

MARKING *CULEX PIPIENS* LINN. LARVAE WITH VITAL DYES FOR LARVAL ECOLOGICAL STUDIES¹

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Adult mosquitoes have been studied extensively for many years because of their annoyance and ability to transmit pathogens. However, detailed studies of the larvae and their habits have been relatively neglected until recently. Information is still lacking in many areas, one such field being the study of naturally occurring larval populations. Knight (1964), in his review of larval survey methods, stated that the literature is scattered and sparse. This is possibly due to the lack of a

quantitative technique to determine absolute population parameters.

Several workers (Horsfall, 1946, Kato *et al.*, 1956, and Edmunds, 1958) have numerically analyzed seasonal fluctuations in larval populations. Their results yielded relative numbers only and the population parameters could not be determined with the techniques used. The work presented here is part of a project designed to determine larval dispersal, instar distribution and population parameter of a locally occurring species.

We needed an efficient, practical, and accurate sampling technique for this study. The only accurate method is to count the entire larval population; however, this is

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practical only in situations where the population is small and can be collected entirely. Other techniques include counting a known proportion of the population by the use of quadrants; mark, release, and recapture; removal; and sequential sampling. We decided to use mark, release, and recapture because this method is accurate and flexible. It can be used to determine absolute sizes and instar proportions of a population and also larval movement and instar distribution.

One of the fundamental problems in the mark, release and recapture technique is the method and material used to mark individuals prior to release. Baldwin *et al.* (1955) used radioactive isotopes for ecological experiments with predators of mosquito larvae. Adult dispersal studies based on radioactive tagging of reared larvae have been carried out by Provost (1957) with *Aedes taeniorhynchus* (Wied.) and Fussell (1964) with *Culex quinquefasciatus* Say.

The first report of marking larvae with vital dyes was by Weathersbee and Hassell (1938). They stained larvae with Giemsa and found it to be effective in their attempt to produce tagged *Anopheles* adults. However, Haddow (1942) repeated their work and did not find the technique satisfactory.

In our search for a suitable marking agent for aquatic larvae we decided that an internal visual marker would be the easiest tag to use both because equipment is not needed for detection and there are no restrictions in its use as with radioactive materials. Therefore, we undertook the following work to determine if any of several dyes would give satisfactory results for population analyses.

MATERIALS AND METHODS. Larvae selected for experimental work were taken from a culture of *Culex pipiens* Linn. maintained at $27 \pm 2^\circ \text{C}$. and 70 ± 5 percent relative humidity. The experimental larvae were reared on dried brewer's yeast in distilled water with a fluctuating temperature of $22\text{--}26^\circ \text{C}$. The times necessary for dyes to adequately stain the larvae were influenced by this fluctuating

temperature, but these variations did not alter the staining properties of any dye. Pre-ecdysis third and post-ecdysis fourth instar larvae were used in the preliminary tests to determine which stains would invade and concentrate in the larval body tissues and which would pass through the gut without entering the tissues.

The dyes used in the preliminary tests are listed in Table 1. Concentrations of 1, 5 or 10 parts per million (p.p.m.) were made from each dye to give approximately equal color intensities of all solutions in the test petri dishes. The treatments were 1.5, 4, 18, and 46.5 hours. Staining characteristics of the dyes were tested on larvae fed dried brewer's yeast and on larvae without yeast.

Further tests were conducted with any dye that stained the larvae adequately in the preliminary experiments. These tests consisted of various combinations of dye concentrations and times in the dye to determine the most efficient concentration and time necessary for the larvae to become stained. The most efficient combination was that from which the lowest mortality and highest percent stained resulted.

We tested the possible influences of food on staining by comparing the speeds of stain pick-up in a dye solution containing food and one without food. In these tests there was an obvious difference in color intensity between the two treatments of each dye solution at completion of the experiment. In all of the treatments with yeast the dye solutions were a lighter color than the treatments without yeast, and the yeast had become intensely stained.

A test was conducted to determine dye absorption by yeast. Nile Blue A and Methylene Blue solutions of approximately 100 p.p.m. were made and their optical densities determined with a Coleman Junior Spectrophotometer Model 6D. Yeast was added to each solution and removed 24 hours later by centrifugation. The optical densities were determined again and were considerably lower compared to the same solutions before the addition of yeast.

We tested the retention of dye under

different conditions by placing stained larvae in distilled water without food, distilled water with food and pond water with a high organic content. Daily observations were made on the loss of visibly detectable stain.

Tests were conducted with Nile Blue A and Neutral Red to determine the movement of these dyes through the food chain and bioenvironment. Ten fourth instar larvae were placed in 100 p.p.m. solution of each dye for 16 hours and were well stained. They were then killed and used to make four treatments. Added to 200 ml. of distilled water with 100 living first and second instar larvae were:

- A. Five homogenized Nile Blue A-stained larvae.
- B. Five whole Nile Blue A-stained larvae.
- C. Five homogenized Neutral Red-stained larvae.

D. Five whole Neutral Red-stained larvae.

The living larvae were examined daily for 6 days to determine any visible concentration of dye.

To study stain movement through the food chain, predators were fed stained larvae. The three types of predators tested were damselfly naiads, dytiscid larvae, and hydrophilid larvae. The mosquito larvae were stained as above and placed live in 200 ml. of water with individual predators. Tests were conducted varying the number of larvae and type of stain given each predator. The predators were examined daily to determine differences between predation on Nile Blue A- and Neutral Red-stained larvae and possible dye pick-up by recently fed predators.

RESULTS AND DISCUSSION. Table I gives concentrations and staining results of all

TABLE I.—Screening for dyes that visibly stain living *Culex pipiens* larval tissues. Tests performed in the presence and absence of dried brewers' yeast.

Dye	Conc. (ppm)	Hours					
		Without yeast				With yeast	
		1.5	4	18	46.5	4	18
Neutral Red	10	0	0	*	***	*	**
Nile Blue A	1	*	*	**	***	**	***
Methylene Blue	1	*	*	**	***	**	***
Safranin o	10	0	0	*	X	0	0
Brilliant Vital Red	10	0	0	0	0	0	0
Crystal Violet	5	0	..	0
Brilliant Cresyl Blue	1	0	..	0
Benzopurpurin	5	0	..	0
Trypan Blue	1	0	..	0
Janus Green B	5	0	..	0
Light Green Crystal	5	0	..	0
Fluorescein	5	0	..	0
Negrosin	1	0
Biebrich Scarlet	10	0
Acid Fuchsin	1	0
Amaranth	10	0
Eosin Y	10	0
Congo Red	10	0
Analine Blue	1	0
Orange G	10	0

* Stain just visible in tissues.

** Light stain in tissues.

*** Dark stain in tissues.

° No stain in tissues.

X Mortality > 80%.

.. No test conducted.

dyes used in the preliminary experiments. Of the 20 dyes tested, only three, Neutral Red, Methylene Blue and Nile Blue A proved suitable for our purposes. Several of the dyes stained the gut, but only the three above invaded and concentrated in the body tissues. Nile Blue A appeared to be best. Neutral Red stained well, but the penetration time was longer and the mortality slightly higher than Nile Blue A. Methylene Blue stained very well, but was eliminated because of the high mortality it caused.

Table 2 gives detailed data for Nile

TABLE 2.—Time necessary for maximum concentration of Nile Blue A in pre-ecdysis 3rd and post-ecdysis 4th instar larvae fed yeast and the resultant mortality.

Dye conc. (ppm)	Time in dye (hours)	% stained	% mortality*
100	7	98	43
10	7	96	13
10	27	100	17
5	51	100	17

* Mortality in dye solution minus mortality of same number of larvae in medium without dye.

Blue A. The best results were with 10 p.p.m. for 27 hours, with yeast for food. Under these conditions all the living larvae were stained with a final mortality of 19.5 percent. Mortality in the absence of dye was 2.5 percent in 27 hours. These data are based on experiments with third and post-ecdysis fourth instar larvae.

Food is an important factor to consider in producing stained larvae since it affects the degree and rapidity with which larvae stain. When yeast is present it absorbs the dye and removes it from solution. Larvae in dye solutions with yeast stain more intensely and two to three times faster than larvae in dye solutions without yeast. This is due to the absorption and resulting concentration of the dye by the yeast particles.

We found that third and fourth instar larvae concentrated the dye and stained best. The first two larval instars were more sensitive to the dye and therefore lower concentrations (0.5 p.p.m.) had to be used to prevent high mortalities. This

resulted in lighter staining. Well-stained third instar larvae would seem to be ideal for population parameter studies. Samples can be taken for the duration of two instars because there would be no loss of marked individuals due to pupation as would be the case with marked fourth instars.

Stained third and fourth instar larvae in distilled water remained tagged for 7 days, even after moulting occurred. The color faded but the larvae were still distinguishable from non-stained larvae. The resulting pupae and adults were visibly stained in some instances. When stained individuals were placed in distilled water with yeast, the dye disappeared in 5-6 days. When larvae were placed in pond water, the dye remained visible for at least 4 days.

The results were negative in tests on movement of stains through the larval food chain. Live larvae did not concentrate dyes to the point of visibility when fed on stained dead larvae, either whole or homogenized. In stain pickup by predators only the gut contents were stained, with no entry into the body tissues as in the mosquito larvae. The gut contents of damselfly naiads fed Nile Blue A-stained larvae remained blue for at least 1 day, with an average of 2.7 days. Larvae dyed with Neutral Red did not seem as susceptible to predation as those stained blue, and if eaten, the naiads' guts remained red for an average of 1 day but never more than 2 days. Dytiscids fed Nile Blue A-stained larvae had green gut contents, the color lasting 1 day. Only dytiscid larvae required more than one Neutral Red-stained prey to color the gut red. The color remained for only 1 day. Nile Blue A-stained larvae usually did not have any color effect upon hydrophilid larvae, but when stain in the gut did show, it remained for 1 day. The results were similar for hydrophilids fed Neutral Red-stained larvae.

The dyes did not appear to seriously affect the habits of the mosquito larvae unless an excess was used. Alarm reactions and feeding habits remained nearly nor-

mal. Dyed undisturbed larvae did, however, seem to move less frequently than unstained larvae. The dyes did have one striking effect on the larvae; a retardation of growth and development. First and second instar larvae kept in a 0.2 p.p.m. solution of Nile Blue A remained as such for more than 3 weeks at 24° C. as compared to normal development in 10-12 days for the entire larval stage of the stock cultures at 25° C. When larvae were removed from the dye solution, normal rates of development resumed after 4-6 days.

CONCLUSIONS. The use of vital stains in ecological studies on mosquito larvae is limited by the effect the stains have on the physiology and behavior of stained individuals.

The greatest value the technique of vitally staining mosquito larvae has is as a tool in studying larval growth, rate of ingestion and assimilation. Further studies in the area are being conducted to determine the mechanism of growth retardation.

Studies on the predators of larval mosquitoes can be made using tagged larvae. Since predators pick up the stain from the prey without having their tissues visibly invaded, an analysis of predation per individual may be made by examination of the alimentary tract of the living predator. Speed of food movement in the gut can be analyzed by following the progress of the stained gut contents through the predator.

Other organisms were stained in studies incidental to the main experiments. Small

organisms, especially copepods, stained as well as mosquito larvae and studies using the vital stain technique could be performed with them.

The use of visual internal stains is limited to certain groups of organisms which possess a desirable speed of development, incomplete opacity of the exoskeleton, and food habits which will enable stains to enter the body and penetrate the tissues.

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