Behavioral evidence for the existence of a region-specific oviposition cue in Anopheles gambiae s.s.

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ABSTRACT: Understanding oviposition behavior is important to behavioral and vector ecologists because of its potential use in developing vector control strategies for insect-borne infectious diseases. Our study compared the oviposition behaviors of Anopheles gambiae s.s mosquitoes from two different regions of East Africa, Mbita Point, Kenya and Ifakara, Tanzania. The work sought behavioral evidence for the presence of an olfactory cue that modulates oviposition behavior in these different regional strains of Anopheles gambiae s.s. Results demonstrated that the larval rearing water of the different mosquito strains produced a signal that yielded a positive oviposition response from Anopheles gambiae s.s. gravid females of the same region. This not only implies the presence of an olfactory determinant of oviposition but it also could be a model for how speciation could arise within related taxa of mosquitoes. Journal of Vector Ecology 33 (2): 321-324. 2008.

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INTRODUCTION

Recent breakthroughs in molecular genetics have identified sub-taxa within Anopheles gambiae s.s (Coluzzi et al. 2002, della Torre et al. 2005) that provide evidence for the onset of the speciation process (della Torre et al. 2002). Evidence for the behavioral consequences of this variation, however, remains scant. Some suggest that understanding Anopheles gambiae behavioral ecology is important as it may compliment the increasing usage of molecular approaches to studying insect population structure, facilitating a more comprehensive understanding of Anopheles gambiae s.s. biology and ecology (Enserink 2002).

One of the candidate behaviors most likely to differ among related taxa of insects is oviposition, as it plays a critical role in sympatric speciation in other insect species (Bush et al. 1977, Weiblen and Bush 2002, Dambroski et al. 2005). Oviposition behaviors have also been studied by vector biologists because of the past success of larval habitat reduction strategies in dengue fever vector control programs (Newton and Reiter 1992). Further understanding of the effectors of oviposition behavior in insect vectors for human disease might contribute to making related vector control strategies more effective. Several such strategies have been recently defined in the context of push-pull mechanisms for integrated pest management, a vector control strategy that attempts to manipulate specifics of insect vector biology to lure insect vectors into traps or other places where they can be captured (Cook et al. 2007, Hassanali et al. 2008). A key step in this process is the identification of behavioral determinants that can be used as a pull, or attractant. Because of its central role in the fitness of mosquito vectors in the wild, site choice for oviposition is a natural candidate behavior where determinants might be identified for potential use in a push-pull vector control strategy.

Here we communicate behavioral evidence for a region-specific olfactory determinant of oviposition behavior in Anopheles gambiae s.s. and briefly discuss possible implications for the findings in the context of the latest data in Anopheles gambiae s.s population structure. We further discuss the impact of these findings on vector control strategies.

METHODS AND MATERIALS

The two groups of mosquitoes compared were the laboratory strains of Mbita and Ifakara-derived populations of Anopheles gambiae s.s. The colony of Ifakara mosquitoes originated in the village of Njage, approximately 70 km south of Ifakara, Tanzania. Ifakara mosquitoes had been maintained under standard laboratory conditions (described below) since April 1996. Mbita mosquitoes originated in the village of Mbita Point, Suba District, in western Kenya. The Mbita colony had been established in 2001 from wild mosquitoes collected from a natural ground pond in Mbita Point. It is not known how the laboratory strains differ from their current wild counterparts, but to ensure that the observed behaviors were specific to mosquitoes from the region of origin, strains were kept separate during all phases of the rearing process. This suggests that there has been no observable gene flow from any other populations since their respective colonies began rearing at the Mbita Point field site.

Mosquitoes in both strains were reared in a screen house (11.5×7.1×4.4 m). The average temperature of the rearing environment was 30° C during the day and 24° C at
night, with relative humidity ranging from approximately 50% during the day to 70% at night. The photoperiod comprised of an equal proportion of daylight and night (12:12). Eggs were deposited in a large bucket of water at a density of approximately 200 larvae in 3 liters of water. These were allowed to progress from the egg state to pupation in the same water bucket. The water was collected from the banks of Lake Victoria, located approximately 1 km from the insectary. The larvae were fed daily with Tetramin™ fish food. Soon after the onset of pupation, pupae were moved to 30×30×30 cm cages where they emerged as adults. Adults remained in the 30×30×30 cm cages after emergence. A solution of 6% glucose was fed to adults as a carbohydrate source. Three-to-four-day-old female An. gambiae mosquitoes were collected, placed in a 30×30×30 cm cage, starved for 12 h and allowed to feed on human arms for a 10-min period on consecutive evenings at 18:00 h. Fully engorged females were left in the cages until they were gravid, moved to a new cage, and used in oviposition assays on the second night after their last blood meal.

Water originated from the banks of Lake Victoria, located near the field site. The water was treated as outlined above for the rearing of the two strains, Mbita and Ifakara. Buckets of water where Mbita mosquitoes were reared were kept separate from the water where Ifakara mosquitoes were reared, though each were reared in close proximity in the same screen house to ensure equivalent ambient conditions. Water from each of these buckets was collected and sieved to remove larvae or pupae. Twenty ml samples of each sample were placed into a 30 ml clear plastic cup and were immediately placed in 30×30×30 cm cages and used for behavioral cage assays.

All cage experiments took place in a screen house insectary with the same ambient conditions as the aforementioned mosquito rearing conditions. Mesh-netted 30×30×30 cm cages were set up with two 30 ml cups to hold the test samples (Ifakara rearing water and Mbita rearing water). Cages were set with a single fully engorged gravid female of either Mbita or Ifakara origin and the two different larval rearing substrates. The cups were set in opposite corners of the cage, the specific corner chosen at random. Cages were set at 17:00 h on each day and the females were allowed to oviposit overnight. At 09:00 h the next morning, mosquitoes were removed, with the number of eggs laid on each substrate recorded.

A binomial test was used to determine the significance in the oviposition patterns of the two strains. More specifically, the binomial test sought evidence that the mosquitoes were making a distinct choice in terms of which sample they preferred to lay eggs on. This was tested in a strain by strain basis and the preference of each of the strains (Mbita or Ifakara) of gravid female mosquitoes for Mbita An. gambiae s.s. rearing water or Ifakara An. gambiae s.s. rearing water was determined. The number of total cages set per day was dependent on the number of fully engorged An. gambiae s.s. gravid females available on a given day, which differed between the two strains over the duration of the experiment for mostly stochastic reasons, and the number of available cages on a given day. A total of 466 different cage bioassays were run from June-July 2003, utilizing 250 Mbita strain gravid females and 216 Ifakara strain gravid females that made a distinct choice of one substrate where all eggs were deposited.

RESULTS AND DISCUSSION

The two study strains of Anopheles gambiae s.s. demonstrated a preference for laying eggs on the water sample used to rear mosquitoes from their own strain relative to rearing water of mosquitoes of another strain. Out of 250 Mbita strain females, 153 chose Mbita strain larval rearing water (61.2%, p<0.001) over Ifakara strain larval rearing water. Out of 216 Ifakara strain females, 143 chose Ifakara larval rearing water over Mbita larval rearing water (66.2%, p<0.001). The only distinguishing characteristic between the two sample substrates was the strain of larvae that were reared in the water samples. The statistically significant preferences from both strains indicate that the signal is likely borne from the eggs and/or larvae reared in the water. There was no statistical relationship between the...
mosquito characteristics that might influence oviposition. This explanation begs further studies on other signals and cues that anopheline mosquitoes use to choose oviposition olfactory cue is just one part of a complex set of specificity might have decreased with time. In this scenario, in an older environment, such as the region of An. gambiae s.s. mosquitoes and further contribute to a fuller picture of oviposition behaviors in An. gambiae s.s.

On a broader scale, these results indicate that over short geographic distances there are observable behavioral differences between regional strains of Anopheles gambiae s.s. This is important given that recent findings have elucidated the genetic structure of An. gambiae s.s sub-taxa (della Torre et al. 2005, Calzetta et al. 2008, Santolamazza et al. 2008). Interestingly, both Ifakara and Mbita mosquitoes exist within the East African region that contains a single chromosomal and molecular sub-type, the FOR/SAV chromosomal form, S molecular form (della Torre et al. 2002). This suggests that behavioral differences not only exist between the molecular and chromosomal sub-taxa as the modern literature suggests, but even within the molecular and chromosomal sub-forms. This lends further credence to the idea that Anopheles gambiae s.s. is remarkably adaptable, and that this adaptability might contribute to the difficulty in establishing a universal set of vector control strategies for malaria, as mosquitoes separated by short geographic distances might differ significantly in relevant behaviors.

Studies on the Ostrinia moth genus provide an example of a potential model for how such regional differences in behaviors might arise. In Ostrinia, a dormant gene became active in a subset of the population, which affected production of a sex pheromone, which is likely to have single-handedly spawned a saltational speciation event (Roelofs et al. 2002, Roelofs and Rooney 2003). Though anopheline mosquitoes differ greatly from Ostrinia moths in many biological characteristics, it is possible that a similar subtle change in production, either in the nature or amount of the insect-borne oviposition determinant, could explain observed behavioral differences between Ifakara and Mbita strains of Anopheles gambiae s.s.

Despite knowledge of olfaction-mediated effectors of oviposition in other insects, the isolation of such a determinant for oviposition behavior in anopheline mosquitoes has remained elusive. This is unlike several mosquitoes in the genus Culex, where an oviposition pheromone was discovered decades ago and has since been

choice made and the clutch size (data not shown). A small fraction of mosquitoes (<5%) laid eggs on both samples. In these situations there was no relationship between choice of sample and clutch size.

The results strongly suggest the existence for an olfactory determinant of oviposition behavior in Anopheles gambiae s.s. and that the signal might be attractive at some concentration, because the effect of both samples was attractive to their respective populations (Mbita larval rearing water OAI = 0.224, Ifakara larval rearing water OAI = 0.324). One important observation involves the magnitude of the measured difference in preference. Though the measured preferences were statistically significant for both strains of mosquito, the percentages were still relatively low. In the case of the Mbita mosquitoes, for example, the percentages equate to them choosing the Mbita rearing water over Ifakara rearing at a ratio of 3:2. A statistically significant preference is not necessarily biologically significant, and it is not known, for example, if this observed preference alone is strong enough to drive species divergence.

There are several explanations for this relatively low preference. For one, maybe we are witnessing the very beginnings of the divergence process, one that would need to be enriched under a set of specific conditions for thousands of generations in order to drive full niche separation. Alternatively, perhaps the observed difference is the remnant of an older preference that the mosquitoes have lost. In this scenario, in an older environment, such oviposition cues might have been useful but have since lost their importance. Because there is less stabilizing selection to maintain this olfactory specificity, the magnitude of specificity might have decreased with time.

A third plausible explanation argues that the purported oviposition olfactory cue is just one part of a complex set of signals and cues that anopheline mosquitoes use to choose an oviposition site. Perhaps the oviposition cage experiment results were important because they localized one aspect of this behavior. This explanation begs further studies on other mosquito characteristics that might influence oviposition behaviors. Differences in cuticular hydrocarbon patterns, mating behaviors, and blood-feeding behaviors might differ between regional populations of An. gambiae s.s. mosquitoes and further contribute to a fuller picture of oviposition behaviors in An. gambiae s.s.

Table 1. Results of the cage bioassay demonstrating the region-specific attraction to the larval sitting water samples. Larval sitting water samples are samples of water where the larvae from the respective strains were reared from the egg stage to adult emergence. The oviposition activity index (OAI) = N_t – N_s / N_t + N_s, where N_t = number of insects laying eggs in test substrate, and N_s = number laying in control substrate, of the respective samples on gravid females. In this circumstance, there is no formal control, since we are testing a preference for one substrate against another. For that reason the control substrate is, in fact, the rearing water from the other strain. In the cages with a Mbita gravid female, the control in the equation is the Ifakara rearing water sample, and in the cages containing an Ifakara gravid female, the control is the Mbita rearing water sample. Positive numbers indicate an attractive effect and negative numbers, a repellent effect (Kramer et al. 1980).

<table>
<thead>
<tr>
<th>Mosquito Strain</th>
<th>Mbita Rearing Water</th>
<th>Ifakara Rearing Water</th>
<th>Total</th>
<th>Prop. Mbita Preferred</th>
<th>Prop. Ifakara Preferred</th>
<th>Exact 2-tailed OAI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mbita</td>
<td>153</td>
<td>97</td>
<td>250</td>
<td>0.612</td>
<td>0.388</td>
<td>0.000397</td>
</tr>
<tr>
<td>Ifakara</td>
<td>73</td>
<td>143</td>
<td>216</td>
<td>0.338</td>
<td>0.662</td>
<td>0.000002</td>
</tr>
</tbody>
</table>
the subject of rigorous analysis and chemical synthesis (Starratt and Osgood 1972, Krasnobrizhii et al. 1980, Millar et al. 1994). Though they are members of a different genus within the family Culicidae, anopheline mosquitoes might modulate their oviposition using a similar mechanism. These results provide the motivation for identifying the chemical blend responsible for oviposition choice. Efforts at isolating anopheline pheromones have been unsuccessful, and this difficulty could reside in the fact that, as compared to the Culex oviposition pheromone, a putative Anopheles gambiae pheromone might be produced in smaller quantity, making the effort to study their structure and function more difficult.

More elaborate extraction methods must be employed to isolate the chemical blends in high enough concentrations for analysis by classical chemical techniques. After the chemical isolation and characterization, different chemical blends from various populations might be compared to see if the observed differences are due to a difference in the structure or concentration of the oviposition pheromone produced.

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