



# *Anopheles gambiae* females readily learn to associate complex visual cues with the quality of sugar sources



Balázs Bernáth\*, Victor Anstett, Patrick M. Guerin

Institute of Biology, University of Neuchâtel, Rue Emile-Argand 11, 2000 Neuchâtel, Switzerland

## ARTICLE INFO

### Article history:

Received 31 March 2016

Received in revised form 29 August 2016

Accepted 31 August 2016

Available online 3 September 2016

### Keywords:

*Anopheles gambiae*

Associative learning

Sugar source

Complex visual cues

## ABSTRACT

The ability to learn plays a key role in tuning and adapting the behaviours of animals for their unpredictable biotopes. This also applies to insect vectors of disease. Anautogenous mosquitoes need to find both sugar and blood for survival and reproduction. Learning processes are expected to contribute to a mosquito's ability to undertake repeated feeding behaviours more efficiently with time, serving to decrease energy demands and avoid hazards. Here we report how visual learning by the Afrotropical malaria mosquito *Anopheles gambiae* allows it to readily associate visual cues with the quality of a sugar source. Circular black and white patterns were used as visual cues. *An. gambiae* females were conditioned in cages with a chequered pattern paired with sucrose and a concentric pattern paired with non-palatable sucrose-NaCl and with reverse combinations. Hours later, significantly higher numbers of feeding attempts were counted on sucrose paired with the chequered pattern following conditioning with the same combination. This was also the case on the concentric pattern paired with sucrose following conditioning with this combination. However, the effect was weaker than with sucrose paired with the chequered pattern. These findings indicate a differential capacity of visual stimuli to induce learning, explained in our experiments by a significantly higher mosquito appetite on sucrose paired with a chequered pattern that mimics floral shape. Training that induced a higher propensity for feeding attempts was found to allow the females to display a fast learning curve (<4 min) on the less suitable concentric pattern paired with sucrose, several hours after conditioning on the chequered pattern paired with sucrose. This has important implications for mosquito behavioural ecology, suggesting that *An. gambiae* shows plasticity in its learning capacities that would allow it to readily turn to alternative sources for a sugar meal once initiated in the process by an appropriate stimulus combination.

© 2016 Elsevier Ltd. All rights reserved.

## 1. Introduction

It is well understood that memory and the ability to learn are not the privileges of higher vertebrates, but rather common assets within the animal kingdom and ubiquitous among insects. While many behavioural patterns of insects are governed by their genetic heritage, individual experiences play a role in tuning and adapting behaviours to unpredictable environments (Dukas, 2008). Insect vectors of diseases in general, and mosquitoes in particular, are no exception to this rule and their learning capacity has elicited a steadily growing interest in the last decade due to their epidemiological importance (McCall and Kelly, 2002; Alonso and Schuck-Paim, 2006). The difficulties of excluding the effects of diverse genotypes of wild mosquito populations on the outcome of experiments combined with the complex spatial structure of the natural

environment in which mosquitoes thrive have served to underline the need for laboratory tests to unravel the learning capacity of mosquitoes. However, no standardised protocols are available for testing the learning abilities of mosquitoes and the body of related knowledge is still meager and somewhat controversial (Alonso and Schuck-Paim, 2006; Menda et al., 2013).

The majority of laboratory studies on mosquito learning have focused on their capacity to associate odours with appetitive stimuli, primarily the blood-meal. This bias is not surprising considering how olfaction is seen as a primary sensory modality of mosquitoes and how learning by individuals during interaction with the host could directly affect vector capacity. Olfactory learning has been reported in medically relevant species of the genus *Culex* (McCall and Eaton, 2001; Jhumur et al., 2006; Tomberlin et al., 2006; Sanford and Tomberlin, 2011; Sanford et al., 2012), *Aedes* (Kaur et al., 2003; Menda et al., 2013; Vinauger et al., 2014) and *Anopheles* (McCall et al., 2001; Chilaka et al., 2012; Lutz and Riffell, 2013; Vantaux et al., 2013, 2014). Taking a

\* Corresponding author.

E-mail address: [balazs.bernaht@unine.ch](mailto:balazs.bernaht@unine.ch) (B. Bernáth).

blood-meal is a relatively rare and hazardous event in the life of a female mosquito and related learning processes are expected to contribute even in the absence of repetition and reinforcement. According to the arguments of Alonso and Schuck-Paim (2006) habituation and sensitisation are hard to distinguish from associative olfactory learning in most cases. To evaluate the true significance of mosquito learning all possible contexts in which resources occur need to be paired with olfactory and non-olfactory stimuli. A versatile learning capacity is expected to emerge that can adequately subserve mosquito survival over successive gonotrophic cycles, during successful incubation of pathogens, location of mates and oviposition sites and dispersal of the vector (McCall and Kelly, 2002).

We raise the question whether mosquitoes capable of memorizing the circumstances of a successful blood-meal are likewise capable to memorize cues concomitant with successful feeding on sugar. Floral and extra-floral nectaries represent an essential resource for mosquitoes, much more abundant and far less risky to exploit than blood (Foster, 1995; Gary and Foster, 2004; Manda et al., 2007a). Plant hosts and resting sites are not strictly identical (Manda et al., 2007a), thus conserving energy by returning to rich sucrose sources is highly advantageous. Female mosquitoes in need of a blood-meal are highly motivated to bite and both the host and its blood provide a rich mixture of specific stimulants that serve to trigger a context-specific learning response. Blood is taken only when warm and salty whereas nectar and sap, practically free of salt, are taken at ambient temperature (Arsic and Guerin, 2008). Feeding events on animal or plant hosts are not comparable and it is far from trivial that memory functions are involved in both situations.

Mosquitoes have well developed compound eyes and there is evidence that they can memorize visual cues associated with food resources (Chilaka et al., 2012; Menda et al., 2013). Lutz and Riffell (2013) reported that *Ae. aegypti* and *An. stephensi* were not able to memorize olfactory cues in the absence of visual stimuli. While the latter finding contradicts earlier studies and requires verification, it underlines the potential importance of visual learning. Studies on this aspect of learning in mosquitoes are scarce. Moreover, there is confusion in the literature regarding the types of visual cues that mosquitoes can memorize. For example, brightness, colour and shape are incorrectly referred as comparable characteristics. These visual cues refer to different physical stimuli involving diverse requirements of the visual system.

It is well established that mosquitoes discern and are attracted to darker objects in their environment (Clements, 1992), such that using simple bright and dark targets for studying mosquito visual learning hardly permits discrimination between simple habituation and sensitisation from associative learning. Colour vision in mosquitoes has hardly been studied at the physiological level. The spectral sensitivity of the eye was measured only in *Ae. aegypti* females (Muir et al., 1992) to reveal a dichromatic system. This renders colour vision possible – that is, the ability to distinguish various wavelengths of light independent of light intensity – but not proven, and does not predict anything about colour vision in other mosquito species. Thus colour cues should be omitted in studying mosquito visual memory in the absence of an adequate body of knowledge on mosquito colour vision. If a role for visual memory is assumed to form part of the suggested memory functions that allow mosquitoes to display host and site fidelity (McCall and Eaton, 2001; McCall et al., 2001; McCall and Kelly, 2002; Alonso and Schuck-Paim, 2006), then the capacity of memorizing complex visual cues composed of shapes and contrasting edges should likewise be assumed. Without this the insect would not be able to recognise characteristic markers or landmarks in its environment. In the design of tests with complex visual stimuli one must also bear in mind that eyes of most mosquito species are

adapted for high sensitivity and have very poor spatial resolution (Kawada et al., 2006). Even at relatively small distances, complex targets are seen as homogenous darker or brighter objects such that in studies dealing with complex targets only patterns with equal portions of neutral dark and bright areas should be used.

Chilaka et al. (2012) found that *An. gambiae* females are able to associate blood quality with visual cues, and possess a long term visual memory that could help a mosquito to recognize appropriate hosts. Here we report how visual learning by *An. gambiae* is more general, as females are also able to associate visual cues with the quality of a sugar source. This indicates that *An. gambiae* possesses the capacity to associate critical resources with common visual cues, a finding that suggests that mosquitoes possess a memorizing capacity allowing them to return to specific points in their environment.

## 2. Methods

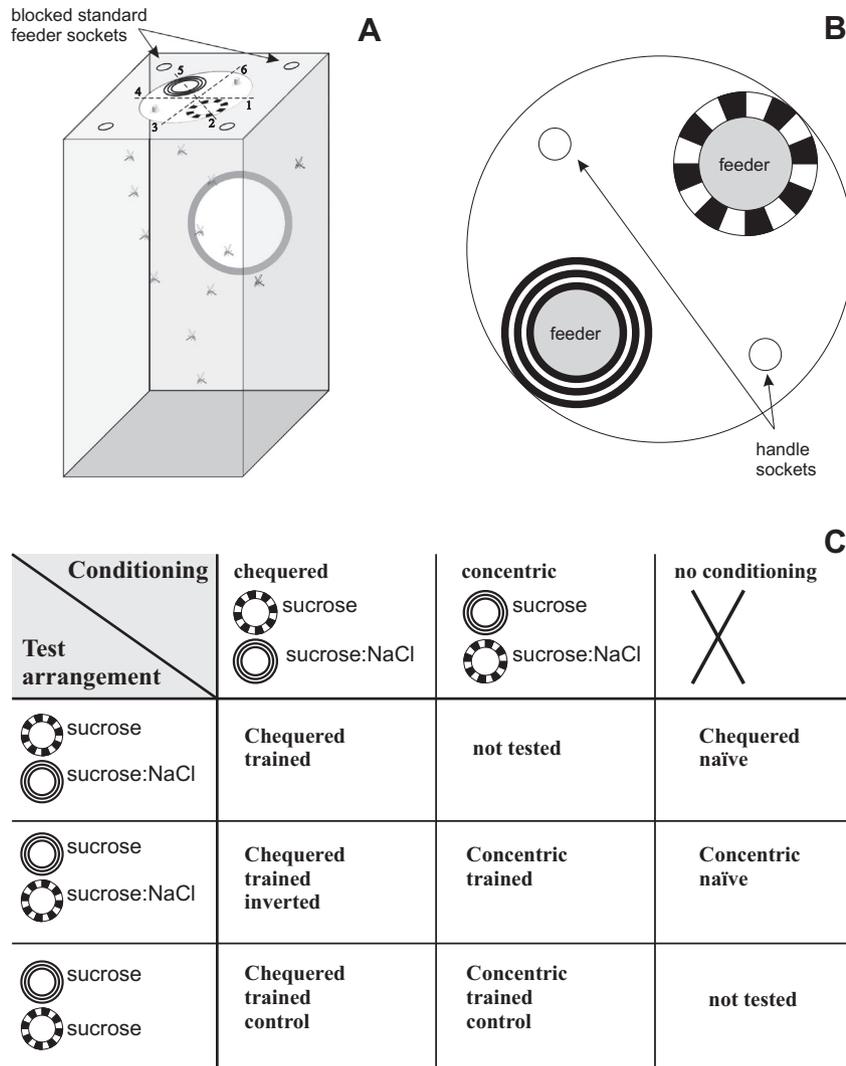
### 2.1. Mosquitoes

Mosquitoes were from a colony of *An. gambiae* (16CSS strain, derived from adults that were wild-caught in 1974 in Lagos, Nigeria) maintained in a walk-in climate chamber at  $28^{\circ} \pm 1^{\circ} \text{C}$  and  $80\% \pm 1\% \text{RH}$  and under a 12:12 h light:dark cycle with 1 h transition periods between the photo and scotophases. The scotophase lasted from 03:00 to 15:00. Light was provided by high frequency fluorescent lighting.

Larvae were raised in plastic trays (300 larvae in 400 ml demineralised water per tray) fed with powdered Tetramin™ nutrient for tropical fish until they pupated. Imagoes emerged from pupae within transparent Plexiglas® rearing cages (W × L × H: 35 × 35 × 55 cm) with a porthole on the front face (diam. 15 cm) to access mosquitoes (Fig. 1A). Sexes were not separated, and females were considered to have mated within 48 h of emergence. Four sugar feeders consisting of 1 cm diam. dental rolls soaked with a 10% sucrose solution in 16 mm diam. 10 ml glass vials were inserted upturned through 17 mm holes along the diagonals of the cage ceiling, each at 10 cm from the nearest corner. The glass vials were prevented from dropping using rubber O-rings. Pure water and a humid refuge was provided by wet cotton placed on a fine metal netting (mesh size 1 mm) that covered a 12.5 cm diam. opening in the centre of the roof of the cage. Six to eight-day-old females that had not taken a blood-meal were used in all experiments.

### 2.2. Conditioning and deprivation

Bulk-training was used for conditioning mosquitoes. Insects were conditioned in the Plexiglas® rearing cage (described above). The 4 sugar feeders (see above) were removed and their sockets were blocked by rubber plugs. The wet cotton on the cage roof was replaced by a white card with two black-and-white circular patterns (outer diam. 5 cm, inner diam. 3 cm) clearly visible through the fine netting. The patterns were printed on standard 80 g/m<sup>2</sup> white paper using a HP LaserJet 600 printer. The distance between the nearest points of the patterns on the cage roof opening was 25 mm. One of the patterns consisted of 3 concentric black circles with widths and separation gaps of 2.8 mm (hereafter the concentric pattern). The second consisted of 16 equally sized alternating black and white sections (hereafter the chequered pattern; Fig. 1B). The patterns were designed to act as neutral visual cues with equal average brightness, identical colour, and comparable cumulative edge lengths. The poor spatial resolution of *An. gambiae* eyes was considered when determining the minimum size for homogenous elements within the patterns. No imitation of any



**Fig. 1.** (A) The pattern-card used both for conditioning and for testing mosquitoes bearing the chequered (upper) and the concentric (lower) pattern. (B) The pattern-cards were placed on the top of the test cage. The patterns could be rotated to one of six randomly selected preset positions for all repetitions. (C) Tests were performed in 7 different arrangements of test patterns combined with sucrose and sucrose:NaCl during conditioning and tests.

specific part of any plant species was intended. Since the patterns were similar to those Chilaka et al. (2012) used for investigating memory functions of *An. gambiae* in relation to a blood meal no innate preference for any of the test patterns was presumed.

Upturned Petri dishes 2.9 cm outer diam. and 1 cm in depth that fitted into the opening in the centre of the visual patterns were used as test feeders (Fig. 1B). They were filled with 0.5 g cotton that could hold about 6.5–7.0 ml of the test solutions. A 10% sucrose solution, identical with that provided for the colony, was offered as a palatable test solution and a solution containing 10% sucrose with 10% NaCl was offered as a non-palatable test solution. Mosquitoes were allowed to feed on the test solutions paired with the visual patterns in the last 9 h of the photophase and up to hour 6 of the subsequent scotophase i.e. for a total of 15 h. After switch off, fluorescent tubes emit faint light the intensity of which decreases exponentially over 2 h. Since the eyes of the nocturnal *An. gambiae* become dark-adapted within an hour or so they are able to perceive light of extremely low intensities. Consequently, one needs to suppose that the patterns were visible to the mosquitoes during the first hours of the scotophase. The mosquitoes were then denied both sugar and water for the last 6 h of the scotophase until tests. For naïve mosquitoes, neither test solutions nor visual

patterns were presented before experiments, but they were likewise denied of sucrose and water in the last 6 h of the scotophase.

### 2.3. Experiments

Tests were performed during the first 2 h of the photophase in a separate Plexiglas® rearing cage (Fig. 1A). Its dimensions were identical with those of the rearing cages (above), with the exception of its central window on the roof had a diameter of 15 cm and was covered with a thin rotatable transparent polyethylene disk that had two openings 2.9 cm diam. for the test feeders and two small openings for M4 screws that served to rotate the disk. Patterns were of the same diameter as for conditioning with a distance between them of 35 mm (Fig. 1B). Freshly prepared test feeders (upturned Petri dishes as above) were placed inside the openings of the polyethylene disk.

All experiments were made in the climatized chamber where the mosquitoes were reared and conditioned. Test feeders were kept in the chamber for a minimum of 1 h before experiments to ensure they reached the temperature of the chamber. For all repetitions, 40 female mosquitoes were released into the test cage with an aspirator, a procedure that introduced some human breath that

**Table 1**

Statistical output of the nested mixed-effect one-way ANOVA ( $X^2$ ) comparing preferences by *An. gambiae* during 5 consecutive 2 min periods (*periods*) for experiments performed in 7 arrangements with N groups of 40 female mosquitoes. To compare the number of feeding attempts in the first 2 min recording period (*period 1*) on the visual patterns presented, the value of the Wilcoxon matched-paired test *W* was calculated for the same groups of mosquitoes. *P* values lower than 0.05 were accepted as significant and are marked in bold.

| Experiments                | Over all periods |                 | Between patterns presented in period 1 |    |                 |
|----------------------------|------------------|-----------------|--|----|-----------------|
|                            | $X^2$            | <i>P</i> -value | <i>W</i>                               | N  | <i>P</i> -value |
| Chequered trained          | 7.7189           | 0.103           | 0                                      | 12 | <b>0.002</b>    |
| Concentric trained         | 7.0391           | 0.134           | 0                                      | 10 | <b>0.018</b>    |
| Chequered trained inverted | 88.256           | <b>2.20E–16</b> | 10.5                                   | 11 | 0.087           |
| Chequered naïve            | 50.31            | <b>3.11E–10</b> | 6.5                                    | 11 | <b>0.020</b>    |
| Concentric naïve           | 3.8622           | 0.425           | 9                                      | 10 | 0.110           |
| Chequered trained control  | 8.3968           | 0.078           | 19.5                                   | 10 | <b>0.033</b>    |
| Concentric trained control | 3.4032           | 0.493           | 25                                     | 10 | 0.080           |

served to activate the mosquitoes. Males were not tested. Groups of 40 mosquitoes were used only once. The porthole on the front face of the test cage was closed with a piece of mosquito netting and turned away from the observer. The polyethylene disk had 6 positions marked at 60° from one other. In this manner the disk could be rotated to move the patterns with the feeders to one of six randomly selected positions during tests (Fig. 1A).

Test solutions and patterns were tested in 7 arrangements with a minimum of 10 groups of 40 females tested per arrangement (Table 1). In **chequered conditioning** sucrose was paired with the chequered pattern and sucrose:NaCl was paired with the concentric pattern, while in **concentric conditioning** sucrose:NaCl was paired with the chequered pattern and sucrose was paired with the concentric pattern (Fig. 1C). In the **chequered trained** test (12 repetitions) mosquitoes in the test cage were presented with the same combination of feeders and visual patterns they had encountered during conditioning, i.e. sucrose paired with the chequered pattern and sucrose:NaCl paired with the concentric pattern. In the **chequered trained inverted** test (11 repetitions) the patterns paired with sucrose and sucrose:NaCl solutions during conditioning were inverted during the test. In the **chequered trained control** test (10 repetitions) mosquitoes that had been conditioned with sucrose paired with the chequered pattern and sucrose:NaCl paired with the concentric one were presented with both visual patterns accompanying sucrose (see the first column in Fig. 1C). In **concentric trained** and **concentric trained control** tests (10 repetitions in each case) sucrose was paired with the concentric pattern and sucrose:NaCl was paired with the chequered pattern during conditioning (see the second column in Fig. 1C). **Chequered** and **concentric naïve** tests (11 and 10 repetitions, respectively) were performed as above for the **chequered trained** and **concentric trained** tests but with mosquitoes that had no previous experience with the visual patterns and the unpalatable test solution (see the third column in Fig. 1C).

All experiments lasted 10 min, the time needed by food-deprived mosquitoes to satiate with sucrose when feeding undisturbed. Tests were divided into periods of 2 min. The patterns and feeders were placed on top of the test cage immediately after introducing the mosquitoes and the number of feeding attempts by mosquitoes on the feeders was counted for 2 min using two hand-counters. Then the feeders were rotated to a randomly chosen new position and the number of feeding attempts by mosquito was counted for another 2 min. This procedure prevented mosquitoes from associating a given position with sucrose and also prevented aggregation by females at a given feeder (Fig. 1A). At the end of the 10 min test the mosquitoes were removed using an aspirator and the interior surfaces of the cage were cleaned with demineralised water. All recordings were made by the same person.

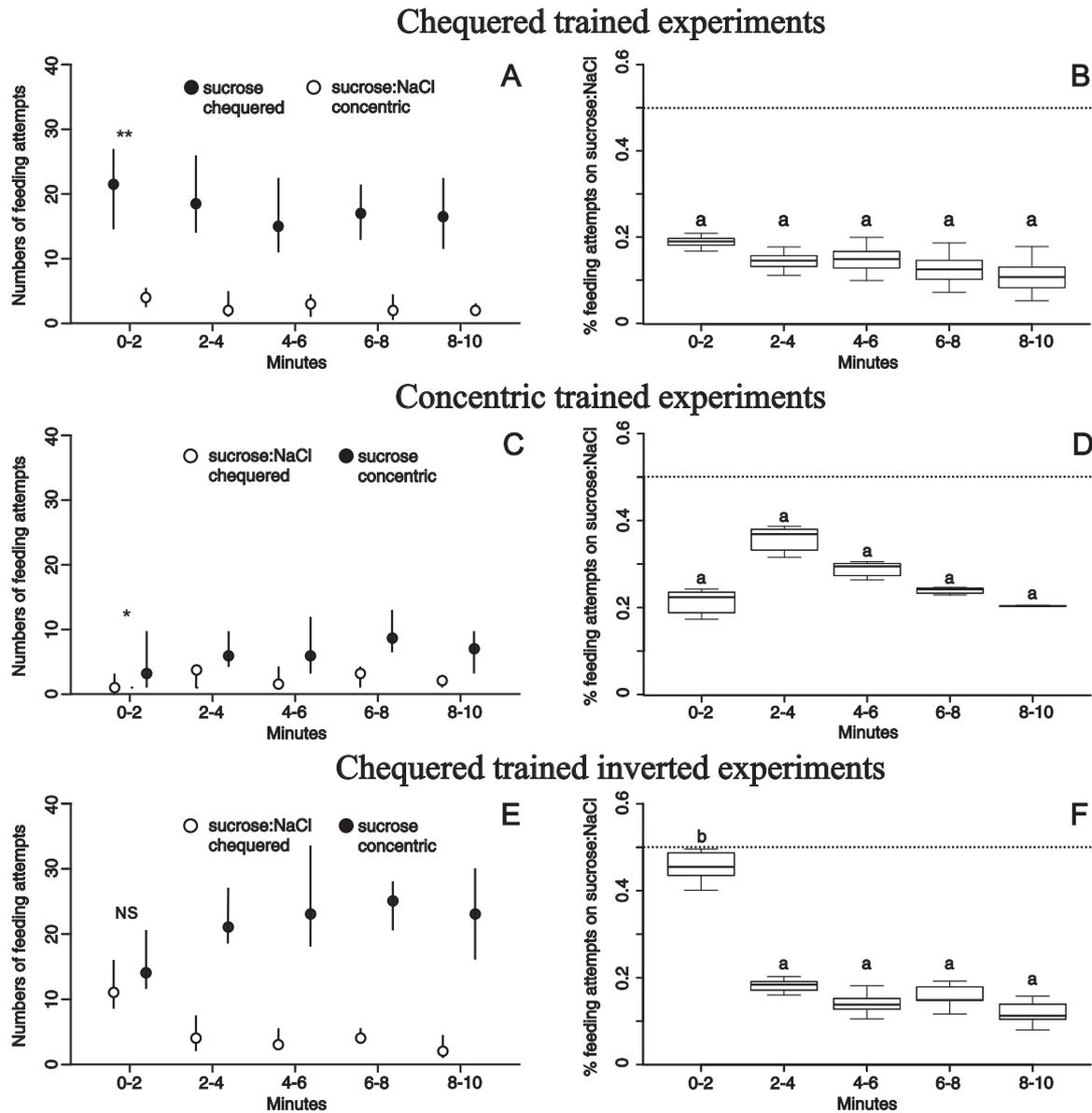
A feeding attempt was counted when the proboscis of the mosquito was seen reaching out to probe the solution-soaked cotton. Simple approaches to the feeders were disregarded. Mosquitoes

seen leaving a feeder and flying farther than about 15 cm, i.e. flying outside the observed space, were considered to be in a new choice situation. Landings by individuals becoming briefly airborne and repositioning themselves at another location on the same feeder were disregarded.

Statistical analyses were performed using R (R Core Team, 2015) R packages MASS, car, MuMIn, effects, party, RVAideMemoire, lmerTest, sjPlot, lattice, lme4. (i) To compare numbers of feeding attempts on treatments in the first 2 min (period 1) for all 7 experiments the Wilcoxon matched-pair test (WMPT) was used. (ii) To analyse for changes in the preference of mosquitoes during a test, the number of feeding attempts on the feeder containing sucrose:NaCl was estimated as a percentage of the total for each 2 min period and these proportions were compared using a nested mixed-effect one-way ANOVA. Consecutive time periods were considered as factors. (iii) To estimate the effect of the chequered and concentric patterns on the efficacy of learning, a nested mixed-effect two-way ANOVA was performed for the above calculated percentages of feeding attempts in the pairs of corresponding **chequered** and **concentric** tests. In this analysis the consecutive time periods and association of either sucrose or sucrose:NaCl with the chequered or concentric pattern were considered as factors. (iv) To compare the total number of feeding attempts on the two feeders between the pairs of corresponding **chequered** and **concentric** tests (chequered vs. concentric) we performed analyses of deviance based on a mixed effects generalized linear model (GLM) with negative binomial errors. This model was adjusted by adding a random effect to account for pseudo-replication for each test period in all 6 analyzed experiments (chequered trained inverted experiment not included). (v) To compare the number of feeding attempts recorded either on the concentric or on the chequered feeders in the **chequered** and **concentric naïve** experiments (concentric in chequered naïve vs. concentric in concentric naïve, and concentric in chequered naïve vs. chequered in concentric naïve) we performed analyses of deviance based on a mixed effects generalized linear model (GLM) with negative binomial errors. This model was adjusted by adding a random effect to account for pseudo-replication for each test period in all 6 analyzed experiments. (vi) To establish if the proportion of feeding attempts on the chequered and concentric patterns, in the **chequered** and **concentric trained control** tests differed from 50:50 distribution we performed a mixed effects GLM with negative binomial errors. Differences were considered to be significant for *P* values lower than 0.05 in all cases.

### 3. Results

In the **chequered trained** experiment significantly higher numbers of feeding attempts were counted on sucrose paired with the chequered pattern during the first two minutes (WMPT  $P = 0.002$ ; Table 1, Fig. 2A). Furthermore, the ratio of feeding attempts on the



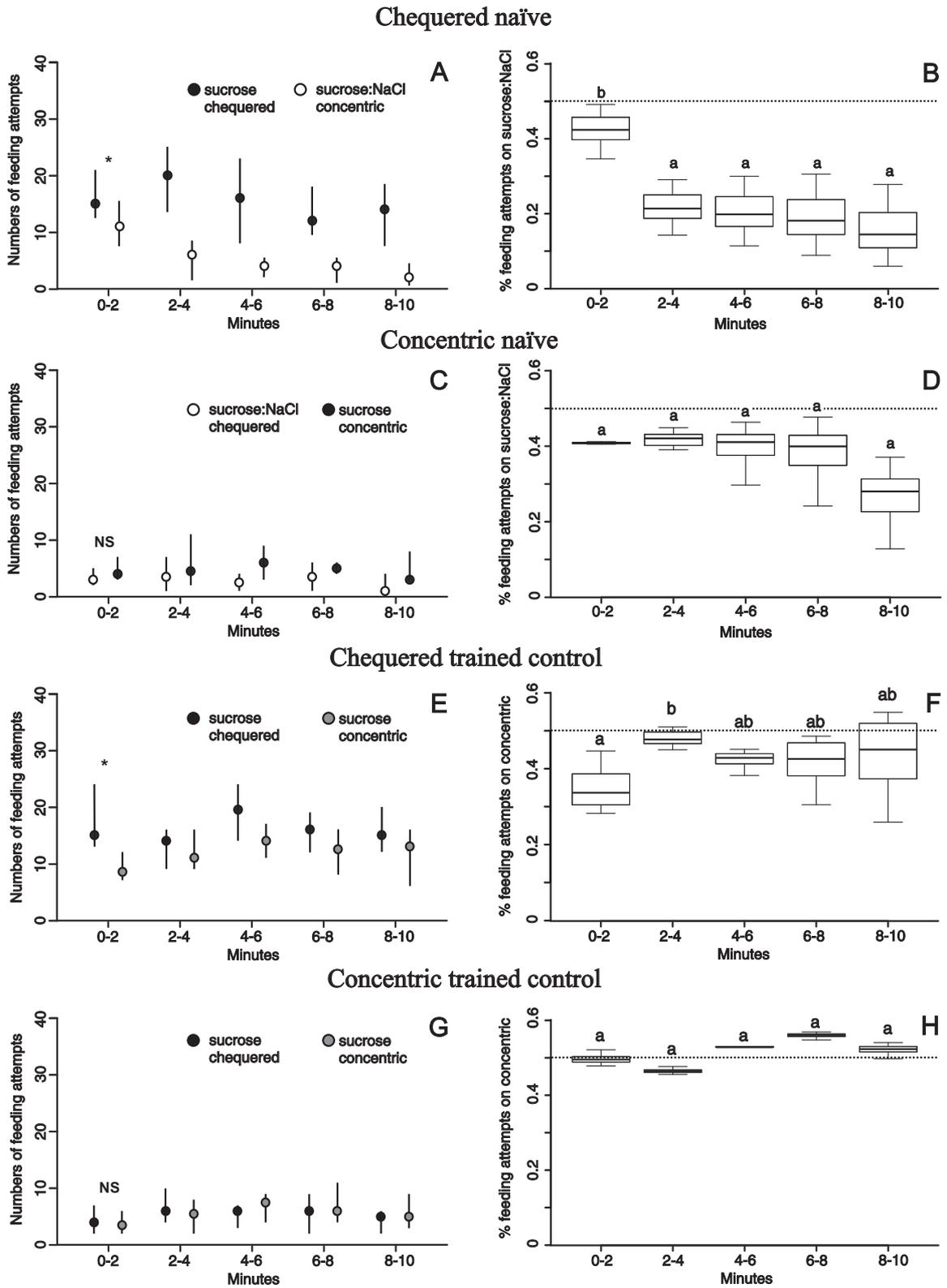
**Fig. 2.** Data plots in the left column show the number of feeding attempts on the sucrose and sucrose:NaCl feeders in (A) **chequered trained**, (C) **concentric trained** and (E) **chequered trained inverted** tests. Circles and vertical lines represent medians and quartiles. Feeding attempts on the chequered pattern are represented by the symbol on the left for each 2 min recording interval. Wilcoxon matched pair tests \* $p < 0.05$ , \*\* $p < 0.01$ . In the right column box plots are presented for the number of feeding attempts on the sucrose:NaCl feeder as a proportion of total feedings attempts (on sucrose plus sucrose:NaCl feeders) (B, D, F). The values were calculated using the log-logistic model fitted to the observed numbers of feeding attempts. The 0.5 proportion (dotted line) indicates equal numbers of feeding attempt on the two feeders and proportions lower than 0.5 indicate a stronger preference for the feeder paired with sucrose. Identical lower case letters indicate test periods during which the proportion of feeding attempts did not differ significantly. Boxes represent medians and quartiles (the range between the lowest and highest 25% of the data).

two feeders did not change over the 5 consecutive two minute recording intervals ( $P = 0.103$ ; Table 1, Fig. 2B). Analogous results were obtained in the **concentric trained** experiment where significantly higher numbers of feeding attempts were counted on sucrose paired with the concentric pattern during the first 2 min

(WMPT  $P = 0.018$ ; Table 1, Fig. 2C) and no significant change was observed over the 5 consecutive recording intervals (GLM  $P = 0.134$ ; Table 1, Fig. 2D). In the **chequered trained inverted** experiment no significant difference between the number of feeding attempts was recorded on the feeders during the first 2 min

**Table 2**  
Statistical output of the nested mixed-effect 2-way ANOVA ( $X^2$ ) comparing changes in preference by mosquitoes for the chequered and the concentric patterns associated with either sucrose or sucrose:NaCl (Experiments) over 5 consecutive 2 min periods (Periods).  $P$  values lower than 0.05 were accepted as significant and are marked in bold.

| Comparison                       | Experiments |                | Periods |                 | Interaction |               |
|----------------------------------|-------------|----------------|---------|-----------------|-------------|---------------|
|                                  | $X^2$       | $P$            | $X^2$   | $P$             | $X^2$       | $P$           |
| Chequered vs. Concentric trained | 22.3634     | <b>2.26E-6</b> | 9.8365  | <b>0.043</b>    | 5.6587      | 0.226         |
| Chequered vs. Concentric control | 6.5874      | <b>0.0103</b>  | 9.3439  | 0.053           | 2.4789      | 0.648         |
| Chequered vs. Concentric naïve   | 8.7094      | <b>3.17E-3</b> | 39.6476 | <b>5.12E-08</b> | 12.9055     | <b>0.0117</b> |



**Fig. 3.** Data plots in the left column show the numbers of feeding attempts on the sucrose and sucrose:NaCl feeders in (A) **chequered naïve**, (C) **concentric naïve**, (E) **chequered trained control** and (G) **concentric trained control** tests. Feeding attempts on the chequered pattern are represented by the symbol on the left for each 2 min recording interval. In the right column box plots are presented for the numbers of feeding attempts on the sucrose:NaCl feeder (B, D) and on the feeder with the concentric pattern (F, H) as a proportion of all feedings attempts. The values were calculated using the log-logistic model fitted to the observed numbers of feeding attempts. The 0.5 proportion (dotted line) indicates equal numbers of feeding attempt on the two feeders, and proportions lower than 0.5 indicate a stronger preference for the feeder paired with sucrose (B, D) and the chequered pattern (F, H). Identical lower case letters indicate test periods during which the proportion of feeding attempts did not differ significantly. Boxes represent medians and quartiles (the range between the lowest and highest 25% of the data).

**Table 3**

Statistical output of the analysis of deviance based on a mixed effect generalized linear model with negative binomial errors comparing the sum of feeding attempts on the chequered and the concentric patterns between pairs of corresponding **chequered** and **concentric** experiments over 5 consecutive 2 min periods (*Periods*). P values lower than 0.05 were accepted as significant and are marked in bold.

| Comparison                       | Experiments |                 | Periods  |               | Interaction |               |
|----------------------------------|-------------|-----------------|----------|---------------|-------------|---------------|
|                                  | $\chi^2$    | <i>P</i>        | $\chi^2$ | <i>P</i>      | $\chi^2$    | <i>P</i>      |
| Chequered vs. Concentric trained | 26.3510     | <b>2.85E–6</b>  | 3.9786   | 0.4089        | 17.7146     | <b>0.0014</b> |
| Chequered vs. Concentric control | 41.8867     | <b>9.67E–11</b> | 5.9007   | 0.2067        | 3.1792      | 0.5283        |
| Chequered vs. Concentric naïve   | 23.8443     | <b>1.05E–6</b>  | 15.3071  | <b>0.0041</b> | 7.0859      | 0.1314        |

**Table 4**

Statistical output of the analysis of deviance in the **chequered** and **concentric naïve** experiments based on a mixed effect generalized linear model with negative binomial errors comparing the number of feeding attempts recorded on the chequered and on the concentric patterns in the two experiments over 5 consecutive 2 min periods (*Periods*). P values lower than 0.05 were accepted as significant and are marked in bold.

| Comparison   | Experiments |          | Periods  |                | Interaction |                |
|--|-------------|----------|----------|----------------|-------------|----------------|
|  | $\chi^2$    | <i>P</i> | $\chi^2$ | <i>P</i>       | $\chi^2$    | <i>P</i>       |
| Concentric ( <i>chequered naïve</i> ) vs. Concentric ( <i>concentric naïve</i> ) | 0.1304      | 0.718    | 31.096   | <b>2.93E–6</b> | 32.47       | <b>1.53E–6</b> |
| Concentric ( <i>chequered naïve</i> ) vs. Chequered ( <i>concentric naïve</i> )  | 1.8093      | 0.1786   | 40.4955  | <b>3.42E–8</b> | 19.5994     | <b>5.99E–4</b> |

(WMPT  $P = 0.087$ ; Table 1, Fig. 2E), but this changed rapidly as the test continued. In the second 2 min. recording period and thereafter, significantly higher numbers of feeding attempts were recorded on sucrose paired with the concentric pattern (GLM  $P < 0.001$ ; Table 1, Fig. 2F).

The efficacy of the chequered and the concentric patterns as memorized cues were found to be significantly different when comparing **chequered trained** and **concentric trained** experiments. While a preference clearly formed for both patterns when paired with sucrose during training, it was weaker in case of the concentric pattern (Table 2, Fig. 2A and C). In the **chequered trained control** experiment the induced preference for the chequered pattern was detectable even in the absence of an unpalatable meal paired with the concentric pattern. This contrasts to what was recorded in the **concentric trained control** experiment (Table 2, Fig. 3E and G). The above observed differences in formed preferences by the mosquitoes were associated with a significantly lower total number of feeding attempts (medians: 3.5 vs. 8.5, respectively) over the duration of the **concentric trained** experiment in comparison to that recorded in the **chequered trained** experiment (GLM  $P < 0.001$ ; Table 3, Fig. 2A and C).

Likewise a significantly lower total number of feeding attempts (medians: 5 vs. 14, respectively) was recorded in the **concentric trained control** test in comparison to that recorded in the **chequered trained control** experiment (GLM  $P < 0.001$ ; Table 3, Fig. 3E and G). In the first 2 min of the **chequered trained control** experiment mosquitoes fed significantly more on the chequered feeder that had been paired with sucrose during conditioning (WMPT  $P = 0.033$ ; Table 1, Fig. 3E), but this preference was significantly weaker by the second 2 min recording period and thereafter (GLM  $P = 0.078$ ; Table 1, Fig. 3F). However, the proportions of feeding attempts between the patterns were always significantly different from 0.5 (GLM  $P < 0.001$ ; Fig. 3F). In the **concentric trained control** experiment no preference for the concentric pattern was detected throughout the tests despite it having been paired with sucrose during conditioning (WMPT  $P > 0.05$ ; Table 1, Fig. 3G and H), and the proportions of feeding attempts on the two patterns were not significantly different from 0.5 (GLM  $P = 0.416$ ; Fig. 3H).

In the **chequered naïve** experiment significantly higher numbers of feeding attempts were recorded on the chequered feeder paired with sucrose during the first 2 min (WMPT  $P = 0.02$ ; Table 1, Fig. 3A). This preference grew to a significantly higher level by the second 2 min test period (GLM  $P < 0.001$ ; Table 1, Fig. 3B) and was maintained thereafter. In the **concentric naïve** experiment no such

rapid change in preference was observed and although mosquitoes did make higher numbers of feeding attempts on the concentric pattern paired with sucrose, this difference was not significant (WMPT  $P = 0.110$ ; Table 1, Fig. 3C) and did not change significantly over the test periods ( $P = 0.425$ ; Table 1, Fig. 3D).

Mosquitoes in **chequered naïve** and **concentric naïve** experiments faced with a choice built a preference for the chequered pattern paired with sucrose within 2 min, but could not build an analogous preference for the concentric pattern paired with sucrose within the 10 min of the tests (Tables 1 and 2). The total number of feeding attempts (medians: 4 vs. 9, respectively) in the **concentric naïve** experiment were significantly lower than in the **chequered naïve** experiment (GLM  $P < 0.001$ ; Table 3, Fig. 3A and C). This difference was formed by the high number of feeding attempts on the chequered feeder associated with sucrose, as no significant difference in number of feeding attempts was recorded on the concentric feeder paired with sucrose:NaCl, the concentric feeder paired with sucrose or the chequered feeder paired with sucrose:NaCl (Table 4).

#### 4. Discussion

The evidence provided here for the ability of *An. gambiae* females to readily associate visual cues with a sugar source is consistent with earlier findings and demonstrate that malaria mosquitoes are able to resort to visual cues that co-occur with vital nutritional resources. This versatile visual memory process should contribute to mosquito site fidelity and survival in an unpredictable everyday environment. Chilaka et al. (2012) have recently reported that *An. gambiae* females readily learn visual and olfactory cues paired with a blood meal and retain such associations in long-term memory for purposes of discernment. We show here that *An. gambiae* females are not only able to associate visual patterns with sucrose and retain this visual memory, but that the memory function is flexible as the learned preferences quickly vanish when mosquitoes encounter a contradictory experience with a given visual pattern. The learning process was not independent from the visual cues; it was much faster when the mosquitoes met the chequered pattern combined with sucrose. It is an open question whether malaria mosquitoes have innate preference for flower-like shapes, which can be strengthened or suppressed, or whether such shapes trigger some more general learning functions.

Different dynamics in feeding attempts and learning were observed with naïve mosquitoes with no prior experience when they were expected to form a preference for the rewarding feeder.

In the **chequered naïve** experiment mosquitoes formed a significant preference for the chequered pattern paired with sucrose within the first 2 min (Fig. 3A), that persisted for the rest of the test (Fig. 3B). However, in the **concentric naïve** experiment no such preference for the concentric pattern paired with sucrose was formed over the whole test. Although a trend forming a slow learning curve is recognisable (Fig. 3D), the unequivocal preference that was recorded in the **concentric trained** experiment (Fig. 2C and D) following several hours of conditioning was not formed in the **concentric naïve** experiment. Fast learning apparently requires a high number of feeding attempts that allows mosquitoes to quickly form a preference. The chequered pattern paired with sucrose was appropriate for inducing intense feeding and thus efficient learning, while the concentric pattern paired with sucrose or chequered pattern paired with sucrose:NaCl was not. Feeding was hindered in the **concentric naïve** experiment on the chequered pattern paired with sucrose:NaCl, on the concentric pattern paired with sucrose and also in **chequered naïve** experiment on the concentric pattern paired with sucrose:NaCl, not permitting the mosquitoes to form a preference (Fig. 3A and C, Tables 3 and 4). The chequered pattern with its similarity to floral shape acted as a releaser for feeding when paired with sucrose resulting in high total numbers of feeding attempts and a fast learning response (Table 3).

In the **chequered trained control** experiment the learned preference for the chequered pattern was confirmed. It was well observable in the first 2 min. Thereafter the number of feeding attempts on the concentric pattern increased, but always represented significantly less than 50% of all feeding attempts, although there was no negative feed-back in the test situation i.e. the absence of reinforcing unpalatable food. In the **concentric trained control** experiment the number of feeding attempts was low such that effective learning in the form of a preference for the concentric pattern formed during previous conditioning was not detectable. The number of feeding attempts on the patterns was equally distributed from the 2 first minutes of the test and thereafter. This means that the former training did not prevent mosquitoes from testing and utilizing the sucrose feeder paired with the chequered pattern, but the increased feeding activity on this feeder seen in other experiments was not displayed in the **concentric trained control** experiment following conditioning with sucrose paired with the concentric pattern.

Training did not induce an irreversible change in the feeding attempts by mosquitoes as they displayed an inherent plasticity in their preference for the feeders. Even in cases where a prior association remained rewarding, as in the case of the **chequered** and **concentric trained** experiments, feeding attempts were consistently observed on the non-rewarding feeder with sucrose:NaCl (Fig. 2A–D, Table 1). However, the strong effect of conditioning was well observable in the **chequered trained** test. The effect was weak but significant in the **concentric trained** test demonstrating that after hours of training the mosquitoes formed a preference for the concentric pattern when paired with sucrose.

Learning and visual memory in mosquitoes were proven also in the **chequered trained inverted** experiment. Although the test situation was identical to that of the **concentric trained** experiment, the initial preference of the mosquitoes was dramatically altered by the previous training on sucrose paired with the chequered pattern (Fig. 2D and F, Table 1). The initial high numbers of feeding attempts on both feeders in the **chequered trained inverted** test show that despite the chequered pattern being paired with an unpalatable solution the mosquitoes displayed a disproportional high level of attention to it compared to the concentric pattern paired with sucrose. Several hours after the last feeding on the chequered pattern paired with sucrose the number of feeding attempts by mosquitoes was still high and they displayed a learning curve similar to that seen in the **chequered naïve** test

(Figs. 2F and 3B). Within 2 min mosquito interest in the chequered pattern decreased and did not change significantly thereafter, marking a rapid modification in the behavioural response through the association between visual cues and food quality. The ability for such a rapid reversal of a learned preference for visual cues associated with a resource has already been reported for the honeybee (Zhang et al., 2006) and is probably an asset for many insect species. Apparently *An. gambiae* females are inherently interested in the chequered pattern. We see it as a rule for displaying intense feeding and effective learning it is apparently necessary for mosquitoes to have had or have access to the chequered pattern paired with sucrose whereas subsequently it is sufficient to have access to sucrose associated with either pattern.

Difference in the intensity of induced feeding behaviour probably originates from the contrast enhancing system of the mosquito eye where the straight edges of the chequered pattern might be more discernible than the set of concentric circles. The poor spatial resolution of mosquito eyes needs to be considered when designing visual patterns (Kawada et al., 2006). Patterns with fine details are seen rather as homogenous patches even from a short distance. Although applying simple homogenous white markers against dark or chequered ones (Chilaka et al., 2012) provides a convenient method, it is a potential source of bias as mosquitoes tend to be attracted to darker zones in their environment (Clements, 1992). Even using patterns with identical spectral reflectance and equal net albedo for studying visual learning by mosquitoes does not guarantee that such patterns are perceived equally and are neutral for mosquitoes.

Increased feeding activity and learning in the presence of floral shapes paired with sucrose and a flexible memory function provide the advantage of efficient feeding on sugar sources on flowers that can be quickly depleted of resources. Learning effects similar to those seen in our tests may or may not occur when sugars other than sucrose are paired with the chequered and concentric patterns. However, *An. gambiae* females show a preference for sucrose over its hexose components fructose or glucose (Kessler et al., 2015). Several sources of sucrose are available for mosquitoes in their natural habitat where they generally feed on floral nectar and their preferred nectar-hosts are *peri*-domestic plants with pale or white-coloured flowers (Yuval, 1992; Foster, 1995; Manda et al., 2007a). Floral nectar is composed of complex mixtures of sucrose and monosaccharides (Manda et al., 2007b), amino-acids (Vrzal et al., 2010) and secondary plant metabolites. Sugar-feeding has a strong influence on female mosquito vectorial capacity through extending longevity (Okech et al., 2003; Impoinvil et al., 2004; Gary and Foster, 2004, 2006), thus enhancing their ability to respond to host odors (Hancock and Foster, 1997) and their biting frequency (Gary and Foster, 2001; Kessler et al., 2015). Availability of sucrose has a considerable impact on the reproductive success of both sexes. In several mosquito species, sugar-feeding increases the number of eggs laid by the female (Foster, 1995) and males, which mainly feed on nectar in their natural habitat, maximize their energy reserves for reproduction (Yuval et al., 1994; Gary et al., 2009; Sawadogo et al., 2013). We can conclude from these earlier findings that it is essential for *An. gambiae* to localize sucrose sources in its environment. The ability to improve the recognition of such ephemeral sources of nutrition through learning their associated optical cues would be a significant advantage to both sexes of malaria mosquitoes.

## Acknowledgements

We are grateful for grant no. 12.354 received by B. Bernáth in the framework of the Scientific Exchange Programme NMS-CH of the CRUS (Conference of the Rectors of Swiss Universities of Switzerland) and for grant no. 943 from the Velux Foundation

(Kirchgasse 42, 8001 Zürich, Switzerland) in support of research on vision in insect vectors of disease at the University of Neuchâtel. The authors are most grateful to Radu Slobodeanu (Institute of Mathematics, University of Neuchâtel), for his advice and aid with statistical evaluation of the data.

## References

- Alonso, W.J., Schuck-Paim, C., 2006. The “ghosts” that pester studies on learning in mosquitoes: guidelines to chase them off. *Med. Vet. Entomol.* 20, 157–165.
- Arsic, D., Guerin, P.M., 2008. Nutrient content of diet affects the signaling activity of the insulin/target of rapamycin/p70 S6 kinase pathway in the African malaria mosquito *Anopheles gambiae*. *J. Insect Physiol.* 54, 1226–1235.
- Chilaka, N., Perkins, E., Tripet, F., 2012. Visual and olfactory associative learning in the malaria vector *Anopheles gambiae* sensu stricto. *Malar. J.* 11, 27.
- Clements, A.N., 1992. The biology of mosquitoes. Development, Nutrition and Reproduction, vol. 1. Chapman & Hall, London.
- Dukas, R., 2008. Evolutionary biology of insect learning. *Annu. Rev. Entomol.* 53, 145–160.
- Foster, W.A., 1995. Mosquito sugar feeding and reproductive energetics. *Annu. Rev. Entomol.* 40, 443–474.
- Gary, R.E., Foster, W.A., 2001. Effects of available sugar on the reproductive fitness and vectorial capacity of the malaria vector *Anopheles gambiae* (Diptera: Culicidae). *J. Med. Entomol.* 38, 22–28.
- Gary, R.E., Foster, W.A., 2004. *Anopheles gambiae* feeding and survival on honeydew and extra-floral nectar of peridomestic plants. *Med. Vet. Entomol.* 18, 102–107.
- Gary, R.E., Foster, W.A., 2006. Diel timing and frequency of sugar feeding in the mosquito *Anopheles gambiae*, depending on sex, gonotrophic state and resource availability. *Med. Vet. Entomol.* 20, 308–316.
- Gary, R.E., Cannon, J.W., Foster, W.A., 2009. Effect of sugar on male *Anopheles gambiae* mating performance, as modified by temperature, space, and body size. *Parasites Vectors* 2, 19.
- Hancock, R.G., Foster, W.A., 1997. Larval and adult nutrition effects on blood/nectar choice of *Culex nigripalpus* mosquitoes. *Med. Vet. Entomol.* 11, 112–122.
- Impoinvil, D.E., Kongere, J.O., Foster, W.A., Njiru, B.N., Killeen, G.F., Githure, J.I., Beier, J.C., Hassanali, A., Knols, B.G.J., 2004. Feeding and survival of the malaria vector *Anopheles gambiae* on plants growing in Kenya. *Med. Vet. Entomol.* 18, 108–115.
- Jhumur, U.S., Dötterl, S., Jürgens, A., 2006. Naive and conditioned responses of *Culex pipiens pipiens* biotype molestus (Diptera: Culicidae) to flower odors. *J. Med. Entomol.* 43, 1164–1170.
- Kaur, J.S., Lai, Y.L., Giger, A.D., 2003. Learning and memory in the mosquito *Aedes aegypti* shown by conditioning against oviposition deterrence. *Med. Vet. Entomol.* 17, 457–460.
- Kawada, H., Tatsuta, H., Arikawa, K., Takagi, M., 2006. Comparative study on the relationship between photoperiodic host-seeking behavioral patterns and the eye parameters of mosquitoes. *J. Insect Physiol.* 52, 67–75.
- Kessler, S., Vlimant, M., Guerin, P.M., 2015. Sugar-sensitive neurone responses and sugar feeding preferences influence lifespan and biting behaviours of the Afrotropical malaria mosquito, *Anopheles gambiae*. *J. Comp. Physiol. A* 201, 317–329.
- Lutz, E.K., Riffell, J., 2013. Visual and Olfactory Learning in *Anopheles stephensi* and *Aedes aegypti* mosquitoes.
- Manda, H., Gouagna, C., Nyandat, E., Kabiru, E.W., Jackson, R.R., Foster, W.A., Githure, J.I., Beier, J.C., Hassanali, A., 2007a. Discriminative feeding behaviour of *Anopheles gambiae* s.s. on endemic plants in western Kenya. *Med. Vet. Entomol.* 21, 103–111.
- Manda, H., Gouagna, L.C., Foster, W.A., Jackson, R.R., Beier, J.C., Githure, J.I., Hassanali, A., 2007b. Effect of discriminative plant-sugar feeding on the survival and fecundity of *Anopheles gambiae*. *Malar. J.* 6, 113.
- McCall, P.J., Eaton, G., 2001. Olfactory memory in the mosquito *Culex quinquefasciatus*. *Med. Vet. Entomol.* 15, 197–203.
- McCall, P.J., Kelly, D.W., 2002. Learning and memory in disease vectors. *Trends Parasitology* 18, 429–433.
- McCall, P.J., Mosha, F.W., Njunwa, K.J., Sherlock, K., 2001. Evidence for memorized site-fidelity in *Anopheles arabiensis*. *Trans. R. Soc. Trop. Med. Hyg.* 95, 587–590.
- Menda, G., Uhr, J.H., Wyttenbach, R.A., Vermeylen, F.M., Smith, D.M., Harrington, L.C., Hoy, R.R., 2013. Associative learning in the dengue vector mosquito, *Aedes aegypti*: avoidance of a previously attractive odor or surface color that is paired with an aversive stimulus. *J. Exp. Biol.* 216, 218–223.
- Muir, L.E., Thorne, M.J., Kay, B.H., 1992. *Aedes aegypti* (Diptera: Culicidae) vision: spectral sensitivity and other perceptual parameters of the female eye. *J. Med. Entomol.* 29, 278–281.
- Okech, B.A., Gouagna, L.C., Killeen, G.F., Knols, B.G.J., Kabiru, E.W., Beier, J.C., Yan, G., Githure, J.I., 2003. Influence of sugar availability and indoor microclimate on survival of *Anopheles gambiae* (Diptera: Culicidae) under semifield conditions in western Kenya. *J. Med. Entomol.* 40, 657–663.
- R Core Team, 2015. <<https://www.R-project.org>>.
- Sanford, M.R., Tomberlin, J.K., 2011. Conditioning individual mosquitoes to an odor: sex, source, and time. *PLoS ONE* 6, e24218.
- Sanford, M.R., Olson, J.K., Lewis, W.J., Tomberlin, J.K., 2012. The effect of sucrose concentration on olfactory-based associative learning in *Culex quinquefasciatus* Say (Diptera: Culicidae). *J. Insect Behav.* 26, 494–513.
- Sawadogo, S.P., Diabaté, A., Toé, H.K., Sanon, A., Lefèvre, T., Baldet, T., Gilles, J., Simard, F., Gibson, G., Sinkins, S., et al., 2013. Effects of age and size on *Anopheles gambiae* s.s. male mosquito mating success. *J. Med. Entomol.* 50, 285–293.
- Tomberlin, J.K., Rains, G.C., Allan, S.A., Sanford, M.R., Lewis, W.J., 2006. Associative learning of odor with food- or blood-meal by *Culex quinquefasciatus* Say (Diptera: Culicidae). *Naturwissenschaften* 93, 551–556.
- Vantaux, A., Dabire, R., Lefèvre, T., Cohuet, A., 2013. Associative learning in blood feeding behaviour of *Anopheles gambiae* mosquitoes. *Pathog. Global Health* 107, 447.
- Vantaux, A., Lefèvre, T., Dabiré, K.R., Cohuet, A., 2014. Individual experience affects host choice in malaria vector mosquitoes. *Parasites Vectors* 7, 249.
- Vinauger, C., Lutz, E.K., Riffell, J.A., 2014. Olfactory learning and memory in the disease vector mosquito *Aedes aegypti*. *J. Exp. Biol.* 217, 2321–2330.
- Vrzal, E.M., Allan, S.A., Hahn, D.A., 2010. Amino acids in nectar enhance longevity of female *Culex quinquefasciatus* mosquitoes. *J. Insect Physiol.* 56, 1659–1664.
- Yuval, B., 1992. The other habit: sugar feeding by mosquitoes. *Bull. Soc. Vector Ecologists* 17, 150–156.
- Yuval, B., Holliday-Hanson, M.L., Washing, R.K., 1994. Energy budget of swarming male mosquitoes. *Ecol. Entomol.* 19, 74–78.
- Zhang, S., Schwarz, S., Pahl, M., Zhu, H., Tautz, J., 2006. Honeybee memory: a honeybee knows what to do and when. *J. Exp. Biol.* 209, 4420–4428.