Oviposition habitat selection by *Anopheles gambiae* in response to chemical cues by *Notonecta maculata*

Alon Warburg[1] ✉, Roy Faiman[1], Alex Shtern[1], Alon Silberbush[2], Shai Markman[3], Joel E. Cohen[4], and Leon Blaustein[2]

[1]*Department of Microbiology & Molecular Genetics, The Institute for Medical Research Israel-Canada, The Kuvin Centre for the Study of Infectious and Tropical Diseases, The Hebrew University of Jerusalem, Jerusalem 91120, Israel, alonw@ekmd.huji.ac.il*
[2]*Institute of Evolution and Department of Evolutionary and Environmental Biology, Faculty of Natural Sciences, University of Haifa, 31905, Israel*
[3]*Department of Biology, Faculty of Natural Sciences, University of Haifa - Oranim, Tivon, 36006, Israel*
[4] *Rockefeller and Columbia Universities, 1230 York Avenue, New York, NY 10065, U.S.A.*

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ABSTRACT: A number of mosquito species avoid predator-inhabited oviposition sites by detecting predator-released kairomones. In the laboratory, we found that when offered de-ionized water and de-ionized water conditioned with *Notonecta maculata*, gravid *Anopheles gambiae* females preferentially oviposited into the former. We then conducted further experiments using two chemical components found in *Notonecta*-conditioned water, chemically pure *n*-tricosane and/or *n*-heneicosane, that was previously shown to repel oviposition by *Culiseta longiareolata.* These hydrocarbons failed to deter oviposition by *An. gambiae* females. Thus, different mosquito species may rely on distinct chemical cues to avoid predators. Identification and chemical characterization of such kairomones could facilitate innovative, environmentally sound mosquito control. ***Journal of Vector Ecology* 32 (2): 421-425. 2011.**

Keyword Index: *Anopheles gambiae*, kairomones, mosquitoes predators, notonectid bugs, oviposition, predation.

INTRODUCTION

A growing body of literature indicates that many mosquito species are able to detect predators of their progeny and avoid oviposition sites infested with these predators (reviewed in Vonesh and Blaustein 2010). Such behavior bears particular relevance for the interpretation of experiments that assess the effects of predators on mosquito larvae in given pools and then infer effects on total adult populations. Frequently, such studies assume that oviposition occurs randomly among larval habitats. If this assumption is incorrect, active avoidance of predator-inhabited pools is likely to cause a gross over-estimation of the role of predation in reducing mosquito populations (Blaustein 1999, Spencer et al. 2002).

Mosquitoes that can detect aquatic predators often do so by sensing predator-released kairomones (review in Vonesh and Blaustein 2010). However, with only one exception (Silberbush et al. 2010), the chemical composition of these predator-released kairomones remains unknown. Characterization of these molecules can facilitate the development of highly specific, ecologically friendly, and inexpensive mosquito control approaches (Silberbush et al. 2010).

Several *Anopheles* spp. have been shown to be sensitive to chemical cues from predators (Petranka and Fakhoury 1991, Munga et al. 2006). Of particular interest is the *Anopheles gambiae* species complex, that include the most important vectors of malaria in Africa (Coetzee et al. 2000). Better understanding of the ecology of *An. gambiae* could lead to improved control methods.

Colonized *An. gambiae* derived from western Kenya have been shown to chemically detect and avoid backswimmers (*Notonecta* sp.) when choosing an oviposition site (Munga et al. 2006). However, this finding does not necessarily reflect the behavior of all the molecular forms within the *An. gambiae* species complex. Larval habitats of the *An. gambiae* complex range from very small, ephemeral puddles with low risk of predation (e.g., hoof prints, tire tracks, drainage ditches) to larger bodies of water with longer hydroperiods and consequently higher risk of predation (e.g., rice fields) (Gillies and De Meillon 1968, Gimnig et al. 2001). Recent studies show that different molecular forms of *An. gambiae* largely segregate according to aquatic larval habitats that differ with respect to hydroperiod and risk of predation (Costantini et al. 2009, Gimonneau et al. 2010). Here, we test whether ovipositing *An. gambiae* females (G3 strain) avoid water conditioned with the predator, *Notonecta maculata*. We further check if the specific *N. maculata*-released hydrocarbons shown to repel oviposition by the mosquito *Culiseta longiareolata* (Silberbush et al. 2010) also repel the G3 strain of *An. gambiae*.

MATERIALS AND METHODS

An. gambiae (G3) were obtained from the Laboratory of Parasitic Disease, NIAID/NIH, Bethesda, MD, U.S.A. G3 is a mongrel stock established with mosquitoes collected in The Gambia in 1975. The colony is characterized by generally good vigor and a "wild" but polymorphic nature (http://www.mr4.org/).

Backswimmers (Notonectidae: Hemiptera) are common and efficient predators of mosquito larvae, worldwide, including larvae of *An. gambiae* (Kweka et al. 2011). Backswimmers have been shown to commonly induce oviposition avoidance by various mosquito species (Chesson 1984, Eitam et al. 2002, Blaustein et al. 2004, Blaustein et al. 2005, Munga et al. 2006). Adult *N. maculata* were collected from freshwater pools on Mount Carmel, Israel, and transported to the laboratory in Jerusalem. Individual *N. maculata* were first starved for four days to clear gut contents. Then, every single adult backswimmer was placed in 150 ml of de-ionized water for 24 h at 26° C to produce *Notonecta*-conditioned water (NCW) used for each replicate in the experiments. For each experiment, four-day-old *An. gambiae* G3 females were fed on an anaesthetized rabbit. Four days thereafter, groups of females were placed in mesh cages (30X30X30 cm) within an insectary. The insectary was darkened between 18:00-07:00 and maintained at a constant temperature of 26° C. In all experiments described below, oviposition was allowed for an 18-h period between 18:00 and 12:00 the following day.

In the first *Notonecta*-conditioned water (NCW) experiment, we determined if the gravid females' oviposition choices were affected by NCW. Two circular oviposition containers (diameter 9 cm, height 8 cm) lined with filter paper were placed in each cage – one containing 150 ml NCW and one with 150 ml de-ionized water. The oviposition containers were positioned in opposite corners of the cage (distance between edges of containers ~20 cm) and in alternate directions in different experimental cages. We have found previously in the field that *Cs. longiareolata* and *Cs. laticinctus* mosquito females, when ovipositing, could distinguish between chemical cues of predator and non-predator pools that were within 20 cm (Blaustein, unpublished data) and 30 cm (Kiflawi et al. 2003) of each other. Moreover, ovipositing mosquitoes have been shown to distinguish between such predator-conditioned water and non-predator water in other laboratory studies with similar among-treatment distances (Hurst et al. 2010). The experiment was repeated over three time periods using a total of 14 experimental cages and 540 gravid females. There were 60 females per cage during the first time period (four cages). To facilitate accurate egg counts, and to reduce the possibility of oviposition "spillover" to less preferred treatments due to density-dependent effects, in subsequent experiments we reduced the number of females to 30 per cage (ten cages).

In two additional hydrocarbon experiments, we tested whether specific hydrocarbons found in NCW affected oviposition by *An. gambiae* G3. Synthetic *n*-heneicosane (0.16 µg) and/or *n*-tricosane (0.97 µg, standard for GC, purity 99.5%; Sigma-Aldrich Chemicals, St. Louis, MO, U.S.A.), dissolved in 50 µl of 99.5% ethyl alcohol (EtOH), were placed in our experimental oviposition containers with 150 ml of de-ionized water. These concentrations of *n*-heneicosane and *n*-tricosane were derived as ecologically relevant concentrations by Silberbush et al. (2010) who conducted chemical analysis on 150 ml of de-ionized water samples that had also contained a single adult backswimmer for 24 h at 26° C after it was starved for four days. To control for any unlikely effect of the minute concentration of the alcohol used to dissolve the hydrocarbons, pure EtOH (50 µl) was also added to the control oviposition container that was filled with 150 ml of de-ionized water. Moreover, we showed previously that EtOH at a similar concentration did not affect oviposition by *Cs. longiareolata* (Silberbush et al. 2010). In both of these two hydrocarbon experiments described below, 30 gravid females were placed in each cage and oviposition took place between 18:00 and 12:00 the following day. The first hydrocarbon experiment comprised five cages with four oviposition containers in each one: one with each experimental substance (H or T), one with a mixture of the two (HT), and one control (C) container with de-ionized water. The four cups were placed in the corners of the experimental cages.

Offering multiple choices to ovipositing insects can mask the effects that a pair-wise comparison might show (Silberbush and Blaustein 2011). Furthermore, in a previous study by Silberbush et al. (2010), both H and T were detected in all NCW samples tested. Thus, an H plus T mixture is more ecologically relevant than either of these two chemicals alone. We therefore conducted an additional experiment that limited females to only two choices: control water (no hydrocarbons, but with the alcohol solvent) and the combination of H plus T (with the alcohol solvent). In the present experiment, we used four cages with two oviposition containers in each one (in opposite corners). The oviposition containers were positioned in alternate directions in the corners of the experimental cages.

Eggs oviposited on numbered filter papers were counted "blindly" (i.e., the person counting did not know the numbers assigned to the different experimental groups). For the NCW experiment, egg numbers from the control (de-ionized water) and NCW oviposition cups in the 14 replicate cages were compared using a sign test. For the four-treatment hydrocarbon experiment (no hydrocarbon, T alone, H alone, and H plus T), we conducted an ANOVA, incorporating treatment effects and block (i.e., cage) effects where the treatment x block effect served as the error term. A Levene's test did not reveal heterogeneity of variance ($p=0.23$). For the "no hydrocarbon" vs "H plus T combination" comparisons, we used a paired t-test.

RESULTS

Notonecta-conditioned water experiment

More eggs (70.2% of total) were laid in control containers (Total eggs in control containers: 15,130)

than NCW containers (Total eggs: 6,444); in pair-wise comparisons within cages, 12 of 14 cages had significantly more eggs in control containers (Sign test: two tailed p=0.0129; Figure 1).

Hydrocarbon experiments

The experiment providing four choices of no chemical, T alone, H alone and T plus H, did not reveal a statistically significant difference in egg numbers (T: $F_{1,12}$=0.236; p=0.636; H: $F_{1,12}$=0.245; p=0.629; T x H: $F_{1,12}$=0.245; p=0.629; Block: $F_{4,12}$=0.571; p=0.689; Figure 2) In fact, egg numbers tended to be higher in containers with the hydrocarbons. Because multiple choice oviposition tests may mask oviposition preference (Silberbush et al. 2010), we decided to run an additional experiment to test only the pairwise choice of the combination of hydrocarbons versus control. This experiment comparing H plus T to control water did not demonstrate a significant difference (paired t-test: t=1.24; df=3; p=0.303; Figure 3). Again, as in the four-treatment experiment, H plus T tended to have more eggs than the control.

DISCUSSION

An. gambiae G3 strain demonstrated a strong oviposition avoidance of NCW. This avoidance of NCW is consistent with the behavior of a number of other mosquito species (Vonesh and Blaustein 2010) and in particular, with an *An. gambiae* colony from western Kenya (Munga et al. 2006). Since different molecular forms within the *An. gambiae* complex prefer different oviposition habitats and demonstrate differential larval vulnerability to predation, not all strains of *An. gambiae* would be expected to demonstrate oviposition habitat avoidance in response to risk of predation as demonstrated in our study and that of Munga et al. (2006). It is thus important to test various strains or molecular forms.

If *An. gambiae* operated according to an ideal-free distribution (Fretwell and Lucas 1969), there might have been more spillover to NCW cups in cages where we used the higher density of mosquito females, assuming density-dependent effects at the densities used. However,

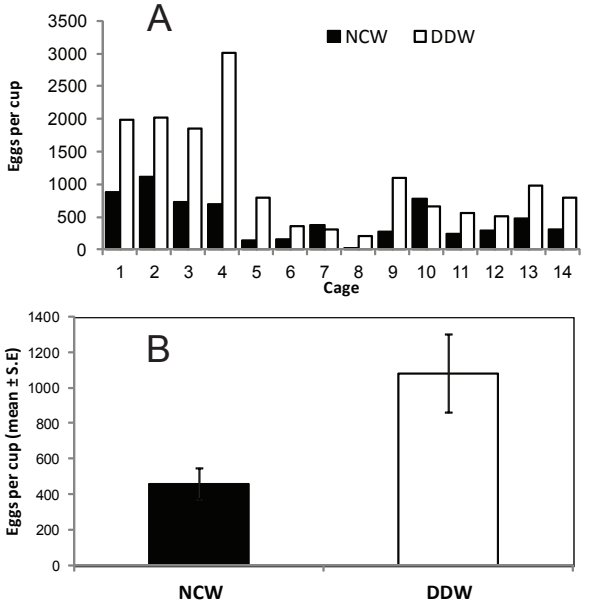

Figure 1. Oviposition of *Anopheles gambiae* G3 in deionized water (control) vs deionized water conditioned with *Notonecta maculata* (NCW): (A) Number of eggs in each container in 14 cages. Sixty females were used in the first four cages while 30 females were used in the next ten cages; (B) mean number of eggs per container in the two treatments, regardless of the number of mosquitoes in each cage. Error bars are ± one standard error. Fewer eggs were laid in NCW containers (Sign test: two tailed p=0.0129).

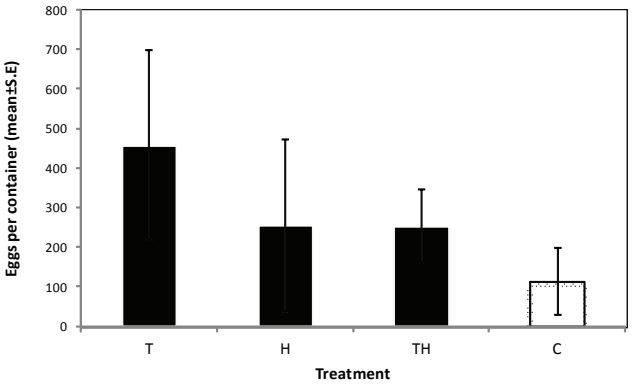

Figure 2. Mean number of *Anopheles gambiae* G3 eggs per oviposition container comparing four different hydrocarbon treatments: T= *n*-tricosane; H= *n*-heneicosane; TH= *n*-tricosane plus *n*-heneicosane; C= control (neither hydrocarbon, but with alcohol solvent). Thirty gravid *An. gambiae* females were placed in each cage (=replicate) and there were five experimental cages, each with four containers (one per treatment). Error bars are ± one standard error. There were no statistically significant treatment effects (T: $F_{1,12}$=0.236; p=0.636; H: $F_{1,12}$=0.245; p=0.629; T x H: $F_{1,12}$=0.245; p=0.629).

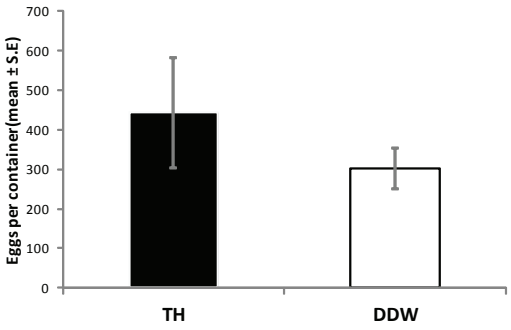

Figure 3. Mean number of *Anopheles gambiae* G3 eggs per oviposition container comparing the two hydrocarbons together plus alcohol solvent (treatment) with Control (no hydrocarbons, alcohol solvent only). There were four cages with two oviposition containers in each one (in opposite corners). Thirty gravid *An. gambiae* females were placed in each cage. TH= *n*-tricosane plus *n*-heneicosane; C= control. Error bars are ± one standard error. There was no statistically significant effect (paired t-test: t=1.24; df=3; p=0.303).

the avoidance response appeared equally strong regardless of the number of gravid females per cage: there were 2.7 times more eggs in controls with 60 female mosquitoes compared to ~two times more eggs in controls with 30 female mosquitoes per cage (Figure 1).

Cs. longiareolata avoided the two specific *Notonecta*-released kairomones also tested in this study n-tricosane and heneiocosane (Silberbush et al. 2010). *An. gambiae* females did not avoid laying eggs in the presence of these two hydrocarbons, suggesting that the two mosquito species do not rely upon the same chemicals to detect the presence of notonectid predators or that they have different thresholds of sensitivity for avoidance or attraction. The hydrocarbons tended to attract more rather than less oviposition, though not significantly so. Gravid *Aedes aegypti*, when exposed to a wide range of heneiocosane concentrations in the laboratory (all concentrations were higher than those we used in our current experiment and in that of Silberbush et al. 2010), oviposited at the same rate as in control tubs in the lowest range of the concentrations tested, then oviposited more in the median concentrations but were repelled by the highest concentrations (Seenivasagan et al. 2009). Importantly, NCW, containing all chemicals released by *Notonecta*, had a stronger repellent effect on *Cs. longiareolata* than synthetic n-tricosane and heneiocosane together (Silberbush et al. 2010). Thus, it appears that *Cs. longiareolata* utilizes additional chemicals for predator cues, and these different mosquito species may possibly recognize some of the same predator-released kairomones.

Determining the chemical identity of these kairomones may have direct use for control of *Anopheles* and other mosquitoes. Adult mosquitoes in general have high daily mortality rates (Silver 2008). Therefore, treating potential larval habitats with oviposition deterrents is likely to prolong the female mosquitoes' search time for suitable oviposition sites, increasing the mortality of gravid females and thereby possibly reducing mosquito populations.

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REFERENCES CITED

Blaustein, L. 1999. Oviposition site selection in response to risk of predation: evidence from aquatic habitats and consequences for population dynamics and community structure. In: S. Wasser (ed.), *Evolutionary Theory and Processes: Modern Perspectives*. pp. 441-456. Kluwer, Dordrecht.

Blaustein, L., J. Blaustein, and J. Chase. 2005. Chemical detection of the predator *Notonecta irrorata* by ovipositing *Culex* mosquitoes. J. Vector Ecol. 30: 299-301.

Blaustein, L., M. Kiflawi, A. Eitam, M. Mangel, and J.E. Cohen. 2004. Oviposition habitat selection in response to risk of predation in temporary pools: mode of detection and consistency across experimental venue. Oecologia 138: 300-305.

Chesson, J. 1984. Effect of notonectids (Hemiptera: Notonectidae) on mosquitoes (Diptera: Culicidae): predation or selective oviposition. Environ. Entomol. 13: 531-538.

Coetzee, M., M. Craig, and D. le Sueur. 2000. Distribution of African malaria mosquitoes belonging to the *Anopheles gambiae* complex. Parasitol. Today 16: 74-77.

Costantini, C., D. Ayala, W.M. Guelbeogo, M. Pombi, C. Y. Some, I.H.N. Bassole, K. Ose, J.M. Fotsing, N.F. Sagnon, and D. Fontenille. 2009. Living at the edge: biogeographic patterns of habitat segregation conform to speciation by niche expansion in *Anopheles gambiae*. BMC Ecol. 9: 16.

Eitam, A., L. Blaustein, and M. Mangel. 2002. Effects of Anisops sardea (Hemiptera: Notonectidae) on oviposition habitat selection by mosquitoes and other dipterans and on community structure in artificial pools. Hydrobiologia 485: 183-189.

Fretwell, S. D. and H. L. Lucas. 1969. On territorial behavior and other factors influencing habitat distribution in birds Acta Biotheoret. 19: 16-36.

Gillies, M. T. and B. J. De Meillon. 1968. The anophelinae of Africa south of the Sahara. The South African Institute for Medical Research.

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

Gimonneau, G., J.R.M.. Bouyer, S. Morand, N.J. Besansky, A. Diabate, and F.D.R. Simard. 2010. A behavioral mechanism underlying ecological divergence in the malaria mosquito *Anopheles gambiae*. Behav. Ecol. 21: 1087-1092.

Hurst, T.P., B.H. Kay, M.D. Brown, and P.A. Ryan. 2010. Melanotaenia duboulayi influence oviposition site selection by *Culex annulirostris* (Diptera: Culicidae) and *Aedes notoscriptus* (Diptera: Culicidae) but not *Culex quinquefasciatus* (Diptera: Culicidae). Environ. Entomol. 39: 545-551.

Kiflawi, M., L. Blaustein, and M. Mangel. 2003. Oviposition habitat selection by the mosquito *Culiseta longiareolata* in response to risk of predation and conspecific larval density. Ecol. Entomol. 28: 168-173.

Kweka, E. J., G. Zhou, T.M. Gilbreath, 3rd, Y. Afrane, M. Nyindo, A.K. Githeko, and G. Yan. 2011. Predation efficiency of *Anopheles gambiae* larvae by aquatic predators in western Kenya highlands. Parasit. Vectors 4: 128.

Munga, S., N. Minakawa, G. Zhou, O.O. Barrack, A. K. Githeko, and G. Yan. 2006. Effects of larval competitors

and predators on oviposition site selection of *Anopheles gambiae sensu stricto*. J. Med. Entomol. 43: 221-224.

Petranka, J.W. and K. Fakhoury. 1991. Evidence of a chemically-mediated avoidance response of ovipositing insects to blue-gills and green frog tadpoles. Copeia 1991: 234-239.

Seenivasagan, T., K.R. Sharma, K. Sekhar, K. Ganesan, S. Prakash, and R. Vijayaraghavan. 2009. Electroantennogram, flight orientation, and oviposition responses of *Aedes aegypti* to the oviposition pheromone n-heneicosane. Parasitol. Res. 104: 827-833.

Silberbush, A. and J. Blaustein. 2011. Mosquito females quantify risk of predation to their progeny when selecting an oviposition site. Funct. Ecol. 25: 1091-1095.

Silberbush, A., S. Markman, E. Lewinsohn, E. Bar, J.E. Cohen, and L. Blaustein. 2010. Predator-released hydrocarbons repel oviposition by a mosquito. Ecol. Lett. 13: 1129-1138.

Silver, J.B. 2008. *Mosquito Ecology - Field Sampling Methods*, 3rd ed. Springer, Dordrecht, Netherlands.

Spencer, M., L. Blaustein, and J.E. Cohen. 2002. Oviposition habitat selection by mosquitoes (*Culiseta longiareolata*) and consequences for population size. Ecology (Washington D C) 83: 669-679.

Vonesh, J. and J. Blaustein. 2010. Implications of predator-induced shifts in mosquito oviposition site selection for vector control: a meta-analysis. Israel J. Ecol. Evol. 56: (in press).