Journal of Medical Entomology, 2017, 1–5
doi: 10.1093/jme/tjw229
Short Communication

A Video-Tracking Analysis-Based Behavioral Assay for Larvae of *Anopheles pseudopunctipennis* and *Aedes aegypti* (Diptera: Culicidae)

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Subject Editor: Lars Eisen

Received 22 June 2016; Editorial decision 2 December 2016

Abstract

Aedes aegypti (L.) is the primary vector of dengue, yellow fever, Zika, and chikungunya viruses, whereas *Anopheles pseudopunctipennis* (Theobald) is the principal vector for malaria in Latin America. The larval stage of these mosquitoes occurs in very different development habitats, and the study of their respective behaviors could give us valuable information to improve larval control. The aim of this study was to set up a bioassay to study basic larval behaviors using a video-tracking software. Larvae of *An. pseudopunctipennis* came from two localities in Salta Province, Argentina, while *Ae. aegypti* larvae were of the Rockefeller laboratory strain. Behaviors of individual fourth-instar larvae were documented in an experimental petri dish arena using EthoVision XT10.1 video-tracking software. The overall level of movement of larval *An. pseudopunctipennis* was lower than that for *Ae. aegypti*, and, while moving, larval *An. pseudopunctipennis* spent significantly more time swimming near the wall of the arena (thigmotaxis). This is the first study that analyzes the behavior of *An. pseudopunctipennis* larvae. The experimental system described here may be useful for future studies on the effect of physiological, toxicological, and chemosensory stimuli on larval behaviors.

Key words: *Anopheles pseudopunctipennis*, *Aedes aegypti*, mosquito, larva, behavior

Anopheles pseudopunctipennis (Theobald) is the most important malaria vector in Central America and South America (Pan American Health Organization [PAHO] 1991). The larvae of this mosquito are found in sun-exposed clean freshwater in association with floating plants and filamentous algae (Manguin et al. 1996). Larval abundance is negatively associated with rainfall and increases during the dry season. Larval habitats are located at altitudes between 200 m to 3,200 m above sea level but are more abundant in the foothills (Fernandez-Salas et al. 1994). Experimental data are scarce for this mosquito because it is difficult to maintain under laboratory conditions owing to its eurygamic status (Lardeux et al. 2007). In striking contrast to *An. pseudopunctipennis*, larvae of *Aedes aegypti* (L.), the most important vector of dengue, Zika, yellow fever, and chikungunya viruses, develop in container habitats in tropical and subtropical urban and suburban areas (Scott et al. 2000, Valença et al. 2013, Ye et al. 2016). An essential strategy to address these diseases is to control the vectors. The most vulnerable point in the life history of mosquitoes is the larval stage. Thus, among the control alternatives proposed, the control of larvae in their development sites is widely accepted and used (WHO 2016).

Locomotor activity impacts complex behaviors such as foraging and navigation. Some behaviors are related to kinesis, increased or decreased unoriented movements (hyper- and hypokinesis, respectively), and taxis, movement directly toward or away from the stimuli, such as thigmotaxis (Wigglesworth 1972, Matthews and Matthews 1978, Clements 1999). In thigmotaxis, animals held in experimental arenas tend to stay in peripheral areas where they can physically touch the walls and avoid the central zones. This behavior has been described in a great variety of animals, including mammals, amphibians, and many invertebrates including insects (Ulanoski and McDiffett 1972, Besson and Martin 2005, Schnörr et al. 2012). Thigmotaxis also may be induced by external agents, such as toxic or pharmacological compounds (Simon et al. 1994, Alzogaray et al. 1997, Hoy et al. 2000, Richendrfer et al. 2012, Denoël et al. 2013).

A number of different methods, with varying degrees of sophistication, have been used to track the behaviors of mosquito larvae, pupae, and adults (Brackenbury 1999, Liu et al. 2010, Kinney et al. 2014, Parker et al. 2015). Video-tracking software programs like EthoVision (Noldus et al. 2001) now affords the opportunity for detailed analysis of a range of behavioral parameters using automated tracking. Although automated tracking cannot replace the intuition and knowledge of a human observer, it can rapidly record large amounts of detailed, precise movement data.

Significant progress has been made in studies of the behavior of adult mosquitoes, but there is a paucity of information for quantification of larval behavior. Such quantitative data are important to

establish reference variables for each mosquito species, and could allow us to evaluate specific behavioral responses under standard laboratory conditions, including to identify olfactory-driven behaviors (Liu et al. 2010), sublethal effects of pesticides (Kembro et al. 2009, Tomé et al. 2014), and antipredator responses among others to develop more specific and effective larval control strategies. Examples include coupling insecticides with larval attractants to improve killing efficacy (Gonzalez et al. 2015), using a larval repellent to eliminate potential larval habitats, and studying sublethal effects of larvicides on the vectorial capacity of the adults.

The behavior of the aquatic stages of culicids largely relates to their nutritional and respiratory needs, and the avoidance of predators (Clements 1999). Anopheline larvae characteristically lie horizontally just below the water–air interface (Christophers and Puri 1929, Merritt et al. 1992, Clements 1999), whereas *Ae. aegypti* larvae hang head downwards from the water surface membrane (Clements 1992). When disturbed, *Anopheles* larvae usually detach themselves from the water surface by a body movement and then, remaining completely still, sink quite rapidly to the bottom. In contrast, *Ae. aegypti* larvae swim actively toward the bottom (Clements 1992).

The objective of this work was to set up a bioassay to study simple behaviors of *An. pseudopunctipennis* and *Ae. aegypti* larvae, including locomotor activity and thigmotaxis, by means of video-tracking software. It was a first step to lay the foundation for a bioassay system to evaluate larval responses to various physiological and toxicological stimuli.

Materials and Methods

Biological Material

Anopheles pseudopunctipennis larvae were collected, using a 0.05-mm mesh size sieve from natural development sites in a subtropical mountainous area in Salta, Argentina. Specific localities included Oran (23° 08′10″ S, 64° 19′20″ W) and Tartagal (22° 30′00″ S, 63° 50′00″ W). Larvae were transported to the laboratory, where they were kept in bowls with dechlorinated water and fed a mixture of rabbit pellets and yeast. *Aedes aegypti* larvae (Rockefeller strain) came from a colony maintained since 1996 in our insectary (25 ± 2°C, 80–90% RH, and a photoperiod of 12:12 [L:D] h). The colony has been free of pathogens and not exposed to insecticides or repellents. *Aedes aegypti* eggs were collected on wet filter paper, dried at room temperature, and stored for at least 30 d before being submerged in dechlorinated water (500 eggs per 2 liter water) at 25 ± 2°C. Eclosion of first-instar larvae were observed 24 h later (Seccacini et al. 2006, Gonzalez et al. 2016), and the larvae were fed a mixture of rabbit pellets and yeast.

Larval Behavior Assay

Early fourth-instar larvae of *An. pseudopunctipennis* and *Ae. aegypti* were used for this study. Larvae were picked and washed carefully in dechlorinated water to eliminate any food particles, and then kept at 27°C and starved for 2 h prior to being used. One larva was transferred to a separate petri dish (9 cm in diameter) filled with 40 ml of water. It was left for 10 min to acclimatize. Then, it was digitally recorded (individually) for 10 min with a video camera (Lumix DMS-LS 80, Panasonic, Kadoma, Japan). The test was conducted under controlled conditions of temperature and humidity (25 ± 2°C, 80–90% RH). A standardized hanging fluorescent light (with a light intensity of 36 W), over the experimental arena, was used to ensure sufficient contrast between insect and background of experimental arena. The recorded video was digitalized, and larval activity was quantified using a video-tracking software (EthoVision XT10.1), which recorded the position of a larva every 0.04 s, to calculate behavioral variables (Noldus et al. 2001).We used dynamic subtraction to identify the larvae from their background. For each species, 10 independent replicates were done.

Movements were assessed as spatial measurements (distance, speed, turning, etc.) that the human observer is unable to accurately estimate (Burešová et al. 1986, Spruijt et al. 1998, Noldus et al. 2001). Activity variables that were quantified included: 1) distance (distance the larvae swam in the experimental arena), 2) velocity (distance the larvae swam per unit time), 3) absolute angular velocity (changes in the direction of movement of the larvae between two consecutive samples, calculated per unit time), and 4) mobility state. The latter was categorized as Highly Mobile (HM), Mobile (M), or Immobile (I), depending on change in pixels of the detected subject between a current image and the previous one. If all the pixels are the same, there is zero mobility. If all the pixels are different, there is 100% mobility (Grieco et al. 2010). The mobility state was established for each sample, according to the mobility value relative to the following thresholds:

-Below the Immobile threshold (<20%), the state is Immobile.
-Between the Immobile threshold (≥20%) and the Highly Mobile threshold (≤60%), the state is Mobile.
-Above the Highly Mobile threshold (>60%), the state is Highly Mobile.

Finally, we defined the "Thigmotaxis Index" (TI) to describe wall-hugging behavior, as:

$$TI = \frac{Tb}{Tb + Tc}$$

where *Tc* is the time in the inner zone (defined as a circle in the center of the petri dish) and *Tb* is the time in a outer zone (the entire arena not assigned to the center zone). The circle of the center zone had a radius of 6.4 cm to equalize its area with the area of the border zone. TI was calculated as the time spent by the larvae in the border zone over the total time of the assay (10 min).

Statistical Analysis

A Principal Components Analysis (PCA) was used to evaluate the association of the two species with the activity variables owing to the high correlation between them (range of absolute value of Pearson correlation coefficient from 0.48 to 1, $P < 0.05$ in all cases). Spearman correlations were used to quantify the associations between the variables and the first Principal Component (PC 1). In these correlations, the threshold for significance was $P < 0.05$. We performed a Generalized Lineal Model (GLM) with the function "Varident," which assigns a different variance to each treatment to model the variance, to analyze differences between the species (Pinheiro and Bates 2000). The variables used in this model were the PC1 obtained from the PCA and the calculated TI. In these cases, the threshold for significance was $P < 0.025$ (after Bonferroni correction). We considered a species to display positive thigmotaxis behavior if the 95% confidence interval of the TI was greater of 0.5. The Infostat/E v2012 package was used for statistical analysis (Di Rienzo et al. 2014).

Results

The results of the PCA for the larval behavior experiments for both *An. pseudopunctipennis* and *Ae. aegypti* are shown in Fig. 1. The first Principal Component (PC 1) of the PCA accounted for 85.7%

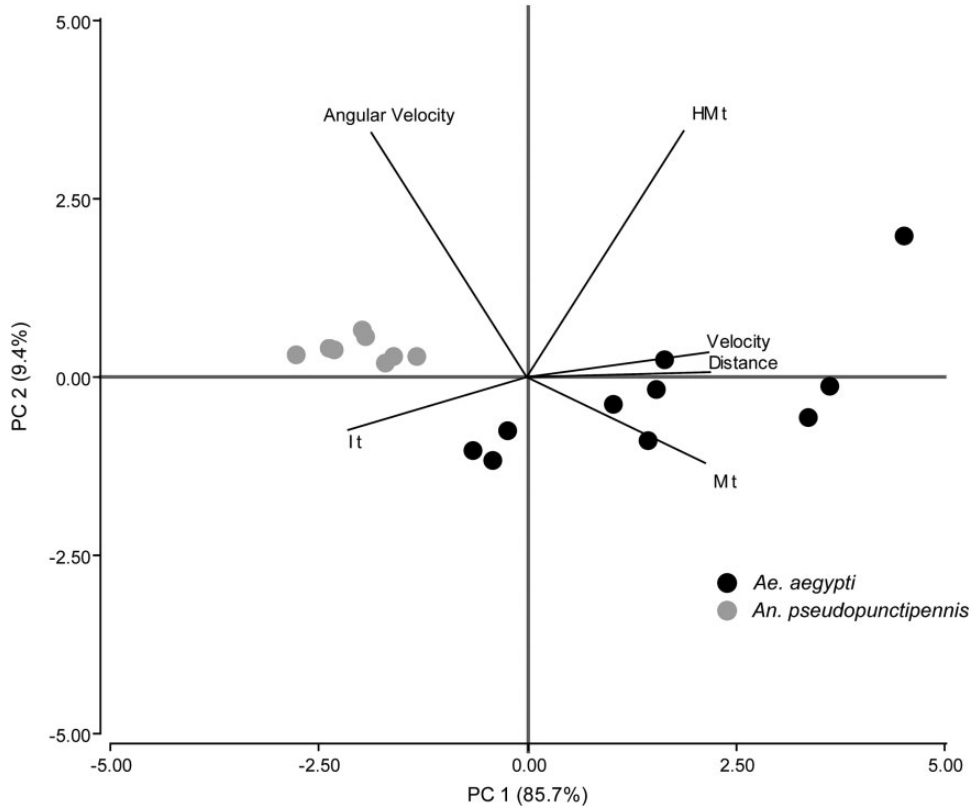

Fig. 1. Bi plot results of Principal Component Analysis of behavior variables and each larva analyzed of *An. pseudopunctipennis* and *Ae. aegypti*. HM t: Highly Mobile time. It: Immobile time. Mt: Mobile time. $n = 10$.

of the variability, whereas the second (PC 2) only accounted for 9.4%. The variables distance, velocity, and time of High Mobility and Mobility had positive significant correlations with PC 1, with coefficients of 0.98, 0.97, 0.84, and 0.95, respectively. The significant correlations between PC 1 and Time of Immobility and Angular Velocity were negative, with coefficients of −0.96 and −0.84, respectively. PC 1, therefore, can be viewed as an activity axis, with greater positive values indicating higher level of activity. In this respect, *Ae. aegypti* displayed significantly higher levels of activity than *An. pseudopunctipennis* ($df = 1$; $F = 37.08$; $P < 0.0001$).

Both *Ae. aegypti* and *An. pseudopunctipennis* displayed positive thigmotaxis (Fig. 2). However, the thigmotaxis was stronger in *An. pseudopunctipennis* than in *Ae. aegypti* ($df = 1$; $F = 5.24$; $P < 0.05$). Our results also showed that the level of movement of *An. pseudopunctipennis* was lower as compared with *Ae. aegypti*.

Discussion

We used an experimental petri dish microcosm together with a video-tracking software to study simple behaviors of larval *An. pseudopunctipennis* and *Ae. aegypti*, and to provide quantitative data for locomotor activity and thigmotaxia. Our study showed that *Ae. aegypti* larvae display more locomotor activity than *An. pseudopunctipennis* larvae, whereas time spent immobile and angular velocity were greater in the latter species. These results could be related to normal species-specific larval development sites with different food supplies, refuges, and predators. While there are no specific studies on the antipredator response of *An. pseudopunctipennis* larvae, our finding that the larvae spent more time immobile than those of *Ae. aegypti* suggest that this behavior could be a response to decrease in the likelihood of being detected by the predator (Ferrari et al. 2007). This "antipredator behavior" being more evident in *An. pseudopunctipennis* than in *Ae. aegypti* is probably owing to the latter species having a different strategy to escape predation (Sih 1986). Predator avoidance behavior is also observed in *Aedes triseriatus* (Say), *Culex pipiens* L., and *Anopheles gambiae* Giles larvae, which react to potential predators by reducing their movement (Kasap 1980, Kasap 1981, Juliano and Gravel 2002, Gimonneau et al. 2012). Furthermore, results obtained for angular velocity were higher for *An. pseudopunctipennis* than for *Ae. aegypti*. This variable could be associated with another antipredator behavior, in the form of an escape response, where larvae quickly change the direction of their movements (Turesson et al. 2009).

In addition, *An. pseudopunctipennis* larvae spent significantly more time swimming near the wall of the petri dish (thigmotaxis) than *Ae. aegypti* larvae. This thigmotaxis behavior with a tendency to maintain in bodily contact with solid objects has been documented also in other *Anopheles* species (Muirhead Thomson 1940, Belkin 1962, Clements 1999, Bugoro et al. 2011). Another study demonstrated that thigmotaxis in *Ae. aegypti* larvae is not as strong as compared with *Aedes albopictus* larvae (Zuharah et al. 2015). Furthermore, thigmotaxis behavior of *Cx. pipiens* larvae has been implicated in antipredator responses (Kasap 1980, Kasap 1981). It is important to study thigmotaxis because a change in this behavior could be useful to identify sublethal effects of insecticidal and repellent substances (Alzogaray et al. 2000, Denoël et al. 2013).

The ability to quantify details of larval behavior via automated processes is an important complement to studies where a human observer collects the data. Repeated human-based measurements can

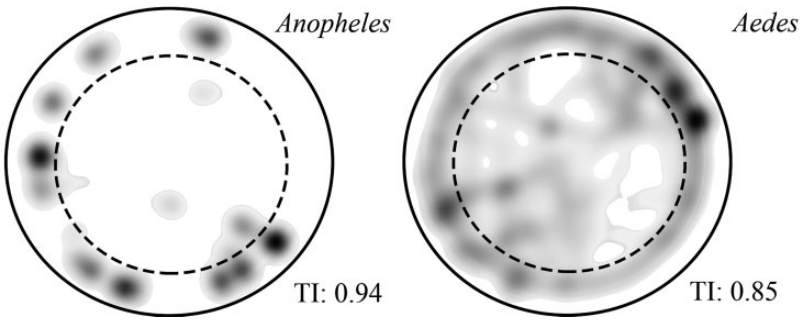

Fig. 2. Location map of free movement of a single larva of *Ae. aegypti* and *An. pseudopunctipennis* during the 10 min of the assay. Results TI (Thigmotaxis Index) are shown. The area delimited by the dotted line indicates the center zone, and the area between the solid line and the dotted line indicates the border zone. The darker gray colors indicate more time spent on this site. $n = 10$.

be time consuming and tedious, may suffer from lack of temporal resolution, and are vulnerable to subjective bias of the data recorder. In addition, observations on instantaneous position, speed, angular velocity, and orientation strategies are beyond a human's ability to measure precisely. Scaling of behavioral measurements, needed for statistical analysis and high throughput screening, is difficult with manual observation (Khurana et al. 2010). In our study, a variety of behaviors were analyzed using a video-tracking software to address these limitations.

Although a petri dish microcosm does not represent the natural larval habitat, it is widely used in behavioral studies (Marechal et al. 2004, Liu et al. 2010, Denoël et al. 2013, Kinney et al. 2014) and allowed us to determine larval movement patterns with great accuracy. Our experimental design is a first step to lay the foundation that in the future will let us evaluate larval responses to many physiological and toxicological stimuli.

In this study, we chose to evaluate only the horizontal movements of the larvae. Future studies can expand to include also their vertical movements in order to provide a more complete picture of the larval movement patterns. Our initial work presents a simple methodology to generate and analyze such data. This is also the first study to analyze the behavior patterns of *An. pseudopunctipennis* larvae. Moreover, our study revealed new and important differences in the larval behaviors of *An. pseudopunctipennis* and *Ae. aegypti*, and expand our understanding of the preadult life stages of these important pathogen vectors.

Acknowledgments

This study received financial support by Agencia Nacional de Promoción Científica y Tecnologica (ANPCyT) of Argentina (PICT 2012-1857). P.G. and A.A.C. have fellowships from CONICET. H.M. is a member of the CONICET scientific staff. Dr. L. Harburguer (CONICET) provided constructive suggestions and criticisms on an earlier version of this manuscript. We also would like to thank two anonymous reviewers for their valuable comments and suggestions, which helped improve the quality of this short communication.

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