Oviposition signals and their neuroethological correlates in the Culex ppiens complex

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A R T I C L E   I N F O

Article history:
Received 25 July 2014
Received in revised form 27 September 2014
Accepted 2 October 2014
Available online xxxx

Keywords:
Apical egg droplet chemistry
MOP
GC-EAD
Oviposition behavior
Molecular identification

A B S T R A C T

Mosquitoes in the Culex ppiens complex (Diptera: Culicidae), especially Cx. ppiens and Culex quinquefasciatus, have successfully exploited the rapid growth of the human population and globalization to their advantage by successfully utilizing man-made habitats, particularly for oviposition. Culex spp. lay over 100 eggs together in a raft. Each egg in the raft produces an apical droplet containing an oviposition attractant, erythro-6-acetoxy-5-hexadecanolide, commonly referred to as Mosquito Oviposition Pheromone (MOP). Here we present a detailed gas chromatography–mass spectrometry (GC–MS) analysis of the apical droplets from six populations that revealed MOP as the most abundant constituent. Subjecting MOP and the remaining 17 most abundant chemical constituents of the droplets from these populations to a Principal Component Analysis (PCA) resolved the populations into two distinct clusters that contained two populations each of Cx. quinquefasciatus and Culex ppiens molestus. The two Culex ppiens ppiens, however, did not resolve into a single cluster, with the Shasta population sorting closer to Cx. quinquefasciatus. Comparing the PCA scores with the genetic evidence from adult females using available molecular markers that have earlier shown to sort various Culex forms, we found that the molecular data support the PCA clustering pattern. Behavioral investigation of the droplet-induced attraction tested in gravid Cx. quinquefasciatus elicited various degrees of oviposition to the droplets from each population. Overall, droplets from all six populations induced higher attraction compared to controls. A detailed time-course analysis of droplet composition in Cx. quinquefasciatus from 6 to 54 h post egg-laying identified MOP again as the main constituent. Finally, our electrophysiological investigation identified MOP as the only biologically active constituent from of the droplets eliciting responses from female antennae. These studies will aid in global efforts to understand the vector biology and evolution that can be exploited to develop novel vector management strategies.

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http://dx.doi.org/10.1016/j.meegid.2014.10.007
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1. Introduction

Mosquitoes in the Culex ppiens complex (Diptera: Culicidae) transmit various pathogens that infect humans such as St. Louis encephalitis virus (SLEV), West Nile Virus (WNV) and filarial worms (Atkinson and Collins, 2014; http://mosquito-taxonomic-inventory.info/simpletaxonomy/term/6165, 2014). This complex is characterized by the presence of a range of species with various biotypes or forms and many hybrids (Harbach, 2012; http://mosquito-taxonomic-inventory.info/simpletaxonomy/term/6165, 2014; Kothera et al., 2013; Kothera et al., 2009; Reisen, 2012; Vinogradova, 2000). Several key traits make members of this complex a formidable vector across the globe. One is their ability as adults to feed on many hosts (Farajollahi et al., 2011), and another is the ability of their larval forms to exploit ‘dirty-water’ rich in organic nutrients generated by humans and their livestock (Bockarie et al., 2009; Vinogradova, 2000). An additional trait that makes Culex female mosquitoes unique among major insect vector species is their habit of laying eggs in clusters of over 100 eggs, called rafts (Clements, 1999). The first clear demonstration of the attractiveness of egg rafts to their conspecific gravid females was demonstrated in Culex tarsalis (Osgood, 1971; Osgood and Kempster, 1971). Subsequent studies reported the attraction of gravid Culex quinquefasciatus to conspecific egg rafts that was later attributed to the presence of apical droplets on the eggs (Bruno and Laurence, 1979). A detailed chemical analysis of the apical droplets by gas chromatography–mass spectrometry (GC–MS) identified the most abundant component in the Cx. quinquefasciatus apical
droplet as erythro-6-acetoxy-5-hexadecanolate, commonly referred to as Mosquito Oviposition Pheromone (MOP) (Laurence and Pickett, 1982). Behavioral studies involving egg rafts/apical droplet or synthetic MOP as a cue revealed the attraction of gravid females under semi-field (Braks et al., 2007) and field conditions (Mboera et al., 2000; Olagbemimo et al., 2004; Otieno et al., 1988; Wachira et al., 2010). MOP is also shown to be behaviorally active in inducing increased oviposition on its own and synergistically higher responses in the presence of organic-material rich water or their constituents (Du and Millar, 1999; Michaelakis et al., 2009; Navarro-Silva et al., 2009; Pickett and Woodcock, 1996).

Olfaction plays a key role in defining oviposition behavior (Bentley and Day, 1989). Mosquitoes are endowed with an elaborate and sophisticated olfactory system sensitive to a diverse array of chemical cues that enables them to detect and discriminate the chemical landscapes. The main olfactory organ in Culex, the antenna, is adorned with thousands of sensilla that house olfactory receptor neurons (ORNs) responding to a variety of volatile chemicals (Hill et al., 2009; Syed and Leal, 2009). The detection of MOP by Cx. quinquefasciatus antenna was demonstrated employing an electroantennogram (EAG) method (Blackwell et al., 1993; Mordue et al., 1992) that approximately represents the summed depolarization of all the ORNs stimulated by a given odorant. An exhaustive screening of all the olfactory sensilla types on the antenna in Cx. quinquefasciatus lead to the identification of a unique sensillum type. All that responded to synthetic MOP by increasing the spontaneous firing frequency of one of the two ORNs housed in the sensillum (Leal et al., 2008). Interestingly, the other co-located neuron responded to indole and methyl indoles, with highest sensitivity to 3-methylindole (skatole), a major constituent in the polluted habitats preferred by ovipositing Culex (Du and Millar, 1999; Millar et al., 1992; Pickett and Woodcock, 1996), thus highlighting the potential significance of these key odorants in spatiotemporal resolution of peripheral coding information needed by the brain (Bruce et al., 2005; Guidobaldi et al., 2014; Ibara et al., 2013; Su et al., 2012).

Targeting vector species offers a credible alternative in managing disease transmission; however, vector manipulation demands a clear understanding of mosquito habits and habitats (Pickett et al., 2010). Even though the first unequivocal evidence for the occurrence of an oviposition pheromone in any disease vectors was in the Culex mosquitoes, there are large gaps in our understanding of this sophisticated yet complex behavior resulting from the interaction of chemical(s) in the egg droplets and their sensory correlates in Culex species. Here we address some of the outstanding questions. How conserved is the egg raft derived MOP among Culex populations, some of which are sympatrically evolving and others that are not? Are there additional chemosensory cues in the egg droplets that are employed by different populations? Can egg droplets (and their derived constituent substances) provide a unique signature that can be used as a marker for resolving the species complex?

In this comprehensive report, we present the results from our detailed chemical analysis using GC–MS of the apical droplets from global populations of Culex species: Cx. pipiens biotype pippens (originating from Shasta, California, USA and South Bend, Indiana, USA), Cx. pipiens biotype molestus (from Chicago, IL, USA and Tokushima, Japan), and Cx. quinquefasciatus (from Johannesburg, South Africa and Boane, Mozambique) that revealed the presence of MOP as the most abundant constituent across biotypes. Using Principal Component Analysis (PCA), we were able to resolve the chemical profiles into species specific groupings based upon the 18 most abundant compounds including MOP. We employed PCR using the biotype specific diagnostic primers to compare and contrast molecular results with PCA. We further evaluated the behavioral consequences of the noted chemical differences among biotypes to determine whether attraction was confined to biotypes. Since the eggs hatch approximately 50 h after being laid and remain attractive to the gravid females, we studied the chemical composition in a time-course analysis spanning from 6 h to 54 h after oviposition. Finally, for the first time, we report the sensory physiological responses of female Cx. quinquefasciatus antenna to the apical droplet constituents as they eluted from a high resolution chromatographic column by GC-linked chromatographic detection (GC-EAD).

2. Materials and methods

2.1. Insects

Mosquitoes from colonies maintained at the University of Notre Dame (Notre Dame, IN, USA) and described elsewhere (Hickner et al., 2013) were used throughout this study. Two Cx. quinquefasciatus populations are originally from Johannesburg, South Africa (CqJHB) and Boane, Mozambique (CqBOA) respectively. CqJHB is the strain that was used in Cx. quinquefasciatus genome project (Arensburger et al., 2010). The four Cx. pipiens populations are from Chicago, IL, USA (CpmCh), Tokushima, Japan (CpmSHI), South Bend, IN, USA (CpPSB) and Shasta, CA, USA (CpPSHA). The CqJHB, CpPSHA and CqBOA strains were originally provided by Anton Cornell, University of California Davis (Cornel et al., 2003) and maintained at the laboratory of David Severson, University of Notre Dame. Shinkura (CpmSHI) strain was originally founded from a single female collected in Tokushima, Japan in 1998 (Hickner et al., 2013). The Chicago strain was collected in 2009 from a below-ground drainage sump (Mutebi and Savage, 2009). The South Bend strain was established from >4000 larvae collected from a single container in South Bend, Indiana, USA in 2009 (Hickner et al., 2013). Colonies were maintained in an insectary at 27 °C with 85% relative humidity and a photoperiod of 16:8 h light:dark cycle with 30 min of dusk and dawn transition.

2.2. Apical droplet analysis by gas chromatography–mass spectrometry (GC–MS)

Egg droplets from the six populations with three to five replicates each were analyzed by Solid Phase Micro-Extraction (SPME) and GC–MS analysis. Each replicate consisted of a freshly laid egg raft. Chemical analysis of the droplets was performed by using SPME fiber (50/30 um DVB/CAR/PDMS Stableflex 23 Ga; Supelco, USA). For each biotype, five droplets from a single egg raft, laid at least 12 h before collection, were collected onto a clean SPME fiber that was conditioned earlier as per the manufacturer’s instructions. The fibers were retracted and injected onto a split–splitless injector of a GC–MS. Chemical analyses were performed on a 7890A GC system (Agilent Technologies, CA) coupled with a 5975C Agilent Technologies mass spectrometer (inert XL MSD with a Triple-Axis Detector). SPME fibers were injected in a splitless mode at 270 °C onto an Agilent HP-5 capillary column (30 m, 0.32 mm ID, 0.25 μm phase thickness). Helium (Ultra High Purity 5.0 Grade; Airgas, USA) was used as the carrier gas at a constant flow rate of 1 ml/min. The column was held isothermally at 40 °C for 1 min, then programmed to increase at a rate of 7 °C per minute until it reached 280 °C where it was held for 2 min and finally the temperature was ramped at 20 °C/min until it reached 300 °C, where it was held for 5 min. MS was operated at 70 eV. Data recording and quantification was performed using Agilent MSD ChemStation software (E02.02.1431).

The ten largest peaks (by abundance) from the Total Ion Chromatograms (TIC) of each droplet sample from the populations were selected for further analyses. These ten compounds were not
the same for each population, thus each of the 10 compounds from every sample were localized in the TICs of each population, resulting in 18 peaks in total. The area under each of the 18 peaks was well above the detection limit of the MS. Finally, due to the possible variation in the sample amounts extracted from different egg rafts in each population, the absolute area under each peak was calculated by summing the areas of all the 18 compounds in each replicate for each population. The percent contribution of each compound to this sum was then transformed and analyzed (below). The identity of the major constituent, MOP, was based on the confirmation of retention time and its mass spectra compared by injecting the synthetic standard (Bedoukian Research Inc., USA).

2.3. Diagnostic PCR for population verification

DNA was extracted from mosquitoes using alkaline method (Rudbeck and Dissing, 1998). In brief, specimens were ground in 80 μL of 0.2 M NaOH, incubated at 75 °C for 10 min and suspended in 1000 μL of pH 8.0 solution containing 0.01 M NaOH and 0.018 M Tris–HCl. Initial diagnostic PCR were carried out to resolve the Culex populations using two sets of primers. The first set used two forward (QACE: TTATAGTTAATTGTTGAGA; PACF: TTATAGTTATGGTGAGAAA) and a reverse primer (B1246: TGGAG CCTCTCTTACCGG) (Aspen and Savage, 2003). QACE and B1246 amplify sequence specific to Cx. quinquefasciatus, whereas PACF and B1246 amplify Cx. pipiens from the acetylcholinesterase gene 2 (Ace-2; spanning full length intron 2 flanked by a part of two adjacent exons). Next, primers pipiens complex was further resolved by employing Bahnck and Fonseca’s protocol (Bahnck and Fonseca, 2006) that used two reverse primers based on the indels flanking the microsatellite region for CQ11 locus (pipCQ11R: CAGTTGAGCTTGGTGGTA; molCQ11R: CACTCCCTAGTTACACAC and a forward (CQ11F2: GATCCTGCAAGCAGGAAA). The CQ11F2 and pipCQ11R primer combination specifically amplifies Cx. pipiens s.s. locus which is 27 bp smaller compared to Culex p. molestus that is amplified by CQ11F2 and molCQ11R combination. PCR reactions were carried out in 25 μL volume containing a final concentration of 1X of Taq buffer (50 mM KCl, 10 mM Tris pH 9.0, 0.1% Triton X), 200 μM of dNTP's, 0.2 μM of forward and reverse primer (0.4 μM for multiplex), 1 unit of Taq polymerase, and 1 μL of template DNA. The PCR condition used were: 94 °C for 5 min, 35 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, concluding with an extension of 72 °C for 5 min. All PCR products were resolved on 1.8% agarose gel and visualized using ethidium bromide stain.

2.4. Apical egg droplets choice assay

Gravid Cx. quinquefasciatus (CqJHB) were used as the test species to evaluate the attractiveness of apical droplets collected from egg rafts of the various populations in a laboratory choice assay (Bruno and Laurence, 1979). Apical droplets from the egg rafts were collected using a freshly drawn out glass capillary (100 mm long and 1.2/0.68 OD/ID mm; 1B120-4, World Precision Instruments Inc., FL) with a tip diameter of 0.10 μm and added to glass distilled hexane (Fisher, >98.5% purity) in a 3 ml Wheaton™ high recovery NextGen™ V-Vial attached with PTFE lined septa caps (Wheaton, NJ) and preserved at −20 °C until use. An injection of this hexane solution (equivalent to SPME amounts) under similar condition (above; for the direct SPME measurements) resulted in the comparable spectra. An aliquot of this solution containing ca. 15 apical droplets equivalent or equal volume of hexane alone as control were added onto a glass coverslip (18 × 18 mm; 48366-045, VWR International LLC, PA). Coverslips were allowed to dry then placed over the water surface in an oviposition bowl (glass, 10 × 4.5 cm). Two bowls with floating cover slips were then placed into a cage (30 × 30 × 30 cm; BioQuip, USA) housing gravid mosquitoes from 5 pm until 9 am the next morning. All tests were done in a controlled insectary kept at 26 °C with 50% relative humidity and a photoperiod of 13:11 h light:dark cycle with an hour of dusk and dawn transition. At the end of the 16 h period, bowls were removed from the cages and the number of egg rafts counted. Up to 5 replicates were carried out for each experiment, and the position of the bowls in the cage was altered between each replicate. The significance of the mean difference between test and control bowls in each experiment was estimated by Student's t test.

2.5. Time-course analysis of apical egg droplet maturation

Gravid females of CqJHB strains were allowed to oviposit in an egg-laying bowl that was removed soon (within 15 min) after the deposition of the first raft. The egg raft was allowed to mature for 6 h as evidenced by the appearance of clearly visible apical droplets. Five droplets were collected onto a SPME fiber (above) at every 6 h interval until 30 h. Two more harvests at 42 and 54 h after deposition were made, since the usual 6 h interval did not result in noticeable apical droplet accumulation. At each time point, 5 apical droplets were removed from the same 5 eggs as the previous collection. The remainder droplets were removed from the egg raft prior to collection in order to avoid over-collection. The samples were subjected to analysis as above on GC–MS. Egg rafts were maintained at 25 °C.

2.6. GC-linked electrophysiological recording from Cx. quinquefasciatus CqJHB females to the apical droplets

The GC-EAD protocol was essentially similar to Syed and Leal (Syed and Leal, 2009). Apical droplets collected in hexane (above) were subjected to GC-EAD analysis using CqJHB female antenna as a detector to locate biologically active constituents from the apical droplets. A GC (7890A; Agilent Technologies, USA) equipped with a capillary column (HP-5 30 m × 0.32 mm, 0.25 μm; Agilent Technologies) was used. The column flow was constant at 3 ml/min, inlet was held at 250 °C, and the Flame Ionization Detector (FID) was maintained at 320 °C. Samples were injected in splitless mode and the program was: 200 °C/min, increased to 300 °C at a rate of 10 °C/min and held there for 5 min. Resolved chemical constituents were split at the end of the capillary column 1:3 between FID and the heated transfer line that carried the effluents to a restrained female mosquito antenna bathing in charcoal filtered humidified airflow. Temperature control unit for the transfer line and oflactometer to deliver clean air were from Syntech (Germany). Signals from antenna were amplified, digitized and recorded on the hard disk of a PC. Hardware and Software to record and analyze the data were also from Syntech (Germany). The ground and recording electrode made from drawn out glass capillary tubes (above) both contained a 0.1 M KCl solution with 0.05% polyvinylpyrrolidone (PVP) and were pulled on a Laser Based Micropipette Puller System (P-2000 Sutter Instruments, CA). Fresh Mosquitoes were used for each recording, and the sensitivity of the antenna at the beginning of the recording and end was verified by challenging it with a 500 ms pulse of nonanal (~2 dilution deposited on to a filter paper) during which 1 ml of the headspace volume from a 5 ml polypropylene syringe was ejected onto the main airflow blowing over mosquito antenna. Mosquitoes with antennal response under 0.5 mV were discarded.

2.7. Statistical analysis

Apical droplet chemistry data was analyzed by Principal Component Analysis (PCA) on the percentile data. Centered Gaussian
normalization was applied in order to bring the percentages of all chemicals into the same scale. The first three principal components (explaining ~72.9% variance) were retained for the subsequent statistical analysis. Each of the remaining principal components (PCs) accounts for proportion of variance of single digits, and therefore they are not kept. Multivariate analysis of variance (MANOVA) was performed with first three PCs being the dependent variables for both experiments (locations and time effects). The post-hoc tests of MANOVA were performed between different locations and are summarized into a p-value matrix. For these 15 comparisons, an alpha value equal to 0.05 was considered significant since less than one treatment can be expected to be a false positive at this alpha value (0.05 × 15 = 0.75). In other words, less than 1 out of 15 comparisons would be expected to turn out significant due to type-I error. Therefore we continued using alpha = 0.05 to interpret the results instead of applying Bonferroni correction, which would be too stringent and conservative here.

All statistical analyses were conducted using R3.1.1 (R-Team, 2012). Python Matplotlib1.3.1 package was used to prepare the 3D PCA plot (Hunter, 2007). Behavioral data was analyzed by paired tests for each trial comparing two treatments (droplets from different populations and hexane as control).

3. Results

3.1. Apical droplet analysis by gas chromatography–mass spectrometry (GC–MS)

Our investigations into egg-droplet chemistry across 6 Culex populations revealed MOP as the dominant peak in every population studied as measured by GC–MS (Figs. 1 and S1). An overall comparison of any variation in the droplet chemical composition was performed using Principal Component Analysis (PCA) that captures as much of the variation in the data as possible. Using the area under each of the 10 largest peaks (by abundance) from every population as the variables, we extracted the PCs and performed post-hoc comparisons across populations. The first three components (PCs) were retained, which together accounted for 72.9% variation (Fig. 2 and Table 1), with PC1 extracting more than 35% of the variation in the dataset (35.6%). We set a PCA factor loadings value above 0.25 (highlighted as bold in the Table 1) to identify the chemicals primarily contributing to the first three PCs. While MOP and the compound with the KI 2501 contributed positively, the PC1 was weighted negatively by compounds of KI 2139, 2267, 2365 and 2417 (Table 1). Separation of species complex (Fig. 2A) can be further illustrated by the major contribution from PC1 alone (Fig. 2B). The proximity of CppSHA (C. pipiens pipiens) to the quinquefasciatus (CqHB and CqBOA), as indicated in Fig. 2A can be broadly explained by PC2 (Fig. 2B) that is positively influenced by compounds with KI 2304, 2342 and 2363, whereas weighted negatively by 2267, 2283, 2306 and 2482.

Multivariate analysis of variance (MANOVA) of the first three principal components revealed highly significant differences among the six mosquito populations in terms of location ($p = 8.317e–10$; Support Table 1). Post-hoc pairwise comparisons among the six populations are summarized in Table 2. In total, 8 out of 15 comparisons turned out to be significant at an alpha value equal to 0.05. No significant differences between the two quinquefasciatus (CqBOA vs. CqJHB; $p = 0.098$) or two pipiens molestus (CpmSHI vs. CpmCHI; $p = 0.160$) populations were noted. Overall, the two quinquefasciatus populations (CqHB and CqBOA) clustered together, as well as the two molestus populations (CpmCHI and CpmSHI), as indicated by the dotted lines in Fig. 2A. The populations of pipiens however did not resolve in discreet clusters, with CppSHA clustering closer to CqJHB as displayed by the higher $p$ value ($p = 0.110$).

3.2. Diagnostic PCR for population verification

Culex populations used in our study were genetically verified by initially resolving them between Cx. quinquefasciatus and Cx. pipiens complexes (Fig. 3A) by using a diagnostic PCR following the Aspen and Savage protocol (Aspen and Savage, 2003). Primers QACEF and B1246 amplified sequences specific to Cx. quinquefasciatus, whereas PACEF and B1246 amplify Cx. pipiens complex by amplifying acetylcholinesterase gene 2. With the exception of CppSHA, that has been earlier categorized as Cx. p. pipiens (Cornel et al., 2003) other populations were resolved accordingly. Our CppSHA colony is derived from the individual used in (Cornel et al., 2003) study. The discrepancy in CppSHA reported here thus can be explained by the reported introgression between Cx. pipiens s.s. and Cx. quinquefasciatus in California (Cornel et al., 2003; Kothera et al., 2013; Strickman and Fonseca, 2012).

Fig. 1. Chemical profiles of the apical egg droplets collected from egg rafts of the members of Culex spp. complex. (A) Total Ion Chromatograms. Arrow indicates the Mosquito Oviposition Pheromone (MOP). Retention time for CqBOA and profiles above are slightly shifted for clarity. (B) Percent composition of the abundant compounds showing MOP and the compound with the KI 2501 contributed positively, whereas the compound with the KI 2304, 2342 and 2363 contributed negatively. CqBOA: Boane, Mozambique; CqJHB: Johannesburg, South Africa; CppSHA: Shasta, CA, USA; CppSBE: South Bend, IN USA; CpmCHI: Chicago, IL USA and CpmSHI: Shinkura, Tokushima, Japan.

3.3. Apical egg droplets choice assay

Egg droplets from the six populations induced varying degree of oviposition from our laboratory Cx. quinquefasciatus CqJHB females. Egg droplets that were dissolved in hexane from each geographical location were tested in a choice oviposition experiment wherein gravid CqJHB females had a choice to lay their eggs between two oviposition bowls, each added with ca. 2 μl of hexane alone that served as control, or with 15 egg droplets equivalent in hexane. Initial tests wherein hexane alone was added in both bowls induced equal oviposition (Fig. 4). Droplets from CqBOA induced the highest oviposition response in CqJHB strains, followed by their own droplets (CqJHB), CppSHA and CpmSHI, respectively. Droplets from CppSBE and CpmCHI did not induce significant increase in oviposition as compared to the solvent.

3.4. Time-course analysis of apical egg droplet maturation

Next we studied in detail the chemical profile of the droplets as they mature in Cx. quinquefasciatus (CqJHB). A full sized droplet appeared 6 h after the egg-laying and the droplet chemistry at that point revealed a full spectrum of chemicals as shown in Fig. 1. Analysis of droplets from the same eggs at 6 h intervals revealed MOP as the most abundant compound all through (Fig. 5A). There was an overall decrease in the amounts of constituent compounds produced with subsequent 6 h sampling period, and we had to allow 12 h interval in sample collection post-30 h to observe the droplets. The two samples at 42 h and 54 h after egg-laying still allowed 12 h interval in sample collection post-30 h to observe the droplets. The two samples at 42 h and 54 h after egg-laying still retained MOP as the major constituent. PCA revealed overlapping components (Fig. 5B and C) and MANOVA of the first three principal components did not reveal any significant differences between the droplet composition at different time intervals (p = 0.06; Support Table 2). Since the omnibus test is not significant, no post-hoc pairwise comparison was performed here. To test if repeated harvesting of apical droplets from the eggs changes the chemical profile, we compared the profile of those five droplets at 54 h after repeated harvests with 5 droplets collected from...
untouched eggs from the opposite end of the same egg raft. Profiles did not reveal any qualitative differences, with MOP being the most abundant compound in both (data not shown). However, untouched droplet over 54 h time period showed overall higher abundance of constituent compounds due to the additive effect of the continual production of compounds over time.

Fig. 4. Apical egg droplets collected from various Culex populations induce variable oviposition response from Cx. quinquefasciatus (CqJHB) gravid females in a laboratory choice test. Hexane is used as control. Pair wise statistical analysis was carried out using two tailed student – t test assuming equal variances, based on Shapiro – Wilk’s normality test (Average ± SEM). n = 3–5, *p < 0.01, **p < 0.001, ns = non-significant.

4. Discussion

Emergence of insecticidal resistance in Culex spp. to the major categories of pesticides, such as organophosphates (Hemingway, 1982) and pyrethroids (Amin and Hemingway, 1989; Ben Cheikh et al., 1998; Chandre et al., 1999) necessitates exploring alternate methods of vector management. Recent advances in our understanding of mosquito physiology and behavior offer unique potential for manipulating the vectors of pathogens during their quest for a host, a mate, and an oviposition site via olfactory detection of semiochemicals (Pickett et al., 2010).

In order to identify the chemosensory basis of the reported gravid female attraction to the oviposition sites across different populations of Culex, we began with a robust chemical analysis of the apical droplets. We observed significant differences in chemical composition between Cx. quinquefasciatus, Cx. p. molestus, and Cx. p. pipiens forms (Figs. 1 and 2). It is interesting to note that the close clustering of CpmCHI and CpmSHI representing Cx. p. molestus (p = 0.160), and CqJHB and CqBOA populations that represent Cx. quinquefasciatus (p = 0.098) in our PCA analysis confirm the earlier classification of these forms based on taxonomic history (Harbach, 2012) and molecular data (Bahnck and Fonseca, 2006; Kothera et al., 2013; Urbanelli et al., 1997a). However, the same PCA analysis placed CppSHA (Cx. p. pipiens form) closer to Cx. quinquefasciatus (p = 0.110), as against the ‘comparable’ Cx. p. pipiens from CppSBE (p = 0.022). These results (Table 2) give further credence to the intermixing of Cx. quinquefasciatus and Cx. p. pipiens in California and indicate that CppSHA strain is indeed a hybrid population (Cornel et al., 2003; Kothera et al., 2013; Strickman and Fonseca, 2012; Urbanelli et al., 1997b). In addition, the diagnostic species specific PCR supported the suggested genetic introgression as CppSHA annealed to the primer specific to the ACE 2 region of Cx. quinquefasciatus (Fig. 3A) and did not anneal to pip-CQ11R, primers specific to Cx. p. pipiens (Fig. 3B). The most significant difference in the apical droplet chemistry (p = 0.005) was noted between CqBOA and CpmSHI.

Specificity or lack thereof, of the Culex apical droplets in inducing oviposition among various forms in Culex species or among genera, has been controversial. Early work defined the droplet as genus-specific rather than species-specific; Cx. quinquefasciatus droplets elicited comparable responses from Cx. tarsalis and Cx. p. molestus as well as Cx. quinquefasciatus (Bruno and Laurence, 1979). Recent data however suggested that at lower doses (1–15 egg rafts) Cx. quinquefasciatus droplets also induce increased oviposition in An. gambiae s.s. and this behavior is reversed at higher raft densities (Wachira et al., 2010).
extended our chemical analyses to the behavioral observations wherein we exposed gravid *Cx. quinquefasciatus* CqJHB to the apical droplets collected from all other *Culex* forms. The numbers of eggs laid by the laboratory CqJHB form were highest in response to the droplets from the *Cx. quinquefasciatus*. However, the droplets from CppSHA and CpmSHI forms also induced significant increase in oviposition compared to controls. Overall, compared to solvent controls, all the extracts received more than 50% of the total eggs deposited in any given test, thus highlighting the plasticity in oviposition response among *Culex* spp. to the droplets induced attraction. It has been earlier shown that *Cx. tarsalis* was less attracted to the *Cx. quinquefasciatus* derived droplets than *Cx. quinquefasciatus* (Hwang et al., 1987). Our bioassays, like many others that have been routinely employed in MOP research over decades, have had a limitation that it measures only the overall effect on total egg deposition without the detailed components leading to the egg laying. One potential confounding effect could be the priming effect of the first egg raft wherein the subsequent gravid females perceive the stimulus as a combination of the applied stimulus (egg droplet in solvent) and the egg rafts laid earlier, possibly resulting in additive or synergistic effects leading to enhanced oviposition (Clements, 1999). However, this is also expected to occur in the field (Braks et al., 2007; Reiskind and Wilson, 2004).

We observed eggs rafts beginning to hatch approximately 50 h after being laid, and it is reported that the egg rafts remain attractive to the gravid females for the period (Clements, 1999). Our time-course analysis of the chemical composition spanning from 6 h to 54 h after oviposition explored the presence of any qualitative differences among the constituent compounds, and results did not yield any significant differences among the time points. Earlier work reported the appearance of the apical droplet soon after oviposition, reaching their maximum size about 24 h later (Laurence et al., 1985; Laurence and Pickett, 1985). Finally, we were the first to utilize the GC-EAD in the long and distinguished history of research on isolation of biologically active constituents from the apical egg droplets (Pickett and Woodcock, 1996). We had anticipated isolating and identifying more constituent compounds other than MOP, the most abundant compound of the droplets, by utilizing a very sensitive detector, the mosquito antenna as the sensing
element. However, we consistently recorded electrophysiological activity in response to MOP alone. This could either be due to the fact that MOP is the only active compound and that the varying amount of MOP induces differential response among different forms, and/or there could be potentially other constituents present in very low abundance unable to induce measurable response in our GC-EAD set-up. From the published work, it appears that the MOP elicited antennal responses only at higher doses (Blackwell et al., 1993; Mordue et al., 1992).

While MOP appears to be the major conserved signal, egg droplets also contained variation in populations, thus enabling the separation of complex into ‘clusters’ (p. piiienis, p. molestus or p. quinquefasciatus) by PCA based on the qualitative and quantitative differences in the droplets chemical composition. Signals and reception rarely arise de novo, and this co-evolutionary process of signaling and reception can be best studied in the evolution of oviposition decisions as less-than-perfect signaling may severely lower both reproductive success of individuals and fitness of the progeny (Herrera-Varela et al., 2014; Refsnider and Janzen, 2010). It will be exciting to correlate the chemical signatures from the Cx. p. piiienis complex egg rafts (noted in this study) with the chemosensory receptor repertoires (esp. olfactory and gustatory receptors) in the Culx spp. genes that potentially act as determinants in the detection and discrimination of host, mate and oviposition habitats. Such co-evolutionary studies have led to novel insights in the model organism, Drosophila, wherein altered ecological niches (defined by unique chemical landscape) of fly species could be directly correlated with the chemosensory genome repertoire from the 12 sequenced fly species (Arguello et al., 2013; McBride, 2007; Sanchez-Gracia et al., 2009). Similar studies await the recent sequencing of 16 Anopheles species (Neafsey et al., 2013). Sequencing the members of Cx. p. piiienis complex will offer exciting possibilities to study the co-evolutionary processes in chemical communication since this mosquito complex has successfully adapted to diverse habitats resulting in widespread global distribution. In addition, members of Culex complex uniquely display a mixed feeding pattern between birds and humans enabling them to transmit several avian pathogens to human. These host-shifts have been suggested to be facilitated by a key olfactory stimulus, nonanal, produced by humans and birds (Syed and Leal, 2009), leading to the intriguing suggestion that “To mosquitoes, we smell like bird” (Enserink, 2009). Given this enormous potential, it is surprising that only one member of the Culex complex, Cx. quinquefasciatus (CqJHB) has been sequenced so far (Arenburger et al., 2010).

Overall, our detailed GC–MS and GC-EAD analyses presents the first clear evidence for the conservation of MOP as the main biologically active constituent of the egg droplets across various Cx. p. piiienis forms, and further reveals a significant similarity between the conspecifics such as Cx. quinquefasciatus (CqJHB and CqBOA) and Cx. p. molestus (CpmCHI and CpmSHI). The only exception was Cx. p. pipiens (CpBE and CpSHA) wherein CpSHA aligned more with CqJHB and CqBOA populations of Cx. quinquefasciatus. This, however, correlates well with the molecular evidence suggesting the introgression of Cx. p. pipiens and Cx. quinquefasciatus in California (Bahnck and Fonseca, 2006; Kothera et al., 2013; Strickman and Fonseca, 2012; Urbanelli et al., 1997a). We believe that our comprehensive analysis of oviposition chemistry across various Cx. p. piiienis forms and the underlying neuroethological observations significantly contribute to the evolutionary understanding of the oviposition trait in the Cx. p. piiienis complex. In addition, our study also suggests MOP as a potential tool in developing effective trapping strategies towards monitoring and managing Culex mosquitoes, especially in areas with mixed populations.

Acknowledgements

We thank Dr. Akio Mori (Prof. David Severson lab, Notre Dame) for providing mosquito colonies. Members of Syed laboratory are acknowledged: Dr. Paul Hickner for assistance with PCR analysis; Dr. Madhura Siddapajji for help in analyzing oviposition data; Nicole Scheidler for comments on the earlier draft of this m/s; Jennifer Topolski and Eric Noakes for help in mosquito care. This research is supported by a grant (to ZS) from the Eck Institute for Global Health, University of Notre Dame (UND). GAS was also supported by College of Science Summer Undergraduate Research Fellowship (UND).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.meegid.2014.10.007.

References


