International Journal of Tropical Insect Science Vol. 24, No. 3, pp. 260–265, 2004 DOI: 10.1079/IJT200433

SHORT COMMUNICATION

Mediation of oviposition site selection in the African malaria mosquito *Anopheles gambiae* (Diptera: Culicidae) by semiochemicals of microbial origin

Leunita A. Sumba[1,2], Tom O. Guda[1], Arop L. Deng[2], Ahmed Hassanali[1], John C. Beier[3] and Bart G.J. Knols[4,*]

[1]International Centre of Insect Physiology and Ecology (ICIPE), Mbita Point Research and Training Centre, PO Box 30, Mbita Point, Kenya: [2]Department of Zoology, Egerton University, PO Box 536, Njoro, Kenya: [3]Department of Epidemiology and Public Health, Highland Professional Building, University of Miami School of Medicine, 1801 NW 9th Ave., Suite 300 (D-93), Miami, FL 33136, USA: [4]Laboratory of Entomology, Wageningen University and Research Centre, PO Box 8031, 6700 EH, Wageningen, The Netherlands

(Accepted 8 April 2004)

Abstract. Laboratory studies were carried out to investigate the role of larval habitat-derived microorganisms in the production of semiochemicals for oviposition site selection by *Anopheles gambiae* Giles *sensu stricto* mosquitoes. Dual-choice bioassays with gravid females were conducted in standard mosquito cages. Field-collected or laboratory-reared mosquitoes, individually or in groups, were offered a choice between unmodified (water or soil from a natural breeding site) or modified substrates (filtered water, autoclaved soil or sterile media to which bacterial suspensions had been added). Egg counts were used to assess oviposition preferences.Mosquitoes preferred to oviposit on unmodified substrates from natural larval habitats containing live microorganisms rather than on sterilized ones. Variable responses were observed when sterile substrates were inoculated with bacteria isolated from water and soil from natural habitats.We conclude that microbial populations in breeding sites produce volatiles that serve as semiochemicals for gravid *An. gambiae*. These signals, in conjunction with other (non-olfactory) chemical and physical cues, may be used by the female to assess the suitability of potential larval habitats in order to maximize the fitness of her offspring.

Key words: oviposition site selection, soil microbiota, semiochemicals, *Anopheles gambiae*

Résumé. Des études de laboratoire ont été conduites afin de déterminer le rôle de substances chimiques attractives de microorganismes présents dans les sites larvaires dans la préférence de ponte des femelles gravides d'*Anopheles gambiae* s.s. pour ces sites. Un test biologique à double choix a été mis en place dans des cages à moustiques standards contenant des femelles gravides. Un choix entre substrat naturel (eau et sol non modifiés provenant des sites larvaires naturels) et substrat modifié (eau filtrée, sol stérilisé, ajout de suspensions bactériennes) est offert individuellement ou en groupe aux femelles

*Current address: Entomology Unit, FAO/IAEA, Agency's laboratories Seibersdorf, A-1400, Vienna, Austria.
Email: B.Knols@iaea.org

collectées sur le terrain ou élevées en laboratoire. Le nombre d'oeufs pondus dans chaque substrat a été utilisé comme critère pour évaluer la préférence des femelles gravides pour un substrat particulier. Les femelles ont préféré pondre dans le substrat naturel contenant des microorganismes vivants plutôt que dans le substrat stérile. Des réponses variables ont été observées lorsque des bactéries provenant des sites larvaires naturels ont été inoculées dans le substrat stérile. Nous concluons que la population microbienne, présente dans les site larvaires, produit des substances chimiques odorantes attirant les femelles gravides d'*Anopheles gambiae* s.s. Ces signaux, associés à d'autres substances non odorantes et à des facteurs physiques, peuvent être utilisés par les femelles pour évaluer le potentiel d'un site larvaire, en vue du développement optimal de leur progéniture.

Mots clés: choix d'un site de ponte, microfaune du sol, substances chimiques attractives, *Anopheles gambiae*

Introduction

Potential breeding sites differ in a range of characteristics, both biotic and abiotic (Minakawa *et al.*, 1999; Gimnig *et al.*, 2001) which, either singly or additively (Beehler *et al.*, 1993) may influence the oviposition behaviour of gravid mosquitoes. Chemical signals associated with these sites can be important mediating factors. For example, gravid *Culex quinquefasciatus* Say (Diptera: Culicidae) are attracted to 3-methylindole, a component of Bermuda grass infusion (Millar *et al.*, 1992). *Aedes aegypti* L. (Diptera: Culicidae) is attracted to hexanoic acid (Knight and Corbet, 1991), an odorous chemical found in decomposing barley straw (Everall and Lees, 1997). *Anopheles gambiae* females prefer to oviposit on turbid rather than clear water (McCrae, 1984).

Although few studies have targeted *An. gambiae*, Blackwell and Johnson (2000) recently observed significant electroantennogram (EAG) responses of *An. gambiae* towards volatile components of water samples from Tanzanian breeding sites. The origin of stimulants in mosquito breeding sites and their mode of action towards gravid females are not fully understood, though it has been suggestively linked to microbial activity (Ikeshoji *et al.*, 1975; Kramer and Mulla, 1979; Benzon and Apperson, 1988; Takken and Knols, 1999; Gimnig *et al.*, 2001).

As an effort towards the identification of larval habitat-derived semiochemicals for anophelines, the study reported here was initiated to demonstrate the effect of breeding site microbiota on oviposition choices of *An. gambiae* mosquitoes. Recent studies with *Ae. aegypti* and *Ae. albopictus* showed an oviposition preference for sites with microbial activity (Trexler *et al.*, 2003) and absence thereof when an antibiotic (tetracycline) was added (Navarro *et al.*, 2003). In line with these findings we hypothesized that (i) a gravid female *An. gambiae* uses volatiles of microbial origin to assess nutrient availability and durability of habitats, both of which are vital determinants for the survival of her offspring and hence, her own fitness and (ii) the absence of such volatiles (in the case of no microbial activity) should lead to a reduction in egg laying or diversion of the female to a different oviposition site ('skip oviposition').

Materials and methods

Mosquitoes

Laboratory-reared mosquitoes were selected from previously established colonies of *An. gambiae* s.s. (Ifakara strain), originally from Njage, south-east Tanzania (colonized since April 1996) and *An. gambiae* s.s. (Mbita strain) from Mbita, Kenya (colonized since February 2001). Adult mosquitoes were kept in standard 30 × 30 × 30 cm rearing cages at ICIPE's Mbita Point Research and Training Centre mosquito insectary at 27 ± 2°C, 65–70% RH and photoperiod of 12:12 (L:D), and offered a 6% glucose solution *ad libitum*. Three- to five-day-old females, kept together with males since emergence, were allowed to feed on a human arm for 10 min on three consecutive nights. Multiple bloodmeals were offered as this has been shown to increase the chance of oviposition by females that are to lay their first batch of eggs (Briegel and Hörler, 1993). Gravid females were then used in the oviposition assays on the second evening after their last blood meal.

Wild, indoor-resting, half-gravid *An. gambiae* s.l. were collected with an aspirator during the early morning from houses in Lwanda village, Suba district, western Kenya. They were immediately transported to the laboratory, provided with 6% glucose solution and used in the experiments the following evening. Previous work has shown this population to consist of *An. arabiensis* Patton at 60–75% of mosquitoes sampled as larvae or adults during various parts of the year (Minakawa *et al.*, 1999).

Cultivation of bacteria

One gram of fresh soil collected from cattle hoof prints found at the edges of a muddy anopheline

larval habitat in Lwanda was added to 10 ml of autoclaved distilled water. The mixture was agitated and 0.1 ml of the soil suspension transferred to standard Trypticase® Soy Agar (TSA) with a sterile 1 ml pipette and spread with a sterile loop. The plates were incubated at 35°C and examined after 24, 36 and 48 h for the presence of colony-forming units (CFUs), which were subsequently subcultured to obtain pure cultures and identified using a rapid biochemical bioassay after Straif *et al.* (1998).

Oviposition substrates

Fresh soil samples from known breeding sites were dried, autoclaved twice for 15 min at 121°C and 1.4 kg/cm^2 pressure and allowed to cool. Water from the larval habitat was sieved to remove debris and then filtered using sterile 0.22 μm filters. To confirm sterility, 0.1 ml of the soil suspension from autoclaved soil and sterile filtered water were applied on TSA plates and incubated at 35°C for 24 h.

Bacteria from fresh soil samples were cultured as described above. After 48 h of incubation at 35°C, autoclaved distilled water was added to six agar plates with bacterial growth and the resulting bacterial suspension transferred to a volumetric flask. The volume was increased to 500 ml using autoclaved distilled water and incubated for 24 h at 35°C (concentrated suspension). An aliquot (250 ml) from the concentrated suspension was further diluted up to a volume of 500 ml with sterile distilled water (diluted suspension). Since the experiments were done on separate days, this procedure was repeated on each experimental day with fresh soil samples from the same larval habitat.

Assay procedures

All dual-choice oviposition bioassays were carried out in the Mbita mosquito insectary in cages measuring 30 × 30 × 30 cm made of white mosquito netting covering a metal frame. Each cage held two oviposition substrates, separated by a distance of 30 cm.

In experiments with autoclaved soil or filtered water the oviposition substrates consisted of two plastic cups ('double-cup' setup; Fig. 1), a larger cup of 8 cm depth, 6 cm diameter and a smaller one of 2 cm depth, 4 cm diameter. The larger cup contained either 150 g of autoclaved or fresh soil or 100 ml of sterile filtered or non-filtered larval site water or autoclaved soil or sterile filtered water inoculated with 15 ml of cultured bacteria suspension. The soils were moistened with 100 ml of autoclaved distilled water previously obtained from Lake Victoria. The smaller cup contained 15 ml of autoclaved distilled water lined with white filter

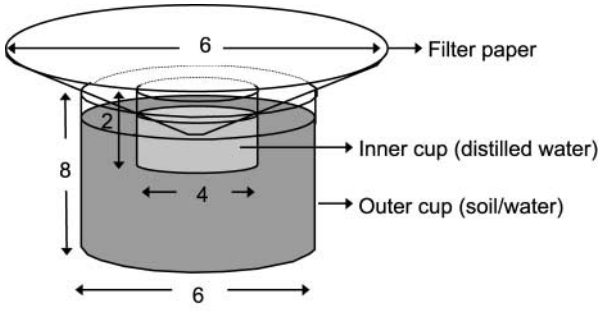

Fig. 1. The 'double-cup' setup used to prevent mosquitoes touching test substrates prior to or during oviposition. Dimensions are in centimetres.

paper (either Whatman® no. 1 or S&S 595) and placed in the larger cup. The filter paper was used so that the mosquitoes did not come into direct (tarsal) contact with the test substrate. The design of these oviposition substrates excluded tactile perception and enabled us to test olfactory responses to the volatiles emanating from the substrates.

On every experimental day at 1700 h, gravid mosquitoes were released into cages, either individually or in groups of 10. Oviposition substrates were introduced at about 1730 h, removed the following morning at 0730 h and the number of eggs oviposited on the filter papers counted under a dissection microscope. Fresh gravid mosquitoes and substrates were used for each experimental day.

Data analysis

SPSS 10.0 for Windows® was used to conduct Wilcoxon signed rank tests for paired samples in order to determine the differences in the number of eggs laid on sterile and non-sterile substrates.

Results

Assays with mosquitoes

Modification of soil or water from a natural anopheline larval habitat, either by autoclaving the soil or filtering the water, affected the choice of substrates by individual and groups of gravid mosquitoes (Table 1). Laboratory females (Mbita strain) laid on average 2.6 (untreated water, $P = 0.01$) and 3.9 times (fresh soil, $P = 0.03$) as many eggs on untreated substrates containing live microorganisms than on sterile ones. Similar findings but with even higher contrasts between the substrates were observed for wild individual females which laid on average 4.6 (fresh soil, $P = 0.03$) to 10.2 (untreated water, $P = 0.02$) times as many eggs on the non-sterile substrates. The number of eggs laid on the autoclaved soil substrate

Table 1. Mean number of eggs oviposited by groups ($n = 10$) of laboratory-reared (Ifakara strain) *Anopheles gambiae* s.s. and wild *Anopheles gambiae* s.l. females or by individual laboratory-reared (Mbita strain) *Anopheles gambiae* s.s. and wild *Anopheles gambiae* s.l. females that were offered a choice between sterile and non-sterile substrates

		Individual mosquito			Groups of mosquitoes		
Mosquito	Substrate	Mean ± SE[1]	N[2]	P[3]	Mean ± SE[1]	N[2]	P[3]
Laboratory	Fresh soil	60.6 ± 10.1			91.3 ± 12.8		
	Autoclaved soil	15.5 ± 8.6	12	0.03	25.7 ± 7.6	30	<0.001
	Non-filtered water	47.5 ± 7.4			78.5 ± 16.1		
	Filtered water	18.4 ± 5.1	24	0.01	17.1 ± 5.4	10	0.03
	Autoclaved soil	39.5 ± 7.3			85.2 ± 16.6		
	Distilled water	22.6 ± 5.5	33	0.18	98.9 ± 16.1	32	0.59
Wild	Fresh soil	84.8 ± 15.0			182.2 ± 25.6		
	Autoclaved soil	18.3 ± 12.5	16	0.03	45.9 ± 14.9	13	0.002
	Non-filtered water	65.5 ± 17.2			147.2 ± 34.9		
	Filtered water	6.4 ± 6.4	10	0.02	5.6 ± 3.1	11	0.03

[1] Standard Error.
[2] Number of paired replicates.
[3] Values represent results of Wilcoxon signed rank test for paired replicates.

did not differ significantly from that on the distilled water ($P > 0.1$).

When groups of 10 females were offered the same choice of substrates, the average number of eggs laid per female dropped sharply compared to batches laid by individual females. Nevertheless, a clear preference for non-sterile substrates occurred and was, as with individual mosquitoes, most pronounced for wild females offered a choice between filtered and non-filtered larval site water.

Microbial growth and identification

Confluent growth was observed on standard TSA plates inoculated with non-sterile suspensions after incubation for 24 h. No growth was observed on plates inoculated with sterile substrates. Both gram-negative and gram-positive bacteria were present in the untreated soil suspension and breeding site waters. Gram-negative bacteria that were oxidase positive were identified as being *Aeromonas*, *Pasturella*, *Pseudomonas* or *Vibrio* species. Oxidase-negative species were identified as either *Acetinobacter* or Enterobacteriaceae species. One colony from breeding site water tested positive on MacConkey and was identified as an Enterobacteriaceae species. All other colonies were non-lactose fermenters. As expected, more bacterial growth was observed on agar plates with concentrated bacterial suspensions than those with the dilute ones.

Assays with cultured bacteria

The attractiveness of sterile soil was restored after inoculation with the concentrated ($P = 0.03$) but not with the diluted ($P = 0.82$) bacterial suspensions. No apparent increase in attractiveness ($P > 0.05$) was observed in the other experiments in which suspensions were added to sterile distilled water (Table 2).

Table 2. Mean number of eggs laid by individual laboratory-reared *Anopheles gambiae* s.s. (Mbita strain) females offered sterile substrates to which bacterial suspensions were added

Substrates	Mean ± SE[1]	N[2]	P[3]
Autoclaved soil + concentrated bacterial suspension	47.2 ± 6.3		
Autoclaved soil	23.6 ± 4.8	29	0.03
Autoclaved soil + diluted bacterial suspension	40.3 ± 10.2		
Autoclaved soil	35.8 ± 10.2	21	0.82
Filtered distilled water + Concentrated bacterial suspension	24.3 ± 8.4		
Filtered distilled water	37.4 ± 12.4	13	0.52
Filtered distilled water + diluted bacterial suspension	49.6 ± 15.1		
Filtered distilled water	17.6 ± 7.1	8	0.16

[1] Standard error.
[2] Number of paired replicates.
[3] Values represent results of Wilcoxon signed rank test for paired replicates.

Discussion

Our results show that the presence of live microorganisms in the soil or water of a natural *An. gambiae* larval habitat affects choices of oviposition substrates by individual or groups of mosquitoes in the laboratory. The double-cup experimental setup allows us to attribute this phenomenon to olfaction.

Bacteria in larval habitats (Walker and Merritt, 1993; Smith *et al.*, 1998; Navarro *et al.*, 2003) may serve as a direct source of food for the larvae (Merritt *et al.*, 1992) or as modifiers of organic matter in breeding waters, which may give rise to constituents ingested by larvae as well as volatile organic compounds of the breeding site waters (Gjullin *et al.*, 1965; Blackwell and Johnson, 2000; Rejmankova *et al.*, 2000). Certain bacterial volatiles have been shown to attract *Cx. fatigans* Coquillet (Ikeshoji *et al.*, 1975), *Ae. aegypti* L. (Hazard *et al.*, 1967) and *Cx. quinquefasciatus* Say (Millar *et al.*, 1992; Poonam *et al.*, 2002). In certain cases, bacterial metabolites (Ikeshoji *et al.*, 1975) were thought to be precursors in the synthesis of the volatile attractants. Our preliminary microbial survey of the larval habitat revealed the presence of microbiota that may mediate the choice of oviposition substrate, but the precise sequence of behavioural events and relative importance of the various cues involved in oviposition site selection by *An. gambiae* needs further investigation. The preference of mosquitoes for unmodified substrates, and the partial restoration of attractiveness of sterile substrates after inoculation with bacterial suspensions indicates that soil microorganisms play an important role in oviposition site-selection. The variable results with bacterial suspensions may be attributed to the fact that female behaviour is influenced by both the substrate type and the concentration of volatiles emanating from the bacterial suspensions. Experiments incorporating bacterial counts and a wide range of concentrations of individual and complex mixtures of bacterial species are therefore recommended.

The preferred breeding sites of *An. gambiae* have been described as transient, sunlit and generally small water bodies (Service, 1993). The durability of such temporary habitats is critical and should, under tropical temperature conditions, be at least 6 days for eggs to develop to the pupal stage and emerge into adult mosquitoes (Minakawa *et al.*, 2001). Gravid females may therefore select sites with a well-developed microbial population that may signal adequate permanence of the habitat to enable completion of the aquatic part of the life cycle.

Much of the behavioural ecology of oviposition by malaria vectors remains unknown, yet elucidation of the principle components affecting site-selection may provide important information for environmental management or larviciding programmes. Our results show that microbial activity is one of these components and identification of key species and the semiochemicals they produce should therefore be a priority. Identification of oviposition stimuli for *An. gambiae* will be useful for the development of odour-baited ovitraps for monitoring anopheline populations, similar to those developed for *Ae. aegypti* and *Cx. quinquefasciatus* (Reiter, 1983; Reiter *et al.*, 1991). Headspace analyses of larval habitats, currently ongoing in Kenya, will further unravel the chemical ecology of oviposition by this important malaria vector.

We conclude that microbial populations in breeding sites produce volatiles that serve as semiochemicals for gravid *An. gambiae*. Such cues may be used by the females, in conjunction with other factors reported before (vegetation cover, turbidity, presence of conspecifics etc., see Gimnig *et al.*, 2001) to assess the suitability and possibly, durability of potential larval habitats in order to maximize the fitness of their offspring.

Acknowledgements

We thank Daniel Impoinvil, Kenneth Okoth, Elisha Obudho, James Kongere and the staff of the malaria vector programme at ICIPE-Mbita for their support. We are grateful to Hortance Manda and Hervé Bossin for translating the abstract. This research was supported by funds from the National Institutes of Health (NIH) grant U19 AI45511, the ABC Fogarty, grant number D43TWØ1142, and the Dutch Scientific Organisation, grant VIDI 864.03.004. LS wishes to acknowledge the PhD scholarship from the German Academic Exchange Service (DAAD) through the African Regional Postgraduate Programme in Insect Science (ARPPIS).

References

Beehler J. W., Millar J. G. and Mulla M. S. (1993) Synergism between chemical factors and visual cues influencing oviposition of the mosquito *Culex quinquefasciatus* (Diptera: Culcidae). *J. Chem. Ecol.* 19, 635–643.

Benzon G. L. and Apperson C. S. (1988) Reexamination of chemically mediated oviposition behavior in *Aedes aegypti* (L.) (Diptera: Culicidae). *J. Med. Entomol.* 25, 158–164.

Blackwell A. and Johnson S. N. (2000) Electrophysiological investigation of larval water and potential oviposition chemo-attractants for *Anopheles gambiae s.s.*. *Ann. Trop. Med. Parasitol.* 94, 389–398.

Briegel H. and Hörler E. (1993) Multiple blood meals as a reproductive strategy in *Anopheles* (Diptera: Culicidae). *J. Med. Entomol.* 30, 975–985.

Everall N. C. and Lees D. R. (1997) The identification and significance of chemicals released from decomposing barley straw during reservoir algal control. *Water Res. J.* 31, 614–620.

Gimnig J. E., Ombok M., Kamau L. and Hawley W. A. (2001) Characteristics of larval anopheline (Diptera: Culicidae) habitats in Western Kenya. *J. Med. Entomol.* 38, 282–288.

Gjullin G. M., Johnsen J. O. and Plapp F. W. J. (1965) The effect of odors released by various waters on the oviposition sites selected by two species of *Culex*. *Mosq. News* 25, 268–269.

Hazard E. I., Mayer M. S. and Savage K. E. (1967) Attraction and oviposition stimulation of gravid female mosquitoes by bacteria isolated from hay infusions. *Mosq. News* 27, 133–136.

Ikeshoji T., Saito K. and Yano A. (1975) Bacterial production of the ovipositional attractants for mosquitoes on fatty acid substrates. *J. Appl. Entomol. Zool.* 10, 239–242.

Knight J. C. and Corbet S. A. (1991) Compounds affecting mosquito oviposition: structure–activity relationships and concentration effects. *J. Am. Mosq. Cont. Assoc.* 7, 37–41.

Kramer W. L. and Mulla M. S. (1979) Oviposition attractants and repellents of mosquitoes: oviposition responses of *Culex* mosquitoes to organic infusions. *Environ. Entomol.* 8, 1111–1117.

McCrae A. W. (1984) Oviposition by African malaria vector mosquitoes. II. Effects of site tone, water type and conspecific immatures on target selection by freshwater *Anopheles gambiae* Giles, sensu lato. *Ann. Trop. Med. Parasitol.* 78, 307–318.

Merritt R. W., Dadd R. H. and Walker E. D. (1992) Feeding behavior, natural food, and nutritional relationships of larval mosquitoes. *Annu. Rev. Entomol.* 37, 349–376.

Millar J. G., Chaney J. D. and Mulla M. S. (1992) Identification of oviposition attractants for *Culex quinquefasciatus* from fermented Bermuda grass infusions. *J. Am. Mosq. Cont. Assoc.* 8, 11–17.

Minakawa N., Mutero C. M., Githure J. I., Beier J. C. and Yan G. (1999) Spatial distribution and habitat characterization of anopheline mosquito larvae in Western Kenya. *Am. J. Trop. Med. Hyg.* 61, 1010–1016.

Minakawa N., Githure J. I., Beier J. C. and Yan G. (2001) Survival strategies of anopheline mosquitoes during dry period in Western Kenya. *J. Med. Entomol.* 38, 388–392.

Navarro D. M. A. F., De Oliveria P. E. S., Potting R. P. J., Brito A. C. and Fital S. J. F. (2003) The potential attractant or repellent effects of different water types on oviposition in *Aedes aegypti* L. (Diptera: Culicidae). *J. Appl. Entomol.* 127, 46–50.

Poonam S., Paily K. P. and Balaraman K. (2002) Oviposition attractancy of bacterial culture filtrates-response of *Culex quinquefasciatus*. *Mem. Inst. Oswaldo Cruz* 97, 359–362.

Reiter P. (1983) A portable battery-powered trap for collecting gravid *Culex* mosquitoes. *Mosq. News* 43, 496–498.

Reiter P., Amador M. A. and Colon N. (1991) Enhancement of the CDC ovitrap with hay infusions for daily monitoring of *Aedes aegypti* populations. *J. Am. Mosq. Cont. Assoc.* 7, 52–55.

Rejmankova E., Harbin-Ireland and Lege M. (2000) Bacterial abundance in larval habitats of four species of *Anopheles* (Diptera: Culicidae) in Belize, Central America. *J. Vect. Ecol.* 25, 229–239.

Service M. (1993) *Mosquito Ecology: Field Sampling Methods*. Chapman and Hall, London. 988 pp.

Smith T. W., Walker E. D. and Kaufman M. G. (1998) Bacterial density and survey of cultivable heterotrophs in the surface water of a freshwater marsh habitat of *Anopheles quadrimaculatus* larvae (Diptera: Culicidae). *J. Am. Mosq. Cont. Assoc.* 14, 72–77.

Straif S. C., Mbogo C. N., Toure A. M., Walker E. D., Kaufman M., Touré Y. T. and Beier J. C. (1998) Midgut bacteria in *Anopheles gambiae* and *An. funestus* (Diptera: Culicidae) from Kenya and Mali. *J. Med. Entomol.* 35, 222–226.

Takken W. and Knols B. G. J. (1999) Odor-mediated behavior of Afrotropical malaria mosquitoes. *Annu. Rev. Entomol.* 44, 131–157.

Trexler J. D., Apperson C. S., Zurek L., Gemeno C., Schal C., Kaufman M., Walker E., Watson D. W. and Wallace L. (2003) Role of bacteria in mediating the oviposition responses of *Aedes albopictus* (Diptera: Culicidae). *J. Med. Entomol.* 40, 841–846.

Walker E. D. and Merritt R. W. (1993) Bacterial enrichment in the surface microlayer of an *Anopheles quadriannulatus* (Diptera: Culicidae) larval habitat. *J. Med. Entomol.* 30, 1050–1052.