Notes

Methods employed for determining Insecticide Resistance in Mosquito Larvae

Submitted by A. W. A. Brown, *Biologist, Division of Environmental Sanitation, World Health Organization, to the seventh session of the WHO Expert Committee on Insecticides*

Two methods for larval testing have recently appeared in mimeographed form. The first, sponsored by the US Army and Navy, and based on recommendations of the Orlando, Fla., Laboratory of the US Department of Agriculture, is applicable to *Anopheles*, *Aëdes* and *Culex*. The second, developed by the Ross Institute of Tropical Hygiene, is designed for *Anopheles* larvae and is based on studies with *A. stephensi*. Gjullin & Peters [a] have published the methods used in resistance studies in California, and Brown and co-authors [b] in Ontario, for *Aëdes* and *Culex* larvae. Krusé, Ludvik & Hawkins [c] of the Tennessee Valley Authority (TVA) have investigated the effect of certain variables in test conditions as affecting the results, using *Anopheles quadrimaculatus*. The US Public Health Service laboratories at Savannah, Ga., and Logan, Utah, carry out routine tests on resistance.

Test larvae. All are agreed that the larvae should be in the early IVth stage, to avoid pupation during the test. R.W. Fay, in a communication to the WHO Expert Committee on Insecticides, finds that similar susceptibility is shown in the IIIrd stage to that in the early IVth. From tests on *Aëdes ægypti*, Burchfield et al. [d] report that the greatest uniformity of response was obtained in the IVth stage, whereas A. H. Parker (personal communication) finds that the IIIrd is the least variable of the stages. The California and Ontario workers collected the larvae from the field in 1-gallon containers (about 4 litres) (preferably Thermos), while the Ross Institute recommends preliminary rearing before testing. Fay reports more uniform results if the larvae are starved for 2-4 hours before being tested.

Container. The Ontario, Savannah and Logan workers have used enamel pans. The Ross Institute and US Army methods recommend glass beakers. The TVA workers had originally used waxed cardboard containers (expendable), but abandoned them on discovering that DDT did not stay in suspension in them. They found the amounts remaining in suspension (from ethanol) after 24 hours were 60% for enamel, 57% for glass and

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a Gjullin, C. M. & Peters, R. F. (1952) *Mosquito News*, **12**, 1

b Brown, A. W. A., Armstrong, J. A. & Peterson, D. G. (1954) *Mosquito News*, **14**, 192

c Krusé, C. W., Ludvik, G. F. & Hawkins, W. B. (1952) *J. econ. Ent.* **45**, 598

d Burchfield, H. B. et al. (1953) *Contr. Boyce Thompson Inst.*, **17**, 317

24% for waxed cardboard. Recently, Hawkins & Kearns [e] have found that the titanium ions in the cardboard remove the zeta-potential of the colloidal micelles of DDT, the wax being of no significance. The flocculation of the colloid may be observed by the colour of the suspension changing from blue to greyish-white.

Fay reports that not only depth of water, but also the shape of the container as determining the water margin, are factors exerting an effect on mortality.

Volume of water. The US Army test uses 200 ml in a 400-ml beaker, and the Ross Institute 100 ml in a 250-ml beaker. The Ontario, Savannah and Logan workers use one litre in an enamel pan 7×11 inches (about 18×28 cm); Gjullin & Peters used 200 ml. Since solutes in water may considerably influence the results, the use of distilled water as a standard is preferable. H. I. Scudder (personal communication) points out that distilled water may be deleterious, and recommends the use of BOD dilution water. [f]

Number of larvae. The US Army test calls for 25 larvae, Gjullin & Peters for 20 larvae, in 200 ml. The Ontario, Savannah and Logan workers used 50 larvae in 1000 ml. The Ross Institute test places 50 *Anopheles* larvae in 100 ml, giving 10 larvae per square inch of surface. Krusé and co-authors found that increasing the larval concentration from 0.1 per square inch to 5 per square inch decreased the mortality of *A. quadrimaculatus* from 80% down to 1.5% (when DDT was applied in a kerosene film at 0.2 μg per square inch). Brown [g] found that larvae at a density of 50 per litre could remove as much as one-third of the DDT from the liquid.

Replicates. Gjullin & Peters used two replicates at each concentration. The US Army method calls for three replicates and the Ross Institute for five replicates at each concentration. Fay has found wider variation between different days of testing than between replicates on the same day, and recommends that tests be performed on a series of days.

Carrier of insecticide. The US Army method, Gjullin & Peters, and the US Public Health Service use acetone as the solvent for the insecticide. The Ross Institute, TVA and Ontario workers use ethanol. The US Public Health Service use 1 ml of acetone carrier to one litre of water, and vary the concentration of DDT in the acetone accordingly. The US Army test varies the amount of acetone solution added between 0.1 ml and 1 ml per 200 ml of water. Krusé and co-authors found that more DDT was removed from the liquid by settling from ethanol suspensions (40% lost in 24 hours) than from xylene-Trex emulsions (20% lost in 24 hours). Hawkins [h] has

[e] Hawkins, W. B. & Kearns, C. W. (1956) *Bull. ent. Res.*, **47**, 197

[f] American Public Health Association (1955) *Standard methods for the examination of water, sewage and industrial wastes*, 10th ed., New York, p. 261

[g] Brown, A. W. A. (1956) *Bull. Wld Hlth Org.*, **14**, 807

[h] Hawkins, W. B. (1956) *J. econ. Ent.*, **49**, 367

found that a tenfold rise in carrier concentration, whether ethanol, acetone or isopropanol, halves the LD_{50} of DDT for *A. quadrimaculatus* larvae.

No information is available on the effect of the method of addition of carrier on the types of colloid suspensoids produced. It is not clear whether the water in the tray or beaker should be stirred or otherwise agitated after the DDT solution has been added by pipette.

Test solutions. The US Army method recommends that 1 : 50 000 and 1 : 500 000 stock solutions be made by serial dilution of an original 1 : 100 stock solution. They thus produce 0.1 p.p.m. (part per million) in 200 ml of water by adding one ml of the 1 : 50 000 stock solution, and produce 0.001 p.p.m. by adding 0.1 ml of the 1 : 500 000 stock solution. Intermediate p.p.m. are produced by adding intermediate amounts of the stock solution. The US Public Health Service and Ontario workers always use 1 ml of stock solution to 1 litre of water. Individual stock solutions are prepared to correspond to the p.p.m. desired in the test, thus 0.1 p.p.m. is produced by dilution of a 1 : 10 000 stock solution, and 0.001 p.p.m. by dilution of a 1 : 1 000 000 stock solution. This method uses more acetone (or ethanol) than the US Army method.

Observation period. The California, Ontario and US Public Health Service workers use a 24-hour observation period. The US Army test recommends 48 hours, but if control mortality is high 24 hours may be taken. The Ross Institute calls for 48 hours, but the larvae are removed from the test insecticide at 24 hours and kept in fresh water, with food added, for the second 24 hours. Fay finds that a 24-hour period is sufficient with DDT, but that with dieldrin there is considerable delayed mortality in the second 24-hour period.

Test temperatures are 25°C for the Ross Institute, 23°C for Ontario, 80°F (27C°) for California. The US Army method does not specify the temperature. A. D. Hess (personal communication) recommends that temperature-mortality curves be obtained in order to interpret results at different temperatures.

Criteria of mortality. The US Army test counts as dead all larvae that cannot rise to the surface after having been forced to the bottom. The Ontario and US Public Health Service count as dead those that do not move on being probed with a needle. The Ross Institute recommends that those that pupate be removed from consideration. A parallel test of mortality in control solutions is required by the Ross Institute and US Army tests; the latter test apparently would tolerate a 25% control mortality, but presumably Abbott's formula should be applied.

Miscellaneous. Larvae are transferred to test solutions by a small net (Ross Institute, US Department of Agriculture, Ontario, US Public Health Service) or a medicine-dropper (US Army). The US Department of Agriculture, US Public Health Service and US Army place the larvae first in

25 ml of the water to be added to the test solution, thus avoiding contamination of the net or dropper. The Ross Institute recommends that glassware be cleaned in chromic acid, but presumably acetone and 10% sodium hydroxide could be used before cleaning with detergents. The Orlando workers heat the glassware above 200°C to destroy the last traces of DDT. The Orlando workers use the purified or technical grade insecticide for test, while in the US Army method even wettable powders are permitted; the TVA and Ontario workers use the purified insecticide. Both Hess and C. H. Smith (in a communication to the WHO Expert Committee on Insecticides) consider it preferable to use the purified insecticides. Smith recommends that the instructions include some specimen figures from which the tester may judge whether his mosquitos are resistant or not.

Conclusions. It is felt that the guiding considerations should be (*a*) simplicity, and (*b*) suitability for both culicines and anophelines, and it is considered unwise to attempt to adhere in all respects to any pre-existing method for its own sake. Desirable characteristics of a test should include:

Container:	Approximately 500 cm^2 in area, preferably enamel pans
Volume of water:	1 litre
Type of water:	Distilled water if possible, tap or field water permissible
Test larvae:	30 larvae in late IIIrd or early IVth stage
Number of tests:	2 replicates at each of 4 insecticide concentrations
Insecticide:	Purified compound
Carrier:	Ethanol: 1 ml to 1 litre
Test solutions:	Series of standard solutions to give required final concentrations
Preparation:	Stirring for 30 seconds with a glass rod
Transfer:	By means of a spoon of wire screening, larvae are transferred into 25 ml of water to be added to test solution
Condition of larvae:	Field-collected or second-generation laboratory rearings
Test period:	24 hours' exposure to insecticide, then transfer to clean water; mortality counts both at end of test period (24 hours) and after another day in clean water (48 hours)
Criterion of death:	Lack of movement on probing; pupae removed from test