Olfactory sensitivity of Anopheles mosquitoes with different host preferences

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Summary

Worldwide, mosquitoes of the genus *Anopheles* are vectors of malaria. Only female mosquitoes transmit malaria parasites when taking a bloodmeal in need of a protein source to develop their eggs. At night, they search for appropriate blood-hosts. In the course of evolution, female mosquitoes of various species have developed different strategies to localise their hosts. Some are highly specialised to take blood from one type of hosts, e.g. humans, cattle, birds or reptiles; others direct their feeding to several types of hosts and show opportunistic biting behaviour.

Anopheles gambiae s.s. is the main vector of malaria in Africa, causing one of the major public health problems there. The species' high capacity to transmit malaria arises from its selective preference to bite a human host, i.e. from its anthropophilic biting behaviour. *An. gambiae s.s.* is a member of the *An. gambiae s.l.* species complex, consisting of 6 closely related African species, which can only be distinguished for certain by their genetic characteristics. In contrast to *An. gambiae s.s.*, the sibling species *An. quadriannulatus* shows a definite preference to feed from animals, i.e. mainly from cattle and other bovines. This zoophilic species hardly ever is a vector of malaria. Another species of the complex, *An. arabiensis*, shows more opportunistic host preferences. Depending on the availability of human or bovine hosts, *An. arabiensis* females will bite one or the other.

The main question of the research project dealt with in this thesis is:

> How do anthropophilic, zoophilic and opportunistic mosquito species of the *An. gambiae s.l.* complex localise and distinguish the different types of hosts during their nightly search for a bloodmeal?

Earlier research has shown that temperature, humidity and carbon dioxide as well as host body odours play an important role in the attraction of mosquitoes to their hosts. An increase in temperature or humidity may be perceived at a few meters from the host. A concentration of CO_2 above background level, due to exhaled breath of warm-blooded animals, may be detected from 15-20 meters. Host-odours are presumably perceived at larger distances, i.e. 50-60 meters, if the mosquitoes possess an olfactory system sensitive to these odour compounds.

> The research described in this thesis aims at comparing the sensitivities of the olfactory organs of *Anopheles* species with different host preferences.

The first step was a study of the morphology of the antennae, the main olfactory organs of the mosquito, using *Scanning Electron Microscopy*. The antennae of male and female mosquitoes of the three African *Anopheles* species, *An. gambiae s.s.*, *An. arabiensis* and *An. quadriannulatus*, were compared.

The antennae of male and female *Anopheles* mosquitoes are 2.2 and 1.5 mm long, respectively. The antennae are movably connected with the head by their scape and globular pedicel, and furthermore consist of 13 flagellar segments. The flagellar segments of the female are covered with different types of sensory hairs, which may serve to perceive either heat or humidity, or tactile stimuli or may have an olfactory function. The first 11 flagellar segments of the male bear whorls of long hairs, which are brought into maximal vibration by the flight-tone produced by a female of the same species. The vibration is perceived by the Johnston's organ in the pedicel. Hence, the male antenna is first of all designed to detect a potential mating partner by sound. The last two segments of the male antennae show a variety of sensory hairs similar to that of the female antennae.

The numbers and distribution of the different types of sensory hairs on the antennae are similar for all 3 *Anopheles* species. The female antennae showed 5 types of sensory hairs: *sensilla chaetica*, *sensilla ampullacea*, *sensilla coeloconica*, *sensilla basiconica* ('grooved pegs') and *sensilla trichodea*. *Sensilla ampullacea* were not observed on the male antennae. *Sensilla trichodea* and grooved pegs have been shown to play a role in olfaction in various other mosquito species. These hairs contain dendrites of olfactory cells which lie at the base of the hair. *Sensilla coeloconica* may have an olfactory function as well. These three types of olfactory sensilla are connected to 95% of the total number of sensory cells present in the female antennae (estimated at 1800 cells) and to 83% of those in the male antennae (800 cells). The number of olfactory cells of bloodfeeding insects is thought to be related to the importance of odours in host-orientation and to the distance the insect has to travel to find a host. The results of the morphological study showed that the number of olfactory hairs, and also the number of olfactory cells, is equal for the anthropophilic, zoophilic and opportunistic *Anopheles* species. Therefore we may assume that the role of the olfactory system is equally important for these species despite their different host preferences.

Because of the demonstrated olfactory function of *sensilla trichodea* and grooved pegs, and the relatively high numbers of these sensilla present on the female antennae, these two types of sensory hairs were the principal candidates for investigation in our electrophysiological research.

The sensitivities of the *sensilla trichodea* and the grooved pegs of the *Anopheles* species were investigated in three electrophysiological studies. By means of electrophysiology, action potentials ('spikes') of the sensory cells at the base of the sensory hairs can be recorded. The olfactory cells of insects usually show a basal continuous firing of spikes. When odour substances are perceived by the olfactory cells, the frequency of the spike-signal changes and either a temporal increase (excitation) or decrease (inhibition) of spikes can be observed.

Existing electrophysiological techniques were adapted to the size and characteristics of the mosquito antennae. For 'single-cell' electrophysiology a detailed view of the olfactory hairs was required, which could be obtained under high magnification (800x) with an inverted microscope. A tungsten electrode with a fine tip (< 1 µm) was carefully positioned at the base of a sensory hair to catch the signal of the sensory cells underlying the cuticula. This signal was thereafter recorded by equipment specifically developed for insect sensory physiology.

Chapter 3 describes a series of electrophysiological measurements on *sensilla trichodea* on the antennae of the 3 African *Anopheles* species and the European *An. maculipennis atroparvus*. The activity of the sensory cells was measured during stimulation with several doses of a number of odour substances, which are emitted by human and bovine hosts. Volatile fatty acids are found in relatively high amounts in sweat and on the human skin and may play a role in the attraction of the anthropophilic *An. gambiae s.s.* Five short-chain fatty acids were tested in these studies. Furthermore, some other substances were chosen, which are known to attract zoophilic bloodfeeding insects: two phenols, mainly found in urine, and 1-octen-3-ol, a compound detected in the breath of cattle.

The cells at the base of *sensilla trichodea* showed clear responses to these odour substances. On stimulation with fatty acids and phenols both excitation and inhibition was found. The cells often responded to more than one compound and the various cells showed different response-profiles. The anthropophilic *An. gambiae s.s.* appeared to have relatively more cells which are excited by fatty acids than the zoophilic *An. quadriannulatus* and *An. m. atroparvus*. Moreover, the cells of the anthropophilic species responded stronger to the fatty acids and to lower doses of them than cells of the zoophilic species. In contrast to this, the *sensilla trichodea* cells of the zoophilic species were more sensitive to 1-octen-3-ol. The cells of the opportunistic *An. arabiensis* showed sensitivities intermediate to those of the other species. The 4 species were equally sensitive to the phenols. The role of phenols in the behaviour of mosquitoes is not quite clear. Apart from their role in host-seeking behaviour they may also be involved in the localisation of oviposition sites.

In chapter 4 the results of electrophysiological measurements on grooved pegs of *An. gambiae s.s.* and *An. quadriannulatus* are described. The majority of the cells at the base of the grooved pegs responded to the odours of ammonia and amines, components evaporating from excretion products (urine and faeces) and to a fatty acid found on the (human) skin; a smaller part of the cells responded to acetone, which is found in breath. Only a few grooved pegs were responsive to phenols and 1-octen-3-ol. In contrast to the results obtained from the *sensilla trichodea*, the responses of grooved peg-cells of the anthropophilic *An. gambiae s.s.* and the zoophilic *An. quadriannulatus* were not significantly different.

More than 50% of the grooved pegs showed excitatory responses to stimulation with water vapour. On the other hand, dry air caused inhibition of the activity of some sensilla. This suggests that grooved pegs play a role in the perception of changes in humidity. These humidity-sensitive responses were never observed from *sensilla trichodea* cells.

In the third series of electrophysiological measurements (chapter 5) the responses of olfactory cells of both *sensilla trichodea* and grooved pegs of *An. gambiae s.s.* and *An. quadriannulatus* to the complete blend of odours emitted by a host (a cow) and its excretion products (urine, manure, skin products) were compared with the responses to individual odour components. The odour of Limburger cheese, which had been shown to attract *An. gambiae s.s.*., was also tested

Grooved peg-cells showed higher responses to urine, manure and ammonia than *sensilla trichodea* cells. The latter responded more strongly to the odours of Limburger cheese, to 1-octen-3-ol and to 3-methyl phenol. No differences were found in the magnitude of response to natural odour mixtures between olfactory cells of the anthropophilic and zoophilic species.

These three electrophysiological studies have made clear that both the olfactory cells underlying *sensilla trichodea* and grooved pegs of *Anopheles* females are able to perceive various odour components emitted by human and bovine hosts. The sensory hairs and underlying olfactory cells are not specialised to detect one odour component only. The cells also do not seem to respond to a specific combination of odour components, at least not for the dozen of odours which were tested in these studies. Cells may respond with inhibition to one compound, and with excitation to another. The large variation of responses and the broad odour spectrum of the cells enlarges the capacity to perceive complex odour mixtures, which are likely to be encountered in the vicinity of the host. The information of the complete cellular network enables the mosquito female to build a detailed 'odour-picture' of the potential host nearby.

On the antennae of the anthropophilic as well as the zoophilic *Anopheles* females around 1700 olfactory cells are found, which altogether show a large diversity of responses to various host odours. The anthropophilic and zoophilic species showed a different level of sensitivity to some odour components which are found in the odour blend emitted by their specific host, i.e. humans or bovines, but they may have similar sensitivities to other components. 'Human odour' and 'bovine odour' cannot be characterised by one or a few odour components, but rather by a specific combination of several odours. The responses of anthropophilic and zoophilic species to complex 'bovine odours' were similar. Species with a specific host preference may not only respond to the odours emitted by their specific host. They also perceive the odour from other potential hosts, and there are indications that these may have a more or less repellent effect on the mosquitoes. In two-choice tests in the field, it was shown that the percentage of *An. gambiae s.s.* females caught in a human odour-baited trap is higher when the mosquitoes have to choose between human odour versus calf odour (99 vs. 1%), than when human odour versus CO_2 is offered (70 vs. 30%). The anthropophilic mosquitoes apparently avoid the calf odour. Similar tests with *An. quadriannulatus* show that females of this zoophilic species may avoid the human odour. Therefore, identification and localisation of a specific host may not only be guided by attraction to odours of the host, but also by repellency by odours of the less preferred host.

In the last chapter of the thesis, the step from physiology to behavioural research is made. 'Actometers', designed for automatic recording of movement of insects, were used to measure the behavioural responses of individual mosquitoes to individual odour components and to odour blends. We compared three African species, *An. gambiae s.s.*, *An. arabiensis* and *An. quadriannulatus*, and the European *An. m. atroparvus*.

First, the spontaneous levels of activity during day and night was recorded of mosquitoes of different physiological states. Males and females of the African species showed similar activity patterns, with peaks of activity during dawn and dusk. During the day the mosquitoes were not active. The European species was mainly active during the evening, in the hours before midnight. These differences are related to climatic differences in the habitats of these species. In the tropics the temperature remains relatively high during the night and early morning, allowing the insects to stay active. In temperate zones, the temperature may decrease during the night below the level where insects can stay active. Interestingly, the activity patterns of the mosquitoes of these *Anopheles*-species have not changed during long-term rearing under stable temperature conditions in the laboratory.

Older mosquitoes (10-14 days) were more active than younger ones (1-4 days). Female mosquitoes showed a higher activity during the dark part of the night than males, and inseminated females were more active than virgins. The nightly activity of hungry, inseminated mosquito females is mainly directed to host-seeking and bloodfeeding. One to two days after bloodfeeding activity was strongly depressed.

To investigate the effect of odour stimuli on the activity of mosquitoes, only females of 4 to 10 days of age were used. An increase in mosquito activity was shown during stimulation with pulses of CO_2 and 1-octen-3-ol and during continuous stimulation with the odour of Limburger cheese. With enduring stimulation, i.e. more than 15 min, the activity decreased. Repeated stimulation with CO_2, after a period of 15 min without stimuli, caused a lower increase in activity than during the first stimulation period. This suggests that some kind of peripheral or central memory may exist.

The responses to 1-octen-3-ol of the zoophilic *An. quadriannulatus* were significantly higher than those of the anthropophilic *An. gambiae s.s.* and the opportunistic *An. arabiensis*. These results confirm the results of the electrophysiological studies, which showed similar differences in the sensitivity of the *sensilla trichodea* to 1-octen-3-ol.

Up to least 12 hours after bloodfeeding, female mosquitoes were not responsive to CO_2 and 1-octen-3-ol, similar to the above-mentioned decrease in activity during one to two days after bloodfeeding. It has been shown that immediately after bloodfeeding host-seeking behaviour is inhibited by 'abdominal distension', i.e. by the enlarged bloodfilled abdomen. Thereafter, if the bloodmeal triggers egg production, the behaviour is inhibited by a hormonal factor in the haemolymph. In An. gambiae the inhibitory phase may be relatively short. Field-data indicate that these mosquitoes may bloodfeed every other night.

The series of activity measurements showed that the 'actometers' used in this study provide a sensitive technique for screening and quantifying the effects of host-odour components on the activity of mosquitoes. In order to identify odour components which may be used to attract mosquitoes in the field, this may be a more efficient method than electrophysiology. Actometer-studies may quickly determine whether mosquitoes perceive an odour and respond to it with increased activity levels. Activation is the first step in the process of host-seeking. However, to entangle the role of the olfactory system in the recognition of the odour-blend of a specific host in mosquitoes with different host-preferences, electrophysiological research such as described here is required. Further research in this line of work should go together with chemical studies identifying specific odour components emitted by different hosts.