EFFECTS OF CHEMICAL STIMULI ON OVIPOSITION OF *CULEX QUINQUEFASCIATUS* (DIPTERA: CULICIDAE) IN TANZANIA

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Summary
Gravid females of the tropical house mosquito *Culex quinquefasciatus* are attracted to waterborne stimuli, products from microbial activity, and to an oviposition pheromone present in the apical droplets of the eggs. Here we report the effects of both stimuli separately and combined in a field study in Tanzania. Stimuli tested were grass infusions, 3-methylindole (skatole) and the synthetic pheromone (*5R,6S*)-6-acetoxy-5-hexadecanolide. Inside a latrine building, adjacent to a natural breeding site of *Cx quinquefasciatus*, a grass infusion, skatole, pheromone and a combination of grass infusion and pheromone or skatole and pheromone, attracted gravid females and elicited oviposition as shown by the production of egg rafts. The combination of the grass infusion and the pheromone caused a significantly enhanced oviposition response compared to the sum of the response to each stimulus separately. This was not the case with the combination of skatole and pheromone. Gravid females were also significantly attracted to grass infusions, the pheromone or their combination. Females were attracted to the chemical stimuli both in the vicinity of a breeding site and away from a breeding site. The proportion of gravid females collected in an odour-baited trap was smaller near the breeding site (63.8-65.8%) than away from it (93.7-97.7%).

INTRODUCTION

The deposition of eggs by mosquitoes is mediated by olfactory cues, which originate from the breeding site as a result of microbial activity (Bentley and Day, 1989; Takken and Knols, 1999). A number of chemical compounds that affect this behaviour have been identified (Millar *et al.*, 1992; Blackwell *et al.*, 1993), and the most active compound is 3-methylindole (skatole). In *Culex quinquefasciatus* Say this behaviour is, in addition, affected by pheromones, which are present in the apical droplet of the eggs. The active pheromone was identified as (*5R,6S*)-6-acetoxy-5-hexadecanolide, one of 4 isomers isolated from the apical droplet (Laurence and Pickett, 1985). (*5R,6S*)-6-acetoxy-5-hexadecanolide has been manufactured synthetically and proven as attractive as the natural product. When the water-borne attractants and the pheromone are combined, so far only an additive effect of the chemicals has been observed (Blackwell *et al.*, 1993; Millar *et al.*, 1994), and the role of each stimulus separately in the oviposition process is not well understood.

We have investigated the effects of grass infusions, skatole and the synthetic oviposition pheromone separately and in combination under field conditions in Tanzania (Mboera *et al.*, 1999; 2000a; 2000b). Here, we summarize the results of these studies and compare the influence of the presence of natural larval habitats on the attractive action of the natural and synthetic oviposition stimuli.

MATERIAL AND METHODS

Study site – The study was conducted in Muheza village, approx. 30 km inland from the coastal town of Tanga, NE Tanzania. The site lies at an altitude of 250 m at the foothills of the eastern Usambara mountains. The climate is tropical, with a short rainy season in November, and longer rains from March to June. The inhabitants of Muheza live in brick houses with corrugated metal roofs. Sanitary facilities consist mostly of an outdoor tap for drinking water and a pit-latrine situated behind the house at a distance of approx. 25 m from the back door. 3 wooden walls, one door and a corrugated iron roof surround pit-latrines.

Mosquitoes – *Cx quinquefasciatus*, the target mosquito species in this study, was abundant in the village, using pit latrines and outdoor soakage pits as breeding sites. Other mosquito species present were *Anopheles gambiae* Giles *sensu lato*, *An. funestus* Patton, *Mansonia uniformis*, *Cx cinereus* Theobald and *Cx tigripes* Grandpré and de Charmoy. For the purpose of this study, only *Cx quinquefasciatus* will be considered.

Chemical stimuli – chemical stimuli used consisted of 3-methylindole (skatole) and (*5R,6S*)-6-acetoxy-5-hexadecanolide and volatiles emanating from a grass infusion. Grass infusions were prepared by soaking 2 kg of finely cut pieces of *Digitaria* spp. in 10 L of unchlorinated tap water in a plastic bucket and allowing fermentation for 5 days. The infusion was then filtered through fine netting and frozen until needed. One gram of skatole (Sigma Chemical Co., St. Louis, USA) was dissolved in 100 ml of 96% ethanol. After stepwise dilution in ethanol, 0.1 ml of a skatole-solution containing 10^{-5} μg of skatole, was added to 1000 ml of unchlorinated tap water. This dose of skatole had previously been shown to elicit the highest oviposition response in *Cx quinquefasciatus* (Mboera *et al.*, 2000b). The synthetic pheromone (*5R,6S*)-6-acetoxy-5-hexadecanolide was kindly provided by Prof. J. A. Pickett, IACR-Rothamsted, UK. Pheromone solutions were prepared as previously described by Dawson *et al.* (1990). Briefly, 20 mg of pheromone dissolved in hexanol was applied to an effervescent tablet. The tablets were placed in unchlorinated tap water, in which they dissolved completely leaving the pheromone behind in the water.

Bioassay – The effect of chemical stimuli on oviposition behaviour was studied in oviposition traps (A) and in traps for the collection of gravid females (B).

<u>A – Collection of egg rafts in odour-baited ovitraps inside a pit latrine building</u> - Odour stimuli were offered in black plastic 1.5 L bowls. Unless otherwise stated, 1000 ml of each solution containing an attractive stimulus was added to each bowl. Bowls were placed in the experiment at 6.00 pm and left overnight. The next morning, egg rafts were collected and bowls were emptied and thoroughly rinsed with clean tap water.
<u>The effect of grass infusion</u>: this experiment was run in six latrine buildings simultaneously, thus producing six replicates for treatment and control. Six bowls containing 800 ml of grass infusion were compared with six bowls containing the same volume of unchlorinated tap water. The bowls were placed on the floor of a pit latrine building 1 m apart at 6.00 pm and the next morning at 8.00 am the number of mosquito egg rafts deposited was recorded.
<u>The effect of skatole</u>: this experiment was run in five latrine buildings simultaneously. Five bowls containing 1000 ml of tap water and 10^{-5} μg of skatole (in 0.1 ml of ethanol) were compared with five bowls containing the equivalent volume of unchlorinated tap water and 0.1 ml of ethanol. Egg rafts were collected at 8.00 am the next morning. Bowls were emptied, rinsed with clean tap water and by evening filled with the test solutions. The experiment was run for six days between 6.00 pm and 8.00 am.
<u>The effect of skatole and pheromone</u>: this experiment was conducted in five latrine buildings simultaneously. Ten black plastic bowls were filled with 1000 ml of

unchlorinated tap water. To five of the bowls was added 0.1 ml of ethanol containing 10^{-5} μg of skatole while the other bowls were treated with a pheromone-impregnated effervescent tablet (20 mg/tablet). In each latrine building a bowl containing skatole and one containing the pheromone were placed 1 m apart on the floor and left overnight. Bowls were emptied, rinsed with clean tap water and by evening filled with the test solutions. The experiment was run for six days between 6.00 pm and 8.00 am.

In another experiment the oviposition response of *Cx quinquefasciatus* to skatole, pheromone or their combination was determined. Three plastic bowls were filled with 1000 ml of unchlorinated tap water. To one of the bowls was added 0.1 ml of ethanol containing 10^{-5} μg of skatole, the second bowl was charged with the pheromone (20 mg/tablet) and the third bowl with 10^{-5} μg of skatole and a pheromone-treated tablet. The three bowls were placed on the floor of a pit-latrine building away from the entrance, 1 m apart. The experiment was replicated three times and ran for six nights. Treatments were alternated between each night. The same experimental design was used to investigate the effects of grass infusion, pheromone and their combination.

<u>B - Collection of gravid females in odour-baited traps</u> – Counterflow-geometry (CFG) traps (Kline, 1999) were baited with chemical oviposition stimuli in the vicinity of a breeding site and away from it.

<u>The effect of grass infusion and pheromone inside a pit latrine building</u>. First, the effect of grass infusion on the attraction of gravid females was compared against tap water. Two CFG traps were hung from the ceiling inside a pit-latrine building, 1 m apart with the lower end just above a white plastic jar. One jar was filled with 800 ml of grass infusion, the other with unchlorinated tap water. Jars were covered with black netting material to prevent mosquitoes from laying their eggs in it. The traps were operated between 6.00 pm and 8.00 am for 8 days. In between days traps were alternated between the sites. The jars were emptied each day, rinsed with tap water and filled again by evening for the next experimental night. Second, the effect of the pheromone on the attraction of gravid females was studied. Two traps were hung in a similar position as with the first experiment. One jar was filled with 800 ml of unchlorinated tap water and one pheromone-treated effervescent tablet. The other jar contained an equivalent volume of water and a tablet treated with hexanol only. Jars were covered with mosquito netting. The experiment ran for eight days, with treatments alternating between the sites each day.

Table 1: Total and geometric mean number of egg rafts of Culex quinquefasciatus *collected from bowls treated with tap water, grass infusion, skatole, pheromone and their combination inside a latrine building*

Treatment	N	Geom. mean and SD
Grass infusion	260	5.6 ± 1.6 a
Tap water	16	0.2 ± 0.7 b
Skatole	271	3.6 ± 2.3 a
Tap water	46	0.9 ± 1.9 b
Skatole	124	2.5 ± 1.5 a
Pheromone	588	9.4 ± 2.5 b
Grass infusion	262	7.6 ± 1.6 a
Pheromone	612	11.0 ± 2.3 a
Grass infusion + pheromone	2060	49.7 ± 1.9 b
Skatole	152	6.6 ± 1.1 a
Pheromone	360	14.3 ± 1.4 b
Skatole + pheromone	503	23.6 ± 0.7 b

Geometric means in the same sub-table followed by different letters are significantly different (*P*<0.05)

The effect of grass infusion and pheromone away from a natural breeding site.
Three CFG traps were placed outdoors in an area away from known breeding sites *of Cx quinquefasciatus*. The nearest human habitation was 200 m away. The treatments, grass infusion, pheromone and tap water, were put in white plastic jars covered with mosquito netting. The traps were hung from wooden poles 10 m apart with their lowest tip touching the netting material. Traps were operated between 6.00 pm and 8.00 am for six days, in a three choice arrangement. Treatments were alternated between the three sites daily.

Data analysis - In the oviposition experiments the egg rafts collected were sorted by shape and take to the laboratory and reared separately to the adult stage when they were identified to species morphologically. All data were log (n+1) transformed and means of treatments were compared using Student *t*-tests. Means of factorial experiments were subjected to ANOVA. An F-test significant at $P<0.05$ was followed by a Least Significant Difference test to sort out differences between treatment means.

RESULTS

Effect of oviposition stimuli in the vicinity of a natural larval habitat
All chemical stimuli offered attracted gravid *Cx quinquefasciatus* and elicited oviposition (Table 1). There was no significant difference between attractiveness of the grass infusion and the pheromone. However, skatole was significantly less attractive than the pheromone ($P<0.05$) (Table 1). Besides *Cx quinquefasciatus*, the ovitraps also induced oviposition of *Cx cinereus* and *Cx tigripes* but in much smaller numbers than *Cx quinquefasciatus* (Mboera *et al.*, 1999)
Gravid *Cx quinquefasciatus* were collected in the CFG traps baited with grass infusion, pheromone or their combination inside a pit latrine building (Table 2). The number of egg rafts deposited were for both stimuli significantly greater than those collected in tap water ($P<0.01$). The proportions of gravid females collected in the traps baited with grass infusion or pheromone were 65.8 (n=2,480) and 63.8% (n=2,059), respectively.

Table 2: Total and geometric mean number of female Culex quinquefasciatus *collected in CFG traps baited with grass infusion and pheromone inside a latrine building*

Treatment	N	Geometric mean and SD
Grass infusion	1632	194.0 ± 1.4 a*
Tap water	370	43.1 ± 1.5 b
Pheromone	1314	140.3 ± 1.9 a**
Tap water	527	57.2 ± 0.7 b

Figures in each sub-column followed by different numbers are significantly different (*:$P<0.001$; **:$P<0.01$)

Effect of oviposition stimuli away from a natural larval habitat
When CFG traps baited with grass infusion or pheromone were placed away from a natural breeding site, they collected significantly greater numbers of gravid *Cx quinquefasciatus* than traps baited with tap water ($P<0.05$). There was no difference in the number of mosquitoes collected with grass infusion or pheromone (Table 3). When grass infusion and pheromone were combined, the combination was significantly more attractive to gravid females than the sum of females attracted to each single stimulus ($P<0.05$) (Table 3). The proportions of gravid females in the traps away from a known breeding site varied from 93.7 to 97.7%, with the highest proportion collected with the combined stimuli.

Table 3: Total and geometric mean number of female Culex quinquefasciatus *collected in CFG traps baited with grass infusion, pheromone and their combination in an area away from a natural larval habitat*

Treatment	N	Geometric mean and SD
Grass infusion	598	94.2 ± 1.9 a
Pheromone	465	71.1 ± 1.7 a
Tap water	46	6.3 ± 1.9 b
Grass infusion	195	30.7 ± 1.4 a
Pheromone	157	24.7 ± 1.4 a
Grass infusion + pheromone	475	77.7 ± 1.2 b

Figures in each sub-column followed by different numbers are significantly different (P<0.05)

DISCUSSION

The results show a strong attraction of gravid female *Cx quinquefasciatus* to all stimuli tested. This was the first time that a synergistic effect of a grass infusion and synthetic oviposition pheromone on oviposition behaviour was demonstrated. It was interesting to note that this effect occurred both adjacent to a natural breeding site (pit latrine) and far away from such a site. This finding suggests that the mosquitoes need both stimuli to achieve an optimum strategy for the location of oviposition sites. Grass infusion and pheromone, in the dose rates used as in this study, appeared equally attractive and induced a similar degree of oviposition. The results strongly suggest that the number of mosquitoes attracted to both stimuli was smaller away from a breeding site than close to it, although we could not, due to our experimental design, provide statistical evidence for this observation. There were clear differences in the proportion of gravid mosquitoes between the study sites: adjacent to a breeding site the proportion of non-gravid mosquitoes collected by the traps was greater than away from breeding sites. This is presumably caused by the number of recently emerged mosquitoes that responded to the olfactory stimuli in the trap. Inside a latrine building, mosquitoes are emerging from the pupa continuously, and perhaps some young non-gravid females may have responded to some of the odour cues emitted by the trap. Indole, a kairomone present in host emanations as well as in grass infusions, may be such a stimulus (Meijerink *et al.*, 2000; Millar *et al.*, 1992). Away from a natural breeding site there would be only few non-gravid mosquitoes visiting an artificial oviposition site, as these mosquitoes are less likely to be attracted to a chemical oviposition cue from a large distance. Only after blood feeding and development of the eggs, would they become responsive to oviposition cues (Takken & Knols, 1999).

The study demonstrated the important role of skatole in the oviposition behaviour of *Cx quinquefasciatus*. Although the total number of mosquitoes attracted to skatole was lower than those attracted to the pheromone, and there was no synergistic action observed when both stimuli were combined, the results confirm previous laboratory studies with skatole (Mordue-Luntz *et al.*, 1992). The present results suggest that skatole acts jointly with other chemical stimuli such as produced by a grass infusion (Millar *et al.*, 1992) or fermented animal droppings (Blackwell *et al.*, 1993) because the synergistic action on oviposition behaviour was observed with grass infusion and pheromone and not with skatole and pheromone.

From the results we conclude that odour-baited traps can be developed as an additional tool for the control of *Cx quinquefasciatus* and possibly other mosquito vectors. Before this idea can be further developed, it is essential that the efficacy of the oviposition traps be established.

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REFERENCES

BENTLEY M. D. AND J. F. DAY. 1989. Chemical ecology and behavioral aspects of mosquito oviposition. Annual Review of Entomology **34**: 401-421.

BLACKWELL, A., A. J. MORDUE (LUNTZ), B. S. HANSSON, L. J. WADHAMS AND J. A. PICKETT. 1993. A behavioural and electrophysiologal study of oviposition cues for *Culex quinquefasciatus*. Physiological Entomology **18**: 343-348.

DAWSON, G. W., A. L. MUDD, J. A. PICKETT, M. M. PILE AND L. J. WADHAMS. 1990. Convenient synthesis of mosquito oviposition pheromone and a highly fluorinated analog retaining biological activity. Journal of Chemical Ecology **16**: 1779-1789.

KLINE, D. L. 1999. Comparison of two American Biophysics mosquito traps: The professional and a new counterflow geometry trap. Journal of the American Mosquito Control Association **15**: 276-282.

LAURENCE, B. R. & J. A. PICKETT. 1985. An oviposition pheromone in *Culex quinquefasciatus* Say (Diptera: Culicidae). Bulletin of Entomological Research **75**: 283-290.

MBOERA, L. E. G., K. Y. MDIRA, F. M. SALUM, W. TAKKEN AND J. A. PICKETT. 1999. Influence of synthetic oviposition pheromone and volatiles from soakage pits and grass infusions upon oviposition site-selection of *Culex* mosquitoes in Tanzania. Journal of Chemical Ecology **25**: 1855-1865.

MBOERA, L. E. G., W. TAKKEN, K. Y. MDIRA AND J. A. PICKETT. 2000a. Sampling gravid *Culex quinquefasciatus* (Diptera: Culicidae) in Tanzania with traps baited with synthetic oviposition pheromone and grass infusions. Journal of Medical Entomology **37: In press.**

MBOERA, L. E. G., W. TAKKEN, K. Y. MDIRA, G. J. CHUWA AND J. A. PICKETT 2000b. Oviposition and behavioural responses of *Culex quinquefasciatus* (Diptera: Culicidae) to skatole and synthetic oviposition pheromone in Tanzania. Journal of Chemical Ecology **26: In press.**

MEIJERINK, J., M. A. H. BRAKS, A. BRACK, W. ADAM, T. DEKKER, M. A. POSTHUMUS, T. A. VAN BEEK AND J. J. A. VAN LOON. 2000. Identification of olfactory stimulants for *Anopheles gambiae* from human sweat samples. Journal of Chemical Ecology **26: In press.**

MILLAR, J. G., J. D. CHANEY AND M. S. MULLA. 1992. Identification of oviposition attractants for *Culex quinquefasciatus* from fermented Bermuda grass infusion. Journal of the American Mosquito Control Organization **8**: 11-17.

MILLAR, J. G., J. D. CHANEY, J. W. BEEHLER AND M. S. MULLA. 1994. Interaction of the *Culex quinquefasciatus* egg raft pheromone with a natural chemical associated with oviposition sites. Journal of the American Mosquito Control Association **10**: 374-379.

MORDUE, A. J., A. BLACKWELL, B. S. HANSSON, L. J. WADHAMS AND J. A. PICKETT. 1992. Behavioural and electrophysiological evaluation of oviposition attractants for *Culex quinquefasciatus* Say (Diptera: Culicidae). Experientia **48**: 1109-1111.

TAKKEN, W. AND B. G. J. KNOLS. 1999. Odour-mediated behavior of Afrotropical malaria mosquitoes. Annual Review of Entomology **44**: 131-157.