

## RESEARCH ARTICLE

# Directional and frequency characteristics of auditory neurons in *Culex* male mosquitoes

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## ABSTRACT

The paired auditory organ of the mosquito, the Johnston's organ (JO), being the receiver of the particle velocity component of sound, is directional by its structure. However, to date almost no physiological measurements of its directionality have been made. In addition, the recent finding on the grouping of the JO auditory neurons into antiphase pairs demands confirmation by different methods. Using the vector superposition of the signals produced by two orthogonally oriented speakers, we measured the directional characteristics of individual units as well as their relationships in physiologically distinguishable groups – pairs or triplets. The feedback stimulation method allowed us to discriminate responses of the two simultaneously recorded units, and to show that they indeed responded in antiphase. Units of different frequency tuning as well as highly sensitive units (thresholds of 27 dB SPVL and below) were found in every angular sector of the JO, providing the mosquito with the ability to produce complex auditory behaviors.

**KEY WORDS:** Primary sensory neuron, Auditory sense, Johnston's organ, Directional sensitivity, Frequency tuning, Harmonic synchronization

## INTRODUCTION

The auditory system of mosquito, the Johnston's organ (JO), is a highly sophisticated system containing thousands of sensory neurons (Boo and Richards, 1975a; Hart et al., 2011). Its complexity should not be surprising as mosquito mating behavior depends on audition (Clements, 1999; Roth, 1948) and hence the ears were developed under high selection pressure. Morphologically, a male mosquito possesses two antennae, designed to be the receivers of air particle velocity (Göpfert and Robert, 2000; Göpfert et al., 1999). The antenna, consisting of the flagellum with many hair-like fibrillae attached to it, originates from the capsule with radially arranged sensillae, or scolopidia, most of which contain two or three bipolar sensory neurons (Boo and Richards, 1975a; Hart et al., 2011). The neurons respond to antenna vibrations by transducing them into electrical potentials, and send the axons to the brain via the antennal nerve. In female mosquitoes, the antenna possesses fewer fibrillae and the JO contains about half as many sensillae compared with males (Boo and Richards, 1975b).

The task of detecting a mate's flight tone, which is believed to be the main function of the mosquito auditory system, does not seem to

be too complicated at first sight. However, the real-life task which is solved by the mosquito JO is much more difficult. First, any external sound blends with the mosquito's own flight tone, which leads to the appearance of multiple mixed harmonics at the receptor input (Gibson et al., 2010; Lapshin, 2011, 2012; Simões et al., 2016, 2018; Warren et al., 2009). The flight tone itself is not stable as it depends on the ambient temperature (Sotavalta, 1952; Villarreal et al., 2017), and, in addition, mosquitoes continuously maneuver, and change their flight velocity and wingbeat frequency. This change is especially remarkable during the courtship 'acoustic dance', when male mosquitoes first produce the rapid frequency modulation (Simões et al., 2016) and then the pair of mosquitoes mutually tune their wingbeats to fit the specific frequency ratio (Aldersley and Cator, 2019; Cator et al., 2009; Gibson and Russell, 2006; Penner et al., 2010; Warren et al., 2009).

A second difficulty that needs to be overcome by the mosquito auditory system is the strong low-frequency deflections of the antenna caused by wind currents, including those produced by the mosquito's own flight maneuvers. Although a common problem for the JOs of flying insects, in mosquitoes, dealing with these low-frequency deflections and wind currents is of particular importance to maintain the extremely high mechanical sensitivity which their ears are famous for (Göpfert and Robert, 2000).

Given all this, the complexity of the mosquito JO is not surprising. The principles of its operation are still to be understood, and there is no reason to consider them to be trivial. From the obvious tasks mentioned above, one can presume that the JO must contain units tuned to different frequencies and, most probably, with different sensitivity to provide high dynamic range. At the same time, even in the most sensitive units there must be a mechanism preventing them from being overloaded by low-frequency antenna deflections. And, last, the JO must detect sounds coming from different directions.


From the engineer's point of view, the mosquito JO is a highly sensitive, wind-resistant microphone which can be tuned to become directional and frequency selective. Recent advances in design of the insect-inspired acoustic micro-sensors (Windmill, 2018; Zhang et al., 2018) shed new light on significance of studies of mosquito audition beyond the obvious task of population control.

The radially symmetrical flagellar JO is said to be inherently directional (Belton, 1974; Robert, 2005). At the level of the sensory neurons, this means that each sensory unit within the population responds selectively to a sound coming from a restricted angular range. Whether the sensitivity and frequency preference of the JO is also symmetrical has never been tested. In several experiments by Belton (1974), the summary responses of the JO to the step deflections of the antenna by the piezoelectric transducer were recorded, but the results, according to the author, were too variable to make unequivocal conclusions.

In the much more simply organized JO of *Drosophila* (>400 mechanosensory neurons), different types of primary sensory neurons have been discovered (Albert and Göpfert, 2015; Kamikouchi et al.,

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2009; Yorozu et al., 2009), as well as interneurons that selectively code the complex stimulus features (Chang et al., 2016; Matsuo and Kamikouchi, 2013). Although the *Drosophila* model allows analysis of the mechanism of action of the JO sensory neurons with tools not currently available in mosquitoes, the results of these studies should be transferred to mosquito audition with some caution given the much higher complexity of the latter and the substantially different acoustic behavior in fruit flies and mosquitoes.

The physiological approach to test the properties of the auditory neurons in the mosquito involves the recording of their responses to sound. However, the method of recording the field potential of the whole JO, which is most commonly applied in studies of mosquito audition, does not allow the testing of hypotheses on the diverse tuning of elements within the JO, either frequency or directional. Recently, we developed a method of recording from small groups of axons of the JO sensory neurons along with controlled acoustic stimulation (Lapshin and Vorontsov, 2013). Although even the fine glass electrode used in that study allowed recording of focal potentials, because of the extremely small diameter of the axons in the antennal nerve, the resolution of recording had to be further improved, which was done by applying positive feedback stimulation. Using this, it was possible to see the frequency preference of a unit situated closer to the electrode tip or possessing the lowest threshold among other units (Lapshin and Vorontsov, 2013).

The first finding which that study brought was the diverse frequency tuning in the JO sensory neurons in female mosquitoes (Lapshin and Vorontsov, 2013), which was followed by a similar discovery in male mosquitoes (Lapshin and Vorontsov, 2017). This was to be expected, keeping in mind the behavioral observations which implied the existence of frequency discrimination in mosquitoes (Aldersley and Cator, 2019; Aldersley et al., 2016; Lapshin and Vorontsov, 2018; Simões et al., 2016, 2018). The individual tuning frequencies were distributed from 85 to 470 Hz in males and from 40 to 240 Hz in females of *Culex pipiens*, while the majority of units were found to be tuned to tones other than the wingbeat frequency of a mate.

A rather unexpected finding, however, was that the adjacent axons in the antennal nerve were grouped in distinct pairs or triplets by the specifics of their response (Lapshin and Vorontsov, 2013, 2017). In each group, the ratio between the individual tuning frequencies varied from 1.28 to 1.34, while the frequencies themselves were distributed more or less randomly within the range of sensitivity. Moreover, the units within a pair demonstrated an antiphase response to the same stimulus (Lapshin and Vorontsov, 2017). Each of the proposed explanations of this last finding was non-trivial: either the axons from the opposite sectors of the JO were pairwise combined in a nerve or the two units belonged to the same sensilla, but nonetheless responded in antiphase as a result of some specific tuning at the mechanotransduction stage.

The weakness of our previous study (Lapshin and Vorontsov, 2017) was in the specifics of the positive feedback stimulation setup, which at that time provided only two variants of the stimulation phase: either 0 or 180 deg relative to the unit response. This limitation meant that we could not tell whether the units that responded in a pair were strictly antiphase (180 deg) or possessed some other close directional ratio, as no directional measurements were performed. This was the primary reason for undertaking another study and using a different method of measuring the directional properties of the JO neurons. Here, we measured the thresholds of auditory neurons of the JO as a function of orientation of the acoustic wave vector relative to the axis of the antenna. Such data plotted in polar coordinates are commonly referred to as the polar pattern or the directional characteristic.

In previous studies, the polar pattern was assessed either by changing the angular position of the speaker relative to the test object (Daley and Camhi, 1988; Vedenina et al., 1998) or by rotating the test object relative to the stationary speakers (Hill and Boyan, 1976; Morley et al., 2012). There exists, however, a third way to study directionality: using a vector superposition of acoustic waves at the point of the receiver, produced by two orthogonally oriented stationary speakers (Theunissen et al., 1996). In this study, we implemented, with modifications, this third method. This allowed us to avoid the mechanical vibrations associated with the movement of objects within the setup, which was in turn important for the stability of recording. Additionally, herein we compared the directional characteristics measured from the same sensory units by the sinusoidal and the positive feedback stimulation, and plotted the distribution of units with different frequency tuning around the axis of the mosquito antenna.

The preliminary study of directional properties of the JO sensory units was done in Chironomidae midges (Lapshin, 2015). Their auditory behavior is generally similar to that of Culicidae: swarming males are attracted to the female wingbeat tone. Morphologically their JOs also have many similarities. These experiments showed that positive feedback stimulation can be successfully used to simultaneously measure the frequency and directional characteristics of the JO sensory neurons.

The central question of this study concerned the directional characteristics of the mosquito JO: is it symmetrical or not, in terms of sensitivity and frequency tuning. However, the experimental data allowed us to put forward hypotheses about the signal analysis performed by the primary auditory neurons of the JO, which may be more generally applicable to the similarly organized mechanosensory organs of other flying insects.

## MATERIALS AND METHODS

The relative threshold characteristics of auditory sensory units of the JO were measured according to the orientation of the acoustic wave vector relative to the axis of the antenna. In parallel, the individual tuning frequencies of units were identified. We used a combination of the two kinds of acoustic stimulation: sinusoidal, which is most commonly used in studies of frequency characteristics, and positive feedback stimulation. While the latter was effective for performing high-throughput measurement of individual tuning frequencies and directional properties of auditory units, only the former allowed us to measure the absolute auditory thresholds in the direction of the maximum sensitivity of a given unit. In addition, sinusoidal stimulation, which was applied to the units previously measured in feedback mode, served as a control against artifacts that could originate from the complexity of the feedback loop. Both kinds of stimulation are described in detail below.

### Animal preparation

Males of *Culex pipiens pipiens* L. ( $n=91$ ) were captured from a natural population in the Moscow region of the Russian Federation. Experiments were conducted under laboratory conditions with an air temperature of 18–21°C at the Kropotovo biological station (54°51'2"N; 38°20'58"E).

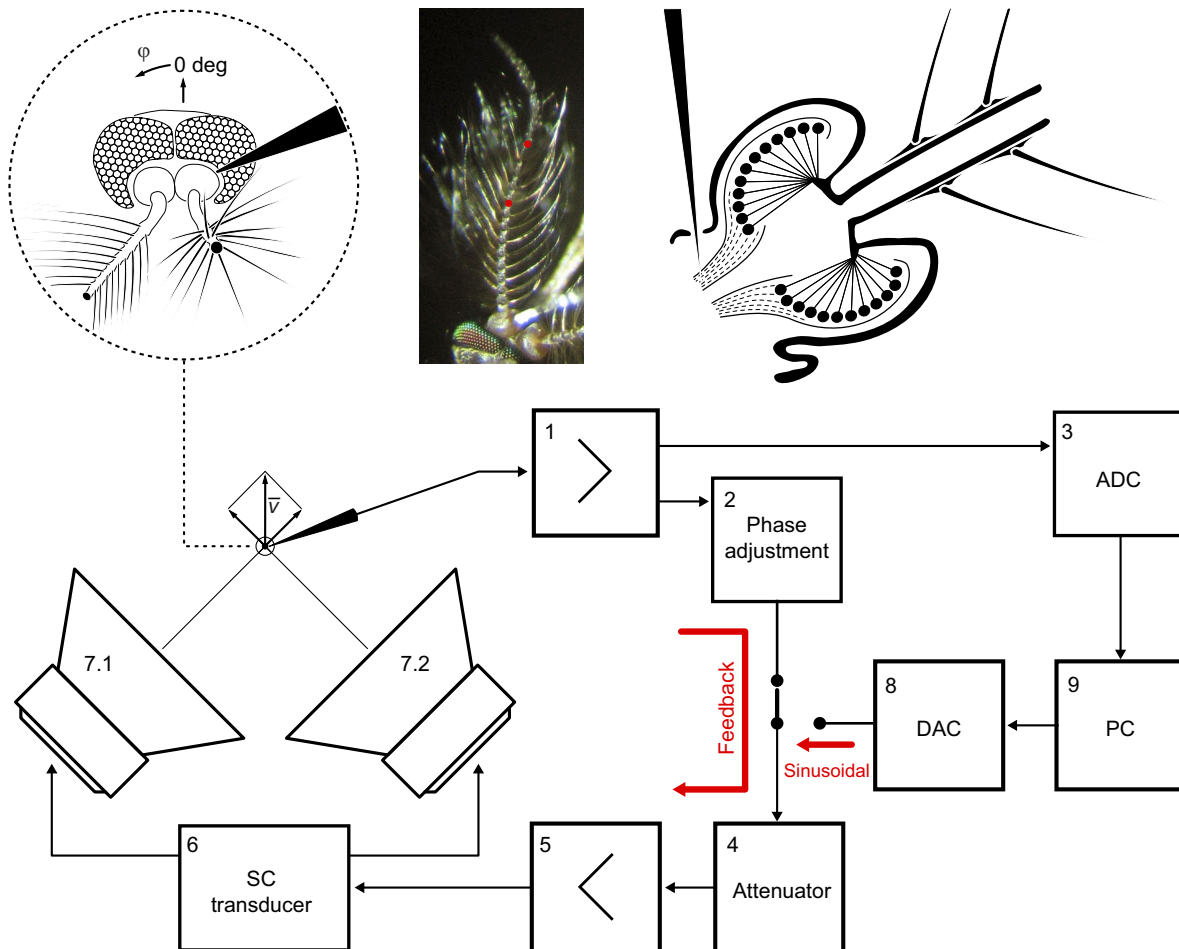
Individual mosquitoes were glued to a small (10×5 mm) copper-covered triangular plate with a flour paste containing 0.15 mol l<sup>-1</sup> sodium chloride. This type of attachment simultaneously served three functions: it ensured good electrical contact of the mosquito with the plate, which was used as a reference electrode, it mechanically fixed the mosquito and it prevented the mosquito from drying out during the experiment. The head of the mosquito

was glued to its body by a bead of nail varnish (partially dried in advance to minimize the drying time) to keep its orientation fixed during the experiment. The mosquito could still move its antennae, but this was visually controlled. The plate with the mosquito was mounted on a holder using a pair of miniature ferrite magnets, which allowed us to position the mosquito at any desired angle relative to the speakers. In most experiments, the mosquito was positioned dorsal side up. However, the constant orientation of the mosquito relative to the recording electrode could result in selective recording of some particular groups of neurons. To avoid this kind of a bias, the orientation was changed from specimen to specimen, either by turning it ventral side up or by rotating it by 180 deg in the horizontal plane; measurements of directional responses were

corrected accordingly. All recordings were made from the left JO because of the constraints dictated by the relative position of the mosquito, stimulating speakers, electrode and microscope. Assuming that the characteristics of a single JO can be asymmetric, we expected the properties of the two JOs to be mirror-like in relation to each other.

### Acoustic stimulation

Sound stimuli were delivered through a pair of Scandinavia 75 dynamic speakers (DLS, Gothenburg, Sweden) positioned with their acoustic axes at a right angle (Fig. 1). The mosquito was fixed at the intersection of these axes in such a way that the left antenna's flagellum was perpendicular to the direction of sound waves



**Fig. 1. Experimental procedure.** The experimental setup for electrophysiological recording and sound stimulation. The mosquito is fixed above two orthogonally oriented speakers (only the proximal parts of the mosquito's antennae are shown). Neuronal responses from the antennal nerve are amplified (1), digitized (analog to digital conversion, ADC; 3) and stored on the PC (9). Sound stimulation is made alternatively in feedback mode [neuronal response after phase adjustment (2) via the attenuator (4), power amplifier (5) and sine-cosine (SC) transducer (6) is fed to the speakers (7.1 and 7.2)] or sinusoidal mode [signal is synthesized on the PC (9) and after the digital-to-analog converter (DAC; 8) is fed to the attenuator (4), amplifier (5), SC transducer (6) and speakers (7.1 and 7.2)]. The mosquito is positioned at the intersection of the axes of the two speakers in such a way that the distal part of the antenna flagellum is horizontal and perpendicular to the axes of both speakers. The resulting direction of the air vibration velocity is determined by the vector superposition of the signals from the two speakers. An increase in angle of stimulation  $\varphi$  corresponds to counter-clockwise rotation of the velocity vector, with the insect's head viewed from the front (as shown in the figure). Accordingly, when viewed from the mosquito's head along the antenna, the clockwise rotation corresponds to an increase in  $\varphi$ . The principle of acoustic vector rotation used in this study can be illustrated by the following examples. When the two speakers are driven by equal electric signals, the resulting acoustic vector  $\vec{v}$  at the position of the mosquito is oriented up (in the coordinates of the figure), which corresponds to 0 deg. If the polarity of the electric signal driving speaker 7.1 is changed to the opposite, then the acoustic vector of its sound will rotate 180 deg and will be directed to speaker 7.1 itself. Then, the resulting vector  $\vec{v}$  will rotate 90 deg towards positive angles. A similar inversion of the polarity of the electric signal on speaker 7.2 only will lead to a rotation of the resulting vector  $\vec{v}$  by  $-90$  deg. If the signals are inverted at the inputs of both speakers, the vector  $\vec{v}$  will be oriented down, i.e. to the angle of 180 deg. To be able to set the resulting vector at all intermediate angles in the range from 0 to 345 deg, the phase of the signal at the input of speaker 7.1 must be adjustable in the range from 45 to  $-135$  deg (the range of negative values of the cosine function), and for speaker 7.2, respectively, from 135 to  $-45$  deg (the range of negative values of the sine function).

originating from each of the two speakers. The flagellum of the mosquito antenna is curved, thus it was impossible to position the whole antenna at a fixed angle relative to the speakers. In our experiments, we favored correctly positioning the distal part of the antenna (approximate position indicated by red dots in Fig. 1) as this part creates the greater torque at the base of the flagellum when affected by sound waves. According to preliminary estimates, the deviation of the antenna flagellum from the position perpendicular to the direction of sound waves within 25 deg would cause less than 1 dB error in the measured auditory thresholds. In the experiments, this deviation was always less than 25 deg.

The speakers were powered from a home-made amplifier (gain=4) via a passive sin-cosine (SC) transducer, which produced two derived signals with amplitudes  $A_1$  and  $A_2$ :

$$A_1 = 0.25 \cdot U \cdot \cos\left(\frac{\pi}{180} \cdot (\varphi + 45)\right), \quad (1)$$

$$A_2 = 0.25 \cdot U \cdot \sin\left(\frac{\pi}{180} \cdot (\varphi + 45)\right), \quad (2)$$

where  $A_1$  and  $A_2$  are the amplitudes of the control signals for the first and the second speaker, respectively;  $U$  is the alternating voltage at the input of the SC transducer; and  $\varphi$  is the angle between the dorso-ventral line passing through the mosquito's head and the vector of vibration velocity of air particles. An increase in  $\varphi$  corresponds to counter-clockwise rotation of the velocity vector, with the insect's head viewed from the front (Fig. 1). Accordingly, when viewed from the mosquito's head along the antenna, the clockwise rotation corresponds to an increase in  $\varphi$ .

The resulting direction of the air vibration velocity in the stimulating system was determined by the vector superposition of the signals from both speakers. Changes in the sound wave direction relative to the mosquito in 15 deg ( $\pi/12$ ) steps were accomplished by coordinated switching of voltage dividers in the SC transducer. For those angles at which the values of the function  $\sin(\varphi+45)$  or  $\cos(\varphi+45)$  were negative, the signal polarity was inverted by switching the terminals of the speakers. This technique of variation of the sound wave vectors did not require any construction elements to be moved inside the test zone during the experiment, so that vibrations which might affect the focal microelectrode recordings could be avoided and, in addition, measurements could be made faster compared with techniques which involve rotation of the speaker or animal.

The moving parts of the speaker had a low resonant frequency (90 Hz). Because of the considerable response lag of the dome of the speaker and its support, the emission phase delay increased with the signal frequency up to the point of inversion. To stabilize the phase delay, a phase correction depending on the stimulation frequency was included in the speaker control circuit.

The sinusoidal stimuli were generated by a digital-to-analog converter (LA-DACn10m1, Rudnev-Shilyaev, Moscow, Russian Federation). Acoustic calibration of the stimulating device was performed with an NR-231-58-000 differential capacitor microphone (Knowles Electronics, Itasca, IL, USA) attached to a micropositioner with axial rotation feature and set in the position of the mosquito. The same microphone positioned 2 cm from the mosquito was used to record the stimulation signals during the recordings.

The differential microphone together with its amplifier was previously calibrated in the far field using a B&K 2253 sound level meter with a B&K 4135 microphone (Brüel & Kjær, Nærum, Denmark). All sound level data in this study are given on a logarithmic scale in dB RMS SPVL (root mean square sound

particle velocity level), with a reference level of 0 dB being equal to  $4.85 \times 10^{-5} \text{ mm s}^{-1}$ , which corresponds in the far field to the standard reference sound pressure of 20  $\mu\text{Pa}$ .

### Microelectrode recordings

Focal recordings from the axons of the antennal nerve were made with glass microelectrodes (1B100F-4, WPI Inc., Sarasota, FL, USA) filled with 0.15 mol l<sup>-1</sup> sodium chloride and inserted at the scape-pedicel joint. In this study, we preferred the extracellular responses to the quasi-intracellular ones because of the stability of the former over the long time interval required for directional measurements.

After penetration of the cuticle, electrodes had a resistance of 15–110 M $\Omega$ . Electrodes were manipulated by means of a micropositioner. The whole setup was mounted on a vibration-isolated steel table. Neuronal responses were amplified using a home-made DC amplifier (gain 10, input resistance >10 G $\Omega$ ). To use the neuronal responses for feedback stimulation (see below) and to measure the response thresholds, 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 an E14-440 A/D board (L-Card, Moscow, Russian Federation) at 20 kHz sampling rate, and LGraph2 software. Digitized recordings were examined with Sound Forge Pro 10 (Sony).

Because the electrode tip and the average diameter of a sensory axon in the antennal nerve were of comparable size ( $\leq 1 \mu\text{m}$ ), we cannot claim that the recordings were made from individual axons. 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 primary sensory neurons of the JO, closely located within the antennal nerve and sharing similar frequency and phase properties, and thus representing a single functional unit. Detailed discussion of this issue can be found elsewhere (Lapshin and Vorontsov, 2013, 2017).

### Measurements of directional sensitivity

During penetration of the antennal nerve by the electrode, the preparation was continuously stimulated with tonal pulses (filling frequency 200–260 Hz, amplitude 60–65 dB SVPL, duration 80 ms, period 600 ms). In this searching procedure, groups of JO neurons situated orthogonal to the antenna oscillation could be overlooked. To avoid this, the vector of the acoustic wave was periodically changed by 90 deg using the switch on the SC transducer.

The threshold measurements were made using either sinusoidal stimulation (to obtain absolute thresholds) or positive feedback stimulation (to obtain relative thresholds). The essence of the latter method is a positive feedback loop established using the amplified in-phase response of a sensory unit as the signal to drive the stimulation loudspeaker. Application of this kind of stimulation to the sensory unit should cause it to 'sing' at a frequency that is close to its intrinsic tuning frequency – we call this effect 'autoexcitation'. With feedback stimulation, the threshold was defined as the signal level that required one more incremental step at the attenuator output (+1 dB) for the system to enter the autoexcitation mode. With sinusoidal stimulation, the criterion of the response threshold was set at 2 dB of sustained excess of response amplitude above the average noise level in a given recording. At each combination of stimulation parameters, the threshold was measured consecutively at least twice.

To distinguish between the above two methods, hereinafter we will use the term 'polar patterns' for the results obtained by the positive feedback stimulation, and 'directional characteristics' for

those measured with sinusoidal stimulation. It is important to bear in mind that the feedback method provides the unipolar response, i.e. it allows the two units responding to the opposite (180 deg) phases of the antenna vibration to be distinguished. The directional characteristics obtained using the sinusoidal stimulation are always bipolar.

The directional characteristics and polar patterns of sensory neurons were obtained by measuring the auditory thresholds at different angles of acoustic stimulation vector, which was changed in 15 deg steps. As the complete set of measurements for a single unit or a pair of units took quite a long time (20–25 min), repeated measurements at certain angles (usually at 45 and 315 deg) were made not less than twice per measurement series to ascertain the stability of the recording.

As a rule, in one specimen, directional measurements were made consecutively from two or more recording sites within the antennal nerve. At each recording site, the polar pattern of one unit (if a single unit was responding), two units (in most cases) or three units was measured together with their tuning frequencies in the mode of feedback stimulation. Then, in the case of a stable recording, the directional characteristics of the same unit(s) were measured using the sinusoidal stimulation.

During the subsequent data processing, the maximum threshold value ( $T_{\max}$ ) was determined for a given recorded unit. Based on this, a set of derived values describing the unit directional characteristic or polar pattern was estimated by the formula  $A_i = T_{\max} - T_i$ . In the curves based on these data, the sectors of the highest sensitivity corresponded to those with the lowest recorded thresholds, and the central zero point corresponded to  $T_{\max}$ . The angles at which no response at the best frequency was observed were given the value  $A_i = 0$ .

The angular sensitivity range of a unit was determined at  $-6$  dB of the maximum sensitivity (in the case of directional

characteristics, the two values from the symmetrical curves were averaged). The best direction of a given unit was determined as the bisector of this range.

As it is known that frequency tuning of the JO, as well as the wingbeat frequency, are highly dependent on the ambient temperature (Costello, 1974), all the frequency data in this study underwent temperature correction to the value of 20°C. For such calculations, a coefficient of 2% per 1°C was used, which was initially calculated based on a previous study (Villarreal et al., 2017) and confirmed in our own behavioral observations (D.L., unpublished).

Statistical analysis was conducted in R (<http://www.R-project.org/>).

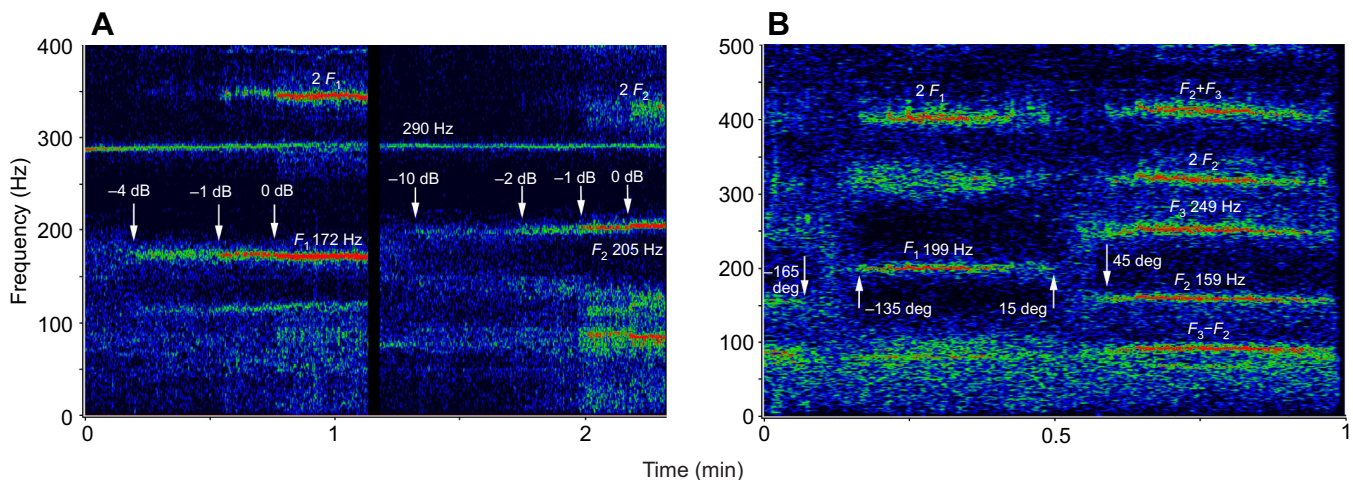
## RESULTS

### Directional properties of the JO units

Recordings were made from 91 male mosquitoes. In total, directional properties of 306 units were measured in the frequency range 114–359 Hz; among them there were 46 single, 85 paired (two units recorded together but responding in antiphase) and 30 triple units. In the last group of recordings, two units responded in phase, but demonstrated different frequency tuning, and one responded in antiphase and was tuned to a third frequency lying between those of the in-phase pair.

Examples of individual responses are shown in Fig. 2. We do not show the waveforms here; they were similar to those described in detail elsewhere (Lapshin and Vorontsov, 2017).

When the power of the feedback was increased from the sub-threshold levels, first a higher level of noise appeared in a narrow frequency range (Fig. 2A), followed by sporadic bursts of activity (from  $-1$  to 0 dB in the example shown). At higher levels of feedback, the response transformed into continuous excitation at the specific frequency, often with higher harmonics also present in the recording. When the direction of the sound wave was switched to



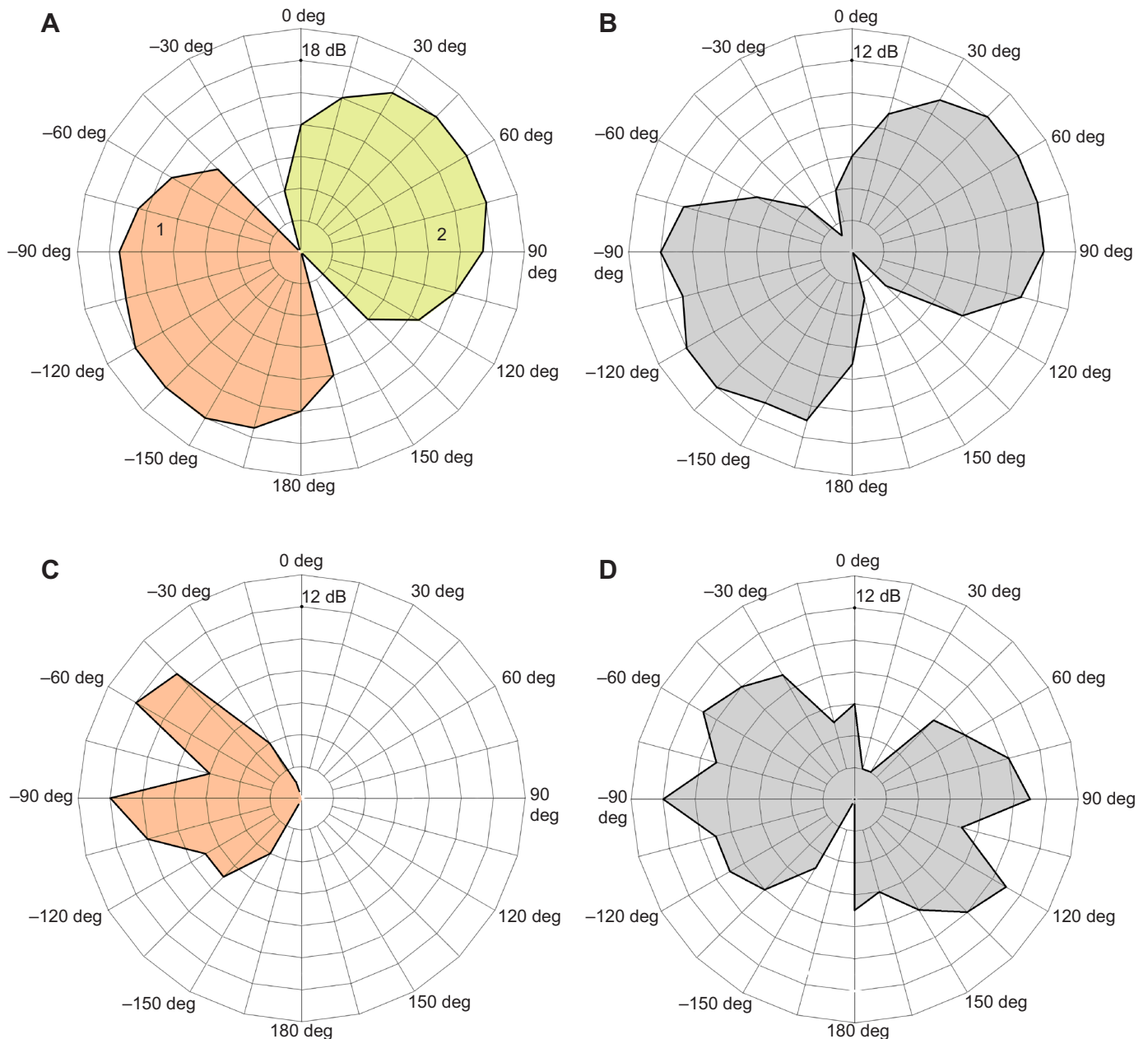
**Fig. 2. Examples of Johnston's organ (JO) unit responses to feedback stimulation.** (A) The direction of the sound wave is set to  $-60$  deg and the feedback power is gradually increased from sub-threshold levels; above  $-4$  dB, the response appears first as the higher level of noise in a narrow frequency range, followed by sporadic bursts (from  $-1$  to 0 dB) and continuous excitation at 172 Hz above 0 dB (absolute threshold of autoexcitation 42 dB SPVL at the fundamental frequency). Then, the stimulation is switched off, the direction of the sound wave is rotated by 180 deg (to 120 deg) and the procedure is repeated starting from  $-10$  dB. The autoexcitation this time appeared at 205 Hz [threshold of autoexcitation 45 dB sound particle velocity level (SPVL) at the fundamental frequency]. The continuous stripe at ca. 290 Hz represents the spontaneous activity in the JO, a correlate of active mechanics of the JO sensory cells, and it produces the combination harmonic (290–205=85 Hz), which can be seen in the right part of the sonogram. (B) An example of a triple-unit system responding to rotation of the sound vector. The feedback level was kept constant 6 dB above the threshold of the first recorded unit ( $F_1$ ); the sound vector was manually rotated by 360 deg in 15 deg steps. The first autoexcitation frequency,  $F_1$ , appeared at  $-135$  deg and disappeared at 15 deg (maximal level 52 dB at the fundamental frequency), then  $F_2$  (159 Hz, 50 dB) and  $F_3$  (249 Hz, 51 dB) appeared at 45 deg and disappeared at  $-165$  deg. Note the combination (mixed) harmonics ( $F_3 - F_2$  and  $F_2 + F_3$ ) when two units were excited simultaneously. Arrows indicate the moments when the autoexcitation appeared and disappeared. Color represents the relative amplitude of the response.

the opposite, the kind of response was similar but the unit(s) excited at a different frequency. We have observed this effect in previous studies (Lapshin and Vorontsov, 2013, 2017) when switching the phase of the signal in the feedback circuit, although here the change of the sound wave direction was done in a different way.

Alternatively, when the feedback power was kept stable above the threshold, and the vector of the acoustic wave was rotated stepwise, the excitation gradually appeared and disappeared depending on the angle of stimulation. In triple-unit recordings, and at certain angles, two frequencies were present simultaneously, producing frequency combination or mixed harmonics (Fig. 2B).

In the case of paired-unit recording, the polar patterns of individual units were opposite ( $180 \pm 10$  deg), mirroring each other,

while their individual tuning frequencies were different (Fig. 3A). For the same pair of units, the directional characteristic measured on the best frequency was bi-directional (figure-of-eight pattern), with its axis (with slight deviation) following the axis of previously measured combined polar patterns (Fig. 3B). The angular orientation of the polar patterns at a given recording site was arbitrary, with no obvious preference across the antennal nerve. Usually, after a slight axial shift of the electrode, another pair or triplet of units started to respond, demonstrating different angular orientation and different tuning frequencies while maintaining the opposite, mirror-like polar patterns. The polar pattern of a single unit had the form of a petal located asymmetrically relative to the center of the polar coordinates.



**Fig. 3. Examples of polar patterns and directional characteristics measured from the JO sensory units.** (A) Polar patterns of a pair of antiphase units; the best frequency of no. 1 is 201 Hz and that of no. 2 is 253 Hz. (B) The same pair of units as in A, obtained with 230 Hz sinusoidal stimulation; the threshold at the best direction is 29 dB SPVL. (C) Polar pattern of the single unit; the best frequency is at 199 Hz. (D) The same unit as in C, directional diagram obtained with 200 Hz sinusoidal stimulation; the threshold at the best direction is 37 dB SPVL. The angle of the sound wave is shown at the perimeter of each diagram, measured from the dorso-ventral axis (see Fig. 1). Relative sensitivity is plotted radially in 3 dB (A) or 2 dB (B–D) steps.

Sometimes, the ordinary petal shape of the polar pattern was distorted, with one or two notches appearing in it (Fig. 3C). Directional characteristics measured from the same units demonstrated some similarity in shape (Fig. 3D).

The average angular sensitivity range of a unit, measured from directional characteristics at  $-6$  dB from the maximum, was found to be  $123 \pm 14.5$  deg (mean  $\pm$  s.d.,  $n=74$ ). That estimated from the polar patterns was slightly narrower:  $100 \pm 16$  deg ( $n=276$ ). This difference in estimates is easily explained, as the positive feedback, on which measurements of the polar patterns were based, was very sensitive to the decrease in transfer coefficient, and this effect had to be most significant at the directions of minimal sensitivity of the unit.

Both frequency tuning and individual sensitivity of the units were found to be more or less evenly distributed around the antenna (Fig. 4A,C). The asymmetry in the angular distribution of units (non-uniform according to Rayleigh test of uniformity,  $P=0.9482$ ) can be explained by the difference in the total number of units recorded in each sector (I and III versus II and IV in Fig. 4B). We could not find a statistically significant difference in the distribution of individual frequencies between the 45 deg sectors of the JO (one-way ANOVA,  $F_{7,301}=0.809$ ,  $P=0.581$ ).

The absolute thresholds of sensitivity at best frequencies varied from 22 to 44 dB SPVL ( $n=74$ ). The distribution of threshold values showed three peaks with 4 dB intervals in between (Fig. 4D: 27, 31 and 35 dB SPVL); however, these data should be treated as preliminary as thresholds were measured at different stimulus frequencies, and thus are not uniform. To a lesser extent, this relates to the left part of the histogram (27 dB SPVL and below): most of the highly sensitive units were tuned within the narrow frequency

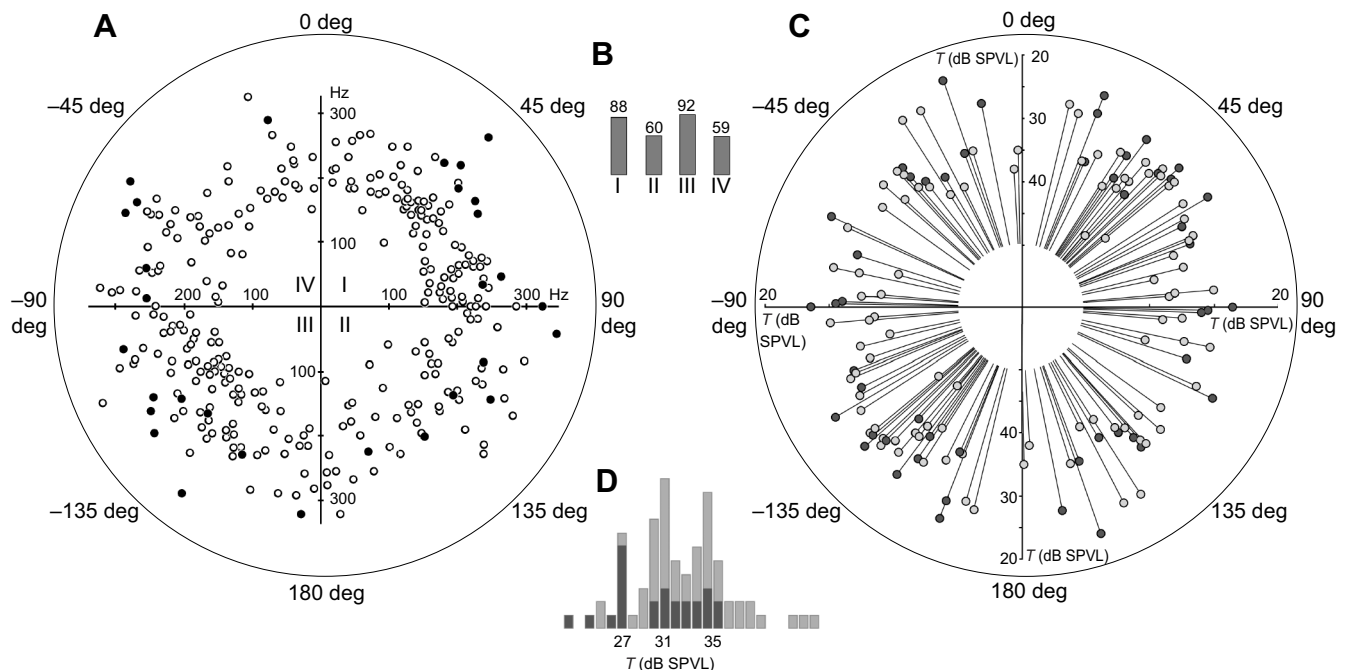
range 185–205 Hz. It should be noted that these highly sensitive units were present in every 45 deg sector of the JO.

### Ratios between individual frequencies

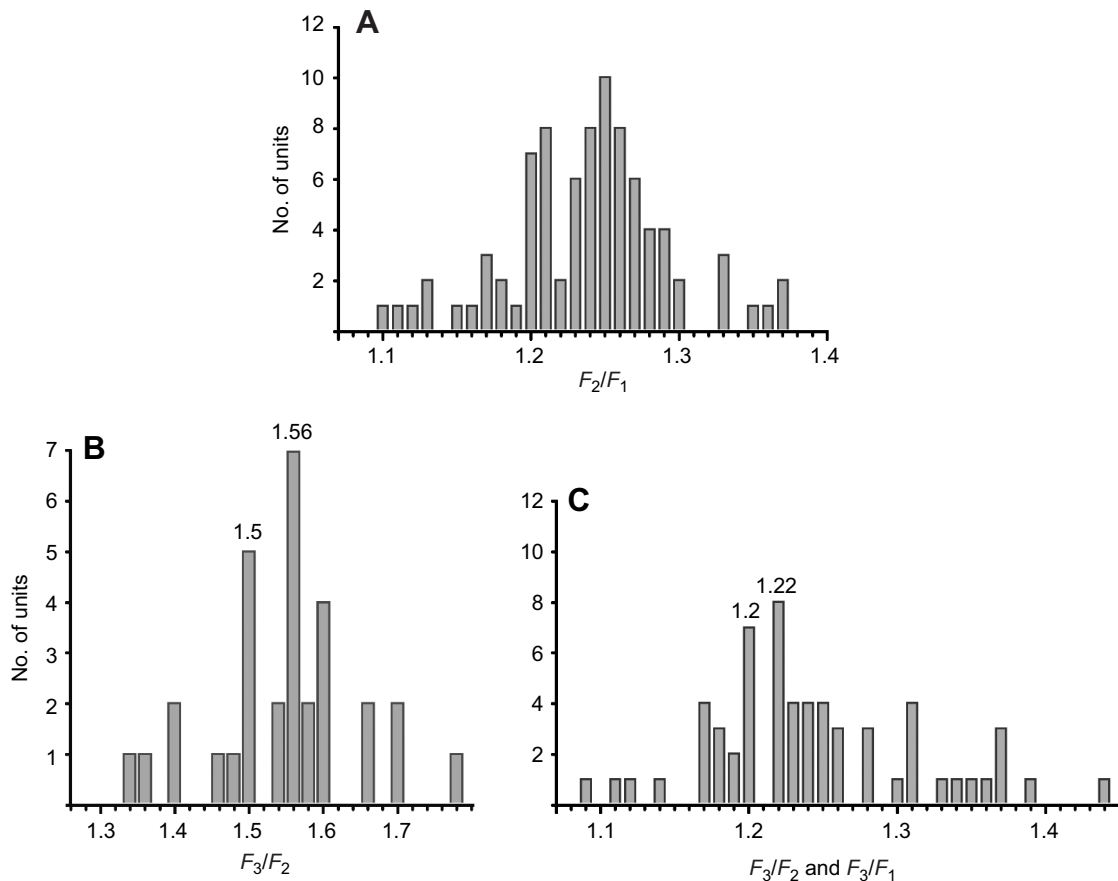
The most remarkable feature demonstrated by the individual tuning frequencies of the JO units is the relationship between those belonging to the same recording site where a pair or a triplet of units responded simultaneously. We divided the whole dataset in two parts, corresponding to the pairs and triplets, and analyzed them independently.

Fig. 5A shows the distribution of the frequency ratio in the antiphase pairs ( $n=85$ , mean  $\pm$  s.d.  $1.24 \pm 0.055$ ). Two major peaks stand out at 1.2–1.21 and 1.23–1.27. The hypothesis of normality cannot be rejected ( $P < 0.95$ ) according to the  $\chi^2$  (Pearson) test.

In triplets of units ( $n=30$ ), two units demonstrated in-phase autoexcitation, meaning that each of the units received not only its own signal, converted by the stimulation system into sound and into vibration of the antenna, but also the one of the second unit, recorded simultaneously. Such a cumulative effect led to the formation of a series of mixed harmonics in the recording. When the ratio between the frequencies was close to 1.5 (i.e. 3/2, one of the peaks in the distribution in Fig. 5B), the resulting spectrum became similar to a single harmonic series, as in Fig. 2B. Such similarity complicated identification of the individual tuning frequencies in the in-phase pair of units; however, the primary frequency could be distinguished from the mixed harmonics by the presence of the corresponding suppression zone at the same frequency, appearing after rotation of the stimulation vector by 180 deg, thus converting the positive feedback into negative feedback for a given unit (Lapshin and Vorontsov, 2017).



**Fig. 4. Directional properties of the JO units.** All results are plotted in polar coordinates with the center corresponding to the axis of the left antenna. (A) Angular distribution of best frequencies of the JO units ( $n=306$ ). Measurements were made from polar patterns, obtained in the feedback stimulation mode; the individual tuning frequency values are plotted radially. Filled circles show the higher-frequency units ( $F_3$ ) belonging to the triple-unit systems. (B) Histogram showing the total number of units recorded in each of the quadrants (I–IV) indicated in A. (C) Angular distribution of individual thresholds ( $T$ ;  $n=74$ ). Measurements were made with sinusoidal stimulation at previously determined individual frequencies; inverse thresholds are plotted radially, so the dots belonging to the more sensitive units are further from the center of the graph. Dark-gray dots belong to the units tuned within the 185–205 Hz range; light-gray dots show all other units in the 145–320 Hz range. (D) The same set of data as in C, showing the distribution of individual thresholds; the threshold amplitude is given on the abscissa. Dark-gray bars show the proportion of units tuned to 185–205 Hz ( $n=25$ ).



**Fig. 5. Distribution of frequency ratios between the units.** Individual frequencies are designated as  $F_1$  and  $F_2$  for pairs, and  $F_1$ ,  $F_2$  and  $F_3$  for triplets, where the  $F_1$  unit is in antiphase to the two in-phase units (lower  $F_2$  and upper  $F_3$  frequencies; an example response is given in Fig. 2B). Numbers above the histograms show the rounded values of the distribution local maxima. (A) Pairs of antiphase units ( $n=85$ ). (B,C) Triplets of units ( $n=30$ ), showing ratios in the in-phase pairs (B) and ratios in the antiphase pairs (C).

Fig. 5B shows the distribution of frequency ratio  $F_3/F_2$ , the in-phase pairs from the triple-unit systems ( $n=30$ , mean±s.d.  $1.54\pm 0.088$ ). The histogram has three major peaks: 1.5, 1.56 and 1.6. The hypothesis of normality should be rejected ( $P>0.95$ ) according to the  $\chi^2$  test; in this particular case, the peak at 1.56 ( $\chi^2=7.07$ ) was critical for the test. Antiphase pairs from the same triple-unit systems (Fig. 5C;  $n=60$ , mean±s.d.  $1.24\pm 0.067$ ) show two peaks at 1.2 and 1.22. The normality hypothesis should be rejected ( $P>0.95$ ), primarily because of the high amplitude of the major peaks: 1.2 ( $\chi^2=6.65$ ) and 1.22 ( $\chi^2=7.49$ ).

## DISCUSSION

### Directional properties of the mosquito JO

The number of sensory units recorded in this study ( $n=306$ ) represents only a minority of their overall number in the JO. However, even this sample of units, most probably, could perform analysis of the sound spectrum in all directions relative to the antenna axis (Fig. 4).

The average width of the directional characteristic is ca. 120 deg (Fig. 3B,D). Based on this estimate, we can conclude that four to five similarly tuned units, evenly distributed around the axis in the JO, would be enough to cover the whole directional range at the given frequency. However, this could be insufficient to provide the required accuracy for determining the angular coordinates of the sound source. According to Belton (1967), male mosquitoes are not attracted to sounds that come from a greater distance, even if these

signals contain conspecific female-like tones. For small flying insects, the most accessible way to estimate the distance to a sound source is to measure its angular position during their own displacement in space (parallax estimation of distance, or triangulation). In calm weather, the swarming *C. pipiens* males fly in large loops within the bounds of the swarm (Gibson, 1985). Performing the triangulation of a sound source, they, apparently, can determine the distance to it. Presumably, mosquitoes will pay attention to the sounds of nearby sources (within the transverse extent of the swarm) while more or less ignoring sounds coming from greater distances. This would help to stabilize the position of the swarm and would increase the noise immunity of the male-female auditory communication channel. However, the task of instant triangulation demands high speed and precision of angular estimates performed by the JO, and can at least in part explain its seemingly redundant complexity: the shorter the triangulation flight path, the higher the angular precision required from the JO, but at the same time the shorter the delay of the response that can be achieved.

Unfortunately, we cannot be sure that the diagrams in Fig. 4A,C show the true angular distribution of the differently tuned JO sensory units. The uncertainty lies in the possibility of selective recording from certain parts of the antennal nerve due to the geometric constraints in the mutual arrangement of the mosquito, the recording electrode and the speaker. However, some asymmetry in the angular distribution of sensory units still may be present, as



mosquitoes with one antenna ablated maintained the ability, although much reduced, to locate a female (Roth, 1948).

### Auditory threshold of units

Our method allowed us to measure the individual threshold at the preliminarily determined best direction of a given unit. Theoretically, the recorded threshold characteristics depend on the mechanical tuning of the antenna and the intrinsic tuning of the sensory cells. The mechanical tuning curve of mosquito antenna has been measured (Göpfert et al., 1999): for *Culex pipiens* males, the maximum is at  $328 \pm 3$  Hz (Warren et al., 2009). In the present study, the lowest thresholds (22–27 dB SPVL) were observed from 185 to 205 Hz (Fig. 4D), while the units tuned to higher frequencies were on average less sensitive. From this it can be concluded that the properties of the sensory cells rather than the mechanical properties of the antenna mainly determine the overall frequency tuning of the JO.

### Pairs and triplets of sensory units

Our measurements show that in the mosquito JO there is a large proportion of pairwise-combined units with different frequency tuning and oppositely oriented polar patterns: 85% of recorded units belonged to the paired- or triple-unit systems.

This finding means that during a single deflection of the antenna, they generate antiphase electrical signals. It is attractive to speculate that this physiological finding corresponds to the well-known morphological fact that most sensillae in the JO contain two or three sensory cells (Boo and Richards, 1975a; Hart et al., 2011) and that their axons keep adjacent position within the antennal nerve. The sinusoidal signals do not allow separation of the responses of these two cells, but the positive feedback stimulation provides an opportunity to study the antiphase units separately and measure their individual best frequencies and polar patterns.

The mechanism of antiphase response in two adjacent sensory cells is currently unknown. We propose two hypotheses: (i) two cells belonging to a single sensilla have different polarity of the mechano-electrical transduction; or (ii) the latency of signal transduction is different in these two cells. The latter mechanism, however, cannot work similarly in a wide range of frequencies. Currently, we discard a third possibility – that the antiphase axons belong to units from the opposite parts of the JO capsule – as there is no morphological evidence for it.

The triple-unit systems, including two units responding in-phase and the third in antiphase, give additional insight into the underlying neuronal morphology and support the above speculations. Because in the triple-unit recordings the polar patterns of the in-phase pair were always oriented similarly, it can be assumed with a high degree of confidence that the units producing these responses are morphologically combined in the capsule of the JO. Moreover, the specific ratio of best frequencies in such pairs and triplets indicates the functional interaction between these units (Fig. 5).

The morphological combination of two or more sensory cells into the sensilla is known for many insect chordotonal organs (Field and Matheson, 1998), including that of *Drosophila*, Chironomidae and mosquitoes. From the widespread occurrence of this phenomenon among insects, one can assume that it must have some general functional significance, not specific to mosquitoes. One of the possible tasks performed by such an organization of the sensillae may be the prevention of responses of the auditory neurons to large low-frequency deflections of the antenna, caused by wind currents during the flight maneuvers of an insect, being sent to the brain. The antiphase pair of sensory cells, having equal sensitivity in the low-

frequency range, can filter out such signals even before they leave the JO or the antennal nerve, provided that these cells are interconnected by gap junctions. This was indeed demonstrated in the JO of *Drosophila* (Sivan-Loukianova and Eberl, 2005). Such a mechanism must be very sensitive to the similarity of parameters of both sensory cells. Combining them into a single sensilla is fully justified in order to ensure equality of their directional characteristics and similarity of metabolism.

In a pairwise combination of specifically tuned antiphase units, there is 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 subsequent information processing in the brain as it allows sounds with a continuous (noise-like) spectrum to be easily distinguished from those with a line spectrum, such as the sound of a flying female, or selectivity for other stimulus features (Chang et al., 2016).

One of the possible reasons for the appearance of single-unit recordings may be the mechanical instability of the preparation due to the muscle contractions. In other words, some of the experiments ceased before measurements were made from all directions. However, not all recordings with only a single unit responding can be explained in such a way. Some of the polar patterns and directional characteristics appeared distorted (Fig. 3C,D). Such distortions can be explained by the presence of axons of other responding units, either in-phase or antiphase, in the area of focal recording, and their effect on the recording quite predictably was more pronounced during feedback stimulation and polar pattern measurements.

### Ratios between the individual frequencies

Analyzing the distributions of Fig. 5A,C, one can roughly estimate the statistical characteristics of the frequency mismatch in associated units. In paired units (Fig. 5A), the mean was 1.24. The same value was obtained for the distribution of frequency ratio in triple units (Fig. 5C). It can be assumed that this is the most probable ratio. At the same time, associated units can demonstrate other ratios, which could originate from the known phenomenon of ‘harmonic synchronization’, which is the specific mode of interaction between the coupled resonant non-linear systems when their frequencies are integrally related to each other. The stability of such synchronization is determined by the local decrease in the energy of the entire system (Yang et al., 2012) and generally decreases with increasing frequency multiplicity. We cannot exclude the hypothesis that the specific frequency ratios, including the ones which manifest themselves in behavior, are just a by-product of energy optimization.

However, the tendency of units in paired- and triple-unit systems to have their best frequency ratios close to the simple integer fractions ( $1.2=6/5$ ,  $1.25=5/4$ ,  $1.5=3/2$ ; see Fig. 5) may be a sign of the mechanism of signal processing, some kind of internal ‘language’ of the system, representing the auditory space of the mosquito. Such a tendency may also explain the multi-modal shape of the overall distribution of individual frequencies, which was demonstrated in our previous study (Lapshin and Vorontsov, 2017).

It should be noted that in triple-unit systems, the ratios between the individual frequencies are interdependent. For example, if the ratio of the in-phase pair  $F_3/F_2=1.5$  and the ratio of any of the antiphase pairs from the same triplet is, for example,  $F_3/F_1=1.25$ , then the ratio of the other antiphase pair should be equal to  $F_1/F_2=1.2$  ( $F_1/F_2=F_3/F_2:F_3/F_1$ ). However, there is a possible alternative, when in triple-unit systems the primary ones are the

ratios in the antiphase pairs. For example, if  $F_1/F_2=1.25$  (5/4) and  $F_3/F_1=1.25$  (5/4; see example in Fig. 2B), then the third ratio between the in-phase units becomes dependent:  $F_3/F_2=25/16=1.5625$ . Remarkably, in Fig. 5B, this ratio fell into the major peak of the distribution. Apparently, the frequency ratios between the individual units in the triple-unit systems do not follow clearly defined criteria (as it seems they do in pairs of units; Fig. 5A), but are limited by the more strict conditions which simultaneously connect three elements; for example,  $F_3/F_2 \times F_3/F_1=1.22 \times 1.22 \approx 1.5$ .

The seeming complexity of frequency ratios in the JO may be explained in the framework of primary signal processing. As every sound, even pure tones, comes to the JO sensory units of a flying mosquito accompanied by mixed harmonics, there must be a need to analyze this complex auditory image and to simplify the input to the brain interneurons. A highly parallel system of JO sensory units supplemented by the ability to instantly discriminate the particular combinations of tones seems to be almost perfectly suited to the task.

Regardless of the functional meaning of harmonic synchronization in mosquito audition, it is possible only if the oscillators express spontaneous activity at their best frequencies and interact, mechanically or electrically. In the JO sensory cells, a good candidate mechanism of interaction would be the active auditory mechanics (Göpfert and Robert, 2001) based on the dynein–tubulin motor of the ciliated sensillae (Warren et al., 2010). Such an interaction can also explain the appearance of mixed harmonics visible in the sonograms of Fig. 2A.

Our recent finding suggests that mosquitoes can potentially demonstrate different kinds of responses to different frequencies of sound. It was shown in behavioral tests that *Aedes diaantaeus* mosquitoes demonstrate fast avoidance response in the frequency range 140–200 Hz (Lapshin and Vorontsov, 2018). In these experiments, mosquitoes that were previously attracted by a sound imitating the wingbeat tone of a female (280–320 Hz) left the stimulation area within 1 s of the onset of the test signal (amplitude 57–69 dB SPVL), flying up, sideways and backward relative to the direction of test signal arrival. This and other behavioral observations, together with our current physiological findings, strongly suggest that the JO of mosquitoes can discriminate tones.

Several conclusions can be drawn from the data presented here. First, the angular distribution of sensory units would allow the JO of *Culex* male mosquitoes to detect and discriminate signals of different frequencies and amplitudes coming from any direction, with angular resolution sufficient for fast triangulation of the sound source. Second, it is very likely that the primary analysis of signals, such as the discrimination of the female tone from other sounds, can be performed by the same primary neurons, specifically interconnected based on their phase and tuning properties. The mechanism of opponent analysis of sounds which we propose based on the antiphase grouping of the JO units would allow maintenance of the high sensitivity and tone selectivity regardless of the mosquito's own flight sound and the wind currents accompanying the flight. This mechanism, as well as the tasks solved with its help, may be common to the chordotonal organs of different flying insects. Our understanding of the principles underlying the functioning of the mosquito JO might allow the design of highly sensitive, wind-resistant directional microphones, far superior in properties to those currently available.

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

Methodology: D.L.; Software: D.V.; Investigation: D.L., D.V.; Writing - original draft: D.L.; Writing - review & editing: D.V.; Supervision: D.L.; Funding acquisition: D.L., D.V.

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