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Artikel: Sense organs in the antennae of "Anopheles Maculipennis Atroparvus" (v. Thiel), and their possible function in relation to the attraction of female mosquito to man
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III. Material and Technique.

The mosquitoes used in the present studies were exclusively *Anopheles maculipennis atroparvus* (v. THIEL), obtained from the laboratory colonies of the Swiss Tropical Institute.

A. Technique used in the morphological and histological work.

For studies on the morphology and distribution of the sense organs in the antennae, adult Anopheles of both sexes were killed with ether and their heads cleared in 10% potassium hydroxide for 24 hours. Then the antennae were separated, washed in distilled water and mounted in Puri solution. They were mounted in between two cover slides in order to be able to examine the antennae from both sides.

For histological examinations the mosquitoes were fixed in Duboscq fluid for 6 hours, dehydrated in alcohol and butyl alcohol, then the antennae were cut off and sectioned 3 to 5 μ thick in paraffin wax (melting point 58°C). Two staining methods were used; Delafield's haematoxylin, counterstained with Erythrosin; and Heidenhain haematoxylin.

All the drawings were made with the help of a drawing mirror, projecting the segments with their sense organs or the sections from the microscope on to the paper in darkness with magnifications of 700× and 3,500×.

B. Technique used in the experimental work.

Physiological condition of female mosquitoes used in the experiments:

THOMSON (1938), working on *Culex fatigans*, found that in many cases the type and intensity of reactions towards temperature and humidity vary according to whether the mosquitoes are newly emerged, starved or blood-fed. We could confirm this for Anopheles. It is thus essential, in order to obtain more or less accurate results in such experiments, to use mosquitoes which are in the same physiological condition. The following precautions were taken to ensure this.

Immediately after the adults emerged, they were fed on honey solution, and about 5 to 8 days later they had one blood meal, which was found to be necessary in order to obtain continuously and regularly the large amount of mosquitoes needed for the experiments. RAHM (1956-58) in his experiments on the attraction of female *Aedes aegypti* employed individuals which had had one blood meal, and they were also quite active towards the attractive stimuli used for test purposes.

The honey solution was removed from the breeding cage for one day. Then, a hand was inserted into the cage and the most active females which landed on the hand and tried to feed were taken away by means of an aspirator. Amputation of the antennal segments followed. The mosquitoes were left to recover from the operation until the next day, when they were used in experiments. On that day, the mosquitoes, aged between 15 and 20 days, had all been starved for two days, and moreover they were in a state of "reaction preparedness"— although the reactions of one group of mosquitoes sometimes showed considerable differences in successive tests (see page 34).

Anaesthetizing and dissecting method:

To immobilize the mosquitoes before amputating various segments of their antennae, we tried at the beginning to use low temperature which had proved effective with *Tenebrio molitor* (PIELOU, 1940), *Drosophila melanogaster* (PERTTUNEN and SYRJÄMÄKI, 1958) and *Aedes aegypti* (RAHM, 1958). With our Anopheles species, however, it was unsuccessful. The mosquitoes used to recover very quickly before amputation could be completed, and even in the few seconds during which they were immobilized their antennae were not motionless [1]. For this reason, we adopted the technique, formerly used by ROTH (1948) on *Aedes aegypti*, of anaesthetizing our Anopheles females with carbon dioxide.

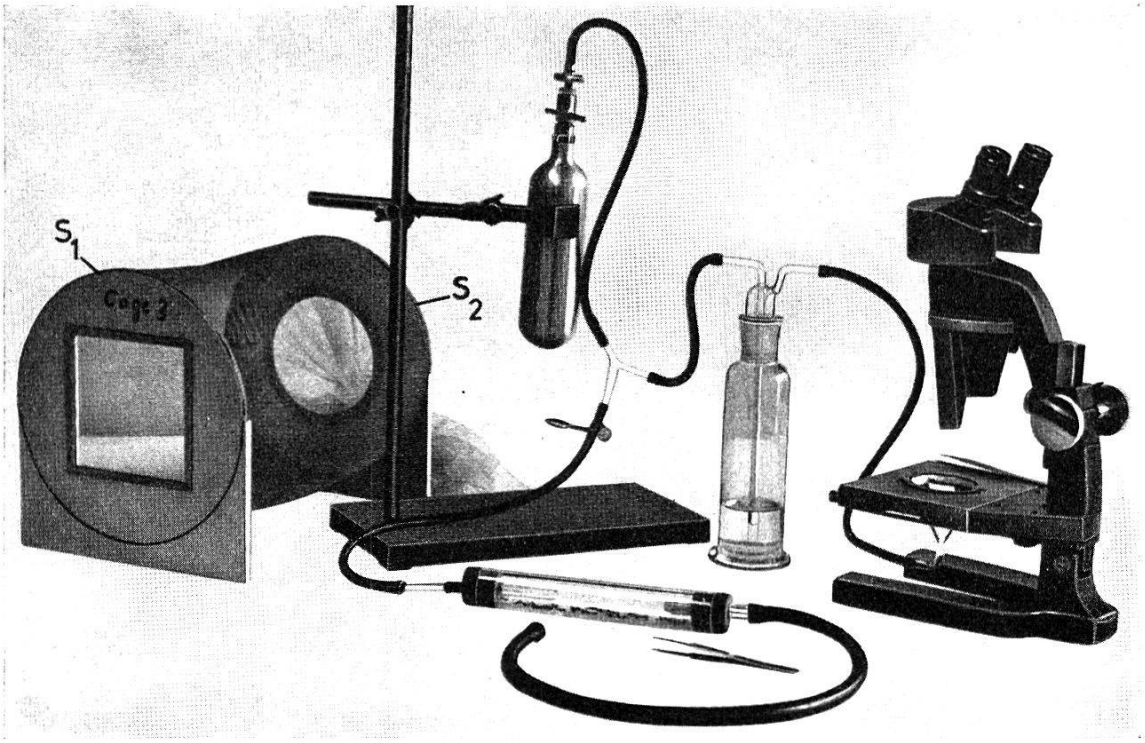

Fig. 1. The anaesthetizing and dissecting apparatus. From the gas cylinder (left) carbon dioxide flows through a rubber tube divided into two lines. One line (at the bottom) leads to the aspirator which contains the mosquitoes, and the second line (at the top) passes through a gas washing bottle (containing water) to the binocular dissecting microscope (right) where the mosquitoes were operated on under continuous anaesthesia. An experimental cage is shown on the left side. S_1 = front base; S_2 = rear base.

The anaesthetizing and dissecting apparatus was made as follows (Fig. 1): The stage of a binocular dissecting microscope was removed and replaced by a wooden plate with a circular opening in the middle. A piece of wire screen was fixed in the opening on which a small square filter paper was laid; a conical funnel was attached underneath the opening. Carbon dioxide obtained from a compressed gas cylinder passed through a rubber tube divided by a "Y" tube into two lines. One line led to the conical funnel after passing through a gas washing bottle which indicated the speed of the gas flow by bubbles in the water, and the other line led to an aspirator containing the mosquitoes. To

[1] Own experiments on immobilizing female *Aedes aegypti* with low temperature gave positive results.

anaesthetize the mosquitoes a pinchcock clamp on the second line was opened to permit a slow flow of carbon dioxide. The anaesthetized mosquitoes were then transferred to the wire screen and the pinchcock clamp was closed. The gas flow was diverted to the other line so that the anaesthetized mosquitoes were completely surrounded by the gas and were under continuous anaesthesia. 30 to 35 mosquitoes were used each time; with a larger number the wire screen would have been overcrowded, and some mosquitoes had to stay longer under anaesthesia, which could have had a fatal effect.

To amputate the different antennal segments, 5 mosquitoes were removed at a time from the wire screen and placed on the white square filter paper. The operation was carried out on them, one by one, with the help of two sharply pointed forceps, used by watch makers. It took only a few seconds to operate on one single mosquito, and when the five had been finished, they were removed and placed in the experimental cage. At the same time, another 5 mosquitoes were taken away from the wire screen without being operated on and put in another similar cage to be used as controls. By treating the control mosquitoes as well with carbon dioxide, we avoid differences in behaviour (if any) due to the effect of the gas. Amputated and control mosquitoes were transferred in their cages to the breeding room where they were kept overnight to recover before being used in the experiments on the next day.

The experimental apparatus:

Our apparatus is in principle the same as the olfactometer described by WIETING and HOSKINS (1939), in which the stimulating factors are carried in an air stream. We also incorporated the modifications of WILLIS (1947) and LAARMAN (1955) for passing the air stream over a part of the host and testing its effect on the behaviour of female mosquitoes. Certain further modifications and simplifications were devised for the purpose of this work.

The apparatus (Fig. 2A) consists of an electric pump (P) drawing outdoor air into the circuit of the apparatus. The air was passed through a cotton filter pad in a flask (FL_1) to eliminate all droplets of oil used for the lubrication of the pump, and its flow was regulated by means of a valve (V) before passing over the palm of the hand (H) in the glass cylinder (C). The hand was fixed by means of a plastic membrane (PM), and the glass cylinder was added only when the chemical stimuli emitted from the hand were needed [2]. The velocity of the air stream was checked by means of a flow-meter (FM) before and during the running of every test. After the air had been checked, it passed through glass helices (GH) in a water bath (WB) with a thermostat, for heating the air stream, and finally it was discharged through a widened glass outlet tube (OT), with an opening of 1.7 cm in diameter into the experimental cylindrical cage (CC) which contained the mosquitoes. When the air had to be moistened, a humidifying flask (FL_2) was inserted in the line; when it had to be dried, a flask with silica gel was inserted. In both cases, the flask was inserted before the flow-meter (FM).

The experimental cage as seen on the left side of Fig. 1 is cylindrical in shape with a length of 30 cm, diameter of 22 cm, and is made mainly out of wire-gauze screen, except for the two bases. The front base (S_1) facing the observer is made of wood with a square glass window measuring 11 $\times$ 11 cm in the middle to allow visual recording of the mosquito reactions, while the rear base (S_2), where the air stream enters, is also made of wood, except that the port consists of a

[2] The hand was washed about 15 minutes before the beginning of each experiment with pure water to avoid any possible external odour.

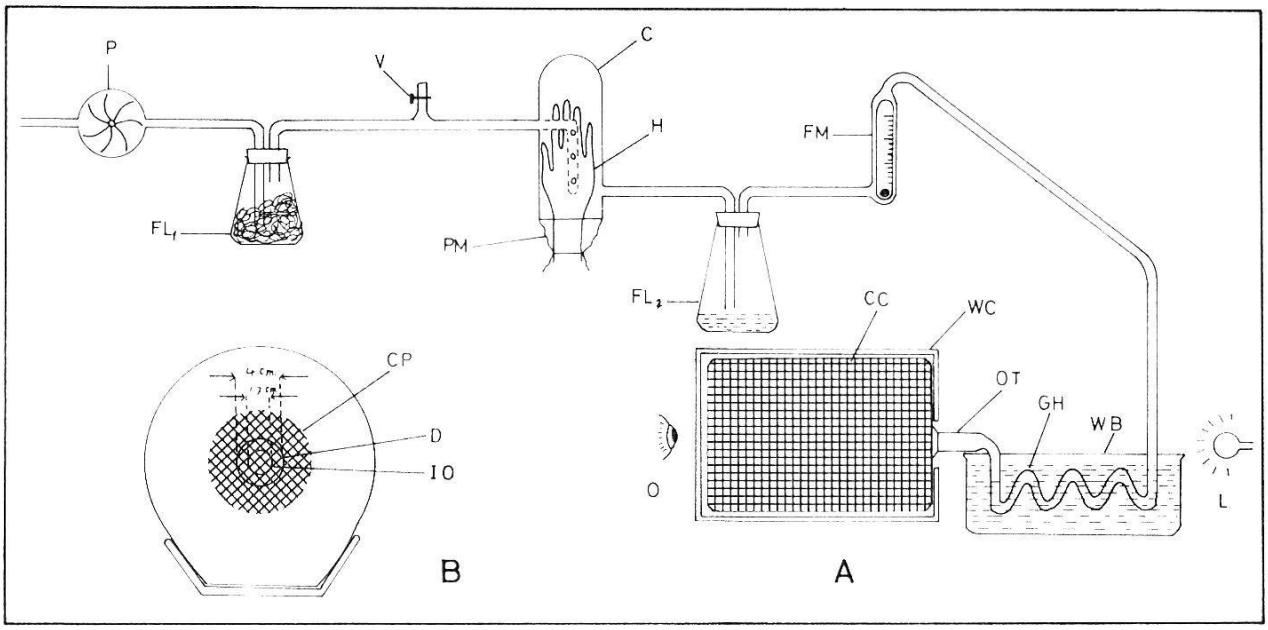

Fig. 2. A. The experimental apparatus. P = electric pump; FL_1 = flask containing a cotton pad; V = valve for regulating the rate of flow; C = glass cylinder; H = hand inserted in the glass cylinder; PM = plastic membrane; FL_2 = flask containing either water for humidifying or silica gel for drying; FM = air current flowmeter; WB = water bath; GH = glass helices; OT = outlet tube; WC = wooden cover enclosing the cage with a glass side facing the observer; CC = cylindrical cage; O = observer; L = lamp of 25 W. B. The aspect of the experimental cage before the outlet as seen by the observer. CP = cage port; D = diaphragm (diameter of 4 cm); IO = air inlet opening (diameter of 1.7 cm).

wire-gauze screen with a diameter of 12 cm. As viewed by the observer (Fig. 2B), the cage's port (CP)has a diaphragm (D) of 4 cm in the centre on which the opening of the outer tube (OT) was fixed (IO) by means of a plastic screw-cap.

During the experiments, the cage with the amputated or control mosquitoes was closed by a well-fitted wooden cover (WC), to eliminate the influence of the observer (O) on the mosquitoes, so that their reactions would be due only to the effect of the experimental factors used. The side of the cover facing the observer was, like the cage, made out of glass for the same purpose of permitting visual recording. A small lamp of 25 W. (L) was placed behind the water bath to illuminate the port and diaphragm.

Our apparatus was constructed for only one line of air stream, instead of two (as used by other authors), in order to enable a single person, who was at the same time using one hand to perform the experiments, to make a more or less accurate record of the mosquito reactions.

As recommended by LAARMAN (1955), a short time was allowed to elapse after the air current had been started and before any observations were recorded; this was done to ensure a steady air current. The air flow was discharged from the outlet against the gauze of the mosquitoes' cage at a constant rate of 3 litres per minute, and the exhausted air escaped through an opening in the top of the wooden cover. The temperature and relative humidity of the air stream were checked at the air outlet before and after the beginning of each test. In all our experiments only two temperature degrees were used: 33.5-34.5°C, which closely approximates to the temperature of the human hand (WILLIS, 1947), and 25-26°C which is equal to the experimental room temperature. To achieve these two degrees at the air outlet, we had to regulate the temperature of the water bath. We invariably found a difference in temperature between the air stream and

the water bath. For this reason the thermostat was constantly regulated. As for the relative humidity, the humidifying flask was set to load the air current with a relative humidity of 75-85%, while the silica gel was used to dry it to 15-25%.

The experiments with certain airborne factors were performed on several groups of mosquitoes, each with a different number of antennal segments missing. The reactions of one single group were studied in a series of 8 experiments which were carried out during the daytime in darkness, using only the small lamp (L) to give the necessary illumination. An interval of one hour was allowed to elapse between every two successive experiments. A single experiment with amputated mosquitoes lasted for 10 minutes and it was always preceded by a control experiment (unoperated mosquitoes) of the same duration. Hence the test consisted of a series of 8 experiments with control mosquitoes and 8 with amputated mosquitoes. Cages containing control and amputated mosquitoes were fixed, each in its turn, in the apparatus at the air outlet.

Sometimes, when many mosquitoes were available, experiments were done by using one control followed by two groups of mosquitoes with different numbers of antennal segments amputated. The number of amputated mosquitoes in every cage varied between 35 and 40, depending on the mortality rate, but the control group always consisted of 40 mosquitoes, those that died being replaced by new ones.

In all our experiments we tested about: 600 mosquitoes in a normal state, 1,600 mosquitoes after amputation and 1,400 mosquitoes as controls. All experiments were carried out in a small room maintained at a temperature of 25-26°C and a relative humidity of 50-60%.

Quantitative recording of the mosquito reactions:

In describing the way we recorded the reactions of the mosquitoes during their attraction to the different stimuli, we should point out that the meaning of the word "attraction" can vary. Since attraction is the result of a number of different reactions, we must bear in mind the difficulty of comparing the respective test results of different authors. But for our type of experiments designed to investigate the attractive function of sense organs receiving the stimuli from a distance, we found that the following two kinds of reactions, used also by LAARMAN (1955), were the most satisfactory:

1. *Hovering*

   Mosquitoes flying towards the air stream outlet, and hovering immediately in front of the outlet.

2. *Alighting*

   Mosquitoes settling on the gauze of the diaphragm, in and around the outlet of the air stream. In most cases, this reaction was followed by typical probing movements.

These two kinds of reaction were measured quantitatively by means of two counters in the hand of the observer. It must be remarked here that this counted number of reactions does not correspond to the number of mosquitoes used, but to the number of hovering and alighting movements of the attracted mosquitoes. In many cases the same individual mosquito was counted more than once, because every mosquito disappearing from the air current in front of the outlet or from the diaphragm and then repeatedly reacting again was counted accordingly.

Mosquitoes which walked from the outside to the inside of the diaphragm were not counted, so as to eliminate the reaction due to the perception of non-directional receptors existing on other parts of the insect body which receive the stimuli when the insect is on or near the source of stimuli (ROTH, 1951 & RAHM, 1958).

IV. The Antennae of Anopheles Maculipennis.

It is a well-known fact that the antennae of males and females differ markedly in Anopheles. They can be distinguished with the naked eye by the very long fibrillae which exist on the different segments of the male antennae. The flagellar segments in the females are loosely articulated, while they are more closely attached to each other in the males. In both sexes the antenna is made up of 15 segments, comprising a scape, a pedicel and 13 flagellar segments.

1. The antenna of the female.

The antenna of the female (Fig. 3) is about 1.6 mm long (average of twelve antennae, minimum of 1.4 mm and maximum of 1.7 mm). The scape is an irregular chitinous ring, connected with a rounded pedicel which measures about 125 μ in diameter and carries a few scales and short articulated thick-walled sensilla (bristles or spines). Both the scape and the pedicel are darkly pigmented and carry many small, slender microtrichia. These are merely cuticular outgrowths and no innervation to them was seen in the histological preparations.

The first flagellar segment fits into the hollow of the pedicel. It is the longest segment of the antenna, measuring about 200 μ in length and 43 μ at its greatest diameter [3]. It bears scales and various lengths of articulated thick-walled sensilla (bristles or spines) on the whole segment except for a small portion at the base. Like the scape and the pedicel it is darkly pigmented and carries many microtrichia.

The second flagellar segment is the shortest of the flagellum, measuring about 80 μ in length and 40 μ in diameter. It carries several short bristles or spines, few thin-walled sensilla and very few microtrichia. It is also darkly pigmented except for a sub-basal colourless ring where long bristles are inserted.

Flagellar segments 3 to 12 are more or less similar to each other, with the segments decreasing slightly in diameter towards

[3] Measurements of all diameters were taken at the widest part of the segments.