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A method for assessing chemically-induced paralysis in headless mosquito larvae

Rafique M. Islam, Jeffrey R. Bloomquist *

Neurotoxicology Laboratory, Department of Entomology and Nematology, Emerging Pathogens Institute, University of Florida, Gainesville,FL 32611, United States

GRAPHICAL ABSTRACT

ABSTRACT

There is a growing interest in studies of mosquito physiology and toxicology due to the heightened need for controlling this group of human disease vectors. In the process of testing a group of polar compounds on mosquito muscles, a novel headless larva bioassay was developed. The heads were removed from fourth instar *Aedes aegypti* larvae, which permitted access of pharmacological agents to the hemocoel while maintaining larval viability. The method allowed effective quantification of the paralytic actions of water soluble compounds that could not ordinarily penetrate the mosquito larva integument and was more easily performed than injection when studying small, soft-bodied aquatic organisms.

The summary of the method is:

- Heads of *A. aegypti* larvae were detached with two pairs of forceps, and the larvae remained responsive for at least 5 h.
- The responsiveness of the larvae was assessed by using a microscope to observe movement after the larvae were probed with an insect pin.
- Drug effects were quantified using either a binary paralysis determination (paralyzed *vs*. not paralyzed), or by counting movement units after probing.

* Corresponding author. Tel.: +1 352 273 9417
E-mail address: jbquist@epi.ufl.edu (J.R. Bloomquist).

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Method details

Introduction

Initial efforts to study the toxicity of water soluble compounds in intact mosquito larvae were not successful due to poor penetration of the test compounds through the larval cuticle. Since the cuticle is known to be a major barrier for chemical penetration in insects [1] and nematodes [2], most investigators bypass this barrier by the use of an injection bioassay, a common protocol used to evaluate the effects of venoms [3]. To overcome the weak response of nicotinic acetylcholine receptor ligands in *Caenorhabditis elegans*, Lewis et al. [4] introduced a cut-worm model, which was further developed by Ruiz-Lancheros et al. [5]. According to the refined method, *C. elegans* were severed at approximately one-third of their length between the anterior end and mid-point, and the method improved response time and reduced the amount of drug used [5]. The hypothesis that detaching the heads of mosquito larvae would provide a diffusion pathway into the hemocoel to facilitate the analysis of toxic chemical properties was tested in the present study.

Preparation of *Aedes aegypti* larvae

Early stage (1st or 2nd instar) *A. aegypti* larvae were obtained from the United States Department of Agriculture, Agricultural Research Service (USDA ARS, Gainesville, FL, USA). Larvae were harvested in unfiltered tap water and held in large trays at approximately 25 °C, and supplemented with food consisting of 3 parts liver powder (MP Biomedical, Solon, OH, USA) and 2 parts Brewer's yeast (MP Biomedical). For all experiments, fourth instar larvae were used. Removal of heads was performed by pressing the neck of the larva against a Sylgard (RTV 615A/B, General Electric Corp., Wafford, NY) surface in a 35 mm dish (Becton Dickinson Labware, Franklin Lakes, NJ, USA) with one pair of forceps and pulling the head away with another pair. Because larvae are actively in motion in water, we reduced this movement by keeping the larvae in a minimal amount of liquid while removing the heads. Headless larvae ($n = 10$) were transferred to a small glass chamber containing 1 ml of a mosquito larval physiological saline solution containing NaCl = 0.9%, $CaCl_2$ = 0.02%, KCl = 0.02%, $NaHCO_3$ = 0.01%, pH 6.9 [6]. The saline was tested each time for correct pH before use. All saline components were purchased from commercial suppliers.

Validation of the assay: larval survival time and behavioral responses

A standard dissecting microscope (World Precision Instruments, Inc., Sarasota, FL, Model PZMT111) and video camera with computer interface (Model DFK 31AU03, The Imaging Source, Bremen, Germany, World Precision Instruments, Inc., Sarasota, FL) was used to observe the movement of the larvae and document behavior. While an intact mosquito larva was highly active and showed rapid swimming motion in water (Video 1), an untreated headless larva tended to be less spontaneously mobile, but responded vigorously when probed with an insect pin (Video 1). The response to probing of headless larvae (Video 1) fell into one of three categories (Fig. 1). A non-paralyzed larva initiated rapid bilateral contractions of the abdominal segments ("active"). The second category was "paralyzed" (or dead) if the larvae did not move after probing with an insect pin. Finally, it was observed that some headless larvae displayed an attenuated response to probing, and were considered "sluggish" but not paralyzed. These sluggish larvae, along with the active larvae, were considered unaffected for the purpose of drug effect quantitation. Over 90% of the untreated, headless larvae were