

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

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Abstract. A membrane feeding assay in which the effects of the antimalarial drugs quinine and artesunate are tested on *Anopheles gambiae* Giles sensu stricto is described. In the present study, 87% of female *A. gambiae* are shown to feed on whole defibrinated bovine blood alone, whereas only 47% and 43.5% feed on saline and on saline + bovine serum albumin (BSA) solutions, respectively, suggesting that additional components in the blood stimulate mosquito feeding. The addition of 1 mM quinine or artesunate to the BSA solution results in a significant reduction in percentage engorgement to 16.2% and 14.1%, respectively. However, the feeding rate is higher when 1 mM artesunate and quinine are mixed in the blood because 67.8% and 78.4% of females engorge on these solutions respectively. Artesunate (10 mM) in the blood reduces percentage engorgement to 20%. Because circulating doses of quinine and artesunate affecting *Plasmodium* in humans are much lower than those affecting feeding by *A. gambiae* in the *in vitro* assay, these two antimalarial drugs should have no effect, or only a minor effect, on the infection rate of mosquitoes feeding on treated patients. Because only the stylets penetrate the membrane and not the labellar lobes, the results of the present study suggest that both blood phagostimulants and feeding deterrents are detected by internal gustatory organs in *A. gambiae*, 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 the present study.

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

Introduction

Mosquitoes feed primarily on floral nectars (Foster, 1995), although females of anautogenous species use blood proteins in addition to plant sugars as an energy resource, as well as to complete egg maturation. Mosquitoes use volatiles to locate a food source at a distance (Foster & Takken, 2004) and other cues, such as vision (Chilaka *et al.*, 2012), temperature and

humidity (Klun *et al.*, 2013). Sensilla on the tarsi are the first taste organs that contact the food source upon landing. This is followed by a patterned search response during which the mosquitoes assess the food source with their labellar and tarsal sensilla (Sanford & Tomberlin, 2011). Tarsal sensilla of *Culiseta inornata* enclose sensory neurones responding to sugars, water and salts (Pappas & Larsen, 1976). In addition, some *Diptera* (e.g. *Drosophila* spp.) are shown to perceive bitter compounds through tarsal taste sensilla (Meunier *et al.*, 2003). On the external surface of the labellar lobes of *C. inornata*, long type 1 (T1) chemosensilla are innervated by one mechanoreceptor cell, as well as by four chemo-sensitive cells sensitive to water, sugars and salts at low and high concentrations. Small type 2 (T2) sensilla house one mechanoreceptor cell, as well as

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one water and one salt receptor cell (Pappas & Larsen, 1976). In *Aedes aegypti*, in addition to the sugar and salt neurones, a bitter sensitive cell is identified in T1 sensilla (Sanford *et al.*, 2013). In *Anopheles gambiae* Giles sensu stricto, the species on which the present study focuses, labellar T1 sensilla enclose a mechanoreceptor and cells sensitive to sugars, water and salts. In this species, both the water and sugar sensitive neurones of T1 sensilla are inhibited by denatonium, quinine and berberine, and this is correlated by the ability of these bitter compounds to inhibit sugar feeding (Kessler *et al.*, 2013). External T1 labellar sensilla are sensitive to nectar constituents such as sugars, salts and bitter tasting secondary metabolites, which are potentially noxious for mosquitoes. Indeed, it is reported that the labellum of *A. aegypti* expresses seven putative sugar receptors and AegGR14 (equivalent to the AgGr2 in *A. gambiae*), a putative bitter receptor (Sparks *et al.*, 2013), identified by sequence homology with *Drosophila melanogaster* (Kent *et al.*, 2008). To date, it is unknown whether internal receptor cells of mosquitoes, 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. Receptor cells in peg organs present at the tip of the labrum of anautogenous female mosquitoes are suggested to be involved in the detection of blood components (Liscia *et al.*, 1993; Werner-Reiss *et al.*, 1999a,b), although they are known to respond electrophysiologically to sucrose in *C. inornata* (Pappas & Larsen, 1976).

In the first part of the present study, to test whether female *A. gambiae* assess the quality of the blood meal through internal taste receptor cells, the phagostimulatory effects on mosquitoes of three different artificial feeding solutions are compared with defibrinated bovine blood alone. This is carried out using a membrane-feeding assay that excludes contact by the external surfaces of the proboscis and by the tarsi with treatments.

Alkaloids, such as quinine, and sesquiterpene lactones, such as artemisinin, in addition to serving as antimalarial drugs, are also defensive compounds of plants, comprising strong feeding deterrents for phytophagous insects (Schoonhoven, 1982; Picman, 1986). In nature, mosquitoes are confronted by bitter tasting and potentially noxious plant secondary metabolites during nectar feeding (Ignell *et al.*, 2010; Kessler *et al.*, 2013). However, it has not yet been established whether non-volatile plant derived compounds can interfere with female mosquitoes feeding on blood. In the present study, the antifeeding effects of quinine and artesunate are compared on female *A. gambiae*, the principal vector of malaria in Africa, foraging for a blood meal. With the help of the membrane-feeding assay, we assess whether female *A. gambiae* can detect, through internal taste receptor cells, quinine and artesunate added to a feeding solution or bovine blood. The results are correlated with the plasma concentration of these two antimalarial drugs found to affect *Plasmodium falciparum* in other studies and are discussed. In addition, a description is provided of the neuroanatomy of gustatory sensilla containing chemo-sensory neurones inside the labellum and on the tip of the labrum that could mediate the perception of feeding stimulants and deterrents by *A. gambiae*.

Materials and methods

Mosquitoes

The *A. gambiae* colony (16CSS strain) was maintained in a climate chamber under a LD 12:12 h photocycle at 28 °C and 80% relative humidity, with 2 h of simulated sunrise and sunset, as described previously by 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 electron microscopy (TEM) and scanning electron microscopy (SEM). For TEM, 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 sodium cacodylate buffer with 4% sucrose. After post-fixation in 1% OsO₄ for 2 h and rinsing in the same buffer, the specimens were block stained with 2% uranyl acetate (pH 3.9) for 1 h at room temperature. The heads were dehydrated through graded series of acetone solutions and embedded in Spurr's resin (Polyscience AG, Switzerland). Ultrathin sections (1 µm) of the first 100 µm from the tip of the proboscis of three females were made on a Reichert Ultratuc S microtome (Reichert-Jung, Austria), stained with uranyl acetate and lead citrate, and examined in a Philips CM 100 electron microscope (Philips Electron Optics, The Netherlands).

For SEM, excised heads of *A. gambiae* were fixed in 70% ethanol, rehydrated and washed in Kodak Photo-Flo (Kodak, France) overnight. After several washes in distilled water, the tissues 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 10 kV using a Philips ESEM XL 30 electron microscope.

Chemicals

Bovine serum albumin (BSA) fraction V was purchased from Roche Diagnostics GmbH (Germany), sodium hydrogen carbonate (NaHCO₃) was purchased from Merck (Germany), and quinine anhydrous, sodium chloride (NaCl), an amino acid solution (RPMI-1640 50X, without L-glutamine, BioReagent, R7131) containing 19 amino acids, artesunate and artemisinin (ART) were purchased from Sigma-Aldrich (Switzerland). Dihydroartemisinin (DHA) was purchased from Biopurify Phytochemicals Ltd (China). The mixture of amino acids was sterile filtered; the purity of all other compounds was ≥98%. Solutions were kept at 4 °C. Molecular structures of drugs were drawn with CHEMBIODRAW ULTRA, version 13.0 (PerkinElmer, Waltham, Massachusetts).

Feeding membranes

A silicone membrane prepared as described in Kröber & Guerin (2007a) was used to feed mosquitoes. Briefly, pieces

of Kodak lens cleaning paper ($7 \times 12.5 \text{ cm}^2$; Eastman Kodak, Rochester, New York) were placed on a layer of kitchen plastic film and impregnated with a mixture of 4.5 g of silicone oil (30% DC 200; Fluka, Switzerland), 0.15 g of Elastosil FL white colour paste and 15 g of silicone RTV-1 Elastosil E4 glue (both from Wacker, Germany). This mixture was rendered less viscous for application by adding 2.9 g of hexane. Excess silicone was removed with an 80 mm wide scraper made from a piece of silicone (thickness 3 mm). Membranes were left to polymerize for approximately 24 h and membrane thickness was $49 \pm 11 \mu\text{m}$ (mean \pm SD, range 30–100 μm).

In vitro feeding experiments

Gorging responses of *A. gambiae* females were recorded on different feeding solutions: 8.75 g L^{-1} NaCl (149.73 mM) + 0.75 g L^{-1} NaHCO_3 (8.93 mM) to provide a saline solution of pH 8.0 (Arsic & Guerin, 2008); 120 g L^{-1} BSA in the saline solution (Arsic & Guerin, 2008); 10-fold diluted RPMI-1640 amino acid mixture in the BSA solution to provide a 5.74 mM solution of L-arginine, the most abundant amino acid in the mixture (see Supporting information, Table S1); 0.38 g L^{-1} artesunate and 0.32 g L^{-1} quinine added,

respectively, to the BSA solution to provide 1 mM solutions of each; freshly collected bovine blood was manually defibrinated at collection in the slaughterhouse; and serial dilutions of between 0.01 and 1 mM quinine and between 0.01 and 10 mM (saturated) artesunate were tested in blood. Solutions 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 haemolysis.

At the beginning of each feeding test 12 ± 4 (mean \pm SD) *A. gambiae* females aged between 3 and 7 days were released into a transparent plastic cylinder (diameter 98 mm, height 52 mm) with an oval opening ($64 \times 22 \text{ mm}^2$) at the top that allowed the mosquitoes to contact the membrane from below (Fig. 1). Mosquitoes were allowed to feed for 30 min on 3.5 mL of the treatment in the dark. This corresponds to the time usually used for *in vitro* mosquito feeding assays (Bousema *et al.*, 2012). Mosquitoes, deprived of water and sucrose, were placed in the cylinder between 1 and 5 h before the experiment. A piece of paper placed over the cylinder opening was pulled out to allow mosquitoes to probe the membrane from below.

All tests were made in a walk-in climate chamber (25°C and 80% relative humidity) during the last 6 h of the scotophase. Water from a bath maintained at $37.4 \pm 0.4^\circ\text{C}$ (Compact-thermostat Typenreihe MT, Germany) was used

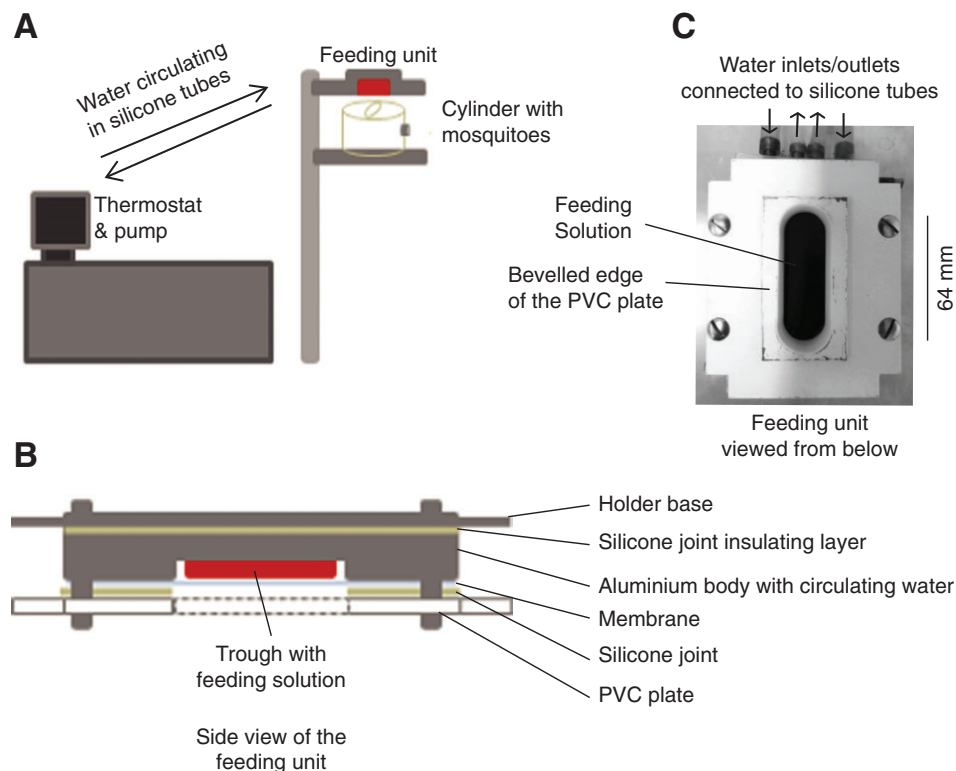


Fig. 1. Outline of the feeding unit assembly: thermostat to control the water bath temperature and pump connected to the aluminium body of the feeding unit by silicone tubes. Mosquitoes were held in a plastic cylinder for feeding assays (A). The feeding unit was made of an aluminium body ($105 \times 65 \text{ mm}^2$) through which warm water circulated. The aluminium body had a trough ($64 \times 22 \text{ mm}^2$; depth 1.5 mm) for the test solutions. A poly(vinyl chloride) (PVC) plate (thickness 6.5 mm) fastened with screws to the heated unit held the membrane in place under a silicone joint (thickness 1 mm). 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 to access the membrane from below (C).

to warm treatments beneath the membrane via a pump connected to the feeding unit through silicone tubes (Fig. 1). The mean \pm SD temperature of the membrane was $35.6 \pm 0.6^\circ\text{C}$ (range $34.8\text{--}39.6^\circ\text{C}$). After each test, mosquitoes were anaesthetized with CO_2 gas to count the percentage of engorged females (visual estimation of abdominal distention as described by Arsic & Guerin, 2008). Only fully engorge mosquitoes were recorded as having fed (Galun *et al.*, 1985a). The climate chamber was ventilated, thus preventing a rise in the ambient CO_2 level.

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

Batches of 36, 31 and 21 5-day-old females were fed as described above on defibrinated bovine blood alone, blood + 1 mM quinine and blood + 1 mM artesunate. In addition, a batch of 34 females fed on blood with 1 mM quinine was tested without subsequently anaesthetizing them. The engorged mosquitoes on each solution were placed in rearing cages ($350 \times 350 \text{ mm}^2$; height 550 mm) with free access to water and 10% sucrose. One day after the blood meal, a crystallizing dish (diameter 100 mm, depth 10 mm) with a filter paper (diameter 90 mm, No. 1001 090; Whatman, U.K.) humidified with 4 mL of demineralized water was placed in the centre 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

Artesunate is not stable in solution and is converted into DHA (Fig. 2A). This reaction is pH dependent and oral doses are rapidly converted at the low pH of the stomach, although artesunate is more stable in plasma at higher pH (Olliaro *et al.*, 2001). In blood, the conversion is assumed to be mediated by plasma and red blood cell esterases (Zhou *et al.*, 1987). To evaluate the kinetics of artesunate conversion in our blood samples, both artesunate 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 containing 1 mM artesunate at $4260g$ 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 of ACN was added to 300 μL of plasma. The supernatant was diluted 100-fold in 50:50 ACN/ H_2O before quantification.

Analyses by UHPLC-QTOFMS were carried out on an Acquity BEH C18 column ($2.1 \times 50 \text{ mm}$; particle size 1.7 μm) from Waters (Milford, Massachusetts) using an Acquity UPLC system (Waters) coupled to a Synapt G2 QTOF mass spectrometer (Waters) through an electrospray (ESI) interface. A solvent gradient programme was employed at a flow rate of $400 \mu\text{L min}^{-1}$: solvent A = water + 0.05% formic acid, solvent

B = ACN + 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 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: capillary and cone voltages +2800 and +25 V, respectively, source temperature 120°C , desolvation gas flow and temperature 800 L h^{-1} and 450°C , respectively, cone gas flow 20 L h^{-1} . Accurate mass measurements were obtained by infusing a 400 ng mL^{-1} solution of the synthetic leucine-enkephalin at a flow rate of $10 \mu\text{L min}^{-1}$ through the Lockspray ESI probe (Waters). The system was controlled by MASSLYNX, version 4.1 (Waters). Artesunate, ART (Fig. 2A) and DHA were quantified based on their $(\text{M} + \text{Na})^+$ ions using extracted ion chromatograms with a mass window of $\pm 0.01 \text{ Da}$: m/z 407.17 for artesunate (retention time 3.20 min), m/z 307.15 for DHA (retention time 2.59 min) and m/z 305.14 for ART (retention time 3.34 min) as IS. Absolute concentrations were determined using calibration curves obtained with artesunate and DHA standards spiked with ART. The concentrations of the calibration points were 0.2, 1, 2 and $5 \mu\text{g mL}^{-1}$.

Statistical analysis

All statistical analyses were conducted using R, version 2.11.1 software (R Development Core Team, 2010). The data from the feeding assay were analyzed with four different generalized linear models (GLM) with a quasi-binomial error distribution and a logit link function. To compare the respective phagostimulatory effects of the saline solution, BSA in saline solution, amino acids in the BSA solution and defibrinated bovine blood alone, a GLM was fitted to the data with the number of engorged versus un-engorged females as the dependent variable and test compounds as the fixed factors. To compare the deterrent effect of doses between 0 and 1 mM of both quinine and artesunate added to blood, the fixed factors comprised the antimalarial drugs and their respective concentrations. Artesunate at 10 mM was not included in the analysis because quinine was not tested at this concentration. To compare the deterrent effect of quinine and artesunate tested at 1 mM on mosquito feeding on the BSA solution or defibrinated bovine blood, the fixed factors were the drugs, their concentrations (i.e. 0 or 1 mM) and the feeding solution (i.e. BSA solution or defibrinated bovine blood). The controls (i.e. BSA or bovine blood without drug) were randomly attributed to 0 mM quinine or 0 mM artesunate for the analysis. Finally, to estimate whether the time at which the experiments were performed, as well as the age of the insects, had a significant effect on the mosquito feeding rate, a GLM was fitted to the data of mosquitoes with access to blood only, with time and age as fixed factors.

To provide an estimate of survival and fecundity on treatments, the number of dead versus live mosquitoes recorded during 6 days after the blood meal was analyzed as a function of the feeding solution with a Cox proportional-hazards model using the survival R package (Therneau, 2013). The number of eggs was divided by the number of females surviving each day. These data

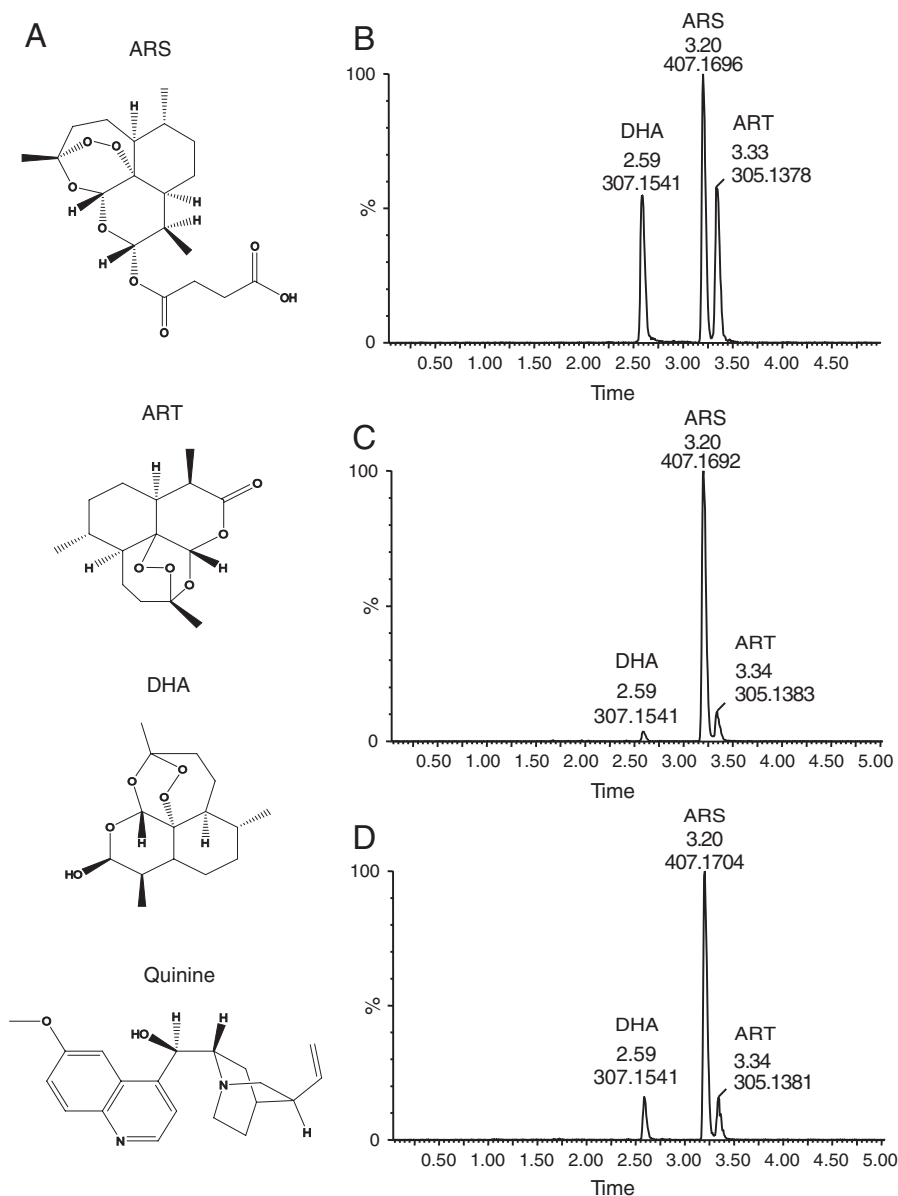


Fig. 2. (A) Molecular structure of artesunate (ARS), artemisinin (ART), dihydroartemisinin (DHA) and quinine. Base peak intensity ultra-high pressure liquid chromatography coupled with quadrupole time-of-flight mass spectrometry chromatograms of the standard mixture of 1 mM DHA, ARS and ART in 50 : 50 acetonitrile/H₂O (B), plasma extract from a freshly made up 1 mM solution of ARS in blood to which 0.1 mM artemisinin was added as internal standard (C), and a plasma extract of a 5-day-old blood solution with the same solutes (D).

are analyzed only descriptively and no statistical analysis was made because the experiment was repeated only once. $P < 0.05$ was considered statistically significant.

Results

Analysis of antimalarial drugs in blood

Concentrations of artesunate of 1.55 and 1.13 mM, respectively, were measured in the serum solution of freshly-made

up 1 mM artesunate in bovine blood and that prepared 5 days earlier. The measured concentrations exceed the 1 mM initially applied as a result of the concentration step at centrifugation. The conversion rate of artesunate to DHA in defibrinated bovine blood was low because only 2.6% (0.04 mM) of the artesunate was converted into DHA in the freshly made solution. This rate was slightly higher for the solution prepared 5 days earlier with 14.2% (0.16 mM) artesunate converted into DHA (Fig. 2B–D). Thus, any deterrent effect can be mainly ascribed to artesunate rather than to DHA, although a synergistic effect between these two compounds cannot be excluded.

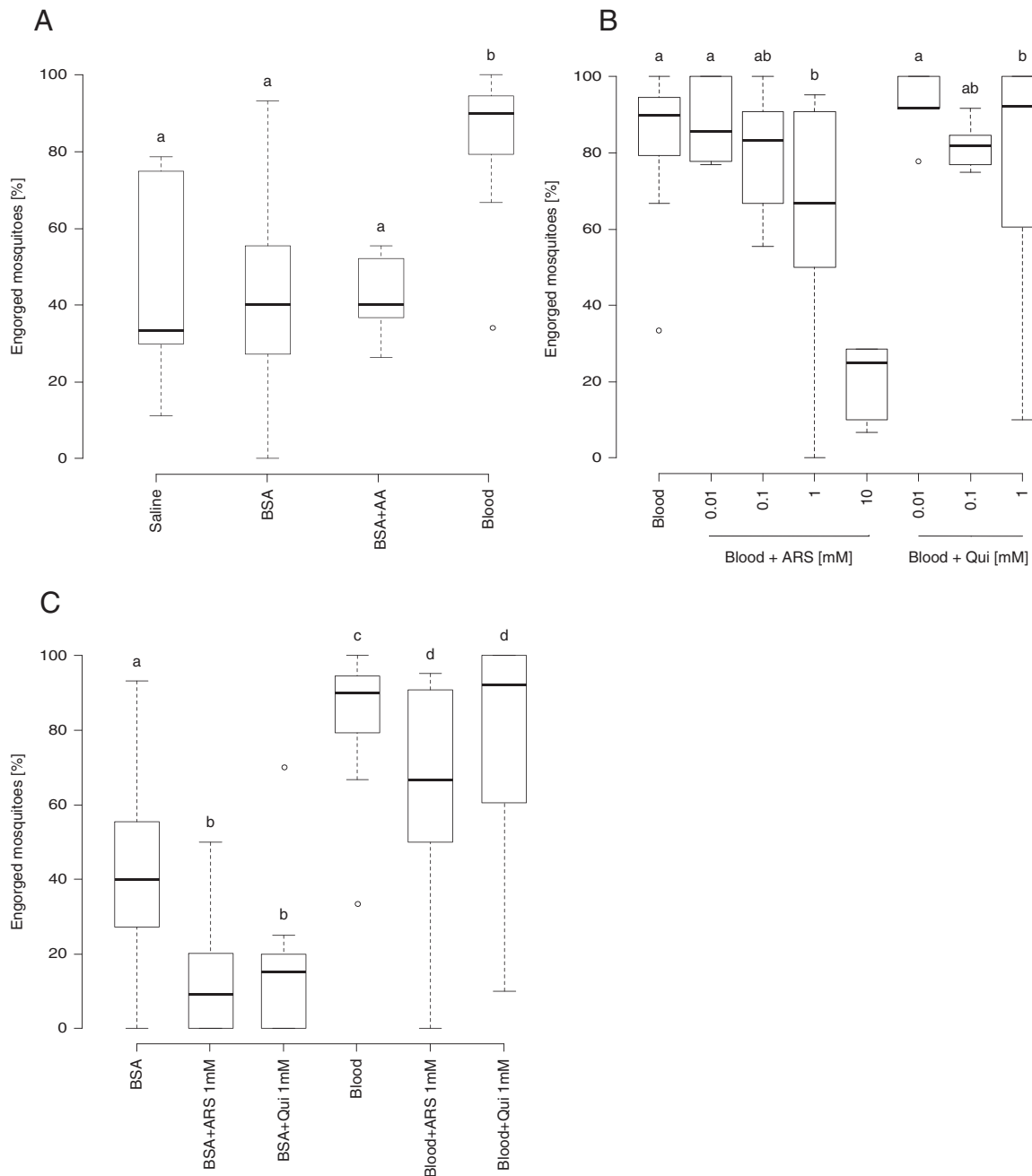


Fig. 3. Box plot representation of the percentage engorged *Anopheles gambiae* females on (A) saline, bovine serum albumin (BSA), BSA + an amino acid mixture (BSA + AA) and bovine blood alone; (B) bovine blood alone, blood + artesunate (ARS) at 0.01, 0.1, 1 and 10 mM, and blood + quinine (Qui) at 0.01, 0.1 and 1 mM; and (C) BSA, BSA + artesunate or quinine at 1 mM, bovine blood alone, and blood + ARS or quinine at 1 mM. Each experiment involved between five and 20 replicates with 12 ± 4 mosquitoes each. 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). Engorgement rates with different letters are significantly different (generalized linear model with a quasi-binomial error distribution, $P < 0.05$).

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

Female *A. gambiae* showed different engorgement responses on the treatments tested (Fig. 3). Females engorged on defibrinated bovine blood alone (86.5%) systematically more than

on saline (47.2%; GLM with a quasi-binomial error distribution, $rd = 93.8$, $d.f. = 37$, $P < 0.001$; Fig. 3A) and BSA solutions (43.5%; $P < 0.001$). Adding 120 g L^{-1} BSA to saline did not increase the feeding response by *A. gambiae* compared with saline ($P = 0.77$). Similarly, adding the amino acid mixture to the

BSA solution did not change the engorging response of females compared with BSA (40.2%; $P=0.6$). All meals were directed to the midgut.

Quinine (Fig. 2A) and artesunate added at 0.01 mM to bovine blood did not change the engorging response of females significantly compared with defibrinated blood alone (92.5% and 87.8%, respectively; GLM with a quasi-binomial error distribution and a logit link function, $rd=169.62$, $d.f.=58$, $P=0.57$), nor at 0.1 mM (81.9% and 80.4%, respectively; $P=0.4$; Fig. 3B). The percentage engorgement dropped significantly after adding artesunate or quinine at 1 mM (67.8% and 78.4%, respectively; $P=0.032$) added to defibrinated bovine blood. No significant differences were found between quinine and artesunate at all doses tested (P values between 0.25 and 0.99). Artesunate at 10 mM added to bovine blood showed a strong feeding deterrent effect because only 20% of the females engorged (Fig. 3B). Quinine at 10 mM was not tested.

Adding either quinine or artesunate at 1 mM to the BSA solution caused a reduction in percentage engorgement to 16.2% and 14.1%, respectively, compared with BSA ($P<0.001$, GLM with a quasi-binomial error distribution and a logit link function, $rd=222.4$, $d.f.=63$; Fig. 3C). The feeding response was similar between 1 mM artesunate and quinine in BSA or in blood ($P=0.18$). At the same concentration of drugs (i.e. 0 or 1 mM), blood solutions were more phagostimulant than BSA solutions ($P<0.001$). Although the drop in the percentage of engorged females was higher when 1 mM of either artesunate (29.4%) or quinine (27.3%) was added to BSA than when added to blood (18.7% and 8%, respectively), no significant interaction was found between the feeding solutions (i.e. BSA solution or defibrinated bovine blood), the antimalarial drugs (i.e. quinine and artesunate) and their concentrations (i.e. 0 or 1 mM; P values between 0.26 and 0.85).

The time at which the experiments were performed within the last 6 h of the scotophase, as well as the age of the insects, had no significant effect on the *A. gambiae* feeding rate on the blood treatment ($P=0.29$ and 0.72, respectively, GLM with a quasi-binomial error distribution and a logit link function, $rd=36.29$, $d.f.=17$). In these assays, 90% of mosquitoes tested during the last hour of the scotophase engorged on blood.

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

After having fed on blood containing 1 mM quinine, 25 of 30 mosquitoes did not recover immediately from anaesthesia. This knockdown was not a result of the interaction of the anaesthetic with the dose of quinine consumed because, in the feeding assay without CO₂ anaesthesia, 26 of the 31 mosquitoes that had fed on 1 mM quinine were knocked down after the blood meal. Recovery from knockdown could take up to 12 h. In total, 36% and 45%, respectively, of mosquitoes knocked down after feeding on blood + 1 mM quinine in the feeding assays with and without anaesthesia did not recover after 24 h and were considered dead. No knockdown effect was recorded on blood alone or on blood + 1 mM artesunate. Six days after the blood meal, the mortality (34%) of mosquitoes fed on defibrinated bovine blood + 1 mM quinine and that had

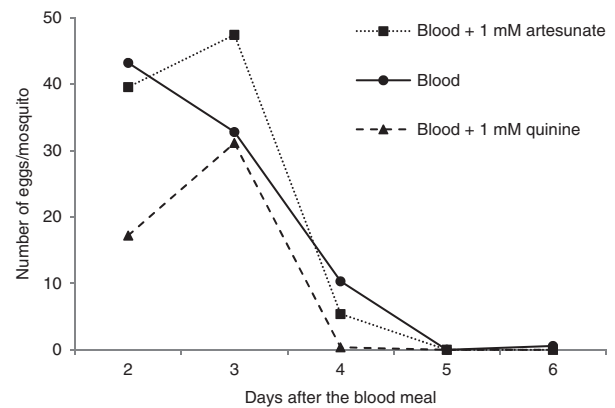


Fig. 4. Number of eggs laid per female *Anopheles gambiae* fed with defibrinated bovine blood alone (solid line with black circles), blood + 1 mM artesunate (dotted line with solid squares) and blood + 1 mM quinine (dashed line with solid triangles).

recovered from knockdown (pooled data from the trials with and without anaesthetic) was higher but not significantly different from that of mosquitoes fed on blood + 1 mM artesunate (15%; Cox proportional hazards regression, $P=0.13$) or those fed on blood alone (23%; $P=0.33$). Females laid eggs between 2 and 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 + 1 mM artesunate than for those fed with 1 mM quinine (pooled data from the trials with and without post-feeding anaesthesia) (Fig. 4). This was particularly evident on the first day of oviposition when the number of eggs laid per female fed on blood alone ($n=43$) or blood + 1 mM artesunate ($n=40$) was almost two-fold higher than for females fed with blood + 1 mM quinine ($n=17$) (Fig. 4).

Ultrastructure of *A. gambiae* labral and labellar sensilla

The labrum of female *A. gambiae* bears a pair of apical peg sensilla at the tip and a pair of subapical peg sensilla situated more proximally on each side (Fig. 5A, B). These sensilla enclose two lymphatic cavities (Fig. 5C). In all, five sensory cells innervate the inner lymph cavity with four dendrites ascending the shaft of the sensillum and a fifth neurone terminating at the base of the hair. This constitutes the basic form of a gustatory sensillum in insects (Altner & Prillinger, 1980). The five dendrites are enclosed at the base by a sheath secreted by the thecogen cell (Fig. 5C). Both apical and subapical sensilla are present only in females. At 50 μ m 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. 5D). No TEM analysis was conducted on this pair of sensilla.

Trichoid sensilla are present on the inner face of the labellar lobes of each sex (Fig. 6). They possess a unique pore at the level of the lateral spur (Fig. 6A, B). The number of branched dendrites enclosed by these sensilla is variable: three or four ascend the inner lymph cavity to the pore and one terminates below the shaft of each sensillum; the presence of a tubular body

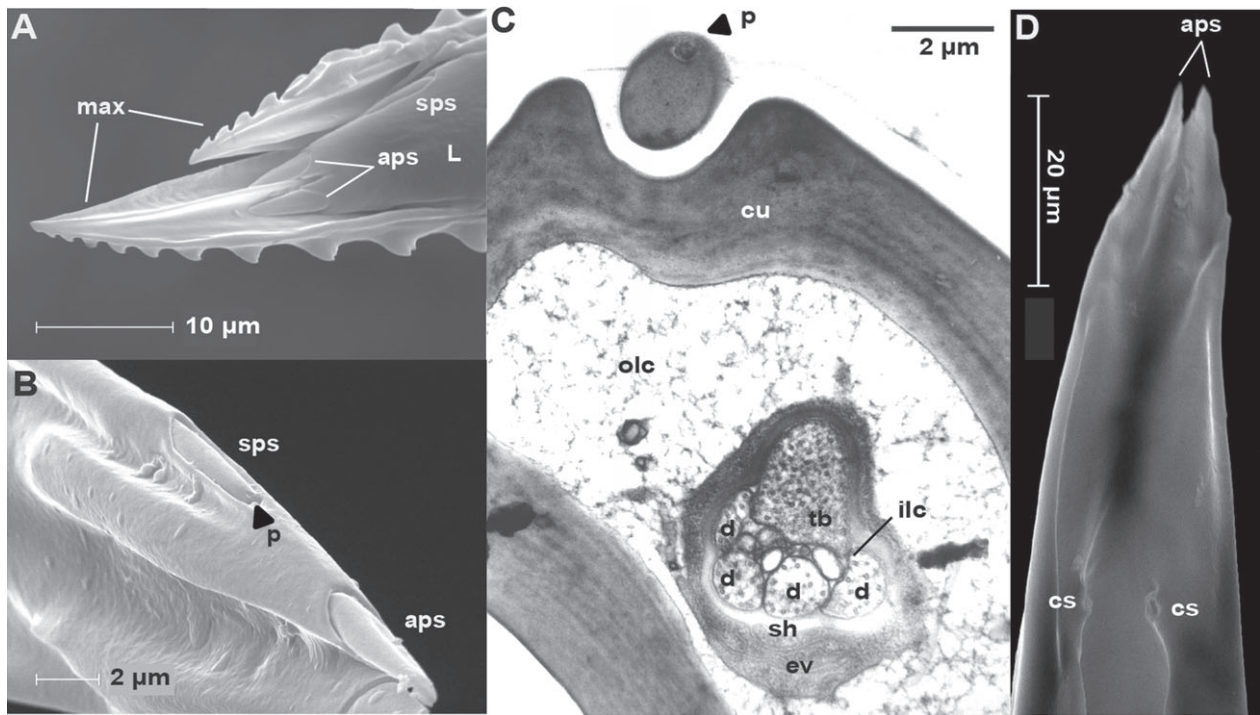


Fig. 5. Ultrastructure of peg sensilla and campaniform sensilla on the labrum of female *Anopheles gambiae*. (A) Scanning electron micrograph (SEM) of the extremities of the maxillary stylets (max) and the labrum (L; dorsal view) showing the position of the apical (aps) and subapical sensilla (sps). (B) SEM of the extremity of the labrum (fronto-latero-ventral view) showing the apical and subapical sensilla. (C) Transmission electron micrograph at the level of the tubular body of an apical sensillum and at the level 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. aps, apical sensilla; cs, campaniform sensilla; cu, cuticle; d, dendrites; ev, enveloping cells; ilc, inner lymph cavity; L, labrum; max, maxillary stylets; olc, outer lymph cavity; p, pore; sh, sheath; sps, subapical sensilla; tb, tubular body.

is shown by the typical arrangement of its microtubules (Fig. 6C, D). Consequently, these gustatory sensilla house between three and five sensory cells.

Discussion

A reliable in vitro feeding assay for mosquitoes

The feeding assay described in the present study represents a reliable and readily accessible method for testing mosquito feeding. The added advantage of the silicone membrane is its capacity to seal once a mosquito has withdrawn its mouthparts, thus avoiding leakage (Kröber & Guerin, 2007b). Because it is possible to treat the silicone membrane, this assay also represents a reliable method for testing compounds acting on contact by mosquitoes. A similar kind of membrane-treated bioassay is reported previously for testing the repellent properties of plant volatiles in mosquitoes (Dube *et al.*, 2011). The percentage of female *A. gambiae* that feed on whole defibrinated bovine blood alone in the feeding assay described in the present study is similar that recorded by Rutledge *et al.* (1964) for *Anopheles stephensi* feeding on a solution of a chick erythrocyte extract + 5 mM ATP through a baudruche membrane.

Blood constituents stimulate feeding in female A. gambiae

Among mosquitoes, anophelines (Culicidae: tribe Anophelini) are assumed to engorge in response to phagostimulants present in the plasma, although culicines (Culicidae: subfamily Culicinae) require cellular components, probably adenine nucleotides (Galun *et al.*, 1985a,b; Werner-Reiss *et al.*, 1999a). By contrast to that reported for *A. gambiae* (Galun *et al.*, 1985a), the percentage of engorged *A. gambiae* is lower in the present study on the saline solution than on blood, indicating the presence of additional phagostimulants in blood. Adding 120 g L⁻¹ BSA to saline does not increase the feeding response by *A. gambiae*, as is noted for *Anopheles dirus* but at a concentration of BSA that is nonetheless 2.4-fold lower (Galun *et al.*, 1985a).

The present data strongly suggest that the presence of internal receptor cells on mouthparts is responsible for the detection of blood phagostimulants. When females pierce a membrane to feed, the stylets enter the feeding medium leaving the labellar lobes bearing external taste sensilla outside. Thus, only receptor cells on the labrum, on the internal face of the labellar lobes and within the cibarium are in contact with blood and can be implicated in the perception of any chemostimulants present in blood. The apical and sub-apical labral peg organs, the first to contact the feeding solution, constitute strategic sites in this regard. Because these sensilla are present exclusively

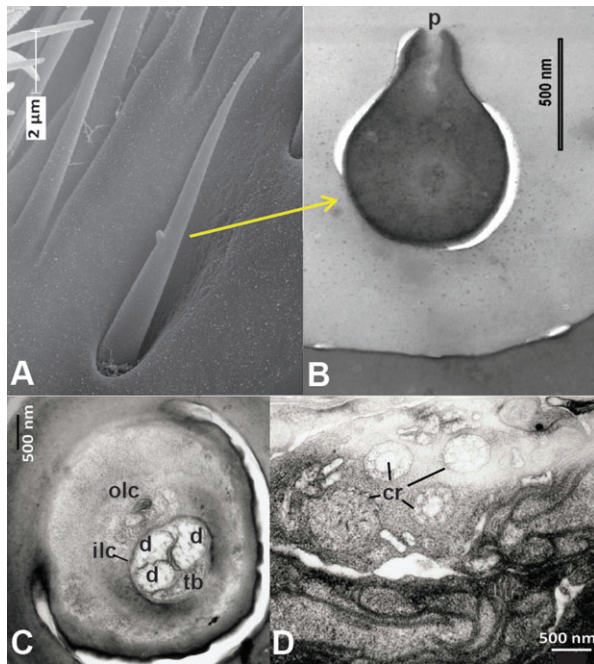


Fig. 6. (A) Scanning electron micrograph of a trichoid sensillum on the inner face of the labellum of *Anopheles gambiae*. Transmission electron micrographs made halfway along such a sensillum showing the lateral pore (B), at the level of the tubular body (C) and at the level of the ciliary root (D). cr, ciliary root; d, dendrite; ilc, inner lymph cavity; olc, outer lymph cavity; p, pore; tb, tubular body.

in females of anautogenous mosquito species (Lee & Craig, 1983b), they appear most likely to carry receptor cells tuned for chemostimulants present in blood. Labral chemoreceptor cells respond electrophysiologically to adenine nucleotides in *Culex pipiens* and *A. aegypti*, as well as to NaCl and to L-alanine, the C-terminal amino acid of albumin, in *A. aegypti* (Liscia *et al.*, 1993; Werner-Reiss *et al.*, 1999a,b).

The gustatory sensilla found on the inner face of each labellar lobe in the present study on *A. gambiae* may also be involved in the detection of chemical stimulants in blood. These sensilla differ from the type 3 (T3) sensilla described on the inner face of the labellum of *C. inornata* by Pappas & Larsen (1976) by the presence of the pore on the spur halfway along the sensillum and by the presence of a tubular body. A sensory cell in the T3 sensilla of *C. inornata* responds to NaCl (Pappas & Larsen, 1976). The morphology of the internal labellar sensilla of *A. gambiae* is similar to the bifurcate palatal papillae found within the cibarium of *C. inornata* (Lee & Craig, 1983a). Cibarial sensilla are not described 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, and campaniform and trichoid sensilla (McIver, 1982; Lee & Craig, 1983a). These papillae probably house chemosensitive cells and are considered to be involved in meal palatability and/or assigning meal destination to the crop versus midgut (McIver, 1982). The campaniform sensilla placed ventrolaterally on the labrum at the entry of the food canal may act as flow detectors (McIver, 1982; Lee & Craig, 1983b).

No phagostimulatory effect of the amino acid mixture in the context of the blood meal

No evidence is provided in the present study for any phagostimulatory effect of the amino acid mixture on female *A. gambiae* feeding on the BSA solution. However, amino acids are also important nectar constituents. As such, amino acids have proven to enhance sugar feeding in *A. aegypti* (Ignell *et al.*, 2010). They are important constituents of the mosquito diet and amino acid constituents of *Lantana camara* nectar are reported to enhance the survival of female *Culex quinquefasciatus* (Vrzal *et al.*, 2010). The amino acids tested in the study by Vrzal *et al.* (2010) are the same as those tested here, although at significantly lower concentrations (between 0.3- and 28.5-fold lower) with L-alanine and L-glutamine added. Ignell *et al.* (2010) have shown that some 'sweet' amino acids such as L-leucine stimulate mosquito feeding by acting synergistically with sucrose. Although such amino acids enhance the 'sweetness' of the sugar diet (Ignell *et al.*, 2010), they might not be critical for females foraging for a blood meal. The presence in the solution tested here of amino acids such as L-asparagine, L-tyrosine, L-aspartic acid and L-histidine (tested at concentrations between 0.48 and 1.89 mM), which are found to deter sugar feeding at 10 mM in *A. aegypti* (Ignell *et al.*, 2010), might serve to mask the phagostimulatory effect of other amino acids that are important elements of the mosquito diet. Although L-alanine and L-leucine at 10 µM stimulate sugar feeding in *A. aegypti* (Ignell *et al.*, 2010), these amino acids are tested in the present study at higher concentrations that could act as a deterrent.

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

The present study shows that quinine and the artemisinin derivative artesunate have a deterrent effect on *A. gambiae* females foraging for a blood meal. Several plant alkaloids and sesquiterpene lactones are already well known to be efficient feeding deterrents in phytophagous insects: doses less than 100 p.p.m. and sometimes even as low as 1 p.p.m. can inhibit feeding (Schoonhoven, 1982; Picman, 1986).

However, although feeding from a glass capillary is almost completely inhibited in *A. gambiae* when 1 mM quinine is mixed with sugar (Kessler *et al.*, 2013), only 8% of females decline to feed on blood to which this dose of quinine is added. This highlights the importance of other stimuli such as mechanical (membrane piercing), thermal and phagostimulants present in blood that have to be counterbalanced by deterrents to prevent blood feeding. Such stimuli are not present in the sugar feeding experiments from a glass capillary. In addition, only internal gustatory organs of the mouthparts, namely the apical and subapical labral peg organs, trichoid sensilla on the internal face of the labellum and sensilla of the cibarium, are in contact with blood and thus can be implicated in the perception of any systemic drugs. By contrast, during the sugar meal taken from a glass capillary in the experiments described by Kessler *et al.* (2013), both external and internal gustatory receptor cells of

the mouthparts are exposed. The present study shows that 1 mM quinine induces high mortality in engorged *A. gambiae*. In addition, mosquitoes that recover after having fed on blood containing 1 mM quinine lay almost half the number of eggs of those fed on blood, highlighting the sub-lethal effects of quinine on mosquitoes. Bitter taste threshold is not always correlated with toxicity (Glendinning, 1994). The percentage of *A. gambiae* declining to feed on blood containing 1 mM quinine is lower but not significantly different from the mosquitoes renouncing to feed on blood + 1 mM artesunate, a dose that does not induce such a lethal effect. In conclusion, in a natural environment, mosquitoes are probably able to detect bitter and potentially noxious compounds quite accurately during nectar feeding using both external and internal gustatory receptor cells on the mouthparts.

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

Artesunate is the first line drug recommended by the World Health Organization (WHO) for the treatment of falciparum malaria and quinine is still used as an alternative drug (WHO, 2010; Achan *et al.*, 2011). However, both artesunate and DHA, as a result of their rapid elimination from humans, and quinine, with its negative side effects relating to long-term treatment, are not considered for prophylaxis. Because artesunate and quinine are used as drugs against malaria, the question arises as to whether mosquitoes can detect the doses circulating in host blood. The present study shows that mosquitoes are affected by quinine and artesunate at doses higher than the therapeutic plasma concentration (i.e. from 1 mM). For example, the extrapolated peak plasma concentration of artesunate 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). In the study by Batty *et al.* (1998), it is shown that, in intravenously treated patients, artesunate is rapidly converted into DHA, which reaches a maximum plasma concentration at 9.3 μM (2.64 mg L^{-1}) with a half-life of 40 min. Similarly, the quinine plasma concentration is 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 & White, 1996). Moreover, it is known that exposing *P. falciparum* gametocytes to a dose of 2.52 μM (816 ng mL^{-1}) quinine before allowing female *A. dirus* to feed on infected blood blocks 90% of the parasite transmission to the mosquitoes (Chotivanich *et al.*, 2006). The ED_{90} is some 2000-fold lower for artesunate than for quinine (1.04 nM, 0.4 ng mL^{-1}). In conclusion, the doses of quinine and artesunate that affect *Plasmodium* are much lower than those affecting feeding by *A. gambiae* in the current assay. Despite the toxic effects of quinine on *A. gambiae*, the effect of quinine with respect to inhibiting blood feeding proves to be very poor on *A. gambiae*. Thus, the feeding deterrence of such a circulating antimalarial drug would have no or only a minor effect on the infection rate of mosquitoes feeding on treated malarial patients.

Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: DOI: 10.1111/phen.12061

Table S1. The 19 amino acids and their respective concentrations constituting the bovine serum albumin + amino acids feeding solution: the concentrations have been calculated from the specifications of the RPMI-1640 50X solution provided by Sigma-Aldrich (Switzerland).

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