back and forth shifts of the electrode were made to demonstrate multiple jumps from one AF to another and back (N=14, example in Fig. 4B).

**Noise suppression during the negative feedback stimulation**

When the phase of the feedback stimulation was inverted following the establishment of sustained autoexcitation at a certain frequency, narrow-band suppression of noise was observed at a similar frequency (or frequencies, if multi-frequency autoexcitation was produced; Fig. 3C,E). This effect was observed in all 42 preparations. The suppression zones corresponded only to the fundamental frequencies (AFs) but not to their higher harmonics. These noise suppression zones can be seen in sonograms as continuous horizontal darker stripes that reflect decreased level of noise within the specific frequency ranges (Fig. 4C). We have observed noise suppression at different frequencies with either 0 or 180 deg phase of stimulation.

**Capture of the autoexcitation frequency by the external stimulus**

The preparation, being already in a state of autoexcitation, was stimulated with additional sinusoidal signals of various frequencies and the amplitude equal to the level of the fundamental frequency of the feedback signal. These experiments, performed in 17 of 42 preparations, demonstrated that capture of AF was indeed possible within the frequency range of 18–25% of the AF (Fig. S4, arrows). The increase of feedback level by 4–6 dB substantially narrowed this range (down to 8%). The effects of capture and release of the AF by the external stimulation occurred similarly during the stepwise increase and decrease of the stimulus frequency when it crossed the autoexcitation range.

**Distribution of autoexcitation frequencies**

The resulting distribution summarizes the 516 measurements of AF recorded from 42 specimens. Individual AFs ranged from 85 to 470 Hz and split up into several groups (L1, L2, L3, A, B, C, D, E, H1 and H2 in Fig. S5). Further analysis of AFs belonging to in-phase and anti-phase pairs of units confirmed the separation of these groups (see below).

The maximal peak of the distribution (the A group) is at 200 Hz; two other major peaks of the distribution, B and C, are around 230 and 260 Hz, respectively. Also, there is a small number of sensory units tuned to lower (85–155 Hz, groups L1–L2) and higher frequencies (350–435 Hz, groups H1 and H2). Intermediate peaks (L3, D and E) were separated on the basis of co-localization of these frequencies with those of the units belonging to the other, more pronounced peaks.

The mixed harmonics in the L1–L2 range were observed in almost every specimen. They were usually equal to the difference of the two higher AFs and repeated the changes of these AFs in the sonogram, often mirroring these changes. Thus any frequency peaks

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**Fig. 5. Summary distribution of autoexcitation frequencies.** The distribution includes the whole set of AF measurements (N=516) from 42 specimens, plotted with a 5 Hz step (see Materials and methods for description of raw data analysis). Prominent peaks (which represent major groups of units) are labeled. Most of the JO sensory units in C. p. piperia males are tuned to 190–210 Hz, while local peaks are over 25–30 Hz apart from each other. Also, there are some units tuned to higher frequencies: 350–435 Hz (groups H1 and H2). It should be noted that the height of the individual peaks in the histogram only indirectly reflects the overall tuning spectrum of the JO, as this distribution shows the representation of differently tuned units in the antennal nerve but not their individual sensitivity. The range of female wingbeat frequency is shown above the distribution (♀).
found in the L1 or L2 ranges were treated as true AFs and included in the distribution only if they could not be explained as a product of non-linear signal transformations in sensory units responding at different frequencies. Taking this into account, there must be an underestimate of the L1–L2 AFs owing to their possible coincidence with the mixed harmonics. The same underestimate must also exist in L3 and in the higher frequency range (the groups H1–H2): the harmonics of lower AFs were often detected in these ranges and according to the principles of data analysis (see Materials and methods), they were also excluded from the distribution.

The analysis of pairwise combination of tuning frequencies revealed a certain pattern: (1) the group A and C units appeared mutually exclusive – within a recording site units belonging to these groups never produced autoexcitation simultaneously, but with high confidence \((N=30, P<10^{-5})\), exact binomial test) were recorded sequentially before and after the inversion of the stimulation phase, i.e. they responded in anti-phase \((F_{AC}/F_A: 1.28\pm0.05, \text{mean}\pm\text{s.d.})\); (2) the same pattern was observed for the groups A and L2 \((N=20, P<10^{-5})\), exact binomial test; \(F_{AL}/F_A: 1.34\pm0.06\); (3) the group B units were co-localized in anti-phase with the low-frequency units of the L3 group \((175–185\ Hz, N=15, P<10^{-4})\), exact binomial test; \(F_{BL}/F_{L3}: 1.29\pm0.03\); (4) the group C units were co-localized with the E group \((N=9, P<10^{-5})\), exact binomial test; \(F_{CE}/F_E: 1.34\pm0.06\).

This pattern of co-localization allowed us to confirm the separation of the individual peaks in the summary distribution (Fig. 5), especially when the frequencies in a pair were separated by a distribution peak in the middle, as in the A–C and C–E pairs.

Besides the anti-phase pairs of sensory units we also recorded in-phase responses, when several units demonstrated autoexcitation simultaneously (examples in Figs 3E and 4C). Their pattern of co-localization was not random: the group E units were often (19 of 28, \(P<10^{-5}\), exact binomial test) paired with the A group units.

**Using the compound response for feedback**

With an electrode residing at the surface of the JO capsule it was possible to record the compound response, the so-called microphonic potential. When used in a feedback loop as a high amplitude (80–90 dB SPVL) stimulation signal, it caused an increase of a narrow-band noise level or even the autoexcitation (seven specimens, compare the spectral peaks in Fig. 3D with the peaks of distribution in Fig. 5). As a rule, several spectral peaks and sets of respective harmonics for each peak could be observed. Following the inversion of the stimulation phase, some peaks were replaced by others, and some retained their initial positions in the frequency range.

**Spontaneous activity of the JO neurons**

The recordings show that some of the JO auditory PSNs projecting to the antennal nerve are spontaneously active. The typical spectrum of spontaneous activity (Fig. 3F) shows the pronounced enrichment in the range 180–360 Hz and a separate peak above 400 Hz. The latter, normally observed in the range from 400 to 560 Hz (mean at 470 Hz) was found in 22 of 42 specimens. Initially we tried to attribute this high-frequency noise to the auto-oscillation activity of neurons that were damaged by an electrode during the previous recordings. To exclude this possibility we performed the analysis of spontaneous activity at the beginning of each experiment, when the number of damaged axons was minimal.

**Audiograms**

Most of the audiograms were of a broadband type (Fig. 6A) with the best sensitivity between 200 and 340 Hz. Many audiograms of this type demonstrated the signs of involvement of several narrow-band units (compare Fig. 5 and the curves of Fig. 6A in the range from 170 to 450 Hz). Besides the main minimum, in 15 of 29 audiograms an additional local resonance was present at higher frequencies; it roughly corresponds to the H2 group in the AF distribution. The individual audiogram of a unit could be obtained when only a single unit responded at a given recording site. In such rare cases \((N=7)\), the tuning characteristic was very narrow \((Q_{10}=7–23, Q_{10}=5–16)\) (Fig. 6B).

**DISCUSSION**

**Non-spiking transmission of signals**

The auditory responses recorded from male mosquitoes, especially the ones without the frequency doubling, confirm our earlier finding in female mosquitoes (Lapshin and Vorontsov, 2013) that axons of the JO PSNs propagate graded amplified receptor potentials rather than all-or-none action potentials. However, given this, we cannot rely on the presence of action potentials as an indicator of intracellular recording. Instead, during the penetration of the axon
we expected to observe a negative shift in potential and substantial rise in the amplitude of the phasic response. These effects were indeed observed (Fig. 2AB), although the shift was not always abrupt. The responses cannot be readily explained by the summation of spikes from a large pool of axons, because (i) even the slight (tens of micrometers) shift of the electrode dramatically changed the amplitude and frequency tuning of response, (ii) high-amplitude (>2 mV) responses did not show frequency doubling and (iii) anti-phase pairs of units with characteristic ratios of frequency tuning were reproducibly found at different recording sites in a single specimen as well as in different specimens.

The evidence we have to date is consistent with the mechanism of graded signal amplification by means of voltage-gated sodium channels (Taylor et al., 1995). The high-amplitude AC component of the response (but not the receptor potentials) is known to be blocked by tetrodotoxin in mosquitoes (Pennetier et al., 2010; Warren, 2010) and Drosophila (Lehnert et al., 2013), as well as by selective RNAi-mediated knockdown of voltage-gated sodium channels in the latter study. In addition, in the proposed theoretical model (Taylor et al., 1995) the axon also acts as a selective frequency filter, possibly explaining the mechanism of the different frequency tunings of the JO PSNs. Based on the studies of sensory cell responses in the hawkmoth JO (Dieudonné et al., 2014; Sane et al., 2007) one can suggest that non-spiking signal transmission in the mosquito auditory system evolved from a spiking one of a common insect ancestor due to the shortening of the primary sensory pathways and extreme multiplication of sensory units, which led to significant decrease of axonal diameter. In such conditions an additional decrease in concentration of voltage-gated sodium channels could prevent the generation of the true all-or-none action potentials, still allowing the non-linear amplification of receptor potentials in analogue mode.

Graded signal transmission has several advantages over the spiking one, for example, the coding of both positive and negative phases of the signal without the need for background activity, or the ability to perform fast pre-interneuronal signal processing in analogue mode. In our opinion, the mosquito JO with its multiple parallel elements possesses several features in common with visual sensory pathways and extreme multiplication of sensory units, which led to significant decrease of axonal diameter. In such conditions an additional decrease in concentration of voltage-gated sodium channels could prevent the generation of the true all-or-none action potentials, still allowing the non-linear amplification of receptor potentials in analogue mode.

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Frequency tuning of the JO

The most remarkable feature of the distribution of tuning frequencies of sensory units in males of culicids (Fig. 5) is the lack of dominant frequency peak corresponding to the range of individual variability of flight tone in C. p. pipiens females from the same mosquito population, 260–350 Hz (Lapshin, 2012). Instead, the major peaks of the distribution range from 190 to 270 Hz, which is exactly the range where the mixed harmonics concentrate. We propose that male mosquitoes predominantly analyse the combination harmonics of their own flight tone with a female tone, $F_1 - F_2$ and $2F_2 - F_1$ [where $F_1$ and $F_2$ are the flight tones (frequencies) of a male and a female, respectively]. The advantage of such an analysis may come from the fact that combination harmonics are substantially less dependent on the air temperature as the latter similarly affects flight tones of males and females (Römer, 1970). Previously, it has been demonstrated in both Culicidae mosquitoes and Chironomidae midges that non-linear processes that give rise to the combination harmonics can strongly influence the reception of acoustic signals (Lapshin, 2010, 2012). Also, our results are in agreement with the recent findings of Simões et al. (2016) that the male JO is tuned not to the female wingbeat frequency per se but to the difference between the male and female flight frequencies. Also, it is worth noting here that the AFs corresponding to the female flight tone (group E) and the ones from the range of combination harmonics (group A) often belonged to the in-phase pairs, implying that these components of the signal are processed together.

A small proportion of sensory units tuned to the flight frequency of a female does not necessarily mean that male mosquitoes are poorly sensitive to it. Their broad tuning can be adequate because of the high individual variation of flight frequency in females. A broadband auditory interneuron tuned to 300 Hz, which was found earlier in C. pipiens males (Lapshin, 2011), also indicates that the units tuned to the female wingbeat frequency exist in the male’s JO and project to the auditory regions of the brain.

The presence of units tuned to 350–435 Hz (groups H1 and H2 in Fig. 5) is further supported by the shape of several audiograms (an example of pronounced optimum at 410 Hz is shown in Fig. 6A). It can be assumed that these groups of units participate in the analysis of mixed harmonics during the mutual convergence of flight tones between a male and a female. In general, our audiograms comply with the ones measured in previous studies of Culex males (Warren et al., 2009; Simões et al., 2016).

The actual diversity of sensory units in the mosquito JO can be underestimated in this study due to the possibility of frequency capture in experiments with positive feedback stimulation (see Fig. S4). This effect has some similarities with a well-known phenomenon, in physics, of locking (or capture) of the oscillator frequency by the external periodic force (van der Pol, 1920), which was also demonstrated in animal auditory systems (Russell et al., 2003). What we interpreted as a single AF can represent the response of a group of PSNs with slightly different individual frequency preferences. The comparison of different kinds of data, i.e. the distribution of intrinsic tuning frequencies of sensory units (Fig. 5), the broadband audiograms measured from the same specimens and also the previously published averaged audiogram with the optimum at 240–250 Hz (Lapshin, 2012), suggest that the units of groups B, C and D make the primary contribution to the integral sensitivity of the JO. However, it should be noted that this distribution shows the representation of differently tuned units in the antennal nerve but not their individual sensitivity.

As for the functional significance of discrete frequency tuning in mosquitoes, we propose three non-exclusive hypotheses. (1) The frequency matching during the courtship behavior is used, as proposed by Pennetier et al. (2010), for mate recognition, while the wingbeat frequencies of male and female mosquitoes of the same species are not strictly determined. To ensure the continuity of such matching, mosquitoes must have a set of sensory units that cover the whole range of frequency variation, probably including the ranges of combination frequencies. (2) Several species of mosquitoes and midges share the same biotopes. Frequency selectivity can serve their reproductive isolation. (3) Little is known about functions of hearing in mosquitoes other than reproductive functions. High numbers of auditory neurons in female mosquitoes suggest that their hearing can also serve other functions such as the evasive behavior against predators or host-seeking behavior.

Estimates of unit bandwidth

We can indirectly estimate the relative frequency bandwidth of a single JO sensory unit either using the effect of AF capture by the external tonal stimulation (Fig. S4A) or by measuring the width of noise suppression zones. However, conclusions based on such data should be drawn with caution, as positive feedback increases the
quality factor of the oscillatory system while negative feedback, on the contrary, reduces it. Without considering these effects, for suppression zones shown in Fig. 3C and E, the \( Q_0 = 3 \) (\( Q_{10} = 2.1; F_0 = 201 \) Hz) and \( 3.6 \) (\( Q_{10} = 2.5; F_0 = 256 \) Hz), respectively. Estimation errors may occur because of the possibility of joint action of several units which have slightly different tuning frequencies (most probably, joint action of several units is responsible for the shape of the suppression zone in the range 240–270 Hz in Fig. 3E). Considering the effect of the negative feedback, the actual quality factor of a single JO sensory unit must be higher, i.e. \( Q_0 > 3.6 \) (\( Q_{10} > 2.5 \)).

Quite naturally, the quality factor of a single sensory unit can be measured from the shape of its audiogram, but only provided that it was not affected by the contribution of other units responding to the same stimulation within the range of the measurements. For narrow-band audiograms (Fig. 6B), \( Q_0 = 7–23 \) (\( Q_{10} = 5–16 \)). High variability of quality factor estimates could arise from the participation of other units in the recording.

If we accept the \( Q_0 \) of the A–C group units being equal to seven as the minimum estimate, then the unit bandwidth will be \( AF = F_0/Q_0 = 29–36 \) Hz (\( Q_{10} = 20–25 \)).

The effect of general adaptation of the JO sensitivity was noticeable in all experiments where two kinds of stimulation were applied simultaneously (Fig. S4B). This effect is in good accordance with the recently described system of efferent neurochemical modulation of the JO sensory cells (Andrés et al., 2016). The adaptation was not frequency specific: during the stepwise frequency increase if the frequency of sinusoidal stimulation consequentially crossed two AFs, it resulted in suppression of autoexcitation at both frequencies. Additionally, within the same time intervals the noise level decreased, which also indicated the overall drop of the JO sensitivity.

Spontaneous activity in the JO

The frequency range of spontaneous oscillations is quite similar to the range of flight tone variation in \( C. p. p i p i e n s \) males within the population from which the mosquitoes were taken for this study: 460–530 Hz with 500 Hz on average (Lapshin, 2012). Keeping in mind that flight vibrations significantly affect the reception of sounds by the mosquito JO, improving its overall sensitivity and frequency selectivity (Lapshin, 2011, 2012), we hypothesize that in a resting mosquito such spontaneously active JO neurons could provide the invariance of the acoustic information processing performed by the higher auditory interneurons. Such spontaneous activity substituting the response to flight vibrations would allow retention of, at least partially, the advantages of the two-frequency acoustic signal processing. At the same time we cannot exclude that spontaneous activity in the antennal nerve could originate from the active mechanical vibration of the antenna flagellum caused by the JO PSN motility (Göpfert and Robert, 2001; Warren et al., 2010).

Pairwise associations of sensory units

In the frequency range from 180 to 315 Hz we have found a strong interrelation between the properties of groups A–C, B–D and B–E, which suggests that PSN axons in the mosquito antennal nerve are orderly arranged according to their frequency and phase properties. Particularly, they maintain a few certain values of frequency ratio in a pair, in most cases responding in anti-phase. Four types of morphological organization of pairs of units responding oppositely to one and the same stimulus can be proposed: (1) a pair of units represents the two PSNs belonging to a single sensilla and their association is preserved further into the antennal nerve; two PSNs respond in anti-phase to the unidirectional shift of the antenna. (2) Both units from an anti-phase pair are attached to the same prong, but to its opposite ends. During the prong movement one of them is stretched while the other one is relaxed. There is morphological evidence supporting this hypothesis (Belton, 1989). (3) Units originating from the opposite parts of the JO capsule are combined into the anti-phase pairs just before entering the antennal nerve or within the nerve.

Together with our further speculations, hypothesis 1 allows us to address the physiological meaning of still unexplained combining of the JO sensory cells into pairs or triplets. The morphological organization of the JO cells, we believe, clearly reflects the mechanisms of primary signal processing in the mosquito auditory system. Hypotheses could be rejected by the direct morphological study, particularly by staining of the sensory cells belonging to a single sensilla from the JO capsule to the axonal nerve, or backwards, or by direct recording of sensory cell responses using the recently developed ‘goggatomy’ technique (Kay et al., 2016).

Studies on \( Drosophila \) showed that in their JO the mechanosensory neurons are specialized not only for hearing, but also for sensing gravity (Kamikouchi et al., 2009) and wind (Yorozu et al., 2009). Using calcium imaging, these authors demonstrated that the static deflection of the antenna produced opposing calcium signals in the anterior and posterior regions of the JO, thus supporting our hypothesis 3. However, recorded courtship songs elicited broadly distributed signals that peaked in or near the central region of the somata array, suggesting either a different kind of response to phasic stimulation or insufficient temporal resolution of the calcium imaging technique.

The fact that the units responding in anti-phase often go in pairs in the antennal nerve gives a plausible explanation for the frequency doubling in the auditory response of the JO, the effect which has been observed and discussed many times (Wishart et al., 1962; Belton, 1974; Clements, 1999; Jackson et al., 2009; Warren et al., 2009). A pair of anti-phase units that have their frequency optima not too distantly separated (as in A–C, B–D or B–E pairs) will demonstrate the signal frequency doubling in the recorded compound response, but each of the two units will not show this effect when recorded separately (Fig. 2, compare responses 1 and 2) or during the feedback stimulation (Fig. 3B). This explanation is supported by all our observations and it does not involve the distant haemolymph currents or the asymmetric response of the sensory cells to stretching and compression. Hypothesis 1 is also supported by the evidence that in the focal recordings from the sensory cell layer only the double-frequency response can be obtained (Simões et al., 2016). The similar mechanism of frequency doubling in the microphonic potential is known for the receptors of the lateral line organ of fish and \( Xenopus \), where neighboring sensory cells face opposite directions and thus respond in anti-phase (Harris et al., 1970; Flock, 1971; Kroese et al., 1980).

Opponent coding hypothesis

More than ten thousand PSNs of the male mosquito JO produce vast amounts of information, and there is an obvious need to analyse and compress it at the earliest stage of signal processing. In a pairwise combination of specifically tuned anti-phase units one can notice an analogy with the opponent coding of color information in the vertebrate retina (Daw, 1973). The opponency of auditory sensory units with different frequency tuning can substantially facilitate the following information processing in the brain as it allows easy distinguishing of sounds with a continuous (noise-like) spectrum.
from ones with a line spectrum such as the sound of a flying female. Additionally, the analysis of signals arising from a pair of anti-phase units with not distant frequency tunings may be the most effective way to prevent the auditory interneurons from responding to the large antenna deflections caused by wind currents during the flight maneuvers of a mosquito.

However, the opponent frequency coding implies that all parameters of both units in a pair except the frequency tuning are stable and identical to allow the analysis of the temporal fine structure of the sound, thus improving the frequency discrimination such as that occurring in mammalian ears (Reichenbach and Hudspeth, 2012). A pair of associated PSNs may compare the phases of signal in two channels tuned to different frequencies. The idea of direct interaction between the JO PSNs is supported by the morphological evidence obtained in Drosophila, where the parallel JO fibers display extensive contacts, including putative gap junctions (Sivan-Loukianova and Eberl, 2005).

The mosquito auditory system must have evolved to be extremely fast and efficient, as the delay and accuracy of the behavioral response directly affects the mating success of a male in a swarm. The multi-channel organization of the mosquito auditory system, both in frequency and spatial aspects, in combination with short pathways of analag signal transduction through the antennal nerve should dramatically speed up the auditory signal processing. The radial arrangement of sensillae in each of the two JOs might allow the fast spatial localization of a female. The sound from a flying female may come from any angle, so each sector of radially arranged sensillae must contain units with different frequency tuning. As the distance to a female and, therefore, the amplitude of its sound changes in a wide range if a male and a female approach each other, one can also assume that the auditory system must contain units with different sensitivity to achieve the high dynamic range combined with efficient mechanism of amplitude adaptation.

References


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