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LABORATORY OF ANIMAL PHYSIOLOGY

THE SUGAR MEAL OF THE AFRICAN MALARIA MOSQUITO *ANOPHELES GAMBIAE* GILES AND HOW DETERRENT COMPOUNDS INTERFERE WITH IT: PERCEPTION MECHANISMS AND PHYSIOLOGICAL IMPLICATIONS

PH.D. DISSERTATION SUBMITTED TO THE
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BY

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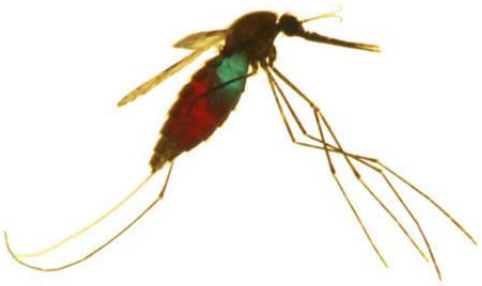

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Titre:

"The sugar meal of the African malaria mosquito *Anopheles gambiae* Giles and how deterrent compounds interfere with it : perception mechanisms and physiological implications"

sur le rapport des membres du jury composé comme suit:

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1. Résumé

Le moustique africain *Anopheles gambiae*, est un vecteur parmi les plus importants de la malaria. Il transmet notamment la forme la plus dangereuse de cette parasitose humaine, *Plasmodium falciparum*.

Les sucres des plantes représentent une source nutritive importante pour *A. gambiae*, alors que le sang des vertébrés est utilisé par les femelles pour la production des œufs. Le but de ce projet de recherche était d'investiguer la physiologie du goût chez *A. gambiae* en utilisant des méthodes comportementales et neurophysiologiques. Plus particulièrement, nous avons tenté de comprendre comment les goûts sucrés et amers sont perçus par les cellules gustatives du labelle du moustique. Nous avons tenté de mettre en relation ces résultats avec la valeur nutritive des sucres testés ainsi que le pouvoir déterrent qu'exercent les substances amères sur la nutrition du moustique.

Nous avons montré que les sucres préférés par les femelles et les mâles *A. gambiae* sont, par ordre décroissant, le saccharose, suivi par le mélange équimolaire de glucose et de fructose, le fructose, le glucose et finalement l'eau. Le saccharose est également préféré au mélézitose, un trisaccharide présent dans certains miellats.

Les sucres maximisant la longévité des femelles et des mâles *A. gambiae* sont le saccharose et le fructose, suivis par le mélange de glucose et de fructose, le glucose, puis l'eau. En revanche, les femelles *A. gambiae* préalablement nourries sur du glucose adoptent un comportement de piqûre plus intense que celles s'étant nourries sur du saccharose. De plus, la masse sèche des femelles maintenues sur le saccharose est plus élevée que celles maintenues sur le glucose, suggérant une plus grande capacité des femelles à accumuler des réserves énergétiques à partir du saccharose. Ces études suggèrent que le saccharose et le glucose peuvent avoir deux effets opposés sur la capacité vectorielle des femelles : celles maintenues sur le saccharose vivent plus longtemps, mais manifestent un comportement de piqûre moins intense par rapport à celles maintenues sur le glucose.

Chez *A. gambiae*, les sensilles trichoïdes T1 du labelle possèdent un pore terminal ainsi que deux cavités lymphatiques, deux caractéristiques typiques des sensilles gustatives. Ces sensilles abritent un mécanorécepteur ainsi que quatre neurones chemo-sensoriels avec des dendrites non-ramifiées. L'un d'entre eux est une cellule gustative percevant les sucres. Un neurone permettant de percevoir l'eau est également présent, excepté dans les sensilles T1 au bout du labelle. Le saccharose est perçu par le biais de l'activation des cellules réceptrices pour les sucres ainsi que par l'inhibition simultanée des cellules réceptrices pour l'eau. Dans les sensilles T1, les cellules gustatives percevant les sucres s'activent à des

concentrations beaucoup plus basses que celles requises pour induire l'engorgement du moustique. Ces cellules gustatives sont plus sensibles pour des petites augmentations de la concentration de saccharose jusqu'à 10 mM.

Les petites sensilles gustatives T2 du labelle d'*A. gambiae* sont également pourvues d'un mécanorécepteur mais seulement d'une seule cellule chemo-sensorielle. Cette dernière répond à l'eau avec des potentiels d'action émis à une haute fréquence et d'une façon tonique. Ce neurone sensoriel ne semble pas être sensible à la pression osmotique car il n'est pas inhibé par 100 mM de saccharose.

Chez *A. gambiae*, les cellules gustatives percevant les sucres des sensilles T1 montrent une forte spécificité pour le saccharose. Cela contraste avec nos résultats des enregistrements neurophysiologiques réalisés sur *Aedes aegypti* et ce qui est connu chez d'autres diptères. Au total, parmi les dix-sept molécules sucrées testées sur les sensilles T1 du labelle d'*A. gambiae*, seul le saccharose, le mélange de fructose et de glucose, ainsi que le mélézitose activent les cellules réceptrices. La capacité des sucres testés à activer les cellules gustatives est discutée en fonction de leur structure respective.

Du fait que le mélange de glucose et de fructose stimule les cellules gustatives et induit l'engorgement du moustique presque aussi bien que le saccharose, nous avons déduit que le glucose et le fructose ont un effet synergique lorsqu'ils sont ensemble en solution.

Nous avons montré que les produits perçus par l'homme comme étant amers ont un effet déterrent sur la nutrition d'*A. gambiae*. Quand 1 mM de quinidine, de quinine ou de benzoate de dénatonium est ajouté à 146 mM de saccharose, la nutrition du moustique est presque complètement inhibée. La berbérine montre un effet moindre et nous n'avons pas observé d'inhibition significative de l'engorgement des moustiques après avoir ajouté 1 mM de caféine. Nous avons démontré pour la quinine, ainsi que pour la quinidine, que l'effet déterrent dépend de la concentration. Des expériences de nutrition menées sur *A. gambiae* en utilisant des micro-capillaires ont montré que les sensilles de contact des pièces buccales ou du cibarium sont suffisantes pour la détection du saccharose ainsi que de la quinine. Les résultats des essais de nutrition avec les produits amers sont en corrélation avec les réponses neurophysiologiques du neurone gustatif percevant le saccharose, ainsi que celui percevant l'eau d'une sensille T1 du labelle. Ces deux neurones sensoriels sont tous deux inhibés par le benzoate de dénatonium, la quinine et la berbérine entre 0.01 et 1 mM, mais pas par les mêmes concentrations de caféine qui n'ont, par ailleurs, aucun effet sur la nutrition d'*A. gambiae*.

En utilisant un essai de nutrition *in vitro* au moyen d'une membrane de silicone, nous avons montré que 87% des femelles *A. gambiae* se gorgent sur du sang de bovin défibriné. Ce

pourcentage est significativement supérieur à celui des femelles se gorgeant sur une solution saline ou sur une solution saline dans laquelle de l'albumine de sérum de bœuf a été ajoutée. Ces résultats indiquent que des composés présents dans le sang exercent un effet phagostimulant chez les femelles moustiques. L'ajout de 1 mM d'artesunate (un sesquiterpène lactone) ou de quinine (un alcaloïde) induit une diminution du pourcentage de moustiques s'engorgeant. Du fait que seuls les stylets pénètrent la membrane et non pas le labelle, ces résultats suggèrent que les produits phagostimulants du sang, ainsi que les produits amers sont détectés par les organes gustatifs internes du moustique, à savoir, les cellules gustatives des sensilles apicales et subapicales du labre, des sensilles de la face interne du labelle ou du cibarium. Etant donné que les concentrations plasmatiques des anti-malarias quinine et artesunate affectant *Plasmodium* chez les humains sont beaucoup plus basses que celles affectant la nutrition d'*A. gambiae* dans notre système *in vitro*, cela suggère que ces deux molécules ne doivent avoir qu'un effet mineur sur le taux d'infection des moustiques se nourrissant sur les patients traités.

Mots clés: insectes hématophages, *Anopheles gambiae*, *Aedes aegypti*, écophysiologie des moustiques, microscopie électronique, sensilles de contacte, goût sucré, goût amer, cellules gustatives, électrophysiologie, nutrition sur le sucre, nutrition sur le nectar, repas sanguin, saccharose, fructose, glucose, eau, osmolarité, phagostimulants, phagodéterrents, médicaments antipaludéens, quinine, artesunate, longévité, fréquence de piqûre, capacité vectorielle.

2. Summary

The African mosquito, *Anopheles gambiae*, is one of the more efficient vectors of the human disease malaria, especially of its more lethal form caused by *Plasmodium falciparum*. Plant derived sugars provide a primary nutrient source for adult *A. gambiae* whereas vertebrate blood provides proteins for egg production in such a blood-feeding mosquito. This research work sets out to gain insight into the physiology of taste in *A. gambiae* by using behavioural and electrophysiological methods. The thesis focuses particularly on sweet taste in relation to the nutritive values of sugars and on bitter taste in relation to the feeding deterrent effect of aversive and potentially toxic molecules.

We show that sucrose is the preferred sugar diet of *A. gambiae* followed, in decreasing order, by an equimolar mixture of glucose and fructose (the two hexose constituents of sucrose) fructose alone, glucose alone and finally water. Sucrose is also prefered over the melezitose, a trisaccharide which can be present in honeydew.

Sugars contributing to the highest longevity in both sexes of *A. gambiae* are sucrose that is equivalent to fructose, followed by the mixture of glucose and fructose, glucose, and finally water. On the other hand, starved female *A. gambiae* previously fed on glucose adopt a more intense biting behaviour on a warm body than those that fed on sucrose. Moreover, the dry mass of females maintained on sucrose is higher than those maintained on glucose suggesting a higher ability of females to accumulate reserves from sucrose. These studies suggest that sucrose and glucose may have two opposing effects on the vectorial capacity of females: those maintained on sucrose have a longer lifespan but manifest a less intense biting behaviour than those maintained on glucose.

Trichoid T1 labellar sensilla of *A. gambiae* are typical gustatory sensilla with two lymphatic cavities and a terminal pore. These sensilla house a mechanoreceptor and four chemosensory receptor cells with unramified dendrites. One of them is a sugar receptor cell. A water sensitive neurone is also present, except in the T1 sensilla at the tip of the labellum. Sucrose is perceived by T1 sensilla through activation of the sugar receptor cells and simultaneous inhibition of the water receptor cells. The electrophysiological response of the sugar receptor cells starts well below the level of sucrose necessary to induce feeding and the receptor cells are most sensitive to small increments in sucrose concentration up to 10 mM.

Small trichoid T2 labellar sensilla of *A. gambiae* have a mechanoreceptor and only one chemosensory neurone responding to water with a high spike frequency and in a tonic

manner. This neurone seems not to be sensitive to osmotic pressure as 100 mM sucrose failed to inhibit it.

The sugar sensitive neurone of T1 labellar sensilla show a strong specificity for sucrose, contrasting to what we found in the yellow fever mosquito, *Aedes aegypti* and what is known in other Diptera species. In fact, over the seventeen sweet tasting compounds tested on the T1 sensilla, only sucrose, the mixture of fructose and glucose and the trisaccharide melezitose activate the sugar receptor cells. The structure-activity relationship of the sugar tested is discussed in this thesis.

In *A. gambiae*, even though sucrose receptor cells of T1 labellar sensilla is not activated by either fructose or glucose alone, equimolar solutions of fructose plus glucose activate the neurone. We conclude that there is a neurophysiological synergism between fructose and glucose leading to a behavioural synergism.

We show that bitter tasting products for humans have a deterrent effect on feeding in *A. gambiae*. When 1 mM quinidine, quinine or denatonium benzoate is added to 146 mM sucrose, feeding is almost totally inhibited. The effect of berberine is lower and no significant inhibition on engorgement occurs for caffeine. The deterrent effect depends on concentration for both quinine and quinidine. Micro-capillary feeding experiments show that contact chemosensilla on the mouthparts or within the cibarium are sufficient for the detection of sucrose and bitter products. The feeding assay findings with deterrents correlate with the neurophysiological responses of the sucrose and the water labellar neurones in a T1 labellar sensillum which are both inhibited by the bitter compounds denatonium benzoate, quinine and berberine at between 0.01 and 1 mM, but not by the same concentrations of caffeine which has no effect on feeding.

Using a membrane-feeding assay, I show that 87% of female *A. gambiae* fed on defibrinated bovine blood, significantly more than on saline or saline + bovine serum albumin solutions indicating the presence of additional components in blood that stimulate mosquito feeding. Adding 1 mM artesunate, a sesquiterpene lactone, or quinine, an alkaloid, in the feeding solution causes a decrease in the percentage of mosquito engorged. Since only the stylets penetrate the membrane and not the labellar lobes, these results suggest that both blood phagostimulants and feeding deterrents are detected by internal gustatory organs, namely sensory cells in the apical and subapical labral pegs, in sensilla on the inner face of the labellar lobes or cibarial receptor cells. The neuroanatomy of the apical and subapical labral pegs and of the sensilla on the inner face of the labellar lobes of female *A. gambiae* is described in this thesis. As circulating doses of the antimalarial drugs quinine and artesunate affecting *Plasmodium* in humans are much lower than those affecting feeding by *A. gambiae*

in the *in vitro* feeding assay used here, I conclude that these two antimalarial drugs would have no or only a minor effect on the infection rate of mosquitoes feeding on treated patients.

Key words: heamatophagous insects, *Anopheles gambiae*, *Aedes aegypti*, mosquito ecophysiology, electron microscopy, contact taste chemo-sensilla, sweet taste, bitter taste, taste receptor cells, electrophysiology, sugar feeding, nectar feeding, blood feeding, sucrose, fructose, glucose, eau, osmolarity, phagostimulants, phagodeterrents, anti-malarial drugs, quinine, artesunate, longevity, biting rate, vectorial capacity.

3. Introduction

3.1 Taxonomy and life cycle of anopheline

Mosquitoes belong to the Diptera, suborder Nematocera, family Culicidae (Fig. 3.1). The life-cycle of these holometabolous insects involves 4 life stages: egg, larva, pupa and adult (Fig. 3.2). Malaria is transmitted only by females of the genus *Anopheles*. Depending on the species, prevailing temperature and larval density, 5 to 14 days are required for anopheline to develop from egg to pupa (Timmermann and Briegel, 1993; CDC, 2012). Eggs of anophelines are laid singly directly on water. Eggs of some anophelines possess floats (Fig 3.2 A and 3.2 B). Eggs hatch after 2-3 days. Larvae develop through four instars. They feed essentially on microorganisms, algae and particles derived from decayed plant tissues present in water and used the flow generated by their “mouth brushes” for feeding (Clements, 1992; Fig. 3.2C). Contrary to culicine and toxorhynchitine, anopheline larvae lack a respiratory siphon (Fig. 3.2C). They breathe through spiracles located on the 8th abdominal segment and, for this reason, position their body parallel to the water surface (Clements, 1992). Because they need air to breathe, larvae and pupae live most of the time at the air/water interface (Clements, 1992). In our rearing, we used a water depth of 0.5 cm for the aquatic stages of *Anopheles* spp. Contrary to that of larvae, the head and thorax of pupae are merged into a cephalothorax which possess a pair of “respiratory trumpets” (CDC, 2012; Fig. 3.2D). After at least one day, the cephalothorax splits and the imago emerges (CDC, 2012). In our laboratory, the development of *Anopheles gambiae* Giles (16cSS strain, derived in 1974 from wild caught adults originating from Lagos, Nigeria, West Africa) from eggs to pupae takes 9 to 10 days at 28°C and 80% relative humidity (RH).

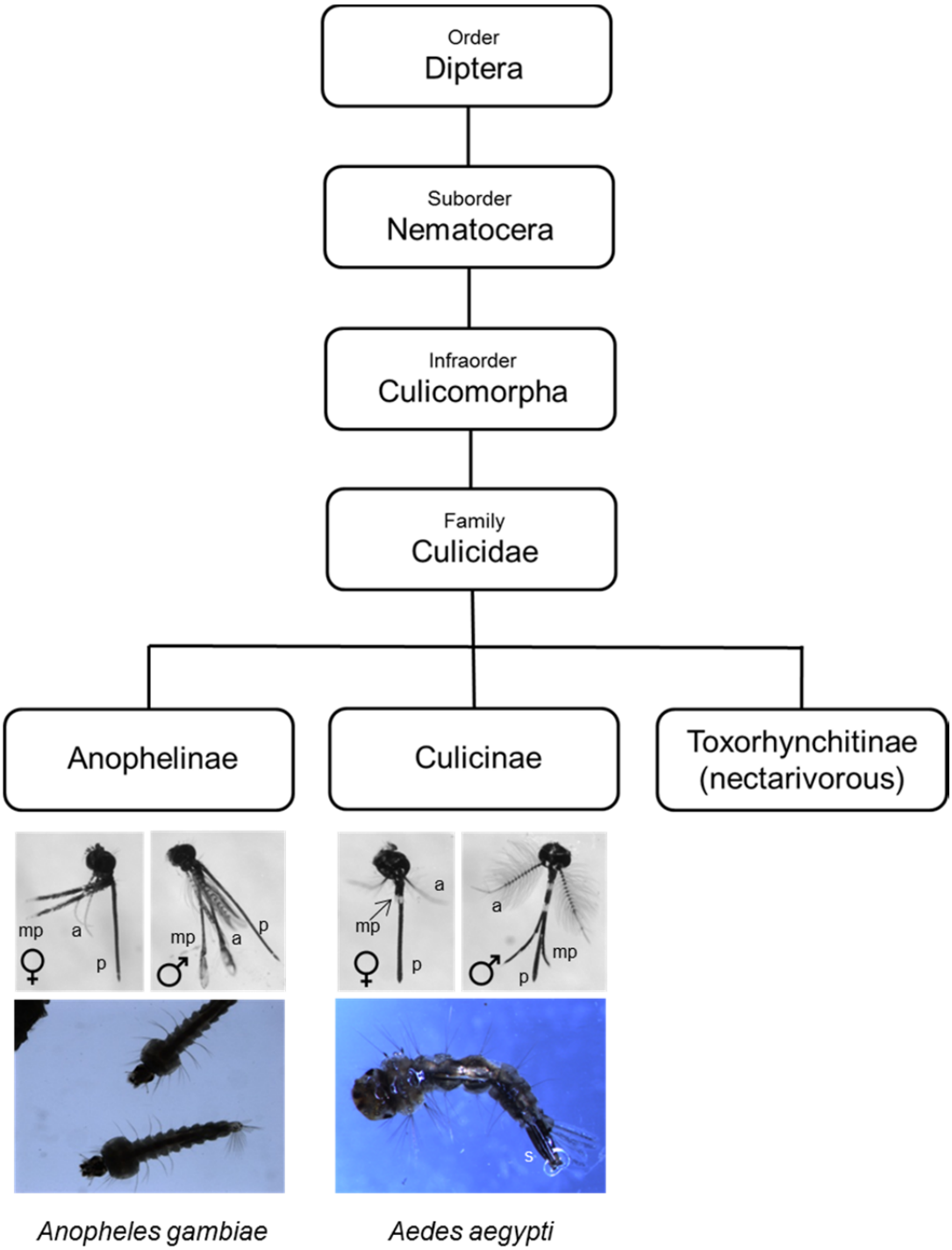

Fig. 3.1: Mosquitoes are classified into three subfamilies. Males are characterized by their feather-like antennae in all species (a). In female *Anophelinae*, the maxillary palps (mp) are as long as the proboscis (p) although they are shorter in female *Culicinae* and *Toxorhynchitinae*. Spiracles of *Culicinae* and *Toxorhynchitinae* are situated at the end of a siphon (s) whereas those of the *Anophelinae* are situated near the end of the abdomen.

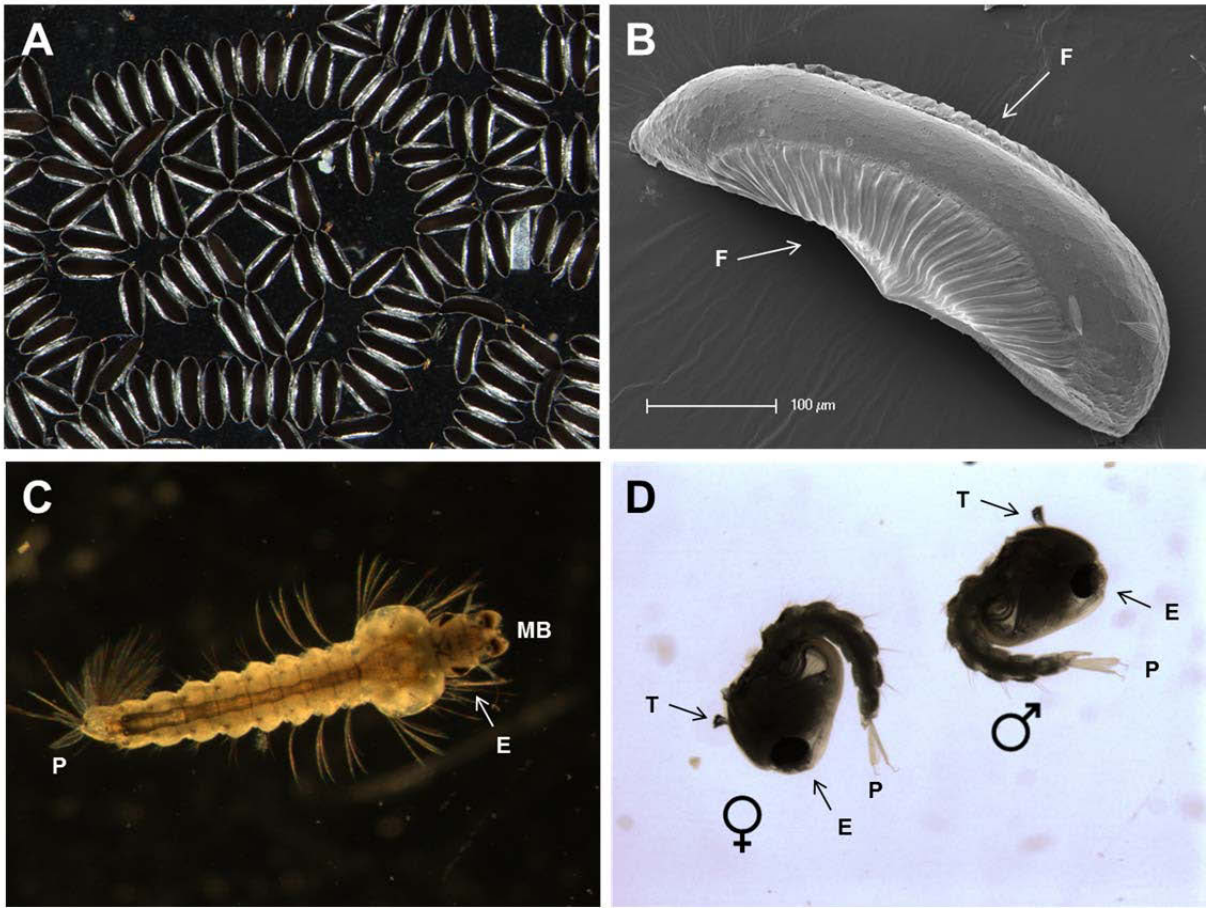

Fig. 3.2: (A) Eggs of *A. gambiae*, **(B)** Scanning electron micrographs of a dorsolateral view of an egg of *A. gambiae*, showing the floats on each side, **(C)** larva and **(D)** female and male pupae of *A. gambiae*. E, eye; F, float; MB, mouth brushes; P, paddle; T, respiratory trumpets.

3.2 The life-cycle of malaria parasites

Malaria is caused in humans by four Apicomplexa protozoans: *Plasmodium vivax*, *P. ovale*, *P. malariae* and *P. falciparum*, the last one being the most pathogenic form responsible of the majority of cerebral malaria cases (Cassier et al., 1998; Garcia and Bruckner, 1997; Smyth, 1994). Causative agents of simian malarias, such as *P. knowlesi*, have also been found to infect humans (White, 2008). Malaria parasites require two hosts: the anopheline mosquito, which is the definitive host in which sexual development occurs, and a vertebrate, defined as the intermediate host (Nsanzabana, 2008). Malaria infection comprises 3 cycles: an exo-erythrocytic and an erythrocytic cycle occurring in hepatocytes and erythrocytes, respectively, of the vertebrate hosts and a sporogonic cycle in the female *Anopheles* (Fig. 3.3). Briefly, female anopheline inoculate several hundred sporozoites to the intermediate host with saliva during the course of an infectious blood meal (Cassier et al., 1998). Rapidly, sporozoites migrate to liver cells, mature into trophozoites, which feed, and became schizonts (exo-erythrocytic schizogony). In *P. ovale* and *P. vivax*, some sporozoites may develop into quiescent stages (called *hypnozoites*) in the liver (Smyth, 1994). The rupture of schizonts release merozoites (after the exo-erythrocytic merogony) into the bloodstream which will then parasitize red cells. Merozoites then develop through the ring stages, tophozoites and schizonts (erythrocytic schizogony). The rupture of erythrocytes containing the pigmented schizonts releases, depending of the species, 6 to 32 merozoites per cell (after the erythrocytic merogony) that invade new erythrocytes (Cassier et al., 1998). All schizonts release their merozoites synchronously every 72 hours (quartan fever of *P. malariae*) or 48 hours (tertian fever of other species) inducing fever paroxysms (Cassier et al., 1998; Garcia and Bruckner, 1997; Smyth, 1994). Within the course of infection, *i.e.* at least 10 days after the first appearance of asexual forms in the case of *P. falciparum* (Nsanzabana, 2008), some merozoites differentiate into sexual stages: the macro-gametocytes (females) and the micro-gametocytes (males). Infectious erythrocytes containing gametocytes could eventually been ingested by a second female mosquito taking her blood meal. Interestingly, it is suggested that the synchronous cyclic occurrence of asexual malaria parasites ensures the presence of mature gametocytes in the blood during mosquito biting activity (Hawking et al., 1968).

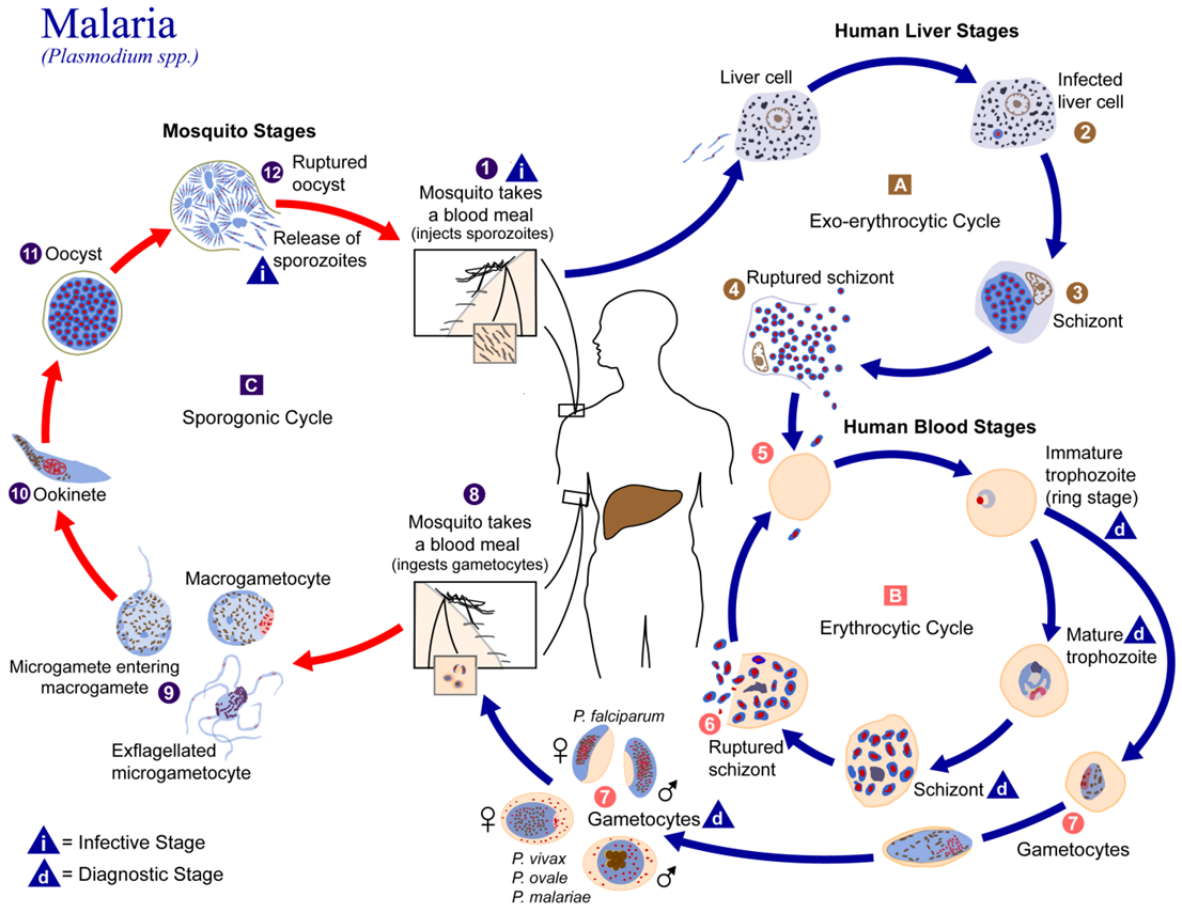

Fig. 3. 3: Life-cycle of malaria parasites (CDC, 2002)

Within the mosquito gut microgametocytes undergo nuclear division to forms 8 haploid microgametes a few minutes after infection. During this process called exflagellation, each microgamete breaks out of the erythrocyte to penetrate a female macrogamete (Cassier et al., 1998; Garcia and Bruckner, 1997; Smyth, 1994). A fertilized macrogamete is called a zygote. The zygote develops into a mobile ookinete, penetrates the peritrophic membrane and the gut wall, and matures into an oocyst between the midgut basement membrane and the epithelium (Cassier et al., 1998; Smyth, 1994; Nsanzabana, 2008). After 10 to 20 days, following meiosis and numerous mitosis events, the mature oocyst breaks out and liberate up to one thousand sporozoites which will reach all the organs of the insect and, particularly, the acinar cells of the salivary glands (Cassier et al., 1998; Smyth, 1994). When an infected mosquito takes a blood meal it will inject less than twenty sporozoites to the vertebrate host and the malaria cycle starts again (Nsanzabana, 2008). To transmit the disease, a female mosquito has to bite at least twice and may survive long enough to allow the formation of sporozoites. Assuming a constant daily survival rate of 0.85 for mosquitoes (Midega et al., 2007; White, 1982) only 10% of females are likely to survive a 14 days extrinsic incubation period. Moreover, those females have to take up a sufficient number of gametocytes during the first blood meal. In fact, a 2754-fold reduction was found between the number of macrogametocytes imbibed by a mosquito and the number of oocysts formed in laboratory strains of falciparum malaria (Jefferson et al., 1992), and a minimum of about 30 ookinetes need to be formed to ensure that the mosquito becomes infectious (Jefferson et al., 1992). These findings show that the female *Anopheles* which may transmit the disease constitute only a minor fraction of the mosquito population that is the target of vector control measures. Despite this, malaria transmission intensity in Africa was found to vary between < 1 to > 1000 infective bites per person per year in sites bearing sometimes extreme prevalence rates up to 94.5% of falciparum malaria (Beier et al., 1999). To enhance its transmission success, *Plasmodium* species seems to manipulate the mosquito hosts. Thus, mosquitoes harboring sporozoites are more persistent and tend to bite more people than uninfected mosquitoes (Koella et al., 1998). On the other hand, a non-transmissible stage of the parasite, e.g. the oocyst, decreases the motivation to take up blood, as biting is risky for mosquitoes (Koella et al., 2002). Moreover, patients harboring gametocytes are about two times more attractive to *A. gambiae* than patients harboring the asexual stages of *P. falciparum* or uninfected patients (Lacroix et al., 2005). This may result from the level of the semiochemicals (body odour, breath components, CO_2) emitted by the host as well its skin temperature and moisture, fundamental stimuli for host recognition by mosquitoes (Gillies, 1980; Klun et al., 2013; Spitzen et al., 2013; Takken and Knols, 1999).

3.3 *Anophelinae* and the malaria transmission

Of the approximately 430 *Anopheles* species only about forty are able to transmit malaria depending on the region and the environment (Kiszewski et al., 2004; Fig. 3.4). In figures 3.4 and 3.5, the correlation between the distribution of *Anopheles funestus* Giles and *A. gambiae* Giles groups and the distribution of falciparum malaria transmission risk, highlights the ability of these two mosquito complexes to transmit the disease in Africa.

The *A. funestus* group comprises at least eleven members: *A. funestus sensu stricto*, *A. vaneedeni* Gillies and Coetzee, *A. leesoni* Evans, *A. rivulorum* Leeson, *A. rivulorum*-like, *A. parensis* Gillies, *A. fuscivenousus* Leeson, *A. aruni* Sobti, *A. brucei* Service and *A. confuses* Evans and Leeson and the Asian *A. fluviatilis* James (MR4, 2011). *A. funestus s. str.* is the most efficient vector of this complex because of its high anthropophilic habit and its endophilic habits (Samson Awolola, Molecular Entomology Research Unit, Public Health Division, Nigerian Institute of Medical Research, Nigeria, personal communication).

A. gambiae sensu lato represents a complex of seven species: *A. bwambae*, *A. merus* (the East African salt-water breeder mosquito), *A. melas* (the West African salt-water breeder mosquito), *A. quadriannulatus* A and B, *A. arabiensis* and *A. gambiae s. str.* (Coluzzi et al., 1979; White, 1974; MR4, 2011). *A. quadriannulatus* is strongly zoophilic and is therefore of little medical interest. *A. bwambae* shows mixed biting preferences. This species is not an important malaria vector although it can be the predominant indoor resting member of the complex in extremely restricted areas. The two salt-water breeding species, *A. merus* and *A. melas*, bite man when other hosts are not available. They are of medical importance in coastal regions where other vectors are scarce (White, 1974). *A. arabiensis* and *A. gambiae s. str.* are known as the most widespread major vectors of malaria in sub-Saharan Africa. Although *A. gambiae s. str.* is mostly anthropophilic and endophilic, the behaviour of *A. arabiensis* is more variable (Samson Awolola, Molecular Entomology Research Unit, Public Health Division, Nigerian Institute of Medical Research, Nigeria, personal communication). These two species occur sympatrically although *A. arabiensis* is more broadly distributed in arid regions (Coetzee et al., 2000) due to a higher body water content which permits a higher desiccation resistance in this species (Gray and Bradley, 2005). It has been shown that two molecular forms, M and S respectively, occur in *A. gambiae s. str.* with strong evidence of reproductive isolation between them (della Torre et al., 2001; Wondji et al., 2002) probably due to male swarm spatial segregation (Diabate et al., 2009).

In addition to malaria, some anophelines also transmit lymphatic worms (filariasis) and arthropod borne viruses (arbovirus) (Becker et al., 2003; White 1974).

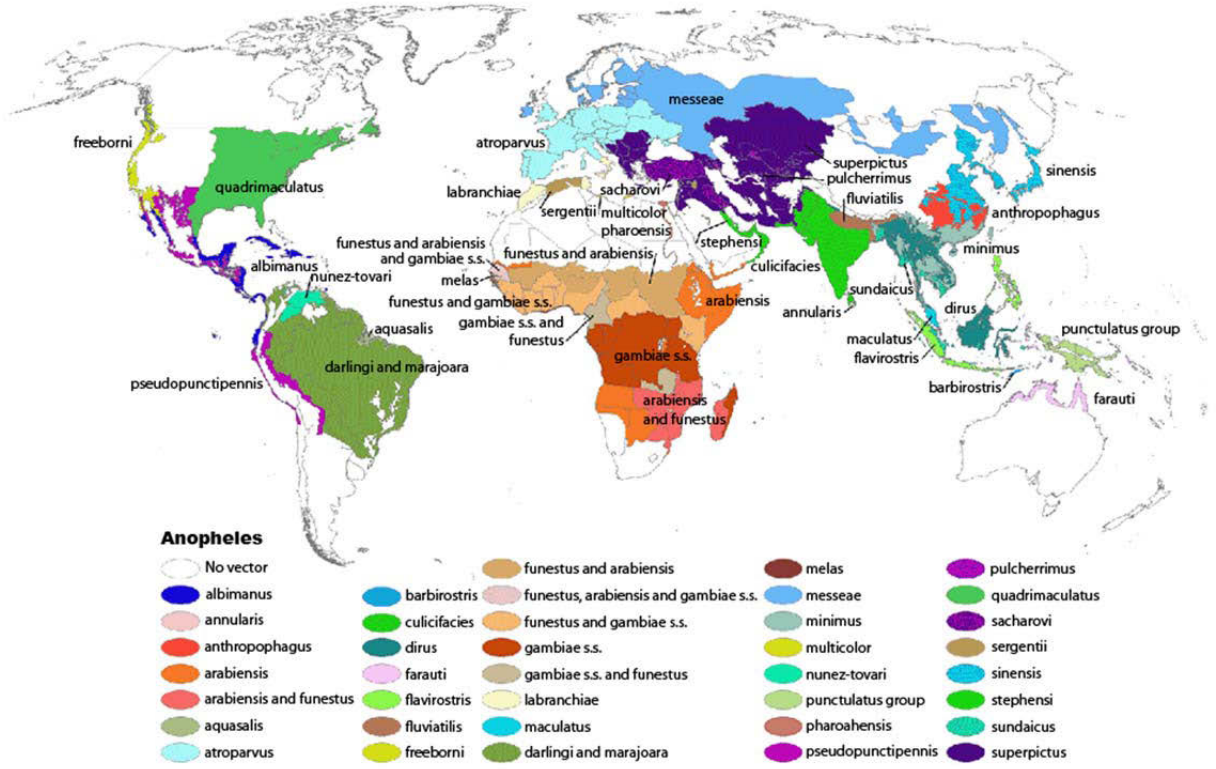

Fig. 3.4: Global distribution of dominant or potentially important malaria vectors (map from Kiszewski et al., 2004, reproduced with permission of the authors).

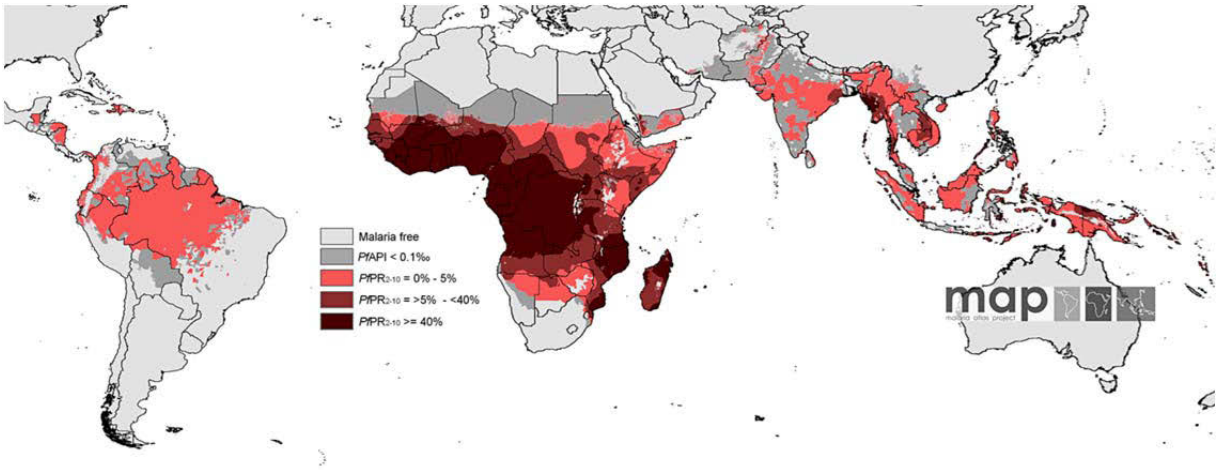

Fig. 3.5: Global distribution of falciparum malaria transmission risk (*Pf*PR = *P. falciparum* parasite rate) in 2007 (map from Hay et al., 2009, reproduced with permission of the editor).

3.4 The mosquitoes: from sensing to behaviour

Sensing stimuli allows animals to avoid predators or harmful compounds, to reach a food source or to locate a mating partner. The four key behaviours in the mosquito life-cycle, e.g. sugar feeding, mating, host seeking and oviposition (Takken and Knols, 1999), depend of both internal factors such as genetic traits, age, energy reserves, gorging state, mating status, gonotrophic stage and its circadian rhythm and the ability of the mosquito to detect external stimuli (Fig. 3.6).

Despite their low visual acuity (Muir et al., 1992), mosquitoes are sensitive to host movements and contrast is important for host finding by both diurnal and nocturnal species (Clements, 1999). Mosquitoes may detect colors as rhodopsins sensitive to green (maximum absorbance more than 500 nm), UV (maximum absorbance less than 400 nm) and blue (maximum absorbance between 400 and 500 nm) have been identified in *A. aegypti* and *A. gambiae* (Hu et al., 2009).

Mosquitoes may use their thermo and hygroreceptor cells to find resting sites with adequate microclimatic conditions (Kessler and Guerin, 2008). Sensilla coeloconica enclosing a pair of thermoreceptive cells (a warm-sensitive and a cold-sensitive neurone) have been identified at the tip of the antennal flagella (Davis and Sokolove, 1975; Gingl et al., 2005). However, how mosquitoes perceive humidity remains unclear (Clements, 1999), although Bar-Zeev (1960) postulates that *A. aegypti* possess hygroreceptors on their antennae and moisture-receptors (e.g. acting by contact) on their tarsi. Both temperature and humidity are important stimuli for the orientation of mosquitoes towards hosts at close range and to elicit probing (Clements, 1999; Klun et al., 2013).

The Johnston's organ found in the pedicel of the antennae provides information during flight such as the relative air flow or the body angle relative to gravity. It also allows males to detect females as the resonance frequency of male flagellum peaks at the fundamental tone of the female wing beat frequency (Clements, 1999).

Mechanoreceptor cells distributed all over the body surface are enclosed in aporous articulated setae or in contact chemosensilla and are activated by air movement or touch to evaluate flight speed, the position of articulations or the structure of a substrate (Clements, 1999).

Although all these stimuli are fundamental, chemical cues are the most developed and probably the most important group of external stimuli affecting mosquito behavior (Takken and Knols, 1999). Chemoperception is generally divided into olfaction (distance-chemoperception) and taste (contact-chemoperception) (de Bruyne and Warr, 2006). However, a strict divide does not occur between these two senses as contact-chemoreceptor

cells can also respond to volatile molecules at short range (Städler and Hanson, 1975). Maxillary palps and antennae constitute the olfactory appendages of mosquitoes. Olfactory receptor cells are enclosed in thin hairs, called sensilla. Double-walled multiporous capitate peg sensilla of the maxillary palps of *A. gambiae* enclose notably receptor cells sensitive to CO_2 and 1-octen-3-ol (Lu et al., 2007). In all, the antenna of *A. gambiae* bears five classes of sensilla. Single-walled multiporous trichoid hairs and double-walled multiporous grooved pegs are the most abundant sensilla on the antennae in *A. gambiae* (Pitts and Zwiebel, 2006). They enclose olfactory neurones responsible for the detection of host volatiles (Carey et al., 2010; Pitts and Zwiebel, 2006; Qiu et al., 2006; Appendix IX). Plant volatiles are also perceived by receptor cells on the antennae of *A. gambiae* (Nyasembe et al., 2012). Odour detection by receptor neurones involves large families of diverse proteins such as olfactory receptors (ORs), the olfactory variant ionotropic glutamate receptors (IRs) and the presence in the sensillar lymph of odorant binding proteins (OBPs) (Biessmann et al., 2010; Liu et al., 2010; Schymura et al., 2010). The specificity of olfactory receptors cells is conferred through the expression of individual olfactory receptor genes (Carey et al., 2010). To date, 79 olfactory receptors (Ors) have been identified in the genome of *A. gambiae* (Carey et al., 2010) and 46 variant ionotropic glutamate receptors (Liu et al., 2010). Members of the Or family are considered to have 7 transmembrane domains (Kent et al., 2008) and are thought to be heterodimers as they are co-expressed with OR7 in *A. gambiae*. IRs form complexes of up three subunits likewise in *Drosophila* (Abuin et al., 2011). Like IRs, it is generally admitted that insect Ors are ionotropic ligand-gated ion channels (Sato et al., 2008), although the involvement of G proteins in the transduction mechanism is still discussed (Pellegrino and Nakagawa, 2009; Silbering and Benton, 2010). In a general manner, ionotropic receptor pathways operate faster than metabotropic ones and allow insects flying upwind to quickly detect the presence or the absence of an odour (Silbering and Benton, 2010). The sense of taste in insects is described in more details below.

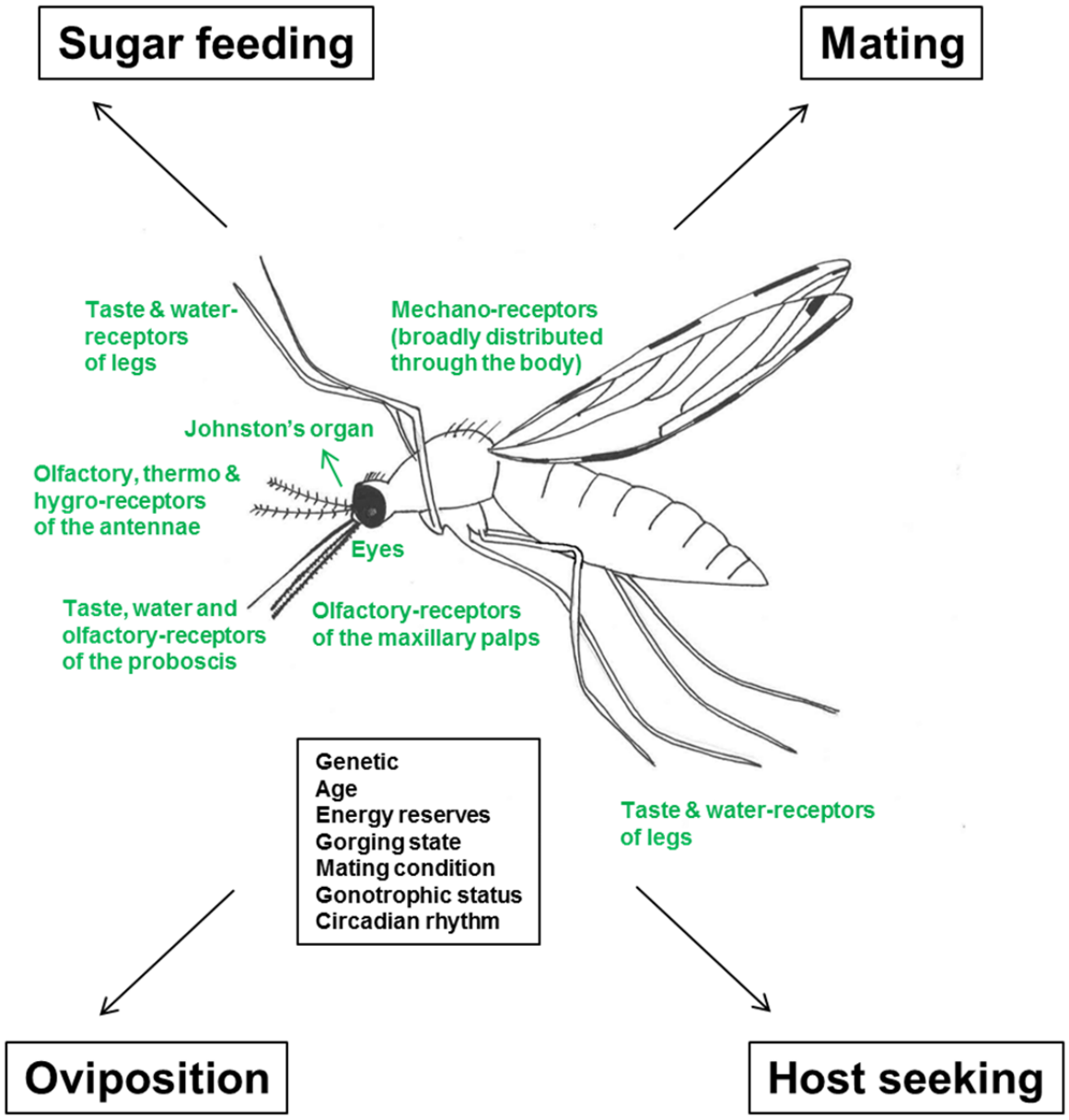

Fig. 3.6: Sensitive (in green) and internal factors (central box) play fundamental roles in the four key behaviours (sugar feeding, host seeking, mating and oviposition) driving the mosquito life-cycle.

3.5 Sugar feeding in mosquitoes

Sugar feeding in mosquitoes has been already extensively reviewed by Foster (1995) and Stone (2011) from whom most of the information summarized in the present section was found.

A characteristic of most blood-feeding *Diptera* is that they also ingest sugar (Stone, 2011), with the exception of the superfamily of *Hippoboscoidea* which comprise the tsetse flies (*Glossinidae*) and the bat flies (*Hippoboscoidae*, *Streblidae* and *Nycteribiidae*). This feeding behaviour does not occur in other orders where heamatophagy has evolved such as fleas (*Siphonaptera*), lices (*Phthiraptera*) or bugs (*Hemiptera*).

3.5.1 Sugar feeding in the mosquito life cycle

Mosquito males depend only on the energy reserves accumulated as larvae and from the plant-derived sugars absorbed after emergence for their flight performance and survival and consequently for their insemination potential (Stone, 2011). Sugar is needed for male *A. gambiae* to survive the maturation period until reproduction and to undertake sexual behaviour such as antennal fibrillae erection and swarming (Gary et al., 2009). Longevity of male *A. gambiae* without a sugar source does not exceed four days in the laboratory (see Chapter 6). Swarming is particularly costly for *A. freeboni*, in which males invest more than 50% of its available calories (Yuval et al., 1994). However, males of this species generally feed on sugar after swarming (Yuval et al., 1994), a temporal pattern also observed in male and female *A. gambiae* (Stone, 2011). Male *A. gambiae* maintained in cages feed at an average frequency of about twice per night (Gary and Foster, 2006). Without sugar, the ability of *A. gambiae* males to inseminate females drops severely (Gary et al., 2009; Stone et al., 2009). Females of the anautogenous species, i.e. females that require a blood meal before oviposition, are mostly opportunistic (Stone et al., 2011): although they need a blood meal to produce eggs, both sugar and blood regimes can permit the development of ovarian follicles of the previtellogenic phase (Fernandes and Briegel, 2005) and flight (Kaufmann and Briegel, 2004). The first night after emergence female *A. gambiae* prefer to feed on sugar although they may feed on blood in the absence of a nectar source (Stone et al., 2011). This could happen in natural conditions where *A. gambiae* and *A. funestus* females are strongly endophilic and are rarely found feeding on sugar (Beier, 1996). During the 5 days following emergence, when sugar is available, the common sequence of behaviour undertaken by female *A. gambiae* is to feed first on sugar, then mate and then have a blood meal (Stone et al., 2011). In laboratory cages, females feed on sugar on average once every 4 nights, between each gonotrophic cycles, before a blood meal (Gary and Foster, 2006). However, females may increase the frequency of sugar meals if an oviposition site or a vertebrate host is not readily accessible (Gary and Foster, 2006). In a laboratory study it was shown than

when sugar is accessible, longevity of females is increased although their blood-feeding rate is decreased. Consequently, total fecundity remains unchanged by sugar meals although without sugar, females achieve a gonotrophic cycle in a shorter time (Gary and Foster, 2001; Stone et al., 2011). However, it appears that the effect of sugar on mosquito populations is underestimated in some of these cage studies (Gary and Foster, 2001) due to the close proximity of mosquitoes. In field or semi-field conditions, *Anopheles* populations are probably not viable without a sugar source, mostly due to the decrease in male performance (Stone et al., 2009).

The major source of sugar is floral nectaries (Fig. 3.7A), although mosquitoes also feed on extra-floral nectaries, honeydew, tree sap, plant phloem and damaged fruits (Fig. 3.8B) or discarded plant material like sugar-cane waste (Foster, 1995). Although mosquitoes can serve as significant pollinators for some plants, they usually "play a minor role in pollination themselves and are essentially nectar thieves" (Foster, 1995). In fact, during field studies, mosquitoes are generally observed to feed on flowers at peak-bloom and at relatively high densities, demonstrating opportunism (Magnarelli, 1979). However, some definite preferences exist.

Both male and female *A. gambiae* feed disproportionately on particular plant species which enhance both their longevity and their fecundity in comparison to discarded plants (Gouagna et al., 2010; Impoinvil et al., 2004; Manda et al., 2007a; Manda et al., 2007b). The signals attracting mosquitoes to the plants are probably the same as for pollinators. Visual, olfactory or gustatory cues could influence mosquito plant preferences. The importance of volatiles for mosquito plant preferences has been recently shown in both females and males *A. gambiae* (Gouagna et al., 2010; Nyasembe et al., 2012). Particularly, mosquitoes are able to associate a sugar reward with an odour (associative learning) (Sanford and Tomberlin, 2011). Little is known about the importance of gustatory cues for nectar foraging in mosquitoes and, to date, no such studies has never been undertaken in *A. gambiae*.

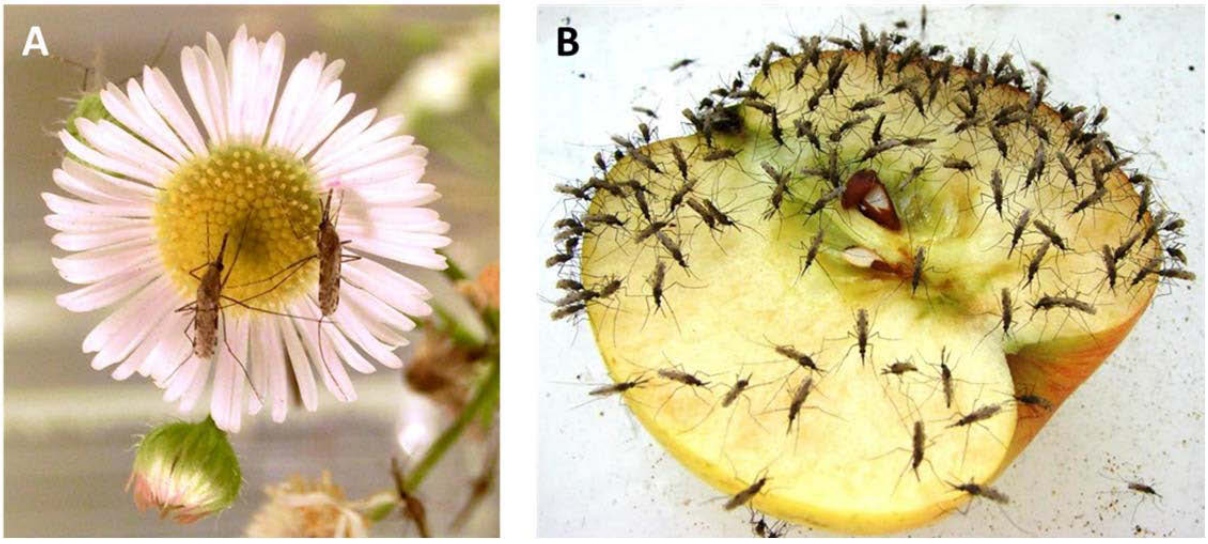

Fig. 3.7: Two reared female *A. gambiae* taking a nectar meal on the flower *Erigeron annuus* (L.) Pers. (A). Starved male and female *A. gambiae* taking a sugar meal on an apple in a rearing cage (B).

3.5.2 Sugar feeding and vector competence

The vectorial capacity (C), firstly described by Garett (1964) defines the efficiency of a vector population to transmist a pathogen. C corresponds to the daily rate at which future inoculations of a pathogen arise from an infective case when all vectors who have fed on this case become infected (Dye, 1986). The vectorial capacity could be translated into the following equation (Dye, 1986; Stone, 2011):

$$C = \frac{m \cdot a^2 \cdot p^n}{-\ln(p)}$$

with *m* being the number of mosquitoes per person and *a* the daily man biting rate, which could be calculated by dividing the proportion of meals taken on humans (the human blood index) by the time interval between meals. Thus, *ma* in this equation represents the number of bites per person and per day. *p* is the female survival rate during the extrinsic incubation period of the parasite *n*. The biting rate *a* and the daily survival rate *p* are the most important factors influencing vectorial capacity. Small changes in these factors result in a large impact on vectorial capacity because of their magnification to the power of 2 and *n* respectively (Stone, 2011).

It was shown that sugar sources have antagonistic effects on the vectorial capacity of *A. gambiae* by increasing the lifespan (Gary and Foster, 2001; Gu et al., 2011; Okech et al., 2003; Stone et al., 2011; Stone et al., 2012) and by decreasing the biting rates of female (Gary and Foster, 2001; Stone et al., 2012).

3.5.3 Storage, absorption and metabolism of the sugar meal

In mosquitoes, sugar is usually stored into the crop, whereas heated blood or physiological solutions presented through a membrane pass directly into the midgut (Fig. 3.8) (Arsic and Guerin, 2008). The destination of a sugar solution depends however of the sugar type and its concentration (Schmidt and Friend, 1991). For example, in *Culiseta inornata* (Williston), cellobiose is directed mainly to the midgut although sucrose is directed to the crop (Schmidt and Friend, 1991). Cibarial gustatory receptors may be involved in the diet destination (McIver, 1982). However, carbohydrates are thought to be absorbed by the midgut rather than by the crop epidermis. In fact, although α-glucosidase, an enzyme involved in the digestion of sucrose, has been found to be produced by salivary glands of aedine mosquitoes (Marinotti et al., 1996; Marinotti and James, 1990), these enzymes are active after passage of the nutrients into the midgut (Souza-Neto et al., 2007). Moreover, the latter authors have shown that three α-glucosidases are produced in the midgut of *Anopheles aquasalis* (Souza-Neto et al., 2007). All three (α-Glu1, α-Glu2 and α-Glu3,) have been found in membrane-bound forms, although α-Glu1 and α-Glu2 are also secreted.

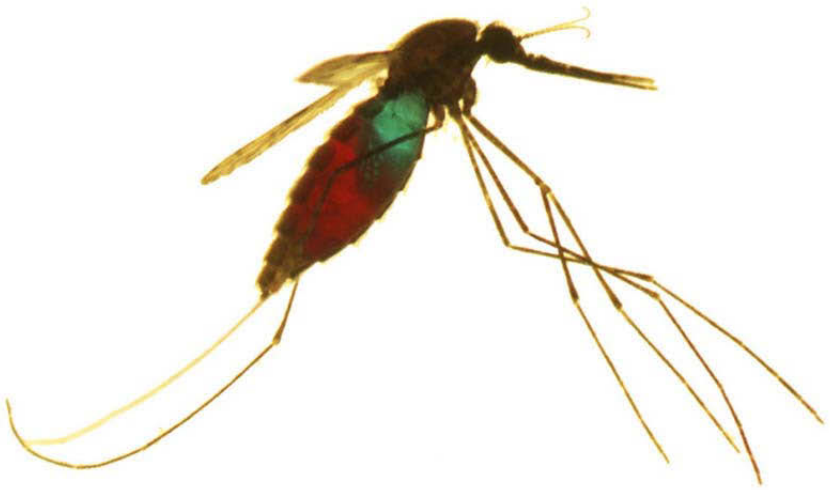

Fig. 3.8: In female *A. gambiae* sugar (colored with a blue food dye) is directed to the crop whereas blood is directed to the midgut.

Only a few studies have been made on carbohydrates absorption in insects (Chapman, 2013). Until recently, it was believed that carbohydrates were absorbed as monosaccharides only by passive diffusion from a high concentration inside the midgut to a lower one in the heamolymph (Chapman, 1998). The concentration gradient is maintained with the rapid conversion of glucose to trehalose within the fat body surrounding the midgut (Chapman, 1998). Absorption of fructose in this passive way is less efficient as its conversion to trehalose is less rapid (Chapman, 1998).

However, there is good evidence that glucose may also be transported in insects across cell membrane by transporters similar to those of mammals. In fact, a sodium-glucose linked transporter (SGLT) and a facilitated glucose transporter 2 (GLUT2) have been identified in the larvae of the hymenopteran parasite *Aphidius ervi* (Giordana et al., 2003). In addition, in mosquitoes, disaccharides are not necessarily digested to monosaccharides before absorption, as enzymes hydrolyzing sucrose have been found in the abdomen of *Aedes sollicitan*s (Walker) from which the alimentary duct was removed (Van Handel, 1968). The fact that sucrose may be absorbed in insects seems to be confirmed by the presence of sucrose transporters recently identified in the hindgut of *Drosophila* larvae (Meyer et al., 2011).

The first step in the metabolism of carbohydrates is the phosphorylation of monosaccharides (e.g. glucose to glucose-6-phosphate) and their isomerization (glucose-6-phosphate to glucose-1-phoshate). Glucose-1-phosphate can then enter either threhalose or glycogen synthesis pathways (Clements, 1999). When the amount of trehalose circulating in the heamolymph rises its synthesis by the fat body is inhibited and carbohydrates are then stored as glycogen (Chapman, 2013). In the flight muscle, trehalose, which diffuses quickly through tissue, is hydrolyzed by the enzyme trehalase to glucose for its further use as an energy source for flight (Clements, 1999). Fructose-1,6-biphosphate could be oxidized to pyruvate through the glycolytic pathway. Pyruvate will lead the production of the two carbon unit Acetyl coenzyme A which can either enter the fatty acids synthesis pathway or the tricarboxylic acid cycle for further energy production.

In *A. gambiae* with *ad libitum* access to 10% sucrose but without a blood meal as in our insect colony maintenance conditions, glycogen and lipid levels reach their respective peaks at 2 and 7 days respectively (Kaufmann and Briegel, 2004). This increase in energy reserves is correlated with an increase in flight performances (Kaufmann and Briegel, 2004). *A. gambiae* provided with sugar utilizes glycogen for survival during the first week post eclosion but not lipids (Kaufmann and Briegel, 2004). Likewise, female *A. aegypti* provided with 10-50% sucrose solutions maintain their lipid reserves above that of their teneral level throughout their life time and the glycogen stock only decreases once the 50% survival level

has been passed (Briegel et al., 2001a). In *A. gambiae*, free carbohydrate levels already increase the first day after emergence because of sugar feeding (Kaufmann and Briegel, 2004). Glycogen, free carbohydrate and lipid reserves constitute the energy sources to sustain flight in sugar fed mosquitoes, including *A. gambiae* (Briegel et al., 2001a; Briegel et al., 2001b; Kaufmann and Briegel, 2004). In mosquitoes, ingested sugars seem to provide an instantaneous energy source for flight (Clements, 1999). For example, after feeding on glucose, *A. taeniorhynuchs* does not utilized glycogen for flight as long free sugar is available (Nayar and Van Handel, 1971). Free carbohydrates were also found to be an important energy source for flight in *A. gambiae* and *Anopheles atroparvus* Van Thiel (Kaufmann and Briegel, 2004). As trehalose is rapidly resynthesized from glycogen during flight (Clements, 1999), the reduction in the free carbohydrate stock measured in the study of Kaufmann and Briegel (2004) reflect the consumption of the ingested sugars by both *A. gambiae* and *A. atroparvus* during flight.

3.6 Bitterness induce avoidance

Plants constitute the richest source of organic chemicals and many of them are protected from most phytophagous insect species by unpalatable substances that humans perceive as bitter (Schoonhoven, 1982). Human bitter-tasting compounds occur as a wide range of chemical classes and prevent animals from eating potentially toxic food. Some of them have been categorized as amines (e.g. denatonium), ureas/thioureas, amino acids (such as L-phenylalanine, L-tryptophan and L-arginine), acetylated sugars (e.g., sucrose octaacetate), isohumulones, phenols (including the flavanols, catechin and epicatechin), carbamates (e.g., phenylthiocarbamide) and ionic salts (e.g. caesium chloride, potassium iodide, magnesium sulfate) (Delwiche et al., 2001; Shallenberger, 1993). However, plant alkaloids such as berberine and quinine are the most common stimuli employed to characterize bitterness.

Nectar contains primary metabolites like sugar and proteinogenic amino acids which attract pollinators but can also contain unpalatable secondary metabolites like phenols, non-proteinogenic amino acids, glycosides or alkaloids. Secondary compounds in nectar are geographically and phylogenetically widespread (Adler, 2000) and occur in at least 21 different plant families (Irwin and Adler, 2008). Toxic nectar has been hypotesised to prevent microbial degradation, encourage specialist pollinators or deter nectar thieves (Adler, 2000). The latter hypothesis is supported by the fact that toxic nectars are mostly found in plant species growing in ecosystems possessing the highest diversity of flower-visitors (Baker, 1977). This is true for the tropics that are home to most mosquito species. Toxic nectar could also have a fitness benefit for the plant in acting on pollinators. It has been shown that secondary metabolites in nectar can reduce nectaring time, nectar volume removed and self-

pollen transfer and so increase the number of visitations by pollinators (Irwin and Adler, 2008; Kessler and Baldwin, 2007).

3.7 The sense of taste

3.7.1 Peripheral coding in mammal taste

In mammals, taste receptor cells are found on the tongue and palate. Taste buds are housed in fungiform, circumvallate and foliate papillae and contain between 50 to 100 receptor cells (Yarmolinsky et al., 2009). Five taste qualities have been identified in humans and are probably common to most mammals: sweet, bitter, sour, salty and umami, the taste of monosodium glutamate (Yarmolinsky et al., 2009). Some receptor cells may respond to multiple taste qualities although this is still discussed (Caicedo et al., 2002). Sweet products and umami are perceived by cells expressing the heterodimeric G protein-coupled receptors (GPCRs) T1R2/T1R3 and T1R1/T1R3 on their microvilli, respectively (Yarmolinsky et al., 2009). The presence of multiple binding sites in each receptor complex may explain the recognition of several ligands by these two receptor complexes (Yarmolinsky et al., 2009). Both T1R2/T1R3 and T1R1/T1R3 expressing cells permit sensitivity to attractive tastants which elicit feeding behaviour (Yarmolinsky et al., 2009). Aversive compounds are recognized by a family of 30-40 GPCRs T2R receptors, the majority of them being expressed in bitter receptor cells (Thorne et al., 2005; Yarmolinsky et al., 2009). However, despite the fact that in mammals bitter-sensitive taste cells express mRNAs for several receptors, each receptor cell seems to respond to a limited number of aversive compounds (Caicedo and Roper, 2001). Moreover, several perception mechanisms for bitter compounds, independent of interactions with any GPCRs, are thought to occur in mammals. For example, quinine has been found to inhibit several ion channel types of rat taste receptors (Chen and Herness, 1997). Salty and sour tastes are evoked by cations sodium and protons, respectively; those either pass through or modulate ion channels (LopezJimenez et al., 2006). Sour taste receptor cells also express Car4, a carbonic anhydrase acting as the main CO_2 sensor of mammals (Yarmolinsky et al., 2009).

3.7.2 Peripheral coding in insect taste

Insect chemoreceptor proteins include both odorant receptors (Ors) and gustatory receptors (Grs) (Kent et al., 2008). Insects Grs, contrary to mammals, are thought to be ionotropic ligand-gated ion channels due to their homology with Ors (Silbering and Benton, 2010). The Gr family was so named because these genes were initially found being expressed in gustatory organs, but some of them are expressed in antennae and are thus putative olfactory receptors (Kent et al., 2008). In all, the genome of *Drosophila* encodes 68 functional

gustatory receptors (Robertson et al., 2003), those of *Aedes aegypti* (L.) 91 and those of *A. gambiae* 90 Grs (Kent et al., 2008).

Numerous studies on insect taste have focused on *Drosophila*. In fact, this species provides a good model for studying taste due to the similarity of its gustatory cues with humans and the readily available molecular tools (Amrein and Thorne, 2005). Insect taste receptor cells are enclosed in sensory hairs called sensilla, usually characterized by two lymphatic cavities and a single pore at the tip (Fig. 3.9) and are not restricted to mouthparts. For example, *Drosophila* carries taste sensilla on the labellum (the mammal tongue equivalent), but also in the pharynx (labral and cibarial organs), on the wings, legs and genitalia (Thorne et al., 2005; Vosshall and Stocker, 2007). In mosquitoes, contact chemo-sensilla have been found on legs, labellar lobes, labrum and cibarium. The contact chemosensory organs of *A. gambiae* are described throughout this thesis.

Tarsal and labellar l and s-type sensilla of *Drososophila* enclose one receptor cell sensitive to sugar, one to water, one to salt at low concentrations (L1) and a fourth that responds to bitter products and salts at high concentration (L2 cell) (Meunier et al., 2003). Labellar i-type sensilla enclose only two receptor cells, one coding for stimuli such as sugar and the other one for aversive stimuli such as bitter products (Hiroi et al., 2004). Within taste sensilla, one receptor cell acts usually as a mechanoreceptor (Falk et al., 1976). In *Drosophila*, chemoreceptor cells in labellar taste pegs have been found to function notably as CO_2 sensors (Fischler et al., 2007; Yarmolinsky et al., 2009) and, recently, the mechanism of sour perception has been highlighted in this species (Charlu et al., 2013). As in olfactory sensilla, binding proteins have been found in taste pegs of *Drosophila* and are thought to carry hydrophobic ligands through the lymph to the Grs (Shanbhag et al., 2001).

In *Drosophila*, labellar sensilla belonging to a same morphological types (L, I and S-type) respond in the same manner to increasing doses of sucrose, glucose, fructose or trehalose, although differences in threshold sensitivity were found between sensillar types (Hiroi et al., 2004). In this species, sugar receptor cells express several Grs for the detection of a large panel of sweet tastants (Dahanukar et al., 2007). For example, Gr5a allows the insect to detect m-α-glucoside, glucose, melezitose and trehalose and Gr64a binds to maltotriose, stachyose, raffinose, leucrose and fructose (Dahanukar et al., 2007). It is likely that sucrose, maltose, turanose, maltitol and palatinose are also detected by other receptors in addition to the Gr64a (Dahanukar et al., 2007) highlighting the fact that, in insects, several different Grs are involved in the perception of a same compound. It is thought that in *Drosophila* distinct classes of sugar sensitive neurones expressing a different Grs assortment are present because Gr61a and Gr64f are coexpressed with Gr5a in some, but not all, sugar-sensitive cells (Dahanukar et al., 2007; Fig. 3.10).

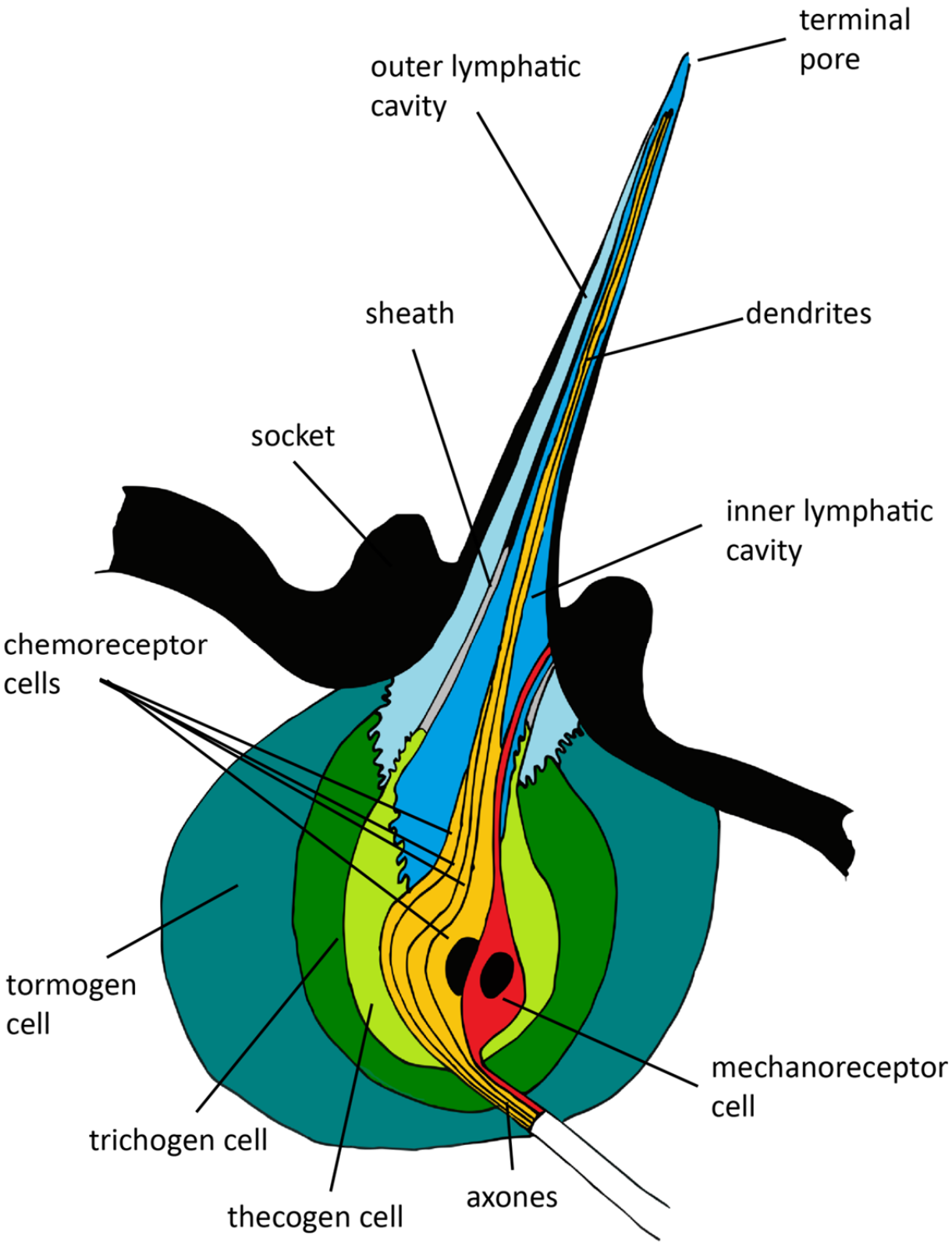

Fig. 3.9: Schematic represention of an insect gustatory-mechano sensillum.

In mosquitoes, *A. gambiae*, *A. aegypti* and *C. pipiens* have 8, 10 and 14 sugar receptor genes respectively among which genes 3 and 1 are pseudogenes (genes not expressed) in *A. aegypti* and *C. pipiens,* respectively (Kent and Robertson, 2009). The ligands for mosquito sugar receptors are difficult to assess from functional studies made on *Drosophila* by gene sequence comparisons as the lineage between the sugar receptors of *Drosophila* spp. and mosquito spp. is not “a simple orthology, but rather a complex pattern of gene duplications and losses” (Kent and Robertson, 2009). These authors hypothesized that fly sugar receptors evolved from a single ancestral receptor gene which further evolved into four major lineages after two simple tandem duplications (Fig. 3.11, orange “1”, purple “2”), before the divergence between Brachycera and Nematocera (~260 Mya).

Bitter products are perceived on the labellum of *Drosophila* through four functional taste neurons expressing distinct Gr panels and which show differential sensitivities to aversive compounds (Weiss et al., 2011; Fig. 3.10). This means that in insects, contact chemosensilla sharing similar morphologies may enclose sensory neurones expressing distinct gustatory receptors and thus may have differential sensitivity for both stimulants and aversive compounds. Thus, insects are able to discriminate between bitter compounds that activate different populations of bitter-sensitive receptor cells (Glendinning et al., 2002; Weiss et al., 2011). In addition, insects are able to discriminate between compounds that elicit different firing temporal pattern within the same taste cell (Glendinning et al., 2002). Both sugar and bitter receptors of *Drosophila* seem to be multimers as the Gr64f has been found to interact with both Gr5a and Gr64a receptors (Isono and Morita, 2010; Jiao et al., 2008) and Gr32a, Gr33a, Gr39a, Gr66a and Gr89a are expressed in all bitter labellar neurones and may function as coreceptors (Weiss et al., 2011). In addition, bitter neurones do not code only for the perception of bitter compounds such as alkaloids but are activated during stimulation with most aversive compounds such as high salt concentrations (Meunier et al., 2003) or acidic solutions (Charlu et al., 2013). This could explain the diversity of Grs expressed on the membrane of bitter taste neurones. Moreover, taste neurones coding for aversive compounds not only mediate feeding avoidance. For example, it was shown that z-7-tricosene, a *Drosophila* sex pheromone inhibiting male-male courtship, is detected by a particular labellar taste neurone that responds to bitter stimuli (Lacaille et al., 2007). Particularly, mutant flies lacking Gr32a (Miyamoto and Amrein, 2008) or Gr33a (Moon et al., 2009), both expressed in labellar bitter neurones (Weiss et al., 2011) display increased male-male courtship.

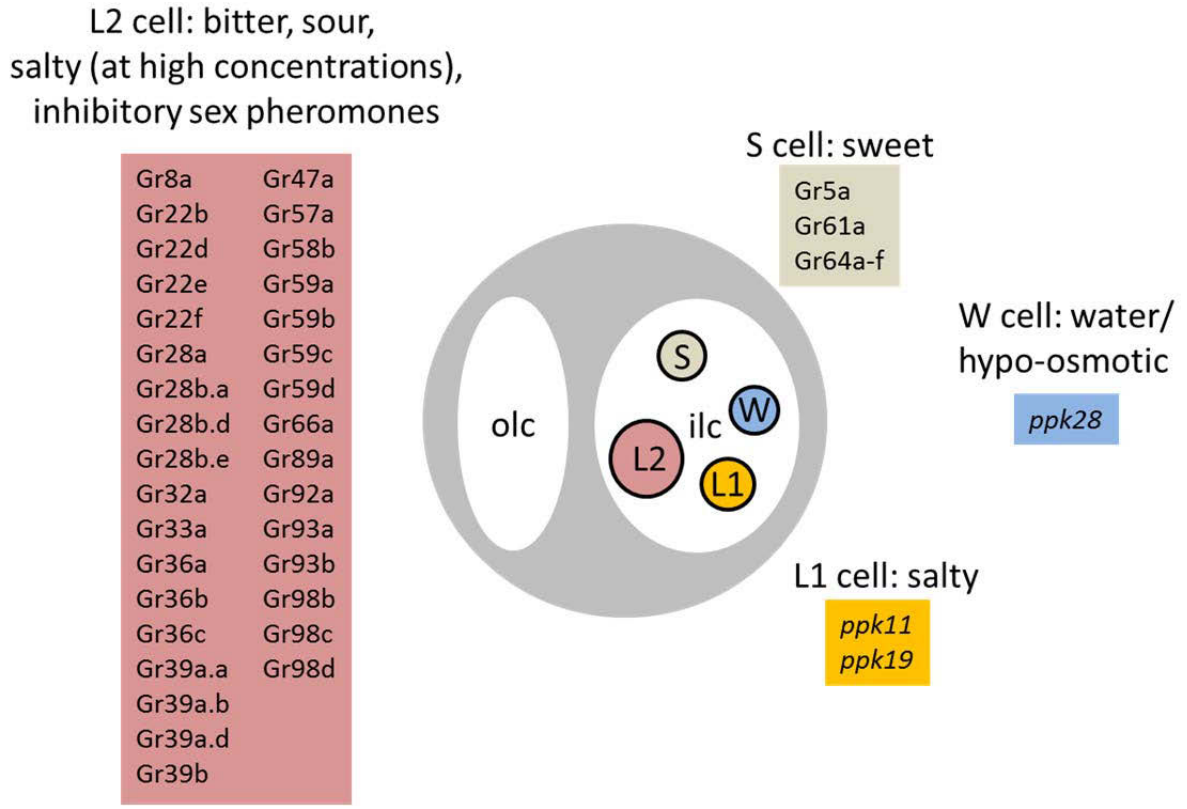

Fig. 3.10: Schematic transversal cut of a taste sensillum of *Drosophila* showing the outer lymph cavity (olc) and the inner lymph cavity (ilc) with the four chemosensory dendrites (s: sweet, w: water, L1: salt and L2: bitter receptor cells) and their associated gustatory receptors (Grs) or amiloride-sensitive *Pickpocket* (*ppk*) ion channels (Charlu et al., 2013; Chen et al., 2010; Dahanukar et al., 2007; Lacaille et al., 2007; Liu et al., 2003; Meunier et al., 2003; Weiss et al., 2011; Modified from Marion-Poll, 2010, Bitter receptors in *Drosophila*, presentation at Institut de Génomique Humaine, Montpellier, France).

As in mammals, the salt response in insects is thought to be mediated by cation-selective amiloride-sensitive pores. Such pores were found to be coded by the degenerin/epithelial sodium channel genes (DEG/ENaC) Pickpocket11 (ppk11) and Pickpocket19 (ppk19) in *Drosophila* (Liu et al., 2003; Fig. 3.10). In the blowflies, the response to salt was not found to be inhibited by amiloride although the labellar sugar receptor cells were strongly inhibited by 0.5 mM of amiloride (Liscia et al. 1997; Sadakata, 2002). An amiloride-sensitive ppk28 channel belonging to the same DEG/ENaC family is thought to be involved in water sensitivity in *Drosophila* (Fig. 3.10). In the present thesis, the neurophysiological activity of amiloride was investigated on the water and on the sugar sensitive neurones of the labellum of *A. gambiae* (Appendix V). In *Phormia*, evidence for the presence of AQglyP in the membrane of the "water" cell which mediates sensitivity to osmolarity was found. Moreover, it is likely that the transduction pathway of the water receptor cell is calcium dependent in both *Drosophila* and *Phormia* (Meunier et al., 2009; Solari et al., 2010; Fig. 3.12).

Sensory information is often coded in arthropods via two antagonistic sensory neurones. For example, the antagonistic responses of warm and cold thermoreceptor cells on mosquito antennae permit an optimal coding of ambient temperature "by providing an excitatory signal to both increasing or decreasing temperatures" (Gingl et al., 2005). The same phenomenon is observed for hygroreceptors in which wet and a dry cells code for an increase and a decrease in humidity respectively (Tichy and Kallina, 2010) or the carbon dioxide receptor cells of the Haller's organ on the tarsus of ticks (Steullet and Guerin, 1992). Also in contact chemo-sensilla, chemical sensitivity is mediated usually through 2 neurones: activation of the salt, the sugar or the bitter neurone and inhibition of the water receptor cell during stimulation with increasing concentrations of salt, sugar or deterrent products, respectively (Fujishiro et al., 1984; Meunier et al., 2003). When mixed with sugars, bitter compounds or salts may depress the response of the "sugar" cell (Fujishiro et al., 1984; Meunier et al., 2003; Schoonhoven, 1982).

This means that information regarding taste quality is already partially achieved at the periphery. In mosquitoes, information from gustatory-mechano sensilla on the labellar lobes and those on the labrum and cibarium projects through neurones in the maxillary-labial nerve and the frontolabral nerves, respectively, to the subesophageal ganglion (SOG) and then to the tritocerebrum (Ignell and Hansson, 2005). In larger flies, the majority of the input to the SOG comes from labellar sensilla although some direct projections from the tarsi have also been accounted for (Mitchell et al., 1999). Gustatory processing is probably one of the major roles of the SOG as a pair of motor neurones that appear to integrate sweet and bitter input and that serve to mediate the proboscis extension reflex were recently identified in the SOG of *Drosophila* (Gordon and Scott, 2009; Yarmolinsky et al., 2009).

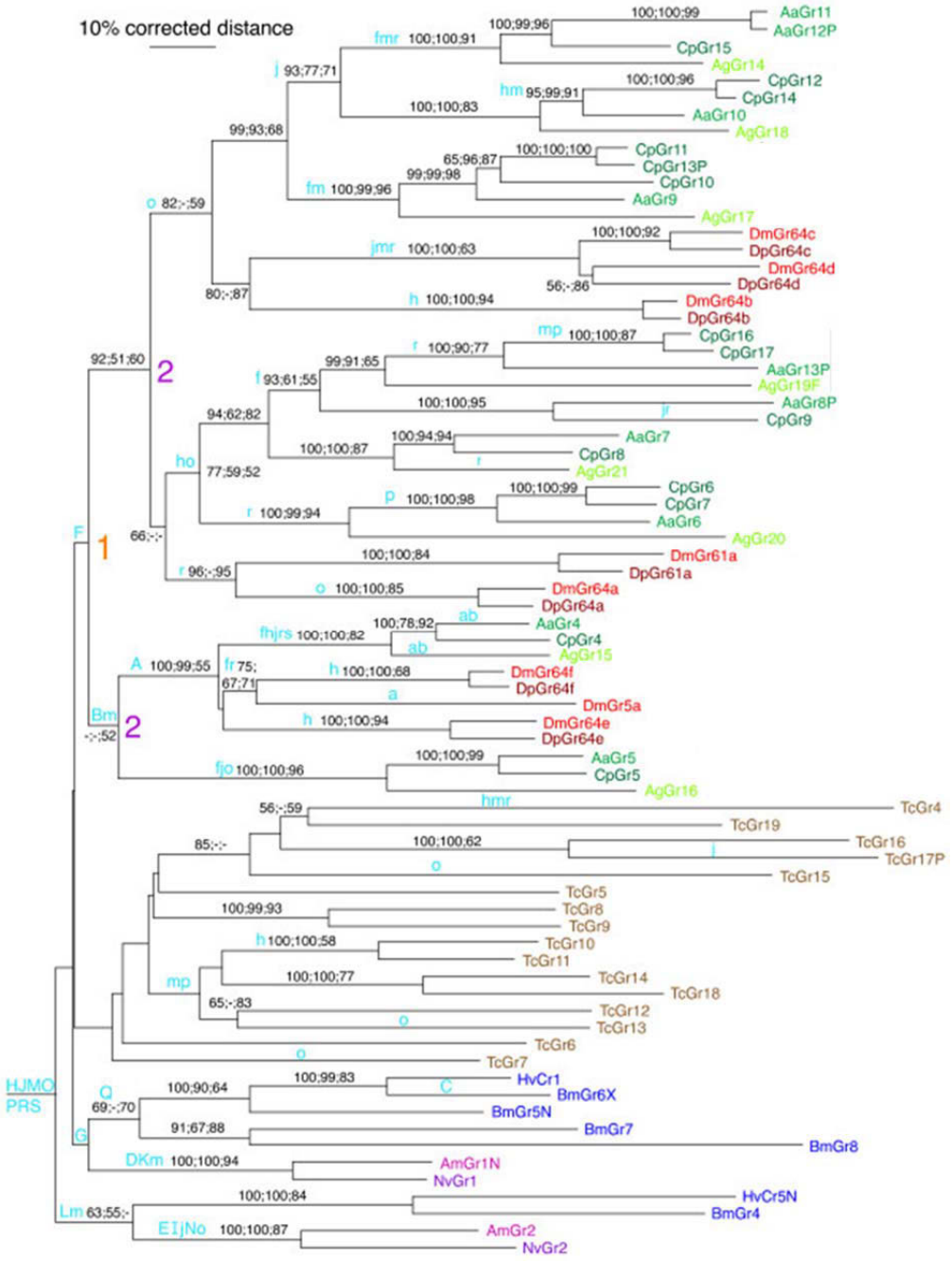

Fig. 3.11: Phylogenetic relationships of sugar receptors in insects. Green: mosquitoes (Aa: *A. aegypti*, Ag: *A. gambiae*, Cp: *C. pipiens*), red: *Drosophila* spp. (Dm: *D. melanogaster*, Dp: *D. pseudoobscura*), blue: *Lepidoptera* (Bm: *Bombix mori*, Hv, *Heliothis virescens*), purple: *Hymenoptera* (Am: *Apis mellifera*, Nv: *Nasonia vitripennis*), brown: *Coleoptera* (Tc: *Tribolium casaneum*). The digits 1 (orange) and 2 (purple) indicate the major tandem duplications hypothesized at the base of the dipteran sugar receptor evolution (modified from Kent and Robertson, 2009, reproduced with permission of the authors).

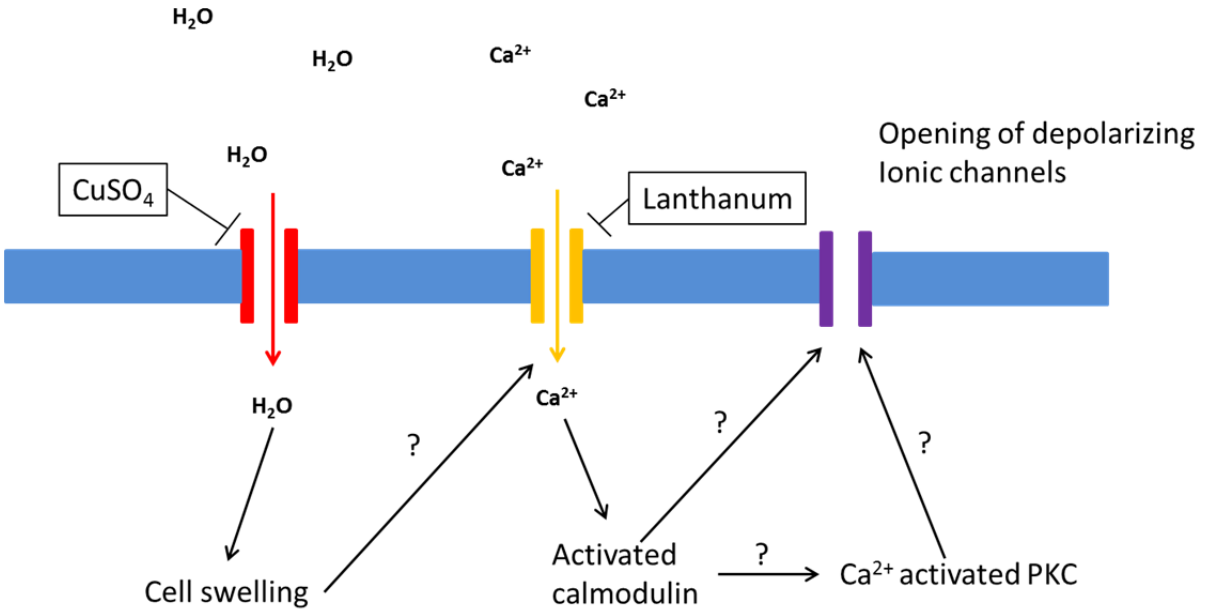

Fig. 3.12: Supposed transduction mechanism in insect water receptor cells. Water or small molecules such as glycerol are thought to cross the membrane (in blue) through aquaglyceroporins (in red) in the blowfly (Solari 2010). This induces opening of calcium channel (in yellow) and entrance of extracellular calcium. Downstream, calmodulin and a protein kinase C (PKC) are likely involved in the opening of depolarizing ion channels (in violet) as suggested for the blowfly and *Drosophila* (Meunier et al., 2009; Solari et al., 2010). $CuSO_4$ is thought to inhibit aquaglyceroporins (Solari et al., 2010; Zelenina et al., 2004) and lanthanum is a broad blocker of calcium-permeable channels (Meunier et al., 2009). Both were utilized in work described in Chapter 5 of this thesis as inhibitors of the water receptor cell of *A. gambiae*.

3.7.3 Contact chemoreception in heamatophagous arthropods

The molecular and cellular architectures of olfactory organs are already well studied in mosquitoes, particularly in the context of host finding (Carey et al., 2010; Lu et al., 2007; Qiu et al., 2006). This makes sense as a major protection tool against mosquitoes focuses on preventing biting by the application of repellents (e.g. DEET, N,N-diethyl-meta-toluamide) that interacts with the mosquito olfactory system and thus disrupts its ability to find its host (DeGennaro et al., 2013). Studies to identify plant semiochemicals attractive for mosquitoes have also been recently undertaken (Gouagna et al., 2010; Nyasembe et al., 2012).

However, despite *A. gambiae* becoming a model for studies on insect olfactory systems, very little is known about taste organs and contact chemoreceptors in this species, despite the importance of this sensory modality in feeding site choice on plants as well on vertebrate hosts. In fact, most literature related to contact chemoreception in heamatophagous insects is relatively old. Few neurophysiological studies have been made on contact chemosensory cells in heamatophagous insects. Sugar and salt sensitive neurons occur on tarsal, labellar and labral sensilla of the winter mosquito *C. inornata* (Pappas and Larsen, 1976). In addition to carbohydrates (sucrose > fructose > glucose = sorbose), the sugar receptor cells on the labellar lobes of the yellow fever mosquito *A. aegypti* respond to high doses of amino acids (Sinitsyna, 1971). Labral apical sensilla contain chemoreceptor cells responding to L-alanine, the C-terminal amino acid of albumin in blood plasma (Werner-Reiss et al., 1999a). In tsetse flies, tarsal receptor cells permit detection of sweat components such as amino acids and uric acid (Van Naters and Den Otter, 1998). Blood platelets are considered to provide a major source of phagostimulants for mosquitoes as their activation causes the release of ADP and ATP (Galun et al., 1993). Indeed, labrum receptor cells show electrophysiological responses to nucleotides in *A. aegypti* (Werner-Reiss et al., 1999b) and such products have an important phagostimulatory effect on feeding in *A. aegypti*, *Culex pipiens* L. and *C. inornata* (Galun et al., 1963; Galun et al., 1988).

Although the feeding dilemma of anautogenous female mosquitoes has been brought to light, namely feed on nectar versus feed on blood, along with the importance of sugar for the maintenance of mosquito population and its influence on vectorial capacity have been demonstrated (Stone, 2011), little is known about mosquito feeding preferences between nectar constituents and their associated nutritive values. Recently feeding choice assays have shown that disaccharides (sucrose > trehalose) are preferred over monosaccharides (fructose > glucose) in *A. aegypti* and that an equimolar mixture of the two monosaccharide constituents of sucrose, glucose and fructose, is as equally appetitive as sucrose (Ignell et al., 2010). The study of Schmidt and Friend (Schmidt and Friend, 1991) on *C. inornata* has also revealed the stronger phagostimulatory effect of di- and trisaccharides, especially

sucrose, over monosaccharides. Amino acids such as alanine, valine, leucine, phenylalanine, threonine and arginine, which can be present in nectar, have also been shown to enhance feeding of *A. aegypti*. However, to our knowledge, nobody has performed neurophysiological recordings on contact chemoreceptor cells of anopheline species and the situation is still unclear with regard to the range of receptor cell types in anopheline species. Likewise, little is known about anopheline feeding preferences on nectar constituents, their associated nutritive values and the impact on the vectorial capacity. Besides contributing to our knowledge on the sensory ecology of the vectors, studying mosquito contact chemoreception could well lead to the identification of neuronal pathways responsible for the detection by mosquitoes of products with low vapour pressures that persist on substrates and which could deter landing and biting responses. Knowledge of contact chemoreception in *A. gambiae* could be fundamental to improve the toxic sugar baits recently developed for controlling populations of this disease vector (Müller et al., 2010).

4. Aims and scope of the thesis research

Chapter 5:

In this chapter, I have studied the feeding response of female *A. gambiae* exposed to the three main sugar nectar constituents, namely sucrose and its two hexose constituents, fructose and glucose tested alone or in combination. For this purpose, mosquitoes were exposed to treated filter papers allowing stimulation of tarsal contact chemo-receptor cells in addition to those of the mouthparts.

To investigate if human bitter tasting products deter sugar feeding in *A. gambiae*, quinine, quinidine, berberine chloride, denatonium benzoate and caffeine were then added to sucrose in the same filter paper feeding-assay.

To investigate whether chemosensilla on the mouthparts alone are sufficient for the detection of sucrose and bitter products, i.e. excluding tarsal chemo-receptors, female *A. gambiae* were fed on sucrose and sucrose + quinine through micro-capillaries.

I describe the ultrastructure of trichoid type 1 (T1) labellar sensilla of *A. gambiae* in this chapter. The response of the sugar and the water sensitive neurones in the lateral No 5 T1 sensillum was studied during stimulation with sucrose, as well as fructose and glucose alone or in combination using the tip recording method (Hodgson et al., 1955). Neurophysiological activities of increasing concentrations of NaCl, KCl, $CuSO_4$, $LaCl_3$ and $CaCl_2$ were studied on the water sensitive neurone using the same method and hypotheses on the action of some of these salts on the transduction pathway were drawn. Electrophysiological recordings with increasing concentrations of denatonium benzoate, quinine, quinidine, berberine chloride and caffeine were performed to investigate the effect of bitter compounds on the sugar and water response of taste neurones in the lateral No 5 T1 sensillum. In addition, recordings with quinine in sucrose or in water were also made on the lateral No 2 T1 sensillum as well on the dorsal No 5 and the ventral No 8 sensilla (Appendix II).

Chapter 6:

In this chapter, the study of the electrophysiological response of labellar receptor cells to sucrose and water has been extended to the other T1 and type 2 (T2) labellar sensilla of female *A. gambiae*.

In addition, 14 sugars as well as glycerol, myo-inositol and the protein thaumatin were tested on T1 sensilla. The structure-activity relationships of the tested sugars are discussed in this chapter.

Feeding preferences between the trisaccharide melezitose, the disaccharide sucrose and the monosaccharides glucose and fructose alone or in combination were evaluated on females

and males *A. gambiae* using a two-choices feeding assay. This assay allows a better discrimination between the phagostimulatory effects of sugars than the no-choice assays described in Chapter 5.

Longevity of mosquitoes maintained on sucrose, glucose, fructose and the mixture of glucose and fructose was measured to evaluate the nutritive value of sugars and the biting response of females maintained on sucrose versus on glucose was measured using the warm body (WB) assay described in Appendix I. As both longevity and biting rate are the most important parameters of vectorial capacity, these experiments allowed me to draw conclusions on the possible action of sugar nectar constituents on the vector competence of *A. gambiae*.

Chapter 7:

In this chapter, a silicone-membrane feeding assay was used to compare the feeding response of female *A. gambiae* exposed to defibrinated bovine blood, saline, a saline + bovine serum albumin (BSA) solution and a saline + BSA + an amino acid (AA) mixture in solution. In addition, the deterrent effects of the two antimalarial drugs quinine (alkaloid) and artesunate (sesquiterpene lactone) was studied on *A. gambiae* feeding on bovine blood and on a saline + BSA solution and the results are discussed in the context of malaria control.

Since only the stylets penetrate the membrane and not the labellar lobes in the membrane-feeding assay, this permitted me to establish if blood phagostimulants and feeding deterrents are detected by internal gustatory organs, namely sensory cells in the apical and subapical labral pegs, in sensilla on the inner face of the labellar lobes or cibarial receptor cells. The neuroanatomy of the apical and subapical labral pegs and of sensilla on the inner face of the labellar lobes of female *A. gambiae* is described in this chapter.

Other achievements of the thesis research:

This thesis contributes to our understanding of gustation in the malaria vector *A. gambiae.* A reliable method to record from *Anopheles* taste receptor cells is described as well as a method to sort spike units (Chapter 5). A complete description of taste sensilla of the proboscis (Chapters 5 and 6, Appendix VII) and of the prothoracic legs (Appendix VIII) was made. Three behavioural assays have been developed to evaluate the feeding response of *A. gambiae* to nectar components. Both the filter paper and the capillary feeding assays permit rapid quantification of the volume ingested from a feeding solution and capillary feeding excludes any tarsal chemosensilla stimulation (Chapter 5). A two-choice assay

permits a more accurate discrimination of *A. gambiae* feeding preferences between solutions (Chapter 6). In addition, results from two *in vitro* bioassays that permit a quantification of the biting (warm body assay, Appendix I) and the blood-feeding (silicone-membrane assay, Chapter 7) responses are presented. As the glass disc used in the warm body assay and the silicone membrane can be readily treated, both assays represent reliable systems to test repellents and deterrents on mosquitoes, in addition to skin attractants and phagostimulants.

5. The sugar meal of the African malaria mosquito *Anopheles gambiae* and how deterrent compounds interfere with it: a behavioural and neurophysiological study

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RESEARCH ARTICLE

The sugar meal of the African malaria mosquito *Anopheles gambiae* and how deterrent compounds interfere with it: a behavioural and neurophysiological study

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SUMMARY

In this study, we show that female African malaria mosquitoes *Anopheles gambiae* starved for 3–5 h start to engorge on sucrose at concentrations between 50 and 75 mmol l^{-1}. Half of the feeding response (ED_{50}) is reached at 111 mmol l^{-1} and the maximum response (0.4 mg) occurs at 250 mmol l^{-1}. Two receptor cells in a trichoid sensillum of the labellum, called the 'sucrose' and 'water' neurones, are activated by sucrose and water, respectively. The electrophysiological response of the sucrose receptor cell starts well below the level of sugar necessary to induce engorgement. The sugar receptor cell is most sensitive to small increments in sucrose concentration up to 10 mmol l^{-1} with a response plateau from 25 mmol l^{-1}. Fructose has a mild phagostimulatory effect on *A. gambiae*, whereas no significant differences in meal sizes between water and glucose were found. However, when 146 mmol l^{-1} fructose plus glucose are mixed, the same engorgement as on 146 mmol l^{-1} sucrose is observed. Likewise, even though the sucrose receptor cell is not activated by either fructose or glucose alone, equimolar solutions of fructose plus glucose activate the neurone. We conclude that there is a behavioural and neurophysiological synergism between fructose and glucose, the two hexose sugars of sucrose. We show that some bitter-tasting products for humans have a deterrent effect on feeding in *A. gambiae*. When 1 mmol l^{-1} quinidine, quinine or denatonium benzoate is added to 146 mmol l^{-1} sucrose, feeding is almost totally inhibited. The effect of berberine is lower and no significant inhibition on engorgement occurs for caffeine. The deterrent effect depends on the concentration for both quinine and quinidine. Capillary feeding experiments show that contact chemosensilla on the mouthparts are sufficient for the detection of sucrose and bitter products. The feeding assay findings with deterrents correlate with the neurophysiological responses of the sucrose and water labellar neurones, which are both inhibited by the bitter compounds denatonium benzoate, quinine and berberine between 0.01 and 1 mmol l^{-1}, but not by the same concentrations of caffeine. In conclusion, sucrose stimulates feeding and activates the labellar sucrose neurone, whereas feeding deterrents inhibit both the sucrose and water neurones. This study provides an initial understanding of the physiological mechanisms involved in sugar feeding in *A. gambiae* and shows how some bitter products interfere with it.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/216/7/1292/DC1

Key words: *Anopheles gambiae*, malaria mosquito, insect gustation, taste neurone, nectar feeding, feeding deterrent.

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INTRODUCTION

Plant-derived sugar is the basic food of adult mosquitoes (Foster, 1995), usually including sucrose, which is also the most common sugar in nectar, along with fructose and glucose (Wykes, 1952; Baker and Baker, 1982; Van Handel, 1984; Petanidou and Lamborn, 2005; Manda et al., 2007b). Carbohydrates are consumed by males to supplement the energy required for their intense swarming (Yuval et al., 1994; Gary et al., 2009). Females of anautogenous mosquito spp. feed on sugar to provide energy for flight and increase longevity, but need blood to produce eggs (Gary and Foster, 2001). The major source of sugar is in floral nectaries, although mosquitoes also feed on extrafloral nectaries, honeydew, tree sap, damaged fruits and leaves or discarded plant material like sugar-cane waste (Foster, 1995). Glucose can also be found in vertebrate blood, although its calorific content is negligible compared with that of blood proteins (Foster, 1995). In the field, mosquitoes are generally observed to feed on flowers in full bloom that are abundant, demonstrating opportunism (Magnarelli, 1979). However, in the African malaria mosquito *Anopheles gambiae* (Giles), some clear preferences exist for particular plants available in the vicinity of human hosts or oviposition sites (Manda et al., 2007b). To date, the physiological basis in *A. gambiae* for discriminating between plant species is still unknown. At medium to long range, plant volatiles probably inform mosquitoes of the presence of a sugar source (Foster and Takken, 2004) as well as those of unsuitable plants, as shown by the strong repellent activity of some plant extracts against female *A. gambiae* searching for a host (Odalo et al., 2005). After landing on the plant, mosquitoes use terminal pore sensilla on their legs and mouthparts to find the nectar source (Pappas and Larsen, 1978) and probably to taste meal quality, such as the sugar concentration or the presence of toxic and unpalatable substances. Behavioural avoidance of noxious allelochemicals is a mechanism of defence developed by insects against plant toxins. Deterrence (feeding inhibition) by bitter-tasting plant secondary metabolites such as alkaloids is known in phytophagous insects such as the moth *Manduca sexta* (Glendinning, 2002), in the scavenger fly *Phormia regina* (Dethier and Bowdan, 1992) and in the fruitfly *Drosphila melanogaster* (Meunier et al., 2003). Recently, it has been shown that the alkaloids quinine and caffeine affect the sucrose feeding response of *Aedes aegypti* (Ignell et al.,

2010). Alkaloids act by activation of a taste neurone called the 'deterrent receptor cell' or *via* inhibition of a receptor cell that responds to phagostimulants (Schoonhoven, 1982).

In recent years, important progress has been made in the understanding of the cellular basis and role of gustatory receptors (Grs) involved in sweet and bitter sensing in *Drosophila* (Dahanukar et al., 2001; Moon et al., 2006; Dahanukar et al., 2007; Jiao et al., 2007; Slone et al., 2007; Weiss et al., 2011). A large family of putative Grs has been identified in the genome of *A. gambiae* (Hill et al., 2002) and the projection patterns of gustatory neurones have been described in detail in the suboesophageal ganglion and the tritocerebrum of this species (Ignell and Hansson, 2005). However, despite the potential importance of deterrent compounds in controlling arthropod-borne diseases, relatively little is known about taste in haematophagous insects. Here we describe feeding experiments with sucrose and its constituents fructose and glucose in *A. gambiae*, and provide insight into sugar perception in this species from electrophysiological recordings performed on a labellar trichoid sensillum containing sucrose- and water-sensitive gustatory receptor cells. We then present the feeding inhibition effects of denatonium benzoate, some quinoline (quinine and quinidine) and isoquinoline (berberine) alkaloids added to sucrose, and account for the inhibitory effect of these compounds on the labellar sucrose- and water-sensitive chemoreceptor cells.

MATERIALS AND METHODS

Anopheles gambiae colony

The *A. gambiae* colony (16cSS strain, derived in 1974 from wild-caught adults originating from Lagos, Nigeria, West Africa) was maintained in a climate chamber [28°C, 80% relative humidity (RH)] under a 12 h:12 h light:dark cycle with 2 h simulated sunrise and sunset. Females fed on a guinea-pig once a week and eggs were recovered on wet filter paper. About 275 larvae were reared in trays in distilled water and fed with pulverized Tetramin fish food. This density provides optimal nutrition for larvae, synchronous eclosion and adults of homogeneous size (Timmermann and Briegel, 1993). Nine to 10 days after oviposition about 800 adult mosquitoes emerged into a rearing cage (350×350×550 mm high) and were provided with 10% (w/v) sucrose and water *ad libitum*.

Test chemicals

D-(–)-fructose, D-(+)-glucose monohydrate, D-(+)-sucrose, berberine chloride, caffeine, quinine anhydrous, denatonium benzoate, NaCl, $CuSO_4$ and $LaCl_3$ were purchased from Sigma-Aldrich (Buchs, Switzerland), (+)-quinidine from Alfa Aesar (Karlsruhe, Germany) and KCl and $CaCl_2$ from Merck (Darmstadt, Germany). Puritiy of all products was ≥98%. Solutions were kept at 10°C, for less than 1 week.

Feeding assays on filter paper

Experiments were performed in a walk-in climate chamber (25°C, 80% RH) during the last 5 h of the scotophase. Four- to six-day-old female *A. gambiae* were individually deprived of water and sugar for 3–5 h in small plastic tubes (15 mm long, 15 mm diameter) closed with two perforated plastic stoppers (diameter 7 mm) with the aperture covered by stainless-steel netting (mesh size: 0.4 mm) to facilitate exchange of air kept in boxes at 95% RH. After this conditioning, the tubes were weighed using a M3 Microbalance (reading precision of ±1 µg; Mettler, Greifensee, Switzerland). For this procedure, the mean standard deviation of weighing five different empty plastic tubes five times was 1 s.d.=2.64 µg. The mosquitoes were then transferred into glass vials (40 mm high, 20 mm diameter) providing adequate space for feeding. Vials were opened and upturned for 30 min on filter paper (55 mm diameter, no. 10 311 807, Whatman Schleicher and Shuell, Dassel, Germany) treated with 600 µl of a test solution in a Petri dish (modified from Arsic and Guerin, 2008). During feeding, a beige plastic container was upturned on the Petri dishes to avoid visual stimuli. Light did not exceed 1 lx under the container. After the feeding experiment, mosquitoes were individually anaesthetized with CO_2 and transferred into the initial plastic tubes and weighed again. The amount of solution ingested was established as the difference in weight before and after feeding. The time between weighings was 60–90 min. Four types of experiments were performed using this bioassay. Firstly, to establish the sucrose level that induces feeding by female *A. gambiae*, solutions of sucrose from 1 to 250 mmol l^{-1} were dissolved in nanopure water. In a second experiment, to measure any phagostimulatory effect of the two hexose sugars that make up sucrose, fructose and glucose were tested individually at 146 and 292 mmol l^{-1}, mixtures of fructose and glucose at 73 mmol l^{-1} each (the same molarity as in 146 mmol l^{-1} sucrose) and at 146 mmol l^{-1} each (i.e. the same mass of glucose and fructose that constitutes 146 mmol l^{-1} sucrose), 146 mmol l^{-1} sucrose (5% w/v) as a positive control and nanopure water as a negative control. To test for feeding inhibition by bitter products, 146 mmol l^{-1} sucrose mixed with solutions of 1 mmol l^{-1} caffeine, berberine, quinine, quinidine, denatonium benzoate and 2 mmol l^{-1} berberine were compared with 146 mmol l^{-1} sucrose as control. In a fourth set of experiments, feeding was quantified for quinine and quinidine at concentrations from 0.01 to 1 mmol l^{-1} in 146 mmol l^{-1} sucrose. Solutions were tested in a random order.

Feeding assays with glass capillaries

Feeding assays were performed under the same environmental conditions as described above from glass capillaries with 4- to 6-day-old female *A. gambiae* at low light (7 lx) to establish if surrounding the mouthparts alone, i.e. excluding tarsal chemosensilla, was sufficient to induce feeding and feeding inhibition by sucrose and quinine presented in sucrose. The mosquitoes were rendered quiescent on ice, and attached by the wings on a glass slide using double-sided adhesive tape 3–7 h before the start of the experiments. During this conditioning period mosquitoes had no access to sucrose or water but were kept in boxes at 95% RH. For these feeding assays, 5 µl glass capillaries (no. 7087 07, Blaubrand, Wertheim, Germany), previously shortened to remove the proximal part without calibration marks, were pulled with an Ealing vertical micropipette puller (no. 50-2012, Harvard Apparatus, Edenbridge, Kent, UK) into two 40 mm segments with a 180 µm internal diameter tip to accommodate the proboscis. Mosquitoes were allowed to feed for 5 min on solutions of 146 mmol l^{-1} sucrose or 146 mmol l^{-1} sucrose + 1 mmol l^{-1} quinine in nanopure water. A blue food colorant (E131) was added to each solution (five drops of 30 mg to 10 ml) to permit visualization of the liquid levels in capillaries. Liquid levels were recorded as images with a scanner (Scanjet 4570c, Hewlett-Packard, Dübendorf, Switzerland) at 600 d.p.i. before and after experiments, and fluid consumption was measured using ImageJ version 1.44 (Rasband, 2011) as described in Sellier et al. (Sellier et al., 2011) using the 1 µl marks on the capillaries as references. To control for possible evaporation within the capillary, the level of liquid in a capillary filled with 146 mmol l^{-1} sucrose solution and manipulated exactly as the test capillaries was likewise measured.

Ultrastructure of trichoid T1 sensilla of the labellar lobes

For transmission electron microscopy, the probosces of *A. gambiae* were fixed in Karnovsky fixative (pH 7.5) overnight at 4°C and rinsed three times in 0.2 mol l^{-1} sodium cacodylate buffer with 4% sucrose. After post-fixation in 1% OsO_4 for 2 h and rinsing in the same buffer, the specimens were stained in block with 2% uranyl acetate (pH 4) in 15% ethanol for 30 min at room temperature, rinsed in 15% ethanol and dehydrated through a graded series of acetone and embedded in Spurr's resin. Ultrathin sections were obtained on a Reichert Ultracut S microtome (Leica, Vienna, Austria) and stained with uranyl acetate and lead citrate. Serial sections were collected every 1 µm from the tip of the first distal trichoid sensillum up to 60 µm of the proboscis from six female and two male *A. gambiae* and were examined in a Philips CM 100 electron microscope (Philips Electron Optics, Eindhoven, The Netherlands) at 60 kV. For scanning electron microscopy, heads of *A. gambiae* were mounted on stubs with Leit-tabs (Plano, Wetzlar, Germany), air-dried for 1 week and coated with a layer of gold in a Sputter coater (Baltec SCD 005; Oerlikon Balzers, Balzers, Lichtenstein). Specimens were examined at 10 kV using a Philips XL 30 scanning electron microscope.

Electrophysiology

Electrophysiological responses of chemoreceptor cells in a trichoid sensillum on the labellum of female *A. gambiae* (Fig. 6A, arrow) were recorded to test stimuli. After being rendered quiescent on ice, mosquitoes were attached by the back on plasticine with two curved tungsten wires, surrounded by moist cotton. After removing the maxillary palps, the mouthparts were fixed with double-sided adhesive tape on a Plexiglas stub. Contact chemosensilla on the mosquito mouthparts were visualized under a microscope (BX51WXIF, Olympus Schweiz, Volketswil, Switzerland) at a magnification of 1000.

Imagoes that had emerged the same morning (0-day-old) were used for electrophysiological recordings when not otherwise specified. Using the method first described by Hodgson et al. (Hodgson et al., 1955), sensilla were stimulated with a recording borosilicate electrode (33 mm long, 14 µm tip diameter) containing the test products diluted in nanopure water. A borosilicate electrode of the same size, mounted on plasticine, filled with haemolymph Ringer solution (Kaissling, 1995), grounded *via* a chlorinated silver wire and inserted into the mosquito head acted as a reference electrode. The recording electrode was connected *via* a chlorinated silver wire to a high impedance 'non-blocking' pre-amplifier (Taste Probe, Syntech, Hilversum, The Netherlands) (Marion-Poll and van der Pers, 1996) mounted on a hydraulic micromanipulator (M0-103, Narishige Scientific Instrument Laboratory, Tokyo, Japan). The electrophysiological signal was amplified ten times with an amplifier in conjunction with a 100–3000 Hz band-pass filter provided in an IDAC-4 intelligent data acquisition controller (Syntech). AC signals were recorded via the IDAC-4 analogue to digital card to a PC equipped with the spike analysis software AutoSpike (version 3.9; Syntech) at a 10 kHz sampling rate. Most concentrations of test products were tested two consecutive times for 2.5–6.5 s at intervals of approximately 2 s. An analysed signal was always the first to be recorded. In cases where a problem occurred during first stimulation (e.g. low signal/noise ratio, insect movement), spikes were quantified from a subsequent stimulation. A delay of 2–5 min was respected between each solution tested

At first, to evaluate the inhibitory effect of salts on the neurophysiological response to water, concentrations from 0.01 to 100 mmol l^{-1} KCl and NaCl, from 0.01 to 1 mmol l^{-1} $CuSO_4$, from 0.01 to 10 mmol l^{-1} $CaCl_2$ and from 0.1 to 1000 µmol l^{-1} $LaCl_3$ in nanopure water were tested in increasing concentration on the sensillum. Each stimulation series started and ended with nanopure water as controls. To check for the presence of a 'sucrose receptor cell', concentrations from 0.1 to 292 mmol l^{-1} sucrose were tested in increasing order in 10 mmol l^{-1} KCl, i.e. at a KCl concentration that inhibits the neurophysiological response to water. Then concentrations from 0 to 100 mmol l^{-1} sucrose in pure water were tested in increasing order to check for the presence of a 'sucrose receptor cell' different from the 'water receptor cell'. This was repeated with 5-day-old mosquitoes, an age that corresponds to the one used in the feeding assays. To study the sugar receptor site(s), fructose and glucose were tested separately at 146 and 292 mmol l^{-1}, mixtures of fructose and glucose (each at 73 mmol l^{-1} and 146 mmol l^{-1}), and compared with 146 and 292 mmol l^{-1} sucrose in 10 mmol l^{-1} KCl as positive controls, and 10 mmol l^{-1} KCl alone as a negative control. The action of bitter products on the responses to water and sucrose by the receptor cells within the trichoid sensillum was investigated by stimulating with concentrations from 0.01 to 1 mmol l^{-1} denatonium benzoate, quinine, berberine or caffeine diluted in nanopure water and in 146 mmol l^{-1} sucrose plus 10 mmol l^{-1} KCl. Each stimulation series started and ended with nanopure water or 146 mmol l^{-1} sucrose in 10 mmol l^{-1} KCl as controls.

Spike discrimination

Spikes were quantified during the 2 s following stimulation. In cases where receptor cells were activated with a delay, spikes were quantified during the 2 s following activation of receptor cells or from a subsequent stimulation showing no delay. All products tested affected receptor cell responses over 2 s. Spikes were discriminated on the basis of amplitudes and shapes using the interactive AutoSpike procedures. Spike amplitudes were sorted using DataView 8.0.0 software (Heitler, 1999) omitting the first 30 ms to avoid the contact artifact. Moreover, to confirm the activation of more than one receptor cell, all recordings were visually inspected for doublets (as presented in Hiroi et al., 2004). Since spike amplitude varies in parallel with frequency in labellar chemosensory cells of *A. gambiae* as in *Drosophila* (Fujishiro et al., 1984), a careful analysis of several recordings was made for different concentrations of products on the same sensillum of the same mosquito (see Results) for the identification of active sensory units.

Statistical analyses

All statistical analyses and graphical representations of data were performed with R 2.11.1 (R Development Core Team, 2010). A logistic model of the type:

$$\text{Meal size} = \text{max}/\left(1+\text{e}\left(\frac{\text{xmid}-\text{conc}}{\text{scal}}\right)\right) \quad (1)$$

was fitted to the meal size data from female *A. gambiae* exposed on filter papers treated with different concentrations of sucrose. In this model max is the maximum response obtained (the horizontal asymptote), xmid is the inflexion point showing the concentration of sucrose at which the meal size is theoretically half of a complete meal (ED_{50}), conc is the sucrose concentration and scal is a numeric scale parameter that is equal to max/(4×slope) at the inflection point of the model.

To establish differences between sugar phagostimulatory effects, the meal sizes of female *A. gambiae* exposed to filter papers treated with sucrose, fructose, glucose and the two mixtures of fructose and glucose were analysed using one-way ANOVA on the ranked values

(discrete data; normal distribution, Shapiro–Wilk test, $P>0.05$; homogeneous variance, Bartlett's test, $P>0.05$). The model was simplified by aggregating non-significant factor levels in a stepwise *a posteriori* procedure. Similar analyses were performed on the meal sizes of female *A. gambiae* exposed to filter papers treated with 146 mmol l^{-1} sucrose and 146 mmol l^{-1} sucrose with bitter products added. To analyse feeding inhibition by quinine and quinidine as a function of concentration and to check for possible interactions between products and concentrations, an ANCOVA based on a linear model was made on the ranked meal sizes (continuous data; normal distribution, Shapiro–Wilk test, $P>0.05$; homogeneous variance, Bartlett's test, $P>0.05$) of female *A. gambiae* exposed to different concentrations of quinine and quinidine added to 146 mmol l^{-1} sucrose. In the feeding assay with glass capillaries, meal sizes (normal distribution, Shapiro–Wilk test, $P>0.05$; homogeneous variance, Bartlett's test, $P>0.05$) of females exposed to 146 mmol l^{-1} sucrose and 146 mmol l^{-1} sucrose with 1 mmol l^{-1} quinine added were compared using the *t*-test.

To study the inhibition of the neurophysiological response to water by salts, the mean of the total spike numbers per 2 s (MTNoS, normal distribution, Shapiro–Wilk test, $P>0.05$, but with inhomogeneous variance, Bartlett's test, $P<0.05$) generated by the water receptor cell for the different concentrations tested were compared with the first water control using Welch's *t*-test. A two-parameter asymptotic exponential model of the type:

$$N \text{ spikes} = \max\,(1 - e^{(-c \times \text{conc})}), \quad (2)$$

was found to be the most suitable to account for the number of action potentials generated by the sucrose-sensitive receptor cell per 2 s to increasing concentrations of sucrose in 10 mmol l^{-1} KCl and water. In this model, max is the maximum response obtained (the horizontal asymptote), *c* is the rate constant and conc is the sucrose concentration. The spike numbers recorded per 2 s for the different fructose and glucose mixtures and sucrose solutions in 10 mmol l^{-1} KCl tested (discrete data; normal distribution, Shapiro–Wilk test, $P>0.05$; homogeneous variance, Bartlett's test, $P>0.05$) were compared by a one-way ANOVA. To analyse sensory inhibition by bitter products and to check for interactions between products and concentrations on sensory cell responses, an ANCOVA based on a linear model (continuous data; normal distribution, Shapiro–Wilk test, $P>0.05$; homogeneous variance, Bartlett's test, $P>0.05$) was applied to the number of action potentials per 2 s as a function of the $\log_{10}$ of the concentration of bitter products added to nanopure water or to 146 mmol l^{-1} sucrose in 10 mmol l^{-1} KCl. The bracketing controls were excluded from the analysis to comply with the requirement of using continuous variables in the linear model employed. A 95% confidence interval level was used for all analyses.

RESULTS

Feeding assays on filter paper

Feeding responses to increasing concentrations of sucrose

The calculated model indicates that starved 4- to 6-day-old female *A. gambiae* start to feed at concentrations between 50 and 75 mmol l^{-1} with meal size estimated at 0.045 mg at 50 mmol l^{-1}, and 0.088 mg at 75 mmol l^{-1} sucrose (Fig. 1). At 250 mmol l^{-1}, mosquitoes engorge 0.376 mg of sucrose solution, close to the asymptote of the model (0.378 mg), an amount corresponding to 30% of their weight prior to feeding. ED_{50} is calculated from the model to occur at 111.09 mmol l^{-1} sucrose with a meal size of 0.19 mg. At 1 and 10 mmol l^{-1} sucrose, mosquitoes imbibe almost nothing (0.01 mg), comparable to the water control [median meal size (MMS), –0.007 mg]. Zero-day-old females can discriminate between water and 292 mmol l^{-1} sucrose like the 4- to 6-day-old females in our assay (data not presented).

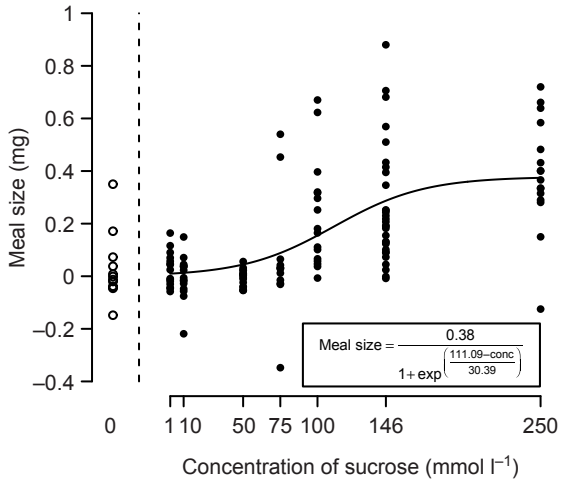

Fig. 1. Logistic model of meal size in female *A. gambiae* following 30 min exposure on filter paper treated with different concentrations of sucrose (conc in the equation). At concentrations of sucrose higher than 250 mmol l^{-1}, meal sizes tend to 0.38 mg and the inflexion point of the model is situated at 111.09 mmol l^{-1}. Water (without sucrose, open circles on left) was not included in the model. Between 12 and 30 mosquitoes were tested per concentration.

Feeding responses to fructose and glucose

Following ANOVA on the ranked meal sizes on fructose, glucose, their binary mixtures and sucrose followed by a stepwise aggregation of non-significant factor levels, three significant contrasts are retained in the final model ($F_{2,159}=35.09$, $P<0.001$, Fig. 2). Meal sizes on water (MMS, –0.021 mg), on 146 or 292 mmol l^{-1} glucose (MMS, –0.0025 and 0.027 mg, respectively) and on 73 mmol l^{-1} glucose plus fructose (MMS, –0.0135 mg) are low and not significantly different. While there are no significant differences between 146 and 292 mmol l^{-1} fructose (MMS, 0.046 and 0.0505, respectively), fructose appears to have a mild phagostimulatory effect on female *A. gambiae* as meal sizes are significantly higher than on water, on 146 and 292 mmol l^{-1} glucose or on 73 mmol l^{-1} glucose plus 73 mmol l^{-1} fructose ($P=0.002$), but lower than on 146 mmol l^{-1} glucose plus 146 mmol l^{-1} fructose and on 146 mmol l^{-1} sucrose ($P<0.001$). The mixture of 146 mmol l^{-1} glucose plus 146 mmol l^{-1} fructose (MMS, 0.133 mg) and 146 mmol l^{-1} sucrose alone (MMS, 0.137 mg) have a similarly strong phagostimulatory effect on starved female *A. gambiae*.

Inhibitory effect of bitter products on the sucrose-feeding response

Except for caffeine, all bitter products tested significantly reduced feeding in *A. gambiae* when added to a solution of 146 mmol l^{-1} sucrose (Fig. 3). Following ANOVA on the ranked meal sizes followed by a stepwise aggregation of non-significant factor levels, three significant contrasts were retained in the final model ($F_{2,156}=101.4$, $P<0.001$). When 1 mmol l^{-1} caffeine is added to 146 mmol l^{-1} sucrose the meal size of starved female *A. gambiae* is not significantly reduced (MMS, 0.184 mg) compared with 146 mmol l^{-1} sucrose alone (MMS, 0.204 mg). Although no significant difference in meal size was found between 1 mmol l^{-1} berberine (MMS, 0.121 mg) and 2 mmol l^{-1} berberine (MMS,

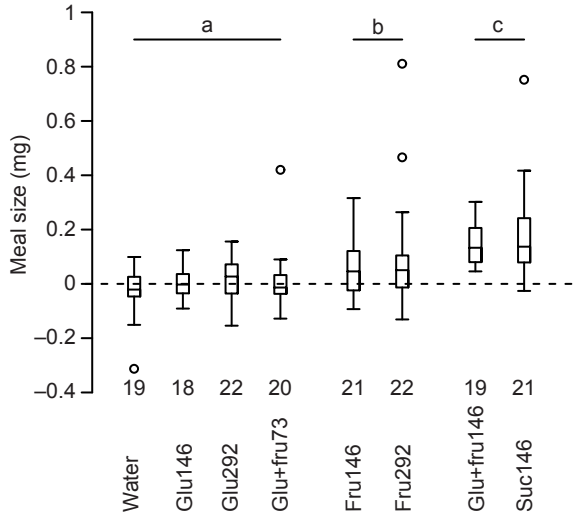

Fig. 2. Meal size of female *A. gambiae* after 30 min exposure on filter paper treated with either water, 146 mmol l^{-1} glucose (Glu146), 292 mmol l^{-1} glucose (Glu292), 73 mmol l^{-1} glucose plus 73 mmol l^{-1} fructose (Glu+fru73), 146 mmol l^{-1} fructose (Fru146), 292 mmol l^{-1} fructose (Fru292), 146 mmol l^{-1} glucose plus 146 mmol l^{-1} fructose (Glu+fru146), and 146 mmol l^{-1} sucrose (Suc146). Groups of products and their mixtures with different letters have a significantly different effect on meal size with a 95% confidence level (ANOVA on the ranked meal sizes; non-significant factor levels were aggregated using a stepwise *a posteriori* procedure). The number of mosquitoes tested is indicated under each box plot. Box plots represent the median (black bars), the 25–75% interquartile range (IQR) intervals (boxes), the lowest and the highest data points still within 1.5 of the IQR (whiskers) and outliers (circles).

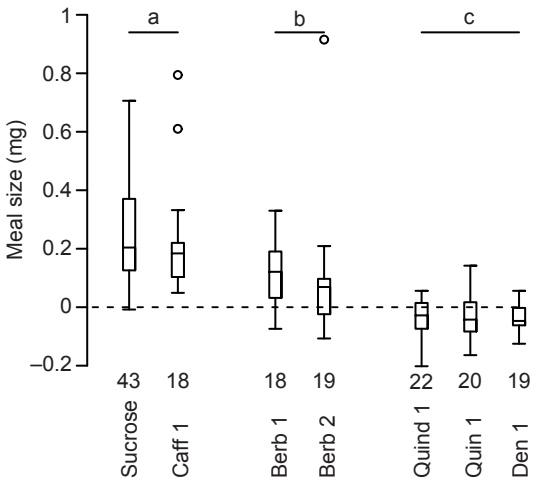

Fig. 3. Meal size of female *A. gambiae* after 30 min exposure on filter paper treated with 146 mmol l^{-1} sucrose and 146 mmol l^{-1} sucrose with 1 mmol l^{-1} caffeine (Caff), 1 mmol l^{-1} berberine (Berb 1), 1 mmol l^{-1} quinidine (Quind), 1 mmol l^{-1} quinine (Quin), 1 mmol l^{-1} denatonium benzoate (Den) or 2 mmol l^{-1} berberine (Berb 2) added. Groups of products with different letters have a significantly different effect on the meal size with a 95% confidence level (ANOVA on the ranked meal sizes; non-significant factor levels were aggregated using a stepwise *a posteriori* procedure). The number of mosquitoes tested is indicated under each box plot. For explanation of box plots, see legend to Fig. 1.

0.069 mg), both concentrations of berberine have an intermediate deterrent effect, with meal sizes significantly lower than 1 mmol l^{-1} caffeine in sucrose and 146 mmol l^{-1} sucrose alone (P<0.001), but significantly higher than 1 mmol l^{-1} quinidine, quinine and denatonium benzoate (MMS, –0.028, –0.0425 and –0.047 mg, respectively, P<0.001) all of which almost completely suppress feeding on 146 mmol l^{-1} sucrose. Although the deterrent effect of both quinidine and quinine clearly depends on concentration (Fig. 4), no significant interactions and no significant differences in the intercepts of the linear equations describing the responses occur between these two products ($F_{3,115}$=40.85, R^2=0.5, P<0.001). Meal sizes on water are not significantly different from meal sizes of mosquitoes exposed to 5% sucrose mixed with 1 mmol l^{-1} quinine, quinidine and denatonium benzoate (ANOVA on the ranked values, P=0.296), allowing us to conclude that mosquitoes do not significantly lose more liquid by diuresis or regurgitation in the presence of the bitter compounds tested.

Feeding assays with glass capillaries

Five minutes are sufficient for starved female *A. gambiae* to engorge on 146 mmol l^{-1} sucrose from glass capillaries (mean ingested volume 0.34 µl; Fig. 5), a level approaching that recorded on filter paper. Quinine at 1 mmol l^{-1} significantly reduced the volume ingested by female *A. gambiae* when added to 146 mmol l^{-1} sucrose (mean ingested volume: 0.15 µl, t=3.13, d.f.=28, P=0.004; Fig. 5). The mean level of evaporated liquid from capillaries was negligible (0.029 µl). As tarsal contact chemosensilla were not in contact with the solution, this indicates that contact chemosensilla either on the mouthparts or in the cibarium are involved in the perception of sucrose and alkaloids. For this reason the electrophysiological recordings described below focused on mouthpart sensilla.

Ultrastructure of trichoid T1 sensilla of the labellar lobes

Long trichoid sensilla of between 30 and 35 µm occur on the external surface of the labellar lobes of both male and female *A. gambiae* (Fig. 6A). These are typical gustatory sensilla with a terminal pore (Fig. 6B) and two lymphatic spaces divided by a cuticular wall (Fig. 6C). The inner receptor lymph cavity (Fig. 6C) contains four chemosensory dendrites and the outer lymph cavity contains an electron-dense fluid (Fig. 6C). In all, five neurones innervate each sensillum (Fig. 6D). One dendrite terminates in a tubular body (Fig. 6D) at the base of the hair, while the other four extend into the inner lymph cavity to the tip of the hair close to the terminal pore (Fig. 6C). At the base, the five outer dendritic segments are surrounded by a sheath, secreted by the thecogen cell, which ends below the cuticle of the socket (Fig. 6D). Further down, the three enveloping cells (thecogen, trichogen and tormogen) encase the five ciliary roots (with 9×2+0 microtubules) and the inner dendritic parts of the sensory cells (Fig. 6E). The morphology of the trichoid sensillum described here on the labellum of female *A. gambiae* appears to be very similar to the long labellar trichoid type 1 sensilla (T1) described by Pappas and Larsen (Pappas and Larsen, 1976) in *Culiseta inornata* (Williston) and those described by Owen (Owen, 1971) and Hill and Smith (Hill and Smith, 1999) in *Anopheles atroparvus* (Van Thiel) and *A. aegypti*, respectively. About 10 T1 sensilla occur on the dorsal and ventral sides of each labellar lobe of female and male *A. gambiae* and no differences have been found between the sexes in either the placement or morphology of T1 sensilla in this species. One dendrite appears to be larger than the other three (measured midway along four different T1 sensilla of three females; Fig. 6C).

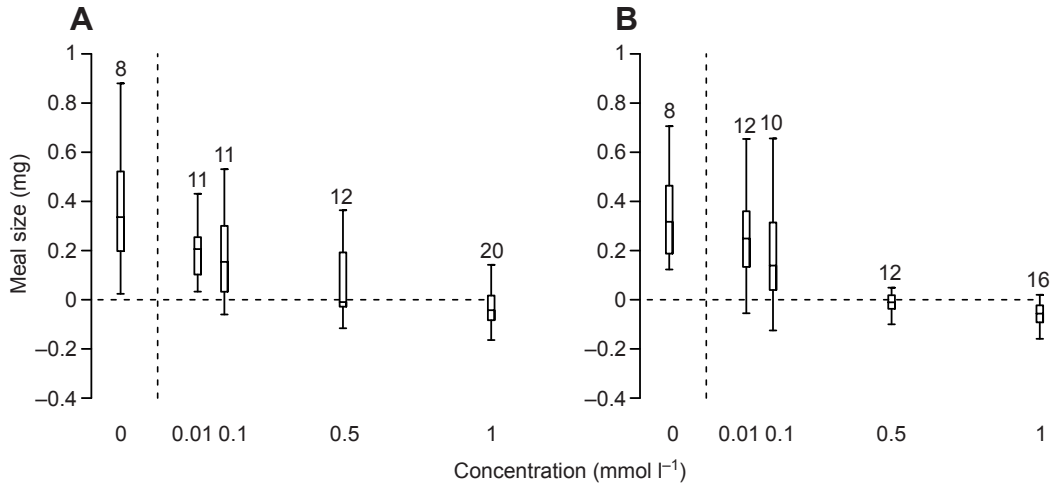

Fig. 4. Concentration–response effects on meal size in female *A. gambiae* following 30 min exposure to filter paper treated with different concentrations of quinine (A) and quinidine (B) in 146 mmol l^{-1} sucrose. The number of mosquitoes tested is indicated above each box plot. Box plots on the left of A and B represent meal sizes on 146 mmol l^{-1} sucrose without an alkaloid. For explanation of box plots, see legend to Fig. 1.

Electrophysiology

Electrophysiological responses to increasing concentrations of salts

When the trichoid sensillum (indicated in Fig. 6A) is stimulated with nanopure water, one receptor cell is activated at a mean frequency of 64.12±1.97 spikes per 2 s. However, this receptor cell is inhibited by increasing concentrations of salts, with $CuSO_4$ and $LaCl_3$ being the most inhibitory (Figs 7, 8). Inhibition is already significant in comparison with the water control at 0.5 mmol l^{-1} $CuSO_4$ (P<0.001) where the mean total number of spikes per 2 s (MTNoS) is reduced by 60% (Fig. 7D). $LaCl_3$ significantly inhibits the water response at 1 mmol l^{-1} (P<0.001) with a drop of 92% in the MTNoS recorded compared with the first water control (Fig. 7F). The inhibitory effects of 1 mmol l^{-1} KCl, NaCl or $CaCl_2$ are lower, with a drop in the MTNoS of 40, 50 and 59%, respectively, but significantly different from the water control (P=0.002, 0.002 and 0.02, respectively; Figs 7, 8). The increase (six spikes) in the MTNoS observed between 0 and 0.1 mmol l^{-1} $CaCl_2$ is not significant (P=0.525). Between 10 and 100 mmol l^{-1} KCl and NaCl the MTNoS increases slightly (10 and 15 per 2 s, respectively; Fig. 7) due to the appearance of predominantly negative-going spikes at these levels of salts (Fig. 7C; Fig. 8A,B). Such spikes could arise as an artifact at high salt levels or represent activation of another cell. All spikes quantified in these experiments could be readily discriminated from the spikes of the mechanoreceptor cell, which are readily visualized at a lower amplitude when the sensillum is pulled proximally (Fig. 8C). In 50% of the mosquitoes tested, the water receptor cell was activated after a delay of up to 2 s following electrode contact with the sensillum tip (Fig. 9A). In such cases a second stimulation was generally made not more than 2 s later and the receptor cell then responded immediately in a phasic–tonic manner (Fig. 9B).

Electrophysiological responses to increasing concentration of sucrose in 10 mmol l^{-1} KCl

The electrophysiological response to increasing concentrations of sucrose was firstly characterized in 10 mmol l^{-1} KCl in order to inhibit the water receptor cell. The action potential frequency is modulated by sucrose concentrations between 0.1 and 292 mmol l^{-1} following the model:

$$N \text{ spikes} = 57.35\,(1 - e^{(-0.17\times\text{conc})}). \quad (3)$$

The mean number of spikes per 2 s (MNS) of the sucrose receptor cell increases strongly from 0.1 mmol l^{-1} (MNS 0.75±0.75) to 50 mmol l^{-1} sucrose (MNS 62.33±3.73) in 10 mmol l^{-1} KCl (Fig. 10; Fig. 11A–C). The frequency of action potentials emitted by the sucrose-sensitive neurone tends to an asymptote (57.35 spikes per 2 s) from 50 mmol l^{-1} in 10 mmol l^{-1} KCl. Half of the maximum frequency (ED_{50}) is estimated at 4.07 mmol l^{-1} sucrose with 28.67 spikes per 2 s. The amplitude of spikes generated by the sucrose receptor cell increases slightly from 3.0±0.2 to 3.8±0.4 mV between 1 and 50 mmol l^{-1}, followed by a drop to 2.0±0.2 mV at 292 mmol l^{-1}, possibly due to the increased resistance caused by the high sucrose concentration in the electrode (Fig. 11D; supplementary material Fig. S1). At 0 mmol l^{-1} sucrose in 10 mmol l^{-1} KCl, no spikes are generated from the sucrose receptor cell. Only spikes of smaller amplitude with a predominantly negative-going shape are observed (Fig. 11A). These spikes could be generated by the water neurone, not totally inhibited by 10 mmol l^{-1} KCl, or by a third sensory neurone sensitive to salt (see above). In Fig. 11B the two types of spike amplitudes are observed during stimulation with 2.5 mmol l^{-1} sucrose in 10 mmol l^{-1} KCl. The smallest spike amplitude class is inhibited as the concentration of sucrose increases and drops to a MNS of 3 at 50 mmol l^{-1}. The same delay, as observed for the activation of the water neurone, was observed for the activation of the sucrose receptor cell.

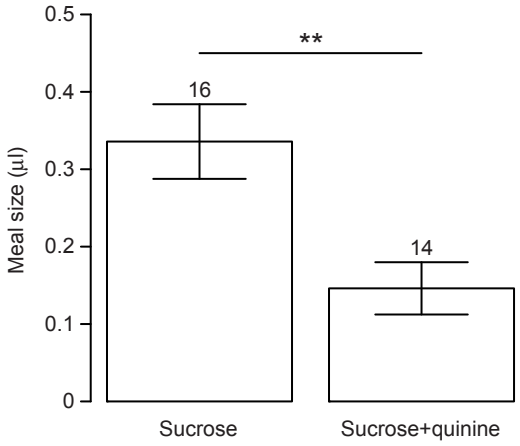

Fig. 5. Mean ± s.e.m. meal size by female *A. gambiae* with their mouthparts inserted for 5 min into glass capillaries containing 146 mmol l^{-1} sucrose and 146 mmol l^{-1} sucrose + 1 mmol l^{-1} quinine. The number of mosquitoes tested is indicated above each bar plot. Mosquitoes fed significantly less from the capillaries with quinine added; t-test, **P<0.01.

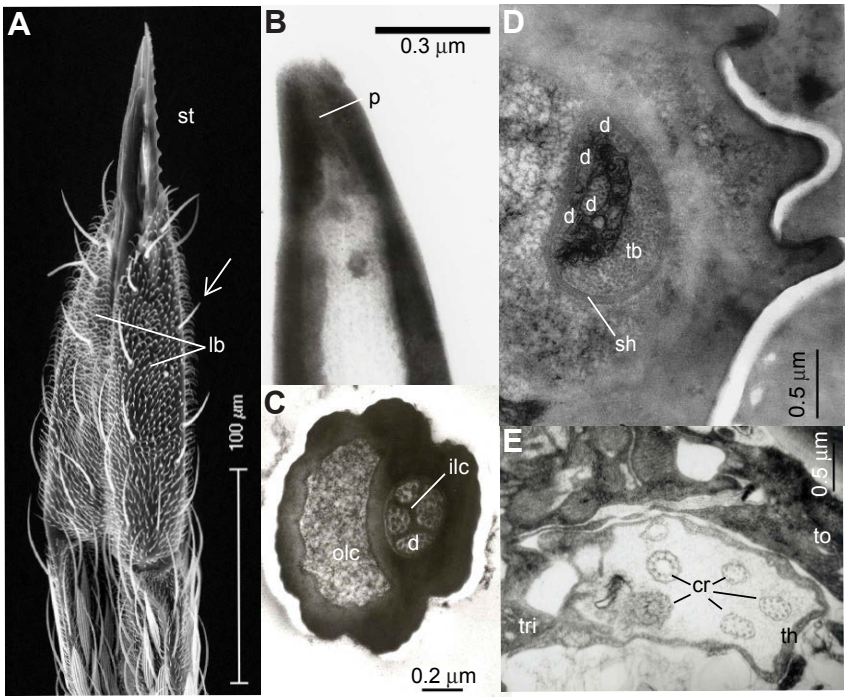

Fig. 6. Ultrastructure of trichoid type 1 sensilla on the proboscis of female *A. gambiae*. (A) Scanning electron micrograph of the extremity of the proboscis (dorsal view) showing the positions of several trichoid T1 sensilla. The arrow indicates the sensillum from which electrophysiological recordings were made in this study. Transmission electron micrographs of a longitudinal section at the tip of a trichoid T1 sensillum (B) and transversal sections at the middle (C), base (D) and at the level of the ciliary roots of a trichoid T1 sensillum (E). cr, ciliary roots; d, chemosensory dendrites; ilc, inner lymph cavity housing the four chemosensory dendrites; lb, labellar lobes; olc, outer lymph cavity; p, terminal pore; sh, sheath; st, stylets; tb, tubular body; th, thecogen cell; to, tormogen cell; tri, trichogen cell.

Discrimination between the water cell and sucrose receptor cell

To determine whether different cells are activated by water and sucrose, increasing concentrations of sucrose between 0.01 and 100 mmol l^{-1} were tested in nanopure water. The water receptor cell fires at a MNS of 61.92±5.35 when the sensillum of 0-day-old mosquitoes is stimulated by pure water. As the sucrose concentration increases, a second spike amplitude category appears from 0.1 mmol l^{-1} with increasing amplitude in parallel with frequency (Fig. 12A,C). At 10 mmol l^{-1} sucrose in water, the amplitude distribution is bimodal in 100% of the recordings (shown in the histograms on the right-hand side of each electrophysiological recording; Fig. 13). This confirms the presence of two activated sensory units. Two electrophysiological traces were removed from the analysis as two sensory units, whose presence was confirmed by the presence of doublets, could not be discriminated on the basis of amplitude, something never observed when sucrose was diluted

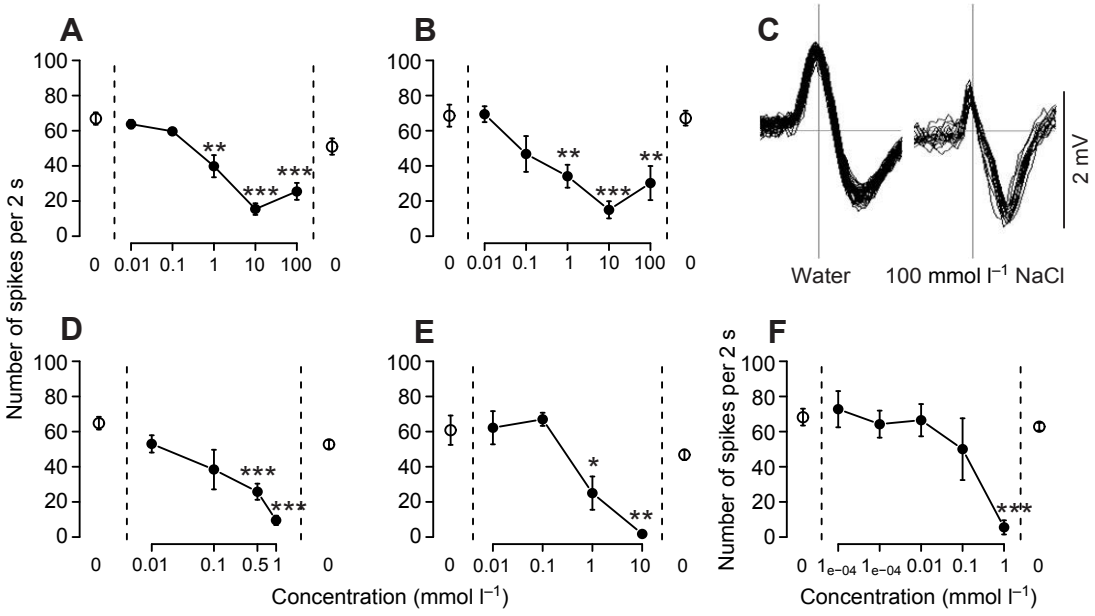

Fig. 7. Mean ± s.e.m. total number of spikes generated by sensory cells within trichoid T1 sensilla on the labellum of female *A. gambiae* during stimulation by water as a function of the $\log_{10}$ concentrations of (A) KCl (12 mosquitoes tested), (B) NaCl (10 mosquitoes tested), (D) $CuSO_4$ (five mosquitoes tested), (E) $CaCl_2$ (five mosquitoes tested) and (F) $LaCl_3$ (four mosquitoes tested). (C) Superimposition of action potentials recorded during stimulation with water (left) and with 100 mmol l^{-1} NaCl (right) displaying differences in shapes and amplitudes of the generated action potentials. Asterisks indicate numbers of spikes generated by the water neurone that are significantly lower than for the initial water controls (presented on the left of each graph); Welch's *t*-test, **P*<0.05, ***P*<0.01, ****P*<0.001.

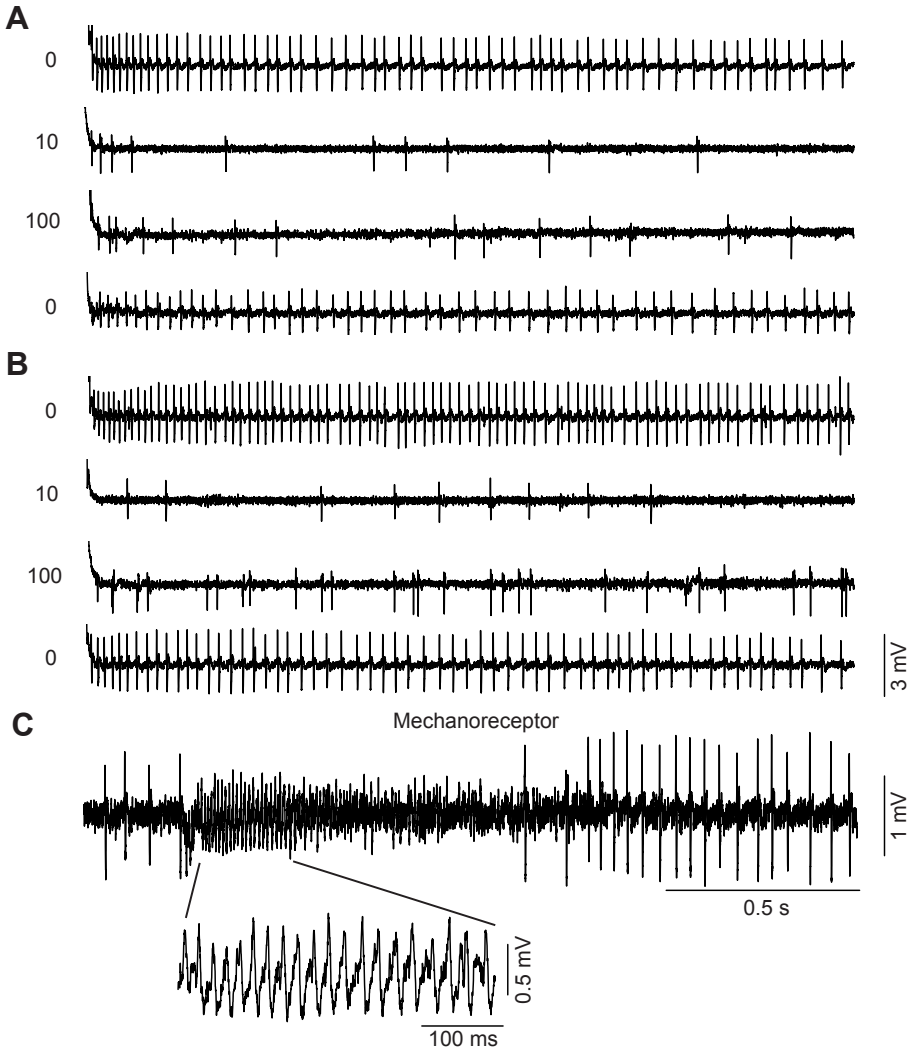

Fig. 8. Representative 2 s electrophysiological responses in trichoid T1 labellar sensilla stimulated with 10 and 100 mmol l^{-1} KCl (A) and NaCl (B). The recording series presented for each salt starts and ends with water (top and bottom traces in each case). (C) Response to water accompanied by mechanical stimulation: when the sensillum is pulled proximally with an electrode filled with pure water the water receptor cell is silenced and the mechanoreceptor is activated. The water-sensitive neurone fires again when the initial position of the sensillum is restored.

in 10 mmol l^{-1} KCl. Attributing spike amplitudes to the sucrose or water receptor cell is based on the fact that spike amplitudes generated by these cells vary in parallel with frequency (Fujishiro et al., 1984). This observation on labellar chemoreceptor cells of *Drosophila* is confirmed here for the labellar sucrose and water cells of *A. gambiae* (Fig. 12A,C). Thus at the sucrose concentration higher than the one where the two spike amplitudes are most similar, the attributions assigned to the two sensory cells is switched for purposes of counting, i.e. the spike amplitude previously assigned to the sucrose cell becomes the water cell and the spike amplitude previously assigned to the water cell becomes the sucrose cell. The concentration at which the mean amplitude is most similar is 5 mmol l^{-1} (Fig. 12A) but this can vary between 1 and 50 mmol l^{-1} depending on the mosquito tested.

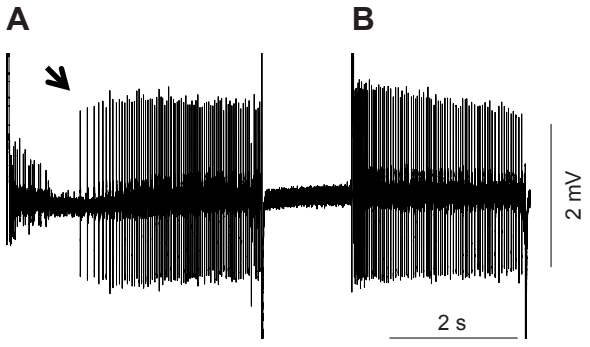

Fig. 9. Two electrophysiological responses to water recorded from a T1 sensillum on the labellum of female *A. gambiae*. (A) On first contact the water receptor cell is typically activated with a delay (arrow at 1.127 s in this example). The mechanoreceptor response is visible during the first 620 ms of this recording, but this is not systematically the case. (B) When a second recording is performed with the same electrode within 1.5 s from the end of the first stimulation, no delay in the response of the water cell is observed. The same phenomenon is observed for the sucrose receptor cell.

Applying this methodology to each recording independently, we calculated the following model to describe the increasing frequency of the sugar receptor cell to increasing concentrations of sucrose dissolved in water:

$$N \text{ spikes} = 53.46\,(1 - e^{(-0.15\times\text{conc})}). \quad (4)$$

An almost identical model was calculated (above) from the data describing the response of the sugar cell to sucrose presented in 10 mmol l^{-1} KCl, i.e. where the water cell is inhibited. This confirms the validity of our spike discrimination method. The number of action potentials per 2 s of the sucrose receptor cell increases strongly from 0.1 mmol l^{-1} (MNS 1.83±0.84) to 25 mmol l^{-1} sucrose (MNS 56.08±5.15) (Fig. 12C). The frequency of the action potentials emitted by the sucrose-sensitive neurone tends to the asymptote (53.46 spikes per 2 s) from 25 mmol l^{-1}. Half of the maximum frequency (ED_{50}) is estimated at 4.62 mmol l^{-1} sucrose in water with

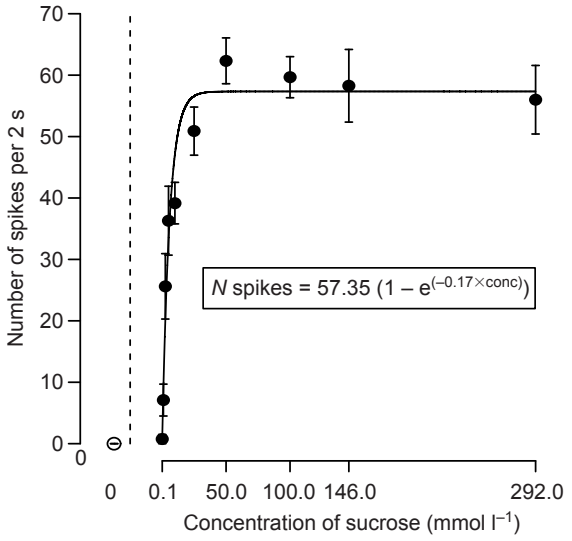

Fig. 10. Asymptotic model of the number of spikes (mean ± s.e.m.) per 2 s emitted by the sucrose-sensitive receptor cell in trichoid T1 sensilla on the labellum of female *A. gambiae* to stimulation with 0, 0.1, 1, 2.5, 5, 10, 25, 50, 100, 146 and 292 mmol l^{-1} sucrose (conc in the equation) presented at increasing concentrations in 10 mmol l^{-1} KCl. The response to 10 mmol l^{-1} KCl alone (open circle on left) is not taken into account in the model (four to 13 mosquitoes tested per concentration).

26.73 spikes per 2 s, close to the ED_{50} calculated for sucrose in 10 mmol l^{-1} KCl at 4.07 mmol l^{-1}. Increasing osmolarity of the stimulating solution by sucrose induces inhibition of the water receptor cell, which generates a MNS of 12.23±4.33 at 100 mmol l^{-1} sucrose (Fig. 12C). No negatively going spikes were recorded with these treatments. Sucrose and water receptor cells were also identified in five 5-day-old female *A. gambiae* by testing increasing concentrations of sucrose between 0.01 and 100 mmol l^{-1} in nanopure water (Fig. 12B,D). The model representing spike frequency of the sucrose receptor cell for the 5-day-old mosquitoes as a function of concentration is:

$$N \text{ spikes} = 96.02\,(1 - e^{(-0.35\times\text{conc})}). \quad (5)$$

This indicates that the asymptote for spikes generated by the sucrose cell of 5-day-old mosquitoes is some two times higher than that of 0-day-old mosquitoes (supplementary material Fig. S2). The model already tends towards the asymptote between 10 mmol l^{-1} (93.05 spikes per 2 s) and 25 mmol l^{-1} (96 spikes per 2 s), and the ED_{50} is situated at 1.99 mmol l^{-1}, more than two times lower than that for 0-day-old mosquitoes.

Electrophysiological responses to fructose, glucose and sucrose

The sucrose receptor cell is hardly activated when the labellar trichoid sensillum is stimulated with either 146 or 292 mmol l^{-1} glucose (MNS 1.2±1.2 and 1.4±1.4, respectively) with 146 or 292 mmol l^{-1} fructose (MNS 0±0 and 4.20±1.91, respectively) in 10 mmol l^{-1} KCl or the control (10 mmol l^{-1} KCl alone; MNS 0±0, P=0.11; Fig. 14). Nevertheless, a mixture of 73 mmol l^{-1} glucose plus 73 mmol l^{-1} fructose (MNS 37.00±3.94), 146 mmol l^{-1} glucose plus 146 mmol l^{-1} fructose (MNS 36.80±5.29), 146 mmol l^{-1} sucrose (MNS 65.20±10.33) and 292 mmol l^{-1} sucrose (MNS 42.25±11.09) activates the sucrose receptor cell (Fig. 14A,B). The number of action potentials recorded per 2 s tend only to be different ($F_{3,15}$=3.04, P=0.06) for 146 mmol l^{-1} sucrose compared with 73 mmol l^{-1} fructose plus 73 mmol l^{-1} glucose, 146 mmol l^{-1} fructose plus 146 mmol l^{-1} glucose and 292 mmol l^{-1} sucrose in 10 mmol l^{-1} KCl (Fig. 14).

Inhibitory effect of bitter products on the sucrose and water receptor cells

Except for caffeine, all the bitter products tested inhibit both the sucrose and water cells at concentrations between 0.01 and 1 mmol l^{-1} (Figs 15, 16), and inhibition is accompanied by a decrease

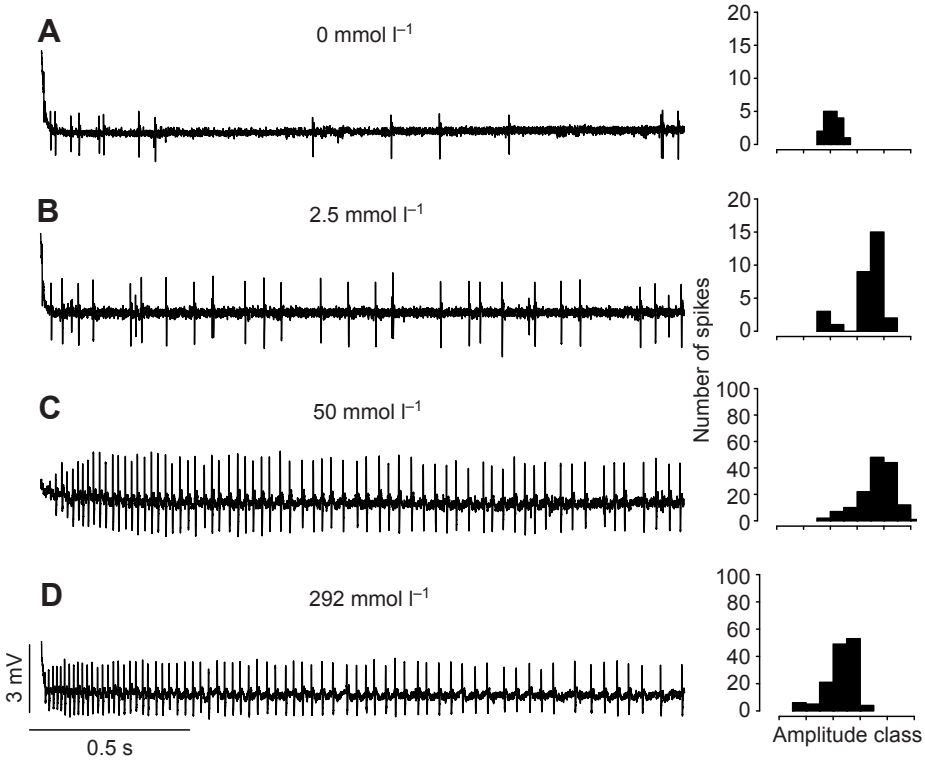

Fig. 11. Examples of recordings from a trichoid T1 sensillum to increasing concentrations of sucrose in 10 mmol l^{-1} KCl and the corresponding spike amplitude histograms (on right; amplitude classes assigned by AutoSpike). With 10 mmol l^{-1} KCl only, spikes with a smaller amplitude than the sucrose cell with a predominantly negative aspect are generated (A). At 2.5 mmol l^{-1}, the sucrose cell is already activated and characterized by spike amplitude greater than those generated in response to 10 mmol l^{-1} KCl alone (as indicated by the bimodal distribution of amplitude classes). At 50 mmol l^{-1} (C) and 292 mmol l^{-1} (D) the sucrose cell dominates and the cell generating small spikes is silenced. Note the decrease in amplitude of the sucrose cell spikes at 292 mmol l^{-1} sucrose.

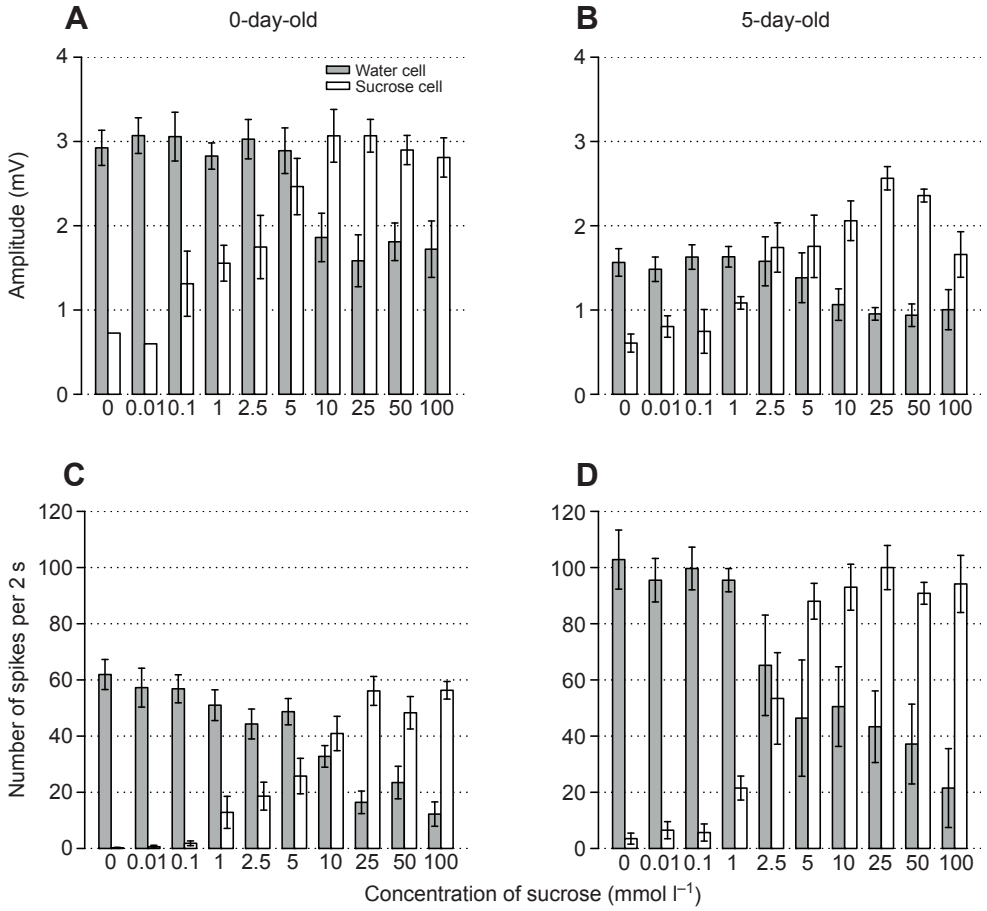

Fig. 12. Mean ± s.e.m. of spike amplitudes recorded from T1 sensilla on the labellum of female *A. gambiae* in response to stimulation with 0, 0.01, 0.1, 1, 2.5, 5, 10, 25, 50 and 100 mmol l^{-1} sucrose in pure water, tested at increasing concentrations on 0-day-old (A) and 5-day-old mosquitoes (B). Number of spikes per 2 s (mean ± s.e.m.) for the corresponding recordings for 0-day-old (C) and 5-day-old mosquitoes (D). The sucrose cell of the 0-day-old mosquitoes is activated in the same way as with sucrose in 10 mmol l^{-1} KCl (see Fig. 10); 5–13 mosquitoes tested per concentration.

in spike amplitude (supplementary material Fig. S3A,B). A linear model fitted to the number of spikes per 2 s as a function of the $\log_{10}$ of concentration of the bitter products proved significant for all inhibitory effects ($F_{9,186}$= 37.34, P<0.001, R^2=0.63; Fig. 15). At the highest concentration tested (1 mmol l^{-1}), one which corresponds to the intercept in the model, nine action potentials less are generated for all bitter products tested in water than in 146 mmol l^{-1} sucrose (P=0.003). The slopes fitted by the model are also significantly steeper for water than for sucrose (P=0.03), suggesting that bitter products are more efficient at inhibiting the water than the sucrose receptor cell. Denatonium benzoate at 1 mmol l^{-1} is significantly more efficient at inhibiting both the sucrose and the water receptor cells (MNS 6.62±2.18; Fig. 15A,E) than quinine (MNS 13.71±3.24, P=0.02; Fig. 15B,F), berberine (MNS 16.42±4.22, P=0.02; Fig. 15C,G) and caffeine (MNS 51±4.94, P<0.001; Fig. 15D,H). The slope of the model is significantly less steep for berberine than for denatonium benzoate (P=0.006) due to the high number of action potentials recorded at 1 mmol l^{-1} berberine in sucrose or water and the greater ability of berberine to inhibit both the sucrose and the water receptor cells at 0.01 mmol l^{-1} in sucrose (Fig. 15C,G). Caffeine has no effect on the electrophysiological response of the sucrose and the water receptor cells with a slope not significantly different from 0 in water or sucrose (P=0.39; Fig. 15D,H). Both the phasic and tonic part of the response of the sucrose and water neurones is affected by bitter products, as shown in supplementary material Fig. S4A,B for inhibition by quinine. No receptor cell appears to be activated by the concentration range of the bitter products tested in the labellar sensillum on which the present study focuses.

DISCUSSION

Sensing and feeding on sucrose by *A. gambiae*

In this study sucrose proved a strong phagostimulant for starved female *A. gambiae*. Mosquitoes start to feed at concentrations between 50 and 75 mmol l^{-1} sucrose in solution. ED_{50} is reached at around 111 mmol l^{-1}, the inflexion point of the feeding model. Small changes in the sucrose concentration at this dose where the slope of the response is at its steepest (0.625%) have the highest effect on meal size. However, the amount of ingested solution reaches a maximum between 146 and 250 mmol l^{-1} sucrose. Since females engorge on a 146 mmol l^{-1} sucrose solution from a glass capillary in a comparable manner, this permits us to conclude that the presence of receptor cells on the mouthparts (eventually within the cibarium) sensitive to sucrose are sufficient to elicit feeding. The glass capillary feeding experiments indicate that tarsal receptor cells are apparently not necessary to induce feeding on sucrose. It should be remembered that *A. gambiae* females feed on NaCl solutions only when heated as in blood and the meal is directed to the mid-gut, whereas a sucrose solution needs to be at room temperature and is directed to the crop (Arsic and Guerin, 2008).

Our electrophysiological study focuses on two labellar receptor cells: one is activated by sucrose in a concentration-dependent manner, and a second is activated by water and inhibited by sucrose and salts. The electrophysiological response of the sucrose receptor cell to sucrose diluted in water starts well below the levels of sugar necessary to induce engorgement. For both 0- and 5-day-old mosquitoes, the slopes of the two electrophysiological models are steepest at 801 and 3326%, respectively, at 0.01 mmol l^{-1} sucrose in water, decreasing to 179 and 103%, respectively, at 10 mmol l^{-1},

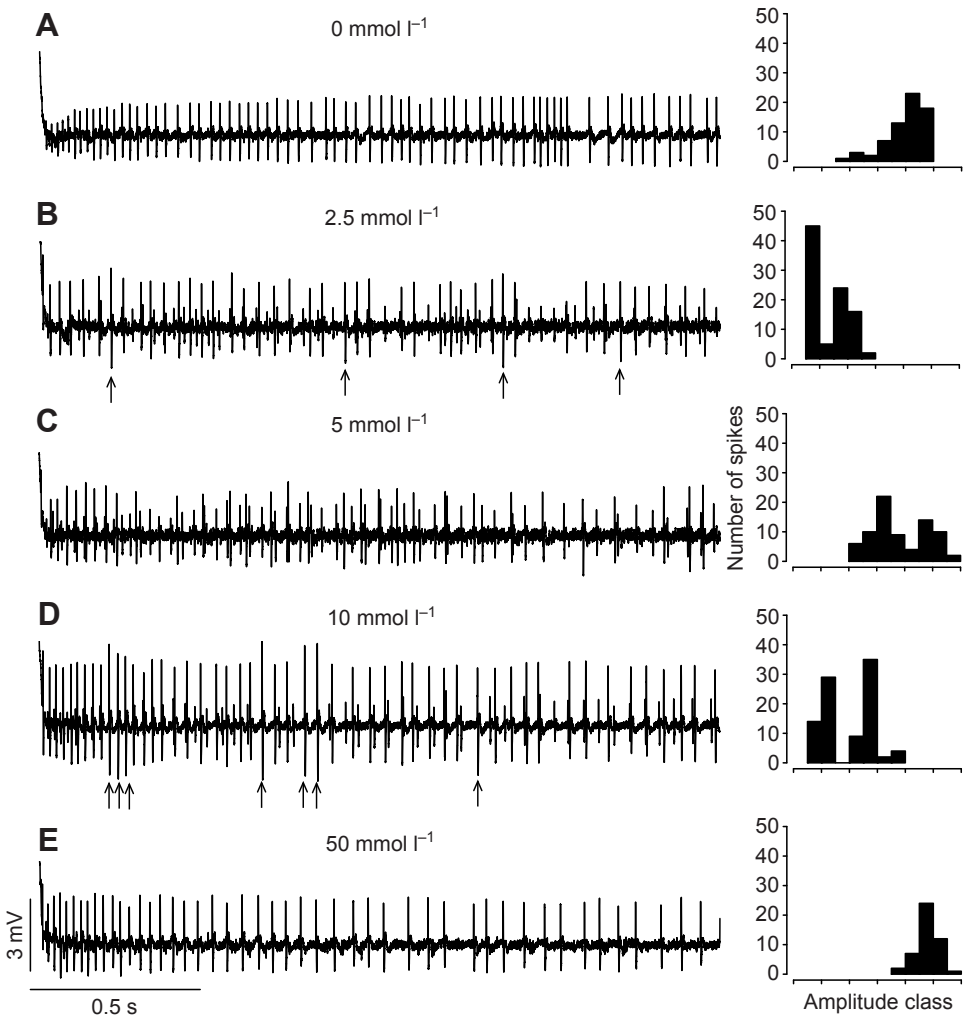

Fig. 13. Examples of recordings from a T1 trichoid sensillum to increasing concentrations of sucrose in pure water and their corresponding spike amplitude histograms (on right; amplitude classes assigned by AutoSpike). At 0 mmol l^{-1} sucrose, only the water cell is active (A). At 2.5 mmol l^{-1} (B) the sucrose cell is already active with a spike amplitude lower than that generated by the water cell (bimodal distribution of amplitude classes). At 5 mmol l^{-1} (C), although the amplitude of the spikes of the sucrose cell is still lower than that generated by the water cell, the amplitudes of both receptor cells move closer. At 10 mmol l^{-1} (D) the sucrose cell is more active with a spike amplitude higher than that generated by the water cell. At 50 mmol l^{-1} (E) the water cell is silenced on this recording and only spikes from the sucrose receptor cell are visible. Superimpositions of spikes are marked with arrows on recordings B and D.

and plateaus from approximately 25 mmol l^{-1} for both ages. This shows that *A. gambiae* is most sensitive to small increments in sucrose concentration up to 10 mmol l^{-1}. An ED_{50} is recorded at 1.99 mmol l^{-1} sucrose for 5-day-old mosquitoes, 56 times lower than the ED_{50} for feeding. For 0-day-old mosquitoes, an almost identical model was calculated for the response of the sugar cell to sucrose presented in 10 mmol l^{-1} KCl and for sucrose presented in water. The sugar cell seems not to be affected by 10 mmol l^{-1} KCl, contrary to its effect on the water cell. Whereas *A. gambiae* is apparently sensitive to sucrose concentrations between 0.1 and 10 mmol l^{-1}, it does not necessarily imbibe such solutions of a suboptimal energy level. Mosquitoes probably use other taste sensilla during their probing-walking responses (Pappas and Larsen, 1978; Sanford and Tomberlin, 2011) to reach a source of sugar at 50 mmol l^{-1} or higher that activates the neurone near its maximum frequency.

In contrast to sucrose, solutions of glucose or fructose alone (the hexose sugars of sucrose) show, respectively, low and intermediate stimulatory effects on feeding. However, female *A. gambiae* engorge on glucose and fructose when combined at 146 mmol l^{-1} each, just as on 146 mmol l^{-1} sucrose, and the feeding response to this mixture is higher than on a 292 mmol l^{-1} solution of either glucose or fructose alone. A direct correlation exists between this feeding response and the sensory input in that an equimolar 146 mmol l^{-1} solution of glucose plus fructose activates the sucrose neurone to the same extent as either 146 mmol l^{-1} or 292 mmol l^{-1} sucrose, whereas no such activation is observed when the sensory neurone is stimulated with either hexose alone. This suggests a synergetic effect between glucose and fructose on the feeding response of female *A. gambiae* that is mediated by the sucrose taste neurone in the labellar trichoid T1 sensillum, a phenomenon already described for the blowfly *Phormia regina* (Dethier et al., 1956; Omand and Dethier, 1969) and the mosquitoes *Culiseta inornata* (Schmidt and Friend, 1991) and *A. aegypti* (Ignell et al., 2010). The fact that the sucrose labellar neurone of *A. gambiae* is activated by sucrose or by a mixture of glucose plus fructose but not by glucose or fructose alone suggests that it expresses distinct domains for glucose and fructose that both require activation to obtain a response from the neurone. It was proposed that the dendritic membrane of contact chemoreceptor cells in *C. inornata* is composed of pyranose sites specific for α-glucopyranose and fructopyranose, and fructofuranose sites (Schmidt and Friend, 1991). It is known that several Grs are co-expressed in insect contact chemoreceptor cells (Isono and Morita, 2010) and sugars are detected through multimeric receptors composed of two or more Grs in *Drosophila* (Dahanukar et al., 2007). Eight genes related to the *Gr5a* of *Drosophila* have already been identified as candidate sugar receptors in the *A. gambiae* genome (Hill et al., 2002; Kent et al., 2008). The low phagostimulatory effect of glucose and fructose on *A. gambiae* is not in agreement with what is known for other mosquito genera where fructose, glucose and sucrose all show strong phagostimulatory effects on their own on *C. inornata*, *A. aegypti* and *Aedes taeniorhynchus* (Wiedemann) (Salama, 1967; Nayar and Sauerman, 1971).

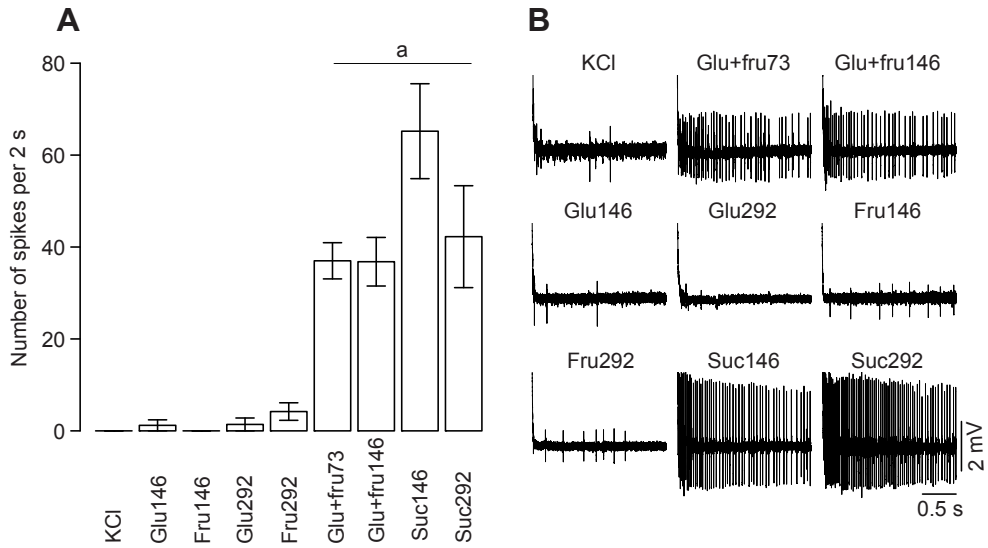

Fig. 14. (A) Mean ± s.e.m. number of spikes recorded from a T1 sensillum per 2 s to 10 mmol l^{-1} KCl and to 146 mmol l^{-1} glucose (glu146), 146 mmol l^{-1} fructose (fru146), 292 mmol l^{-1} glucose (glu292), 292 mmol l^{-1} fructose (fru292), 73 mmol l^{-1} glucose plus 73 mmol l^{-1} fructose (glu+fru73), 146 mmol l^{-1} glucose plus 146 mmol l^{-1} fructose (glu+fru146), 146 mmol l^{-1} sucrose (suc146) and 292 mmol l^{-1} sucrose (suc292) in 10 mmol l^{-1} KCl. Recordings were made from four to five female mosquitoes. The number of spikes per 2 s recorded for the mixtures of glucose plus fructose and to sucrose at the two concentrations tested is marginally not significant (a, ANOVA, P=0.06). (B) Corresponding electrophysiological recordings in response to stimulation with KCl, glucose, fructose, glucose plus fructose, and to sucrose.

Although mosquitoes can drink water to maintain their water balance (Benoit and Denlinger, 2010), the amount of pure water drunk in our feeding assay by mosquitoes maintained at a high relative humidity was low. In our assay, negative values for meal size are probably related to body water loss when the mosquitoes did not feed at all (Benoit et al., 2010). Our results suggest that the sugar receptor cells are responsible for food acceptance rather than the water neurones when mosquitoes are exposed to sucrose diluted in water. In the blowfly *Protophormia terraenovae* (Robineau-Desvoidy) it was shown that labellar water neurones function as osmometers since saline solutions, hypertonic with respect to the intracellular medium, cause cell shrinkage contrary to hypotonic solutions, such as that of glycerol, which cause cell swelling (Solari et al., 2010). Cell swelling coupled to a calcium influx leads to a transient receptor potential and action potential generation (Liscia et al., 1997). The possibility that the water receptor cell functions as an osmometer in *A. gambiae* is indicated by the strong inhibitory effect on the labellar hair neurone found here for $CuSO_4$ at 1 mmol l^{-1} (a supposed aquaglyceroporin AQP3 inhibitor) (Zelenina et al., 2004). A similar effect has been recorded for $CuSO_4$ on the water cell on the labellar lobes of *P. terraenovae* (Solari et al., 2010). Despite the strong inhibitory effect found here for $LaCl_3$ on the labellar water neurone of *A. gambiae*, a broad Ca^{2+}-permeable channel inhibitor, no significant activation of the neurone was observed when the extracellular Ca^{2+} concentration was increased by stimulating the sensillum with $CaCl_2$, as observed in the tarsal water neurones of *Drosophila* (Meunier et al., 2009). Whereas 1 mmol l^{-1} KCl is used as the reference to characterize the water cell in labellar and tarsal taste sensilla of *D. melanogaster* (Hiroi et al., 2004; Meunier et al., 2009), the labellar neurone of *Anopheles* appears to be more sensitive to KCl as this concentration reduced the number of spikes generated by the water cell of the T1 sensillar

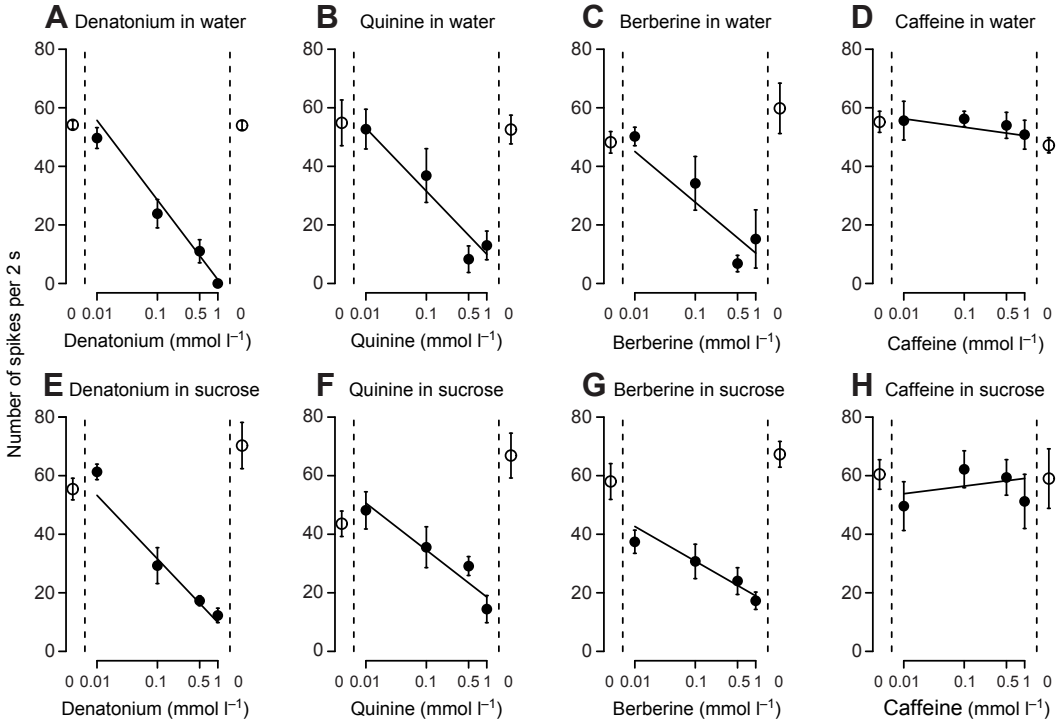

Fig. 15. Linear model ($F_{9,186}$=37.34, P<0.001, R^2= 0.63) and mean ± s.e.m. number of spikes generated per 2 s by the water cell (upper row of graphs) and by the sucrose cell in T1 sensilla (lower row of graphs) as a function of the $\log_{10}$ concentration of denatonium benzoate (six and seven mosquitoes tested), quinine (seven mosquitoes tested in both conditions), berberine (five and seven mosquitoes tested) and caffeine (five mosquitoes tested in both conditions) added to water (A–D) and to 146 mmol l^{-1} sucrose in 10 mmol l^{-1} KCl (E–H).

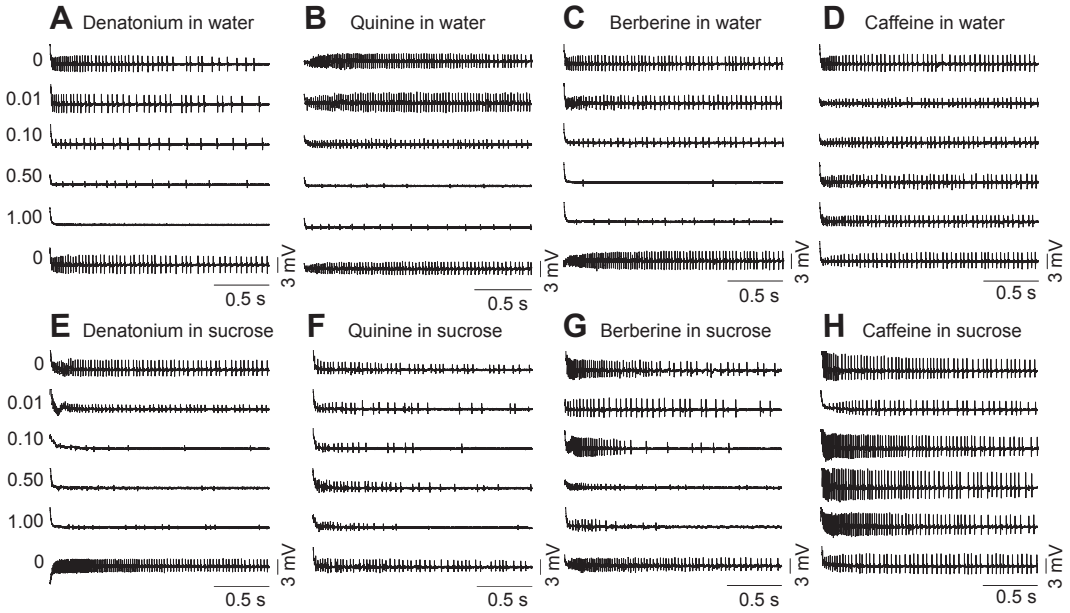

Fig. 16. Representative 2 s electrophysiological responses of the sucrose and water cells in T1 labellar sensilla of *A. gambiae* to water (A–D) and 146 mmol l^{-1} sucrose in 10 mmol l^{-1} KCl (E–H) each with increasing concentrations (0.01–1 mmol l^{-1}) of denatonium benzoate, quinine, berberine and caffeine added. Each recording series starts and ends with water (A–D) or 146 mmol l^{-1} sucrose in 10 mmol l^{-1} KCl (E–H). Except for caffeine, spike amplitude decreases with increasing concentration of all products tested.

receptor cells by 40%. Water, salt and sugar neurones have been identified in labellar trichoid T1 of *C. inornata* (Owen et al., 1974; Pappas and Larsen, 1976). The functionality of the lateral labellar T1 sensillum neurones in *A. gambiae* on which this study focused seems to be similar as separate sugar and water neurones have been identified in the present study. However, the functionality of the other labellar T1 sensilla of *A. gambiae* still need to be characterized.

Availability of sugars for *A. gambiae* in its habitat

Anopheles gambiae feeds preferentially on nectars from *Ricinus communis* (Euphorbiaceae) and *Tecoma stans* (Bignoniaceae) in its natural habitat (Manda et al., 2007b). In the case of *R. communis*, sucrose, glucose and fructose are present at a concentration of about 30% (w/v, 876 mmol l^{-1} for sucrose and 1665 mmol l^{-1} for the two hexoses) each in extrafloral nectar (Baker et al., 1978), and fructose and glucose are present at a concentration of around 50% (2775 mmol l^{-1}) each in floral nectar of *T. stans* whereas its sucrose content is only 2% (Freeman et al., 1985). The ratio between sucrose and hexose sugars can vary in floral nectar but it is rare to find nectar in which only one sugar occurs (Baker and Baker, 1982). Of the five Kenyan endemic plant species on which *A. gambiae* were observed feeding most frequently, fructose or glucose were never detected alone in floral or extra-floral nectaries (Manda et al., 2007a). In the same study these authors have shown that longevity in days of female *A. gambiae* that had access to 6% (w/v) glucose increased by more than fivefold over females that had access only to water. If *A. gambiae* is to exploit glucose or fructose alone as a resource, then individuals need to be starved for more than the 3 h period used in this study to compensate for the lower phagostimulatory effect of these monosaccharides. This is quite a plausible scenario since a steady diet of 10% sucrose during the days prior to feeding assays described here allowed *Anopheles* to increase its carbohydrate and lipid reserves (Briegel, 1990). Some plant nectars are more suitable at increasing *A. gambiae* longevity and fecundity over others (Manda et al., 2007a; Manda et al., 2007b). Such differences among plant species in meal quality could be due to optimal sugar levels combined with other essential nutrients or to the absence of toxic compounds, whereas plant preferences could be related to absence of deterrents.

Bitter products as feeding deterrents for *A. gambiae*

Except for caffeine, all bitter products tested significantly reduced sugar feeding in *A. gambiae*. The glass capillary feeding experiment including quinine allows us to conclude that proboscis and/or cibarial gustatory receptor cells are sufficient for the perception of bitter products. Ignell et al. (Ignell et al., 2010) found that the concentration of quinine that starts to inhibit feeding in *A. aegypti* is affected by the sucrose level presented, with only 0.001 mmol l^{-1} quinine required at 10 μmol l^{-1} sucrose but with 0.1 mmol l^{-1} required at 1 mol l^{-1} sucrose. The latter concentration of quinine is proportional to what starts to be effective at inhibiting feeding by *A. gambiae* in our no-choice assay, i.e. 0.01 mmol l^{-1} quinine in 146 mmol l^{-1} sucrose. However, whereas Ignell et al. (Ignell et al., 2010) found that quinine only affects feeding by female *A. aegypti*, quinine and quinidine were found to affect the feeding response of male and female *A. gambiae* in a comparable manner in our assays (data not shown). In most cases alkaloids inhibit feeding in insects (Dethier and Bowdan, 1989; Ramaswamy et al., 1992; Zhang and Mitchell, 1997; Meunier et al., 2003). Bitter products are not all equally toxic, and, to date, it is not clear if there is a direct correlation between bitterness and toxicity of these products for insects (Glendinning, 2002; Weiss et al., 2011).

Bitter products inhibit sucrose-feeding in *Anopheles* through sensory inhibition

The concentration-dependent inhibitory effects of denatonium benzoate, quinine and berberine on the electrophysiological responses of labellar sucrose and water receptor cells to water or sucrose confirms the critical role of sensory inhibition in the perception of deterrent compounds in *A. gambiae* as in other insects (Schoonhoven, 1982). These compounds also inhibit feeding, whereas caffeine, which fails to inhibit feeding even at 1 mmol l^{-1} by *A. gambiae* on sucrose, shows no inhibitory effect on the electrophysiological responses of the neurones. Inhibition of the receptor cell responses is stronger when denatonium benzoate, quinine and berberine are presented in pure water than in 146 mmol l^{-1} sucrose, indicative of a competitive phenomenon between sucrose and these bitter products. This suggests that the bitter compounds induce inhibition of the sucrose cell by acting on specific Gr(s) or on a specific ion channel type. It was suggested

that the water receptor cells in *Drosophila* are inhibited by bitter products through an osmolarity-based mechanism (Meunier et al., 2003; Weiss et al., 2011). In our study we show that both the labellar sucrose and water neurones would appear to be involved in the detection of all products showing feeding deterrent activity in *A. gambiae*. Nevertheless, the numbers of bitter compounds we have tested is not exhaustive. To date, we have not succeeded in characterizing receptor cells in sensilla on the proboscis or on the tarsi of *A. gambiae* that are selectively activated by bitter products, as is known in *Drosophila* (Meunier et al., 2003; Hiroi et al., 2004; Weiss et al., 2011). It has recently been shown that labellar neurones responding to bitter products in *Drosophila* can be segregated into four functional classes, each possessing a specific response pattern to 16 bitter products and expressing a specific combination of 33 Grs (Weiss et al., 2011).

In summary, this study highlights how bitter-tasting products have a deterrent effect on sugar feeding by *A. gambiae*. The diversity of bitter compounds in nature has led to a wide diversity of Grs involved in the detection of unpalatable substances in *Drosophila* (Weiss et al., 2011). Despite this, we have identified two neurones on the labellar lobes of *A. gambiae* that are activated by palatable compounds and inhibited by unpalatable ones. These neurones could be the target of future tests to uncover novel deterrents for *A. gambiae* and to identify phagostimulants to better understand the ecology of feeding in this species.

LIST OF ABBREVIATIONS

ED_{50}	effective dose that induces 50% of the maximum response
MMS	median meal size
MNS	mean number of spikes per 2 s generated by only one receptor cell
MTNoS	mean of the total number of spikes per 2 s counted without discriminating between the receptor cells of the sensillum

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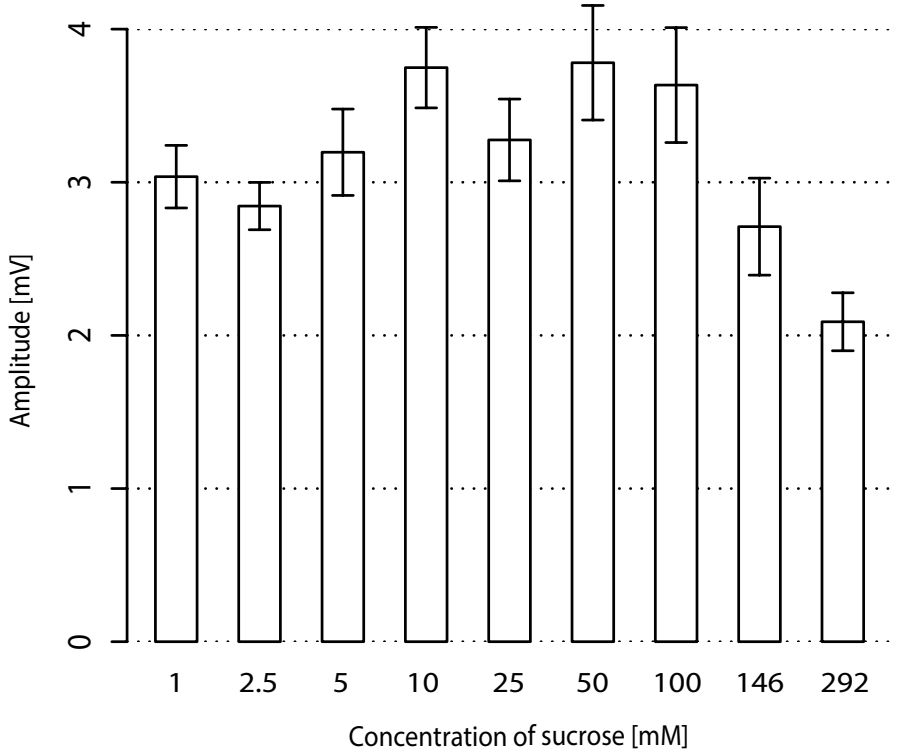

Fig. S1. Mean ± s.e.m. spike amplitudes generated by the sucrose-sensitive cell to increasing concentrations of sucrose in 10 mmol l^{-1} KCl (data set as in Fig. 10).

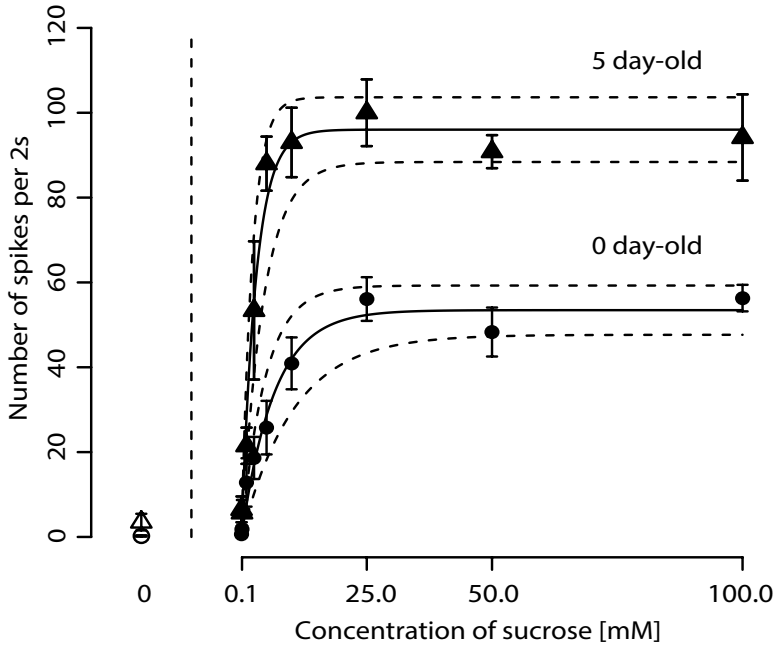

Fig. S2. Asymptotic models of the number of spikes (mean ± s.e.m.) per 2 s emitted by the sucrose-sensitive receptor cell in trichoid T1 sensilla on the labellum of 0-day-old (circles) and 5-day-old (triangles) female *A. gambiae* to stimulation with 0, 0.01, 0.1, 1, 2.5, 5, 10, 25, 50 and 100 mmol l^{-1} sucrose presented in increasing concentrations in water. The dotted lines indicate the 95% confidence intervals of the models. The response to water alone (open symbols on left) is not taken into account in the models (five to 13 mosquitoes tested per concentration, data sets as in Fig. 12).

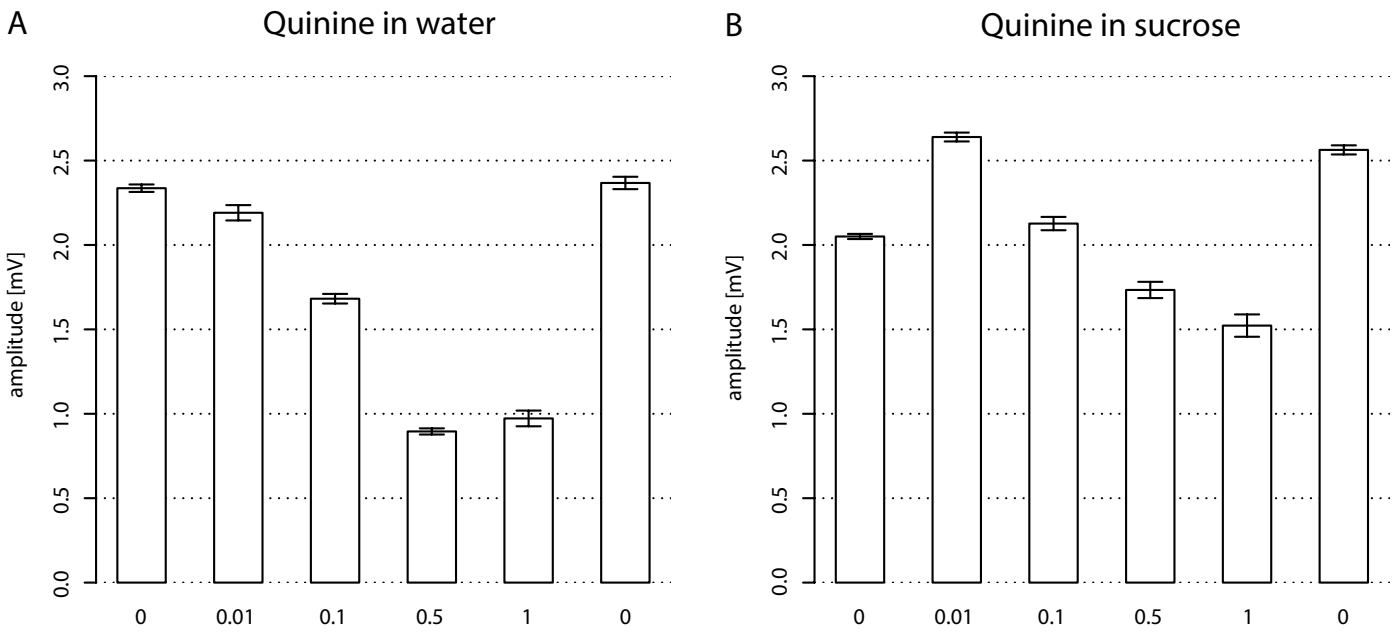

Fig. S3. Mean ± s.e.m. spike amplitudes of the water receptor cell (A) and the sucrose receptor cell (B) as a function of increasing concentrations of quinine (data sets as in Fig. 15B,F).

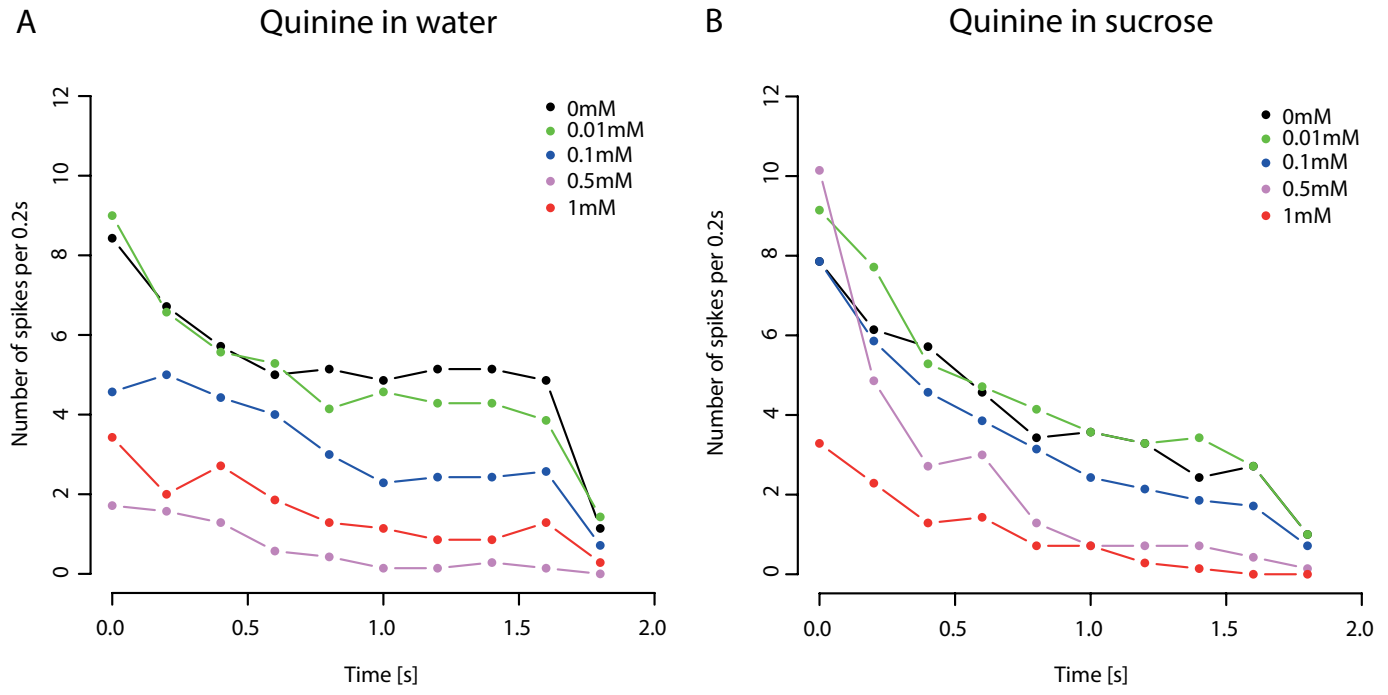

Fig. S4. Mean number of action potentials per 0.2 s in 2 s recordings from the water cell (A) and the sucrose cell (B) for 0, 0.01, 0.1, 0.5 and 1 mmol l^{-1} quinine diluted in water (A) and in 5% sucrose plus 10 mmol l^{-1} KCl (B) (data sets as in Fig. 15B,F).

6. The sugar meal of the African malaria mosquito *Anopheles gambiae*: from chemoreception to vectorial capacity

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6.1 Abstract

Mosquitoes feed in a discriminative manner on floral and extrafloral nectars and their fitness depends upon which plants they have fed, suggesting that prefered plants have a higher nectar value. In this study, the presence of a water- and a sugar-sensitive neurone was confirmed in most long labellar trichoid type 1 (T1) and some short type 2 (T2) sensilla on the labellum of *Anopheles gambiae*. In all, seventeen sweet-tasting compounds were tested using electrophysiological recordings from T1 sensilla in *A. gambiae* and also in *Aedes aegypti*. The findings show that sucrose is the best stimulant for the labellar sugar sensitive neurones of *A. gambiae* and *A. aegypti*. In *A. gambiae* the sugar sensitive neurones of the T1 sensilla on the tip of the labellum which possess no water sensitive neurone react to increasing concentration of sucrose with an ED_{50} value one half lower (2.46 mM) and with higher numbers of spikes than sensilla more proximally situated which possess both a sugar sensitive neurone and a water sensitive neurone (ED_{50} = 5.21). To bind to the receptor, both the glucopyranoside and fructofuranoside residues of the sucrose molecule are required in *Anopheles* along with the anomeric form α of the glucopyranoside. In choice experiments, sucrose is also the preferred diet of *A. gambiae* followed by an equimolar mixture of glucose and fructose, fructose alone and finally, glucose alone. Sugars contributing to the highest longevity in both sexes were sucrose, equivalent to fructose, followed by the mixture of glucose and fructose, and finally glucose. As mosquitoes fed on sucrose live longer than those fed on a mixture of the two monosaccharides glucose and fructose, this suggests the presence of a different absorption process and/or a metabolic pathway for assimilation of disacchrides than for monosaccharides. On the other hand, female *A. gambiae* that have fed on glucose adopt a more intense biting behaviour on a warm body than those that fed on sucrose. The dry mass of females maintained on sucrose increased between day 4 and day 10 and was significantly higher at both ages than the dry mass of females maintained on glucose that dropped between day 4 and day 10. This study suggests that sucrose and glucose may have two opposing effects on the vectorial capacity of females: those maintained on sucrose have a longer lifespan but manifest a less intense biting behaviour than those maintained on glucose. The question arises as to how the nectar quality of plants in the peridomestic environment influences the vectorial capacity of an anthropophilic mosquito species like *A. gambiae*.

Key words: Mosquitoes, sugar-feeding, sweet taste, water receptor cell, mosquito feeding preferences, mosquito survival, mosquito biting behaviour, vectorial capacity.

6.2 Introduction

In Nematocera, haematophagy has probably evolved from ancestral entomophagous or nectarivorous groups equipped with piercing-sucking mouthparts (Balashov, 1999). Mosquitoes are positioned at the interface between heamatophagy and phytophagy: although females of anautogenous species need a blood meal to produce eggs, all species retain their nectar feeding habit and plant sugars consitute the main energetic source for adult mosquitoes (Foster, 1995). It is the only resource consumed by males, but females of anautogenous spp. also require sugars as nutrients, despite the selective pressure on females of anthropophilc species which live in sugar-scarce habitats to feed only on blood (Gary and Foster, 2001). Research by Manda et al. (Manda et al., 2007b) and Nyasembe et al. (Nyasembe et al., 2012) have shown that the afrotropical vector of malaria, *Anopheles gambiae* Giles feeds on plants in a discriminative manner. Moreover, mosquitoes fed on prefered plants have a higher fitness (Gary and Foster, 2004; Impoinvil et al., 2004; Manda et al., 2007a). Although the sugar constituents of these plant nectars have been identified and include sucrose, fructose, glucose, gulose and maltose (Manda et al., 2007b; Nyasembe et al., 2012), nothing is known about taste sensitivity and the nutritive values of these carbohydrates in this species. The strong influence of sugar feeding on lifespan and biting rate, two key factors of vectorial capacity, has already been shown in *A. gambiae* (Gary and Foster, 2001; Stone et al., 2011; Stone et al., 2012). Fecundity and flight capacities have also been investigated in this context (Gary and Foster, 2001; Kaufmann and Briegel, 2004; Stone et al., 2012). However, to our knowledge, no one has studied these parameters in *A. gambiae* populations maintained on different sugar diets. In this study, structure-activity relationships of thirteen sugars and some of their mixtures, along with other sweet-tasting compounds, were investigated by single sensillum recordings on labellar sugar receptor cells of *A. gambiae*. Complementary recordings were made from the similar sensilla of *Aedes aegypti* (L.), the yellow fever mosquito. Feeding preferences between glucose, fructose, sucrose and melezitose were investigated and the nutritive value of sugar diets is examined in the context of lifespan and biting behaviour of *A. gambiae*.

6.3 Material and methods

Mosquito colonies

A. gambiae (16cSS strain) are maintained in a climate chamber (28°C, 80% RH) under a 12:12 light-dark cycle with 2 h simulated sunrise and sunset. *A. aegypti* (UGAL strain) are maintained in a second walk-in climate chamber under a 12:12 h light-dark cycle at 26 ±2°C and 65 ±2% RH during the day and 22 ±2°C and 90 ±2% RH during the night. Females take a blood meal from a Guinea pig and eggs are recovered on wet filter papers. About 275 *Anopheles* larvae are reared in trays containing 400 ml distilled water (water depth: 0.5 cm) and about 200 *Aedes* larvae are reared in 800 ml water (water depth: 1 cm). This density provides adults of homogeneous optimal size (Timmermann and Briegel, 1993). Larvae of both species are fed with pulverised Tetramin® fish food. In this manner, about 800 adult mosquitoes emerge into rearing cages (350 x 350 x 550 mm high) and are provided with 10% (w/v) sucrose and water *ad libitum*.

Test chemicals

D-(-)-fructose, D-(+)-glucose monohydrate, L-(+)-gulose, 1-kestose, D(+)-maltose monohydrate, D-(+)-melezitose monohydrate, methyl α-D-glucopyranoside, methyl β-D-glucopyranoside, D-(+)-raffinose, D-sorbitol, L-(-)-sorbose, D-(+)-sucrose, thaumatin, D-(+)-trehalose, D-(+)-turanose were purchased from Sigma-Aldrich (Buchs, Switzerland), glycerol 87% from AppliChem (Darmstadt, Germany) and KCl from Merck (Darmstadt, Germany), all with a purity ≥97%, except glycerol which was mixed with water and thaumatin which was bought with an unknown purity. Solutions were kept at 4°C for less than 1 week.

Ultrastructure of labellar sensilla

Scanning (SEM) and transmission electron microscopy (TEM) procedures used were the same as described in Kessler et al. (Kessler et al., 2013). Although the ultrastructure of type 1 (T1) trichoid sensilla has been described in detail in the precedent study (Kessler et al., 2013), SEM was used here to map the ventral and dorsal T1 sensilla as well as to describe the external morphology of trichoid type 2 (T2) sensilla on the labellar lobes. Ultrathin sections (1 µm) of the first 100 µm from the tip of the proboscis of three female *A. gambiae* were subjected to TEM to study the ultrastructure of the T2 sensilla.

Electrophysiological recordings from A. gambiae *and* A. aegypti

Electrophysiological recordings from T1 and T2 sensilla on the labellar lobes of *A. gambiae* and *A. aegypti* have been made as described in Kessler et al. (Kessler et al., 2013). A tastePROBE (DTP-02, Synthech, Hilversum, The Netherlands) was used in "pass-through" mode, i.e. without compensation for the DC offset, omitting the first 10 – 20 ms of recordings. The DC offset was suppressed by high pass filtering (100 Hz). In all, four different experiments were made. In the second experiment, where increasing doses of sucrose were tested in *A. gambiae* and *A. aegypti*, spikes generated in the T1 sensilla by the sugar sensitive neurone and those generated in response to 10 mM KCl were differentiated by their shapes as described in a preceding study (Kessler et al., 2013). In all other recordings, the total number of spikes was counted without any discrimination between the different firing units. We analyzed spikes in the first 2 s from recordings made for 4-6 s on the T1 sensilla. In the case of the T2 sensilla of *A. gambiae*, the first 10 s of recordings of up to 30 s could be analyzed due to the highly tonic response patterns obtained from the receptor cells to water.

Responses of T1 and T2 labellar sensilla to sucrose and water in A. gambiae *and* A. aegypti

To search for the presence of water and sugar sensitive neurones in T1 and T2 labellar sensilla of female *A. gambiae*, nanopure water and 146.07 mM sucrose (5%) in 10 mM KCl were tested on 13 trichoid T1 sensilla on each labellar lobe of females. The presence of water and/or sugar sensitive neurones within such sensilla was confirmed following recordings made from labellar sensilla of three different mosquitoes which showed a response to water and/or to 146.07 mM sucrose in 10 mM KCl, and absence of a water sensitive neurone was confirmed from recordings on at least seven sensilla of seven mosquitoes which showed no response to water but responded to sucrose. Water and 100 mM sucrose were also tested on four T2 sensilla of four different female *A. gambiae.* Pure water was also tested on the T1 sensilla Nos 4, 7, 9 and 10 (numbered following Hill and Smith, 1999) of 3-5 female *A. aegypti*.

Dose dependent responses of T1 labellar sensilla to sucrose in A. gambiae *and* A. aegypti

Sensitivity of the lateral No 5 sensillum of *A. gambiae* to increasing concentrations (0.1 to 292.14 mM) of sucrose in 10 mM KCl has already been investigated in Kessler et al. (Kessler et al., 2013). In the present study, we extend this to the lateral No 2 sensillum (see Fig. 6.1), a sensillum which possess both a water and a sugar sensitive neurone, and to the ventral No 8 and the dorsal No 5 sensilla, namely two sensilla which possess a sugar sensitive neurone but no water sensitive neurone. We also compared the spiking frequency of the sugar

sensitive neurone of the lateral No 5 sensillum in female and male *A. gambiae*. Sucrose at between 0.1 to 100mM in 10 mM KCl was also tested on the ventral sensilla Nos 4, 7, 9 and 10 (numbered following Hill and Smith, 1999) of female *A. aegypti*.

Labellar receptor cell sensitivity to glucose and fructose as constituents of sucrose in A. gambiae *and* A. aegypti

It is already established that the sugar sensitive neurone of the lateral labellar sensillum No 5 of female *A. gambiae* can be activated by sucrose or by a mixture of its two hexose components glucose and fructose, but not by glucose or fructose alone (Kessler et al., 2013). To evaluate in more detail the affinity of the labellar sugar receptor cells of female *A. gambiae* for fructose and glucose, mixtures of the two hexoses at 5:15, 15:5 and 10:10 mM were tested on the ventral No 8 and the dorsal No 5 sensilla and compared with the stimulatory effectiveness of 10 and 20 mM glucose or fructose alone and with 5, 10 and 20 mM sucrose. As glucose is present in the sucrose molecule in its α anomeric form, methyl α-D-glucopyranoside and methyl β-D-glucopyranoside mixed with fructose at 10:10 mM were tested on the same sensilla to test the selectivity of the sugar receptor cells for the anomeric form of glucose.

In female *A. aegypti*, fructose and glucose were tested at 10, 50 and 100 mM, mixtures of fructose and glucose at 25:75, 75:25 and 50:50 mM, and sucrose at 50 and 100 mM sucrose in 10 mM KCl on the ventral sensilla Nos 2, 4, 5, 7, 9 and 10 (numbered following Hill and Smith, 1999).

Sensitivity of labellar receptor cells to other sweet tastants

Insect sugar sensitive neurones express several gustatory receptors (Grs) responsible for their broad tuning (Dahanukar et al., 2007). In this study, we tested several human sweet tasting compounds at 100 mM on the ventral No 8 and the dorsal No 5 sensilla to assess the specificity of *Anopheles* sugar sensitive neurones. Data from stimulations with 100 mM fructose, glucose, gulose and maltose, four carbohydrates identified in the nectar of plant species on which *A. gambiae* feeds in nature (Manda et al., 2007b; Nyasembe et al., 2012), are presented. Two plant carbohydrates sorbose, and its alcohol, sorbitol, have been added to this series as well as trehalose, a storage form of carbohydrates in insects which has been found to bind specifically the Gr5a of *Drosophila* (Dahanukar et al., 2007). Turanose, melezitose and kestose were also tested to evaluate the specificity of the sugar sensitive neurones. Turanose is a sucrose isomer (Fig. 6.7) known to elicit strong responses from sugar receptor cells in *Drosophila* (Dahanukar et al., 2007) whereas melezitose and kestose

are two trisaccharides composed of a sucrose unit with either α-D-glucopyranoside or a β-D-fructofuranoside, respectively (Fig. 6.7). Data for increasing doses of several other human sweet tastants tested on the ventral No 8 and dorsal No 5 sensilla and the lateral Nos 2 and 5 sensilla of *A. gambiae* are presented in supplementary material (Figs. S6.1 and S6.2).

Sugar diet feeding preferences of A. gambiae

A. gambiae males and females of between 5-6 days-old (20±3 of both sexes) previously maintained on 10% sucrose were released into Makrolon® cages (200x260x180 cm) for 21.5 h (comprising the last 3 hours of the scotophase, the 14h the photophase and the first 4.5 h of the following scotophase). Experiments were performed in the mosquito rearing climate chamber under the same temperature, humidity and light conditions as described above. During choice tests, mosquitoes had free access to water presented on a saturated cotton wool pad (1.5 cm thick) covering a 3.5 x 13 cm opening on the roof of the cage and to two sugar solutions. In these experiments, glucose and fructose were tested at a molarity two times higher than sucrose, and melezitose was tested at the same molarity as sucrose. Solutions of 292.14mM sucrose (10%, wt:vol) and melezitose (14.74%), 584.28 mM fructose (10.53%), 584.28 mM glucose monohydrate (11.58%), a mixture of 292.14 mM glucose + 292.14 mM fructose and water were provided in two 10 ml test tubes closed by 12 mm diameter dental rolls (No. 17 301 00, Hartmann AG, Neuhausen, Switzerland). The test tubes were inserted upturned through 1.8 cm diameter holes 16 cm apart on the roof of the cage whereby the cotton rolls were impregnated with the test solutions to allow the mosquitoes to imbibe *ad libitum*. Sugar solutions were coloured with blue (E131) or red (E122) food dyes (one drop of 30 mg in 2 ml). The dyes were interchanged between the test solutions and the position of each solution was alternated between cages. After 21.5 h exposure, the mosquitoes were cold anaesthetized and the solution imbibed by each was determined by visual inspection of the dissected crop and midgut (Ignell et al., 2010): i.e. either red, blue or violet in cases where the mosquitoes have imbibed both solutions. All colour estimations were made by the same person.

A. gambiae *longevity on different sugar diets*

The effect of different sugars on *A. gambiae* longevity was tested in the rearing climate chamber under the environmental conditions described above. Adult *A. gambiae* of the same generation emerged from pans containing 200 pupae in Plexiglas® rearing cages (35x35x55 cm). Cages had an opening (15 cm diameter) on one side, closed by nylon netting to provide access. A circular opening (12 cm diameter) on the top of the cages closed by stainless steel

netting (1 mm mesh) allowed mosquitoes free access to a moistened cotton pad (1.5 cm thick) of the same size as the opening. Solutions of either 292.14 mM sucrose, 584.28 mM fructose, 584.28 mM glucose monohydrate, a mixture of 292.14 mM glucose + 292.14 fructose or water were provided in four test tubes 21 cm apart on the top of the cages (as described above). Sugar solutions and the moistened cotton were replaced weekly. The sex ratios among treatments are shown in table 1. The position of the 5 cages was changed each day in a circular manner. Dead mosquitoes were counted each day and were removed from cages using gloves to prevent females from taking a blood meal.

Biting response as a function of sugar diet

The effect of feeding glucose versus sucrose on the biting response of *A. gambiae* was tested at 4 and 10 days after emergence. For this experiment, 200 pupae were placed in small rearing cages (200 x 260 x180 mm high). In the days before testing their biting response mosquitoes had access *ad libitum* to water and to 10% sucrose or 10% glucose solutions on cotton rolls suspended from test tubes on the roof of the cage (as described above). The day before the warm body test (see below), females were individually deprived of water and sugar for 12 h in glass vials (40 mm high, 20 mm diameter) closed with perforated plastic stoppers with a 7 mm diameter aperture carrying stainless-steel netting (mesh size 0.4 mm) and kept in boxes at 95% RH as already described (Kessler et al., 2013). After this starvation period, females were allowed to feed for 30 minutes by upturning the opened tubes on filter paper (55 m diameter, no. 10 311 807, Whatman Schleicher and Shuell, Dassel, Germany) treated with 600µl of 292.14 mM sucrose or 584.28 mM glucose monohydrate, i.e. on the diet on which they had been maintained. Sugar-engorged females with a distended crop were selected to test their biting response. This was evaluated by using the warm body bioassay 3 to 6 hours after the feeding period. During this second starvation period, the mosquitoes were enclosed in the same glass vials at 95% RH, 25 °C and 14 lux.

The biting response of mosquitoes fed on either sucrose or glucose diet was quantified by counting the number of landings and the time spent probing by females on a warm body (WB, Kröber et al., 2010). Attraction of individual mosquito enclosed in a Makrolon cage (200 x 260 x180 mm high) was measured during 5 minutes to a sandblasted glass disc heated to skin temperature by the WB (60 mm diameter disk, 20 mm thick) inserted through a port (155 mm long) into the cage. The biting behaviour was measured in a climate chamber at 25 °C, 80% RH and 14 lux, 5 hours into the scotophase. Females were released individually into test cages at least 10 minutes before the start of an experiment. A white filter paper disc (60

mm diameter cut from 185 mm Ederol filters N° 15) was inserted between the glass and the black WB surface to visualize mosquito landings. A PT100 temperature controller maintained the temperature of the WB at 37±0.5°C, assuring a temperature of 35±0.5°C on the surface of the glass. A 0.2 sec pulse of pure CO_2 (volume 6.25 ml, 99.99% CO_2, H_2O < 200 ppm) from a pressurized cylinder (Carbargas, Switzerland) was released into the cage using a solenoid valve to activate the mosquito just before the WB with the attached glass disc was introduced. The mosquitoes were filmed at 60.1 images/s with a GigE Monochrome Camera (DMK 23G618, The Imaging Source, Bremen, Germany) mounted on a rail behind the cages. A CVI STAR-RL-100/69-IR850-12V (Stemmer Imaging AG, Pfäffikon, Switzerland) provided infrared light (850 nm). A foot switch synchronized both the activation of the solenoid valve and the switch on the camera. The number of landings by the mosquito was counted by the same experimenter for all assays. Mosquitoes only land on the heated glass disk to probe and were never observed resting on the glass disc of the WB. The time spent probing was measured after experiments using the "analyze particles" tool of ImageJ (Rasband, 2011). A macro was designed to transform the pictures into an 8 bit grayscale representation from which a black and white image was generated by adjusting the image threshold to 0/76. Each particle of between 120 to 500 px^2 was counted as a mosquito. In this manner, only mosquitoes which landed on the WB and that had closed their wings were counted. The time spent probing by mosquitoes tested individually was estimated by multiplying the number of images on which a mosquito was counted by the time of successive frames:

$$Time\ spent\ probing = Npictures \cdot 0.01664s \qquad (1)$$

Immediately after the test, mosquitoes were anesthetized on ice and their wet mass was recorded using a M3 Microbalance (reading precision of ±1 µg, 1 SD = 2 µg; Mettler, Greifensee, Switzerland). The dry mass was recorded after overnight desiccation at 70°C in an oven.

Statistical analyses

All statistical analyses were performed with R 2.11.1 (R Development Core Team, 2010). Graphical representations were made with R and Excel (Microsoft Office 2007). As stipulated in results, in some electrophysiological experiments, data recorded from several sensilla of the same mosquito were pooled, introducing pseudo-replication. A 3-parameter log-logistic model (function LL.3) was fitted to the number of spikes as a function of increasing concentrations of sucrose using the *drm* command (with a continuous probability distribution) in the *drc* package (Ritz and Strebig, 2013). Responses of different sensilla of *A. gambiae* and *A. aegypti* were pooled if no significant difference in deviance were observed between

models (F tests). In addition, the electrophysiological response thresholds for sucrose between the sexes and among female sensilla were calculated using the ratios of the estimated ED_{50} values. Statistical differences between ED_{50} thresholds were assessed by the SI function in the *drc* package.

The number of spikes generated by the receptor cells within the ventral No 8 and the dorsal No 5 T1 labellar sensilla during stimulation with KCl, glucose, fructose, α and β-methyl-glucosides, the mixtures of glucose and fructose, and sucrose were analyzed by a generalized linear model (GLM) with a Gamma error distribution and a reciprocal link function followed by Tukey HSD post hoc test. The total number of spikes generated by the receptor cells within these two sensilla to 100 mM of each sugar was compared to those generated during stimulation with 10 mM KCl by a GLM with a Gamma error distribution and a reciprocal link function.

A choice index (CI) according to Ignel et al. (Ignell et al., 2010) was used for the representation of the sugar diet preferences:

$$CI_{dietA} = \frac{[(n_{dietA}) + (n_{dietA+dietB})]}{n_{total}} \qquad (2)$$

$$CI_{dietB} = \frac{[(n_{dietB}) + (n_{dietA+dietB})]}{n_{total}} \qquad (3)$$

The number of mosquitoes that choose only diet A and those who choose only diet B was analyzed by a GLM with a quasibinomial error distribution and a logit link function. The number of mosquitoes that choose a particular diet was treated as the dependent variable and sugar diets and sexes as the fixed factors. Significant differences in the percentage of mosquitoes that fed on both solutions as a function of diet and sex was assessed in a separate analysis using a GLM with a binomial error distribution and a logit link function. Mosquito longevity as a function of sugar diet was analyzed with a Cox proportional-hazards model using the *survival* R package (Therneau, 2013) and data were graphed using Kaplan-Meier survivorship curves.

In the biting response experiment, differences between the dry masses and the ratios between water content and dry mass were subjected to two 2-way ANOVA with sugar diet and age as the explanatory factors. The effects of dry mass, water content, the ratio between water content and dry mass and starvation time on the number of landings and duration of probing attempts were investigated with linear models. Separate 2-way ANOVA were made to test the effects of the sugar diet and age on the number of landings and on the duration of probing attempts, respectively. For parametric analyses, homogeneity of variances was

controlled with F-tests. All models were checked for the appropriate distribution of residuals. A 95% confidence interval level was used for all analyses.

6.4 Results

Ultrastructure of labellar sensilla in A. gambiae

Trichoid type 1 (T1) sensilla on each labellar lobe (Fig. 6.1A and B) contain 4 chemoreceptor cells and one mechanoreceptor cell (Kessler et al., 2013). Shorter trichoid type 2 (T2) sensilla 5 µm long are found proximally on the ventral and dorsal side of each labellar lobe from around 40 µm from the tip of the labellum (Fig. 6.1A and B). They arise from a cylindrical socket (Fig. 6.1C) and two receptor cells inervate these sensilla: just below the shaft, a neurone terminates and a second neurone extends its single dendrite to the tip of these hairs (Fig. 6.1D-F). A prominent groove is present on their cuticular surface (Fig. 6.1C and F). Trichoid T1 sensilla are similar to those described by Pappas and Larsen (Pappas and Larsen, 1976) in *Culiseta inornata* (Williston), those described by Owen (Owen, 1971) in *Anopheles atroparvus* (Van Thiel) and by Hill and Smith (Hill and Smith, 1999) in *A. aegypti*. The external morphology of the T2 hairs of *A. gambiae* appears to be similar to those described for *A. aegypti* (Hill and Smith, 1999) and those described in *C. inornata* (Pappas and Larsen, 1976) although 3 neurones inervate these sensilla in *Culiseta*. To our knowledge, no transmission electron microscopic studies has been made on the T2 sensilla of *A. aegypti*

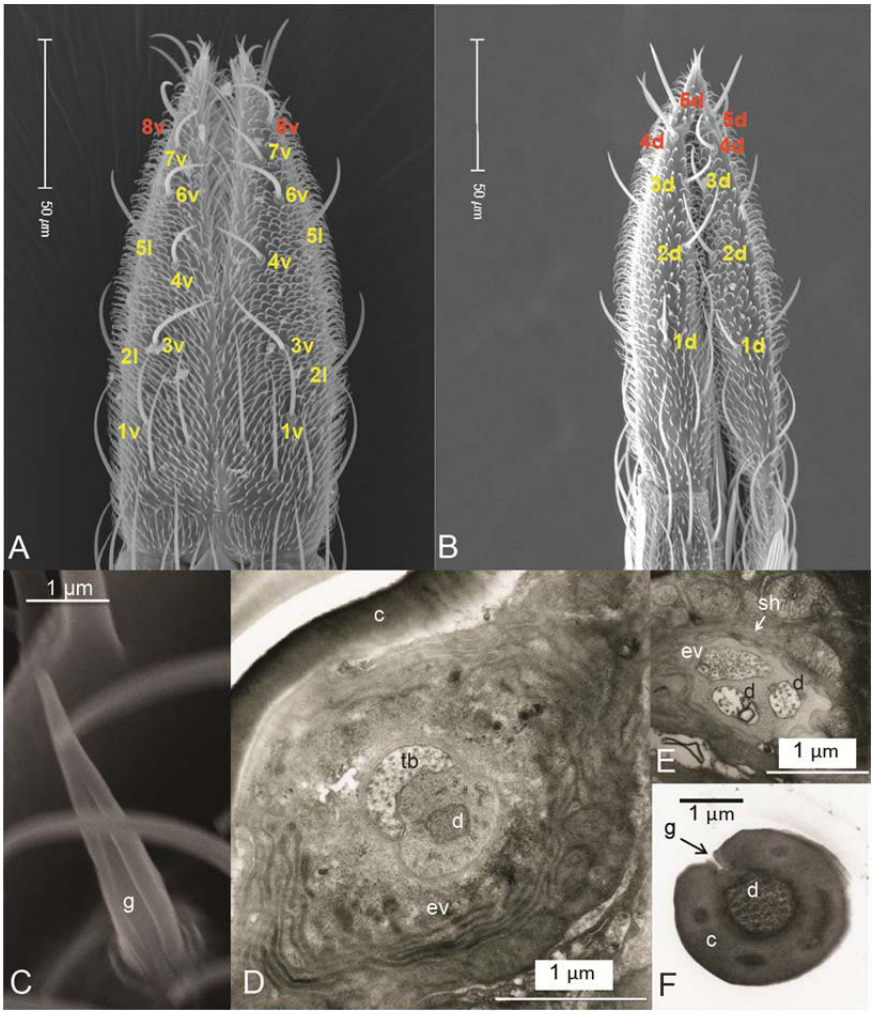

Fig 6.1: Scanning electron micrographs of the labellar lobes of female *A. gambiae*, (A) ventral view and (B) dorsal view. Trichoid type 1 sensilla which possess both sugar and water sensitive neurones are shown in yellow and those which have a sugar sensitive neurone but no water sensitive neurone are shown in red; d dorsal; l lateral; v ventral. T2 sensilla were found proximally from 40 µm from the tip of the labellum. Scanning electron micrograph of a labellar trichoid T2 sensillum (C) and transmission electron micrographs of transverse sections at the base (D), above the ciliary root (E) and in the middle of the sensillum (F); c cuticle; d dendrite; ev enveloping cells; g groove, sh sheath; tb tubular body.

Responses of T1 and T2 labellar sensilla to sucrose and water in A. gambiae *and* A. aegypti

In *A. gambiae*, both a water and a sugar receptor cell were found on the 10 proximal T1 sensilla tested on each labellar lobe following at least three positive recordings (Fig. 6.1 sensilla marked in yellow, and Fig. 6.2). In the 10 proximal sensilla, between 63-100% of the water neurones and between 75-100% of the sugar neurones responded to stimulation with water or 146.07 mM sucrose, respectively. No water sensitive neurone was found in the three distal trichoid T1 sensilla following 7 to 17 recordings from each sensillum but a receptor cell within these sensilla responded to sucrose in 75-100% of the mosquitoes tested as in the more proximal sensilla (Fig. 6.1 sensilla marked in red, and Fig. 6.2).

Water elicits a strong tonic response from a receptor cell in the T2 sensilla (Fig. 6.3A and B) that is not significantly affected by presence of 100 mM sucrose (Fig. 6.3A and C; note the overlapping standard errors of means in 6.3A). When these hairs are bent, a second spike category, characterized by lower amplitude and a phasic response pattern is generated, accompanied by inhibition of the water response (Fig. 6.3D and E). In female *A. aegypti* no water sensitive neurone was found within the ventral sensilla Nos 4, 7, 9, 10 following stimulation with pure water on 3-5 different mosquito preparations although these sensilla were found to respond to sugars (Fig. 6.6).

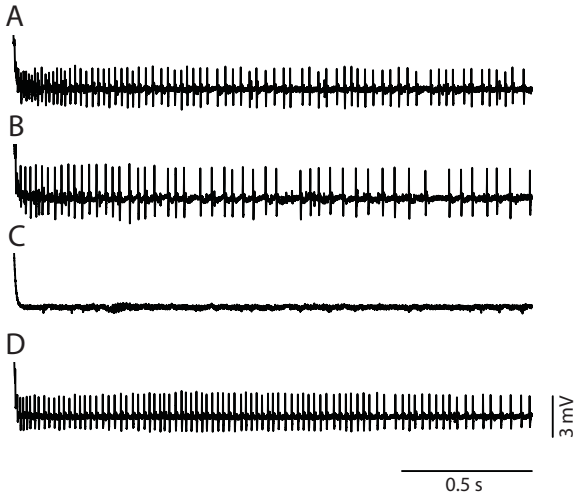

Fig 6.2: Representative electrophysiological responses in the lateral No 5 and the ventral No 8 trichoid type 1 sensilla of *A. gambiae* during stimulation with water (A and C, respectively) and during stimulation with 146.07 mM sucrose in 10 mM KCl (B and D, respectively).

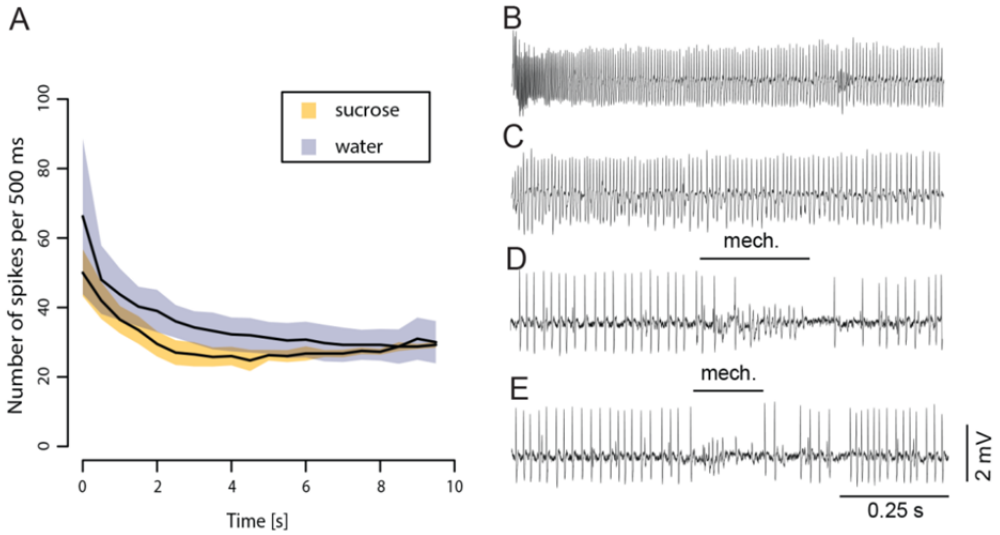

Fig 6.3: Adaptations curves (mean ±s.e.m., N = 4) of action potential frequency in trichoid type 2 sensilla of *A. gambiae* recorded each 500 ms during 10 s of stimulation with water or 100 mM sucrose (A). Representative 1 s electrophysiological responses during stimulation with water (B) and 100 mM sucrose (C). (D) and (E) are responses recorded to water accompanied by mechanical stimulation: where the sensillum was bent with an electrode filled with water: the water receptor cell is silenced when the mechanoreceptor cell (mech.) is activated. The water-sensitive cell fires again when the initial position of the sensillum is restored.

Dose dependent responses of T1 labellar sensilla to sucrose in A. gambiae *and* A. aegypti

The sugar sensitive neurone in lateral No 5 sensilla of males apparently respond in the same manner to sucrose as in females (Fig. 6.4A). Although the ED_{50} for females (4.51 mM) is 1.55 times higher than the corresponding value for males (2.90 mM), this difference is not significant ($P = 0.096$). Similarly, the ED_{75} value for females (11.25 mM) and males (4.15 mM) are marginally not significant ($P = 0.06$). However, the 3-parameter log-logistic models accounting for the data for males and females separately differ significantly from the model where both sexes are pooled ($P < 0.001$).

3-parameter log-logistic models calculated for the action potential frequencies generated as a function of dose of sucrose by the sugar sensitive neurones of the lateral sensilla Nos 2 and 5 (the sensilla type enclosing both a “sucrose” and a “water” neurone) and the ventral No 8 and dorsal No 5 sensilla (the sensilla type with a “sucrose” neurone but no “water”neurone) are shown in figure 6.4B. The ED_{50} of the action potential frequency of the ventral No 8 and dorsal No 5 sensilla (2.46 mM) corresponds to one half of the ED_{50} value for the lateral Nos 2 and 5 sensilla (5.21 mM), and this is higly significant ($P < 0.001$). For the ventral No 8 and dorsal No 5 sensilla the response plateaus at 73 spikes per 2s from around 10 mM and the response of the lateral sensilla Nos 2 and 5 plateaus at 60 spikes per 2 s from around 50 mM sucrose. No significant differences were found between the models fitted to the data for each sensillum tested independently and those fitted where the ventral No 8 and the dorsal No 5 sensilla as well the lateral Nos 2 and 5 were pooled ($P = 0.214$). This means that sugar sensitive neurones in both the ventral No 8 and the dorsal No 5 sensilla and the lateral sensilla Nos 2 and 5 respond similarly to increasing doses of sucrose. However, the two groups differ, i.e. ventral 8 and dorsal 5 sensilla differ from lateral 2 and lateral 5 as the model taking into account the two groups was significantly different ($P < 0.001$) from the model with the four sensilla pooled.

In *A. aegypti*, no significant differences were found between the logistic models fitted to the data for sugar sensitive neurone responses to sucrose in each sensillum tested independently, *i.e*. Nos 1, 4, 7 and 9, and those where the ventral Nos 4, 7 and 9 were pooled ($P = 0.202$, Fig. 6.4C). However, the two groups differ, i.e. ventral Nos 4, 7 and 9 differ from No 1 ($P < 0.001$). With the pooled data from sensilla Nos 4, 7 and 9 we calculated the maximum frequency at 61 spikes per 2 s and the ED_{50} at 1.14 mM sucrose. However, the response pattern from the ventral sensilla No 1 was significantly different ($P < 0.001$): the maximum frequency was estimated at 104 spikes per 2 s and the ED_{50} was situated at 3.03 mM sucrose, significantly higher than for the other sensilla tested ($P < 0.001$).

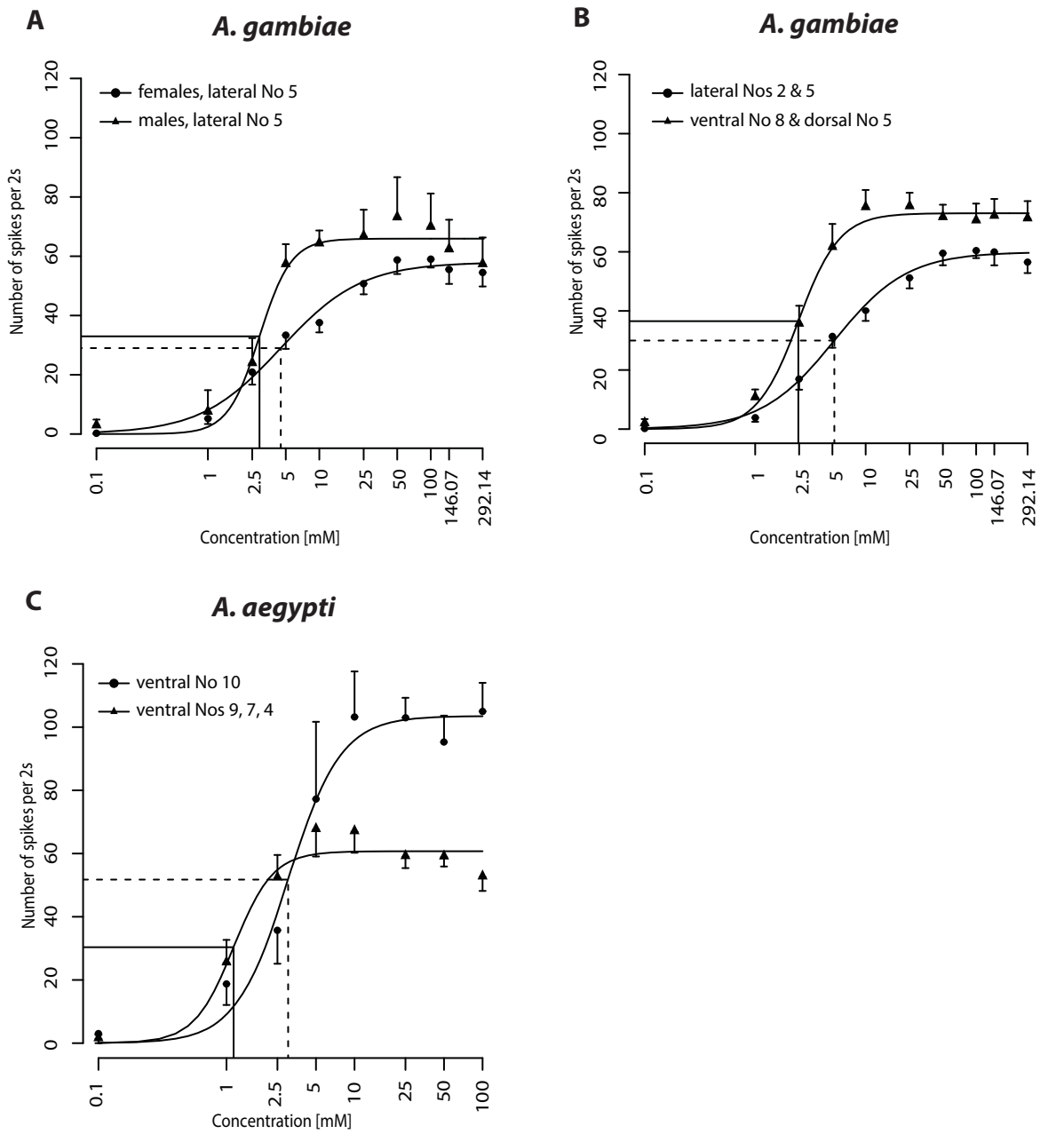

Fig 6.4: Means ±s.e.m. and fitted 3-parameter log-logistic models of the number of spikes generated per 2 s by the trichoid type 1 sucrose cells in the lateral labellar sensillum No 5 of female (circles) and male (triangles) *A. gambiae* (A), in the ventral No 8 and dorsal No 5, namely the sensilla possessing a "sucrose" neurone but no "water" neurone (triangles) and the lateral Nos 2 and 5, namely the sensilla possessing both a "sucrose" and "water" neurone (circles) of female *A. gambiae* (B) and in the ventral No 10 (circles) and the ventral Nos 9, 7, and 4 sensilla (triangles) of female *A. aegypti* (C). The sensilla were stimulated with increasing concentrations of sucrose in 10 mM KCl. Full and broken straight lines indicate the ED_{50} values. Between 3 to 4 *A. aegypti* and 5 to 18 *A. gambiae* were tested for each sensillum.

Labellar receptor cell sensitivity to glucose and fructose as constituents of sucrose in A. gambiae *and* A. aegypti

In *A. gambiae* the total number of spikes recorded per 2 s during stimulation of the ventral sensillum No 8 and the dorsal sensillum No 5 with 10 and 20 mM glucose, fructose, methyl β-D-glucospyranoside or methyl α-D-glucopyranoside in 10 mM KCl was not significantly different from 10 mM KCl alone (GLM with a Gamma error distribution, rd 154.99, df 239, followed by a Tukey HSD post hoc test, *P* values between 0.393 and 1, Fig. 6.5). The three mixtures of glucose and fructose tested generated a total number of spikes lower but not significantly different to 5, 10 and 20 mM sucrose (*P* values between 0.059 and 1, Fig. 6.5). The equimolar mixture 10 mM fructose plus 10 mM glucose generated a mean number of spikes (45 ±4) higher than 15 mM fructose plus 5 mM glucose (34 ±5, $P = 1$) or 5 mM fructose plus 15 mM glucose (27 ±4, $P = 0.875$) although these differences were not significant (Fig. 6.5). Interestingly, the equimolar mixture of 10 mM fructose with methyl α-D-glucopyranoside elicited a spike frequency from the sugar sensitive neurones comparable to those generated by sucrose or with the mixtures of glucose and fructose (*P* values between 0.6 and 1). However, the number of spikes recorded during stimulation with the equimolar mixture of 10 mM fructose and methyl β-D-glucopyranoside was significantly lower than for KCl ($P < 0.05$, Fig. 6.5).

In *A. aegypti*, fructose and glucose tested alone at 10, 50 and 100 mM, mixtures of fructose and glucose at 25:75 mM, 50:50 mM and 75:25 mM, 50 and 100 mM sucrose in 10 mM KCl elicited spike frequencies significantly higher than 10 mM KCl alone (poolled data from all sensilla, GLM with a Gamma error distribution, rd 195.05, df 359, $P < 0.001$). The mixtures of fructose and glucose at 25:75 mM (45.08 ±2.76 spikes), 75:25 mM glucose (46.41 ±3.1 spikes) and 50:50 mM (42.97 ±3.25 spikes), all diluted in 10 mM KCl, generated a similar mean number of spikes ($P = 0.59$, 0.73 and 0.838 respectively). The spike frequencies recorded during stimulation with these three mixtures were significantly higher than recorded for 100 mM fructose (23.68 ±2.34 spikes, $P < 0.001$ in the three cases) but barely not significantly different to 100 mM glucose (35.13 ±3.13 spikes, $P = 0.075$, 0.054 and 0.149, respectively), or sucrose tested at either 50 mM (49.56 ±3.96 spikes, $P = 0.54$, 0.681 and 0.356, respectively) or 100 mM (51.89 ±3.57 spikes, $P = 0.312$, 0.439 and 0.177 respectively) in 10 mM KCl. The response heat map shows heterogeneity in sugar sensitivity between labellar sensilla of *A. aegypti* (Fig. 6.6), but the overall image needs to take into account the low sample size and the high variability in the responses obtained.

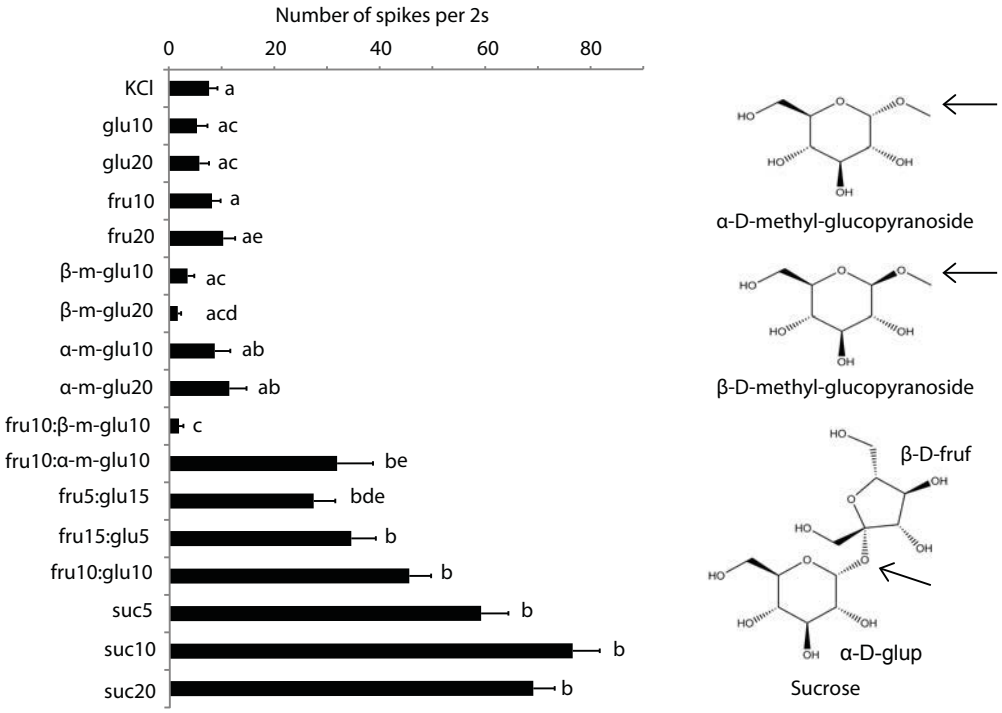

Fig. 6.5: Mean ± s.e.m. of the total number of spikes generated per 2 s by chemoreceptor cells in the ventral No 8 and the dorsal No 5 sensilla of female *A. gambiae* during stimulation with 10 mM KCl, 10 (glu 10) or 20 mM glucose (glu 20), 10 (fru 10) or 20 mM fructose (fru20), 10 (β-m-glu 10) or 20 mM methyl β-D-glucopyranoside (β-m-glu 20), 10 (α-m-glu 10) or 20 mM methyl α-D-glucopyranoside (α-m-glu 20), 10 mM fructose plus 10 mM methyl β-D-glucopyranoside (fru10:β-m-glu10), 10 mM fructose plus 10 mM methyl α-D-glucopyranoside (fru10:α-m-glu10), 5 mM fructose plus 15 mM glucose (fru5:glu15), 15 mM fructose plus 5 mM glucose (fru15:glu5), 10 mM fructose plus 10 mM glucose (fru10:glu10), and to 5, 10 or 20 mM sucrose each diluted in 10 mM KCl. Action potential frequencies with different letters are significantly different; GLM with a binomial error distribution, followed by a Tukey post-hoc test, $P < 0.05$. Between 8 to 20 sensilla were tested per treatment. Arrows on the molecular structures show the α or β position of the anomeric groups of the glucopyranosides.

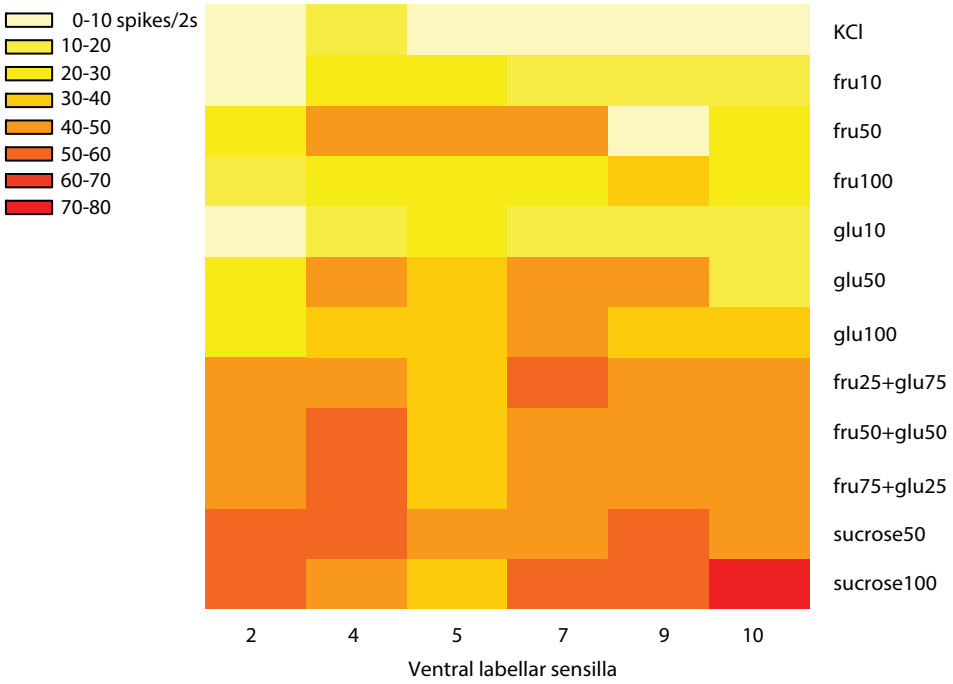

Fig 6.6: Heat map of the mean number of spikes per 2 s recorded during stimulation of the ventral trichoid sensilla on the labellum of female *A. aegypti* (numbered according to Hill and Smith, 1999) with 10 mM KCl and to 10 mM fructose, 50 mM fructose, 100 mM fructose, 10 mM glucose, 50 mM glucose, 100 mM glucose, 25 mM fructose plus 75 mM glucose, 50 mM fructose plus 50 mM glucose, 75 mM fructose plus 25 mM glucose, 50 mM and 100 mM sucrose, each diluted in 10 mM KCl. Between 2 to 7 mosquitoes were tested for each sensillum.

Selectivity of labellar receptor cells to sweet tastants

In recordings where various sugars where tested on the ventral No 8 and the dorsal No 5 sensilla of *A. gambiae*, only melezitose and sucrose at 100 mM in 10 mM KCl elicited spikes (18.8±5.5 and 53.4±5.1 spikes respectively) in significantly higher numbers than 10 mM KCl (4.6 ±2.5 spikes, GLM with a Gamma error distribution, rd 76.413, df 76, $P < 0.05$ and $P < 0.01$ respectively, Fig. 6.7). Despite the fact that the number of spikes generated by 100 mM fructose (10.8 ±2.1 spikes) or 100 mM glucose (12 ±2.6 spikes) was higher than by 10 mM KCl, these differences were not significant ($P = 0.129$ and 0.0915 respecitvely). However, the change in spike shape (monophasic versus biphasic, Fig. 6.7) and the presence of some doublets (with the cell responding to KCl) when doses between 0.1 to 100 mM were tested suggest a marginal but non-systematic activation of the sugar sensitive neurones in the ventral No 8 and dorsal No 5 sensilla by these two monosaccharides. The other sugars tested failed to activate receptor cells in these two sensilla at 100 mM (Fig. 6.7) or at lower doses (Figs. S6.1 and S6.2). No differences in specificity were found between the sugar sensitive neurones of the ventral No 8 and the dorsal No 5 sensilla (Figs. 6.7 and S6.1). Although the sugar sensitive neurones in the lateral sensilla Nos 2 and 5 seem to react as those of the ventral No 8 and dorsal No 5 sensilla, strict differences in sensitivity to sugars was difficult to assess by counting the total number of spikes, i.e. without discriminating between the receptor cell units. There was a slight activation of the water sensitive neurone in some recordings from lateral sensilla Nos 2 and 5 (Fig. S6.2). The human sweet-tasting compounds myo-inositol and glycerol also failed to activate these sugar sensitive neurones (Figs. S6.1 and S6.2). A receptor cell in the ventral No 8 and dorsal No 5 sensilla was slightly activted during stimulation with 1% thaumatin although this result was hardly reproducible due to the poor contact obtained at such a high concentration of the protein and without any evidence as to wether it was the sugar sensitive neurone or not (Figs. S6.1 and S6.2).

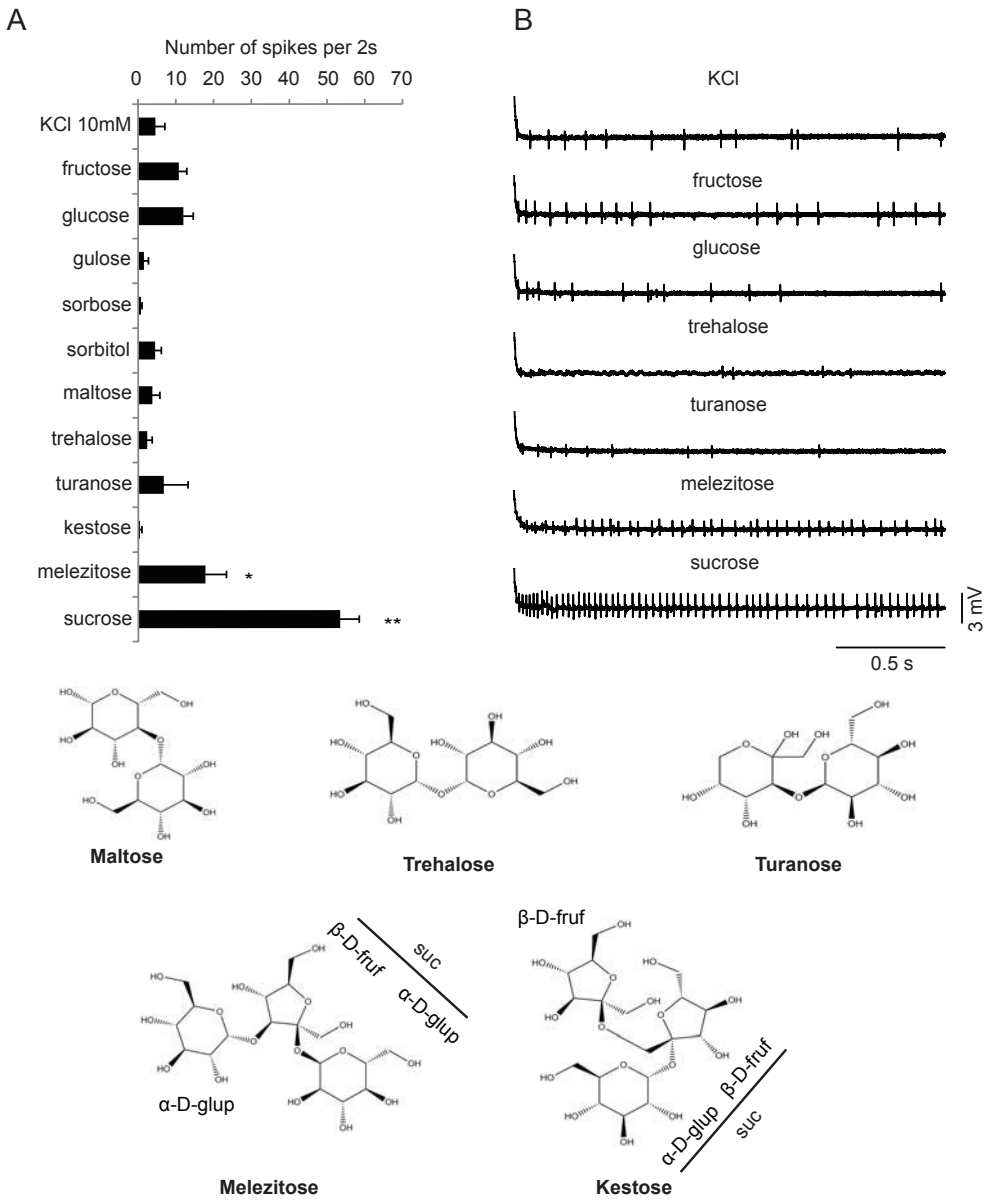

Fig 6.7: Mean ± s.e.m. total number of spikes per 2 seconds generated by labellar neurones housed in the ventral No 8 and dorsal No 5 sensilla of female *A. gambiae* during stimulation with 10 mM KCl or with 100 mM fructose, glucose, gulose, sorbose, sorbitol, maltose, trehalose, turanose, kestose, melezitose or sucrose, each diluted in 10mM KCl (A). Only receptor cells responding to 100 mM sucrose in 10 mM KCl, tested as a positive control at the end of the stimulation series, were included in the analysis. Only melezitose and sucrose show a number of spikes significantly higher than 10 mM KCl. Between 4 to 10 sensilla were tested per treatment; GLM with a Gamma error distribution, $*P < 0.05$, $**P < 0.01$. (B) Representative 2s electrophysiological responses in the ventral No 8 or in the dorsal No 5 sensilla to 100 mM sugar solutions in 10 mM KCl. Structures are depicted in the lower part of the figure to show the residues that composed each polysaccharide.

Sugar diet preferences of A. gambiae

Sugar diets were preferred in the following order by *A. gambiae*: sucrose > the mixture of glucose and fructose > fructose > glucose > water (GLM with a quasibinomial error distribution, rd 125.34, df 90; Fig. 6.8). Sex has no significant effect on the sugar diet preference and was consequently removed from the model. Mosquitoes largely prefer sucrose than its monosaccharide constituents glucose or fructose alone ($P < 0.001$ in both cases). Of the mosquitoes that choose only one diet, 3.05% of mosquitoes fed only on glucose when tested against sucrose and 10.88% fed on fructose tested against sucrose. The preference for sucrose was significantly lower when sucrose was tested against fructose rather than against glucose ($P < 0.05$) as mosquitoes prefer to feed on fructose rather than on glucose when offered a choice between these two monosaccharides ($P < 0.001$). When offered a choice between a sucrose solution and a mixture of glucose and fructose, mosquitoes showed a less marked preference for sucrose as 31.12% of the mosquitoes that fed only on one diet choose the mixture of glucose and fructose and this is significantly different from 50% ($P < 0.001$). Mosquitoes became slightly less discriminating when given the choice between either fructose or glucose and the mixture of glucose and fructose, compared to what they do when they have the opportunity to feed on sucrose: on average, 21.88% of mosquitoes fed on fructose and 8.51% fed on glucose when the monosaccharides were compared to the mixture of glucose and fructose. These percentage tend to be higher than when these monosaccharides were presented with sucrose ($P < 0.05$ and $P = 0.072$ respectively). *A. gambiae* also prefer to feed on the 292.14 mM sucrose solution rather than on a solution of melezitose at the same molarity, comparable to what was observed when 292.14 mM sucrose was compared to 584.28 mM fructose ($P = 0.121$) or 584.28 mM glucose ($P = 0.475$).

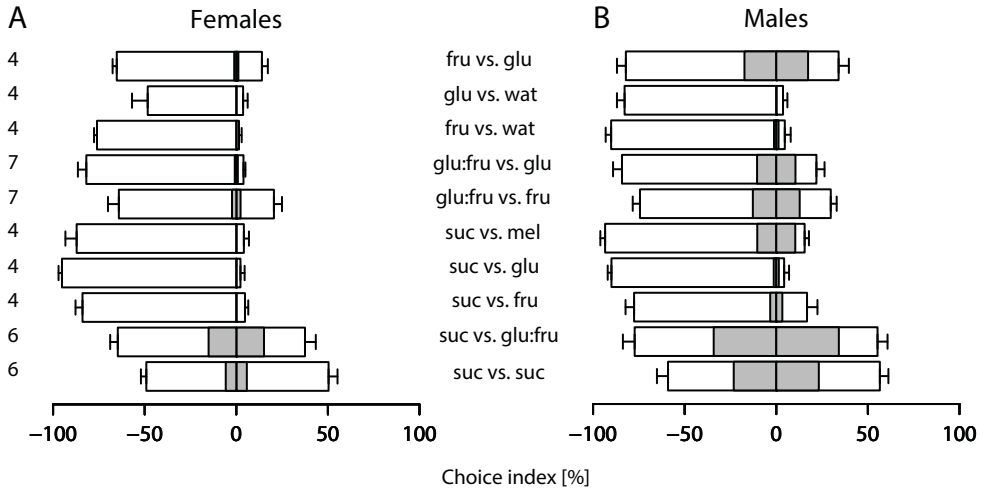

Fig 6.8: Mean ± s.e.m. of sugar choice indices by female (A) and male (B) *A. gambiae* for a mixture of 584.28 mM fructose versus 584.28 mM glucose; either 584.28 mM glucose or 584.28 mM fructose versus water; 292.14 mM glucose with 292.14 mM fructose versus 584.28 mM glucose alone or versus 584.28 mM fructose alone; 292.14 mM sucrose versus 292.14 mM melezitose, 584.28 mM glucose or 584.28 mM fructose; 292.14 mM sucrose versus a mixture of 292.14 mM glucose with 292.14 mM fructose or the same solution of 292.14 mM sucrose. The number of choice tests is shown on the left. Percentages of mosquitoes tested that fed on both solutions are marked in grey.

For the percentage of mosquitoes that fed on both solutions, interactions between the choices and sexes were not significant (*P* between 0.09 and 0.936) and were consequently removed from the model. However, males were less discriminating as the numbers feeding on both solutions were higher for all choices ($P < 0.001$, GLM with a binomial error distribution, rd 54.443, df 89). The percentage of female and male *A. gambiae* tested that fed on both solutions was higher when the mosquitoes had a choice between a sucrose solution and the mixture of glucose and fructose (25.32%) and between two sucrose solutions (13.53%) rather than in the other choices tested (between 0 to 9%; $P < 0.05$), except for the choice between two sucrose solutions and the choice between 584.28 mM glucose versus 584.28 mM fructose (9%; $P = 0.206$). The percentage of mosquitoes tested that fed on both solutions in the choice between the mixture of glucose with fructose versus glucose (5.8%) was significantly higher than the percentage of mosquitoes that feed on both solutions when given the choice between sucrose and glucose (0.6%, $P < 0.05$). Likewise, the percentage of mosquitoes tested that fed on both solutions in the choice between the mixture of glucose with fructose versus fructose (7.7%) was significantly higher than the percentage of mosquitoes that fed on both solutions when given the choice between sucrose and fructose (1.8%, $P < 0.05$)). This suggests that discrimination is more arduous for mosquitoes when monosaccharide solutions are tested against the mixture of fructose and glucose rather than against sucrose. After the 21h30 feeding period, the percentage of the tested mosquitoes considered as unfed was unsurprisingly higher in the choice between the less preferred glucose and water (31.1%) rather than for the other treatments (between 5.1 to 14.6%).

Longevity on different sugar diet

Sugars contributing to the highest longevity were in decreasing order, sucrose = fructose > glu&fru > glucose for both sexes of *A. gambiae* (Fig. 6.9). Water alone did not allow mosquitoes to survive longer than 4 days, shorter than for any of the sugars. Mosquito longevity between sugar treatments differed significantly (Cox proportional hazards regression, $P < 0.01$), except between fructose and sucrose ($P = 0.48$). The maximum longevity was observed for females (28 days) on sucrose and fructose and for males on fructose (35 days). A significant interaction between sugar diets and sex was found: males survived on average 4 days longer than females on fructose and 3 days more on sucrose ($P < 0.001$ in both cases), whereas females survived on average half a day longer on glucose ($P < 0.05$) but the longevity of both sexes was the same on the mixture of glucose plus fructose or on water ($P = 0.103$ and 0.145, respectively).

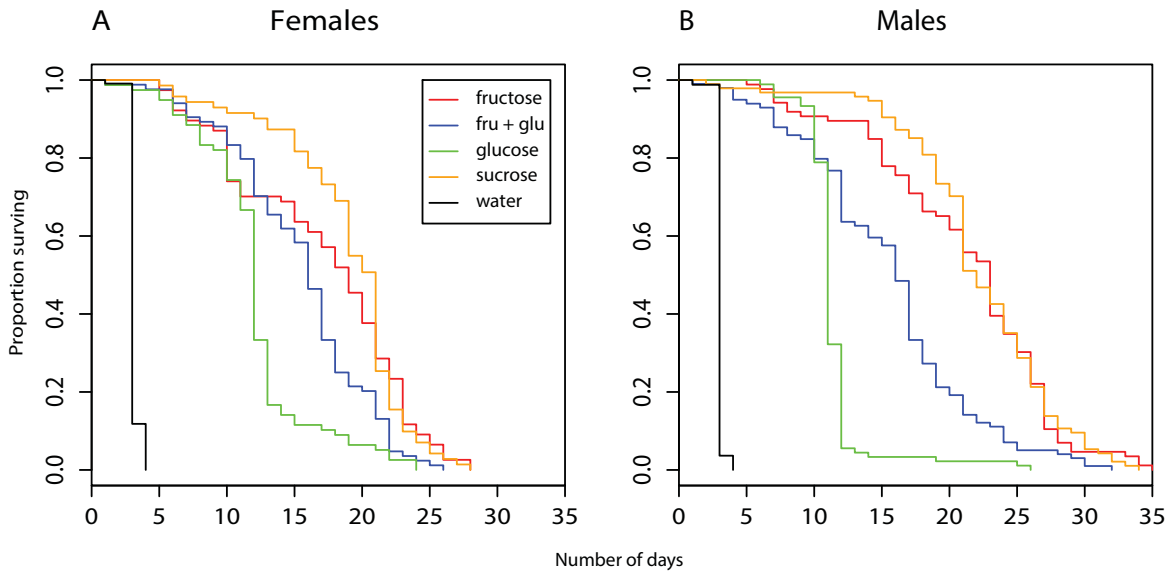

Fig 6.9: Kaplan-Meyer analysis of survival in days of female (A, between 71 to 110 individuals tested) and male (B, between 86 to 99 individuals tested) *A. gambiae* fed on different 10% sugar solutions.

Biting response as a function of sugar diet

After a 12 h starvation period, 78 and 84% of 4 day-old females tested engorged on sucrose and glucose solutions respectively, the diets on which they had been maintained. At 10 days old, 91% of females fed on sucrose although only 34% fed on glucose. This means that 10 day-old mosquitoes became more selective as most of them renounced to feed on glucose.

We measured the dry mass, as an indicator of the energetic state of the insects, the body water content and also calculated the ratio between body water content and dry mass after the mosquitoes have been tested in the WB test. Females maintained on sucrose have a higher dry mass (0.45 ±0.01 mg) but tended only to have a higher water content (0.83 ±0.02 mg, 1-way ANOVA, $F_{1,82} = 4.066$, $P = 0.047$) than those maintained on glucose (0.34 ±0.01 mg and 0.79 ±0.01 mg). Consequently, the ratio between the water content and the dry mass is higher for females maintained on glucose (2.36 ±0.05) than those maintained on sucrose (1.88 ±0.02, Fig. 6.10). The interactions between dry mass and age and, consequently, the water content/dry mass ratio and age are significant but not for the body water content (Fig. 6.10). The dry mass is lower for 10 day-old females (0.31 ±0.01 mg) than for 4 days-old females (0.36±0.1 mg) maintained on glucose but higher for 10 day-old (0.47 ±0.02 mg) than for 4 day-old (0.43 ±0.01 mg) females maintained on sucrose (2-way ANOVA, effect of sugars $F_{1,80} = 58.411$, $P < 0.001$; effect of age $F_{1,80} = 0.135$, $P = 0.71$; effect of the interaction $F_{1, 80} = 10.406$, $P < 0.01$). Consequently, the water content/dry mass ratio is lower for 4 day-old females (2.25 ±0.04) maintained on glucose than for 10 day-old females (2.51 ±0.09) and higher for 4 day-old females (1.93 ±0.02) maintained on sucrose than for 10 day-old females (1.82 ±0.03, 2-way ANOVA, effect of sugars: $F_{1, 80} = 93.32$, $P < 0.001$, effect of the age: $F_{1,80} = 2.5$, $P = 0.118$, effect of the interaction: $F_{1,80} = 13.5$, $P < 0.001$).

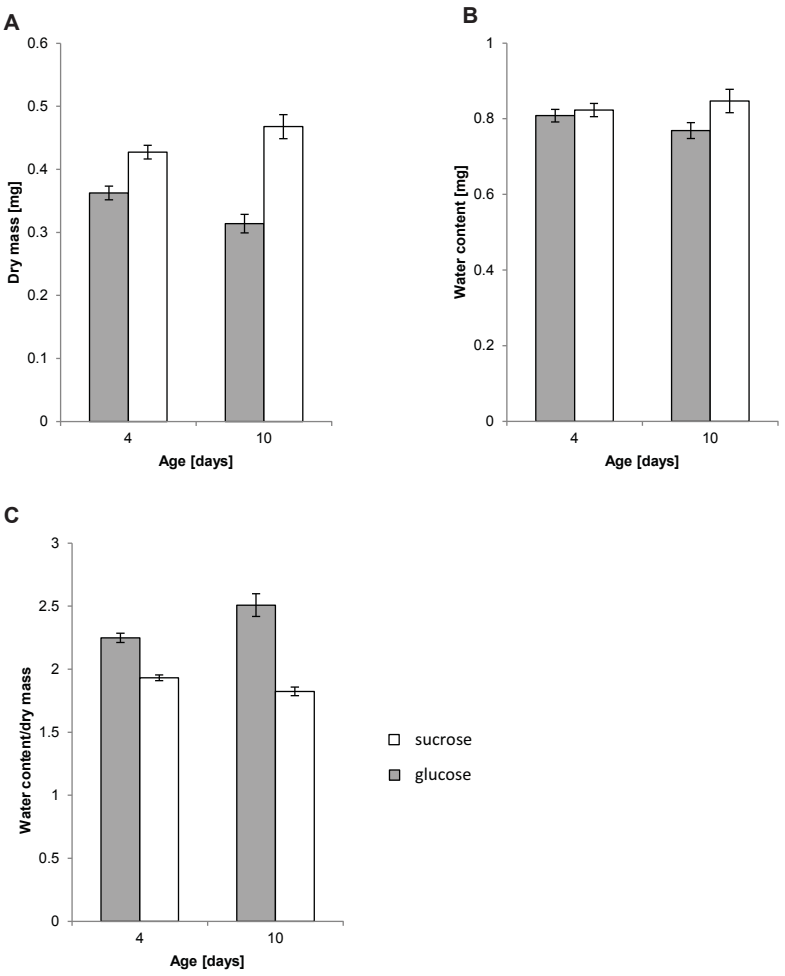

Fig 6.10: Mean ± s.e.m. of the dry mass (A) the water content (B) and the ratio between water content and dry mass (C) of 4 and 10 day-old female *A. gambiae* maintained on glucose (grey bars) or on sucrose (white bars) following tests of their motivation to bite in the warm body assay (see Fig. 6.11). Between 19 and 24 mosquitoes were tested with each treatment.

We found no correlation between the numbers of landings or the time spent probing and dry mass, body water content or the ratio between body water content and dry mass of females (linear models, all *P* between 0.309 and 0.81). The starvation time (the time between the end of the feeding period and the WB test) has a tendency to be correlated with the number of landings on the WB (linear model, $F_{1,107} = 3.165$, $P = 0.078$, slope = 0.044, $R^2 = 0.02$) and with the time spent probing (linear model, $F_{1,108} = 4.154$, $P = 0.044 < 0.05$, slope = 0.105, $R^2 = 0.03$). From the measured parameters, the sugar regime had a significant effect on the number of landings on the WB (1-way ANOVA, $F_{1,\ 107} = 10.15$, $P < 0.01$, Fig. 6.11a) and the time spent probing by the mosquitoes (1-way ANOVA, $F_{1,108} = 6.299$, $P < 0.05$, Fig. 6.11b). The effect of age and the interaction between the sugar regime and age were not significant and were removed from these two models. On average, females land 16.8 ±1.66 times and spent 40.69 ±3.59 s to probe over 5 minutes on the WB when fed on sucrose but landed more frequently (25.22 ±2.06 times) and spent more time to probe (54.60 ±4.47 s) when fed on glucose.

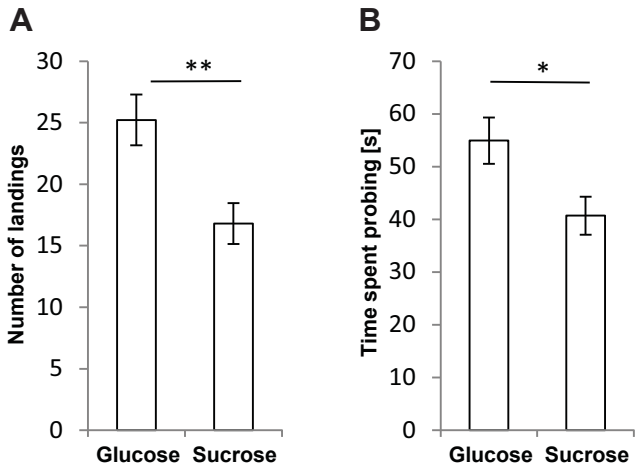

Fig 6.11: Mean ± s.e.m. of the number of landings (A) and the time spent probing (B) by female *A. gambiae* (pooled data of 4 and 10 day-old mosquitoes) on a warm body heated to skin temperature. Prior to tests, the mosquitoes were fed with 10% glucose or 10% sucrose; 55 mosquitoes tested in each treatment (1-way ANOVA, $^{**}P < 0.01$, $^{*}P < 0.05$).

6.5 Discussion

In this study, we show that the neurones of the labellar T1 sensilla of *A. gambiae* are specifically tuned for sucrose. This disaccharide is also preferred as a diet over glucose or fructose. Our data suggest that sucrose and glucose diets may have two opposite effect on the vectorial capacity of females: sucrose allows a higher survival rate than the monosaccharide glucose although the biting response is lower in females with exclusive access to sucrose than for those with access only to glucose.

Neurophysiology of sugar sensitive neurones

All long trichoid T1 sensilla on the labellum of female and male *A. gambiae* tested respond to sucrose. Those from the labellum tip do not house a water sensitive neurone and their sugar sensitive neurone respond to sucrose at higher spike frequencies than those in sensilla situated more proximaly on the labellum which also possess a water receptor cell. Although various sugars, in addition to glucose, fructose and sucrose, have been identified in the leaves and flowers of plant species fed on by *A. gambiae* (Manda et al., 2007b; Nyasembe et al., 2012), the sugar sensitive neurone of the T1 labellar sensilla seems to be specifically activated by sucrose. Except for sucrose, the mixure of glucose and fructose, the mixture of α-D-methylglucopyranoside and fructose and to a lesser extent melezitose, all the other sugars tested in this study do not significantly affect sugar receptor cell activity.

No water sensitive neurone was found in the T1 labellar sensilla of *A. aegypti* tested although they do house a sugar sensitive neurone which appears to be more broadly tuned than those of *A. gambiae*, in that it responds preferentially to sucrose, but also responds, to a lesser extent, to glucose and fructose. As in *A. gambiae*, the neurones in the most distal labellar sensilla seem to generate a maximum spike frequency in response to sucrose that is higher than for neurones in the other sensilla tested.

In *A. gambiae*, the action potential frequency generated by the sugar sensitive neurones appears to be lower during stimulation with the 5:15, 15:5 and 10:10 mM mixtures of glucose and fructose than during stimulation with sucrose, although the differences were not significant. Moreover, neuronal activation seems to be slightly higher during stimulation with the 10:10 mM equimolar mixture than during stimulation with the 5:15 mM or the 15:5 mM mixtures. Although the ligand of the sugar receptor (Sr) is probably sucrose, the two monosaccharides could presumably bind to this site weakly. Morita and Shiraishi (Morita and Shiraish, 1968) have already proposed a heterodimeric model for the receptor sites of the labellar sugar sensitive neurones in the fleshfly *Boettcherisca peregrina* Robineau-Desvoidy, composed of two subunits that are simultaneously occuped by one disaccharide or two

monosaccharide molecules. Synergism between glucose and fructose in activating the sugar sensitive neurone in the ventral No 8 and dorsal No 5 sensilla is demonstrated in the present work and has already been described for the sugar sensitive neurone in the lateral No 5 sensilla (Kessler et al., 2013). As such, it seems to be common to all sugar sensitive neurones in long trichoid labellar sensilla (T1) of *A. gambiae*. Despite the fact that spike frequencies recorded during stimulation with the three mixtures of fructose and glucose (25:75, 75:25 and 50:50 mM) were higher than during stimulation with 100 mM fructose or 100 mM glucose in *A. aegypti*, the concentrations tested were too high to establish any differences between the three mixtures or to discriminate for a synergetic effect between fructose and glucose in this species. In *A. gambiae*, the anomeric form α of glucopyranosyl appears also to be fundamental for binding the receptor as a mixture of α-D-methylglucopyranosyl with fructose activates the sugar sensitive neurone whereas the mixture of β-D-methylglucopyranosyl with fructose does not. In α and β-D-methylglucopyranosyls the hydroxyl group of the C1 is methylated whereas in sucrose this group is condensed to form the bond with the β-D-fructofuranoside. Apparently, methylation of this hydroxyl group does not affect binding to the receptor. Turanose, an isomer of sucrose constituted of an α-D-fructofuranoside linked by its C3 to the C1 of the α-D-glucopyranosyl (sucrose is formed by a β-D-fructofuranoside linked by its C2 to the C1 of the α-D-glucopyranosyl) fails to activate the sugar sensitive neurone. The trisaccharide melezitose which is composed of a molecule of sucrose linked to glucose activates the neurone to a lesser extent than sucrose does, due to the presence of the supplementary α-D-glucopyranosyl linked to the C3 of the β-D-fructofuranoside. On the contrary, kestose, another trisaccharide composed of a molecule of sucrose linked to a supplementary β-D-fructofuranoside via the C1 of the β-D-fructofuranoside failed completely to activate the sugar sensitive neurone.

The strong specificity of the labellar sugar receptor cells of *A. gambiae* to sucrose contrasts with *A. aegypti* and to what is known in other dipteran species like *Drosophila*. In the latter, even though sucrose seems also to be the best activator of the sugar sensitive neurone of L-type labellar sensilla*,* these taste cells also appear to be activated by the disaccharides maltose, turanose or trehalose tested at 100 mM, or by the monosaccharide, m-α-glucopyranoside which appears to be a better ligand than glucose or fructose (Dahanukar et al., 2007; Hiroi et al., 2002). In *Drosophila*, the response to glucose, methyl α-D-glucopyranoside, trehalose and melezitose is mediated by neurones expressing Gr5a, although Gr64a is required to detect fructose and to a less extend sucrose, maltose or turanose molecules (Dahanukar et al., 2007). A complex pattern of co-expression of the eight Sr genes occurs in taste neurones of *Drosophila* (Dahanukar et al., 2007; Kent and

Robertson, 2009). Seven and eight functional Sr genes are present in the genome of *A. aegypti* and *A. gambiae,* respectively, but no direct orthology have been established for Srs between *Drosophila* and mosquitoes (Kent and Robertson, 2009). This could explains the differences in sweet taste sensitivity between *A. gambiae and A. aegypti* on the one hand and *Drosophila*.

Neurones in the T2 sensilla displaying a strong tonic response when stimulated with water were not found to be affected by 100 mM sucrose. The dendrite extending to the tip of each T2 sensillum fills the lumen of the hair (Fig. 6.1 F). This is typical of insect hygroreceptors that use a hygromechanical transduction mode (Tichy and Kallina, 2010). This transduction process is based on swelling or shrinking of the hygroscopic cuticle of the sensilla (Tichy and Kallina, 2010). These kinds of hygroreceptor cells respond to both liquid water and to variation in relative humidity of the air and, as shown here for the T2 sensilla of *A. gambiae*, their responses are affected by movements of the sensilla (Yokohari, 1978; Fig. 6.3 D and E). Although the neurone ending below the shaft seems to be a mechanoreceptor cell sensitive to deflection of the hair, as already noted in *C. inornata* (Pappas and Larsen, 1976), the presence of a dry cell or a thermoreceptor cell was not tested on the T2 sensilla. It could be interesting to test if the neurone sensitive to water in the T2 sensilla can be activated by an increase in RH. It was shown recently that floral humidity is a reliable indicator of nectar volume used by pollinators during foraging (von Arx et al., 2012). Moreover, water vapour in air interacts synergetically with temperature to induce the biting behaviour of tsetse flies which possess hygroreceptor cells on their maxillary palps (Chappuis et al., 2013). It could be that neurones in the T2 sensilla of *A. gambiae* are involved in the perception of water vapour which was recently confirmed as being involved in blood feeding behaviour of mosquitoes (Klun et al., 2013).

Sugar preferences

Results from the sugar feeding preference assays correlate well with the electrophysiological findings. Sucrose and the mixture of its two monosaccharide constituents, glucose and fructose, which both activate the labellar sugar sensitive neurones are prefered over fructose alone or glucose alone, both of which are weaker neuronal activators. Likewise sucrose, which activates the sugar sensitive neurone at a slightly higher frequency than the mixture of glucose and fructose, is prefered over the monosaccharide mixture. After a 24h starvation period, Salama (Salama, 1966) found in no-choice capillary feeding experiments with *A. aegypti* similar median acceptance tresholds for sucrose and fructose (23 and 20 mM respectively) but a higher one for glucose (108 mM). Despite the fact that melezitose

activates labellar sugar sensitive neurones in *A. gambiae*, no significant reduction in the preference for sucrose was observed in the presence of either melezitose, glucose or fructose. Salama found a median acceptance threshold for melezitose of 113 mM comparable to that of glucose but much lower than for the other trisaccharide, raffinose (445 mM) in *Aedes*. Also, in *C. inornata,* Schmidt and Friend (Schmidt and Friend, 1991) found that sucrose was the best sugar-feeding stimulant followed by the trisaccharide melezitose and the equimolar mixutre of glucose and fructose. These authors suggest that the best sugars are composed of α-glucopyranoside and fructoside residues. Accordingly, in *C. inornata*, melezitose, raffinose and turanose are strong phagostimulants. Interestingly, maltose and trehalose also proved to be very strong phagostimulants for *C. inornata*. Fructose and glucose alone were found to be "less potent but still phagostimulatory" in *C. inornata*. Synergism between glucose and fructose as feeding stimulants was found in *A. aegypti* (Ignell et al., 2010), *C. inornata* (Schmidt and Friend, 1991) and in this study for *A. gambiae* and seems to be a general rule in dipterans as it was also recorded for the fly *Phormia regina*, Meigen (Dethier et al., 1956). Although sucrose was the preferred sugar in our two-choice assay, *A. gambiae* will feed on fructose or on glucose presented alone against water. This finding strongly suggests expression of Srs somewhere else other than on the labellar lobes. Mosquitoes possess taste neurones sensitive to sugars on their legs which initiate probing with the mouthparts when stimulated as shown by Pappas and Larsen (Pappas and Larsen, 1976) and Pappas and Larsen (Pappas and Larsen, 1978) for *Culiseta.* In this species, the inner face of the labellum carries sensilla that when stimulated with a sucrose solution in conjonction with the peg organs on the labrum tip serve to initiate pumping. Peg organs on the tip of the labrum of mosquito spp. are described in Lee and Craig (Lee and Craig, 1983) but most authors have argued that these chemosensory units are probably involved in blood-feeding (Lee and Craig, 1983; McIver, 1982).

The fact that males were found less discriminating than females might be explained by their lower teneral reserves and body size (Briegel, 1990), their lower ability to synthetise energy reserves from sugar meals (Gary et al. 2009) and their higher foraging activity (Gary and Foster. 2006). Females, on the other hand, are not restricted to feed on nectar and can also synthetitise energy reserves from blood meals (Gary et al. 2009).

Tastants do not always rhyme with nutritive reward

Although sucrose provides the mosquitoes a longer lifespan and glucose appears to be a less suitable sugar, mosquito longevity recorded on sugar diets does not fully correlate with the results obtained from the feeding-choice assays. No significant differences were found

between sucrose and fructose for *A. gambiae* in terms of longevity, as already shown in *Aedes communis* (Degeer) (Andersson, 1992), and the mixture of glucose and fructose is significantly less suitable for *A. gambiae* survival than sucrose or fructose but better than glucose alone or water. The lowest survival time was mesured on water, followed by glucose. While 4 day-old females previously fed on glucose imbibe the sugar rather well after one night of starvation, at 10 days they hardly fed on glucose that provides a relatively low nutritive value, thus accelerating the drop in their energy reserves. Altough the mixture of fructose and glucose has a higher phagostimulatory effect than fructose on *A. gambiae*, the longevity of mosquitoes maintained on the mixture of fructose and glucose was lower than for those maintained on fructose alone, probably due to the replacement of half of the molecules by glucose in the mixture. So, differences in longevity between sugar diets seems to be due to a differential ability of *A. gambiae* to absorb or to metabolize sugars. An equimolar mixture of fructose and glucose was found to be as suitable for survival in *Drosophila* as sucrose (Hassett, 1948). In *C. inornata*, sucrose, fructose and glucose provide comparabe 50% values of longevity of 30, 28 and 24 days. Mosquito survival depends on both the amount of sugar ingested and the rate of sugar metabolism which can vary between species (Nayar and Sauerman, 1975).

It is generally accepted that monosaccharides are absorbed in insects by passive diffusion and the absorption of glucose depends of the rate of conversion of absorbed glucose into trehalose to maintain a concentration gradient between the gut lumen and haemolymph (Treherne, 1958). Sucrose digestion by α-glucosidase does not start before entry into the midgut (Souza-Neto et al., 2007). If disaccharides are completely hydrolized before absorption then the mixture of glucose and fructose should have the same nutritive value as sucrose, unless if *A. gambiae* metabolizes preferentially the fructose constituent of sucrose and that the glucose moiety is incorporated to oligosaccharides by transglucosylation, as has already been shown in aphids (Ashford et al. 2000). In addition, there is good evidence to suggest that disaccharides are also absorbed by insects. Recently, a sucrose transporter was identified in the hindgut of *Drosophila* larvae (Meyer et al., 2011) and enzymes hydrolizing sucrose have been found in the abdomen of mosquitoes from which the alimentary duct had been removed (Van Handel, 1968). In our study on *A. gambiae*, the capacity of sucrose to serve as the best stimulant for the labellar sugar receptor cells seems to be correlated with behavioural preferences and nutritional value. This is not always the case in insects. For example, arabinose is a sweet-tasting compound for *Drosophila* but has no nutritional value (Burke and Waddell, 2011). Similarly, sorbose was reported as a non-metabolizable phagostimulant in *Aedes taeniorhynchus* (Wiedemann) (Nayar and Sauerman, 1971). Despite sorbitol being tasteless but metabolisable in *Drosophila*, flies prefer to feed on

100 mM sorbitol than on water although mutants lacking the Gr43a, a fructose receptor identified in their brain, do not (Miyamoto et al., 2012). Although taste plays a role in the establishment of short-term memory, this result suggests that a post-ingestive reinforcement pathway is fundamental for the establishment of a long-lasting memory to evaluate metabolic reward which, in this manner, influences food choice by insects (Burke and Waddell, 2011; Wright, 2011). In *A. gambiae*, the nutritive value of other sugars such as maltose, gulose or raffinose that are present in plant nectar on which this vector has been observed to feed but that fail to activate the labellar sugar sensitive neurone are still to be evaluated. Due to the importance of odours for *A. gambiae* plant choice (Nyasembe et al., 2012) and the ability of mosquitoes to associate an odour with sugar reward (Sanford and Tomberlin, 2011), it could be interesting in a future study to evaluate the ability of this vector to associate an odour with the taste and nutritive qualities of a particular plant nectar.

Sugar diet influences A. gambiae *biting behaviour*

In this study, we show that females maintained on glucose, which possess a lower nutritional value, show a more intense biting behaviour on the WB. Females have more energetic reserves, represented by a higher dry mass, when maintained on sucrose rather than on glucose. However, no linear correlation was found between the dry mass and the number or duration of landings on the WB. Moreover, the dry mass of females maintained on glucose decreases between 4 to 10 days-old, while those of females maintained on sucrose increases with age. Because no significant interactions between age and number of landings were found, it is most likely that the differences measured in dry weights reflect the diferent ability of females to accumulate reserves from glucose and sucrose rather than a difference in reserve consumption to stustain a more intense flight activity on glucose. Kaufmann and Briegel (Kaufmann and Briegel, 2004) found that maximum lipogenesis in *A. gambiae* having access to *ad lib* 10% sucrose is reached after 7 days and maximal glycogenisis within 2 days (Kaufmann and Briegel, 2004).

Roitberg et al. have already shown that the attack rate by *A. gambiae* is nonlinearly correlated with the energy state: “mosquitoes of intermediate energy state (i.e. 1-day-starved) show lower attack rates than 2-day food deprived or nondeprived mosquitoes” (Roitberg et al., 2010). By measuring only the dry weight, we were not able to discriminate between the glycogen versus the lipid reserves. In mosquitoes, free carbohydrates, including glucose, constitute the first fuel used to sustain flight, followed by glycogen and lipids used for longer flight periods (Kaufmann and Briegel, 2004; Nayar and Sauerman, 1971; Nayar and Van Handel, 1971). In our study, the higher biting response observed in female

A. gambiae fed on glucose could be explained by the fact that glucose is probably used immediately to substain flight, altough it constitutes a weaker energy substrate for the acculmulation of reserves to support survival. Consequently, females fed on glucose would try to compensate for the lower energy reserve by actively seeking a blood meal resulting in a more intensive biting behaviour on the vertebrate host.

Sugar diets may have opposite effects on vectorial capacity

Survival rate and biting rate are the most important factors influencing vectorial capacity and small changes in these factors result in a large impact on vectorial capacity because of their magnification in the equation (Dye, 1986; Garrett-Jones, 1964; Stone, 2011). Although the number of landings and the time spent probing by mosquitoes measured in our WB assay differ from biting frequency which corresponds to the number of bites/host/time unit by each mosquito (Dye, 1986), our measured parameters are directly linked to the intensity of biting behaviour. We show that sugar diets have antagonistic effects on vectorial capacity: sucrose enhances mosquito survival but decreases the intensity of biting behaviour by females in comparison to glucose which supports a lower survival but stimulate females to bite.

It has already been shown in laboratory, semi-field and field studies that the presence of artificial or natural sugar sources have antagonistic effects on vectorial capacity by increasing the lifespan (Gary and Foster, 2001; Gu et al., 2011; Okech et al., 2003; Stone et al., 2011; Stone et al., 2012) but by decreasing the biting rate by *A. gambiae* (Gary and Foster, 2001; Stone et al., 2012). Our study provides new insight into the influence of sugar feeding on vectorial capacity of this species by providing evidence that nectar sugar constituents will also impact on parameters in the equation. This is particularly important for an endophilic vector like *A. gambiae* that feeds on plant communities maintained by man. Nectar quality depends on the carbohydrates present in it, but all nectars do not possess the same phagostimulatory and nutritive values and differ also in the presence of deterrent secondary metabolites (Kessler et al., 2013). Our results show that sucrose is one of the most suitable sugars for consumption by *A. gambiae*. This could correspond to the field situation as it is probably the most common sugar consumed by mosquitoes, as sugested by Souza-Neto et al. (Souza-Neto et al., 2007). The correlation between plant preferences and nectar quality, as well as the influence of various kinds of nectars on vectorial capacity remain to be investigated in field or semi-field studies. It could be that the effect of nectar quality is negligible in an antropophilic species like *A. gambiae* that lives in a sugar-scarce environment under a selective pressure to feed on blood only (Beier, 1996; Gary and Foster, 2001; Souza-Neto et al., 2007). Recently, an important effort has been made to control some

local populations of *Anopheles* using attractive sugar baits. Understanding the sensory and physiological basis of sugar feeding and assimilation will prove to be fundamental for this approach to control mosquito vectors (Beier et al., 2012).

6.6 Acknowledgments

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6.7 Funding

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6.8 Supplementary material

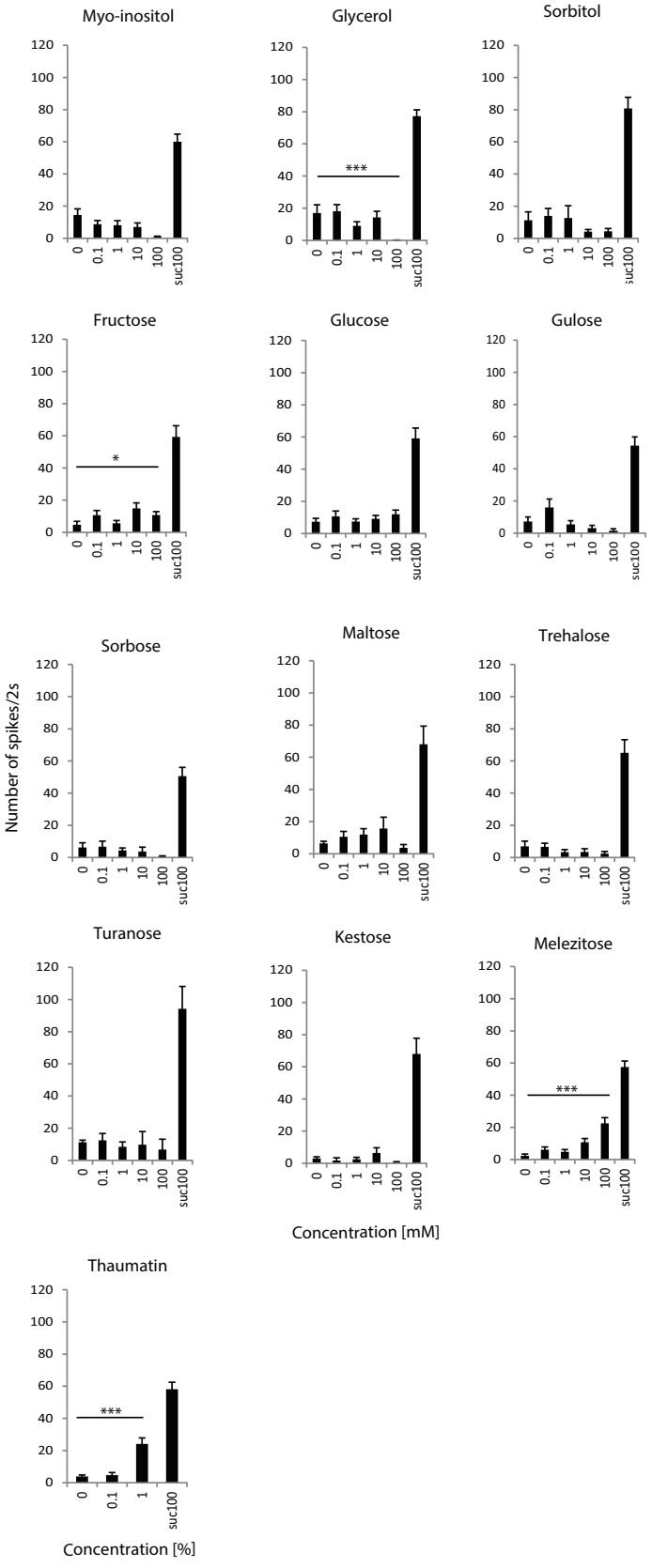

Fig S6.1: Mean ± s.e.m. of the total number of spikes per 2 s generated by receptor cells housed in the ventral No 8 and the dorsal No 5 sensilla on the labellum of *A. gambiae* during stimulation with increasing concentrations of the sugar alcohols myo-inositol, glycerol and sorbitol, the monosaccharides fructose, glucose, gulose and sorbose, the disaccharides maltose, trehalose and turanose, the trisaccharides kestose and melezitose and the protein thaumatin, all diluted in 10 mM KCl. Between 3 to 14 mosquitoes were tested per treatment. Each stimulation series ended with 100 mM sucrose in 10 mM KCl as positive control. The number of spikes recorded during stimulation with 100 mM sucrose (suc100) was significantly higher than for all other compounds at the highest concentration tested (Wilcoxon test, $P < 0.05$). Glycerol showed significant inhibition of the response to 10 mM KCl (Kruskal-Wallis test, $P < 0.001$). Significant differences were found between doses of fructose (Krukal-Wallis test, $P < 0.05$) with the highest number of spikes recorded per 2 s at 10 mM fructose. Spike frequency increased significantly as a function of the dose of melezitose and thaumatin (Kruskal-Wallis test, $P < 0.001$).

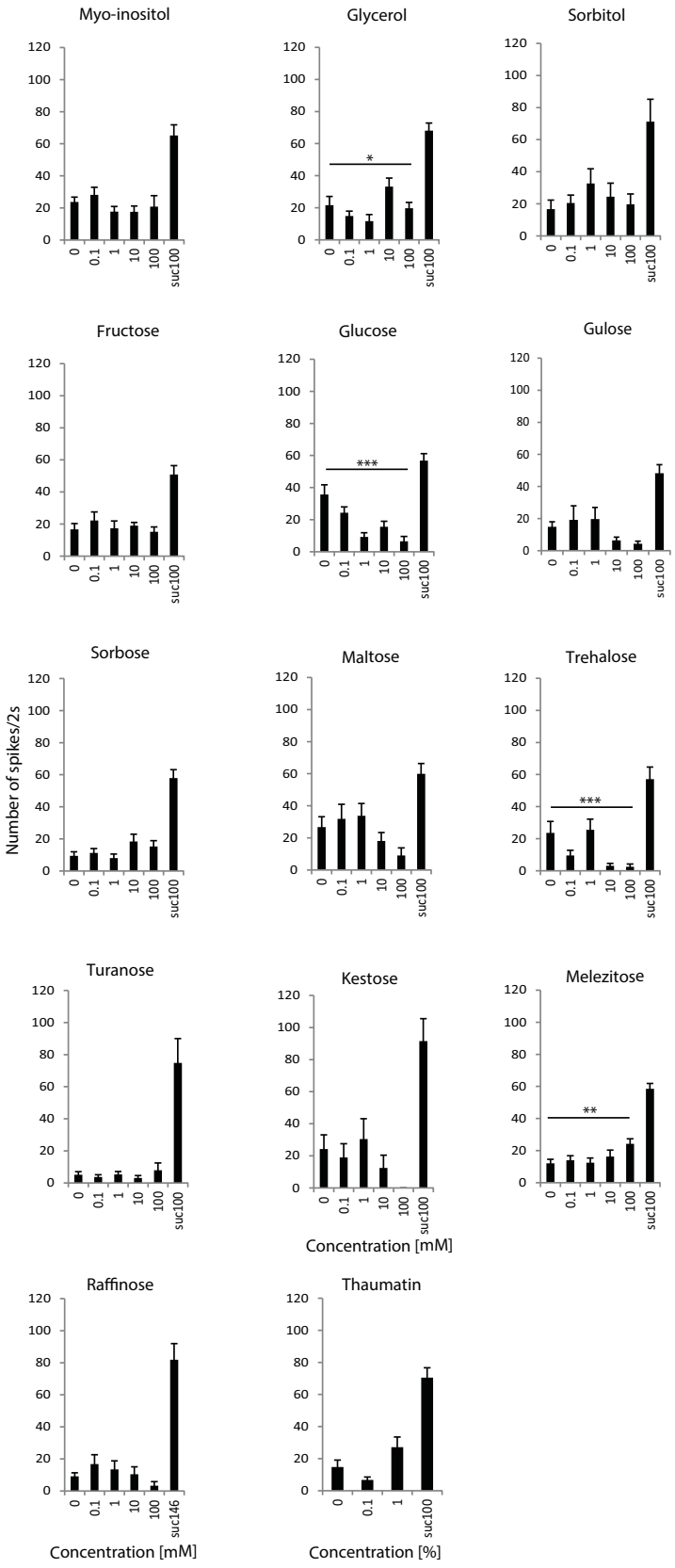
Myo-inositol
Glycerol
Sorbitol
Fructose
Glucose
Gulose
Sorbose
Maltose
Trehalose
Turanose
Kestose
Melezitose
Raffinose
Thaumatin
Number of spikes/2s
Concentration [mM]
Concentration [%]
0
0.1
1
10
100
suc100
suc146
*
***
**

Fig S6.2: Mean ± s.e.m. of the total number of spikes per 2 s generated by receptor cells housed in the lateral No 2 and No 5 sensilla on the labellum of *A. gambiae* during stimulation with increasing concentrations of the sugar alcohols myo-inositol, glycerol and sorbitol, the monosaccharides fructose, glucose, gulose and sorbose, the disaccharides maltose, trehalose and turanose, the trisaccharides kestose, melezitose and raffinose and the protein thaumatin diluted in 10mM KCl. Between 4 to 17 mosquitoes were tested per treatment. Each stimulation series ended with 100 mM or 146 mM sucrose in 10 mM KCl as a positive control. The number of spikes recorded during stimulation with 100 mM sucrose (suc100) was significantly higher than for all other compounds at the higher concentration tested (Wilcoxon test, $P < 0.05$). Glucose and trehalose show significant inhibition of the water sensitive neurone that was not completely inhibited by 10 mM KCl (Kruskal-Wallis test, $P < 0.001$). This inhibition was marginally not significant for maltose ($P = 0.069$). Spike frequency generated by the sugar sensitive neurone increased significantly as a function of the dose for melezitose (Kruskal-Wallis test, $P < 0.01$). Significant differences in the spike frequency generated by the water sensitive neurone (the response to KCl is inhibited by glycerol, see Fig. S6.1) were found between doses of glycerol (Krukal-Wallis test, $P < 0.05$) with the highest number of spikes recorded per 2 s at 10mM glycerol. Solari et al. (Solari et al., 2010) suggested that glycerol has the ability to activate the water sensitive neurone of insects by crossing the receptor cell membrane through aquaglyceroporines, thus inducing cell swelling.

7. Quinine and artesunate inhibit feeding in the African malaria mosquito *Anopheles gambiae*: the role of gustatory organs within the mouthparts

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7.1 Summary

We describe here a membrane feeding assay in which the effects of the antimalarial drugs quinine and artesunate (ARS) were tested on *Anopheles gambiae*. 87% of female *A. gambiae* fed on whole defibrinated bovine blood whereas only 47% and 43.5% fed on saline and on saline + bovine serum albumin (BSA) solutions, respectively, indicating that additional components in blood stimulate mosquito feeding. Adding 1 mM quinine or artesunate to the BSA solution caused a significant reduction in the percentage engorgement to 16.15% and 14.1%, respectively. However, blood phagostimulants reduced the deterrent effect of both 1 mM ARS and quinine as 67.8% and 78.4% of females engorged on these solutions. Ten mM of ARS in blood reduced the percentage engorgement to 20%. As circulating doses of quinine and ARS affecting *Plasmodium* in humans are much lower than those affecting feeding by *A. gambiae* in our assay, these two antimalarial drugs would have no or only a minor effect on the infection rate of mosquitoes feeding on treated patients. Since only the stylets penetrate the membrane and not the labellar lobes, our results suggest that both blood phagostimulants and feeding deterrents are detected in *A. gambiae* by internal gustatory organs, namely sensory cells in the apical and subapical labral pegs, in sensilla on the inner face of the labellar lobes or by cibarial receptor cells. The neuroanatomy of gustatory sensilla on the apical and subapical labral pegs and on the inner face of the labellar lobes of female *A. gambiae* is described in this study.

Key words: *in vitro* feeding assay, mosquito blood meal, phagostimulants, feeding deterrents, alkaloids, sesquiterpene lactones, antimalarial drugs.

7.2 Introduction

Malaria is estimated to have caused the death of 660'000 people in 2010, mostly in sub-Saharan Africa (WHO, 2012) where the mosquito *Anopheles gambiae* Giles is one of its more efficient vectors. The anthropophilic host preference of this species, its endophilic and endophagic habits as well its adaptation of urban environments guarantee a persistent host-vector interaction necessary for a high intensity of parasites transmission (Geissbuhler et al., 2007; Oduola et al., 2013; Takken and Knols, 1999). The cellular and molecular architecture of the olfactory system used by *A. gambiae* to find its host is already well studied (Carey et al., 2010; Lu et al., 2007; Qiu et al., 2006) and how repellent molecules interact with the olfactory system of *Drosophila* species and mosquitoes is well investigated (DeGennaro et al., 2013; Kwon et al., 2010; Pellegrino et al., 2011; Syed et al., 2011). However, despite mosquitoes becoming models for studies on olfaction, little is known about taste organs and contact chemoreception in these important vectors.

Chemo-sensory cells in contact sensilla on labellar lobes as well as those on the legs have been found to respond electrophysiologically to sugars, amino acids and salts in *Aedes aegypti*, *A. gambiae* and *Culiseta inornata* (Williston) (Elizarov and Sinitsina, 1974; Kessler et al., 2013; Pappas and Larsen, 1976). Receptor cells in peg organs present at the tip of the labrum of anautogenous females are suggested to be involved in the detection of blood components (Liscia et al., 1993; Werner-Reiss et al., 1999a; Werner-Reiss et al., 1999b), although they were already found to respond electrophysiologically to sucrose in *C. inornata* (Pappas and Larsen, 1976). The role of mouthpart taste neurones in the perception of bitter compounds and especially the perception of quinine by sensory cells in sensilla on the external surface of the labellar lobes has been established in *A. aegypti* (Sanford et al., 2013) and *A. gambiae* (Kessler et al., 2013). However, it is unknown whether internal receptor cells, namely, those of the labrum, the internal part of the labellum and those of the cibarium, are involved in the perception of aversive and potentially toxic compounds. In our earlier study we showed that feeding on sugar is inhibited in *A. gambiae* by denatonium benzoate, quinine, quinidine and berberine when the proboscis was enclosed in the tip of a capillary containing the treatments (Kessler et al., 2013). The aim of this study was to understand the role of internal taste receptor cells in the detection of potentially deterrent products in female *A. gambiae*.

Reducing transmission of malaria gametocytes could prove to be crucial both to control malaria as well as to limit the spread of strains resistant to antimalarial drugs (Chotivanich et al., 2006). Thus, inhibiting vectors to feed on infected patients as well as reducing the lifespan of those mosquitoes that do succeed to feed could be important. Retention of chloroquinine in *Anopheles stephensi* Liston fed on Rhesus monkey blood containing the

antimalarial drug has already been investigated (Langer et al., 1968). In comparison to circulating artesunate, an artemisinin derivative, quinine has lower effects on both *Plasmodium falciparum* gametocyte transmission-blocking and the gametocyte carrying rate (Chotivanich et al., 2006; Lelievre et al., 2012; Pukrittayakamee et al., 2004). However, blood-feeding deterrent activity of antimalarial drugs on the insect vectors has to our knowledge not yet been investigated. Alkaloids like quinine and sesquiterpene lactones like artemisinin, in addition to serving as antimalarial drugs, are also defensive products of plants playing a role as strong feeding deterrents for phytophagous insects where doses less than 100 ppm and sometimes even as low as 1 ppm can inhibit feeding (Picman, 1986; Schoonhoven, 1982). Here we compare the antifeeding effects of quinine and artesunate. We show how both products inhibit feeding by *A. gambi*ae with the help of a membrane-feeding assay that excludes contact by the external surfaces of the proboscis and by the legs with treatments. In addition, we provide a description of the neuroanatomy of gustatory sensilla containing chemo-sensory neurones inside the labellum and on the tip of the labrum that could mediate perception of feeding stimulants and deterrents by *A. gambiae*.

7.3 Material and methods

Mosquitoes

The *A. gambiae* colony (16cSS strain, derived in 1974 from wild-caught adults originating from Lagos, Nigeria, West Africa) was maintained in a climate chamber at 28°C, 80% relative humidity (RH), under a 12h:12h light:dark cycle, with 2h simulated sunrise and sunset as previously described by Kröber *et al.* (Kröber et al., 2010).

Ultrastructure of sensory organs inside the mouthparts

The same procedures as described in Kessler et al. (2013) were used for transmission and scanning electron microscopy. For transmission electron microscopy, heads of female *A. gambiae* were fixed in Karnovsky fixative (pH 7.4) overnight at 4°C and rinsed three times in 0.2 mM Na cacodylate buffer with 4% sucrose. After post-fixation in 1% OsO4 for two hours and rinsing in the same buffer, the specimens were block stained with 2% uranyl acetate (pH 3.9) for 1 hour at room temperature. They were dehydrated through graded series of acetone solutions and embedded in Spurr's resin. Ultrathin sections (1 µm) of the first 100 µm from the tip of the proboscis of three females were made on a Reichert Ultracut S microtome, stained with uranyl acetate and lead citrate and examined in a PHILIPS CM 100 electron microscope.

For scanning electron microscopy, excised heads of *A. gambiae* were fixed in 70% ethanol, rehydrated and washed in KODAK Photoflo overnight. After several washes in distilled water, they were dehydrated gradually in ethanol solutions and air dried. The heads with extended stylets were mounted on stubs and coated with a gold layer and examined at 10kV using a PHILIPS ESEM XL 30 electron microscope.

Chemicals

Bovine serum albumin (BSA) fraction V was purchased from Roche Diagnostics GmbH (Mannheim, Germany), $NaHCO_3$ from Merck (Darmstadt, Germany), quinine anhydrous, NaCl, an amino acid (AA) solution (RPMI-1640 50X, without L-glutamine, BioReagent, R7131) containing 19 AAs the major one being L-Arginine (10 g/l, 57.41 mM), artesunate (ARS) and artemisinin (ART) were purchased from Sigma-Aldrich (Buchs, Switzerland). Dihydroartemisinin (DHA) was purchased from Biopurify Phytochemicals Ltd. (Chengdu, China). The mixture of AAs was sterile filtered. The purity of all other products was ≥98%. Solutions were kept at 4 °C.

Feeding membranes

We used a silicone membrane prepared as described in Kröber & Guerin (Kröber and Guerin, 2007a) to feed mosquitoes. Briefly, pieces of Kodak lens cleaning paper (7 x 12.5 cm, Eastman Kodak, Rochester, NY, USA) were placed on a layer of kitchen plastic film and impregnated with a mixture of 4.5 g silicone oil (30% DC 200; Fluka, Switzerland), 0.15 g of Elastosil® FL white colour paste and 15 g silicone RTV-1 Elastosil® E4 glue (both from Wacker, Burghausen, Germany). This mixture was rendered less viscous for application by adding 2.9 g hexane. Excess silicone was removed with an 80 mm wide scraper made from a piece of silicone (3 mm thick). Membranes were left to polymerize for about 24h and membrane thickness was on average 49±11 µm (range 30-100 µm).

In vitro *feeding experiments with antimalarial drugs*

Gorging responses of *A. gambiae* females was recorded on different feeding solutions: 8.75 g l^{-1} NaCl (149.73 mM) plus 0.75 g l^{-1} $NaHCO_3$ (8.93 mM) to provide a pH 8.0 saline solution (Arsic and Guerin, 2008); 120 g l^{-1} BSA in the saline solution (Arsic and Guerin, 2008); 10 times diluted RPMI-1640 amino acid mixture in the BSA solution to provide a 57.4 mM solution of L-arginine, the most abundant amino acid in the mixture; 0.38 g l^{-1} ARS and 0.32 g l^{-1} quinine added, respectively, to the BSA solution to provide 1 mM solutions; freshly

collected bovine blood was manually defibrinated at collection in the slaughterhouse; and serial dilutions of between 0.01 to 1 mM quinine and ARS were tested in defibrinated bovine blood. Solutions were prepared between 1 h and 5 days before the start of the experiments, were sonicated for 10 min at 40 °C and held at 4°C. This treatment permitted minimal heamolysis.

At the beginning of each feeding test about 10 *A. gambiae* females of between 3 to 7 days old were released into a transparent plastic cylinder (98 mm diameter x 52 mm high) with an oval opening of 64x22 mm at the top that allowed the mosquitoes to contact the membrane from below (Fig. 7.1). Mosquitoes were allowed to feed for 30 minutes on 3.5 ml of a treatment in the dark. This corresponds to the times usually used for *in vitro* mosquito feeding assays (Bousema et al., 2012). Mosquitoes were placed in the cylinder between 1-5 hours before the experiment, deprived of water and sucrose. A piece of paper placed over the cylinder opening was pulled out to allow mosquitoes to probe the membrane.

All tests were made in a walk-in climate chamber (25°C, 80% RH) during the last 6 hours of the scotophase. Water from a bath at 37.4±0.4°C (Compact-thermostat Typenreihe MT, Lauda-Königshofen, Germany) was used to warm treatments beneath the membrane via a pump connected to the feeding unit through silicone tubes (Fig. 7.1). The temperature of the membrane was on average 35.6±0.6°C (range 34.8-39.6°C). After each test, mosquitoes were anesthetized with CO_2 to count the percentage of engorged females (visual estimation of abdominal distention as described in Arsic & Guerin, 2008). Only fully engorge mosquitoes were recorded as having fed (Galun et al., 1985a).

Recording of survival and fecundity of A. gambiae *on antimalarial drugs*

Batches of 36, 31 and 21 five day-old females were fed as described above on defibrinated bovine blood alone, blood plus 1 mM quinine and blood plus 1 mM ARS. In addition, a batch of 34 females fed on blood with 1 mM quinine was tested without subsequently anesthetizing them. The engorged mosquitoes on each solution were placed in rearing cages (as above) with free access to water and 10% sucrose. One day after the blood meal, a crystallizing dish (100 mm diameter, 10 mm deep) with a filter paper (Whatman, 90 mm diameter, No 1001 090) humidified with 4 ml of demineralized water was placed in the center of each cage as an oviposition site and the filter papers were changed each day. In this manner, eggs numbers and dead insects were counted each day for 6 days after the blood meal.

Analysis of antimalarial drugs in blood

ARS in solution is not stable and is converted into dihydroartemisinin (DHA). This reaction is pH dependent and oral doses are rapidly converted at the low pH of the stomach although ARS is more stable in plasma at higher pH (Olliaro et al., 2001). In blood, the reaction is thought to be mediated by plasma and red blood cell esterases (Zhou et al., 1987). In order to evaluate the kinetics of ARS in our blood samples, both ARS and DHA were quantified by ultra-high pressure liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UHPLC-QTOFMS). Two samples, prepared as described above, were analyzed: a freshly made solution and a solution prepared 5 days earlier. Plasma was obtained by centrifuging blood samples containing1 mM ARS at 4260 rpm for 10 min to which 0.1 mM ART diluted in 50:50 acetonitrile (ACN)/ H_2O was added as an internal standard (IS) just before centrifugation. For protein precipitation, 600 µl ACN was added to 300 µl plasma. The supernatant was diluted 100-fold in 50:50 ACN/H_2O before quantification.

UHPLC-QTOFMS analysis was carried out on an Acquity BEH C18 column (2.1x50 mm, 1.7 µm particle size) from Waters (Milford, USA) using an Acquity UPLC™ system (Waters) coupled to a Synapt G2 QTOF mass spectrometer (Waters) through an electrospray (ESI) interface. The following solvent gradient program was employed at a flow rate of 400 µL/min: solvent A = water + 0.05% formic acid, solvent B = acetonitrile + 0.05% formic acid; 20-80% B in 5.0 min, 80-100% B in 1.0 min, held at 100% B for 1.5 min, and re-equilibrated at 20% B for 1.0 min. The temperature of the column was maintained at 25°C. The injection volume was of 2.5 µL. The QTOF mass spectrometer was operated in positive ion mode over a range of 85-600 Da with a scan time set to 0.4 s. Source parameters were as follows: capillary and cone voltages +2800 and +25 V, respectively, source temperature 120°C, desolvation gas flow and temperature 800 L/hr and 450°C respectively, cone gas flow 20 L/hr. Accurate mass measurements were obtained by infusing a 400 ng/mL solution of the synthetic leucine-enkephalin at a flow rate of 10 µL/min through the Lockspray™ ESI probe (Waters). The system was controlled by Masslynx™ V.4.1 software. ARS, ART and DHA were quantified based on their $(M+Na)^+$ ions using the following extracted ion chromatograms (EIC) with a mass window of ± 0.01 Da: *m/z* 407.17 for ARS (retention time (RT) 3.20 min), *m/z* 307.15 for DHA (RT 2.59 min) and *m/z* 305.14 for ART (RT 3.34 min) as IS. Absolute concentrations were determined using calibration curves obtained with ARS and DHA standards spiked with ART. The concentrations of the calibration points were 0.2, 1, 2, and 5 µg/mL.

Statistics

All statistical analyses were made with R 2.11.1 software (R Development Core Team, 2010). Graphical representations were made with R and Microsoft Excel 2010. The feeding responses of females were analyzed by a generalized linear mixed model (GLMR) with a binomial error distribution (glmer function in the *lme4* package, Bates et al., 2011). The number of engorged versus un-engorged females was treated as the dependent variable, test products were the fixed factor and the feeding trials constituted the random factor. To provide an estimate of survival and fecundity on treatments, the number of dead versus alive mosquitoes recorded six days after the blood meal was analyzed as a function of the feeding solution with a generalized linear model (GLM) with a binomial error distribution and a logit link function. The number of eggs was divided by the number of females surviving each day. These data are analyzed only descriptively and no statistical analysis was made as the experiment was repeated only once. The significance level for all tests was set at $P = 0.05$.

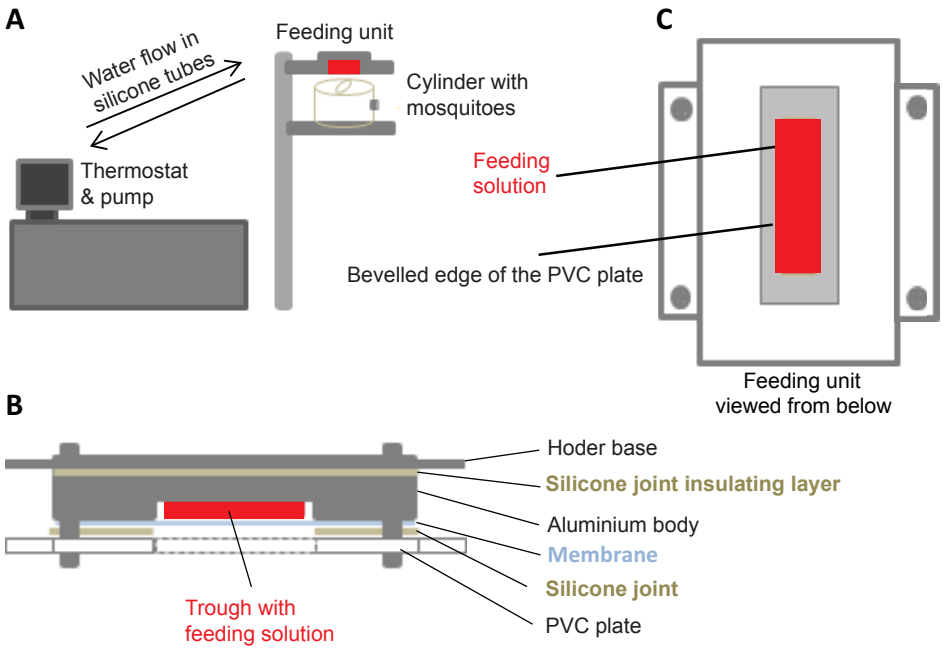

Fig. 7.1 Outline of the feeding unit assembly: Thermostat to control the water bath temperature and pump connected to the aluminium body of the feeding unit through silicone tubes; mosquitoes were held in a plastic cylinder for feeding assays (A). The feeding unit was made of an aluminium body (105x65 mm) through which warm water circulated. The aluminium body had a trough (63x20x1.5 mm deep) for the test solutions. A PVC plate (6.5 mm thick) fastened with screws to the heated unit held the membrane in place under a silicone joint (1 mm thick). A second silicone joint was placed between the aluminium body and the holding base to insulate the feeding unit (B). Both the PVC plate and silicone joint had an opening, the size of the trough to allow mosquitoes access to the membrane from below (C).

7.4 Results

Analysis of antimalarial drugs in blood

A concentration of the artemisinin derivatives (ARS + DHA) of 1.55 and 1.13 mM was measured in the serum of the freshly-made up 1 mM solution in bovine blood and the one prepared five days earlier, respectively, i.e. at higher levels than initially tittered due to the concentration step at centrifugation. The conversion rate of ARS to DHA in defibrinated bovine blood was low as only 2.6% (0.04 mM) of the ARS was converted to DHA in the freshly-made solution (Fig. 7.2B). This rate was slightly higher for the solution prepared 5 days earlier as 14.2% (0.16 mM) of the ARS was converted in DHA (Fig. 7.2C). Thus, we can consider that any deterrent effect can be mainly ascribed to ARS rather than to DHA, although a synergetic effect between these two compounds cannot be excluded.

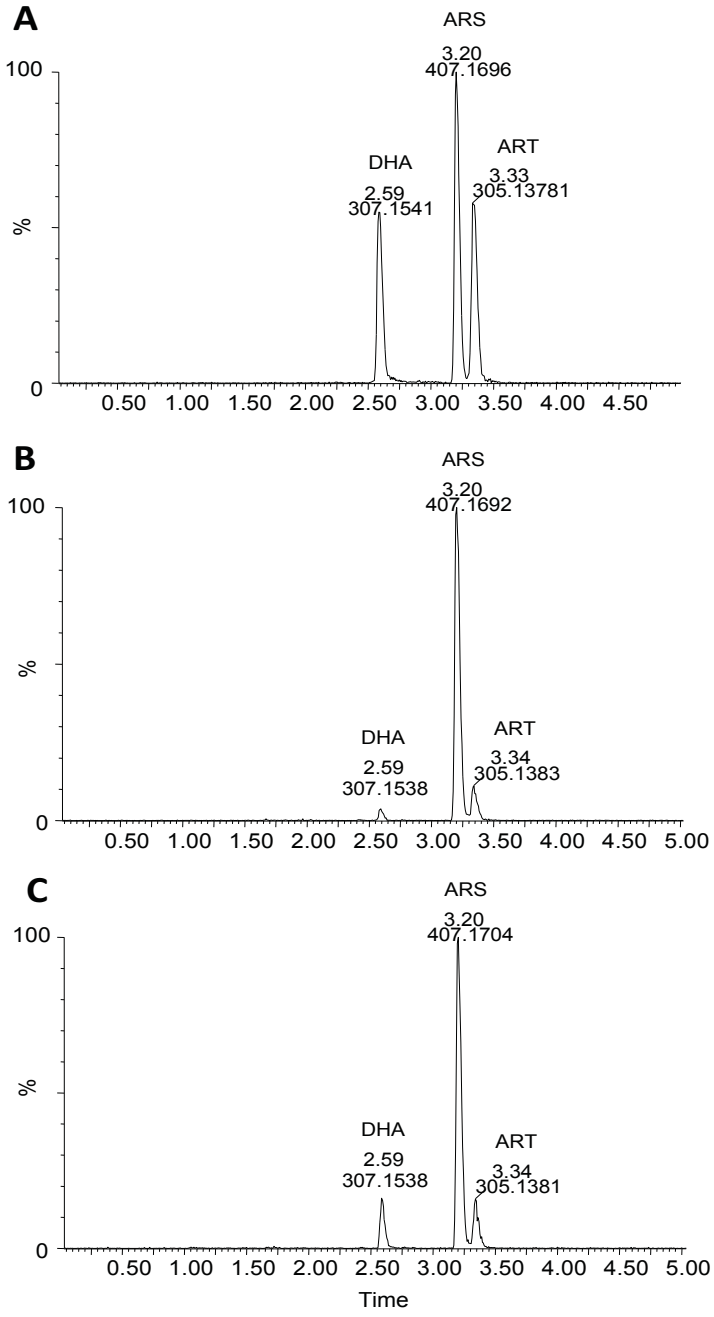

Fig. 7.2: Base peak intensity UHPLC-QTOFMS chromatograms of (A) the standard mixture of 1 mM dihydroartemisinin (DHA), artesunate (ARS) and artemisinin (ART) in 50:50 acetonitrile/H_2O. (B) Plasma extract from a freshly made up 1 mM ARS in blood to which 0.1 mM artemisinin was added as internal standard and (C) a plasma extract of a 5-day old blood solution with the same solutes.

Deterrent effects of antimalarial drugs on A. gambiae *feeding*

Female *A. gambiae* show different engorgement responses on the treatments tested (Fig. 7.3). Females engorged on defibrinated bovine blood (87%) systematically more than on saline (47%, $P < 0.001$) and BSA solutions (43.5%, $P < 0.001$). Adding 120 g l-1 BSA to saline did not significantly increase the feeding response by *A. gambiae* compared to saline ($P = 0.84$). Likewise, adding the amino acid mixture to the BSA solution did not change the engorging response of females compared to BSA (40%, $P = 0.968$). Adding either quinine or ARS at 1 mM to the BSA solution caused a reduction in the percentage engorgement to 16.15% and 14.1%, respectively compared to BSA ($P < 0.001$ in both cases) and both treatments had the same effect ($P = 0.72$). All meals were directed to the midgut.

Quinine and ARS added at 0.01 mM l^{-1} to bovine blood did not significantly change the engorging response of females compared to defibrinated blood alone (92.5%, $P = 0.34$ and 87.8%, $P = 0.78$, respectively), nor at 0.1 mM (81.9%, $P = 0.46$ and 80.4%, $P = 0.44$). The percentage engorgement dropped significantly after adding ARS at 1 mM (67.8%, $P = 0.006 < 0.001$), but not for quinine (78.4%, $P = 0.25$) added at the same dose to defibrinated bovine blood. However, the feeding responses of *A. gambiae* between 1 mM ARS and quinine added to blood were not significantly different ($P = 0.13$). Ten mM ARS added to bovine blood showed a strong feeding deterrent effect in comparison to blood alone, as only 20% of the females engorged ($P < 0.001$). Ten mM quinine was not tested due to the lack of solubility of quinine at this dose.

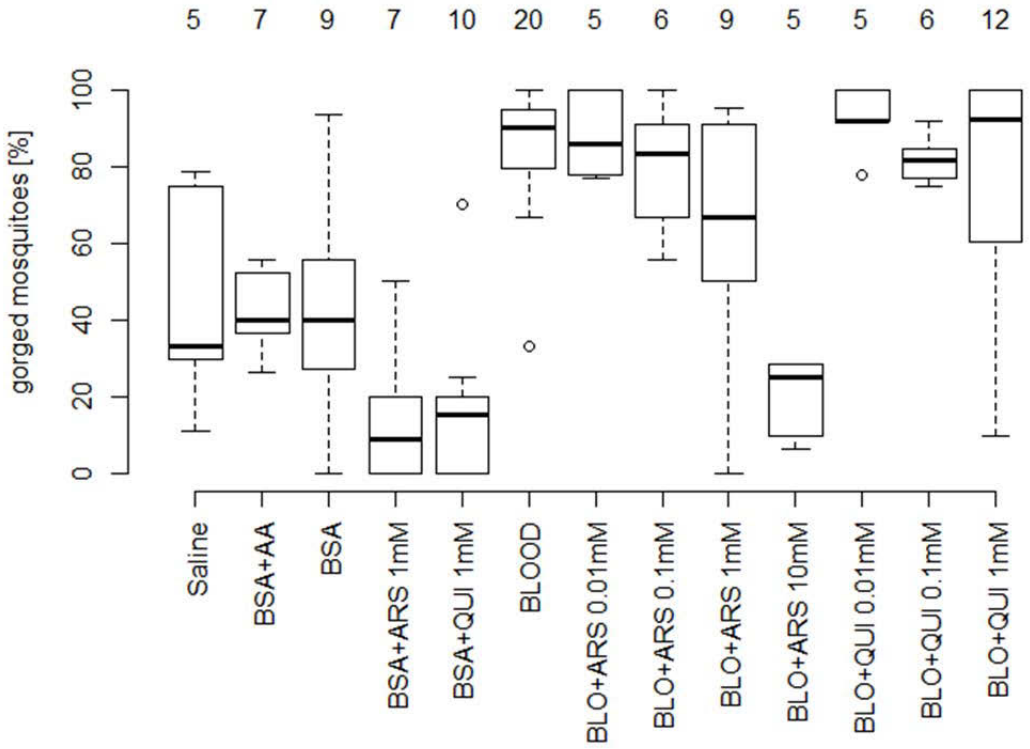

Fig. 7.3: Box plot representation of the percentage engorged *A. gambiae* females for the following treatments: saline, bovine serum albumin (BSA), BSA plus an amino acid mixture (BSA+AA), BSA plus artesunate (BSA+ ARS 1 mM), BSA plus quinine (BSA+QUI 1 mM), bovine blood, blood plus ARS (BLO+ ARS) at 0.01 mM, 0.1 mM, 1 mM and 10 mM and blood plus quinine (BLO+QUI) at 0.01 mM, 0.1 mM and 1 mM. The number of repetitions for each treatment is indicated over the boxes. Box and whisker plots represent the median (black bars), the 25-75% interquartile range (IQR, boxes), the lowest and the highest data points still within 1.5 of the IQR (whiskers) and outliers (circles).

Survival and fecundity of A. gambiae *on antimalarial drugs*

After having fed on blood containing 1 mM quinine, 25 of 30 mosquitoes that had fed did not recover immediately from anesthesia. This knock down was not due to the interaction of the anesthetic with the dose of quinine consumed as in the feeding assay without CO_2 anesthesia 26 of the 31 mosquitoes that had fed on 1 mM quinine were knocked down after the blood meal. Recovery from knock down could take up to 12 h but 36% and 45% of the mosquitoes knocked down after having fed on blood plus 1 mM quinine in the feeding assays, respectively, with and without anesthesia did not recover after 24 h and were considered as dead. No knock down effect was recorded on blood or on 1 mM ARS. Six days after the blood meal the percentage mortality (34%) of mosquitoes fed on defibrinated bovine blood + 1 mM quinine and that had recovered from knock down (data from the trials with and without anesthetic were pooled) was higher but not significantly different to that for mosquitoes fed on blood plus 1 mM ARS (15%, $P = 0.13$) or those fed on blood alone (23%, $P = 0.29$). Females laid eggs between 2 to 4 days after the defibrinated bovine blood meal. The number of eggs laid per females was higher for mosquitoes fed with defibrinated blood alone or blood plus 1 mM ARS than for those fed with 1 mM quinine (data from the trials with and without post feeding assay anesthesia were pooled; Fig. 7.4). This was particularly evident on the first day of oviposition, when the number of eggs laid per females fed on blood alone (43) or blood plus 1 mM ARS (40) was almost two times higher than for females fed with blood plus 1 mM quinine (17; Fig. 7.4).

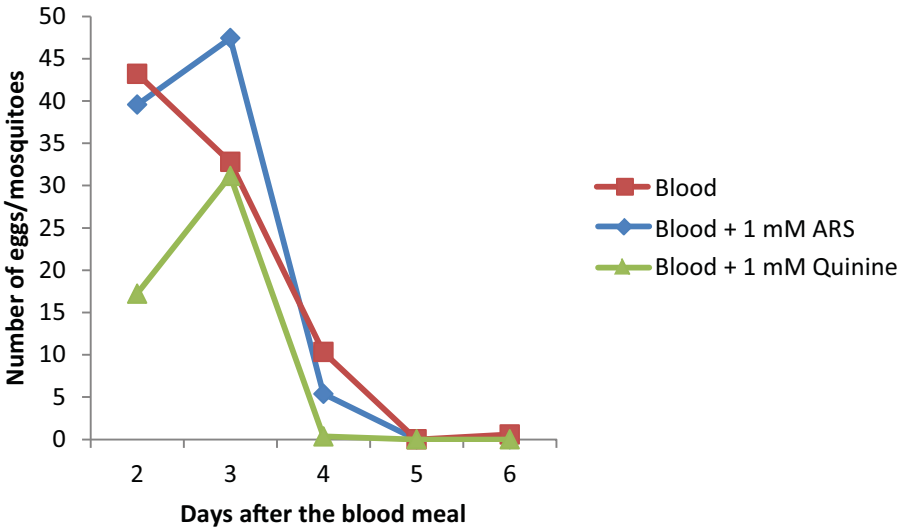

Fig. 7.4: Number of eggs laid per female *A. gambiae* fed with defibrinated bovine blood alone (red squares), blood plus 1 mM ARS (blue diamonds) and blood plus 1 mM quinine (green triangles).

Ultrastructure of A. gambiae *labral and labellar sensilla*

As in other anautogenous mosquitoes (Lee and Craig 1983), the labrum of female *A. gambiae* bears one pair of apical peg sensilla at the tip, and one pair of subapical peg sensilla situated more proximally on each side (Figs. 7.5A and B). These sensilla enclose two lymphatic cavities (Fig. 7.5C). In all, 5 sensory cells innervate the inner lymph cavity with 4 dendrites ascending the shaft of the sensillum and a fifth neurone terminates at the base of the hair. This constitutes the basic form of a gustatory sensillum in insects (Altner and Prillinger, 1980). The five dendrites are enclosed at the base by a sheath secreted by the thecogen cell (Fig. 7.5C). Both apical and subapical sensilla are not present in males. At 50 µm from the tip of the labrum, a pair of campaniform sensilla is present on the ventral side in both sexes, near the entry of the food canal (Fig. 7.5D). No transmission electron microscopy analysis was performed on this pair of sensilla.

Trichoid sensilla are present on the inner face of the labellar lobes of each sex (Fig. 7.6). They possess a unique pore at the level of the lateral spur (Fig. 7.6A and B). The number of unramified dendrites enclosed by these sensilla is variable: three to four ascend the inner lymph cavity to the pore and one terminates below the shaft of each sensillum; the presence of a tubular body is shown by the typical arrangement of its microtubules (Fig. 7.6C and D). These gustatory sensilla therefore house between three to five sensory cells.

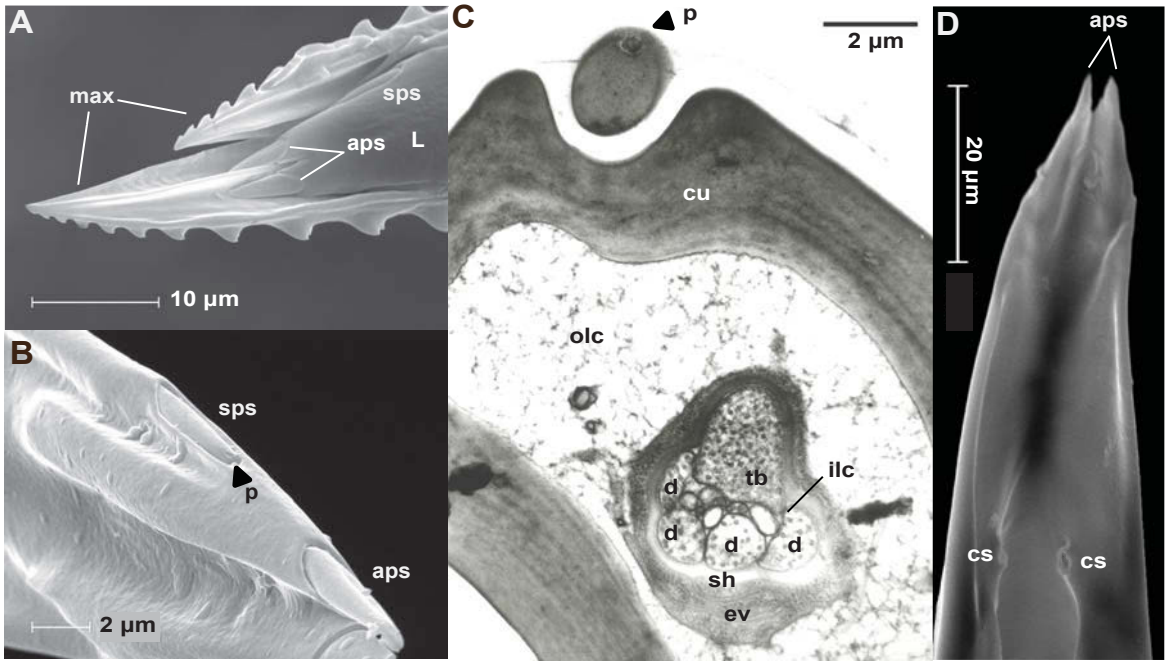

Fig. 7.5: Ultrastructure of peg sensilla and campaniform sensilla on the labrum of female *A. gambiae*. (A) Scanning electron micrograph (SEM) of the extremities of the maxillary stylets (max) and the labrum (L; dorsal view) showing the position of the apical sensilla (aps). (B) SEM of the extremity of the labrum (fronto-latero-ventral view) showing the apical (aps) and subapical sensilla (sps). (C) Transmission electron micrograph at the level of the tubular body of an apical sensillum and at that of the pore of a subapical sensillum. (D) SEM of the ventral side of the labrum showing the position of the two apical sensilla and the two campaniform sensilla (c). Abbreviations: aps, apical sensilla; cs, campaniform sensilla; cu, cuticle; d, dendrites; ev, enveloping cells; ilc, inner lymph cavity; max, maxillary stylets; olc, outer lymph cavity; p, pore; sh, sheath; sps, subapical sensilla; tb, tubular body.

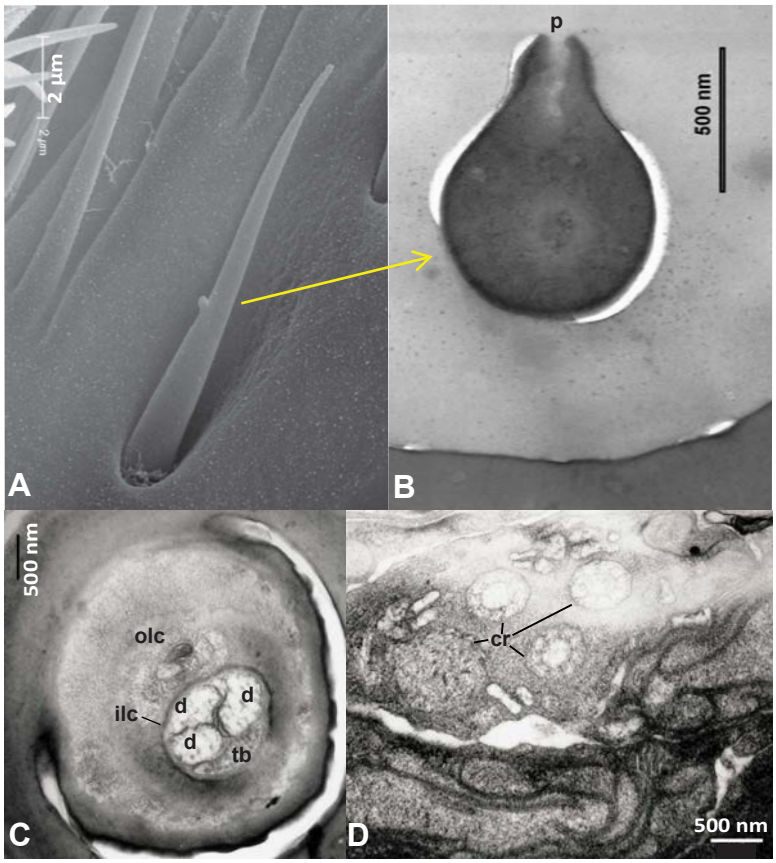

Fig. 7.6: (A) Scanning electron micrograph of a trichoid sensillum on the inner face of the labellum of *A. gambiae*. Transmission electron micrographs made at half way along such a sensillum showing the lateral pore (B), at the level of the tubular body (C) and at the level of the ciliary root (D). Abbreviations: cr, ciliary root; d, dendrite; ilc, inner lymph cavity; olc, outer lymph cavity; p, pore; tb, tubular body.

7.5 Discussion

A reliable in vitro *feeding assay for mosquitoes*

In our feeding assay, 87% of female *A. gambiae* tested fed when they were exposed to whole defibrinated bovine blood. This percentage is similar to the 86% of *A. stephensi* fed on a solution of a chick erythrocyte extract plus 5 mM ATP through a baudruche membrane recorded by Rutledge et al. (Rutledge et al., 1964). The assay also allowed us to discriminate between treatments as blood consistently outperformed saline or saline plus BSA as feeding stimulant for *A. gambiae*. The feeding assay described here represents a reliable and readily accessible method to test feeding by mosquitoes. The added advantage of the silicone membrane is its capacity to seal once a mosquito has withdrawn its mouthparts thus avoiding leakage (Kröber and Guerin, 2007b). As it is possible to treat the silicone membrane, this assay also represents a reliable method to test products acting by contact on mosquitoes. In this manner, contact insecticides applied to the membrane or systemics dissolved in the blood (e.g. ivermectin; Chaccour et al., 2010) may be tested as already described for ticks (Kröber and Guerin, 2007a).

Blood constituents stimulate feeding in female A. gambiae

Among mosquitoes, anophelines are thought to engorge in response to phagostimulants present in the plasma although culicines require cellular components, probably adenine nucleotides (Galun et al., 1985a; Galun et al., 1985b; Werner-Reiss et al., 1999a). Contrary to what was found by Galun et al. for *A. gambiae* (Galun et al., 1985a), the percentage of engorged *A. gambiae* was lower in this study on the saline solution than on blood, indicating some additional phagostimulants in blood. Adding 120 g l^{-1} BSA to saline did not significantly increase the feeding response by *A. gambiae*, as already found for *Anopheles dirus* Peyton & Harrison but at a concentration of BSA nonetheless 2.4 times lower (Galun et al., 1985a). No evidence was found here for any phagostimulatory effect of the AA mixture on imagoes of *A. gambiae* when added to the BSA in saline. This may be due to the presence in the solution of AAs such as L-leucine, L-asparagine, L-tyrosine, L-aspartic acid or L-histidine (tested at doses between 0.48 mM and 2.15 mM), which were found to deter sugar feeding at 10 mM in *A. aegypti* (Ignell et al., 2010). Although L-alanine and L-leucine were identified as feeding stimulants for *A. aegypti* when added to a sugar solution (Ignell et al., 2010), these AAs were tested here at higher doses which could be deterrent. Such overdosed AAs might mask the phagostimulatory effect of other AAs that are important elements of the mosquito diet. AAs constituents of *Lantana camara* L. nectar were found to enhance survival of female *C. quinquefasciatus* (Vrzal et al., 2010). The AAs tested in the study of Vrzal *et al.*

were the same as those tested here but at doses nonetheless lower (between 0.3 and 28.5 times lower) and to which L-alanine and L-glutamine were added.

Feeding deterrent effect of antimalarial drugs on A. gambiae

The percentage of females refusing to feed was significantly higher when 1 mM of either ARS (29%) or quinine (27%) was added to BSA than when added to blood (19% and 8%, respectively, $P < 0.001$ in both cases). Thus, in our assays, phagostimulants in blood serve to reduce the anti-feeding effect of both ARS and quinine on feeding in *A. gambiae*. The feeding response was similar between 1 mM ARS and quinine in BSA ($P = 0.72$) or in blood ($P = 0.13$).

Plants constitute a highly rich source of organic molecules and among them, some alkaloids and sesquiterpene lactones are known feeding deterrents for phytophagous insects (Picman, 1986; Schoonhoven, 1982). The alkaloid quinine and the sesquiterpene lactone artemisinin, two molecules known for their antimalarial activity, are derived from plants. Quinine was originally isolated from the bark of *Cinchona* trees (Achan et al., 2011) and artemisinin from *Artemisia annua* L. (deVries and Dien, 1996). In this study, we show that the artemisinin derivative ARS has a deterrent effect on *A. gambiae* females foraging for a blood meal but the effect of quinine was not significant. It is generally assumed that the bitter taste threshold is correlated with toxicity (Glendinning, 1994). However, we found an inverse relation between deterrence and toxicity. In fact, 1 mM quinine which did not significantly reduce *A. gambiae* blood feeding induced high mortality in fed mosquitoes. In addition, mosquitoes that recovered after having fed on blood plus 1 mM quinine laid almost twice fewer eggs than those fed on blood, highlighting sub-lethal effects of quinine on mosquitoes. In contrast, although 32.2% of mosquitoes renounced to feed on blood plus 1 mM ARS no toxic effects of this product were detected in *A. gambiae* that fed on this dose.

Feeding deterrent compounds are perceived by internal gustatory cells in A. gambiae

It was recently shown that doses of between 0.01 to 1 mM quinine decrease sugar feeding in *A. gambiae* and this is correlated with inhibition of the sugar sensitive neurone in external type 1 (T1) sensilla on the labellar lobes (Kessler et al., 2013). A modest activation of a “deterrent” receptor cell has been recorded in the same sensilla in response to doses of quinine in *A. aegypti* (Sanford et al., 2013). In this study, only the stylets penetrated the membrane. Therefore the data suggest that both ARS and quinine are additionally detected

by internal gustatory organs of the mouthparts. These are the apical and subapical labral peg organs, trichoid sensilla on the internal face of the labellum and sensilla of the cibarium.

Although feeding from a glass capillary is nearly completely inhibited in *A. gambiae* when 1 mM quinine is mixed with sugar (Kessler et al., 2013), only an insignificant percentage (8%) of females renounce to feed on blood to which this dose of quinine was added. This highlights the importance of other stimuli such as mechanical (membrane piercing), thermal and possibly phagostimulants present in blood which have to be counterbalanced by deterrents to prevent blood feeding. Such stimuli were not present during sugar feeding from the glass capillary tip. When females pierce a silicone membrane to feed the stylets enter the feeding medium leaving the labellar lobes bearing the T1 sensilla outside. Thus, only receptor cells on the labrum, on the internal face of the labellar lobes and within the cibarium are involved in the perception of any stimulants and deterrents. In the sugar meal taken from the glass capillary tip (Kessler et al., 2013) both external and internal receptor cells of the mouthparts were exposed. As most feeding deterrent compounds are found on plants by mosquitoes, it seems logical that the deterrent effect of quinine should be higher when associated with the sugar meal than with the blood meal.

Feeding deterrent effects of antimalarial drugs on A. gambiae *in the context of malaria control*

Since ARS and quinine are used as drugs against malaria, the question arises whether the doses circulating can be detected by mosquitoes. Both ARS and DHA, because of their rapid elimination from humans, and quinine, because of its negative side effects in long term treatment, are not considered for prophylaxis. ARS could be considered as a prodrug, as it is almost entirely converted into its active metabolite DHA before absorption of oral doses and is immediately converted after intravenous administration (Batty et al., 1998). The concentration of ARS inhibiting 50% maximum growth (IC_{50}) of *Plasmodium falciparum in vitro* lies between 1.66 to 2.18 nM, and that of DHA between 0.36 to 7 nM (deVries and Dien, 1996). As recommended by WHO, ARS is administered orally, in combination with mefloquine for the treatment of uncomplicated falciparum malaria or parenterally in severe cases (WHO, 1995). The extrapolated peak plasma concentration of ARS measured in patients treated intravenously (120 mg, 312.5 µmol) against *P. falciparum* reaches 11 mg l^{-1} (29.5 µM) but is rapidly cleared with a half-life of 2.7 min. (Batty et al., 1998). This dose is 34 times lower than the 1 mM that is marginally deterrent against *A. gambiae* feeding. In the study by Batty et al. (1998) it was shown that in intravenously-treated patients ARS is rapidly converted in DHA that reached a maximum plasma concentration at 9.3 µM (2.64 mg l^{-1}) with

a half-life time of 40 min. Quinine is still used for the treatment of malaria in areas where multiple-drug resistance occurs. A single dose of 600 mg of various quinine salts administered to subjects without malaria results in a mean plasma concentration of between 3.4 mg l^{-1} (10.481 µM) and 6.2 mg l^{-1} (19.111 µM) (Krishna and White, 1996). These doses are around 100 to 50 times lower than the highest concentration of quinine tested in the present study (1 mM) which had no significant deterrent effect on feeding by *A. gambiae* on defibrinated bovine blood. Such a dose of quinine has to be administrated in adults every 8 hours for 3, 7 or 10 days (WHO, 1995), thus increasing the plasma concentration. However, quinine plasma concentration was never found to exceed 17.9 mg l^{-1} (55.176 µM) in infected patients treated orally or parenterally with quinine in various pharmacokinetic studies (Krishna and White, 1996). It was already shown that exposing *P. falciparum* gametocytes to a dose of 2.52 µM (816 ng ml^{-1}) quinine prior to allowing female *A. dirus* to feed on infected blood blocks 90% of the malaria transmission to the mosquitoes (Chotivanich et al., 2006). The ED_{90} was situated at some 2000 times lower for ARS than for quinine (1.04 nM, 0.4 ng ml^{-1}). In conclusion, the doses of quinine and ARS affecting *Plasmodium* are much lower than those affecting feeding by *A. gambiae* in our assay. Contrary to ARS, quinine has little effect on the gametocytes (Chotivanich et al., 2006; Lelievre et al., 2012; Pukrittayakamee et al., 2004). Unfortunately, despite the toxic effects of quinine on *A. gambiae*, the effect of quinine in inhibiting blood feeding proved to be very poor on *A. gambiae*. Thus, the feeding deterrence of a circulating antimalarial, particularly that of quinine, would have no or only a minor effect on the infection rate of mosquitoes feeding on treated patients.

Blood stimulates internal mouthpart gustatory receptor cells in females A. gambiae

Our data strongly suggest the presence of internal receptor cells on mouthparts responsible for the detection of blood phagostimulants. The apical and sub-apical labral peg organs, the first to contact the feeding solution, constitute strategic candidates in this regard. Because these sensilla are present exclusively in females of anautogenous mosquito species (Lee and Craig, 1983b), they appear most likely to carry receptor cells tuned for chemostimulants present in blood. Labral chemoreceptor cells were found to respond electrophysiologically to adenine nucleotides of *Culex pipiens* L. and *A. aegypti* as well as to NaCl and to the C-terminal AA of albumin, L-alanine, in *A. aegypti* (Liscia et al., 1993; Werner-Reiss et al., 1999a; Werner-Reiss et al., 1999b).

Furthermore, the gustatory sensilla found on the inner face of each labellar lobe in this study on *A. gambiae* may also be involved in blood chemical detection. These sensilla differ by the presence of the pore on the spur half way along the sensillum and by the presence of a

tubular body from the type 3 (T3) sensilla described by Pappas and Larsen in *C. inornata*, which were found to respond electrophysiologically to NaCl (Pappas and Larsen, 1976). The morphology of the internal labellar sensilla of *A. gambiae* appears like the bifurcate palatal papillae found within the cibarium of *C. inornata* (Lee and Craig, 1983a). Cibarial sensilla were not investigated in this study on *A. gambiae*. However, it is already established that, in all, five types of cibarial sensilla occur in mosquitoes: palatal, dorsal and ventral papillae, campaniform and trichoid sensillum (Lee and Craig, 1983a; McIver, 1982). These papillae probably house chemo-sensitive cells and are thought to be involved in meal palatability and/or assigning the destination of the meal to the crop versus the midgut (McIver, 1982). The campaniform sensilla placed ventrolaterally on the labrum at the entry of the food canal may act as flow detectors (Lee and Craig, 1983b; McIver, 1982).

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8. Discussion and research perspectives

8.1 Laboratory feeding preferences of *A. gambiae* may reflect the availability of sugars in nature

Plant-derived sugars are the basic food for adult mosquitoes. This is true also for the endophilic malaria vector *A. gambiae*. The three main sugar nectar constituents (Wykes, 1952) are preferred by this species in the following order: sucrose > fructose > glucose, as already shown for the yellow fever mosquito *A. aegypti* (Ignell et al., 2010), the winter mosquito *C. inornata* (Schmidt and Friend, 1991) or another dipteran, the blowfly *P. regina* (Dethier et al., 1956).

In addition, the results from our feeding assay have confirmed that the two hexoses glucose and fructose, have a synergistic effect on feeding in *A. gambiae* as in *A. aegypti*, *C. inornata* and *P. regina*. This could mirror the field reality where fructose and glucose are often present at the same proportions in plant nectars (Ignell et al., 2010).

Sucrose was also preferred in the feeding assay over melezitose by both male and female *A. gambiae*. Melezitose is a constituent of some honeydews and was found to be preferred by ants feeding on *Aphis* species producing honeydew (Volkl et al., 1999). Although *A. gambiae* is able to feed on honeydew, Gary and Foster (2004) have measured a survival rate that is higher for mosquitoes maintained on floral nectar of manioc (*Manihot esculenta* Crantz) or *Ricinus communis* L. than for mosquitoes maintained on honeydew, suggesting that floral nectars are more nutritive for *A. gambiae*.

8.2 Sensitivity of labellar sugar sensitive neurones of *A. gambiae* in comparison with other species

Sugar sensitive neurones of type 1 labellar trichoid sensilla are activated by sucrose or by an equimolar mixture of its hexose constituents glucose and fructose. Although the mixture of fructose and glucose elicits a number of spikes slightly lower than sucrose, differences were never significant at the doses tested. However, contrary to *A. aegypti*, fructose and glucose tested alone do not elicit an increase in spike frequency as a function of dose (0.1-292 mM) in the labellar sugar sensitive neurones tested in *A. gambiae*. The synergism between glucose and fructose has already been shown for the labellar sugar sensitive neurones of *B. peregrina* and *P. regina* as well for the receptor cells in the galeal sensilla of the honey-bee (Morita and Shiraish, 1968; Omand and Dethier, 1969; Whitehead and Larsen, 1976). Although sugar sensitive neurones are also more sensitive to sucrose in these species, they also react to glucose or fructose alone. In flies, the labellar sugar sensitive neurones are more sensitive to fructose at low doses although they react better to glucose at high doses (>250 mM in *B. peregrina* and >1 M in *P. regina*) (Morita and Shiraish, 1968; Omand and Dethier, 1969). Inversely, below 1 M, the sugar receptor cell sensitivity of galeal sensilla of

A. mellifera is sucrose > glucose > fructose although, at higher concentrations (>2 M), the sugar receptor cells show more sensitivity to fructose than to glucose (Whitehead and Larsen, 1976). In the honey-bee the sensitivity of sugar receptor cells in sensilla chaetica of the labial palps is sucrose > glucose = fructose (Whitehead, 1978).

Thus, the synergism between glucose and fructose induced on the sugar sensitive neurones of *A. gambiae* is much stronger to what is known for other species. For example, a difference of only 8.4 spikes/s was found between the average of the response to a mixture of 500 mM fructose plus 500 mM glucose and the sum of the response of each hexose sugar presented separately to the labellar receptor cells of *P. regina* (Omand and Dethier, 1969).

We hypothesise that the sucrose receptors expressed by the labellar sugar receptor cell of *A. gambiae* consist of heterodimeres, composed of two subunits that are simultaneously occuped with either one disaccharide molecule or two monosaccharide molecules as previously suggested by Morita and Shiraish (1968) for the fleshfly.

Although we found no activity as a function of dose in labellar sugar sensitive neurones of *A. gambiae* to glucose and fructose, it should be noted that these monosaccharides were never tested at doses higher than 292 mM. They also remain to be tested on the other labellar sensilla than the four investigated in this thesis. Nonetheless, the strong specificity of the labellar sugar receptor cells of *A. gambiae* contrasts to what is known in other species and to the fact that several Grs are usually expressed in insect sugar sensitive neurones (Dahanukar et al., 2007).

Interestingly, we have found that the sugar sensitive neurones in the distal sensilla of the labellar lobes of *A. gambiae*, namely the ventral No 8 and the dorsal No 5 sensilla that lack water sensitive neurones, respond with a higher number of spikes to increasing concentrations of sucrose than the lateral sensilla Nos 2 and 5. Particularly, the sugar sensitive neurones of the ventral No 8 and the dorsal No 5 sensilla appear to be most sensitive to small increments of sucrose up to 5 mM although the lateral Nos 2 and 5 sensilla appear to code better for sucrose between 5 and 50 mM. Thus, in *A. gambiae* the threshold sensitivity for sucrose seems to increase from proximal to distal labellar sensilla, as the sensitivity for water decreases. In *A. aegypti*, the sugar sensitive neurone in the ventral No 10 sensilla, the most distally situated, responds with a maximum number of spikes higher than neurones in the more proximally situated ventral sensilla Nos 9, 7 and 4. Here, however, the sugar sensitive neurone in the ventral Nos 9, 7 and 4 seems to be more sensitive to low doses of sucrose (between 0.1 and 2.5 mM) whereas ones in the ventral No 10 responds better to higher doses (2.5 mM and 25 mM). Generally speaking, in both *A. gambiae* and *A. aegypti*, sugar sensitive neurones on the tip of the proboscis, i.e. the first

to come into contact with nectar, appear to have a higher resolving capacity than the more proximally situated ones. Interestingly, variation in sensitivity with position is also found in bees, in which taste receptor cell sensitivity in sensilla chaetica on the labial palps depends on the segment on which they are found (Whitehead, 1978).

8.3 Relationship between sugar receptor cells and feeding behaviour

The maximum engorgement of 3-5 h starved females *A. gambiae* on sucrose was measured from 250 mM. However, the maximum sensitivity range of sugar sensitive neurones of labellar T1 sensilla to increasing concentrations of sucrose was recorded between 0.1 to 25 mM; afterwards the response plateaus. Thus, sugar sensitive neurones of T1 sensilla are more likely to be involved in the probing-walking response (Sanford and Tomberlin, 2011) displayed by mosquitoes searching for a more concentrated sugar source, rather than in the gorging response. The behavioural threshold of *A. gambiae* for sugars remains to be evaluated. This could be done by testing different concentration of sugars against water in the two-choice assay which is more sensitive than the filter paper or the capillary no-choice assays.

Taste neurones enclosed in taste sensilla within the mouthparts or within the cibarium may be involved in the crop filling response. For example, in *C. inornata*, when sensilla on the inner face of the labellum stimulated with a 1 M sucrose solution in conjunction with the peg organs on the labrum tip serve to initiate pumping (Pappas and Larsen, 1978). Sugar sensitivity of tarsal taste neurones as well those enclosed in sensilla at the tip of the proboscis remain to be explored. In addition to the perception of high sucrose concentrations possessing adequate nutritive value, these internal receptors may also be involved in the perception of other carbohydrates like fructose or glucose.

As internal mouthpart receptors are not accessible for electrophysiological recordings, molecular biology may provide useful tools for this purpose. With respect to the low transcription level of Gr genes (Scott et al., 2001), real-time quantitative PCR performed on specific mosquito organs would constitute an easier method than *in-situ* hybridization to detect where sugar receptors are expressed, although this would not provide information about the sugar ligands as such. Heterologous expression of Grs coupled with patch-clamp recordings or calcium imaging may allow the determination of their respective ligands. This is possible as sugars are well soluble in the medium bath and because Gr proteins are ligand-gated ion channels and seem to respond with an influx of extracellular Ca^{2+} in a G-protein independent manner. This was successfully done recently on the BmGr-9, a fructose receptor of the silkworm *Bombyx mori* (Sato et al., 2011). Testing *A. gambiae* mutants with

selected sugar receptor genes silenced in the feeding assay presented in this thesis would prove to be a powerful tool to discover ligands for sugar receptors. Mutant *Drosophila* lacking specific sugar receptors have already been created as well as mutant *A. gambiae* (Liu et al., 2010) and *A. aegpyti* lacking Ors (Pellegrino et al., 2011).

8.4 Sensing water by T1 and T2 labellar sensilla receptor cells

A water sensitive neurone has been identified in all labellar sensilla of *A. gambiae*, except in the 6 more distally situated. Sugars and salts inhibit these water sensitive neurones, indicating that they are sensitive to osmotic pressure. The strong inhibitory effect of $LaCl_3$, a broad Ca^{2+} channel inhibitor, appears to indicate the involvement of extracellular Ca^{2+} in the transduction pathway as already suggested for the water receptor cells of *Drosophila* (Meunier et al., 2009) and the blowfly *P. terraenovae* (Solari et al., 2010). Nonetheless, no significant increase in the firing rate was observed in *A. gambiae* during stimulation with increasing concentrations of $CaCl_2$, contrarily to what was found in *Drosophila* (Meunier et al., 2009). In fact, although $CaCl_2$ increases the extracellular concentration of Ca^{2+}, it also increases two fold more that of Cl^- and the osmotic pressure. The strong inhibitory effect of $CuSO_4$ may indicate the involvement of an aquaglyceroporin, as $CuSO_4$ is supposed to be an inhibitor of AQP3 (Solari et al., 2010; Zelenina et al., 2004). This would appear to be confirmed by the higher spike frequency of the water receptor cell recorded during stimulation with increasing concentrations of glycerol in 10 mM KCl. Solari et al. (Solari et al., 2010) suggested that glycerol has the ability to cross the membrane of the water receptor cell through aquaglyceroporines, thus inducing cell swelling and the depolarisation of these neurones.

I show in Chapter 6 that a sensory neurone in T2 sensilla generates a response in a highly tonic pattern and at a high spike frequency to water. When the sensillum is bent, a second spike unit with lower amplitude is generated by the mechanoreceptor. This confirms that the response observed during stimulation with water is generated by the hygroreceptor cell. This sensory neurone seems to be sensitive to water and not to osmotic pressure; contrary to the water receptor cell of the T1 sensilla, 100 mM sucrose does not inhibit the neurone in T2 sensilla. Assuming a hygromechanical transduction mode of the neurone, the groove present on the surface of the T2 sensilla may allow swelling or shrinking of the cuticular wall.

In *C. inornata*, two putative chemoreceptor cells and one mechanoreceptor cell innervate the T2 sensilla (Pappas and Larsen, 1976) whereas in *A. gambiae*, only one sensory cell is present in addition to the mechanoreceptor. Interestingly, Kwon et al. (2006) have found that the chemoreceptor cell in T2 sensilla of *A. gambiae* responds also to odorants related to humans such as isovaleric acid, butylamine and several ketones and oxocarboxylic acids.

Moreover, 24 Ors were also found by the latter authors to be expressed in the proboscis. Moreover, neuronal projections from the proboscis target both the subesophageal ganglion and the antennal lobes suggesting that this appendage have olfactory functions in addition to carrying hygroreceptor and mechanoreceptor cells (Kwon et al., 2006). In a future study, it could be interesting to test if the chemoreceptor cell can be activated by an increase in RH. In fact, it was shown recently that water vapour is involved in the biting behaviour of both tsetse flies and mosquitoes (Chappuis et al., 2013; Klun et al., 2013).

No T2 sensilla were found on the distal 40 µm of the labellar lobes and no receptor cells respond to stimulation with water in the 6 more distally situated T1 sensilla. Nonetheless, the four trichoid sensilla, not classified within the T1 group, found at the tip of the labellum (Appendix VII) house a water receptor cell. As all T1 sensilla enclose four chemo-sensory receptor cells, the ligand(s) that activate the cells which replace the water sensitive neurones in these distal T1 sensilla remain to be identified.

8.5 Nectar quality may influence vector competence of *A. gambiae*

In this thesis, we have shown that not only presence or absence of nectar may influence the vector competence of *Anopheles* but also the sugar constituents of nectar play a role, mainly by acting on mosquito survival and on their motivation to bite. In our cage studies the effect of sugar may be underestimated (Stone et al., 2011), due to the high energy reserves that mosquitoes accumulate as larvae, the close proximity of mating partners and the proximity of the warm body mimicking the skin of a potential vertebrate host. Thus, under natural conditions, differences between the biting behaviour and survival of mosquitoes fed on nectar of different nutritive values may be higher. Testing artificial nectars or plants with different nutritive values in mesocosms could prove to be important (Stone et al., 2011).

I have shown in Chapter 6 that mosquito feeding preferences are correlated to the nutritive value of sugars. Likewise, *A. gambiae* prefers to feed on plants producing nectar thus maximizing their survival and their fecundity (Manda et al., 2007a; Manda et al., 2007b). In addition, the fact that mosquitoes are able to associate odours and visual simulation with food rewards (Chilaka et al., 2012; Sanford and Tomberlin, 2011) probably helps them in nature to rapidly locate plants producing nectars of high nutritive value.

Differences in longevity between mosquitoes maintained on sucrose and the mixture of its two hexose constituents, glucose and fructose, as well the difference in weight observed between mosquitoes maintained on sucrose versus glucose suggest that sucrose, fructose and glucose are absorbed and/or metabolized through different pathways. Unfortunately, sugar assimilation in insects is still a black box despite its primarily physiological importance.

The nutritive value of other nectar constituents such as amino acids remains to be evaluated in *A. gambiae*.

8.6 Taste stimuli from hosts

Our membrane feeding assay highlights the importance of blood tastants in the gorging response of female *A. gambiae*. However, these phagostimulants remain to be identified. As labral taste pegs are unique to females, they constitute strong candidates to enclose receptor cells sensitive to blood compounds (Werner-Reiss et al., 1999a; Werner-Reiss et al., 1999b). The silicone membrane used in this feeding assay presents the advantage to be treatable by skin extracts, for example. Moreover, camera-assisted recording of behaviour is readily available for this assay. Comparing the feeding preferences of the vector between a treated versus an untreated zone on the membrane would allow us to understand the involvement of contact chemoreceptor cells in feeding site choice of mosquitoes. In this regard, it may be interesting to compare the feeding preferences of a mostly zoophilic species such as *A. stephensi* Liston with a mostly anthropophilic species such as *A. gambiae*.

8.7 Bifurcate sensilla, a specific taste organ in females

The ultrastructure of tarsal bifurcate sensilla is described in detail in the present thesis. As these sensory organs are specific to females, they may be involved in the recognition of host skin stimuli, oviposition site stimuli or, eventually, of mating partners. They appear to be common to all *Anophelinae,* as we also observed them on the tarsi of female *A. atroparvus* and *A. stephensi,* but seem to be absent in *Culicinae* as they were not found in females *A. aegypti*. Bifurcate sensilla were first described in the female blackflies *Simulium venustum* which share a similar biology to mosquitoes (McIver et al., 1980). In fact, in *Simulidae*, only females feed on blood whereas males feed on nectar and, as in mosquitoes, larval development is aquatic.

8.8 Bitter taste: towards new mosquito deterrents

In the fifth Chapter, I show that bitter compounds that inhibit sugar feeding in *A. gambiae*, namely denatonium benzoate and the alkaloids quinine, quinidine and berberine, are perceived through inhibition of receptor cells involved in the perception of sucrose or water. The inhibition of these two taste receptor cells appears to be specific as no significant effect was recorded for caffeine. Likewise, caffeine also fails to inhibit sugar feeding in *A. gambiae*.

Inhibition of both sugar and water receptor cells occurs at the range of concentrations that inhibit feeding (0.01-1 mM). Bitter and potentially toxic compounds have an effect at doses situated well below those of sugars. Sugars are detected by taste neurones at doses that start to have a nutritive value, whereas bitter compounds are detected at lower doses, but

which are already toxic for the mosquitoes. In Chapter 7, a high mortality was recorded for mosquitoes fed on blood to which 1 mM quinine was added.

Bitter products such as quinine may act as ion channel inhibitors (Chen and Herness, 1997). However, the genome of *A. gambiae* encloses in all 90 putative Grs (Kent et al., 2008) of which only 8 are estimated to be sugar receptors (Kent and Robertson, 2009). Thus, it is likely that a broad number of Grs serve *A. gambiae* to detect the various plant secondary metabolites that may be present in nectars. I did not succeed to characterize a deterrent receptor cell in the lateral T1 sensillum No 5 on the labellar lobes using denatonium benzoate, quinine, quinidine, berberine chloride or caffeine. Neither did 1 mM quinine activate a bitter receptor cell in the lateral sensillum No 2, the ventral sensillum No 8 nor the dorsal sensillum No 5 (Appendix II). However, in *A. aegypti*, a deterrent receptor cell in T1 sensilla is slightly activated by quinine at 1 mM and the same receptor cell appears to be activated by the two mosquito repellents DEET and icaridin (Sanford et al., 2013). In *A. gambiae*, two receptor cells seem to be activated by the repellent icardin in the lateral sensilla Nos 2 and 5 but only one was found to be activated in the ventral No 8 sensillum and in the dorsal No 5 sensillum (Appendix III). Moreover, DEET at high concentration also activates a receptor cell in the lateral sensillum No 5 (Appendix IV). This indicates the presence of deterrent receptor cells in labellar sensilla of *A. gambiae*, but, as already shown for *Drosophila*, sensitivity to deterrents seems to vary between sensilla (Weiss et al., 2011). In a future study, it could be interesting to investigate if this is related to a differential expression of Grs by deterrent-sensitive receptor cells. In Chapter 7, I show behaviourally that deterrent are detected by receptor cells in internal mouthpart gustatory organs, namely labral peg organs, sensilla on the inner face of the labellar lobes or cibarial receptor cells. Such taste organs could well enclose receptor cells selectively activated by deterrents.

In our feeding assay, caffeine at 1 mM was not found to inhibit sugar feeding of *A. gambiae*. This dose also failed to inhibit both the sugar and the water sensitive neurones. Similarly, in *A. aegypti*, 1 mM caffeine failed to stimulate the deterrent receptor cells. All plant secondary metabolites do not act on insects by inhibiting feeding. For example, it was shown recently that caffeine is present in some nectars at doses that have no deterrent effect and alters the behaviour of pollinators by enhancing their memory of reward, thus increasing the probability of the plant being pollinated (Wright et al., 2013).

Investigations on the gustatory responses of mosquitoes to bitter compounds could well lead to the identification of products with low vapor pressures that persist on substrates to deter landing and biting responses of mosquitoes. In this perspective, it could be interesting to test other alkaloids and to extend the range of compounds tested to other chemical classes such as terpenes for example (Schoonhoven, 1982).

8.9 Effect of insecticides on *A. gambiae* taste neurones

I have tested the neurophysiological activity of two insecticides used to control adult mosquitoes on taste labellar neurones of female *A. gambiae* (Appendix VI).

Malathion is an organophosphate which acts as an acetylcholinesterase inhibitor and cypermethrin is a pyrethroid that maintains sodium channels of neurones open (IRAC 2010). No effect of malathion, which acts at the level of the synapse, was observed on the sugar and the water sensitive neurones. However, cypermethrin is deleterious to these two neurones. No receptor cells were found to be activated by these two insecticides in these preliminary data. However, this study would need to be extended to other taste neurones, notably those of the legs, and other insecticide classes remain to be tested. Insecticides could also been tested at sub-lethal doses. Should taste receptor cells mediating deterrence behaviour be activated, this could limit contact of the insect with the insecticide and thus facilitate the development of resistances.

9. Remerciements

Un grand merci à toutes les personnes qui ont contribué par leurs compétences et leur disponibilité à la réalisation de ce projet de thèse.

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10. Appendix I: An *in vitro* assay for testing mosquito repellents that employs a warm body and carbon dioxide as a behavioural activator

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AN IN VITRO ASSAY FOR TESTING MOSQUITO REPELLENTS EMPLOYING A WARM BODY AND CARBON DIOXIDE AS A BEHAVIORAL ACTIVATOR

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ABSTRACT. We describe here an in vitro behavioral assay for testing mosquito repellents applied in a dose-based manner to a warm body (34°C) in test cages. The system was used to assess the sensitivity of 4–6-day-old *Anopheles gambiae* to the insect repellent diethyl methyl benzamide (deet). These tests were made in the absence and presence of additional carbon dioxide (CO_2) applied as a pulse to activate mosquitoes in the cages. In the absence of the CO_2 pulse the mosquitoes hardly responded to the warm body. Increasing the CO_2 level in the cage by 1,000 parts per million caused a 25-fold increase in the number of landings by mosquitoes on the warm body in 2-min tests. This mosquito activation allowed the measurement of a significant reduction in the number of landings to bite on the warm body with increasing doses of deet (0.4 to 3.8 μg/cm^2). An asymptotic nonlinear model fitted to the repellency data in the presence of CO_2 allowed estimation of the effective dose of deet that reduced landings to bite by 50% (ED_{50}) at 0.95 μg/cm^2 (5 nmol/cm^2) and the corresponding ED_{95} at 4.12 μg/cm^2 (21.5 nmol/cm^2). This in vitro bioassay has the advantage of permitting a fast throughput of test products under standardized conditions and is suitable for screenings designed for the purpose of discovering lead products with as yet unknown human toxicological and dermatological profiles.

KEY WORDS In vitro repellent assay, mosquito, insect vector, *Anopheles gambiae*, deet

INTRODUCTION

Diethyl methyl benzamide (deet) is the most widely used repellent against a wide range of biting arthropods including mosquitoes (Debboun et al. 2007), although new N-acylpiperidine products with longer lasting repellence against *Aedes aegypti* (L.) have been recently described (Katritzky et al. 2008). Estimation of repellency is usually based on comparison between the number of bites on a treated versus an untreated forearm or leg exposed to mosquitoes for a finite period (Granett 1940). The repellent efficacy of a product is estimated as the rate of bites per minute or the time required to obtain the first bite (protection time) on an individual from a test population of mosquitoes. To evaluate the effective dose (ED) required to repel a given percentage of an ectoparasite test population, a ''dose-response method'' has been adopted where several doses of a repellent can be tested simultaneously on a test subject's limb exposed to mosquitoes (Buescher et al. 1982, Klun and Debboun 2000). However, in vivo experiments are not always ideal, since mosquito attraction can vary between test subjects (Carlson et al. 1992). In addition, pharmacokinetic studies on the skin of different mammals revealed that portions of the initial dose of deet applied, which ranged from 4 to 500 μg/cm^2, were absorbed over a time interval of 10 h to 5 days (Qiu et al. 1998) and, as such, did not evaporate from the skin. Although repellents designed to protect humans must eventually be evaluated in vivo, this is not necessarily the case for screenings designed for the purpose of discovering products with as yet unknown human toxicological and dermatological profiles. Bar-Zeev and Schmidt (1959) were the earliest to use a membrane, derived from the outermost layer of ox cecum, to evaluate repellent product efficiency. Subsequently, the Klun and Debboun module (2000) was adapted for in vitro tests on repellents using Baudruche® and collagen membranes (Klun and Debboun 2005).

In this study, we recorded in the test cages the number of landings by female *Anopheles gambiae* Giles, activated by a CO_2 pulse, on a warm body treated with increasing doses of deet and showed that the number of mosquitoes landing decreased with an increasing dose of the repellent. This in vitro assay, which uses a dose-based approach, can be readily adopted in screenings to identify novel repellent molecules and to establish threshold levels above which such molecules can affect mosquito behavior.

MATERIALS AND METHODS

Mosquitoes: The *An. gambiae* colony (16cSS strain, derived in 1974 from wild caught adults originating from Lagos, Nigeria, West Africa) was maintained in a climate chamber (28°C, 80% relative humidity [RH]) under a 12:12 light:dark cycle with 2 h simulated sunrise and sunset. Females were fed on a Guinea pig (*Cavia*

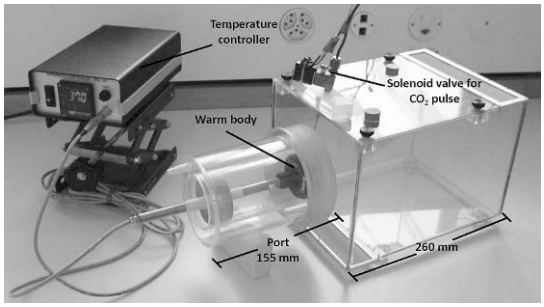

Fig. 1. The bioassay setup with the mosquito cage, warm body inserted into the port, temperature controller and solenoid valve for CO_2 pulse delivery.

porcellus L.) once a week and eggs were recovered on wet filter paper. About 250 larvae were reared in trays with 400 ml distilled water (8 mm deep) and fed with pulverized Tetramin® fish food. About 800 adult mosquitoes emerged into rearing cages (350 × 350 × 550 mm high) and were provided with 10% sucrose and water ad libitum. For the bioassay, we used 50 ± 6 *An. gambiae* females between 4 and 6 days old that were attracted to a hand in the rearing cages. Jones and Gubbins (1978) have shown an increasing flight activity of inseminated *An. gambiae* females during the scotophase from 4 days old. Bioassay cages containing the mosquitoes were positioned at least half an hour before the tests in the experimental climate chamber, to avoid mechanical stimulation of the mosquitoes through handling of the cages before tests.

Bioassay cages: The bioassay cages were made of polycarbonate (Makrolon®; Borotec, Bern, Switzerland; 200 × 260 × 180 mm high; Fig. 1). They had a front side opening (110 mm diameter) in which a port was fitted permitting the introduction of the warm body (below) in line with the wall of the cage without escape of mosquitoes. The port was made of 2 concentric acrylic glass Gevacril® tubes (110 mm outside diameter, 63 mm inside diameter, and 155 mm long; Melzo, Italy) into which the warm body was inserted during 2-min experiments. Otherwise a plastic "stopper" was put into the port. Two openings, 1 at the bottom and 1 on the top of each cage (200 × 50 mm), closed by netting (1 mm mesh size) facilitated exchange of air.

Warm body: Attraction of female mosquitoes was evaluated using a cylindrical warm body (WB, 60 mm diameter disk, 20 mm thick) as a heat source (Fig. 1). A low voltage electrical current (power 33 Watts) was split in parallel over 3 resistors fixed in a triangular arrangement to the inner wall of the black anodized aluminum corpus of the WB. A glass Petri dish (60 mm diameter) with a sandblasted floor was attached to the face of the warm body by metal springs. A PT100 temperature controller maintained the temperature of the WB to within 37°C ± 0.5°C, assuring a temperature of 34°C ± 0.5°C on the surface of the Petri dish. A white filter paper disk (55 mm diameter, Whatman No. 10 311 807) was inserted between the Petri dish base and the black warm body surface to visualize mosquito landings.

Bioassays: Experiments were conducted in a walk-in climate chamber (25°C ± 1°C and 80% ± 3% RH) during the last 6 h of scotophase. The sides of the bioassay cages were covered with white cardboard to avoid activation by visual stimuli due to the presence of the experimenter. Dim light (4 Lux) was provided from above by fluorescent tubes (Philips TLD, 32 Watts at 36 KHz). The Petri dish floor was treated with 100 µl pure ethanol (Merck, Darmstadt, Germany) as control or with 100-µl ethanol solutions containing of 0.1, 0.3, or 1 µg/µl of deet (Riedel de Haen, Pestanal®, Seelze, Germany) providing doses of 0.4, 1.1, and 3.8 µg/cm^2 on the Petri dish floor. After evaporation of the solvent (40 s) the warm body with the Petri dish attached vertically to its face was introduced through the port such that the Petri dish base was in line with the vertical wall of the cage.

In the case of experiments performed in the presence of CO_2, a 0.2-sec pulse of pure CO_2 (volume 6.25 ml, 99.99%, $H_2O \leq 200$ parts per million [ppm]) from a pressurized cylinder (Carbagas, Switzerland) was applied using a solenoid valve just after the warm body with the attached Petri dish was introduced (Fig. 1). This caused the CO_2 level in the cage to rise from 500 ppm to 1,500 ppm to activate the mosquitoes. Such an increment ensured that all the mosquitoes in the cage were activated. The CO_2 pulse was delivered 40 mm above the center of the warm body and at 10 mm in front of it. CO_2 levels were measured using a gas analyzer (model Li-820; LiCor, Lincoln, NE) near the wall on the opposite side of the cage and at 90 mm from the top. CO_2 values were measured during each experiment at 1-sec intervals and stored on a personal computer. To monitor the number of landings, the warm body was filmed with a low light sensitive black and white charge-coupled device camera (model WV BP310; Panasonic, Osaka, Japan) equipped with a TV zoom lens (model J6 × 12, 12.5–75 mm 1:18; Canon, Tokyo, Japan) placed at the opposite side of the cage.

Doses of deet at 0.4, 1.1, and 3.8 µg/cm^2 (2, 6, and 20 nmol/cm^2, respectively) were tested without an additional CO_2 pulse in 12, 14, and 18 cages, respectively. These 44 tests without the addition of CO_2 were preceded by ethanol controls. The same doses of deet were also tested in the presence of the CO_2 pulse in 12, 17, and 22 cages, respectively, preceded by a test with ethanol as control with a CO_2 pulse in all 51 cages. The minimum time interval between a

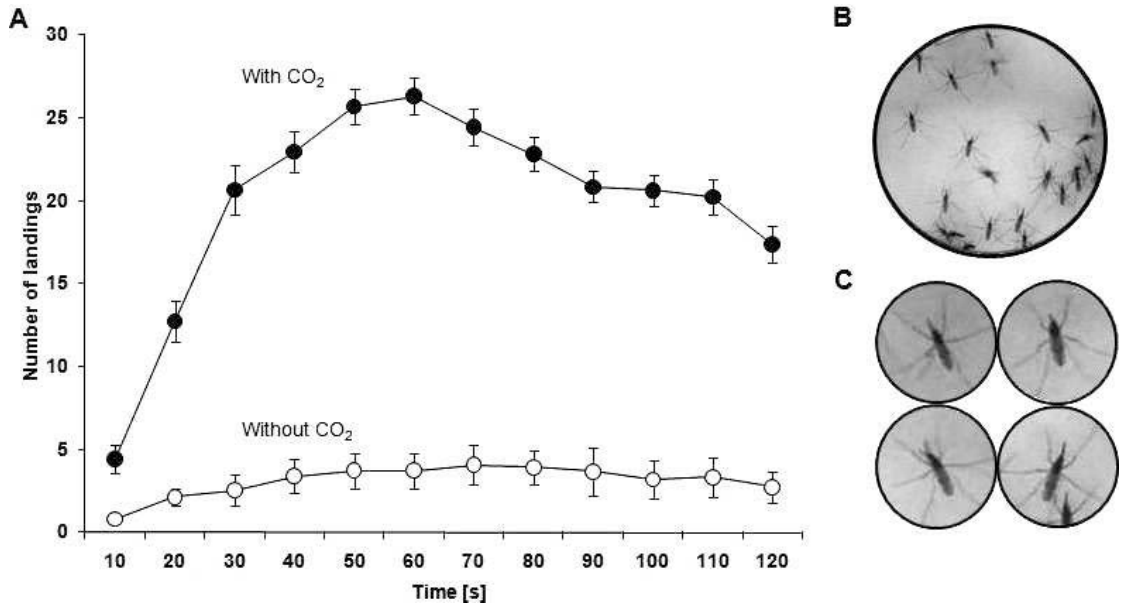

Fig. 2. (A) Mean number of landings made by *An. gambiae* females at 10-sec intervals on the warm body heated at 34°C in the presence of a CO_2 pulse in cages with 50 ± 6 mosquitoes (n = 39 cages, solid circles) compared with the number of landings on the warm body in absence of the CO_2 pulse (n = 15 cages, open circles). The presence of CO_2 as an activator causes significantly increased visits by *An. gambiae* to bite on the warm body over the first 30 sec; bars = 1 SE. **(B)** *An. gambiae* landing on the WB surface. **(C)** Different proboscis positions depicting probing attempts by *An. gambiae* females on the WB surface in the presence of CO_2.

control and a deet treatment was never shorter than 20 min for a batch of mosquitoes. Dekker et al. (2002) allowed *An. gambiae* to acclimatize for 20 min in an olfactometer before testing. The different doses of deet were tested at random, and each cage was used only once for each dose. All cages were furthermore exposed to the WB plus a CO_2 pulse after a series of the aforementioned experiments to confirm that the mosquitoes still showed appetence (data not shown).

Data analysis: The total number of mosquito landings over 2 min on the vertical surface of the Petri dish was counted for ethanol controls and for the 3 doses of deet tested with and without the CO_2 pulse. In addition, we counted the number of mosquito landings in controls on the Petri dish treated with ethanol alone on the warm body for 10-sec intervals during 2 consecutive minutes in 39 cages in the presence and in 15 cages in the absence of the CO_2 pulse. Most of the mosquitoes left the warm body after 1 to 15 sec but landed again after a short period of time elsewhere on the Petri dish floor. As such, multiple landings by the same mosquitoes were counted as independent events.

For each cage, a repellency index for deet was calculated using the formula

$$\frac{L_{\text{control}} - L_{\text{deet}}}{L_{\text{control}}} \times 100$$

where L was the number of landings on control or the test surfaces. An asymptotic nonlinear model

$$\text{repellency} = 100 \times \{1 - \exp\,[-\exp\,(A \times \text{dose})]\}$$

using an equation passing through the origin (no repellent effect when no deet was applied) and with a fixed asymptote converging to 100% repellency was fitted to the data set of repellency indices recorded for each cage with a nonlinear regression (NLS in R version 2.9.0). This software was also used for graphical representation of data.

RESULTS

In the presence of the WB alone the visit rate by *An. gambiae* females reached a mean level of 3 per 10-sec interval with 10% to 60% of the mosquitoes in each cage responding, and this barely changed over the 2 min of observation (Fig. 2A open circles). Injection of a pulse of CO_2 into the cage induced a drastic increase in the number of mosquitoes visiting the WB over 30 sec to reach a plateau at a mean level of 22 visits per 10-sec interval with 95% of the individuals responding to the warm body (Fig. 2A solid circles). Remarkably, most landings on the Petri dish base were immediately followed by several attempts of probing with mouthparts (Fig. 2B, 2C), similar to the biting pressure observed in tests on human skin. Probing attempts were too difficult to count accurately.

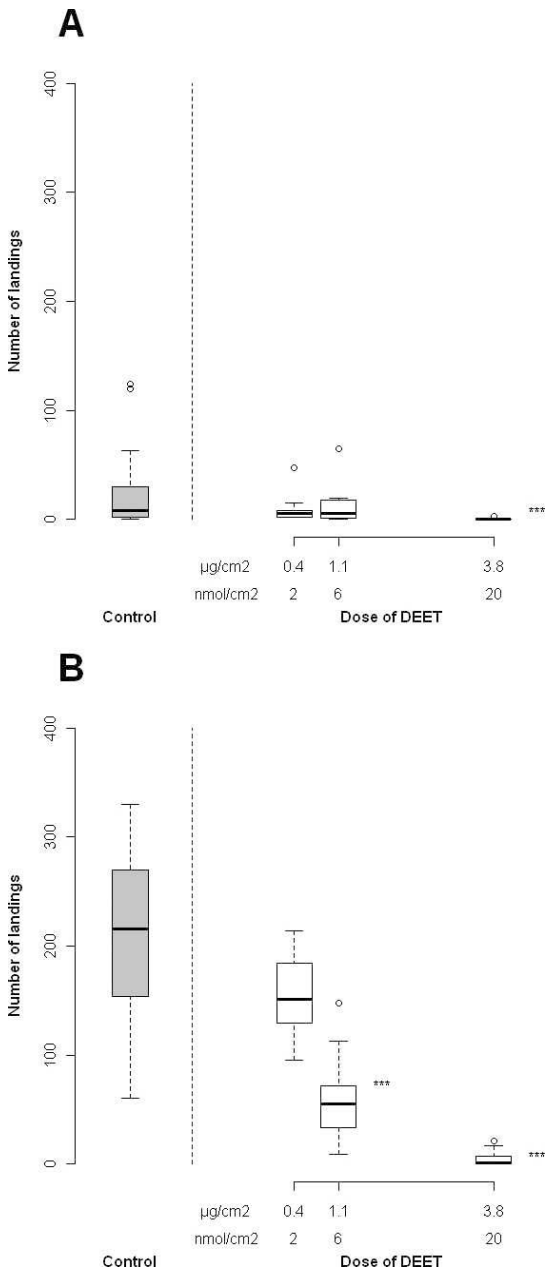

Fig. 3. Number of landings during 2-min experiments made by female *An. gambiae* on the warm body treated with increasing doses of deet (A) in absence and (B) in presence of a CO_2 pulse. Controls (0 µg/cm^2) are depicted in gray, and the 3 different doses of deet tested are shown in white. Box plots represent the median (black bar) and the 25%–75% interquartile interval (box), whiskers depict the 10th–90th percentile, and the points depict outliers. *** Doses different from the control at $P < 0.001$.

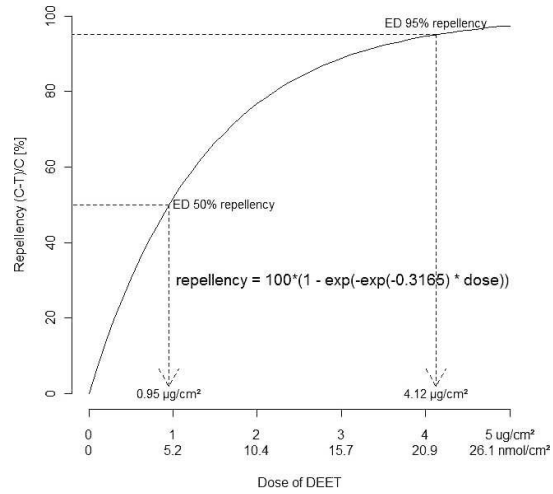

Fig. 4. Dose-response curve describing the repellency of deet on a warm body to *An. gambiae* females. The curve was fitted using an asymptotic nonlinear regression model passing through the origin. The arrows indicate the estimates of the effective doses that cause 50% and 95% repellency.

In treatments with deet but without the CO_2 pulse, the cumulative number of females landing on the WB treated with doses of 0.4 or 1.1 µg/cm^2 of deet were not different from the control (Kruskal–Wallis test, $P > 0.05$). Only at 3.8 µg/cm^2 was a significant drop in landings established (Mann–Whitney *U*-test, $P < 0.001$; Fig. 3A). In the presence of the CO_2 pulse, female *An. gambiae* showed a clear dose-dependent reduction in the number of landings in response to deet (Fig. 3B). A strong interaction between CO_2 level and deet concentration was confirmed by a Friedman 2-way analysis of variance ($P < 0.05$). The 0.4 µg/cm^2 dose of deet approached the lower amount of this product that affected landings by female *An. gambiae* on the WB (Mann–Whitney *U*-test, $P = 0.32$, compared to the control; Fig. 3B). The 1.1 µg/cm^2 dose induced a 4-fold reduction in the median number of landings in the presence of the CO_2 pulse (Mann–Whitney *U*-test, $P < 0.001$; Fig. 3B). The response of *An. gambiae* to the WB treated with the highest dose of deet in the presence of the CO_2 pulse was even higher compared with the situation without the CO_2 pulse (Mann–Whitney *U*-test $P < 0.001$; Fig. 3A and B). Despite this response, the median number of landings on the WB with CO_2 added was only 1 per 2-min experiment at the 3.8 µg/cm^2 dose (Fig. 3B).

The nonlinear asymptotic regression model indicates a highly significant dose-dependent effect of deet on *An. gambiae* in the presence of an augmented level of CO_2 (natural log rate constant 0.3165, *t*-value 2.957, residual standard error 19.82, df 50, $P \leq 0.01$; Fig. 4). The effective

dose to reduce landings by 50% (ED_{50}) was estimated at 0.95 μg/cm^2, and the corresponding ED_{95} at 4.12 μg/cm^2.

DISCUSSION

The mosquitoes increased their visits on the WB heated to 34°C when the CO_2 level increased by some 1,000 ppm in the bioassay cage. We chose to add 1,000 ppm CO_2 to assure adequate stimulation (Grant et al. 1995). This permitted the establishment of a clear dose-dependent response by *An. gambiae* females to the different doses of deet tested in the presence of the CO_2 pulse. In the absence of CO_2 as an activator, the WB was largely disregarded even in controls. Although heat is important for the approach of mosquitoes to a nearby host (Khan et al. 1966, Gillies and Wilkes 1969), this physical cue alone only elicited a response from a few individuals in our cages.

Khan and Maibach (1966) and Khan et al. (1966) have already shown that attraction to a WB heated to 34°C is increased for *Ae. aegypti* when CO_2 is released in its vicinity. A brief CO_2 pulse can increase sensitivity to skin odors in female *Ae. aegypti* and increase the number that find this odor source in a wind tunnel (Dekker et al. 2005). Furthermore, Mayer and James (1969) have demonstrated in an olfactometer that adding 500 ppm CO_2 increases the percentage of *Ae. aegypti* attracted to an arm treated with 1 ml of 5% deet by 3-fold. The 3.8 μg/cm^2 dose of deet showed a high repellency index against *An. gambiae* in our assay even in the presence of the CO_2 pulse. We also performed trials with *An. stephensi* Liston and *Ae. aegypti* using this assay and found these 2 species show responses similar to *An. gambiae*. At 3.8 μg/cm^2, the repellency index of deet reached 74% for *An. stephensi* and 94% for *Ae. aegypti*.

Our results confirm the effectiveness of deet in repelling mosquitoes. Its effect is related to the dose applied, with increasing doses causing decreasing numbers of *An. gambiae* landings on the WB in the presence of the CO_2 stimulus. The response curve established from our experiments suggests that the dose of 0.4 μg/cm^2 (2 nmol/cm^2) is near the lower amount of this product that affects the landing behavior of female *An. gambiae* on the WB. The effective dose that repels 50% of a test population of *An. gambiae* (ED_{50}) on rabbits has been estimated at 2.5 μg/cm^2, and the ED_{95} has been estimated at 12.8 μg/cm^2 (Robert et al. 1991). The ED_{95} for biting reduction by *Ae. aegypti* was estimated at 23 nmol/cm^2 (4.4 μg/cm^2) on human volunteers (Klun et al. 2004). The effective doses of deet required for repellency on humans are higher, probably because of the anthropophilic preferences of species such as *An. gambiae*. Indeed, the ED_{50} of deet for the same 16cSS strain of *An. gambiae* used here has been estimated at 19.9 nl/cm^2 (19.82 μg/cm^2) and the ED_{90} at 71.2 nl/cm^2 (70.92 μg/cm^2) for humans (Curtis et al. 1987). This means that the dose inhibiting 95% of the landings in our assay (4.12 μg/cm^2) is some 20 times lower than that required to affect 90% of individuals in an *An. gambiae* population attempting a blood feeding on humans. This difference is probably due to the fact that the WB does not carry the numerous chemicals associated with the skin of the vertebrate host. We have compensated for this lower attractiveness of the WB by releasing CO_2 to simulate the increasing concentration of this metabolite encountered by mosquitoes near a potential human host that releases about 45,000 ppm CO_2 at each expiration (Gillies 1980).

There is a range of in vivo tests involving volunteers to evaluate repellence as described by the US Environmental Protection Agency (US EPA 1999) and the one used recently in development of novel repellents for mosquitoes by Katritzky et al. (2008). The bioassay described here has the advantage of permitting a faster throughput of test products under standardized conditions. The rearing and test condition for this study were such that there was no shift in the phenological state of the mosquitoes over the period of the study, i.e., adult emergence and daily activity rhythm were constant. In addition, the experimental design involved controls on each day of tests.

This assay is already being used to test products affecting *An. gambiae* behavior in the framework of the EU-sponsored European Network for Advanced Research on Olfaction for Malaria Transmitting Insect Control (www.enaromatic.org).

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11. Appendix II: Electrophysiological stimulations of *A. gambiae* labellar sensilla with quinine

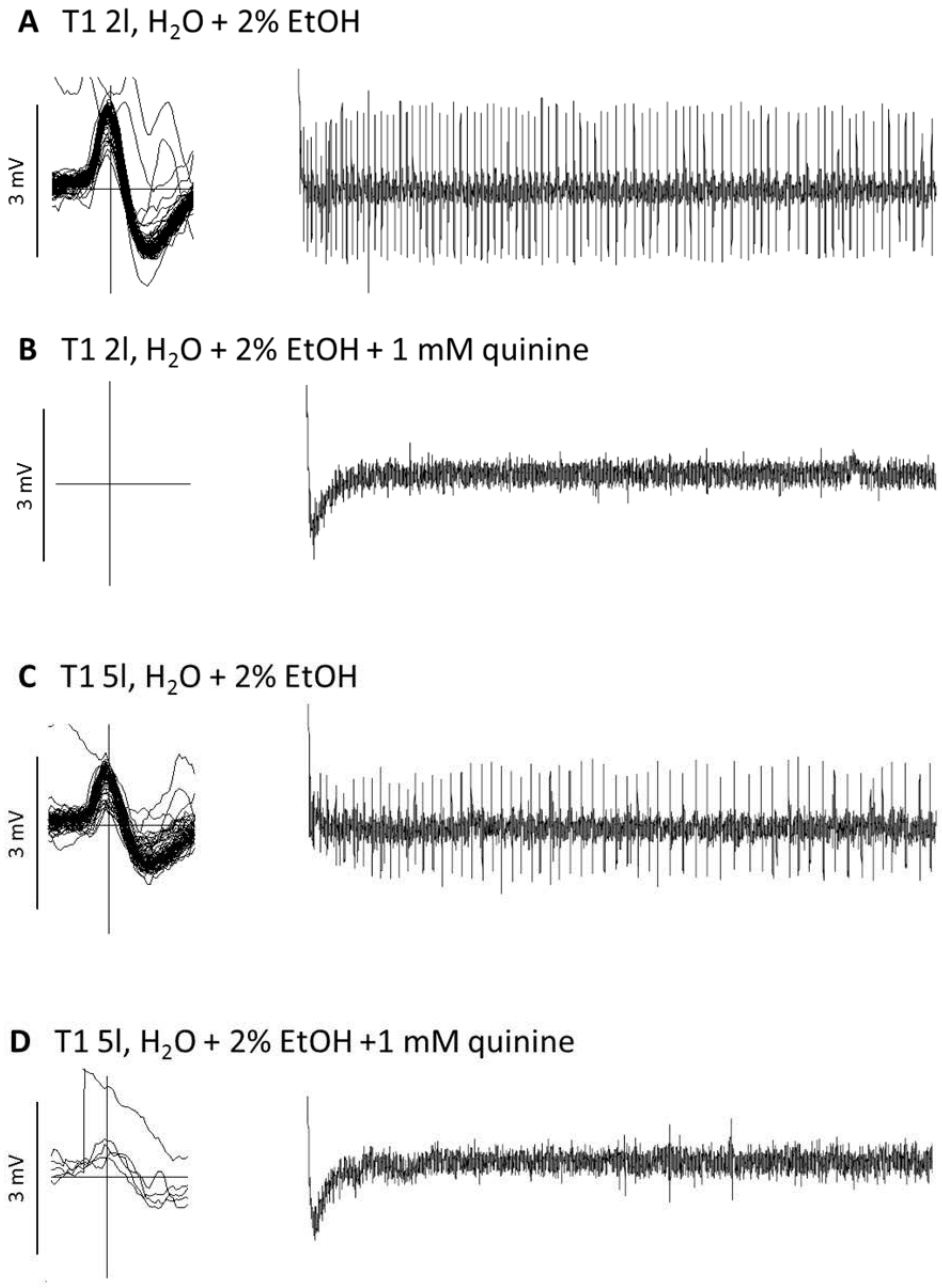

Fig. II.I: 2 s electrophysiological recordings from the lateral T1 labellar sensilla Nos 2 (A and B) and 5 (C and D) in response to water + 2% ethanol (A and C) and water + 2% ethanol + 1 mM quinine (B and D). Quinine was first dissolved in ethanol to facilitate dilution. 2% ethanol had no effect on spike activity. Quinine inhibits the water sensitive neurone in both sensilla but does not activate a receptor cell. Recordings are typical for between twelve and thirteen 0-5 day-old mosquitoes tested for each sensillum.

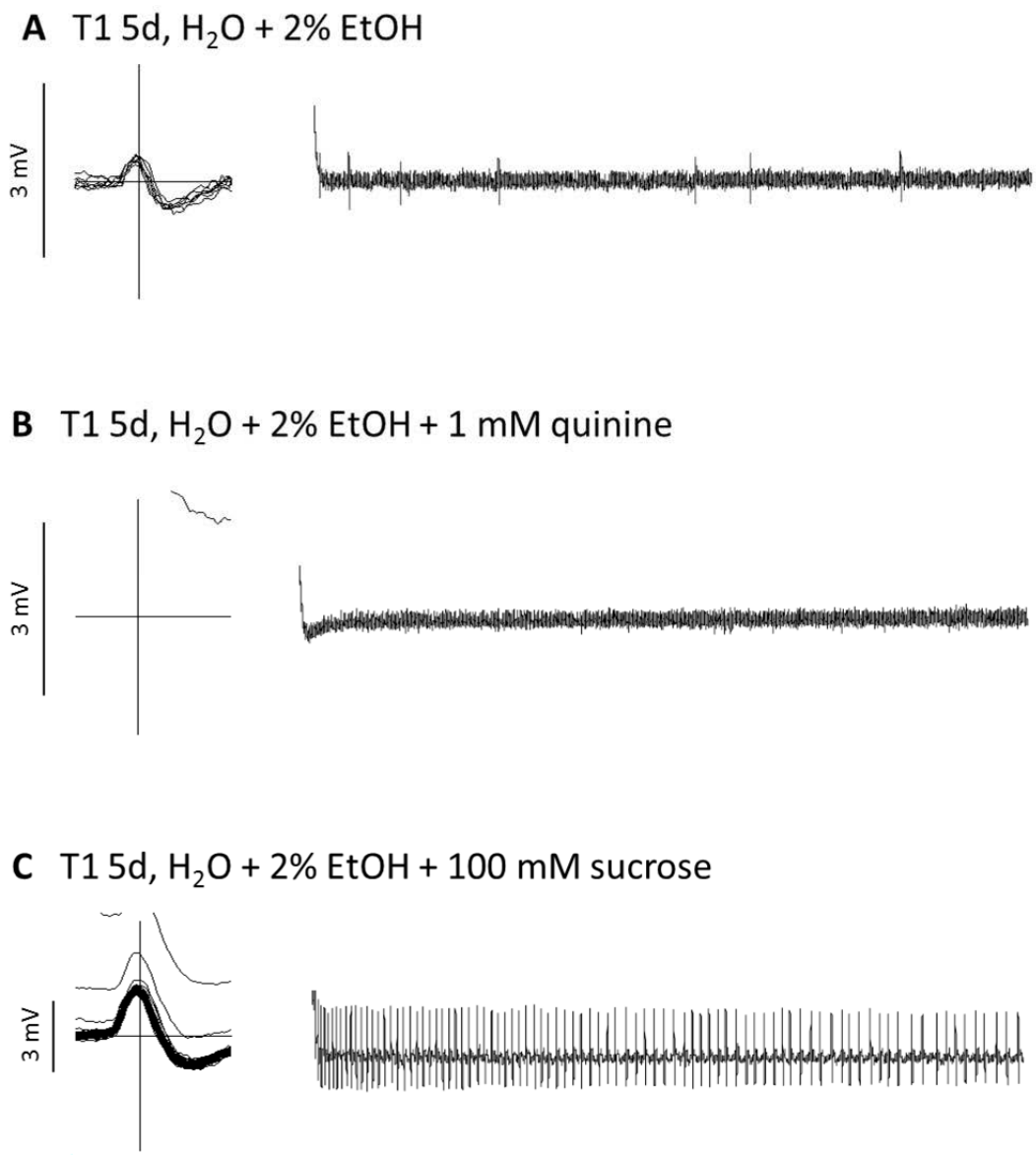

Fig. II.II: 2 s electrophysiological recordings from the T1 dorsal labellar sensillum No 5 in response to water + 2% ethanol (A), water + 2% ethanol + 1 mM quinine (B) and water + 2% ethanol + 100 mM sucrose (C). Quinine was first dissolved in ethanol to facilitate dilution. 2% ethanol had no effect on spike activity. No receptor cell is activated by water (A) or quinine (B) although 100 mM sucrose activates the sugar receptor cell (C). In recording A, the spikes generated do not represent a specific response to water as the frequency is low and are not systematically observed during stimulation with water. Recordings are typical for the nine 0 day-old mosquitoes tested.

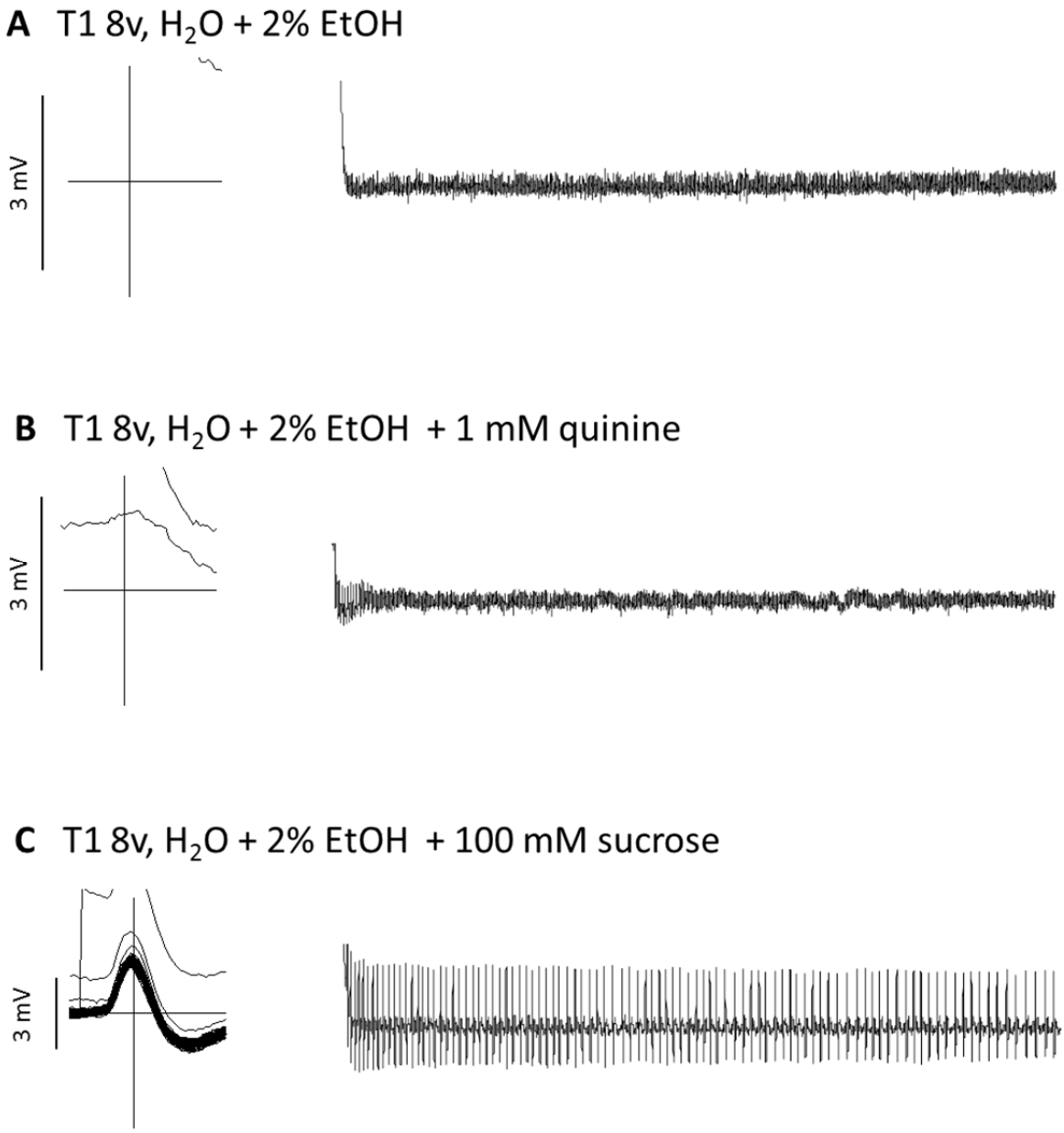

Fig. II.III: 2 s electrophysiological recordings from the T1 ventral labellar sensillum No 8 in response to water + 2% ethanol (A) water + 2% ethanol + 1 mM quinine (B) and water + 2% ethanol + 100 mM sucrose (C). Quinine was first dissolved in ethanol to facilitate dilution. 2% ethanol had no effect on spike activity. No receptor cell was activated by water (A) or quinine (B) although 100 mM sucrose activates the sugar receptor cell (C). Recordings are typical for the nine 0 day-old mosquitoes tested.

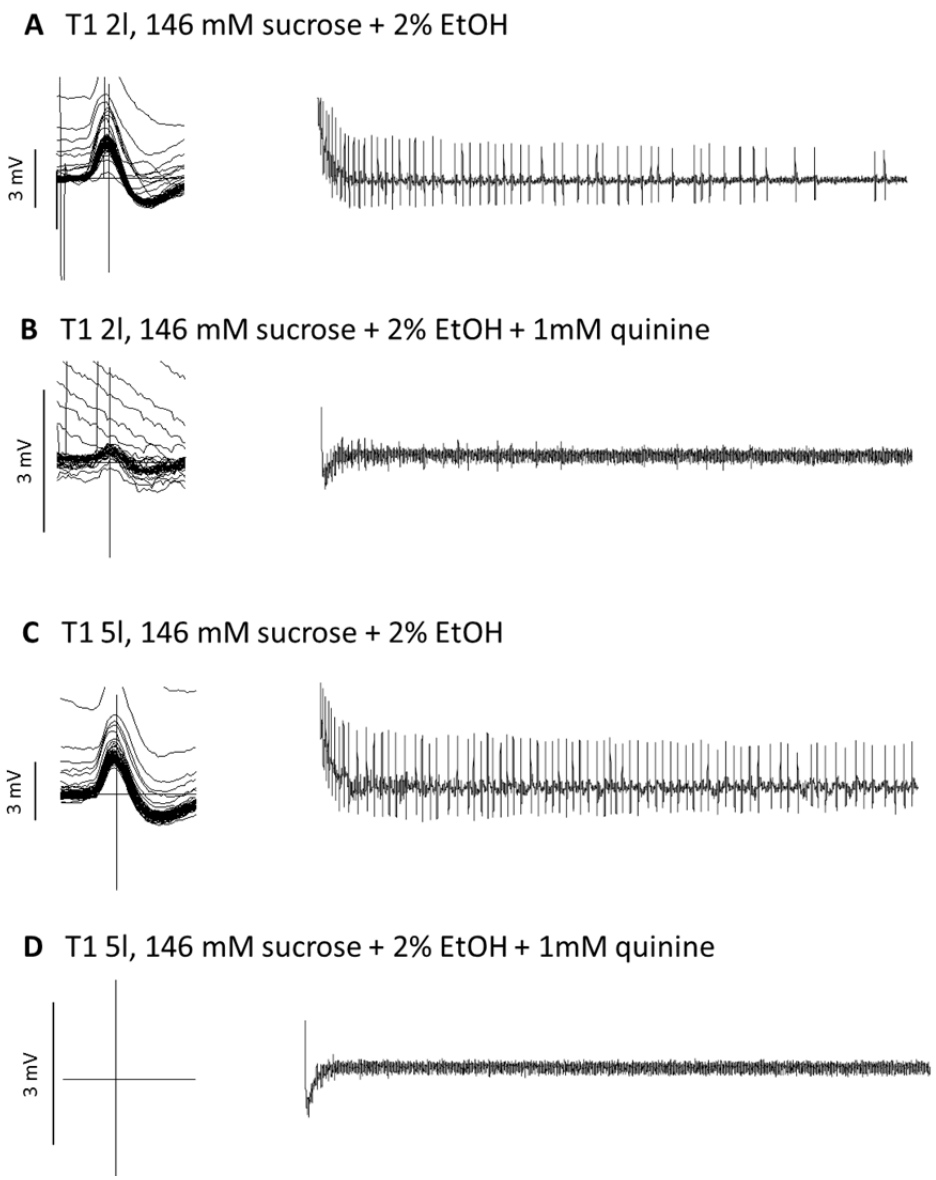

Fig. II.IV: 2 s electrophysiological recordings on the lateral T1 labellar sensilla Nos 2 (A and B) and 5 (C and D) in response to 146 mM sucrose in 2% ethanol (A and C) and 146 mM sucrose in 2% ethanol + 1 mM quinine (B and D). Quinine was first dissolved in ethanol to facilitate dilution. 2% ethanol had no effect on spike activity. Quinine inhibits the sugar sensitive neurone in both sensilla (B and D) but does not activate a receptor cell. The partial inhibition of the sugar receptor cell in recording B is accompanied by a decrease in spike amplitude. Recordings are typical for the three 0 day-old mosquitoes tested for each sensillum.

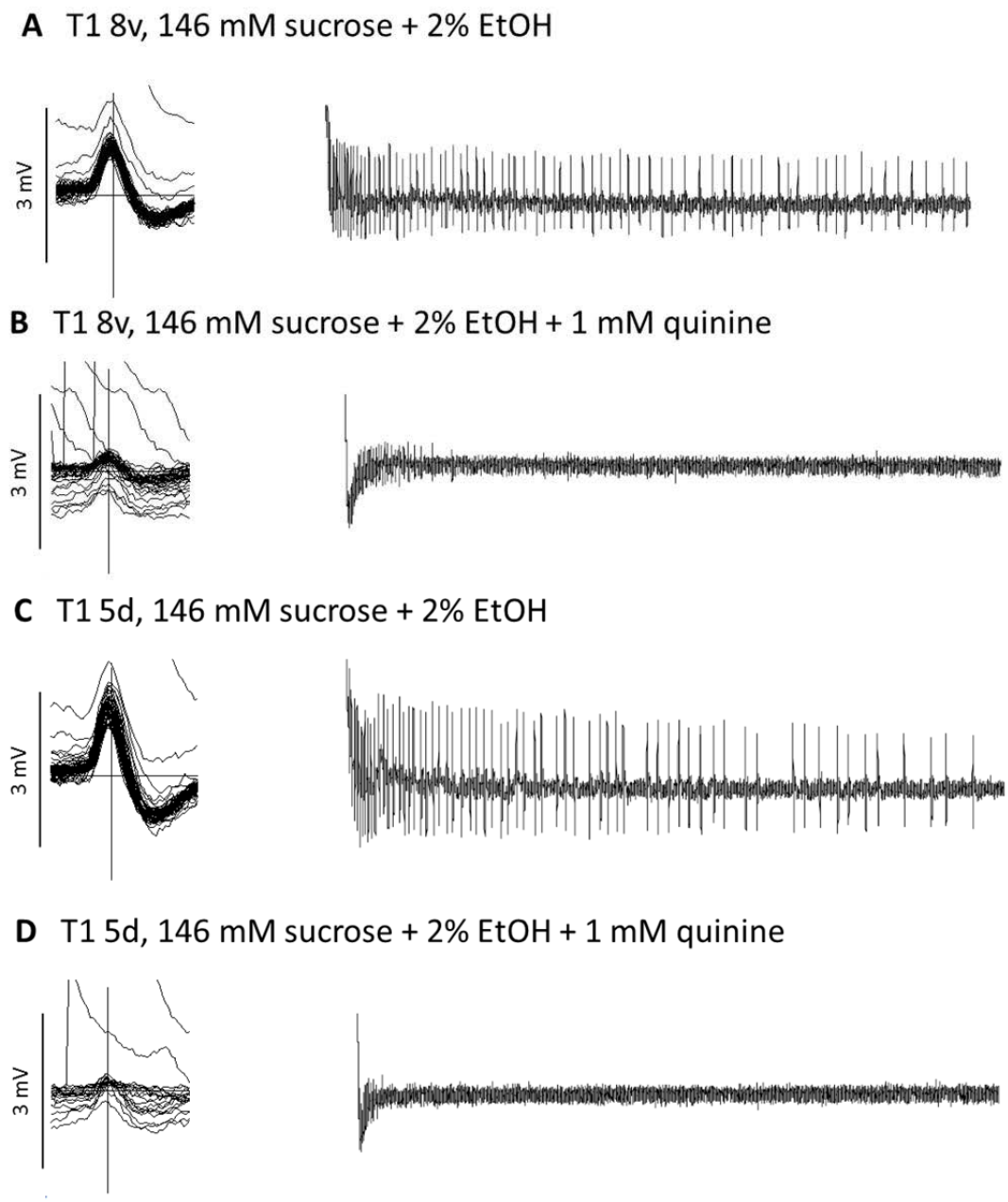

Fig. II.V: 2 s electrophysiological recordings from the ventral T1 labellar sensillum No 8 (A and B) and the dorsal No 5 sensillum (C and D) in response to 146 mM sucrose in 2% ethanol (A and C) and 146 mM sucrose in 2% ethanol + 1 mM quinine (B and D). Quinine was first dissolved in ethanol to facilitate dilution. 2% ethanol had no effect on spike activity. Quinine inhibits the sugar sensitive neurone in both sensilla (B and D) but does not activate a receptor cell. The partial inhibition of the sugar receptor cell in recording B and D is accompanied by a decrease in spike amplitude. Recordings are typical for the three 0 day-old mosquitoes tested for each sensillum.

12. Appendix III: Electrophysiological stimulations of *A. gambiae* labellar sensilla with icaridin

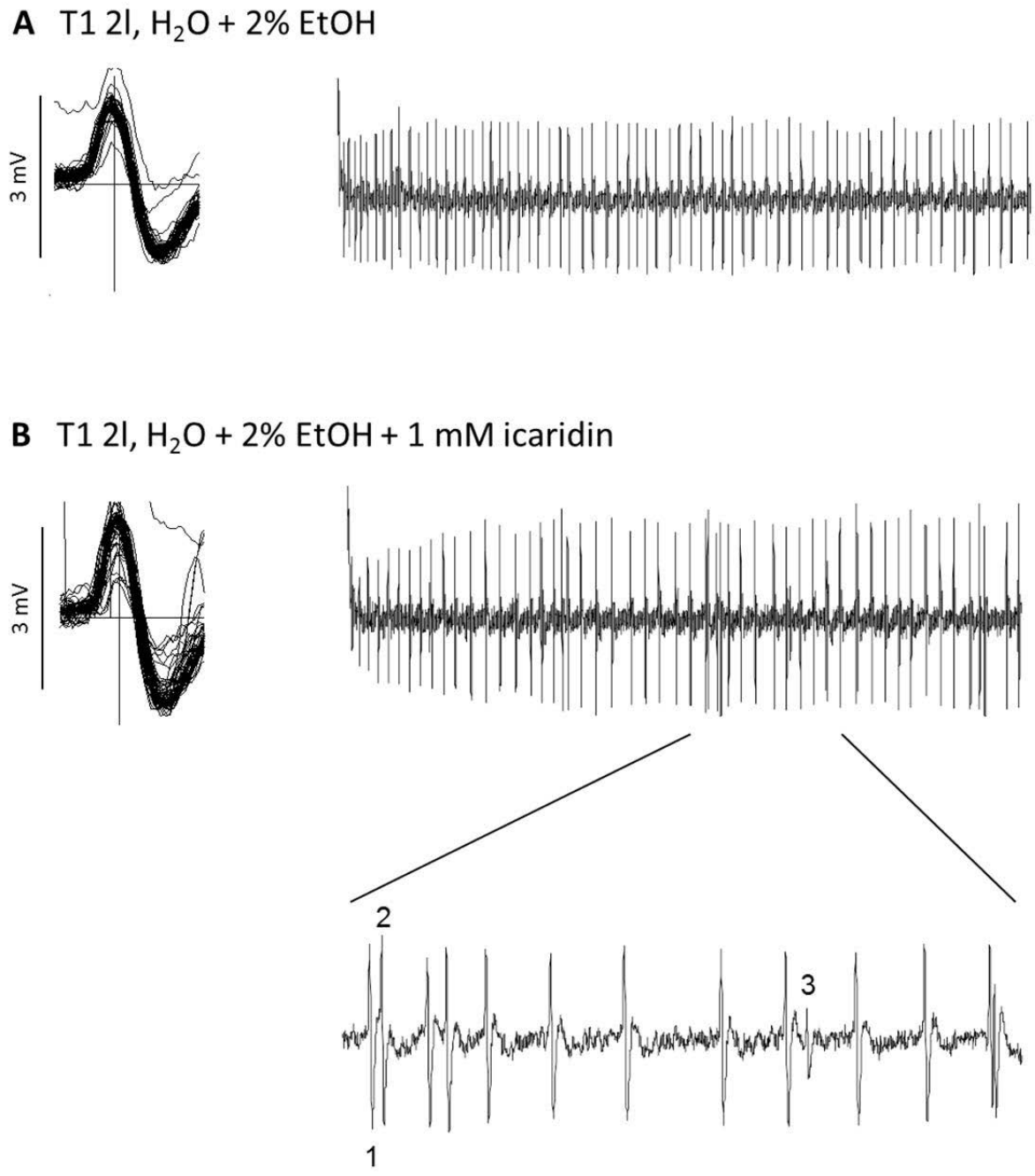

Fig. III.I: 2 s electrophysiological recordings from lateral T1 labellar sensillum No 2 in response to water in 2% ethanol (A) and water in 2% ethanol + 1 mM icaridin (B). Icaridin was first dissolved in ethanol to facilitate dilution. 2% ethanol had no effect on spike activity. 1 mM icaridin activates 2 receptor cells in addition to the water sensitive neurone: in recording B two generated spikes are of the same size whereas the spikes of the third cell are lower in amplitude. Recordings are typical for the four 0 day-old mosquitoes tested.

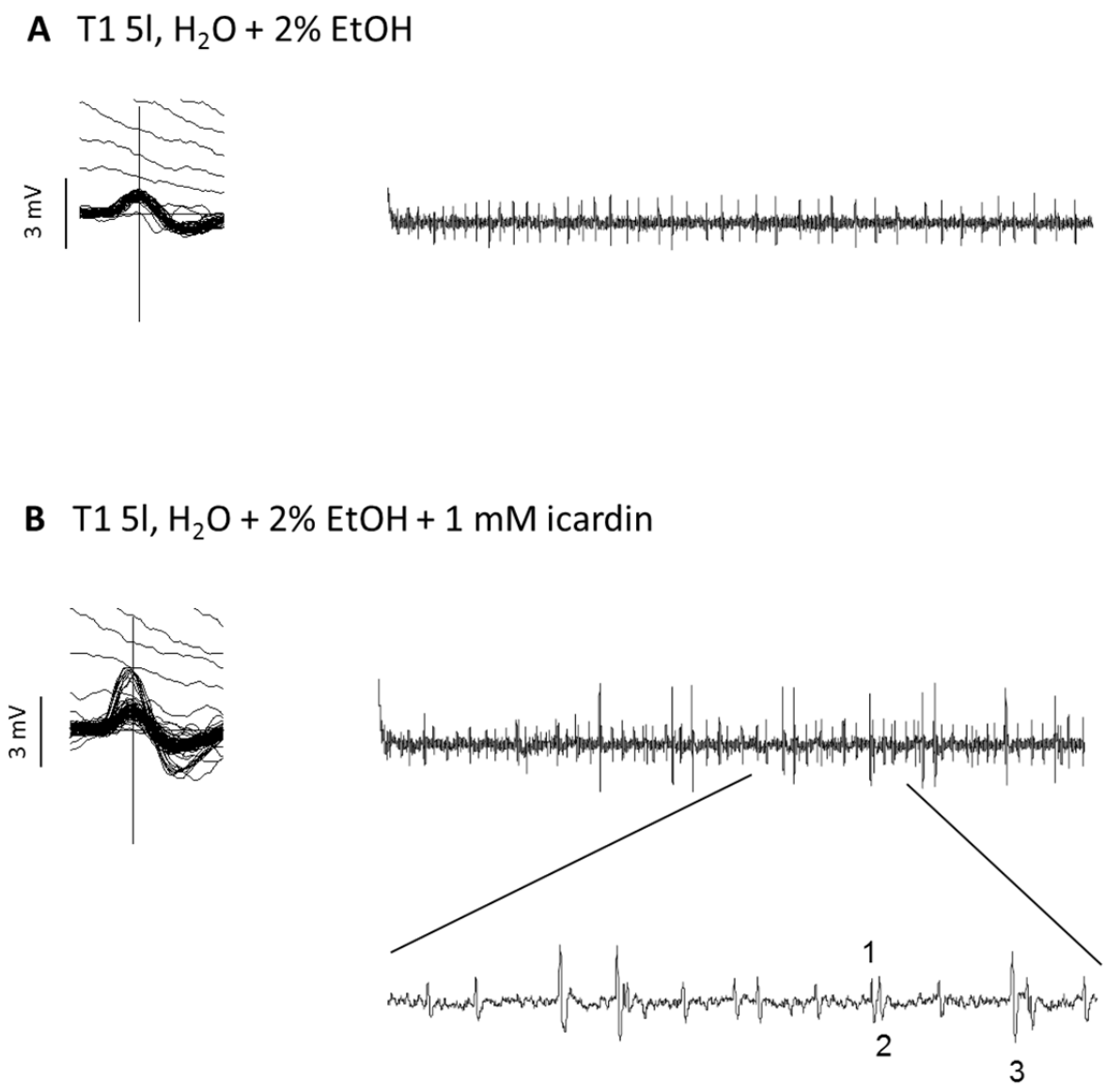

Fig. III.II: 2 s electrophysiological recordings from the lateral T1 labellar sensillum No 5 in response to water in 2% ethanol (A) and water in 2% ethanol + 1 mM icaridin (B). Icaridin was first dissolved in ethanol to facilitate dilution. 2% ethanol had no effect on spike activity. 1 mM icaridin activates 2 receptor cells in addition to the water sensitive neurone: in recording B two generated spikes are of the same size whereas the spikes of the third cell are higher in amplitude. Recordings are typical for four 0 day-old mosquitoes tested.

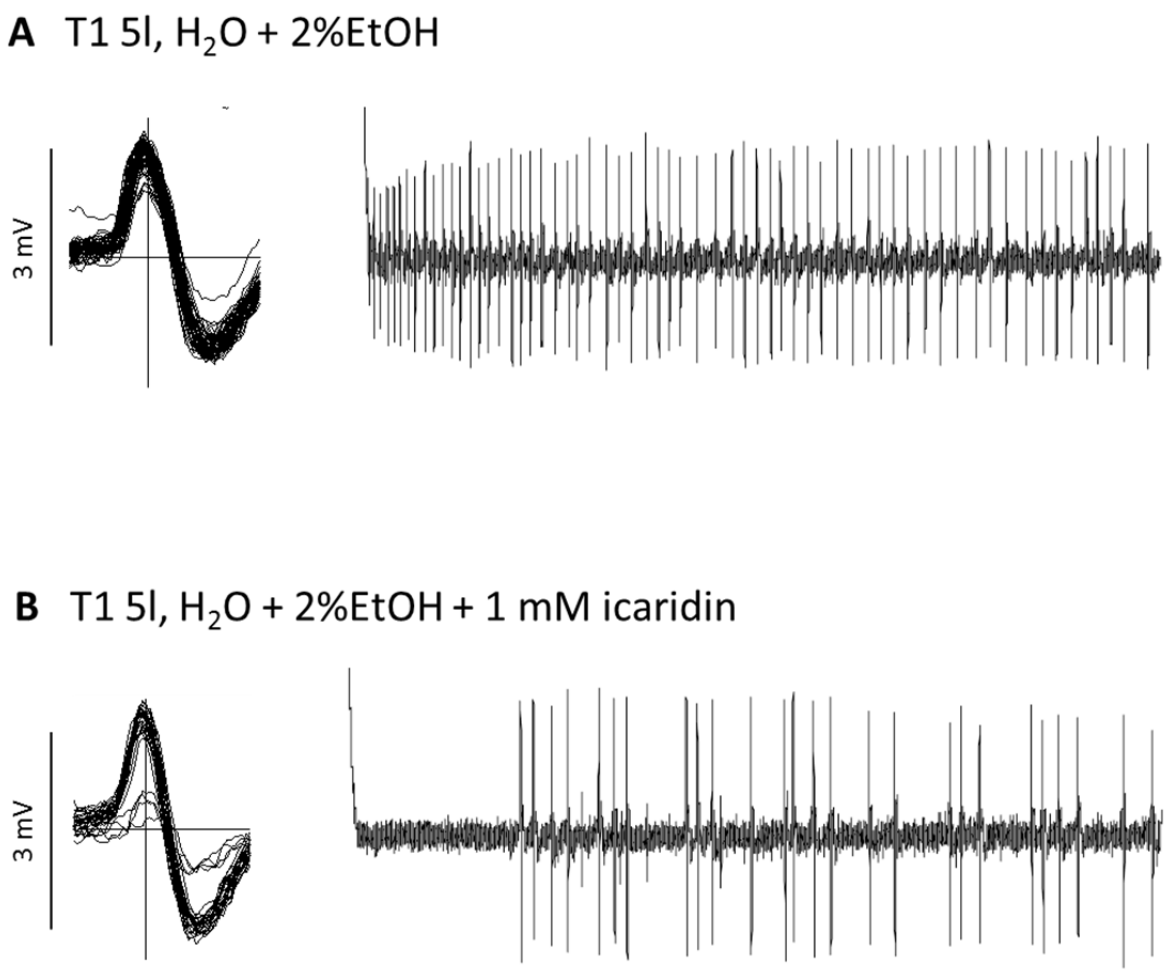

Fig. III.III: 2 s electrophysiological recordings on the lateral T1 labellar sensillum No 5 in response to water in 2% ethanol (A) and water in 2% ethanol + 1 mM icaridin (B). Icaridin was first dissolved in ethanol to facilitate dilution. 2% ethanol had no effect on spike activity. 1 mM icaridin was found to inhibit the water sensitive neurone in lateral T1 labellar sensilla No 2 (data not shown) and No 5 (as in recording B) in 4 mosquitoes of 6 tested in addition to the activation of the two receptor cells.

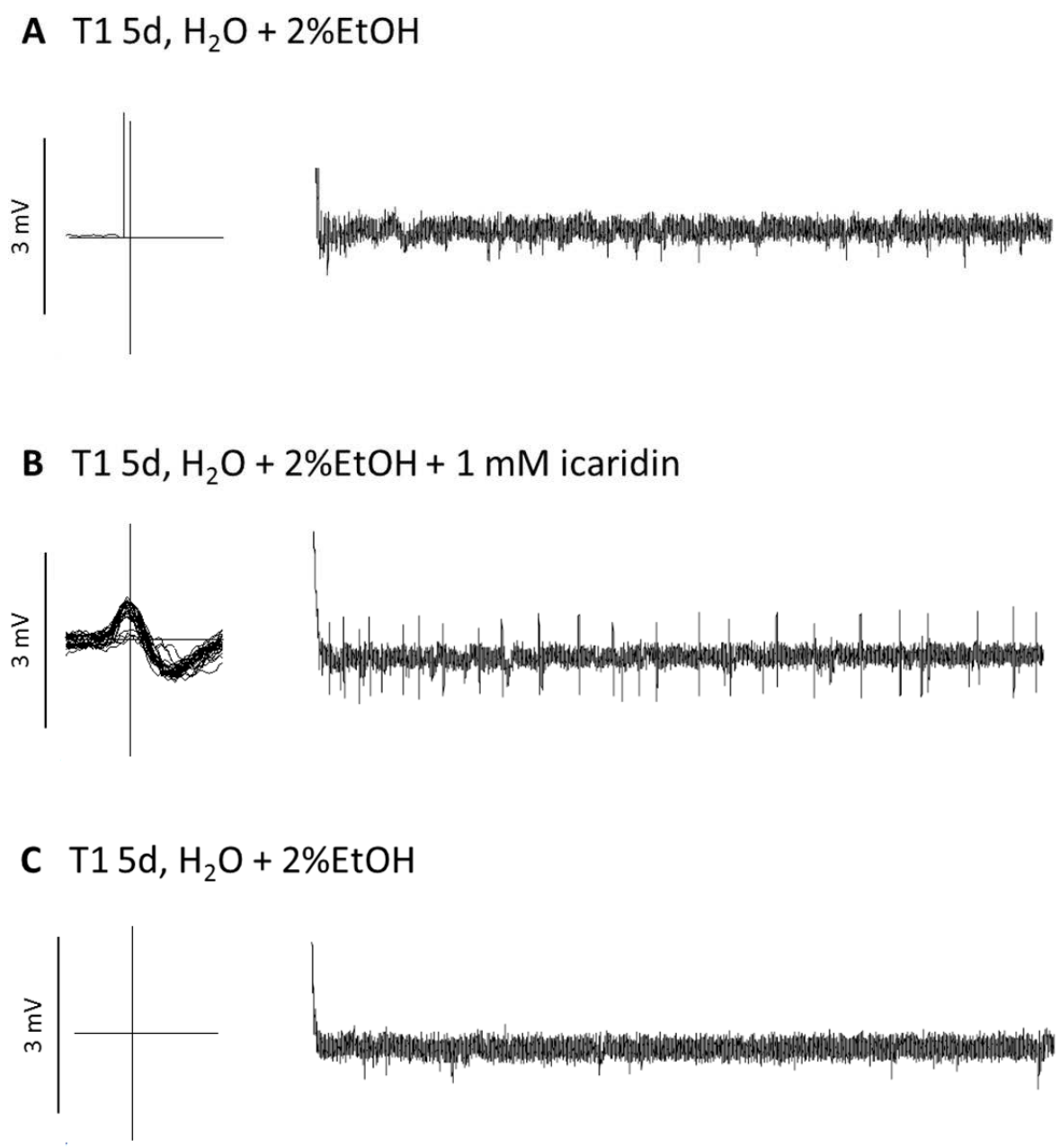

Fig. III.IV: 2 s electrophysiological recordings from the dorsal T1 labellar sensillum No 5 in response to water with 2% ethanol (A and C), respectively, before and after stimulation with water with 2% ethanol + 1 mM icaridin (B). No water sensitive neurone is present in this sensillum (A and C). Icaridin was first dissolved in ethanol to facilitate dilution. 2% ethanol had no effect on spike activity. 1 mM icaridin was found to slightly activate a receptor cell in the dorsal T1 labellar sensillum No 5 in 4 of 6 mosquitoes tested and in the ventral T1 labellar sensillum No 8 in 1 mosquito (data not presented).

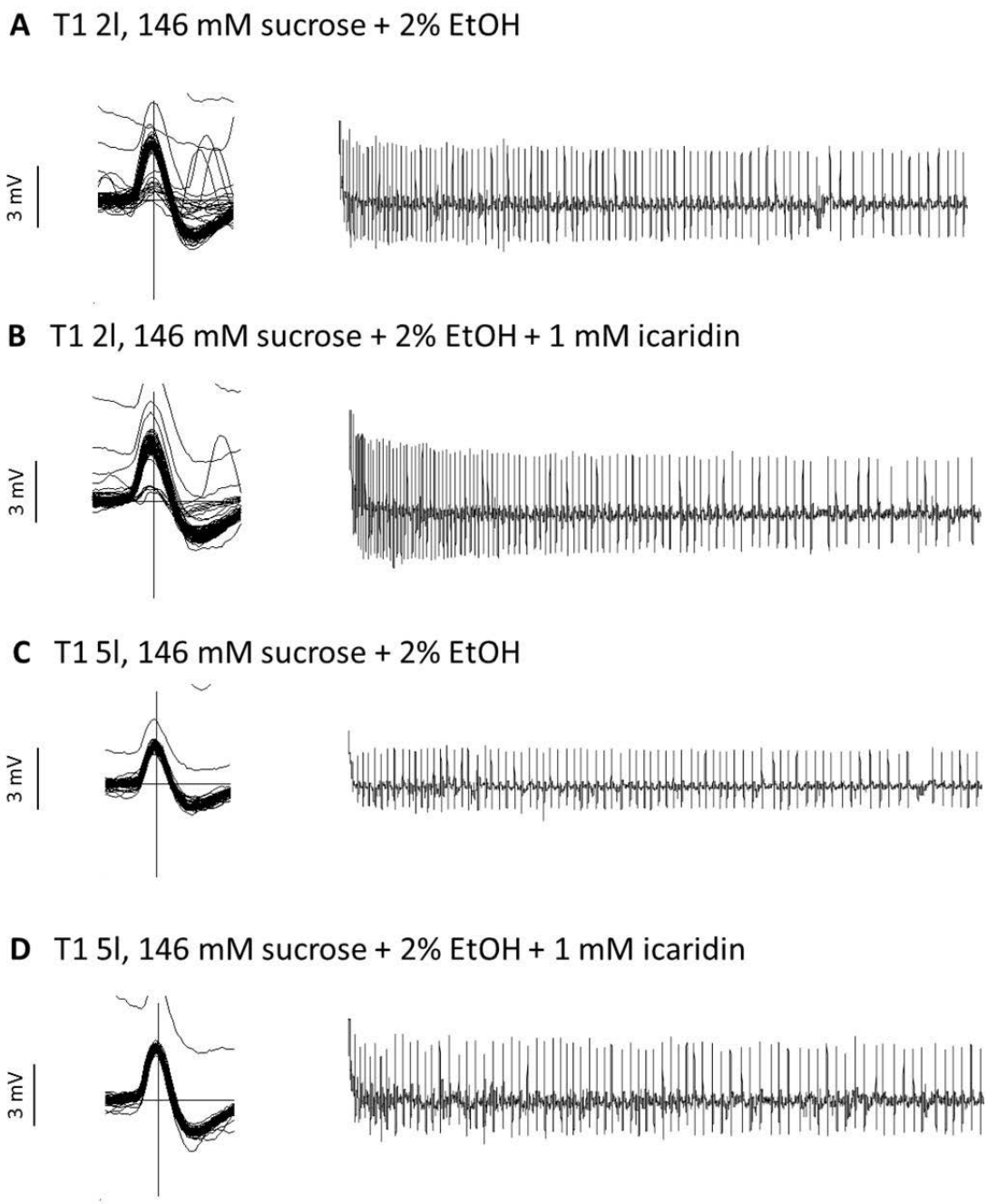

Fig. III.V: 2 s electrophysiological recordings from the lateral T1 labellar sensilla Nos 2 (A and B) and 5 (C and D) in response to 146 mM sucrose in 2% ethanol (A and C) and 146 mM sucrose in 2% ethanol + 1 mM icaridin (B and D). Icaridin was first dissolved in ethanol to facilitate dilution. 2% ethanol had no effect on spike activity. When presented to the sensilla in 146 mM sucrose, 1 mM icaridin failed to activate the two receptor cells that are activated in these sensilla when icaridin is presented only in water. Contrary to the water sensitive neurone, the sugar sensitive neurone is not inhibited by 1 mM icaridin. Recordings are typical for four 0 day-old mosquitoes tested for each sensillum.

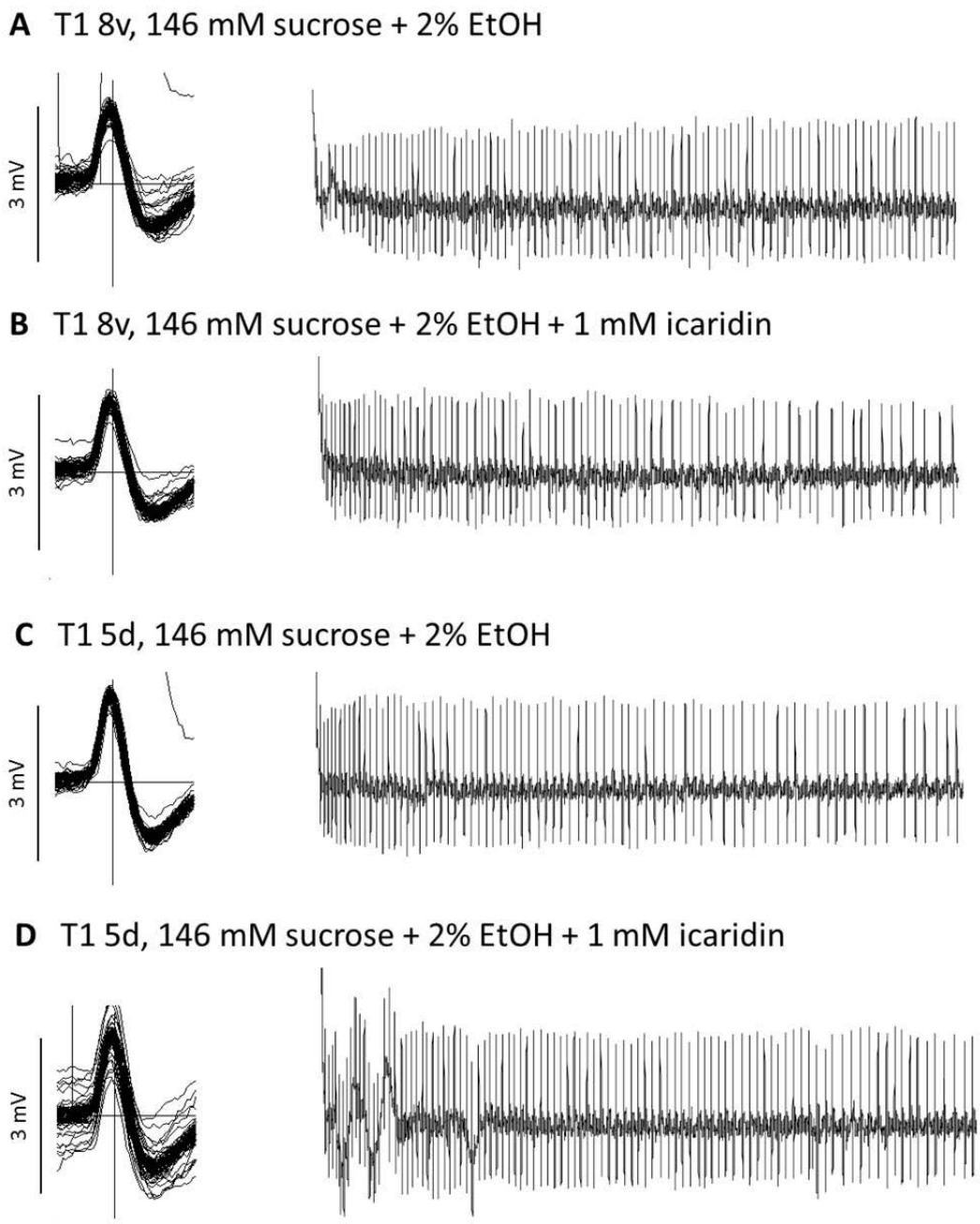

Fig. III.VI: 2 s electrophysiological recordings on the T1 ventral sensillum No 8 (A and B) and dorsal No 5 sensillum (C and D) in response to 146 mM sucrose in 2% ethanol (A and C) and 146 mM sucrose in 2% ethanol + 1 mM icaridin (B and D). Icaridin was first dissolved in ethanol to facilitate dilution. 2% ethanol had no effect on spike activity. When presented to the sensilla in 146 mM sucrose, 1 mM icaridin failed to activate the receptor cell that is activated in these sensilla when icaridin is presented only in water (see Fig. III.IV). The sugar sensitive neurone is not inhibited by 1 mM icaridin. Recordings are typical for three 0 day-old mosquitoes tested for each sensillum.

13. Appendix IV : Electrophysiological stimulations of *A. gambiae* labellar sensilla with DEET

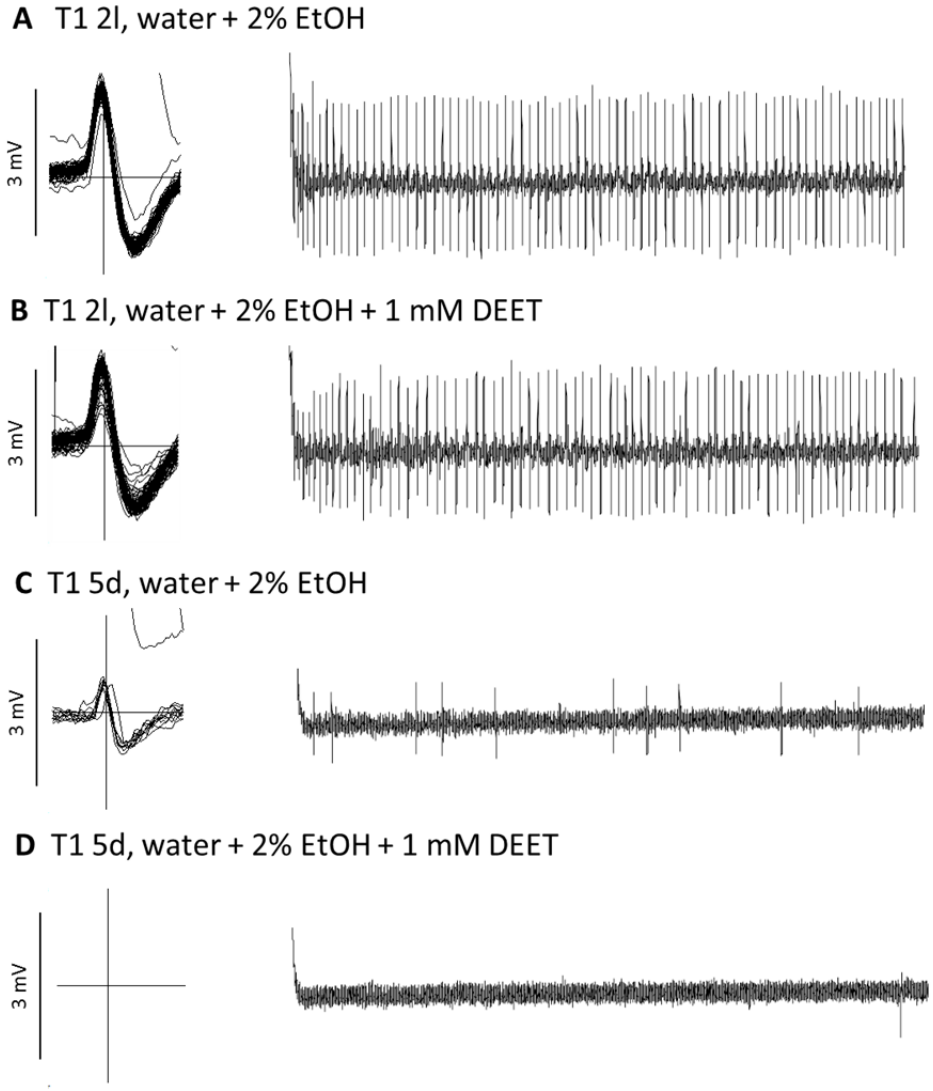

Fig. IV.I: 2 s electrophysiological recordings from the T1 lateral sensillum No 2 (A and B) and dorsal No 5 sensillum (C and D) in response to 2% ethanol in water (A and C) and water + 2% ethanol + 1 mM DEET (B and D). No water sensitive neurone is present in the dorsal sensilla No 5. In recording C, the generated spikes are not in response to water as the frequency is low and such spikes were not systematically observed during stimulation with water. 1 mM DEET does not inhibit the response to water in lateral sensillum No 2. No receptor cells were found to be activated by 1 mM DEET in either sensillum. DEET was first dissolved in ethanol to facilitate dilution. 2% ethanol had no effect on spike activity. Recordings are typical for three 0 day-old mosquitoes tested for each sensillum.

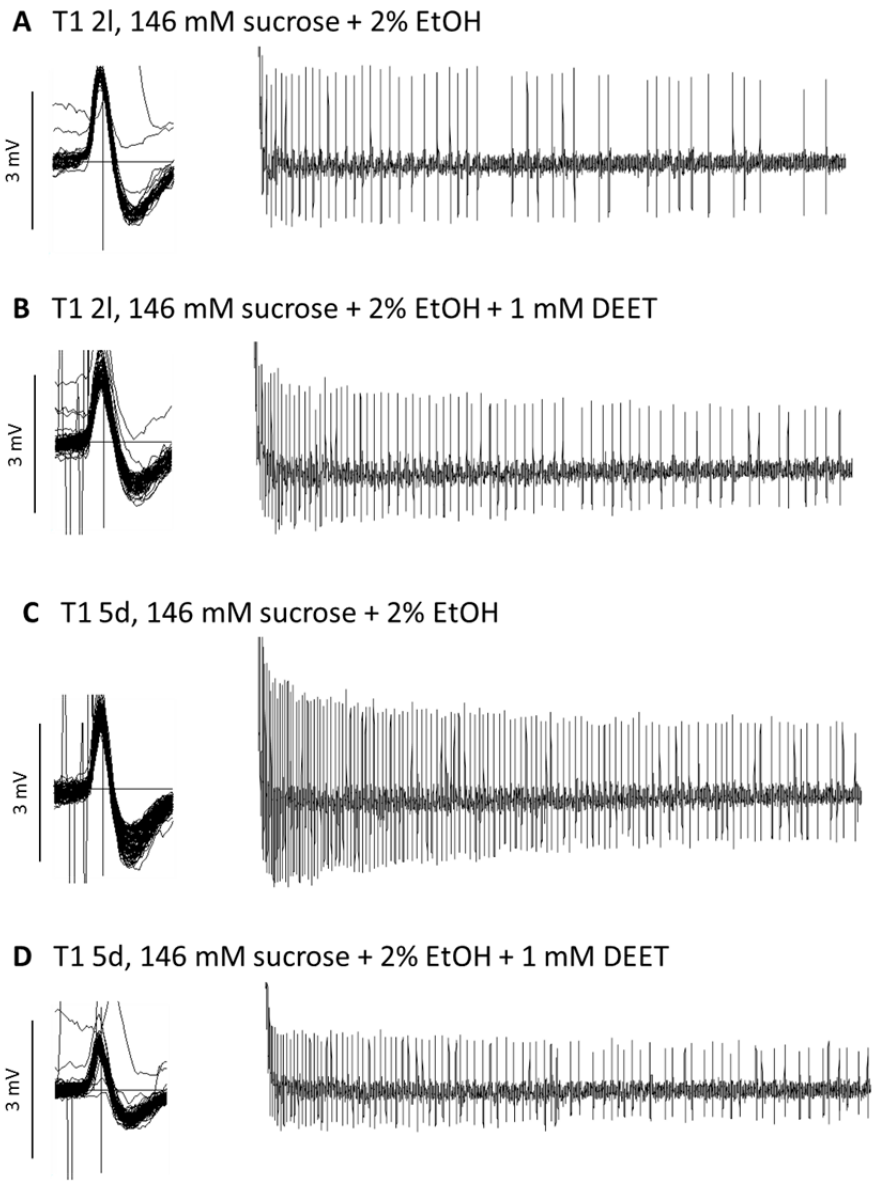

Fig. IV.II: 2 s electrophysiological recordings from the T1 lateral sensillum No 2 (A and B) and dorsal No 5 sensillum (C and D) in response to 146 mM sucrose + 2% ethanol (A and C) or 146 mM sucrose + 2% ethanol + 1 mM DEET (B and D). 1 mM DEET does not inhibit the response to sucrose nor does it activate any other receptor cells in either of these sensilla. DEET was first dissolved in ethanol to facilitate dilution. 2% ethanol had no effect on spike activity. Recordings are typical for three 0 day-old mosquitoes tested for each sensillum.

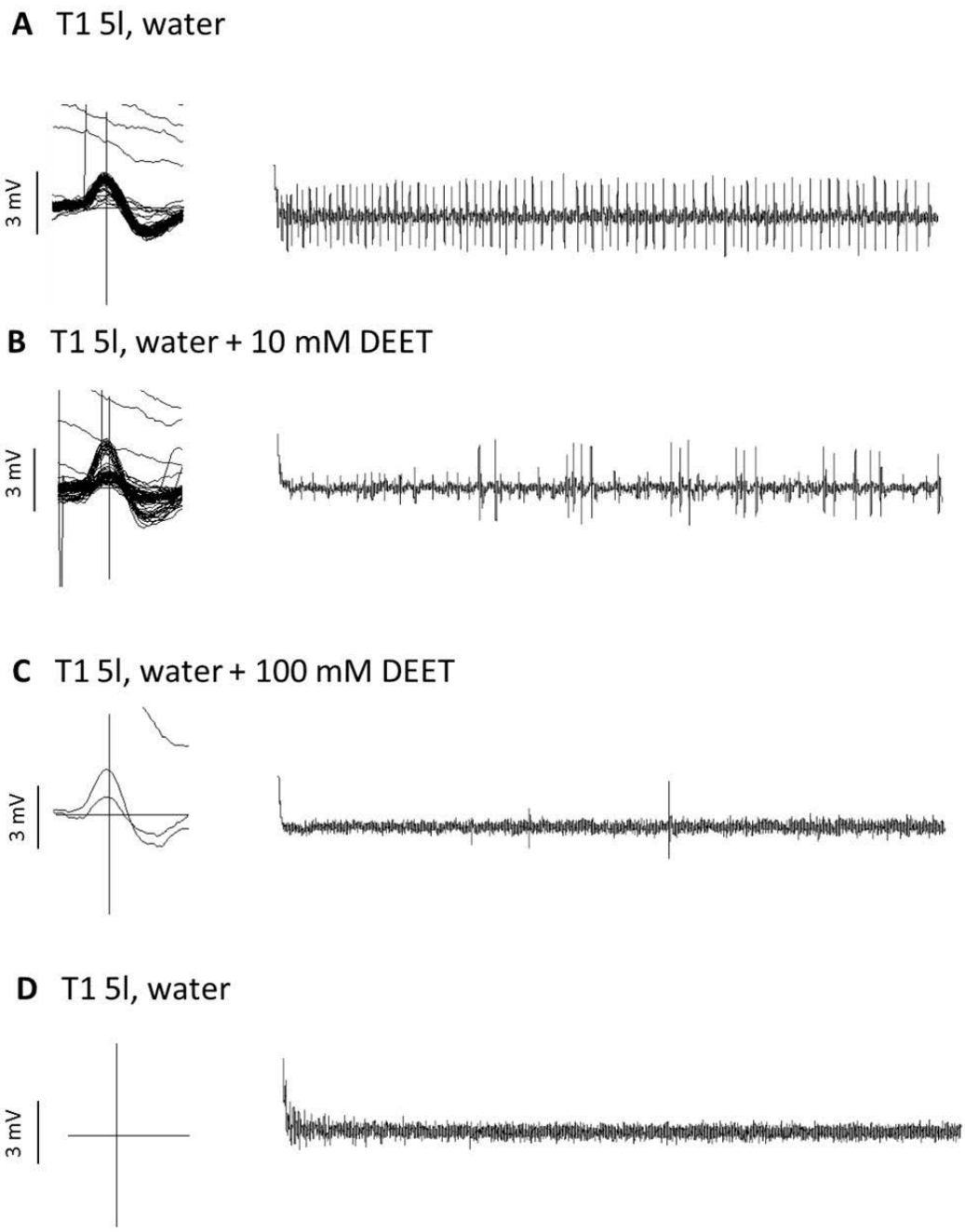

Fig. IV.III: 2 s electrophysiological recordings from the T1 lateral sensillum No 5 in response to water (A and D), and to water + 10 mM (B) and water + 100 mM DEET (C). High doses of DEET inhibit the response to water. A receptor cell is slightly activated by 10 mM DEET although the spike frequency of this receptor cell drops at 100 mM. After stimulation with 100 mM DEET, the response to water was not restored in sensilla of five 0 day-old mosquitoes tested indicating a potential neurotoxic effect of DEET on this receptor cell.

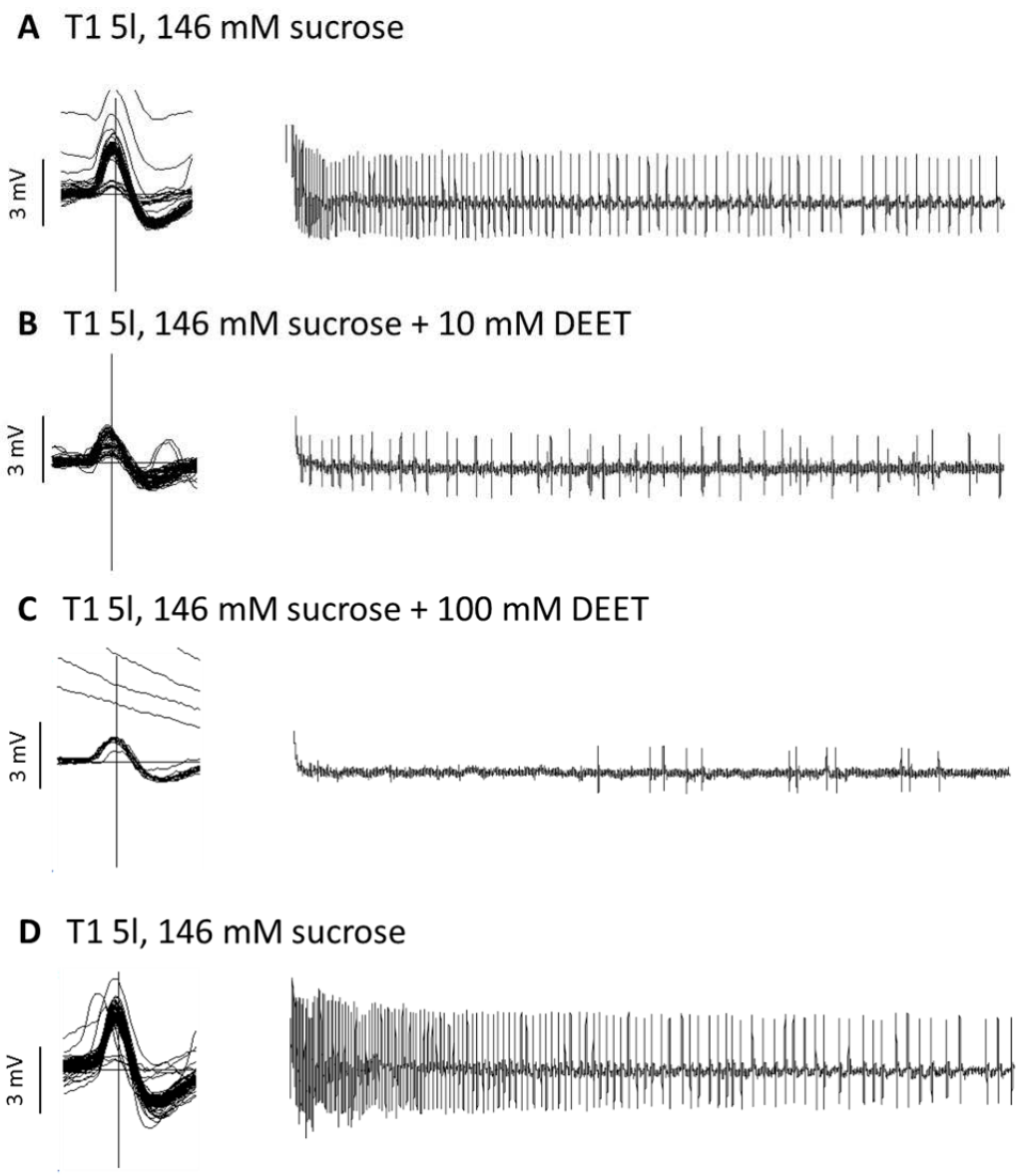

Fig. IV.IV: 2 s electrophysiological recordings from the T1 lateral sensillum No 5 in response to 146 mM sucrose (A and D), and to 146 mM sucrose + 10 mM (B) and 146 mM sucrose + 100 mM DEET (D). High doses of DEET inhibit the response to sucrose. Another receptor cell is slightly activated by 10 mM DEET although the spike frequency of this receptor cell drops at 100 mM. After stimulation with 100 mM DEET, contrary to the water sensitive neurone (see Fig. III.III), the response to sucrose was present in the three 0 day-old mosquitoes tested.

14. Appendix V: Inhibition of the *A. gambiae* labellar water and sugar sensitive neurones by amiloride

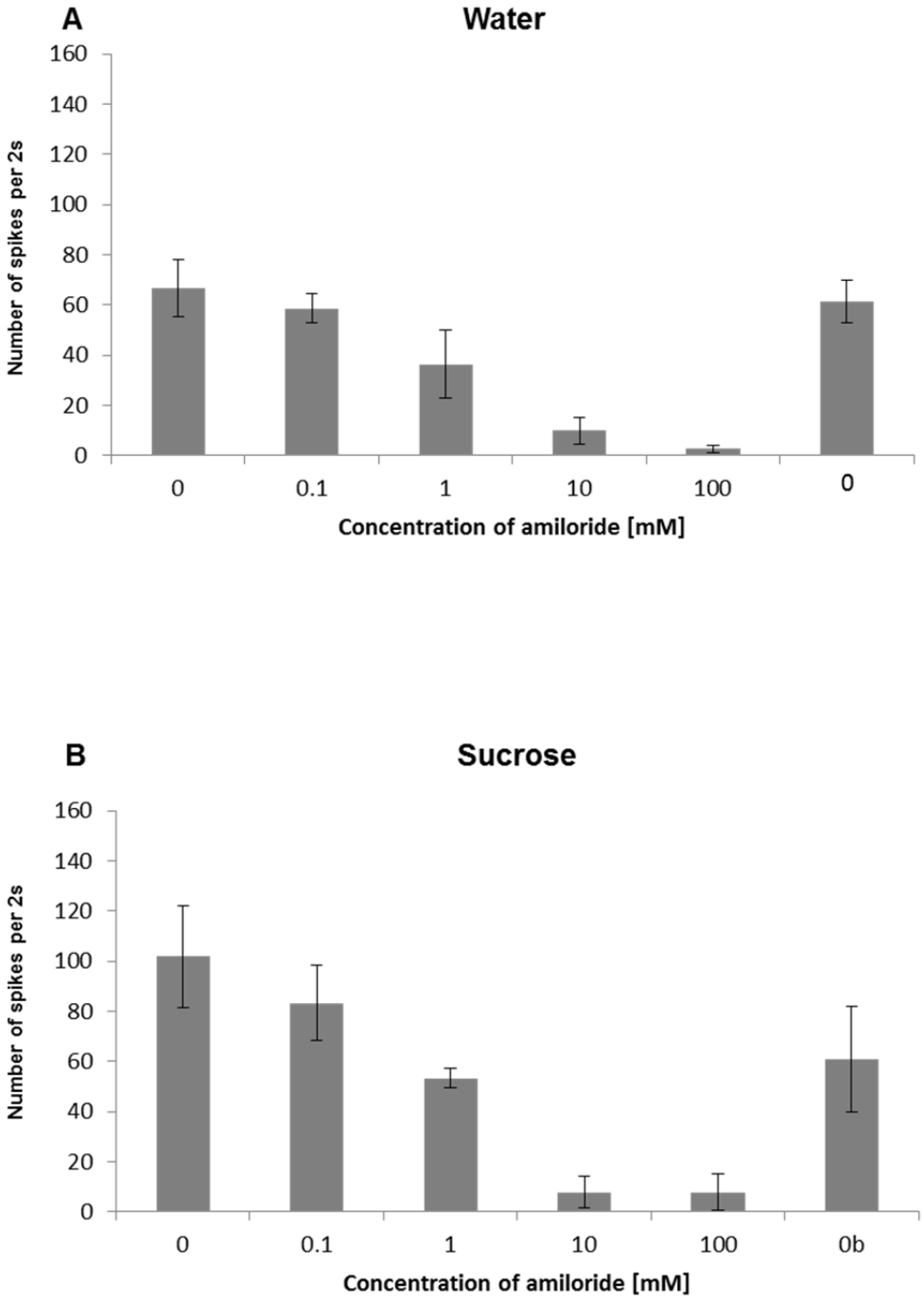

Fig. V.I: Mean ± s.e.m. total number of spikes generated by sensory cells within the two lateral trichoid T1 sensilla Nos 2 and 5 on the labellum of female *A. gambiae* during stimulation by water (A) and 146 mM sucrose in 10 mM KCl (B) as a function of concentration of amiloride hydrochloride. Both cells are inhibited by amiloride at high doses in a concentration-dependent manner.

15. Appendix VI: Effect of the insecticides malathion (organophosphate) and cypermethrin (pyrethroid) on the response of *A. gambiae* labellar receptor cells to water and to sucrose

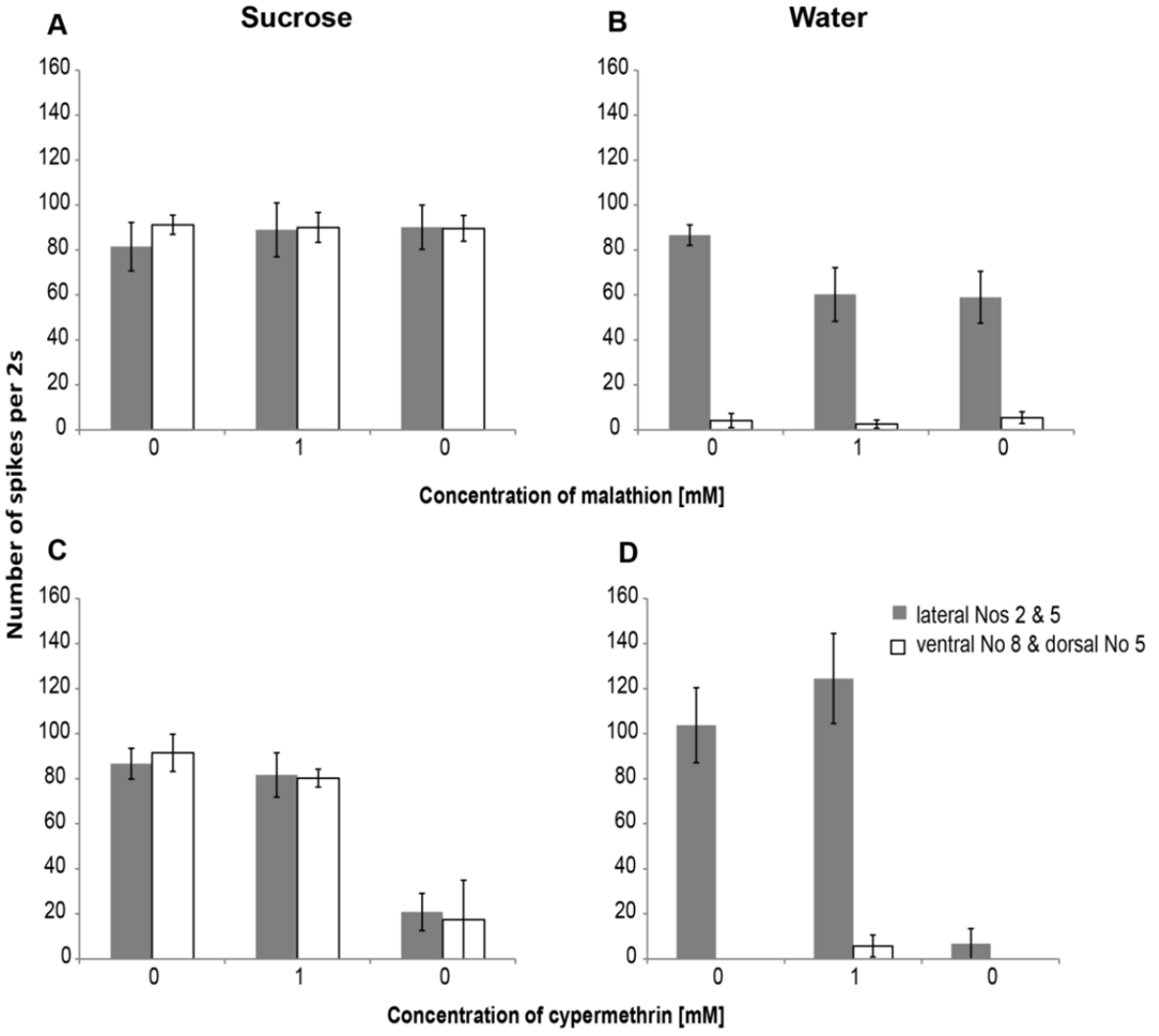

Fig. VI.I: Mean ± s.e.m. total number of spikes generated by sensory cells within trichoid T1 lateral sensilla Nos 2 and 5 (in grey) and ventral No 8 and dorsal No 5 sensilla (in white) on the labellum of female *A. gambiae* during stimulation by 146 mM sucrose + 2% EtOH (A and C) or water + 2% EtOH (B and D) before and after stimulation with 1 mM of the organophosphate malathion (A and B) or the pyrethroid cypermethrin (C and D) diluted in either 146 mM sucrose or water + 2% EtOH, respectively. Sensilla were stimulated successively for periods of 4-6 s with the insecticides for a mean duration of 15.33 s (between 4.5 to 36.3 s). No water sensitive neurone is present in the ventral No 8 and the dorsal No 5 sensilla (B and D). No receptor cells were found to be activated by these two insecticides in all sensilla tested. Malathion had no effect on the sugar receptor cells of both types of sensilla (A, one-way ANOVA followed by a Tukey post-hoc test, all $P > 0.05$). In comparison to the first water control, the spike frequency tends to be lower when 1 mM malathion is added to water (B, one-way ANOVA followed by a Tukey post-hoc test, $P = 0.0999$) and during the second water control ($P = 0.0820$). The sugar sensitive neurones in both sensilla types are inhibited after stimulation with 1 mM cypermethrin in sucrose (C, $P < 0.001$ for the lateral Nos 2 and 5, $P < 0.01$ for the ventral No 8 and dorsal No 5 in comparison to the first control). In comparison to the first water control, the slight increase in spike frequency generated by the water sensitive neurones in lateral sensilla Nos 2 and 5 when 1 mM cypermethrin is added is not significant (D, one-way ANOVA followed by a Tukey post-hoc test, $P = 0.628 > 0.05$). However, the water sensitive neurone is significantly inhibited ($P < 0.01$) during the second water control following exposure to 1 mM cypermethrin. Between 3 and 8 sensilla from 2 and 4 mosquitoes tested per treatment and per sensilla type; data for water and sugar receptor cells were pooled for lateral sensilla Nos 2 and 5, and for ventral No. 8 and dorsal No. 5 sensilla.

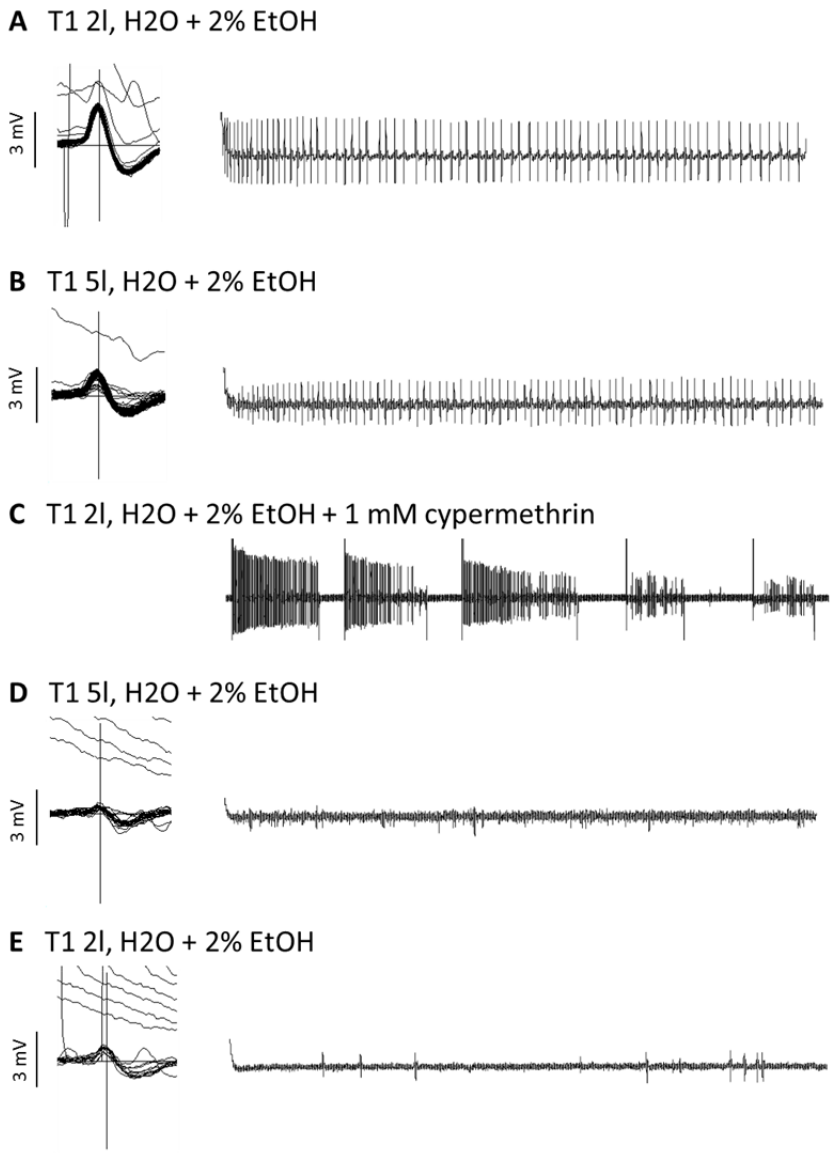

Fig. VI.II: 2 s electrophysiological recordings from the T1 lateral sensilla Nos 2 and 5 in response to water + 2% EtOH, respectively, before (A and B) and after (D and E) exposure for 41 s of the lateral sensillum No 2 to 1 mM cypermethrin diluted in water + 2% EtOH (C). Exposure of lateral No 2 sensillum to 1 mM cypermethrin affected receptor cells in neighbouring labellar sensilla. In the lateral sensillum No 5, which was not exposed to the insecticide, the water receptor cell did not respond to water after the lateral sensillum No 2 exposed to cypermethrin, whereas the mosquitoes were still alive; Two mosquitoes were tested.

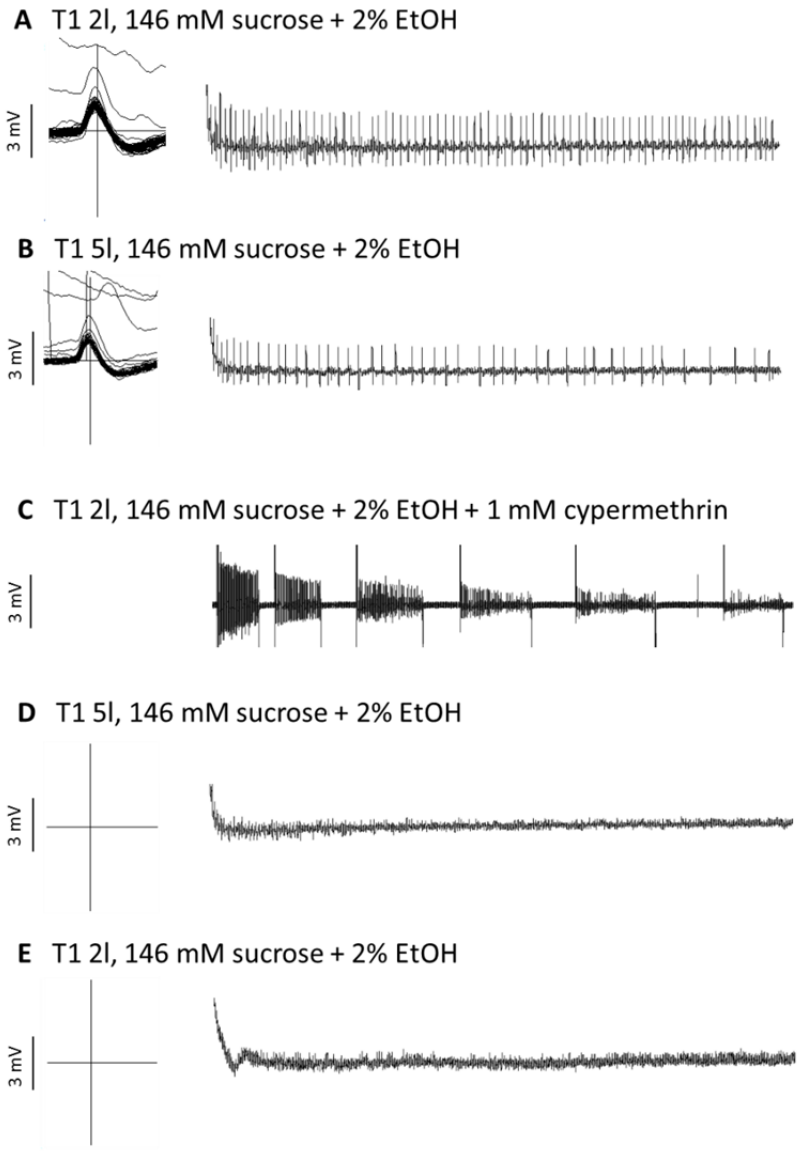

Fig. VI.III: 2 s electrophysiological recordings from the T1 lateral sensilla Nos 2 and 5 in response to 146 mM sucrose + 2% EtOH, respectively, before (A and B) and after (D and E) exposure to 33 s of lateral sensillum No 2 to 1 mM cypermethrin diluted in 146 mM sucrose + 2% EtOH (C). Exposure of lateral No 2 sensillum to 1 mM cypermethrin affected receptor cells in neighbouring labellar sensilla. In lateral sensillum No 5, which was not exposed to the insecticide, the sugar receptor cell did not respond to sucrose after the lateral sensillum No 2 was exposed to cypermethrin, whereas the mosquitoes were still alive; four mosquitoes were tested.

16. Appendix VII: Ultrastructure of trichoid sensilla on the tip of the labellum of *A. gambiae* and preliminary electrophysiological recordings

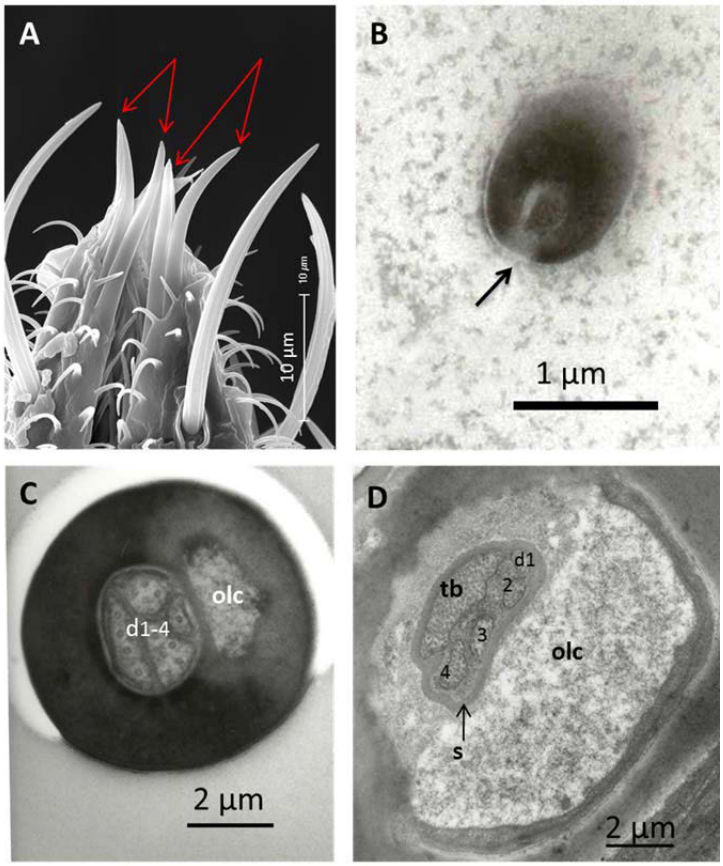

Fig. VII.I: Four long trichoid sensilla 28 µm in length (arrows, A) are found on the dorsal surface at the tip of the labellum of female and male *A. gambiae*. These are typical gustatory sensilla with a terminal pore (arrow in B) and two lymphatic spaces divided by a cuticular wall (C). The inner receptor lymph cavity contains four chemosensory dendrites (C). In all, five neurones innervate each sensillum (D). One dendrite terminates in a tubular body at the base of the hair, while the other four extend into the inner lymph cavity to extend to the tip of the hair close to the terminal pore (D). At the base, the five outer dendritic segments are surrounded by a sheath, secreted by the thecogen cell (D); d, dendrites; olc, outer lymph cavity; s, sheath; tb, tubular body.

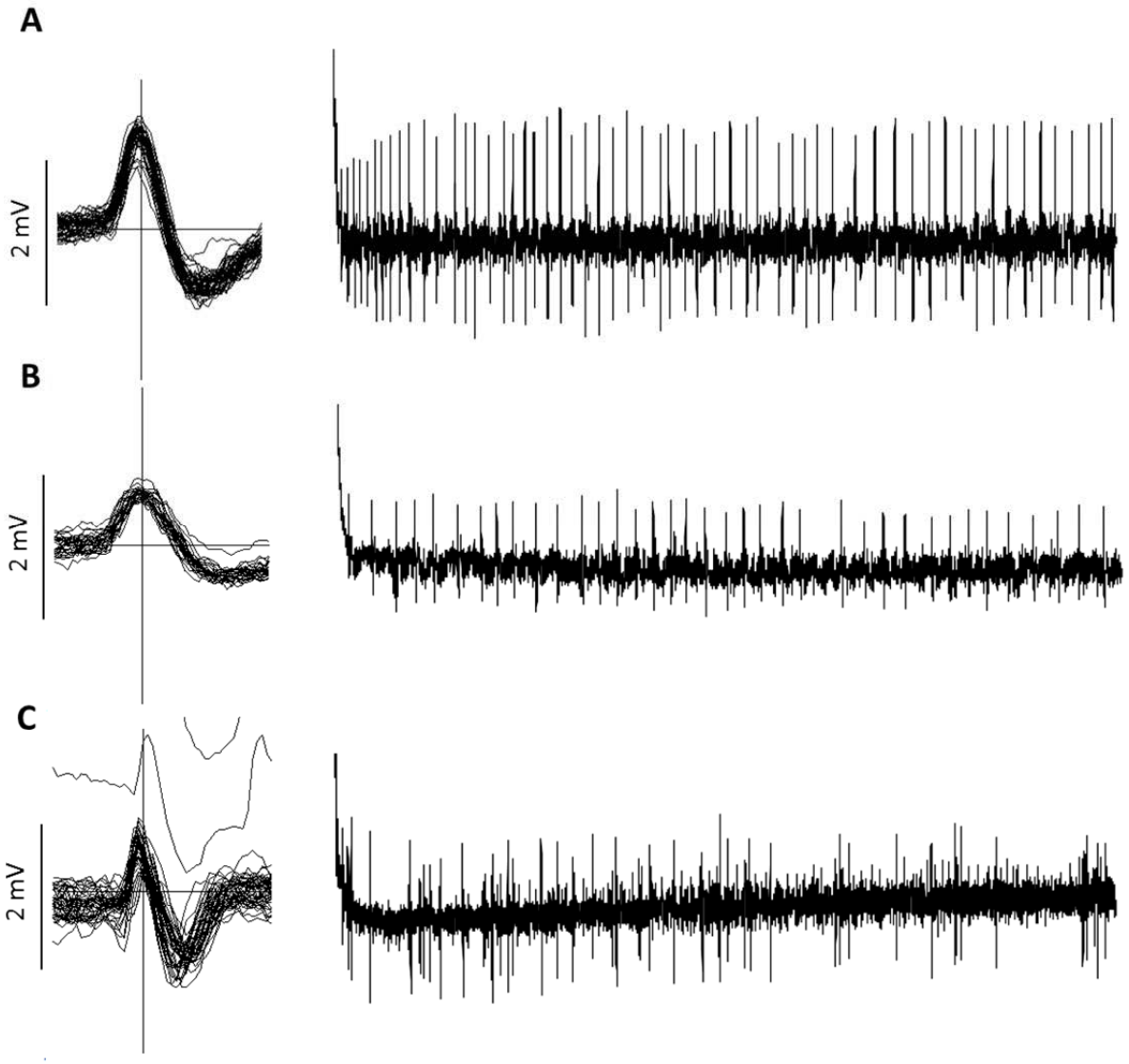

Fig. VII.II: 2 s electrophysiological recordings from trichoid sensilla at the tip of the labellum with water (A), sucrose 100 mM (B) and 100 mM KCl (C). Only one receptor cell responds to water and 100 mM sucrose, although at least two cells respond to 100 mM KCl (two spiking units stand out from noise in recording C). As no dose-response recordings were made, it is unknown wether the receptor cell responding to 100 mM sucrose is the water sensitive neurone or a sugar sensitive neurone.

17. Appendix VIII: Gustatory sensilla on the prothoracic legs of *A. gambiae*

As already observed for *A. aegypti* (McIver and Siemicki, 1978) the prothoracic tarsi of *A. gambiae* are divided into five tarsomeres which are densely covered with scales, except the ventral part of tarsomere 5 of males (Fig. VIII.I.B). The posttarsus bears an empodium, two pulvilli and a double claw (unguis) but no arolium. In females, the two claws are the same size and are not digitized (Fig. VIII.I.A). In males, the external claw is atrophied while the other is digitized (Fig. VIII.I.B).

On tarsomere four and five of both sexes 5 types of sensilla are found: non-innervated thin hairs, spines and long hairs with a tubular body (similar to type A hairs of *A. aegypti*), trichoid sensilla (Fig. VIII.I, ts, like type C2 of *A. aegypti*), and bifurcate sensilla (similar to bifurcate sensilla of *Simulium venustum,* McIver *et al.* 1980, Fig. VIII.II).

Trichoid sensilla on the prothoracic legs of A. gambiae

Trichoid sensilla are long pointed hairs (20-32 µm), shorter on the ventral face (10-18 µm) with a thin wall and set into a socket. As already described for *C. inornata* (Pappas and Larsen, 1976), the external surface of tarsal hairs is scalloped (Fig. VIII.I.E). The tip of the hair has a multiporous cuticle 1 µm in length (Fig. VIII.I.C and D); the diameter of the pores is 30 nm. Beneath the pores a small lymphatic cavity joins the lymphatic space with 4 dendrites (Fig. VIII.I.D). The hook-shaped tip of the ventral trichoid sensilla is not an artifact due to desiccation of the specimens, but represents the real shape of these sensilla in live insects (Fig. VIII.I.C). The hairs are typical gustatory sensilla composed of two lymphatic spaces divided by a cuticlar wall (Fig. VIII.I.E, cu). The inner receptor lymph cavity (Fig. VIII.I.E, ilc) contains 4 chemosensory dendrites and the outer lymph cavity (Fig. VIII.I.E, olc) contains an electron dense fluid. In all, five neurons innervate the sensillum (Fig. VIII.I.F). One dendrite terminates in a tubular body (Fig. VIII.I.F, tb) at the base of the hair while the other four extend to the tip of the hair shaft close to the pores (Fig. VIII.I.E and D). At the base, the five outer dendritic segments are surrounded by a sheath, secreted by the thecogen cell which ends below the cuticle of the socket (Fig. VIII.I.F). Deeper, two enveloping cells (tricogen and tormogen, Fig. VIII.I.G, ec) encase the five ciliary roots (with 9x2+0 microtubules, Fig. VIII.I.G, cr) and the inner dendritic parts of the sensory cells. Just below the posttarsus of the prothoracic legs of both females and males two trichoid sensilla are situated dorsally and two laterally. On the ventral side of the female prothoracic tarsomere 5 a group of three trichoid sensilla are present just below the posttarsus and three others are also present at the middle of tarsomere 5. However, in males, only three trichoid sensilla are found on the ventral side

of prothoracic tarsomere 5 (Fig. VIII.I.B, ts). On the distal half of females tarsomere 4 there two trichoid sensilla are placed laterally just behind the articulation, then two ventrally and one more proximal on the ventral side.

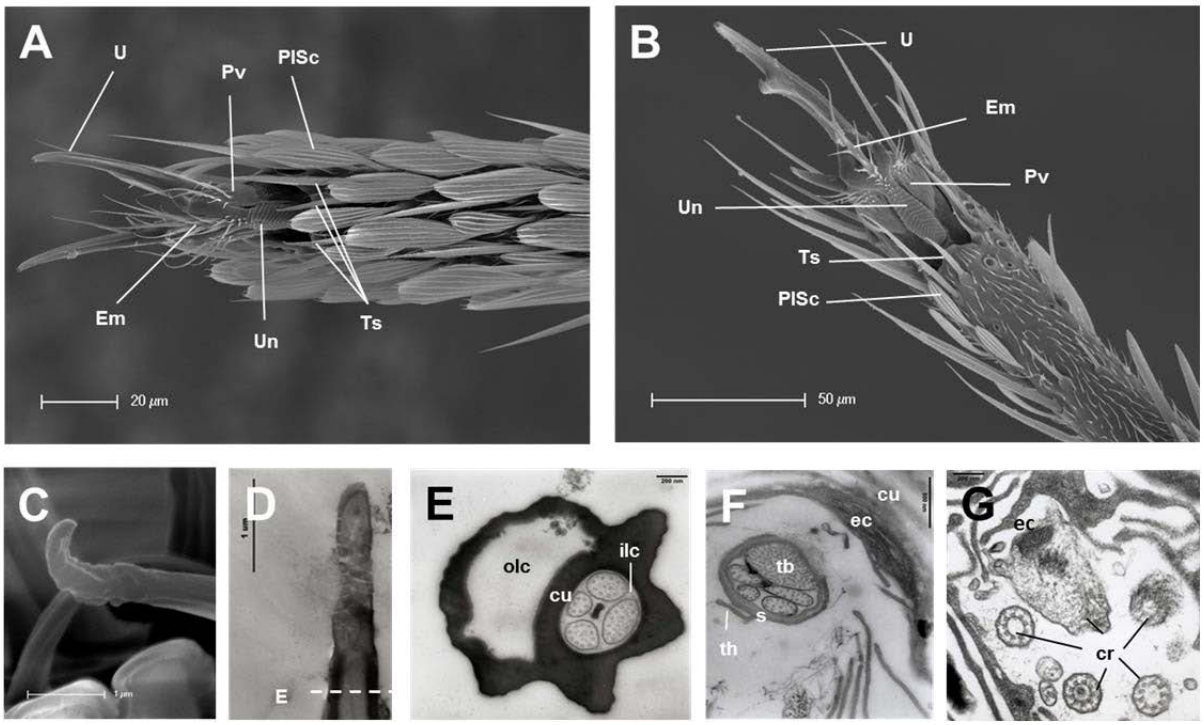

Fig. VIII.I: Scanning electron micrographs of *A. gambiae* female (A) and the male (B) left prothoracic leg. Scanning electron micrograph of the hook-shaped tip of a ventral trichoid sensillum of a female (C) and transmission electron micrographs of longitudinal (D) and transverse sections of the same sensillum type (E, F and G). The dashed line on D represents the transversal section in E. F is a section at the base of the sensillum and G a section at the level of the ciliary roots. Cr, ciliary root; cu, cuticle; ec, enveloping cells (tormogen, tricogen and thecogen); Em, empodium; ilc, inner receptor lymph cavity; olc, outer receptor lymph cavity; PlSc, plume scale; Pv, pulvillus; s sheath; tb, tubular body; th, thecogen cell, Ts, trichoid sensilla; U, unguis (claw); Un, unguitractor plate.

Bifurcate sensilla of the prothoracic legs of female A. gambiae

As already described by McIver et al. (1980) for *Simulium venustum*, bifurcate sensilla are chemosensory organs specific to females. We have also observed this kind of sensillum on female tarsi of *A. atroparvus* and *A. stephensi* although there are absent in *Aedes aegypti*. They occur in two rows of six to ten sensilla on the ventro-proximal side of tarsomeres 2 and 3 (Fig. VIII.II.A). One bifurcate sensillum is also present, just proximal to the articulation, on tarsomeres 1 and 2, between the two grooved spines. They are short hairs 10 µm long set into a socket. The distal cuticle bifurcates into lobes and forms a funnel. A single pore opens into a groove at the base of the lobes (Fig. VIII.II.B and C, arrowed). In the lobe, an electron dense median layer is visible in the transmission electron micrograph of the frontal section (Fig. VIII.II.C, edl). Each sensillum is innervated by four unbranched neurones which terminate proximal to the pore (Fig. VIII.II.C and D). As for other gustatory hairs, the sensillum has two lymphatic spaces one of which contains the four chemosensory dendrites.

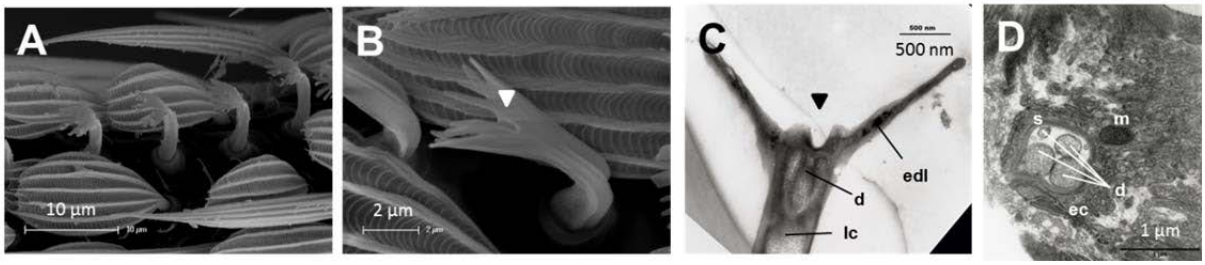

Fig. VII.II: Scanning electron micrographs (A and B) of bifurcate sensilla of a female *A. gambiae* and transmission electron micrographs of a frontal section (C) and of a transverse section (D) at the base of a bifurcate sensillum. The unique pore situated at the base of the lobes is indicated with an arrow (B and C). d, dendrite; ec, enveloping cells, edl, electron dense median layer; lc, lymph cavity; m, mitochondrion; s, sheath.

18. Appendix IX: Antennogram responses of *A. gambiae* to host-associated volatiles

18.1 Method

GC-EAG

For electroantennogram (EAG) recordings, the head of each 0–6 day-old female *A. gambiae* was excised at the occipital opening and placed on the reference glass electrode containing 100 mM KCl with 0.5% polyvinylpyrrolidone. The EAG response was recorded via a glass electrode filled with the same saline solution as for the indifferent electrode and brought into contact with the terminal antennal segment whose tip was cut off (Biessmann et al., 2010). As described in Schmidt-Büsser (2008), the recording electrode was connected to a high impedance preamplifier (gain: 10x). The electrophysiological signal was then amplified 100 times with a universal AC/DC amplifier (UN-03, Syntech, NL). The gas chromatograph (GC, Carlo Erba Instruments 5300) was equipped with a precolumn (BGB, fused silica capillary column; length 1 m, I.D. 320 µm, O.D. 450 µm, deactivated with OV-1701-OH) connected to a polar column (BGB-FFAP, a polyethylene glycol phase esterified with terephthalic acid, length 30 m, I.D. 0.25 mm, film thickness 0.25 µm). The carrier gas used was H_2. The column effluent was split in two flows using a glass secure Y connector (Restek®, Germany). One outlet was directed to the flame ionization detector (FID) and the other into a charcoal-scrubbed and humidified air stream (95% RH, 1m/sec) that blew over the mosquito antenna. 1 µl of the test solution was injected on-column. The oven was held at 40 °C for 5 min then heated at 15 C°/min to 225 C° and held for 5 min. EAG responses were recorded from the products eluting from the GC column or from an air puff of vapour over 10 µl of a solution containing products at between 0.01 to 10 µg/µl in CH_2Cl_2 (DCM) applied to a filter paper strip in a 5 ml plastic syringe connected to the air stream. The FID and EAG-responses were recorded simultaneously on a PC using GC-EAG 4.6 software (Syntech). Dose-response curves using the stimulus syringe were established using the software EAG-Pro 2.0 (Syntech). The antennae were tested using 0.1 to 100 µg of the tested compounds on filter paper delivered as puffs from the stimulus syringe before and after GC-EAG recordings.

Tested compounds

Solutions of standard products were tested with GC-EAG: the two first contained 100 ng/µl and 10 ng/µl, respectively, of butyric acid, isovaleric acid, 3-mercapto-1-hexenol, geranylacetone, octanoic acid and (*R/S*)-3-hydroxy-3-methyl hexanoic acid (HMHA)

dissolved in DCM. The third contained 100 ng/µl of (*R/S*)-3-mercapto-3-methyl-1-hexanol (transpirol™), 3-mercapto-1-hexenol and p-cresol and the fourth solution was 100 ng/µl solution of HMHA, hexanoic acid and p-cresol. Moreover p-cresol, geranylacetone, skatole, octanoic acid, hexanoic acid, (*E/Z*)-3-methyl-2-hexenoic acid (MHeA), HMHA, transpirol and 3-mercapto-1-hexenol were tested using the stimulus syringe in order to establish dose-responses curves and to test the antennal sensitivity before performing GC-EAD. Geranylacetone, hexanoic acid and p-cresol were purchased from Sigma-Aldrich (Switzerland, purity ≥98%), DCM from Merck (Germany, SupraSolv®) and the other compounds tested were kindly provided by Firmenich SA (Geneva, Switzerland).

18.2 Results and conclusions

Whereas there are clear dose-response relationships for p-cresol, geranylacetone, skatole and octanoic acid, this is not the case for either (E/Z)-3-methyl-2-hexenoic acid, (R/S)-2-hydroxy-3-methyl-hexanoic acid nor for 3-mercapto-1-hexenol (Figs IX.I, IX.III, IX.IV). The latter 3 last products also elicited the lowest proportion of positive EAG responses from the mosquito antennae (Table IX.I). For hexanoic acid, EAG responses were elicited only at 10 and 100 µg applied to the filter paper strip, the two highest doses tested (Figs IX.I and IX.IV and Table IX.I). There was a slight tendency of a response as a function of dose for transpirol (Fig. IX.I). For the 7 mosquitoes tested, no EAG-responses were recorded for 100 ng hexanoic acid, (R/S)-2-hydroxy-3-methyl-exanoic acid, 3-mercapto-1-hexenol or transpirol™ injected onto the GC column (Figs IX.II, IX.III and IX.IV).

Table IX.I: Percentage of positive electroantennogram (EAG) responses recorded from female *A. gambiae* antennae to an air puff applied to the antennal preparation from a 5 ml syringe containing the products tested. N is the number of antennae tested.

Products	Concentration [µg]	Percentage of positive EAG responses	N
p-cresol	0.1	62.5	8
	1	87.5	8
	10	100	8
	100	100	9
geranylacetone	0.1	37.5	8
	1	87.5	8
	10	100	8
	100	100	9
skatole	0.1	20	5
	1	80	5
	10	100	7
	100	87.5	8
octanoic acid	0.1	20	5
	1	60	5
	10	100	6
	100	100	8
hexanoic acid	0.1	0	3
	1	0	4
	10	60	5
	100	100	5
E/Z-3-methyl-2-hexenoic acid	0.1	0	3
	1	0	3
	10	0	4
	100	60	5
HMHA	0.1	0	3
	1	33.3	3
	10	0	4
	100	20	5
transpirol™	0.1	0	3
	1	33.3	3
	10	44.4	9
	100	62.5	8
3-mercapto-1-hexenol	0.1	33.3	3
	1	0	3
	10	25	8
	100	62.5	8

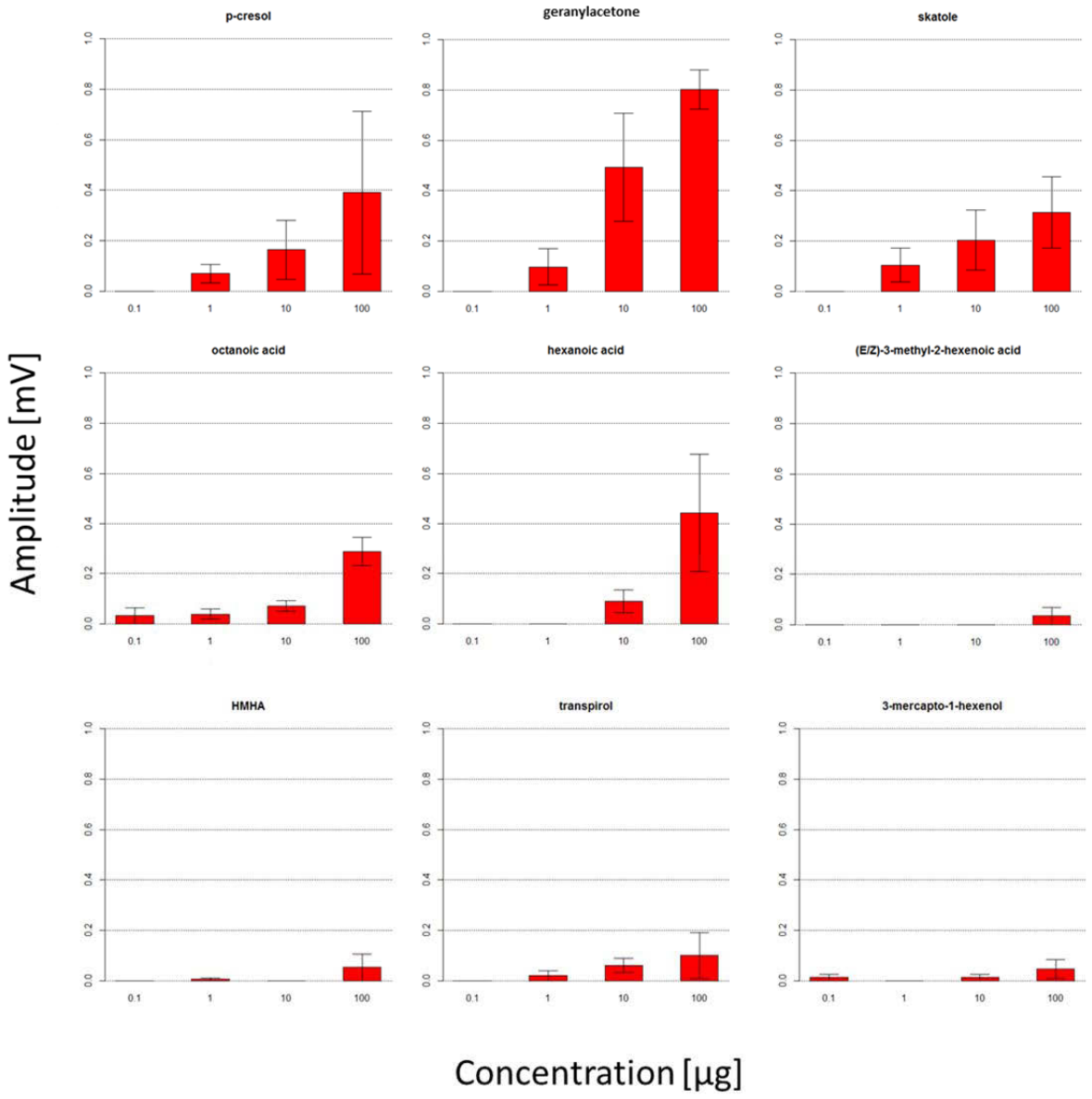

Fig. IX.I: Amplitude (mean ±S.E.M.) of the electroantennogram responses of *A. gambiae* antenna stimulated with an air puff containing increasing doses of test compounds. Between 3 to 9 female *A. gambiae* were tested per dose.

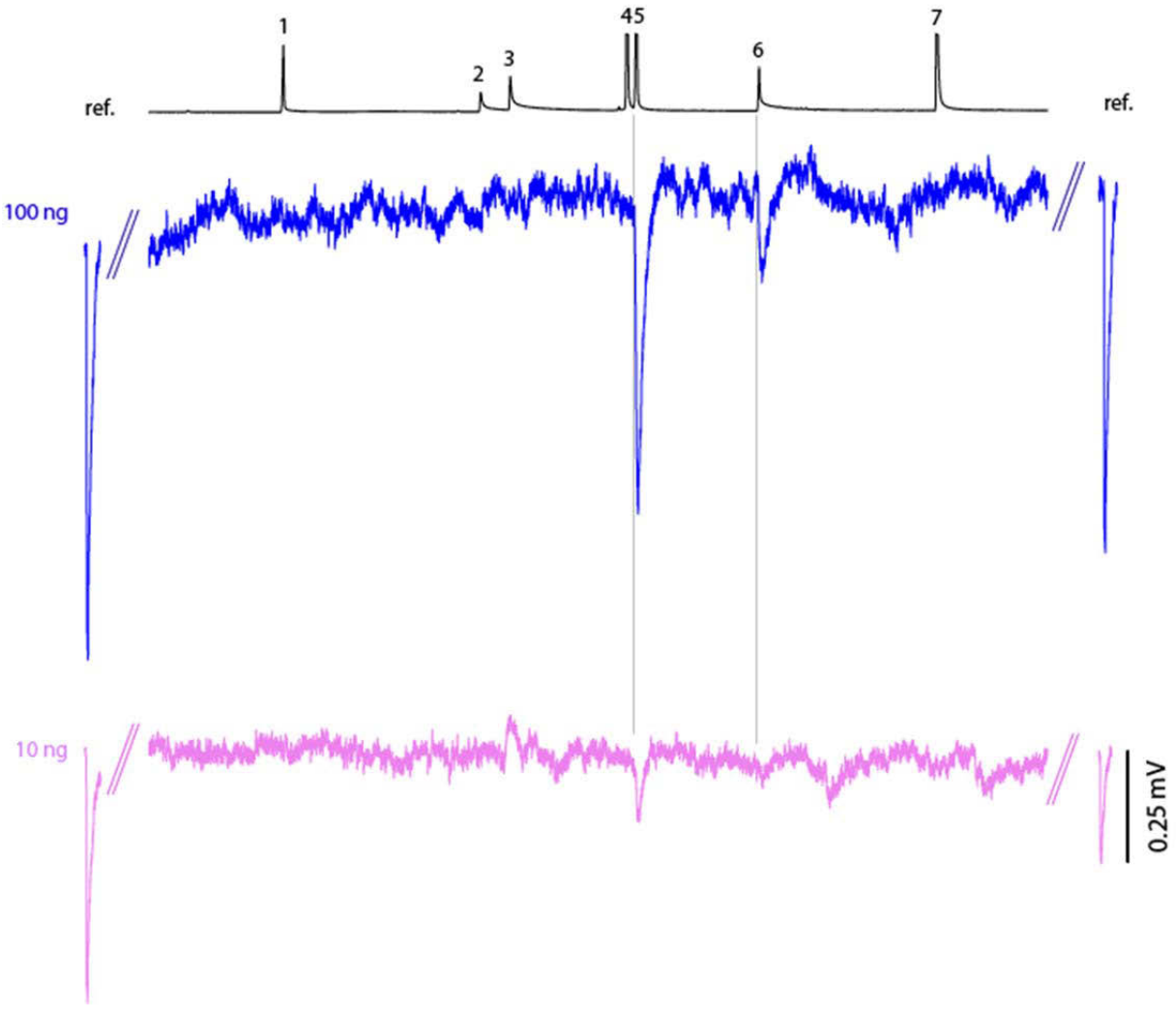

Fig. IX.II: Electroantennogram (EAG) responses of a one-day old female *A. gambiae* to a mixture of nonanal **(1)**, butyric acid **(2)**, isovaleric acid **(3)**, 3-mercapto-1-hexenol **(4)**, geranylacetone **(5)**, octanoic acid **(6)** and (R/S)-3-hydroxy-3-methylhexanoic acid **(7)** eluting from the chromatographic column (upper trace). Mixtures of the compounds containing 100 (upper EAG trace) and 10 ng/µl (lower EAG trace) in CH_2Cl_2 were injected onto the chromatographic column. Only geranylacetone and octanoic acid were found to elicit EAG responses. **Ref.** is the response to an air puff from a 5 ml syringe containing 100 µg geranylacetone on a filter paper strip delivered to the antennal preparation at the start and end of each analysis; mV scale common to both EAG traces.

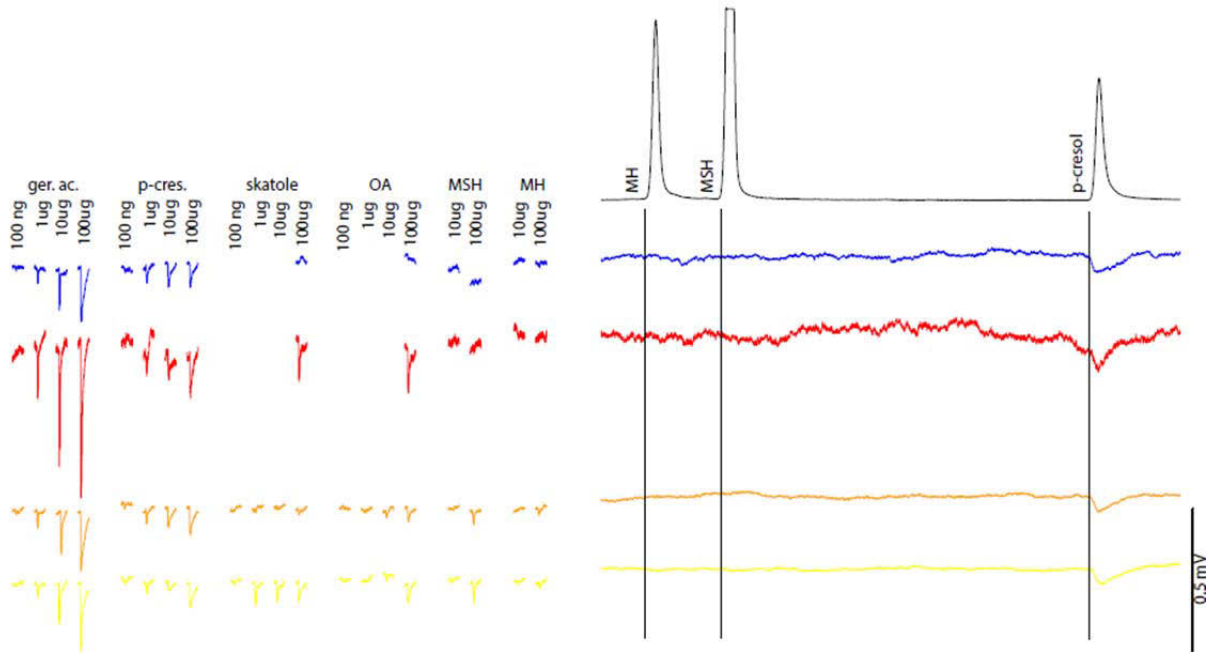

Fig. IX.III: Electroantennogram (EAG) responses of 4 female *A. gambiae* antennae to different doses of geranylacetone, p-cresol, skatole, octanoic acid (OA), (R/S)-3-mercapto-3-methyl-1-hexanol (MSH, transpirol™) and 3-mercapto-1-hexenol (MH) presented as air puffs from 5 ml syringes (left hand EAG traces) and to 100 ng of 3-mercapto-1-hexenol (MH), transpirol™ and p-cresol injected onto the chromatographic column (upper chromatographic trace on right). The EAG and GC-EAG recordings were made in the sequence presented; mV scale common to all recordings.

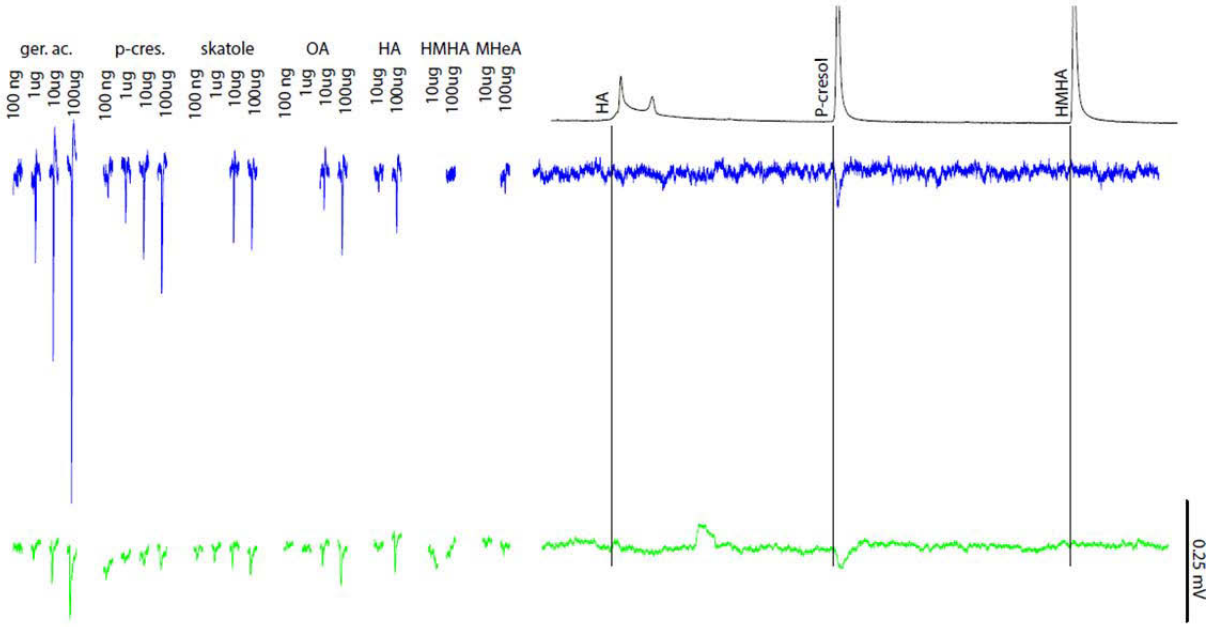

Fig. IX.IV: Electroantennogram (EAG) responses of 2 female *A. gambiae* antennae to different doses of geranylacetone, p-cresol, skatole, octanoic acid (OA), hexanoic acid (HA), (R/S)-3-hydroxy-3-methyl-hexanoic acid (HMHA) and (E/Z)-3-methyl-2-hexenoic acid (MHeA) presented as air puffs from 5 ml syringes (left hand EAG traces) and to 100 ng of HA, p-cresol and HMHA injected onto the chromatographic column (upper chromatographic trace on right). The EAG and GC-EAG recordings were made in the sequence presented; mV scale common to all recordings.

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