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LETTER

Predator-released hydrocarbons repel oviposition by a mosquito

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Abstract

Prey species commonly use predator-released kairomones (PRKs) to detect risk of predation, yet the chemical identity of PRKs remains elusive. Chemical identification of PRKs will facilitate the study of predator–prey interactions and the risk of predation, and when the prey are pests, will potentially provide environmentally friendly means of pest control. In temporary pools of the Mediterranean and Middle East, larvae of the mosquito *Culiseta longiareolata* Macquart are highly vulnerable to the common predatory backswimmer, *Notonecta maculata* Fabricius. We demonstrate that *N. maculata* releases two hydrocarbons, *n*-heneicosane and *n*-tricosane, which repel ovipositing females of *C. longiareolata*. In behavioural tests with environmentally relevant chemical concentrations in outdoor mesocosm experiments, the repellent effects of the two compounds were additive at the tested concentrations.

Keywords

Aquatic habitats, backswimmer, mosquito, *n*-heneicosane, *n*-tricosane, oviposition habitat selection, predator-released kairomones, risk of predation.

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INTRODUCTION

Kairomones are information-carrying chemicals produced by an individual of one species (the donor) and received by an individual of a different species (the receiver), benefiting the receiver and often disadvantaging the donor (Dicke & Sabelis 1988; Pohnert *et al.* 2007). They are pervasive. In aquatic systems, kairomones are reportedly the most prevalent type of chemical cue mediating ecological interactions (Burks & Lodge 2002). Many prey species detect predators via predator-released kairomones (PRKs) and these kairomones can play an important role in predator–prey interactions (Kats & Dill 1998; Brönmark & Hansson 2000; Dicke & Grostal 2001; Van Donk 2007). PRKs induce diverse responses by prey: examples include physiological and morphological responses such as increased body size or armour, and behavioural responses such as alterations in foraging, mating and oviposition (Kats & Dill 1998; Kusch 1999; Brönmark & Hansson 2000; Whitman & Blaustein 2009). Aquatic organisms, given commonly turbid conditions, may rely more on chemical cues than on visual cues, compared with organisms using the air medium (Pohnert *et al.* 2007).

Despite the wide occurrence and importance of kairomones, the chemical identity of PRKs remains largely undetermined (Kats & Dill 1998; Pohnert *et al.* 2007). Although a handful of studies have made significant inroads into identifying and demonstrating that certain chemicals act as PRKs (Tollrian & von Elert 1994; von Elert & Loose 1996; Kusch 1999; von Elert & Pohnert 2000; Akkas *et al.*

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2010), none involves PRKs that inhibit oviposition by prey. Furthermore, previous studies do not provide a complete chemical characterization combined with a demonstration that the putative causal chemical acts as a PRK at environmentally relevant concentrations (e.g. Pohnert & von Elert 2000).

Gravid females of a number of amphibian and insect species with aquatic larval stages and terrestrial adult stages detect aquatic predators of their progeny when selecting an oviposition site (reviewed by Skelly 2001; Vonesh & Blaustein 2010). Unlike aquatic stages of prey that detect their aquatic predators via PRKs in the water, ovipositing females may not necessarily need contact with the water to detect a predator. Instead, they may be capable of detecting chemicals of aquatic predators from above the water surface (Silberbush & Blaustein 2008). Knowledge of the chemical identity of these kairomones can provide an important tool in the study of predator–prey interactions and a better understanding of the role of kairomones in shaping food webs (Burks & Lodge 2002; Fink 2007). Moreover, when a PRK repels pest oviposition, it can have consequences for pest population size (Spencer *et al.* 2002) and community and metacommunity dynamics (Binckley & Resetarits 2005) and chemical identification can be used to develop environmentally friendly means to reduce pest populations.

Many species of mosquitoes detect chemical cues of predation risk when choosing oviposition sites (e.g. Angelon & Petranka 2002; Blaustein *et al.* 2004; Van Dam & Walton 2008; reviewed in Vonesh & Blaustein 2010). Notonectids, which are common and effective predators of mosquito larvae, chemically repel oviposition of some mosquito species. For example, the backswimmer *Notonecta maculata* repels ovipositing females of *Culiseta longiareolata*, *Culex laticinctus* (Kiflawi *et al.* 2003a,b; Blaustein *et al.* 2004; Arav & Blaustein 2006) and the malaria vector, *Anopheles gambiae* (Munga *et al.* 2006). However, the chemical identities of these PRKs remain unknown. Behavioural assays demonstrated that all or some of the chemicals used by *C. longiareolata* to detect *N. maculata* are volatile because they are detected from above the water surface (Silberbush & Blaustein 2008). Here, we chemically identified two compounds isolated from *Notonecta*-conditioned water (NCW) that repel oviposition by *C. longiareolata* at ecologically relevant concentrations in outdoor artificial pools.

MATERIALS AND METHODS

Screening for potential volatile kairomones

To identify candidate volatile chemicals that might serve as PRKs, we conducted automatic headspace solid-phase microextraction (HS-SPME) comparing water containing *N. maculata*, water containing the larval dragonfly *Anax imperator* Leach and control water (no predator). *Anax imperator* is also an effective predator of *C. longiareolata* larvae but this predator does not repel oviposition by *C. longiareolata* via chemical cues (Stav *et al.* 2000). Thus emitted chemicals, if any, that *A. imperator* had in common with *N. maculata* could be ruled out as the putative kairomones.

Adult *Notonecta* and *Anax* larvae used in the experiments were netted from natural pools (with a permit from Israel Nature & Parks Authority). *Notonecta* and *Anax* individuals were kept without food for 4 days prior to using them in the studies. Single live starved adult *N. maculata* (14 replicates) or single live starved larval *A. imperator* (eight replicates – four of early and four of late-instar larvae) or control (no predator) water (six replicates) were placed, each one separately, in 10 mL of water in a 20-mL headspace glass vial (Supelco SU860097, Bellefonte, PA, USA) with an inert material septum as a lid especially designed to accommodate the SPME sample. The SPME fibre was inserted into the vial headspace without touching the water. The vials were sealed and kept at room temperature for 24 h until analysis. The volatiles were adsorbed for 30 min by automatic HS-SPME at 35 °C by a 65-μm PDMS/DVB fibre (polydimethylsiloxane/divinylbenzene; Supelco, Bellefonte, PA, USA). The fibre was inserted into the injection port of the gas chromatography-mass spectrometry (GC-MS) for 5 min (splitless) for desorption of the volatiles (further details in Davidovich-Rikanati *et al.* 2008).

Determination of concentrations of potential *Notonecta*-released kairomones

To determine concentrations of the potential kairomones, five live, starved (for 4 days) adult *Notonecta* were placed together in a single 500-mL vial containing 250 mL of water and sealed for 24 h. The *Notonecta* were removed and the remaining water was extracted with 250 mL of methyl *tert*-butyl ether (MTBE) and 5 μg of isobutyl benzene. The isobutyl benzene was an internal standard that was added, at the beginning of every chemical analysis also in all the following relevant procedures stated next, by vigorous shaking (250 rpm) on a shaker apparatus overnight at room temperature. The upper MTBE layer was separated, dried with sodium sulphate and concentrated to a volume of 0.5 mL under a gentle stream of nitrogen. The samples were kept at 4 °C until analysis. A 1-μL aliquot of the concentrated MTBE extract was injected into a GC-MS in the splitless mode.

GC-MS analysis of volatiles

Concentrated MTBE extracts (1 μL) were introduced into a Gas Chromatograph-Mass Spectrometer Detector (GC-MSD) [(Aligent, Santa Clara, CA, USA, equipped with a

Rtx-5SIL MS column (30 m length × 0.25 mm i.d., 0.25-μm film thickness, stationary phase 95% dimethyl- 5% diphenyl polysiloxane)]. Helium (0.8 mL min^{-1}) was used as a carrier gas with splitless injection. The injector temperature was 250 °C, and the detector temperature was 280 °C. The conditions used were as follows: initial temperature was 50 °C for 1 min followed by a ramp of 50–280 °C at a rate of 5 °C min^{-1}. A quadrupole mass detector with electron ionization at 70 eV was used to acquire the MS data in the range of 41–350 m/z. A mixture of straight-chain alkanes (C7–C26) was injected into the column under the above conditions to calculate retention indices. The quantification analysis was followed by an internal standard (isobutyl benzene) correction and the results were corrected due to losses and differential extraction efficiencies. Volatiles were identified by comparing their retention indices with those from the literature and by comparison of spectral data with authentic standards. Component amount in each sample was calculated as peak area × internal standard response factor divided by response factor × internal standard peak area (further methodological details in Davidovich-Rikanati *et al.* 2008).

General protocol for field experiments

Three field mesocosm experiments described next to assess oviposition repellency of specific *Notonecta*-released chemicals and NCW were conducted between April and June in 2007 and 2008 at the Hai Bar Nature Reserve, Mt. Carmel, Israel (32°44′ N, 35°01′ E). Mesocosm pools were green plastic tubs (width × length × height: 40 × 50 × 20 cm). To each pool in each experiment, we added 15 L of aged tap water, and 1 g of 'Sera pond' fish food pellets (32% protein) as a nutrient source. Water volume in the pools was maintained using aged tap water. Each week, the pools were emptied and replenished with new aged tap water and food pellets to avoid build-up of algae, other organisms and chemicals that might influence the effects of potential PRKs. Conservatively, despite emptying and refilling, we treated a single pool over the entire experiment as a single unit, not as different units after each refilling with water.

Based on our chemical analyses, we found two candidate chemicals that were never found in the control or the *Anax* samples, but were the only compounds found in all *Notonecta* samples*: n*-heneicosane and *n*-tricosane (Table 1), hereafter referred to as H and T, respectively. Synthetic H and T (Sigma-Aldrich Chemicals, St Louis, MO, USA) used in the experiments were standard for GC (purity ≥ 99.5%), and were dissolved in 99.5% ethyl alcohol (EtOH). Synthetic hydrocarbons and NCW were added to appropriate pools *c.* 1 h before dusk to minimize evaporation of compounds before gravid females, which oviposit between dusk and dawn, arrived. To assure that the minute amounts of EtOH added (30 μL of EtOH in 15 L of water) did not affect oviposition, we ran separate replicated outdoor mesocosm experiments at three different sites comparing oviposition in control water with control water plus alcohol using five replicates per treatment at the first site (15 nights), five at a second site (15 nights) and nine at a third site (10 nights). We analysed the number of mosquito egg rafts oviposited per pool per night with a two-way ANOVA. Site had a significant effect ($F_{2,32} = 5.06$, $P = 0.012$) but neither alcohol ($F_{1,32} = 0.03$, $P = 0.858$; number of egg rafts per pool per night [standard error]: control=1.74 [0.57]; alcohol=1.58 [0.27]) nor the alcohol × location interaction had a significant effect ($F_{2,32} = 0.95$, $P = 0.958$).

Culiseta longiareolata was the only mosquito oviposited in sufficient amounts to analyse statistically for these oviposition experiments. This mosquito species oviposits egg rafts at night (Van Pletzen & Van Der Linde 1981). Mosquito egg rafts, which are easily visible, were removed from the experimental pools every morning and taken to the laboratory where eggs hatched and individuals from each egg raft were grown to fourth instar larvae for identification.

Table 1 Compounds identified in headspace over *Notonecta maculata*-conditioned water (NCW) and the frequency (%) of these compounds in the headspace over the 14 *N. maculata*, 8 *Anax imperator* and 8 control samples tested. The traces obtained for total ion count, typical for these hydrocarbons, are shown in Fig. 1

Group	Compound	Frequency (%) of *N. maculata* samples	Frequency (%) of *A. imperator* samples	Frequency (%) of control samples
Alkanes	*n*-tricosane	100	0	0
	n-heneicosane	100	0	0
	n-dodecane	21	12.5	0
	n-nonadecane	7	0	0
	n-undecane	7	0	0
	2,6,10,14-tetramethylhexadecane	7	0	0
Others	Nonanal	14	12.5	0
	Dodecanoic acid (lauric acid)	7	0	0

For each experiment, all pools were placed *c.* 50 cm apart along a rectangular perimeter. Previous studies showed that this inter-pool distance was sufficient for *C. longiareolata* to distinguish between control and *Notonecta* pools (e.g. Kiflawi *et al.* 2003a,b; Eitam & Blaustein 2004). Preliminary studies at this site demonstrated that specific location of a pool importantly affected oviposition rates. To control for this expected variation, we did the following. First, along this perimeter, we grouped sets of pools where each group contained one replicate of each treatment randomly assigned within the group. Second, each day, we rotated all pools, moving each pool clockwise by one spot along the rectangular perimeter. As, for each of these three experiments, the number of nights tested exceeded the total number of pools in the experiment, each pool occupied each location in the perimeter for at least one night. Thus, groups were not traditional blocks with fixed location. The group effects were not statistically significant, and we absorbed them into the error terms for analyses. We used Levene's test to test for homogeneity of variance. The data passed this test except in one case where a natural-log transformation was needed to make the data satisfy homogeneity of variance.

Treatment descriptions and concentrations for the field experiments

The following treatments were added to each appropriate pool containing the 15 L of aged tap water:

- *Control*: 100 mL of aged tap water.
- *NCW*: 100 mL of aged tap water that had held three starved adult *Notonecta* for 24 h. Prior to adding the NCW to the experimental pools containing the 15 L of aged tap water, *Notonecta* were first fed on *Daphnia,* then starved in individual cups for 96 h, then placed without food in 100 mL of aged tap water for an additional 24 h. This starvation period of 4 days prior to allowing excretion in the 100 mL of water reduced, and probably eliminated, chemicals directly from their ingested prey.
- *H*: 0.49 μg of synthetic *n*-heneicosane dissolved in 30 μL of EtOH (this is the amount of *n*-heneicosane measured in 100 mL of aged tap water that held three starved adult *Notonecta* for 24 h) plus 100 mL of aged tap water.
- *T*: 2.99 μg of synthetic *n*-tricosane dissolved in 30 μL of EtOH (this is the amount of *n*-tricosane measured in 100 mL of tap water that held three adult *Notonecta* for 24 h) plus 100 mL of aged tap water.
- *H* + *T*: A mixture of 0.49 μg of *n*-heneicosane and 2.99 μg of *n*-tricosane dissolved in 30 μL of EtOH plus 100 mL of aged tap water.

Field Experiment 1: Time course of effects of H + T combination and NCW on oviposition

The first field experiment consisted of three treatments: control, H + T mixture, and NCW. Each treatment was replicated six times. H + T mixture and NCW, in the amounts described before, were added every other day. In the data analysis, we distinguished between egg raft data collected on first nights (immediately following chemical application) and second nights (starting 24 h after chemical application). By adding materials only every other day, but measuring oviposition every day, we were able to assess any differences between the synthetic compounds and NCW in repelling oviposition over time. The experiment began on 12 April 2007, and lasted 23 days. After excluding nights in which there was no oviposition at all, we used data from 9 first and 9 second oviposition nights. Total *C. longiareolata* egg rafts for a given pool across all dates for either first or second nights served as the response variable. In total, 138 egg rafts were deposited during the experiment. We analysed the first and second oviposition nights separately by one-way ANOVA and then used Tukey's HSD *post hoc* pairwise comparison test for detecting significant differences among treatments.

Field Experiment 2: Comparing effects of all five treatments

The second field experiment assessed oviposition when mosquitoes were simultaneously offered all five treatments: control, H alone, T alone, H + T and NCW. Each treatment was replicated with five pools. NCW and synthetic hydrocarbons were added every day just prior to sundown (in contrast to field Experiment 1 where chemicals were added only every other day) and egg rafts were collected every morning. Data were collected between 10 May and 21 June 2007 (41 days). Total *C. longiareolata* egg rafts for a given pool across all dates served as the response variable. In total, 115 egg rafts were deposited during the experiment. Data were analysed in two ways: (1) one-way ANOVA compared all five treatments with Tukey's HSD to assess for specific treatment differences, and (2) two-way ANOVA, excluding the NCW treatment, compared the presence or absence of each of the two hydrocarbons crossed with the other.

Field Experiment 3: Effects of each hydrocarbon alone

Ovipositing *C. longiareolata* females can quantify the risk of predation when given pairwise choices (Silberbush 2010) but only showed a qualitative assessment of presence or absence when simultaneously given multiple choices of various risks of predation (Eitam & Blaustein 2004). To

examine more closely than we did in Experiment 2 how each hydrocarbon separately might affect oviposition, in the third field experiment, we reduced the number of simultaneous choices to three: control, H alone and T alone (5 pools per treatment, 15 pools in total). Hydrocarbons were added every evening and egg rafts removed every morning. Egg raft data were collected for 20 days between 16 April and 5 May 2008. Total egg rafts for a given pool across all dates served as the response variable. In total, 103 egg rafts were deposited during the experiment. Data were analysed as a one-way ANOVA and Dunnett's test was used to compare the effect of each hydrocarbon alone against the control.

Measuring decay of H and T concentration under field conditions

To measure the decay of these hydrocarbons under similar experimental field conditions, we set up nine pools placed in three blocks on 20 September 2008. Pools were placed in a shady location and filled with 15 L of aged tap water. Water was aged for 24 h and then three treatments were assigned randomly within each block: (1) control – nothing added; (2) alcohol – 30 μL of chemically pure EtOH (min 99.5%); and (3) H + T – 0.49 μg of *n*-heneicosane plus 2.99 μg of *n*-tricosane dissolved in 30 μL of EtOH similar to the H + T treatment applied in field experiments. Chemicals were added close to dusk.

A 500-mL sample was taken from each pool 1 and 24 h after treatments were added. These samples were returned to the laboratory for immediate chemical analysis. Exactly 500 mL of water from each pool for each of the sampling times were placed in a sealed 1000-mL bottle containing 500 mL of MTBE and 20 μg of isobutyl benzene as an internal standard. The chemicals were extracted by vigorous shaking (250 rpm) on a shaker apparatus overnight. The upper MTBE layer was separated, dried with sodium sulphate and concentrated to a final volume of 0.5 mL under a gentle stream of nitrogen. The samples were kept at 4 °C until analysis. A 1-μL aliquot of the concentrated MTBE extract of the sample from each pool was injected into a GC-MSD in the splitless mode.

RESULTS

HS-SPME analysis showed that the common compounds found in the headspace of NCW were mostly C11–C24 alkanes (Table 1) as well as several unidentified compounds displaying a prominent ion 69. All compounds that appeared in the SPME analyses were present also in the solvent extraction of NCW at various concentrations.

Two hydrocarbons, *n*-heneicosane and *n*-tricosane (H and T, respectively), were the only ones present in the air samples above all headspace vessels containing water conditioned by *N. maculata* and not present in any headspace above control water or larval *Anax*-conditioned water samples (Fig. 1 and Table 1). The average (±SE) concentrations were 0.82 ± 0.01 μg for H and 4.98 ± 0.13 μg for T per 250 mL of water sample.

Our field experiments were designed to test the individual and combined effects of these two hydrocarbons as well as NCW on *C. longiareolata* oviposition habitat selection at environmentally relevant concentrations. In the first experiment, H + T mixture and NCW had very similar, statistically significant repellent effects on oviposition (*c.* 65% reduction compared with control pools) on nights immediately following chemical application ($F_{2,15} = 9.67$, $P = 0.002$; see Fig. 2 indicating *post hoc* differences between treatments). However, on the second nights after chemical application, the NCW repellent effect remained strong but H + T no longer repelled mosquito oviposition ($F_{2,15} = 6.53$, $P = 0.009$; see Fig. 2 indicating *post hoc* differences between treatments). These results suggested that at least one of the hydrocarbons repelled oviposition, but by the second night, hydrocarbon concentrations were lowered to a level insufficient to repel oviposition. Consistent with this hypothesis, water samples taken from outdoor pools from the hydrocarbon decay experiment with the same initial concentrations of H + T as in this field experiment indicated a statistically significant reduction (by *c.* 37%) in the concentrations of these chemicals after 24 h (paired *t*-test, $t = 3.23$, d.f. = 2, $P = 0.042$).

The second outdoor mesocosm experiment simultaneously offered five choices. One-way ANOVA (natural log-transformed [$y + 1$]) indicated that the mean reductions in oviposition resulting from T alone and H alone (by *c.* 35% and 44% compared with control pools, respectively) were not statistically significant, whereas H + T mixture (*c.* 65% reduction) and NCW (*c.* 88% reduction) both reduced oviposition statistically significantly ($F_{4,20} = 11.42$, $P < 0.001$; see Fig. 3 indicating *post hoc* differences between treatments). When four treatments except NCW were analysed as a two-way ANOVA, both main effects of H ($F_{1,16} = 7.64$, $P = 0.014$) and T ($F_{1,16} = 4.30$, $P = 0.055$) exhibited reduced oviposition that was statistically significant or near significance, and there was no significant interactive effect ($F_{1,16} = 0.27$, $P = 0.610$). These results suggested that each compound had a repellent effect and the effects of both were additive at these concentrations.

The third, three-choice field experiment comparing each hydrocarbon alone with control demonstrated that both compounds individually repelled oviposition; significantly fewer egg rafts were laid in outdoor mesocosms with T only (*c.* 58% reduction) or with H only (*c.* 47% reduction) compared to mesocosms with control water ($F_{2,12} = 5.92$, $P = 0.016$; see Fig. 4 for Dunnett's *post hoc* comparisons).

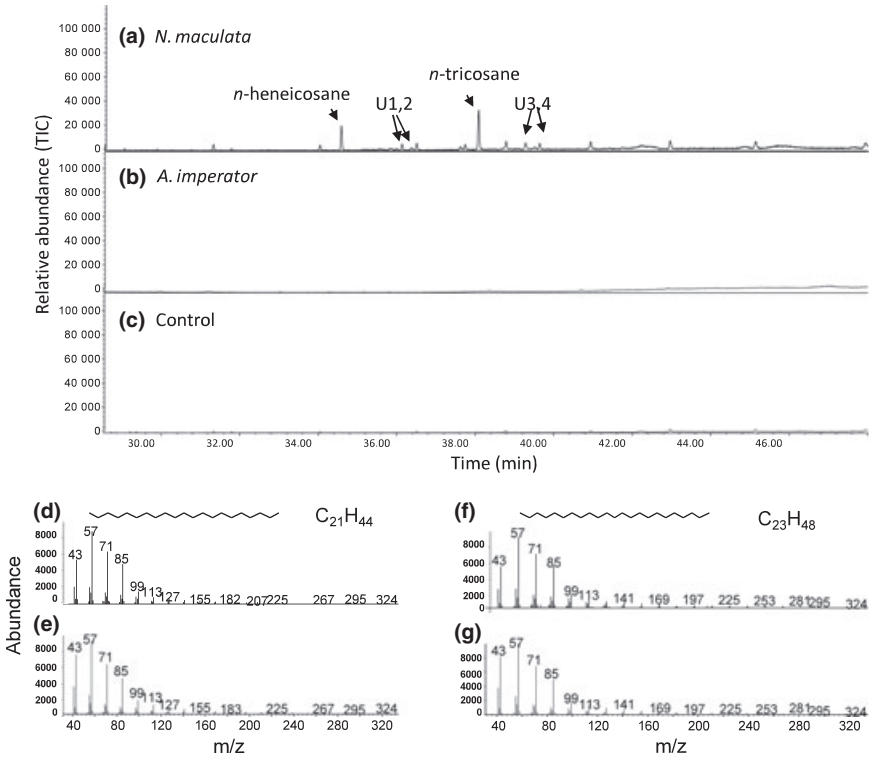

Figure 1 Hydrocarbon emissions from water conditioned with mosquito larvae predators. Chromatograms of the headspace volatiles released by (a) a single *Notonecta maculata* adult in water, (b) a single *Anax imperator* nymph, (c) water only (control) from headspace solid-phase microextraction coupled to gas chromatographic mass spectrometry (total ion count, TIC). The major compounds identified from headspace over *Notonecta*-conditioned water and not found in headspace over other water treatments are *n*-heneicosane and *n*-tricosane. Other unidentified compounds (U1–U4) exhibiting major ion masses at 69 and 111 are also shown. Mass spectra of *n*-heneicosane (d) and *n*-tricosane (f) released by *N. maculata* in our headspace samples and mass spectra of the authentic *n*-heneicosane and *n*-tricosane (e and g), which had identical retention times and mass spectra. The trials were replicated twice with identical results.

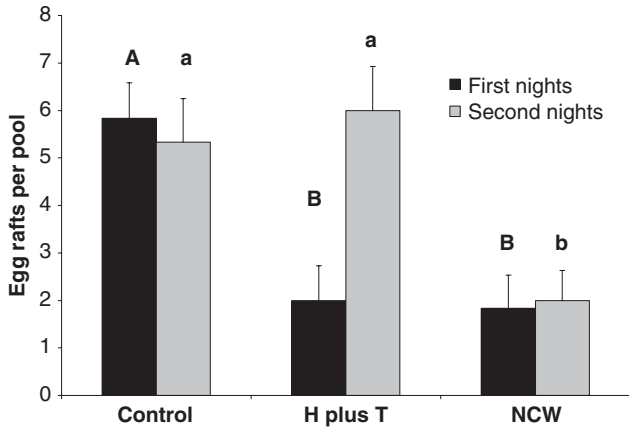

Figure 2 Oviposition habitat selection by *Culiseta longiareolata*: outdoor mesocosm Experiment 1. Female mosquitoes faced an array of outdoor aged tap water pools containing the following treatments: (1) control pools; (2) *n*-heneicosane plus *n*-tricosane (H + T); (3) *Notonecta*-conditioned water (NCW). H + T and NCW (see text for concentrations) were reinoculated every 2 days and oviposition was followed on first nights following pre-dusk inoculations and on second nights after inoculations. On first nights, oviposition (mean number of egg rafts ± SE per pool across the entire experimental period) decreased in H + T and in NCW compared with control pools. On second nights oviposition decreased only in NCW-treated pools. Different letters (capital letters for first nights and small letters for second nights) signify statistically significant differences ($P < 0.05$) by Tukey's HSD.

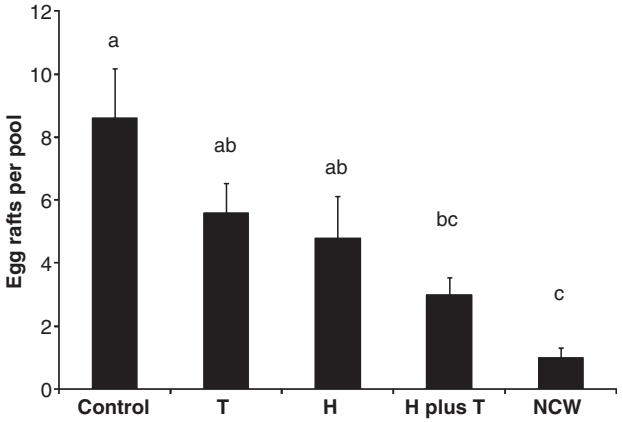

Figure 3 Oviposition habitat selection by *Culiseta longiareolata* measured by mean number of egg rafts ± SE per pool across the entire experimental period: outdoor mesocosm Experiment 2. Female mosquitoes faced an array of outdoor aged tap water pools containing the following treatments: (1) control pools; (2) T (*n*-tricosane); (3) H (*n*-heneicosane); (4) H + T; (5) *Notonecta*-conditioned water (NCW; see text for concentrations). When analysed as one-way ANOVA, number of egg rafts per pool over the entire experimental period significantly decreased in the H + T combination and in NCW compared with control. Different letters signify statistically significant differences ($P < 0.05$) by Tukey's HSD.

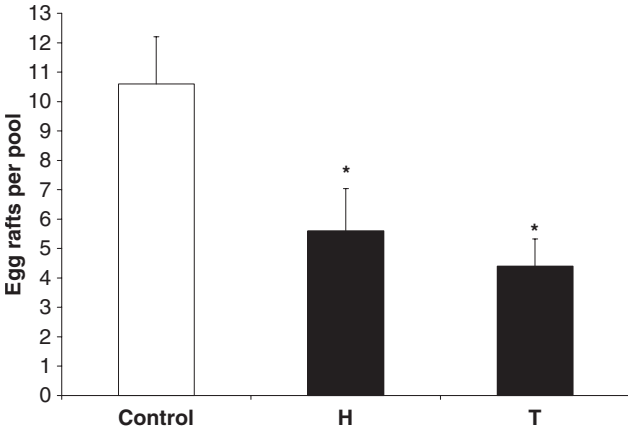

Figure 4 Oviposition habitat selection by *Culiseta longiareolata*: outdoor mesocosm Experiment 3. Wild females faced an array of outdoor aged tap water pools containing one of the following treatments: (1) control pools; (2) H; (3) T (see text for concentrations). Data are means ± SE egg rafts per pool across the entire experimental period. * signifies $P < 0.05$ compared with control pools, one-way ANOVA, Dunnett's *post hoc* test.

DISCUSSION

PRKs are ubiquitous and highly influential on prey responses (Kats & Dill 1998; Brönmark & Hansson 2000; Dicke & Grostal 2001; Van Donk 2007). Current reviews on infochemicals (Dicke & Grostal 2001; Burks & Lodge 2002; Pohnert *et al.* 2007) all point to the extreme lack of information on the chemical identity and on the importance of chemical identification in understanding better the roles of these compounds in shaping induced responses, ecological interactions and food web dynamics. Only a handful of studies have made inroads into chemically identifying PRKs (non-olefinic hydroxy carboxylic acid excreted by *Chaoborus* larvae and detected by *Daphnia*, Tollrian & von Elert 1994; non-olefinic molecule excreted by fishes and detected by *Daphnia*, von Elert & Loose 1996; a peptide 'A-factor' excreted by *Amoeba* and detected by *Euplotes*, Kusch 1999). To our knowledge, our study provides the most complete picture of any PRK: we have chemically identified two compounds, determined environmentally relevant concentrations, used synthetic chemicals to eliminate possibilities that the prey is reacting to other compounds and determined that they repel mosquito oviposition under field conditions at environmentally relevant concentrations. Moreover, the chemical and concentration determinations of these PRKs, combined with extensive context-dependent studies of this *Notonecta–Culiseta* predator–prey system done previously (Spencer *et al.* 2002; Kiflawi *et al.* 2003a,b; Blaustein *et al.* 2004; Eitam & Blaustein 2004; Arav & Blaustein 2006; Silberbush & Blaustein 2008) make the *Culiseta–Notonecta* interaction perhaps the best understood example of PRK-mediated decision-making by prey searching for an oviposition site.

Either hydrocarbon, H or T, alone significantly reduced oviposition. Together, they had an additive effect at these concentrations. Given that the *Notonecta* were starved for 4 days prior to using them, it is likely that the hydrocarbons do not arise from excretions originating from the ingested prey. However, definitive studies to test this are needed. In addition to the H and T identified here as the PRKs, other compounds may also contribute to repel oviposition by this mosquito. Evidence comes from the following. In the first experiment, NCW (without *Notonecta* present) continued to repel oviposition in the second night whereas the synthetic hydrocarbons alone did not repel oviposition by the second night. Our second experiment also suggested that NCW may have reduced oviposition rates more than the combined H + T mixture, although this difference in reduction of oviposition was not significant. Finally, NCW reduced oviposition by *C. longiareolata* for *c.* 1 week (Blaustein *et al.* 2004), far longer than the H + T mixture in this study.

Our HS-SPME analyses of the air above NCW samples revealed H and T in every sample. These compounds, although not classified as highly volatile, are volatile to a certain degree at room temperature (Chickos & Hanshaw 2004). Mosquitoes are physiologically equipped to detect minute concentrations of air-borne chemicals (Clements 1992). In particular, Seenivasagan *et al.* (2009) demonstrated by electroantennagram that the mosquito *Aedes aegypti* can detect *n*-heneicosane in the air. Our findings in this article, combined with our previous finding that *C. longiareolata* can detect NCW without touching the water (Silberbush & Blaustein 2008), suggest that this mosquito is capable of detecting these two hydrocarbons in the air above the water. The decay of hydrocarbon concentration that we found (37% in 24 h) is consistent with the absence of oviposition repellency by these compounds after 1 day. The decay does not necessarily imply that all decay was due to the volatility. The measured concentration could have dropped due to degradation, adsorption to surfaces and intake or breakdown by microorganisms. Other studies of PRKs that affected oviposition by mosquito prey showed persistence times similar to that of H and T found in this study (Blaustein *et al.* 2005; Hurst *et al.* 2010).

Detection of predators in the water from above the air–water interface, rather than on the interface itself, likely has advantages for the female mosquito. In addition to avoiding ovipositing into a pool with high predation risk, the female can also avoid being preyed upon by aquatic predators (McGuffin *et al.* 2006). Although detection from above the water can reduce predation risk, it can also be to a mosquito's demographic and evolutionary advantage to assess the presence of less volatile PRKs together with more

volatile compounds, to increase the likelihood of detecting unsuitability of a pool, especially when water bodies are scarce.

Notonectids chemically induce a variety of responses by a wide range of prey species (e.g. Barry 2000; Blaustein *et al.* 2004). It remains to be determined if the hydrocarbons identified as PRKs here are also responsible for some of these other prey responses. Given that emission of these kairomones incurs a cost to the predator of prey avoidance, these compounds may be necessary for the predator for other reasons or they may be necessary by-products of metabolic pathways. *n*-alkanes and other long-chain hydrocarbons like H and T are important components of insect cuticle and cuticular hydrocarbon composition is species-specific (Lockey 1991). Cuticular hydrocarbons can have various roles including preventing desiccation, providing physical protection and reducing permeability. They can also be important semiochemicals, acting as recognition cues in social insects and sex pheromones in non-social insects (Blomquist & Bagnères 2010) and as signals for task allocation among social insects (Greene & Gordon 2003). Little is known about the composition and roles of cuticular hydrocarbons in aquatic insects although Alarie *et al.* (1998) showed that the composition of cuticular hydrocarbons from an aquatic beetle species is similar to the composition in terrestrial beetle species. Nakashima *et al.* (2004) found that a terrestrial predatory beetle emitted various hydrocarbons including *n*-tricosane, which repelled a parasitoid. This response was beneficial for both species as it avoided predation on the parasitoid wasp eggs and avoided competition between the beetle and parasitoid for aphid hosts.

Adult *Aedes aegypti* mosquitoes are attracted to *n*-alkanes such as heneicosane excreted by conspecifics when ovipositing, thus acting as a pheromone (Mendki *et al.* 2000). In this particular case, this is not necessarily a tradeoff with increased risk of predation because *A. aegypti,* a small-container breeder, and *Notonecta,* a pool or pond inhabitant, do not generally use the same aquatic habitat. Nevertheless, this suggests that some alkanes, including heneicosane, act as both pheromone and kairomone, and may have a potential dual role in influencing intraspecific (pheromones) and interspecific (kairomones) communication. This raises the general question of potential double roles that similar chemicals may play in creating tradeoffs.

Chemically identifying PRKs will pave the way for more precise studies of how risk of predation affects prey behaviour or food web interactions. For example, using specific chemical concentrations rather than predator densities can make for more precise, lower within-treatment variation in assessing whether prey can quantify risk of predation rather than react to it qualitatively. Chemically identifying the kairomones that influence oviposition by *C. longiareolata* and other mosquitoes that are important disease vectors may provide an environmentally friendly means of reducing mosquito populations. The ability to detect a risk of predation may increase individual fitness and increase population size compared with random oviposition (Spencer *et al.* 2002). However, gravid female mosquitoes that avoid predator-free pools containing only PRK are at risk of death while searching for an oviposition site: mosquito adults generally exhibit 20% mortality per day (Service 1993). Repelling mosquitoes from a significant fraction of predator-free pools could increase mortality of adults because they would have to search longer for an acceptable pool, and the concentration of egg rafts in fewer pools could cause greater intraspecific larval competition (Kiflawi *et al.* 2003a). Identifying less volatile PRKs that reduce oviposition might improve a potential commercial oviposition repellent based on compounds such as H and T. Other mosquitoes including *Anopheles gambiae* chemically detect and avoid NCW (Munga *et al.* 2006). Our study should serve as a stepping stone for chemically identifying the PRKs used by other medically important mosquitoes.

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