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# RETARDATION OF LARVAL DEVELOPMENT OF *AEDES AEGYPTI* (L.) BY THE VITAL DYE, NILE BLUE SULFATE (A) [1]

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## INTRODUCTION

In a recent paper, Peters and Chevone (1968) reported the successful internal marking of *Culex pipiens* L. larvae with vital dyes. The work had been undertaken to tag mosquito larvae for ecological investigations based on mark, release and recapture studies, a sampling technique essential for determining absolute population sizes. Of the three vital dyes found suitable as marking agents, Nile Blue Sulfate gave the best results; concentrations of 10.0 p.p.m. yielded 100 percent staining in 27 hours with a mortality only 10.0 percent greater than normal laboratory mortality.

During these studies we noted that larval development was retarded if the larvae remained in Nile Blue Sulfate solutions for extended periods. Kolyer (1966) reported larvae of *Colias* spp. (Lep.) matured into stunted adults when fed plant materials containing Nile Blue Sulfate or Neutral Red.

The following work was initiated to determine the effects of Nile Blue Sulfate on larval development of *Aedes aegypti* (L.), and thereby evaluate the potential use of this dye as a marking agent. A significant change in behavior or physiology of marked larvae by the dye would alter distribution patterns of the larvae. The mark, release, and recapture technique is based on the assumption of normal redistribution of marked individuals. Therefore, if the dye significantly altered the physiology of the larvae, it would introduce errors into population estimates. The investigations undertaken included (1) histological determination of the tissues and organs concentrating the dye in IV instar larvae, (2) effect of low dye concentrations on length of development to pupation, and (3) comparison of oxygen consumption rates of stained and unstained IV instar larvae.

## MATERIALS AND METHODS

CULTURE REARING TECHNIQUES. The source of experimental animals was a laboratory culture of *A. aegypti* obtained

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from Rutgers University 6 months prior to experimentation. Adults were kept in a glass enclosed cage in an environmentally controlled room at 27±2.0° C, 60 ±15 percent R.H. and a 15–9 hour light dark period. Blood meals from hamsters or *Coturnix* quails were administered during the photoperiod every 4–5 days for 1–2 hours. Eggs, deposited on cones of filter paper emerging from distilled water, were stored at 75 percent R.H. and 26.5° C. All eggs were vacuum-hatched at 20 psi for 15 minutes, yielding larvae of uniform age. The larvae were reared in enamel pans (300–400/pan) containing 2 liters of distilled water at 26.5±1.0° C water temperature. The larvae fed on a mixture of ground dog chow (Gaines Meal ®) and dried brewer's yeast. Pupation occurred in 5–6 days with a larval mortality of 5–6 percent. Adults emerged about 2 days after pupation and first blood-fed 2–3 days later. Oviposition began 2–3 days after the blood meal.

EXPERIMENTAL TECHNIQUES. Experimental rearing techniques were similar to culture rearing techniques and standard for all tests. No eggs older than 8 weeks were used in the tests. After the eggs were vacuum-hatched for 15 minutes, 50 larvae per dish were placed in petri dishes with 100 ml. of distilled water. Each petri dish constituted a replicate in an experiment. Dried brewer's yeast, the only food, was added volumetrically on a per milliliter basis in the following amounts in mg./ml./day: 0.5 on days 1–2 (I–II instars); 1.0 on day 3 (III instars); and 1.5 on days 4–6 (IV instars). The larvae developed in the culture room at a water temperature of 26.5±0.86° C.

A standard solution of 0.1 percent Nile Blue Sulfate (Harleco, C. I. No. 51180, 2nd Ed., 83 percent pure dye content) was prepared by dissolving 0.1205 gram of dye in 100 ml. of distilled water. This standard was pipetted into the test petri dishes as required to give the desired concentrations. The water was changed daily in extended experiments to prevent dye concentration resulting from water evaporation. Peters and Chevone (1968) had shown that dye uptake and subsequent deposition in the larval tissues primarily occurred by the larvae feeding on stained brewer's yeast. Consequently, all studies employed simultaneous use of the dye and yeast.

HISTOLOGY. Histological studies were conducted to determine rate of dye uptake and sites of dye deposition in IV instar larvae. Rate of dye uptake was determined by placing IV instar larvae, reared under standard conditions for 92–94 hours, in a 2.5 p.p.m. dye solution with 2.5 mg./ml. of brewer's yeast. After 30 minutes, 4 larvae were removed periodically from the dye solution each ½ hour for 3 hours. They were examined whole under the dissecting microscope and entry of dye into the gut and fat body tissue was followed in a series of specimens for 3½ hours. Sites of dye deposition were determined by examining IV instars stained *in vivo*. Larvae, reared under standard conditions for 92–94 hours, were starved for 12 hours and then placed in a 2.5 p.p.m. dye solution with 2.5 mg./ml. of brewer's yeast. The starvation period facilitated increased feeding, accelerating dye entry into the body tissues, and ensured uniform feeding rates by the larvae.

After 12 hours, the larvae were removed from the dye and divided into two groups. One group was examined live under the dissecting microscope; the other was killed, fixed and differentiated following Mallory's (1944) procedure for Nile Blue Sulfate staining. In these latter specimens, the midgut, extending from the thorax to abdominal segment V, was dissected out and mounted whole. The remaining body tissues were dissected and both temporary and permanent slides made. The mounts were studied under both light and phase contrast illumination at powers up to and including oil immersion (960X).

EFFECTS OF THE DYE ON LARVAL DEVELOPMENT. A preliminary experiment was conducted to determine the gross effects of low dye concentrations on larval development. Concentrations of 0.25, 0.50, 0.75,

1.00, 2.50, 5.00, 7.50, and 10.00 p.p.m. were tested against a control. Three replicates of each treatment (except 10.00 p.p.m. with 2 replicates) were run. Time for development to pupation was the criterion used to measure the dye's effects upon length of the larval stage. Observations were recorded approximately every 24 hours after the first pupa appeared.

Information gained in the preliminary test aided in designing an experiment to give more definitive data of dye effects on larval development to pupation. Concentrations of 0.25, 0.50, 0.75, 1.00 and 2.50 p.p.m. were tested against a control. Three replicates of each treatment were run. Observations on number of larvae molting were recorded at 1-hour intervals whenever possible.

RESPIROMETRY. Respirometry studies were conducted to determine if the dye affected the oxidative metabolism of IV instar larvae. A refrigerated Gilson differential respirometer, Model GR 14, was used for these studies. The operating conditions were: water bath at 27° C., 10 IV instar/test flask, 2 ml. distilled water/flask, 20 drops of 10 percent KOH in the center wells, and 4 replicates/treatment. Larvae to be tested for dye effects were reared under the previously discussed conditions for 92–94 hours and then placed in a 2.5 p.p.m. dye solution with 2.0 mg./ml. of brewer's yeast for 24 hours. Larvae used as controls were hatched 6 hours later than the test larvae to approximate the dye's inhibitory effects on the test larvae (see fig. 1). After the 24-hour staining period, all larvae were placed in the flasks and the system was allowed to equilibrate for 30 minutes. Gentle shaking occurred throughout the test. Readings, accurate to 0.1 $\mu$., were recorded at ½-hour intervals for 4 hours. At termination of the test, the larvae were killed by boiling and then dried at 100° C. for 1½ hours. Each replicate of 10 larvae was weighed on a Mettler single pan balance to the nearest 0.1 mg. The $O_2$ recordings were converted to $\mu$l./mg. dry wt/½ hours.

## RESULTS AND DISCUSSION

RATES OF DYE ENTRY. Thirty minutes after 92–94 hour old larvae had been placed in the dye solution, stained yeast appeared in the gut lumen from anterior proventriculus to posterior mesenteron except for the lumen of the caecae. The anterior midgut epithelium had stained very lightly.

At 1 hour, a diffuse blue color was present throughout the hindgut. Occasionally, large stained particles, presumably fecal material, were present. No stain appeared in either the lumen or the epithelial cells of the Malpighian tubules. Caecal and midgut epithelium had stained completely and the color had darkened. Lateral visceral fat body tissues in abdominal segments I–III had stained.

At 2 hours, initial staining occurred in parietal fat body tissue of the thorax and anterior quarter of abdominal segments I–III. The entire gut lumen from proventriculus to anus contained dye; however, midgut epithelium was the only region of gut wall that stained.

At 3 hours, lateral and dorsal portions of fat body in abdominal segments IV–V began to stain. Staining sequence of fat tissue in abdominal segments I–V began on the lateral anterior quarter of a segment and gradually spread dorsoventrally and slightly posteriorly. Abdominal segments I–III stained completely first. In abdominal segments VI–VIII, staining first appeared on the anterior quarter of a segment and then spread ventrally and posteriorly. Whether or not this sequence of staining reflects deposition of food reserves is speculative.

At 3½ hours, parietal fat body of all abdominal segments had stained; the intensity of color diminished progressively posteriorly. Other distinguishable tissues and cells that stained were: pericardial cells and longitudinal abdominal muscles (2 hours) and supraoesophageal ganglion (3½ hours).

SITES OF DYE DEPOSITION. Since a description of intensity and pattern of staining

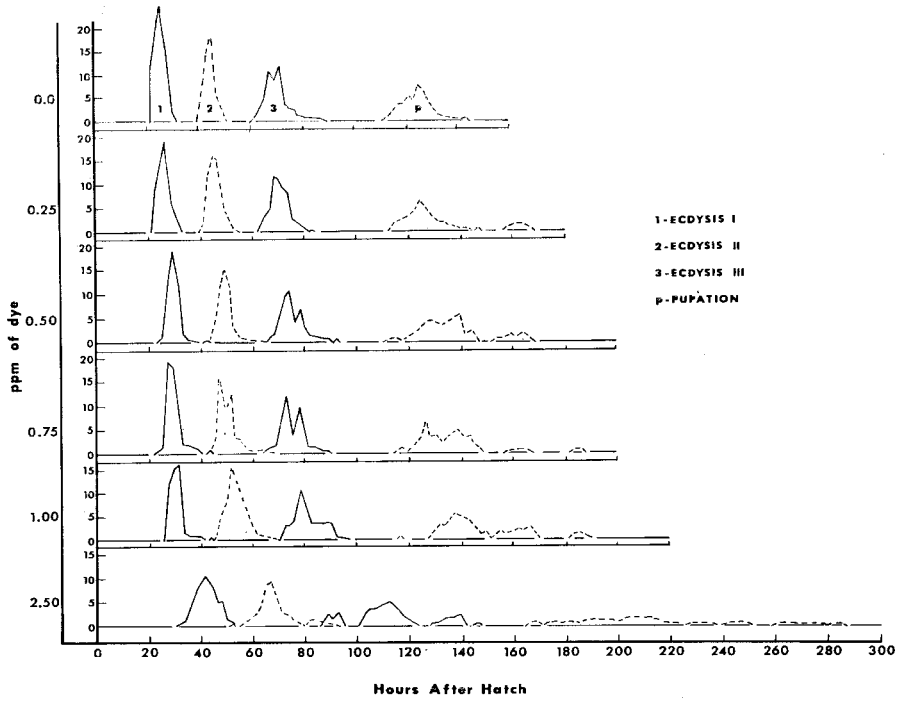


FIG. 1.—Ecdysial rates of *A. aegypti* larvae reared in different concentrations of Nile Blue Sulfate (A).

within cells is subjected to the interpretation of the observer, a summary only of the staining properties of those tissues and organs examined is presented. The foregut, hindgut, Malpighian tubules, tracheal system, hemolymph, heart wall and the exoskeleton did not noticeably stain. The muscle tissue (dorsal and ventral longitudinal muscles of the abdomen), imaginal buds, and supraoesophageal ganglion stained lightly. Oenocytes, plasmocytes, pericardial cells (dorsal nephrocytes), lipid stores in the trophocytes of the fat body and midgut and gastric caecal epithelium stained intensely. Overall bluish appearance of the larvae was due predominantly to stained fat body tissue, since in relative proportions, this was the most extensive of the stained tissues.

The trophocytes contained small spherical bodies, located toward the cell periphery, that stained intensely deep purple to black. These bodies have not been described in the literature and the nature of these objects is speculative. The hemocytes and pericardial cells were the only other cells that showed a similar intensity of staining. These cells function in phagocytosis and have been shown to pick up particulate materials injected into the hemolymph of mosquito larvae (Wigglesworth, 1965). Intense staining in hemocytes and pericardial cells probably resulted from a high concentration of Nile

Blue Sulfate. Intense staining of the spherical bodies in the trophocytes suggests that these objects may also be similar concentrations of Nile Blue Sulfate.

RETARDATION OF LARVAL DEVELOPMENT. A preliminary experiment gave little information on the dye's effect on length of larval development. The 24-hour observation interval was too long for statistically significant data on retardation of development at dye concentrations below 5.0 p.p.m. However, the dye's toxicity to the larvae was determined. Table 1 presents mortality figures of larvae reared to pupation in different dye concentrations. Larval mortality in dye concentrations up to 2.50 p.p.m. was not significantly greater ($p < .05$) than normal laboratory mortality. High mortality occurred in 7.50 p.p.m. (53.17 percent) and in 10.00 p.p.m. (92.31 percent) concentrations and consequently these concentrations were eliminated from the subsequent experiments.

In experiment II, hourly observations (whenever possible) gave quantitative data of the dye's inhibitory effects on development time of each instar. A statistical summary of results is presented in Table 2. A general trend of increasing range, mean and S.D. of an ecdysial period occurs with increasing dye concentration at all ecdysial levels. Data for the 5.00 p.p.m. concentration are lacking beyond ecdysis II since ecdysis III and pupation were incomplete at the termination of the experiment (about 12.5 days after hatch). Mean pupation times were delayed from normal by the different dye concentrations as follows: 0.25 p.p.m., 3.5 hr; 0.50 p.p.m., 13.0 hr; 0.75 p.p.m., 12.0 hr; 1.00 p.p.m., 20.9 hr; 2.50 p.p.m., 77.2 hr (3.2 days) and 5.00 p.p.m., 6.5 days.

Raw data for each ecdysial period in this experiment were modified to perform an analysis of variance test. In each molting period, the hour of initial ecdysis (appearance of the first cast skin) in any treatment was designated hour 1. Each larva was then assigned a number corresponding to the hour that it molted after initial ecdysis. These figures adequately described comparative rates of larval development in the various dye solutions. The modified data were analyzed using a nested one-way classification (Steel and Torrie, 1960). A highly significant difference, $p < .01$, was found among treatments at all ecdysial levels.

Since dye concentrations below 1.00 p.p.m. did not adequately stain larvae for mark, release and recapture studies, Duncan's Multiple Range test was used to determine the dye concentration that significantly altered normal larval development in each stadium. Table 3 presents results of this analysis. A complete test was not run at each ecdysial level. Information was desired on only the lowest dye concentration significantly different from the control, and relationships among the various dye concentrations were not considered. Table 3 shows a significant difference, $p < .05$, occurred between the control and treatment 0.25 p.p.m. at ecdysial levels, I, II and IV. At all ecdysial levels, a highly significant difference $p. < 01$, occurred between the control and treatment 0.50 p.p.m. The analysis shows the dye significantly retarded larval development at concentrations ½, and in most cases ¼, of the concentration (1.00 p.p.m.) necessary for adequate staining for mark, release and recapture studies.

Figure 1 gives the mean (of 3 replicates)

TABLE 1.—Mortality of *A. aegypti* larvae reared to pupation in Nile Blue Sulfate solutions.

| | dye concentration (p.p.m.) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0.00 | .25 | .50 | .75 | 1.0 | 2.5 | 5.0 | 7.5 | 10.0 |
| Dead | 6 | 10 | 6 | 6 | 10 | 5 | 16 | 76 | 96 |
| Live | 144 | 138 | 146 | 143 | 138 | 145 | 138 | 67 | 8 |
| Total | 150 | 148 | 152 | 149 | 148 | 150 | 154 | 143 | 104 |
| % Mort. | 4.00 | 6.75 | 9.95 | 4.03 | 6.75 | 3.39 | 10.39 | 53.17 | 92.31 |

ecdysial rates of *A. aegypti* larvae reared in different concentrations of Nile Blue Sulfate. A graph of the 5.00 p.p.m. treatment is not included since ecdysis III and pupation were incomplete at termination of the experiment. Since hourly observations could not be made, the number of larvae molting at the missing hours was approximated. A cumulative ecdysial curve was constructed from the recorded observations of each ecdysis. The number of larvae molting at the missing hours was then interpolated from these curves. The general trend of increasing developmental time with increasing dye concentrations is evident. Duncan's Range test shows that a significant retardation from the control first occurs at ecdysis I at all concentrations tested.

RESPIROMETRY. Table 4 presents the $\mu$l $o_2$ consumed/mg. dry weight/½ hour by stained and unstained IV instar larvae over a 4-hour period. Analysis of these data, using a split plot design, showed no significant difference, $p<.01$, between the $o_2$ consumption of test and control larvae. Mean rates in $\mu$l./mg. dry weight/hour were 7.83 $\mu$l. for unstained larvae and 8.04

TABLE 2.—Means, range, standard deviation, and coefficient of variation of ecdysial periods of *A. aegypti* larvae reared in Nile Blue Sulfate solutions at 26.5° C.

| p.p.m. of dye | Range (hours after hatch) | Mean (rate of ecdysis) | Standard Deviation | Coefficient of Variation |
|---|---|---|---|---|
| | | Ecdysis I | | |
| 0.00 | 24–30 | 3.32 | 1.57 | 47.28 |
| 0.25 | 24–32 | 3.97 | 1.85 | 46.59 |
| 0.50 | 26–43 | 7.49 | 3.66 | 48.86 |
| 0.75 | 24–43 | 7.10 | 3.64 | 51.52 |
| 1.00 | 28–44 | 8.04 | 3.77 | 46.89 |
| 2.50 | 31–49 | 19.30 | 3.69 | 19.11 |
| 5.00 | 42–193 | 55.15 | 42.40 | 76.88 |
| | | Ecdysis II | | |
| 0.00 | 41–50 | 4.67 | 2.15 | 46.03 |
| 0.25 | 42–54 | 6.30 | 2.57 | 40.79 |
| 0.50 | 43–66 | 10.47 | 3.76 | 35.91 |
| 0.75 | 43–66 | 10.25 | 4.85 | 47.31 |
| 1.00 | 47–66 | 13.46 | 4.65 | 34.54 |
| 2.50 | 50–91 | 30.88 | 10.47 | 33.90 |
| 5.00 | 91–282 | 124.15 | 1051.09 | 846.66 |
| | | Ecdysis III | | |
| 0.00 | 66–91 | 5.50 | 4.41 | 80.18 |
| 0.25 | 66–91 | 5.94 | 3.47 | 58.41 |
| 0.50 | 66–91 | 12.74 | 7.33 | 58.78 |
| 0.75 | 66–91 | 12.03 | 7.09 | 58.43 |
| 1.00 | 71–114 | 19.72 | 7.89 | 40.01 |
| 2.50 | 91–139 | 49.43 | 12.89 | 26.07 |
| 5.00 | 139–303+ | ..... | ..... | |
| | | Pupation | | |
| 0.00 | 114–144 | 14.91 | 9.91 | 66.46 |
| 0.25 | 115–164 | 18.44 | 10.54 | 57.15 |
| 0.50 | 114–183 | 27.91 | 12.91 | 46.25 |
| 0.75 | 116–183 | 26.15 | 11.17 | 42.71 |
| 1.00 | 117–183 | 35.81 | 15.52 | 43.33 |
| 2.50 | 143–287 | 92.06 | 24.59 | 26.71 |
| 5.00 | 230–303+ | ..... | ..... | |

..... incomplete at termination of experiment.

TABLE 3.—Duncan's Multiple Range test for mean development rates of *A. aegypti* larvae reared in Nile Blue Sulfate solutions.

| Ecdysis I | | | | Ecdysis II | | |
|---|---|---|---|---|---|---|
| p.p.m. dye | 0.00 | 0.25 | 0.50 | p.p.m. dye | 0.00 | 0.25 |
| Mean (hr) | 3.32 | 3.97 | 7.49 | Mean (hr) | 4.67 | 6.30 |
| | .... | | | | .... | |
| | | .... | | | | .... |
| | | | .... | | | |
| **Ecdysis III** | | | | **Ecdysis IV** | | |
| p.p.m. dye | 0.00 | 0.25 | 0.50 | p.p.m. dye | 0.00 | 0.25 | 0.50 |
| Mean (hr) | 5.50 | 5.94 | 12.75 | Mean (hr) | 14.91 | 18.49 | 27.91 |
| | ..... | | | | ..... | | |
| | | ..... | | | | ..... | |
| | | | | | | | ..... |

——— indicates no significant difference at p. 0.01.
..... indicates no significant difference at p. 0.05.

μl. for stained larvae. The dye did not significantly affect $O_2$ consumption of IV instar larvae over a 4-hour period. Buffington (1968) showed that oxidative metabolism in *Cu. pipiens* larvae is based on a circadian rhythm. Whether such an endogenous rhythmic activity is present in *A. aegypti* larvae is not known.

The above results show that Nile Blue Sulfate significantly retards normal development of *A. aegypti* larvae in concentrations at least as low as 0.25 p.p.m. and that the dye does not affect the oxygen consumption rate of IV instar larvae over a 4-hour period. The following discussion elaborates on these results.

Ecdysial curves of *A. aegypti* larvae developing under optimal conditions show a successive increase in duration of each molting period from ecdysis I to pupation (Table 1). In this work, at 27° C., ecdysis I occurred within a 6-hour interval and pupation within a 30 hour interval. A corresponding decrease in the hourly number of molting larvae also occurs (Fig. I), a relative comparison showing a maximum of 75 larvae/hour during ecdysis I and only 36 larvae/hour during pupation. A separation of events due to individual growth rates in a population, whether resulting from sex or other genetic differences or biological or physical factors,

TABLE 4.—Oxygen consumption of stained and unstained IV instar larvae of *A. aegypti* expressed as μl $O_2$/mg dry weight/half hour.

| | | Half Hours | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Treat. | rep | 0.50 | 1.00 | 1.50 | 2.00 | 2.50 | 3.00 | 3.50 | 4.00 | Total |
| Normal | 1 | 4.92 | 3.88 | 4.43 | 3.98 | 3.62 | 3.80 | 3.88 | 3.75 | 32.26 |
| | 2 | 4.38 | 3.69 | 4.30 | 3.84 | 3.49 | 3.72 | 3.77 | 3.42 | 30.62 |
| | 3 | 4.23 | 3.53 | 4.03 | 3.72 | 3.51 | 3.70 | 3.70 | 3.57 | 29.86 |
| | 4 | 4.79 | 3.86 | 4.46 | 4.36 | 2.56 | 4.05 | 3.88 | 3.56 | 32.46 |
| Total | | 18.32 | 14.96 | 17.22 | 15.84 | 14.18 | 15.27 | 15.10 | 14.31 | 125.20 |
| Stained | 1 | 5.25 | 4.32 | 4.93 | 4.11 | 3.79 | 4.20 | 4.20 | 4.42 | 35.22 |
| | 2 | 3.49 | 3.83 | 4.51 | 3.72 | 3.63 | 3.83 | 3.73 | 3.94 | 30.68 |
| | 3 | 4.62 | 3.93 | 4.18 | 3.91 | 3.58 | 3.88 | 3.66 | 3.93 | 31.69 |
| | 4 | 4.18 | 4.00 | 4.22 | 3.83 | 3.38 | 3.88 | 3.66 | 3.92 | 31.67 |
| Total | | 17.54 | 16.08 | 17.84 | 15.57 | 14.38 | 15.79 | 15.25 | 16.21 | 128.66 |

occurs over time in the normal larval development of this mosquito in the laboratory.

A population of *A. aegypti* larvae reared in a 2.50 p.p.m. Nile Blue Sulfate solution under otherwise identical conditions as above, required 18 hours to complete ecdysis I (versus 6 hours normally) and 123 hours to complete pupation (versus 30 hours normally). Corresponding relative maxima of the number of larvae molting/hour were 33 larvae/hour (75 larvae/hour normally) for ecdysis 1 and 9 larvae/hour (36 larvae/hour normally) for pupation. Population sizes under normal and test conditions were nearly identical and a comparison of these rates and initial onsets of ecdysis is valid. Since the dye inhibited the onset of each ecdysial period and the duration of an ecdysial period has been shown to increase over time, comparisons of molting periods occurring during similar age groups rather than similar developmental stages are most valid. In this work, such comparisons are possible. Initial ecdysis III of a normally reared population occurred 66 hours after hatch and lasted only 25 hours, whereas initial ecdysis II of a dye-reared population (2.50 p.p.m.) occurred 55 hours after hatch and lasted 38 hours. Likewise, initial pupation of a normally reared population occurred 114 hours after hatch and lasted only 30 hours whereas initial ecdysis III of a 2.50 p.p.m. dye-reared population occurred 91 hours after hatch and lasted 48 hours.

If dye inhibition of development occurred by a mechanism affecting all larvae essentially equally, such as reduced metabolic rate due to low temperatures, one would expect the ecdysial ranges of similar age groups to be nearly identical. That is, no differential rate of growth would occur above that expected from a normally reared population taking an equivalent length of time to mature. However, this did not occur in our experiments. Larvae reared in 2.50 p.p.m. dye, 11 hours younger than normally reared larvae, required 13 hours longer to complete an ecdysial period. Also, dye-reared larvae (2.50 p.p.m.), 23 hours younger than normally reared larvae required 18 hours longer to complete an ecdysial period. The dye appears to differentially affect developmental rates of larvae.

This differential effect is further exemplified by comparing the four ecdysial periods in larval development of dye-reared and normally reared larvae. Normally, ecdysial periods in *A. aegypti* are distinctly separate (10 hours minimum at 27° C. between the end of one ecdysial period and the onset of the next) with never more than two instars simultaneously present in a culture. Occasionally in our laboratory we have found exceptions to this, with a few larvae developing much slower than the majority of the culture. However, the number of larvae representing this group is less than 1 percent. Normally, ecdysis I occurs over a 6–10 hour period beginning 22–24 hours after hatch; ecdysis II over a 10–12 hour period beginning 42–48 hours after hatch; ecdysis III over a 20–25 hour period beginning 66–72 hours after hatch and pupation over a 25–30 hour period beginning 114–120 hours after hatch. At dye concentrations of 0.50 p.p.m., overlap of ecdysial periods begins to occur. At 5.00 p.p.m., this overlap has occurred to such an extent that I, II, III, and IV instars are simultaneously present in a culture (Table 2). A differential dye effect is evident when some larvae 193 hours old are still I instars and other larvae 193 hours old are IV instars.

Respirometry studies showed no significant difference in rate of oxygen consumption between stained and unstained larvae, in fact $O_2$ consumption of stained larvae was slightly higher than that of normal larvae. The dye inhibited larval growth but did not affect the oxidative metabolic processes of the larvae. Therefore, it appears that stained and unstained larvae utilize the same amount of energy but in different ways. The normal larvae grow, the stained larvae do also, but at a much slower rate. The dye somehow diverts or utilizes energy larvae normally use for growth and development, and this diversion of energy occurs to different degrees in different larvae.

Histological studies show unknown peripherally located spherical bodies in trophocytes of the fat body. These bodies stained intensively blue-black. Intensity of color was similar to cytoplasmic inclusions in hemocytes. Hemocytes are known to function actively in phagocytosis and have been shown to pick up ammonium carmine dyes. Therefore, it appears that these bodies in the trophocytes are concentrated deposits of the dye. The dye's inhibitory action on larval development suggests the use of this dye as a possible control agent. The dye could be applicable to situations where retarded larval development would be detrimental to the species, i.e., temporary ponds that dry rapidly or northern breeding areas where prolonged larval development results in adult emergence after the onset of cold weather. Also those mosquito species that are multivoltine could be partially controlled by continual treatment of the breeding sites throughout the breeding season. No information is available of dye affects on adults. Whether or not the dye affects adult longevity, fertility, fecundity or egg viability is not known.

## SUMMARY

The vital dye, Nile Blue Sulfate, significantly inhibits larval development at the minimum concentration (1.00 p.p.m.) necessary for adequate staining of *A. aegypti* larvae for mark, release and recapture studies. The observed effects of the dye have made possible a discussion of inhibitory action and possible economic value of this dye or related substances. Histological and respirometric studies indicate that the dye interferes with normal energy utilization in the developing larvae. The appearance of intensely stained blue-black spherical bodies in trophocytes of the fat body is tentatively associated with the dye.

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