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The Risler Manuscript

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Abstract

An unpublished manuscript "The Auditory Organ of Male Mosquitoes (Culicidae) (Studies on Aëdes vexans Meigen 1830)" describes the structure of the pedicels and flagella of male and female *Aedes vexans* (= *Aedimorphus*[1] *vexans*) from scanning and transmission electron micrographs. We reproduce diagrams from the manuscript showing an in-depth section of the pedicel and first few flagellar segments, a hinge involved in extending and collapsing the long fibrils on the male flagellum that has not been described before in the Culicinae and the structure and arrangement of two different types of sensory units (scolopidia), one of which we speculate might be involved in vibrating the flagellum and increasing the sensitivity of Johnston's organ.

Key words: *Aedes vexans, Aedimorphus vexans,* Johnston's organ, hearing, antennae, fibril extension.

Introduction

During a review of the literature dealing with the acoustic properties of mosquito antennae we came across an unpublished manuscript by the late Professor Helmut Risler (1914-1995). Risler gave a copy of this manuscript to Dr. A.N. Clements in 1984, with permission to use micrographs from it in his upcoming book (Clements, 1999). The manuscript was never published and after Risler's death, Clements deposited it in the library of the Natural History Museum, London (Risler, 1984). Because this subject is changing rapidly and producing very interesting new findings, we consider it important to point out some of Risler's hitherto overlooked results and review the topic for the general mosquito biologist.

In 1955 Risler published his first diagram of a longitudinal section of a male culicid antenna showing long setae (fibrils) extending outward from 3 basal flagellar segments (inset upper right in Fig. 1) and it is still one of the most commonly seen illustrations in major references (e.g. Clements, 1999 Fig. 26.5). In 1955 there was good behavioural evidence that males of many species use their highly sensitive antennae to detect and fly toward the sound of the female's wingbeat. In many species this occurs in crepuscular swarms where groups of males fly over visual markers; Marshall (1938) includes several early descriptions of females flying into the swarms, where they

[1] Proposed species names follow editorial policy to reflect the creation of new generic and subgeneric rankings in ongoing internal classification of the Culicidae, as published by John Reinert and colleagues in the *Zoological Journal of the Linnean Society* and elsewhere.

are quickly surrounded by a cluster of fast flying males and mate with one of them. Whereas the swarming behaviour was well known, the physiological and physical aspects of the use of sound were not. In many species of several genera the 500 or so long fibrils on the flagellar segments of the male antennae are folded parallel to the flagellum (as they are when they emerge from the pupae) except when they are swarming. There is a blood vessel in the flagellum of the species so far examined which led Downes (1969) to suggest that males extended and collapsed their fibrils through changes in blood pressure. This was disproved, at least in *Anopheles stephensi,* by Nijhout & Sheffield (1979) who showed convincingly that a deeply-grooved crescent of protein becomes hydrated and opens to extend the two groups of fibrils on either side of the first 12 segments of the male flagellum. In this paper, the authors caused confusion by concluding that "Mating is limited to the period of antennal hair erection since this is the only time males can perceive the female" but Wishart *et al.* (1962) had already shown the contrary, that removing half the fibrils made no significant difference to the electrical activity they recorded from Johnston's organ (JO) of males in response to sound.

Until the beginning of this century it was believed that females, although possessing JO's only slightly smaller than those of males with identical sensilla that respond to sounds, did not change their behaviour in response to them. Now it is known that the females of several mosquito species are attracted to the sounds of their amphibian hosts (Borkent & Belton, 2006; Toma *et al.,* 2005; Bartlett-Healy *et al.,* 2008). There is also convincing evidence that in at least four genera, both males and females can hear and adjust to each other's wingbeat frequency (Gibson *et al*., 2010).

A third recent discovery may also be relevant to Risler's description of Johnston's organ. That is the finding that the neurons, or at least some of them, in the movement-sensitive organelles (scolopidia) of both males and females can vibrate the flagella (Göpfert & Robert, 2000). Risler made careful counts and comparisons of the four different types of scolopidia in male and female *Aedes vexans* and we point out here some of their characteristics that may be related to vibration of the flagellum.

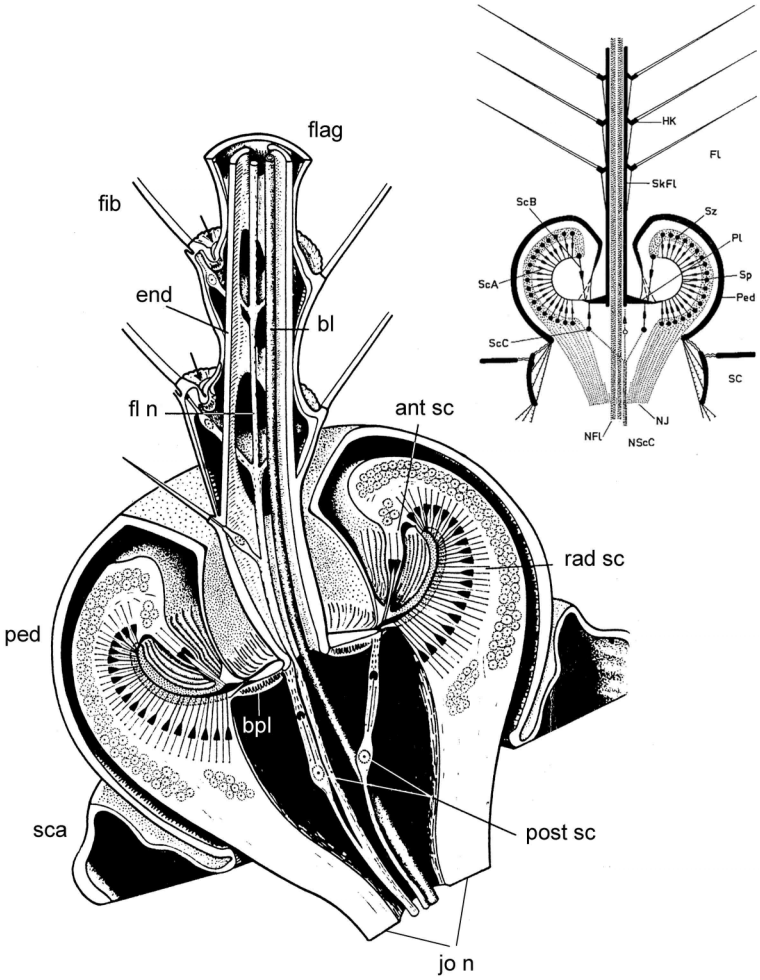
Material and methods

Mosquitoes were from a stenogamous strain of *Aedes vexans* from the Rhein River Valley, near Mainz, Germany. They were reared for the Institute of Genetics, Johannes Gutenberg University (Kuhn, 2002). Adult mosquitoes were fixed and sectioned conventionally with osmium tetroxide fixation and lead citrate staining. For scanning electron microscopy, partly dissected heads were dried at the critical point and sputter-coated with gold. Micrographs were made with a Zeiss EM9a or a Siemens Elmiscop 1A for transmission and a Cambridge Mark II for scanning microscopy. Some of the micrographs were taken of partly sectioned heads with the Araldite resin partly removed (Mayor *et al.,* 1961).

Results

We will restrict this summary to descriptions of the diagrams in the Risler manuscript because the original transmission and scanning micrographs have not been found. We replaced the original abbreviations in the diagrams with more usual ones corresponding to the terms used by Clements (1999) and show the position of the fragmented inner wall of the pedicel in Fig. 3 to make our explanation of its probable function clearer.

Figure 1. Diagram of a section of the male pedicel and basal flagellar segments of *Aedes vexans*, it is clearly related to Risler's (1955) earlier version of the same region (inset upper right).



The first diagram shows the interior of the pedicel (ped) attached to the scape (sca) and the basal segments of the flagellum (flag) in depth. The hollow nerve from JO (jo n) is nearly 70μm in diameter and probably contains over 30,000 axons. Separate nerves run from the single scolopidia (post sc) attached to the underside of the basal plate (bpl) and the sensilla on the flagellum. A blood vessel (bl) runs alongside the inner nerve into the flagellum and small tracheae are present here and between the neurons and outer pedicel wall (Risler, 1953) but not shown in this diagram. The inner surface of the pedicel shows vertical grooves corresponding to the 58 (mean) prongs that curve out and up from the basal plate. Only 19 radial (rad sc, type A) scolopidia are shown diagrammatically attached to the outer surface of each of the two opposite prongs. Risler estimated that there are actually more than 250 scolopidia of this type attached to the outside of each prong, a total of 15,000 arranged radially in each JO.

The suspension of the basal plate must be extremely flexible because the antennae of the males are thought to be among the most sensitive hearing organs in the animal kingdom. More detail is given in Clements' clear description (1999) but the thin septa that separate the prongs and the fine connecting distal filaments of the scolopidia are shown on the inner surface of the relatively massive 'doughnut' of scolopidia and neurons in this figure. Anatomically distinct scolopidia (ant sc), the anterior or type B series are drawn within the prongs. Two are shown on each prong but in the electron micrographs there are four filaments running down (in this orientation) to the inner surface of the base of each prong where it thickens and attaches to the margin of the basal plate. Risler estimated 232 of these scolopidia (exactly four per prong with a mean of 58 prongs). There is the same arrangement of this type of scolopidium attached to the inner surface of each prong near the basal plate in the female JO.

An endoskeleton (end) is shown originating midway up the first flagellar segment. Oval windows in each segment allow axons from the sensilla to pass through the skeleton and join one of the two flagellar nerves (fl n). The basal 40-50μm of the long fibrils (fib) is shown in deep sockets, their length decreases from 600-300μm toward the apex of the flagellum and those on the inner (medial) side are shorter than those on the outer. There are up to 60 fibrils on each segment arranged in two equal groups dorsal and ventrally on raised crescents of cuticle. Each group has a deep groove on its inner surface that Risler terms 'cuticular ring' shown on the left-hand fibrils in this figure (arrows). This is shown in detail in the next figure.

Figure 2. Diagram of the articulation of a fibril and the structure of its mechanoreceptive sense cell.

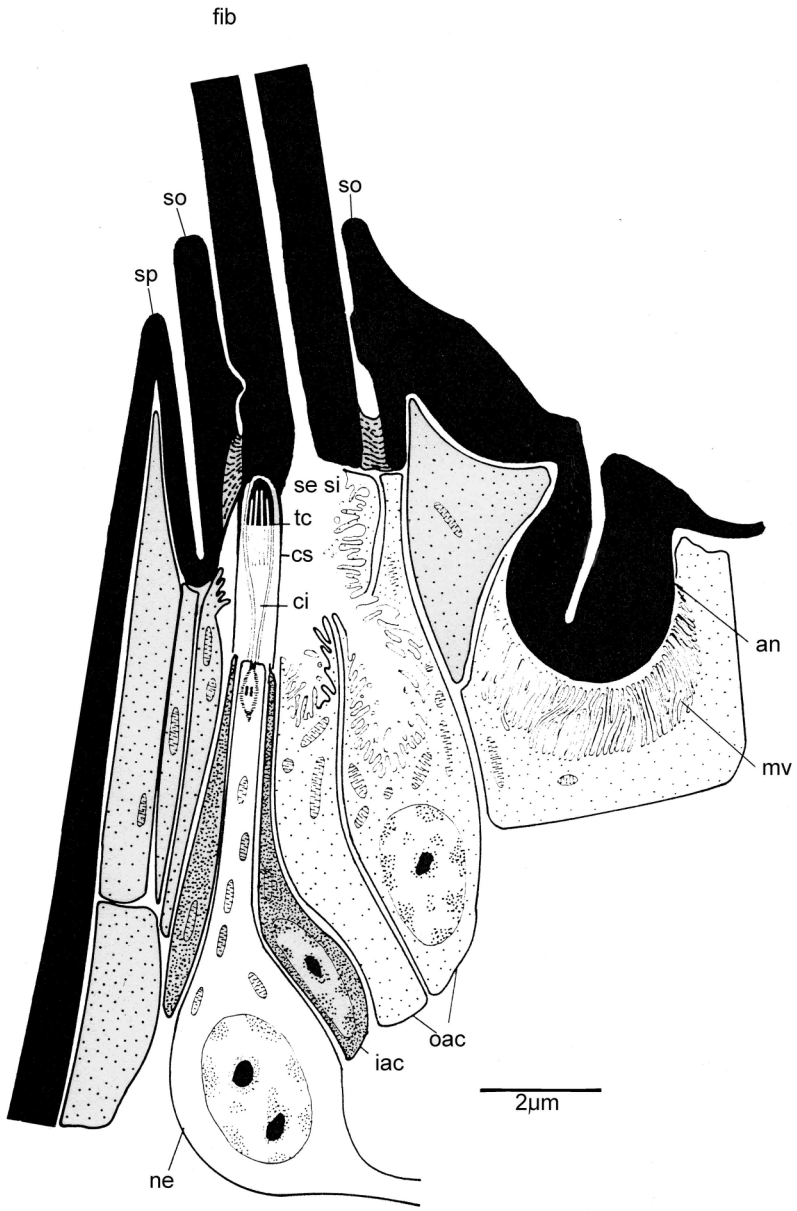


Figure 2 shows the base of a typical long fibril with its mechanoreceptive neuron. The sensory neuron has a typical structure at its apex with a cilium (ci) ciliary sheath (cs) and terminal cap (tc). Below the fibril (fib) is a sensory sinus (se si) and the neuron has inner and outer accessory cells (iac and oac). On the right is the deeply grooved electron-dense annulus (an) and below it a large cell with dense microvilli (mv) and numerous mitochondria.

Risler's scanning micrographs show a scaly process (sp) that overlaps the socket (so) of the adjacent fibril and presumably keeps the fibrils in line as they extend and close. He suggests that the large cell below the inner electron-dense groove of the annulus is secretory.

Figure 3. Diagram showing the differences between the radial (A) and anterior (B) scolopidia

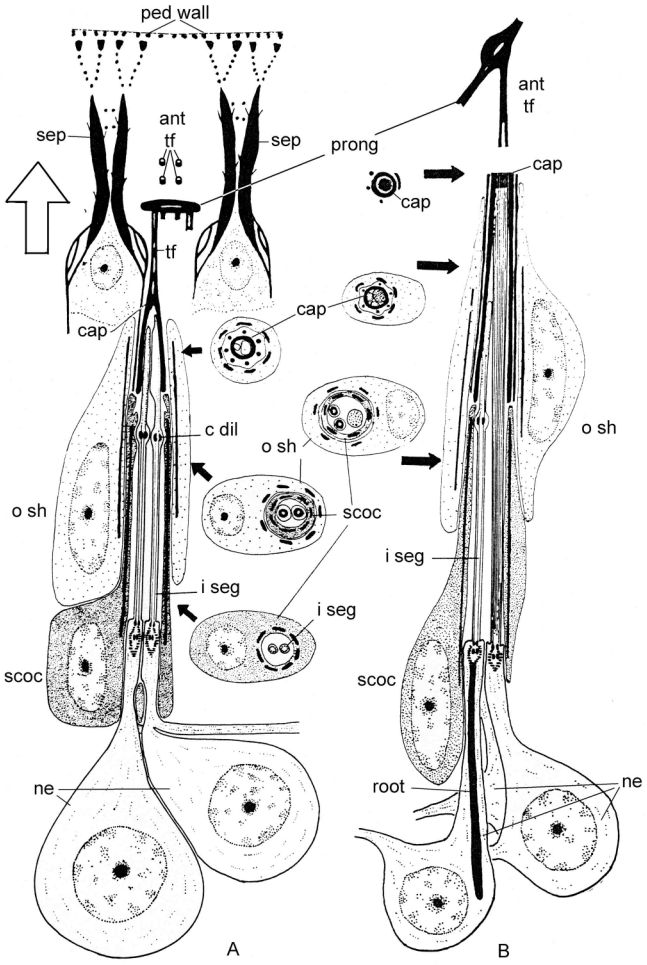
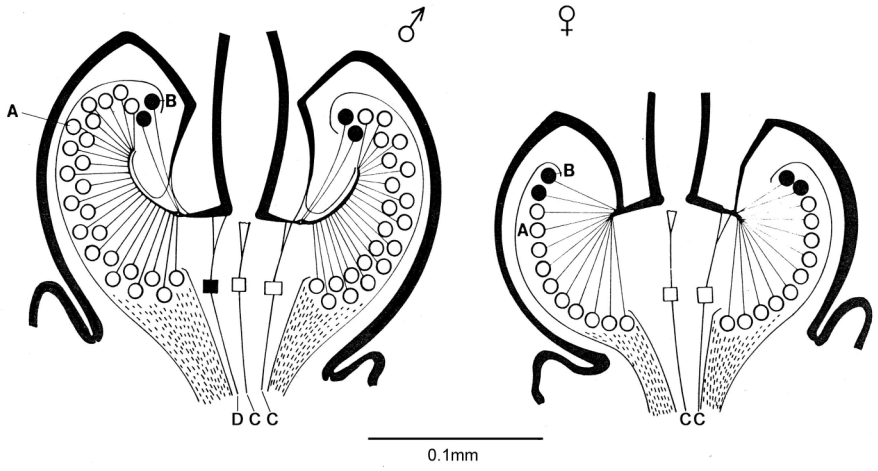


Figure 3 shows a radial scolopidium on the left with two similar neurons (ne) enclosed in a scolopale cell (scoc). The scolopidia are shown in transverse section at the arrows in the centre of the figure. The scolopale cell contains a 'cage' of seven electron-dense rods, and is enclosed distally by an outer sheath cell (o sh) with a similar arrangement of overlapping rods. The outer sheath envelops a third cage of rods, (bars of Boo & Richards, 1975) that connect to the septa (sep) and the inner wall of the pedicel (ped wall) The two neurons have typical ciliary inner segments (i seg) and distal ciliary dilations (c dil) and appear to be firmly attached apically to a conical cuticular cap (cap). The apical cap tapers to a fine terminal filament (tf) that attaches to the outer surface of the flattened prong that curves up (anteriorly) from the basal plate. Three neurons are contained in each anterior (B) scolopidium, two of them are similar to the ciliated type in A, but the dendrite of the third is packed with microtubules. One of the ciliated neurons has a long electron-dense root process (root) extending close to the nucleus of the neuron. The anterior scolopidia have much longer terminal filaments (ant tf) (broken on the right) joining the inner surface at the base of each prong very close to its origin at the margin of the basal plate. The electron-dense scolopale rods are shown in sixes rather than sevens around the transverse sections of the three dendrites but the number is variable from scolopidium to scolopidium. Curiously, the number of rods in the sheath and envelope cells always seems to be the same as in the scolopale cell.

Risler has clearly combined the results of his former student Schmidt with features taken from the literature and his own micrographs in this diagram. We include them because they allow a side-by-side comparison of the two main types of scolopidium. It is hard to visualize the arrangement of prongs and septa in two dimensions and there is a more complete description in Clements (1999) for those interested in more detail. We will simply point out that when sound moves the prongs in the direction of the open arrow on the left, the terminal filament pulls the tips of the cilia with them and away from the scolopale rods that evidently remain fixed to the interspersed septa. The scolopale rods of the B units are presumably also attached to septa, but this is not yet clear from the micrographs.

Figure 4 shows the very different arrangement of scolopidia along the prongs in the two sexes. Risler points out that the number of B scolopidia is similar (about 220 in females and 232 in males) but that the type A are much fewer in the female (about 2,750 compared with 14,500 in males). The distal filaments of the A and B units in the female are similar in length, but as in the male the B scolopidia are attached closest to the basal plate. The single type C and D scolopidia are probably not involved in hearing (Clements, 1999) although the type D has only been seen in males. The drawings are to the same scale.

Figure 4. A scale diagram comparing male and female Johnston's organs. Cell bodies of the type B scolopidia in black, the more numerous type A clear. As in Fig. 1, two opposite prongs are shown with only a few of the scolpidia, but in their correct alignment. The much longer terminal filaments of the B scolopidia in the male are shown attached to the inner surface of the prongs close to the basal plate. Two septa are shown extending from the inner surface of the pedicel above the prongs of the male. The prongs of the female are tiny by comparison and curve downward. The single D (black) and C (clear) scolopidia are shown below the basal plate.



Discussion

Considering that the males of many species of mosquito in at least five genera keep their long antennal fibrils closely appressed to the flagellum except when they swarm and mate, it is surprising that the method of their movement has not been followed up since Nijhout & Sheffield's study of *Anopheles stephensi* in 1979. In his study of *Aedes vexans,* Risler shows the large cells on the inner margins of the fibril sockets that Nijhout investigated in *Anopheles* very clearly (Figs 1 & 2) and later realized their function. At a meeting in 1989 Risler describes in *Ae. vexans*: "einer dicken, polsterartigen Gelenkfalte am innenrand der Kränze" – 'a thick bolster-shaped hinge on the inner margin of the fibrils' (our translation), (Risler, 1990). We have evidence that the hinge is composed of a protein similar in properties to that of *An. stephensi* in another aedine that extends its fibrils, *Aedes* (=*Tanakaius*[1]) *togoi*, (Hart *et al.,* 2010). Their fibrils are closely appressed in heads immersed in saline with low pH and expand in alkaline (pH 8) saline, exactly like those of *An. stephensi*. Evidently this protein absorbs water when alkaline and

increases in size, widening the deep groove in the hinge and extending the groups of fibrils. The large cells below the hinge, with their mitochondria and mass of microvilli evidently secrete the liquid. This is the only known hydration device in the animal kingdom (Vogel, 1988) and it would be interesting to make comparisons with other genera of mosquitoes that collapse and extend their fibrils.

The arrangement and structure of the two main types of scolopidia, A and B, were also of great interest to Risler. He believed that the type B, more numerous in the male and arranged radially, was involved in hearing and finding the female. The A scolopidia, with similar numbers in both sexes but less numerous than type B he thought had a more general function, perhaps detecting air currents. This is a logical conclusion, but the discovery that both male and female Johnston's organs can vibrate their flagella and perhaps change their tuning lead us to suggest a different function. Little is known of the movement of sensory cilia, but as there are no muscle fibres in the pedicel that could move its flagellum, some or all of the scolopidia must be motile. We believe the type A scolopidia with their third dendrite packed with microtubules might be prime suspects. If they can contract, their distal filaments are attached closest to the basal plate in both sexes and would thus produce a greater amplitude of vibration than if they were attached to a more distal region of a prong. It may also be significant that the type A and B scolopidia are attached to opposite sides of a prong. With that arrangement, if they both respond to stretch, movement of the flagellum in one direction would excite the type A ciliary dendrites and relax the type B. and movement in the other would excite type B and relax type A. If the type A scolopidia included a stretch sensitive microtubular motor, it is not difficult to visualise how they could cause oscillation at the resonant frequency of the antenna. The radially arranged scolopidia of both types are stimulated maximally in the direction of vibration and those at 0° longitude will move in the opposite direction to those at 180°. Different hypotheses involving this phase difference are possible and have already been proposed to explain spontaneous vibration (Avitabile *et al.,* 2010). There is an interesting parallel in the mammalian ear, where there are two sets of hair cells, the inner with a sensory function and the outer capable of vibrating and increasing the sensitivity of the ear. The outer hair cells have a motor innervation however, and there is no such nerve supply known in insects but the physiological similarity is remarkable. Warren *et al.,* (2010) have shown that dynein and tubulin, molecules found in cilia, are involved in the movement, and that when they are blocked, some sensory cells can still respond to sounds.

The arrangement of the electron-dense and presumably rigid rods around the dendrites of neurons in the scolopidia is still not completely understood, but is more clearly shown in Risler's diagram (Fig. 3) than elsewhere. Several recent models of the scolopidia seem to assume that the whole organelle shortens and stretches (Avitabile *et al*., 2010 for example). It should be obvious that the sensory cilium can only be stretched relative to the surrounding arrays of rods, and that the rods are firmly connected to the filamentous septa and inner wall of the pedicel. The same applies to movement. If the cilia move they can only shorten by movement of the distal filament of the cilia with respect to the scolopale rods and septa.

The attachment of A and B scolopidia on opposite sides of the prongs has so far been described in *Anopheles*, *Culex* and *Aedes* (*Stegomyia*) (Risler, 1955); *Aedes* (*Aedimorphus*) (Risler, 1984) and probably also in *Toxorhynchites* (Göpfert & Robert (2001). Of these, male *Anopheles* (Gibson *et al*., 2010) and male and female *Culex* (Warren *et al*., 2010) and *Toxorhynchites* (Göpfert & Robert, 2001) vibrate their flagella spontaneously, and it seems likely that these characteristics and the ability to fold the fibrils on male antennae evolved with the Culicidae and are plesiomorphic.

Risler's ambidextrous artistic ability has influenced generations of researchers interested in mosquito hearing. His simple diagram of Johnston's organ has been reproduced in almost every text that mentions the subject, sometimes attributed wrongly to Autrum (1963), one of the first to use and acknowledge it. We believe this description of his manuscript could stimulate further investigations of the use of sound by mosquitoes using histological, molecular, or electrophysiological techniques and perhaps their systematic relationships within and outside the culicid family.

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