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Frequency tuning of individual auditory receptors in female mosquitoes (Diptera, Culicidae)

D.N. Lapshin [a], D.D. Vorontsov [b,*]

[a] Institute for Information Transmission Problems of the Russian Academy of Sciences (Kharkevich Institute), Bolshoy Karetny Per. 19, Moscow 127994, Russia
[b] Koltzov Institute of Developmental Biology Russian Academy of Sciences, Vavilova 26, Moscow 119334, Russia

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ABSTRACT

The acoustic sensory organs in mosquitoes (Johnston organs) have been thoroughly studied; yet, to date, no data are available on the individual tuning properties of the numerous receptors that convert sound-induced vibrations into electrical signals. All previous measurements of frequency tuning in mosquitoes have been based on the acoustically evoked field potentials recorded from the entire Johnston organ. Here, we present evidence that individual receptors have various frequency tunings and that differently tuned receptors are unequally represented within the Johnston organ. We devised a positive feedback stimulation paradigm as a new and effective approach to test individual receptor properties. Alongside the glass microelectrode technique, the positive feedback stimulation paradigm has allowed us to obtain data on receptor tuning in females from three mosquito species: *Anopheles messeae*, *Aedes excrucians* and *Culex pipiens pipiens*. The existence of individually tuned auditory receptors implies that frequency analysis in mosquitoes may be possible.

1. Introduction

The sensing of acoustic stimuli by mosquitoes was first reported by Johnston (1855) when he described a sensory organ in the second segment of the insect antennae, which was subsequently named Johnston's organ (JO). Since then, considerable morphological, physiological and behavioural evidence has been accumulated about audition in mosquitoes.

1.1. Behaviour

It has been established that males are able to detect conspecific females by their flight sounds – the acoustic waves that are generated by wing strokes during flight (Belton, 1974; Clements, 1999; Roth, 1948; Tischner and Schieff, 1955; Wishart et al., 1962). Because female mosquitoes produce a relatively low-amplitude sound (Mankin, 1994), the male auditory system must be highly sensitive. Many studies have been performed to explain the mechanisms of hearing in male mosquitoes. Meanwhile, until the beginning of this century, it was believed that female mosquitoes themselves did not demonstrate any behavioural response to sound. Most behavioural experiments were conducted using male attraction to sounds of different frequencies, but this experimental paradigm proved to be ineffective for females. Quite recently, it was found that females of several mosquito species are attracted to the sounds of their amphibian hosts (Bartlett-Healy et al., 2008; Borkent and Belton, 2006; Borkent, 2008; Toma et al., 2005).

The recent finding that male and female mosquitoes mutually alter their wing-beat frequencies when they hear the conspecific signal (Cator et al., 2009; Gibson and Russell, 2006) allowed for the measurement of behavioural tuning curves measured from Toxorhynchites mosquitoes using alterations in wing-beat frequency as a response to sound stimulation. The tuning curves of males and females of this species are similar in shape, with their best frequencies being close to 400 Hz; males are approximately seven times more sensitive than females (Gibson and Russell, 2006).

When analysing the tuning curve of the auditory organs of a mosquito, it is generally believed that its shape is determined as acoustic vibrations are transduced into brain signals by the mechanical tuning of the antenna, tuning properties of sensory cells and active feedback mechanisms. Below, we briefly discuss each of these steps.

1.2. Mechanical frequency tuning

In both males and females, the antennae are resonantly tuned mechanical systems that move as simple forced-damped harmonic

Abbreviations: JO, Johnston Organ; AF, autoexcitation frequency; *pV*, particle velocity; SPVL, sound particle velocity level.

* Corresponding author.
E-mail addresses: dmitry.lapshin@gmail.com (D.N. Lapshin), colupaika@gmail.com (D.D. Vorontsov).

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oscillators when acoustically stimulated. The resonant frequency of the female antenna is approximately 230 Hz. That of the male is approximately 380 Hz, which corresponds to the fundamental frequency of female flight sounds (Göpfert et al., 1999). In recent years following the discovery of the mutual wing beat frequency adjustment in males and females of *Toxorhynchites brevipalpis* (Gibson and Russell, 2006) and *Aedes aegypti* (Cator et al., 2009), much attention has been paid to the nonlinearity of response characteristics in the antennal mechanical complex (Gibson et al., 2010; Jackson and Robert, 2006; Pennetier et al., 2010; Warren et al., 2009).

In addition to the passive mechanical properties of the antenna, the mosquito JO and the ears of many other animals demonstrate active mechanics that enhance the sensitivity and tuning of the flagellar mechanical response in physiologically intact animals, amplifying sound-induced vibrations at specific frequencies and low intensities (Avitabile et al., 2010; Göpfert and Robert, 2001; Nadrowski et al., 2011)

1.3. Auditory neurons

Each of the two Johnston's organs of a male mosquito contains approximately 15,000 mechanosensory cells (Boo and Richards, 1975a), with approximately half that number present in females (Boo and Richards, 1975b). These mechanosensory neurons are grouped into radially arranged sensory units, called the scolopidia, each of which consists of one to four bipolar sensory neurons and typically three types of supporting cells (Belton, 1974; Boo and Richards, 1975a; Hart et al., 2011). The sensory neurons mechanoelectrically transduce the nanometre-scale sound-induced vibrations of the antennal flagellum into electrical signals (Göpfert and Robert, 2000) and pass them on to the brain *via* the antennal nerve.

To date, the majority of physiological data from the mosquito auditory organ has been obtained by impaling the JO with tungsten electrodes and recording acoustically evoked field potentials. The results of these experiments highly depend on the filtering method used during the recording (Cator et al., 2009; Pennetier et al., 2010) and the author's choice from different components of the complex potential to measure auditory thresholds. Nevertheless, whatever method was used to obtain the frequency threshold curve (audiogram), it was tacitly implied that the tuning characteristics of individual receptor cells were similar to each other and to the tuning of the entire JO. In other words, it means that mosquitoes, despite possessing a comparably large number of auditory receptors, are not capable of distinguishing different frequencies.

However, there have been recent observations that have contradicted this assumption:

(1) Behavioural auditory tuning curves that are based on the minimum threshold level of sound that elicits a change in wing-beat frequency in response to pure tones were found to be sharper than the mechanical tuning of the antennae (Gibson and Russell, 2006).
(2) We have recorded responses from auditory interneurons in the brain neuropile of *Culex pipiens pipiens* males (Lapshin, 2012, 2011). The use of a glass microelectrode technique allowed us to record from individual interneurons and to measure their frequency characteristics. In addition to the expected broadband frequency responses that fit the summary response of the JO, we found a significantly different type of neurons that demonstrated very narrow-band responses (peak width approximately 30 Hz at Th+10 dB).

In light of these facts, it was natural to ask whether all of the auditory receptors have the same broadband frequency response similar to the one that is usually observed in the extracellular field potential recorded from JO. In other words, from where did these sharp tuning curves appear?

Here, we present evidence that individual receptors within the JO have different and discrete frequency tuning characteristics. We have studied females from three common mosquito species that are known to have different behavioural and feeding preferences (Silver, 2007). Female mosquitoes were chosen instead of males due to obvious gaps in our knowledge about their auditory system and due to their relevance to humans. Similar results that we have observed from male mosquitoes are currently being prepared for publication.

2. Methods

2.1. Animal preparation

Female mosquitoes of *Anopheles messeae* Falleroni (33 specimens), *Aedes excrucians* Walker (20 specimens) and *Culex pipiens pipiens* L. (46 specimens) were collected from the wild in the Moscow region of the Russian Federation. Mosquitoes were collected in June (*Ae. excrucians*) and August–September (*An. messeae and Cx. p. pipiens*) at the Kropotovo biological station (105 km south-west from Moscow).

Individual mosquitos were glued to a small (10 × 5 mm) copper-covered triangular plate by a flour paste with 0.15 M sodium chloride added. This type of attachment simultaneously serves three functions: it ensures good electrical contact of the mosquito with the plate, which was used as a reference electrode, mechanically fixes the mosquito and prevents it from drying during the experiment. The head of the mosquito was glued to its body by a bead of varnish. The plate with the mosquito was mounted on a micropositioner within the sound stimulation chamber using a pair of miniature magnets (Fig. 1A).

2.2. Microelectrode recordings

Recording from individual receptor axons requires a very sharp electrode tip; we found the conventional tungsten electrode technique inapplicable. Glass microelectrodes can be pulled to a very sharp tip but one that often breaks during the puncture of a hard cuticle. Nevertheless, in our preliminary experiments, we found an area at the lower edge of the pedicel where the cuticle was thinner and softer (Fig. 1B), and adjusted the glass pulling conditions to obtain an electrode tip that, after puncturing the cuticle, was still sharp enough to record from a single or several neighbouring receptor axons.

Recordings from the antennal nerve were made with borosilicate glass microelectrodes (1B100F–4, WPI Inc.) filled with 0.15 M sodium chloride. We used sodium rather than potassium ions because most of our recordings were made extracellularly and to minimise the damage caused by the solution flowing out from the electrode onto the neurons of the antennal nerve.

The position of the microelectrode tip within the antennal nerve was roughly estimated according to the reconstruction of mosquito's deutocerebrum neuronal structure provided by Ignell et al. (2005) and supported by the observation of the characteristic response to the acoustic stimulation.

Freshly prepared electrodes had a resistance of 60–80 MΩ. During the puncture of the cuticle the tip of the electrode broke giving a larger diameter and lower resistance which usually remained constant at approximately 10 MΩ. Most of the recordings (extracellularly measured tuning curves and autoexcitation experiments) were made with these low-resistance electrodes. Often we replaced an electrode in the course of a single experiment using