

MARKING TECHNIQUE OF *SIMULIUM ORNATUM*

MEIGEN, 1818 (COMPLEX) LARVAE

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ABSTRACT – Methylene blue was previously used in our laboratory in a mixture with larval feed to color the adults of *Culex pipiens* complex mosquitoes. Trials with simuliids were conducted in order to follow the downstream movement of *S. ornatum* larvae and evaluate the carry of B.t.i. in the Danube River. The section of the Danube in Vojvodina is characterized by huge stream flow (average 2800 m³/s, maximum 9290 m³/s), inconstant flow velocity, meandering, and irregular configuration of the river banks. The carry of B.t.i. can be assessed more accurately if methylene blue is administered during larvicide application. Third to sixth instar larvae of *S. ornatum* were colored in the laboratory and degrees of intestinal and haemolymph coloration were recorded, together with larval mortality of mosquitoes. Two doses of vital dye (25 and 50 mg/l H₂O) were used in the trials. Degrees of coloration of living larvae, larvae treated with hot water, and ones treated with hot water and ethanol were assessed both visually and under a dissecting microscope.

KEY WORDS: Simuliidae, larval marking, dying, methylene blue

INTRODUCTION

Widely used for *in vivo* staining of the simuliid nervous system, methylene blue preferentially stains the frontal ganglion and labral and antennal nerves (CRAIG, 2005). Methylene blue was previously used in our laboratory in a mixture with larval food to color the adults of *Culex pipiens* complex mosquitoes. Our interest in vital staining of simuliid larvae developed through the need for a simple method that could be used to follow the downstream movement of *Simulium ornatum* larvae and evaluate the carry of B.t.i. in the Danube River. The section of the Danube in Vojvodina is characterized by huge stream flow (average 2800 m³/s, maximum 9290 m³/s), variable flow

velocity, meandering, and irregular configuration of the river banks. For flows that do not lend themselves easily to depth and velocity measurement, “flow visualization” with methylene blue dye should be applied (SOLUK AND CRAIG, 1988; CRAIG 2002). The carry of B.t.i. can be assessed more accurately if methylene blue is administered during larvicide application.

MATERIALS AND METHODS

Third to sixth instar larvae of *S. ornatum* were collected in nearby streams (5-10 km from our laboratory at the Faculty of Agriculture in Novi Sad - 45°14.790'N 19°51.182'E) and the larvae transferred to glass vials containing 400 ml of water taken from the breeding site and aerated by aquarium water pumps. From ten to 20 larvae were pipetted into each vial for every trial and/or replicate. Four doses of methylene blue (2.5, 5, 25, and 50 mg/l H₂O) were used with exposure time of 10 minutes and/or 24 hours. Larval mortality was recorded, at four hours and one, two, three, and four days after exposure. The degree of coloration was observed in three groups of larvae: living, treated with hot water, and treated with hot water followed by 70% ethanol. Time intervals were set at 30 min and 4 h after exposure in the same and separate trials, and larvae were checked both visually and under a the dissecting microscope. Data were analyzed using the SPSS 10 statistical set.

RESULTS

Comparison of four different doses of methylene blue (Table 1) showed that 25 and 50 mg/l gave much better coloration (95 and 91% respectively) than 2.5 and 5 mg/l (30 and 45%) 10 minutes after exposure. Lower concentrations colored a substantial percent of larvae after long exposure (24 h) (Table 1). However, since short exposure is a prerequisite for “carry” experiments and more convenient for “released marker recapture” assays, higher rates of methylene blue were chosen for further testing. Adult mosquitoes sampled after the larvae were treated with methylene blue in concentrations of 0.05 and 5 mg/l for 2 hr, and in concentration of 0.5 and 50 mg/l for 1hr were recognized as colored by the naked eye in the following percentages of cases: 0, 42.7, 83.7 and 80.2 %, respectively (unpublished data). Among many stains tested on the mosquito species *Culex pipiens* biotype *molestus* and *Aedes vexans* methylene blue, particularly at dosages of 50 mg/l for one

Table 1. Coloration and mortality of simuliid larvae subjected to different rates and times of exposure to methylene blue.

Methylene blue g/l	Time after exposure	Number of larvae	Colored		Mortality	
			number	percent	number	percent
2.50	10 min	20.00	6.00	30.00	0.00	0.00
2.50	24 h	20.00	16.00	80.00	7.00	35.00
5.00	10 min	20.00	9.00	45.00	0.00	0.00
5.00	24 h	20.00	13.00	65.00	12.00	60.00
25.00	10 min	100.00	95.00	95.00	0.00	0.00
25.00	24 h	100.00	95.00	95.00	3.00	3.00
50.00	10 min	100.00	91.00	91.00	0.00	0.00
50.00	24 h	100.00	91.00	91.00	8.00	8.00

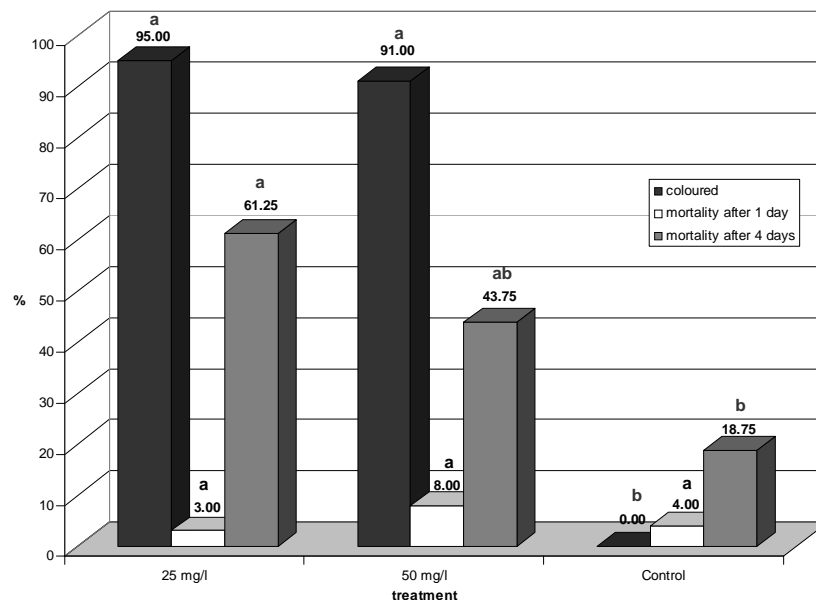


Fig. 1. Percentage of colored simuliid larvae and mortality rates after methylene blue staining.

hour and 5 mg/l for two hours of larval exposure, was the most effective one.

Figure 1 shows that there were no significant differences in the level of larval coloration between 25 and 50mg/l or in the overall mortality rate one day after methylene blue application (25 mg/l – 3%, 50 mg/l – 8%, control – 4%). However, mortality recorded after four days was significantly higher in replicates of 25 mg/l (61.25%) than in the five control replicates (18.75%), but similar to that recorded in replicates of 50 mg/l (43.75%). There was no significant difference in the average numbers of dead larvae in the 50 mg/l and control variants. Levels of larval and pupal mortality were similar in the control and after treatments with 0.05 and 5 mg/l for 2 hrs and with 0.5 and 50 mg/l for 1 hr (5.00, 9.00, 13.93 and 14.00 %, respectively). Significantly higher mortality in comparison with the control was registered for continuous exposure to concentrations of 5 mg/l (90 %) and 50 mg/l (100 %).

Because it is difficult to examine energetically moving larvae, they were immobilized by hot water treatment; surprisingly, there was a gain in the visibility of coloration. Figure 2 shows results of comparing coloration observed by the naked eye and with binoculars for living larvae, larvae treated with hot water, and ones treated with 70% ethanol after hot water treatment. It is plainly evident both to the naked eye and under binoculars that hot water increases intensity of the dye in larval bodies.

Finally, we compared the recovery rate of colored larvae in the three aforementioned groups as assessed by a person who dealt with coloration right from the beginning of the experiment and by newcomer who had no experience with simuliid larvae. There were no significant differences between their observations (Fig. 3). It was possible to observe coloration shortly after stain treat-

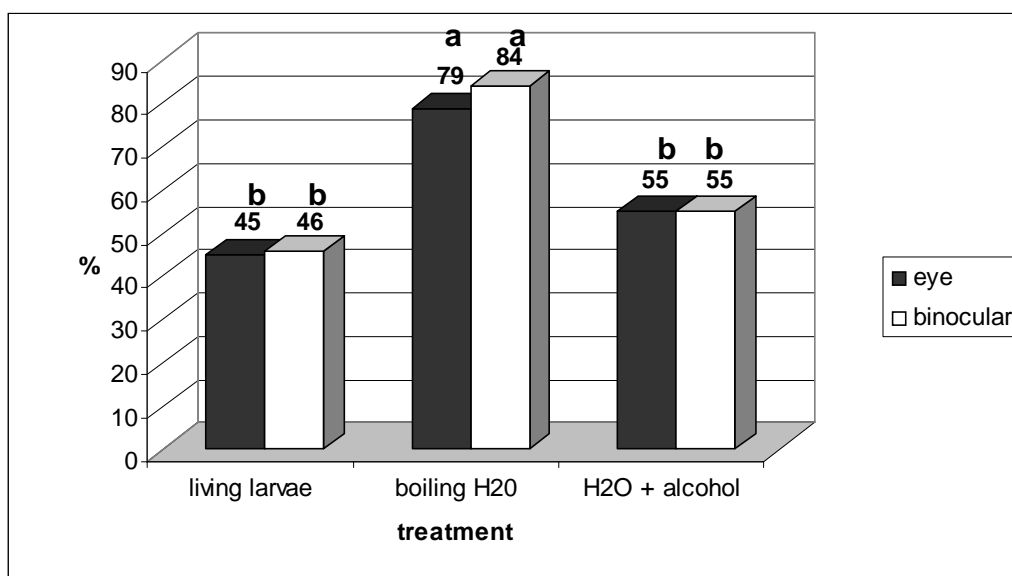


Fig. 2. Coloration intensity in living larvae, larvae treated with hot water, and ones treated with hot water plus ethanol.

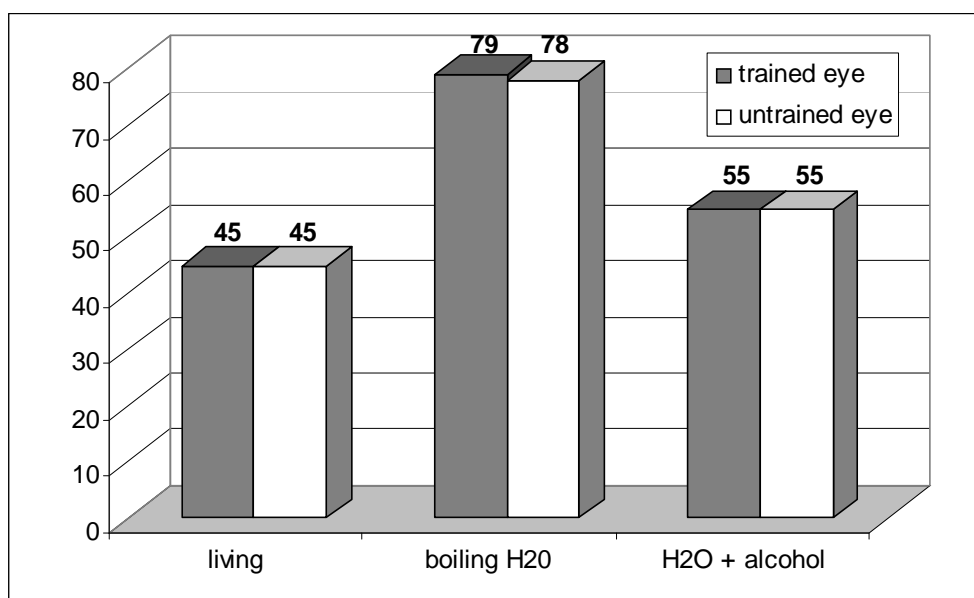


Fig. 3. Number of stained larvae as estimated by an “expert” and by a “layman”.

ment. This suggests that the technique can be extended to larvae in order to follow the dispersal of free-living populations and the carry of particles in the Danube River. The expediency of this way of using methylene blue is supported by the results of KAY AND MORTTRAM (1986), who found better *in vivo* staining of *Aedes* mosquito species by methylene blue using pre-stained yeast for stain application.

ACKNOWLEDGEMENTS

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