ENTRY AND MOVEMENT OF PETROLEUM DERIVATIVES IN THE TRACHEAL SYSTEM OF MOSQUITO LARVAE

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ABSTRACT. The presence of certain petroleum derivatives throughout the extracellular tracheal system of living mosquito larvae was observed by exposing them to fluorescein-labelled materials. These hydrocarbons continued to enter such larvae when the tracheal trunks were collapsed. Numerous specimens were able to complete development to adults after hexadecane was present throughout the respiratory system.

During the approximately 75-year period that petroleum-type larvicides have been used in mosquito control, there has been considerable disagreement concerning the relationship between their entry and movement within the tracheal system, their mechanism of action, and the subsequent mortality in mosquito larvae exposed to water surface treatments. A review of the literature reveals that the conflicting findings stem from vast differences in composition of the petroleum derivatives and techniques used, as well as in mosquito species studied (Hagstrum and Mulla 1968, and Micks 1968a). In fact, practically all of these derivatives of petroleum were specifically developed for their fuel characteristics, and not for their mosquito control properties. It is not surprising, therefore, that the majority of these larvicidally active compositions share the disadvantage of being toxic to the various other forms of life in the aquatic environment. This situation, coupled with increasing problems with insecticide-resistance and reversals in malaria eradication programs (Micks and Chambers, 1974), led us to team up with industry for the purpose of developing new kinds of mosquito control agents from petroleum which would be effective against larvae and pupae, biodegradeable, non-resistance inducing, and have a very low order of acute toxicity, being essentially inert (Micks et al. 1967; Micks 1968a; Micks et al. 1968b; Micks et al. 1969; Micks, 1970; Micks et al., 1972).

During the course of this work we were recently able to observe in living larvae the movement of petroleum hydrocarbons throughout the entire, extracellular tracheal system. We obtained evidence that suggests that these petroleum derivatives may continue to enter larvae exposed to surface films when they are already present in all portions of the tracheal system and when the tracheal trunks are collapsed. Also, we found that rather large numbers of larvae were able to complete their development to adulthood after their main tracheae were full of one of these materials.

Materials and Methods. The specimens of the southern house mosquito, *Culex pipiens quinquefasciatus* Say, and of the yellow fever mosquito, *Aedes aegypti* (L.), were obtained from laboratory colonies reared under standardized conditions in our laboratory since 1956.

Batches of 25 fourth-instar larvae of *Cx. quinquefasciatus* and *Ae. aegypti* were exposed in 250 ml of distilled water in 400 ml beakers to 4 μl and 2 μl water surface treatments of hexadecane (an essentially pure straight chain hydrocarbon) both with and without a spreading agent (Triton X-45), and FLIT® MLO (an alkyl cycloparaffinic petroleum derivative) respectively, for periods ranging from 1 minute to 2 hours. At the end of the exposure period, the tracheal system of each specimen was examined microscopically to determine the location of the Oil Red A marker incorporated into the test materials. The larvae were placed in beakers of clean water immediately thereafter and reexamined daily as long as they survived. Larval mortality and adult emergence were both recorded. Other batches of larvae were similarly exposed to these petroleum hydrocarbons with a fluorescein label. Individual specimens were removed at various intervals of time for *in vivo* examinations under the fluorescence microscope and photographed at 100 X and 200 X magnification using Tri-X Pan film with 15-second exposures.

Still other batches of larvae were exposed to the unlabelled test materials for periods ranging from 3–17 hours, and then immediately transferred to clean beakers wherein the specimens were exposed to the fluorescein-labelled hydrocarbons for similar periods of time. Each larva was then examined microscopically for the presence of tracheal system fluorescence. Appropriate untreated controls were included in all experiments.

Results and Discussion. Hexadecane (with Oil Red A) could be observed in the tracheal trunks and main branches of the tracheal system but not in the finer branches and tracheoles due to their small diameter and the corresponding decrease in color density. The red coloration of the tracheae frequently persisted for several days. Surprisingly, from 44–83% of the *Cx. quinquefasciatus* larvae with hexadecane throughout the tracheal trunks completed their development and emerged as adults (Fig. 1). This finding is contrary to the views of earlier investigators (Murray 1936, Watson 1941) and some mosquito control workers today, i.e., that once an "oil" enters the respiratory system, death invariably ensues. Whereas the percentage of larvae with hexadecane present tended to increase with exposure time, the percent of those developing to adulthood was variable but high. Because of its much greater biological activity, we exposed *Cx. quinquefasciatus* larvae to FLIT MLO for shorter periods of time. Although some larvae were able to survive exposures of 8, 10, 15 and 60 minutes long enough to complete their development, in general, once the hydrocarbon was present in the siphon, mortality was complete (Fig. 2). The development of specimens reaching adulthood following the entry of hexadecane or FLIT MLO was delayed from 3–7 days as compared with untreated controls. The adults were able to make and produce viable eggs. Very similar results were obtained with *Ae. aegypti* in these various experiments except that the overall mortality was slightly less than in *Cx. quinquefasciatus.* Consequently, we have not included details of those findings.

For the first time, the fluorescein-labelling of these two petroleum hydrocarbons enabled us to determine that they travel throughout the entire tracheal system to the finest branches in spite of the high viscosity of hexadecane and its poor spreading characteristics on water surfaces. In fact, the addition of a 1% spreading agent to the hexadecane did not increase the mortality of exposed larvae even though water surface coverage and movement within the tracheal system were greatly enhanced. Thus it is clear that penetration of this system is not simply

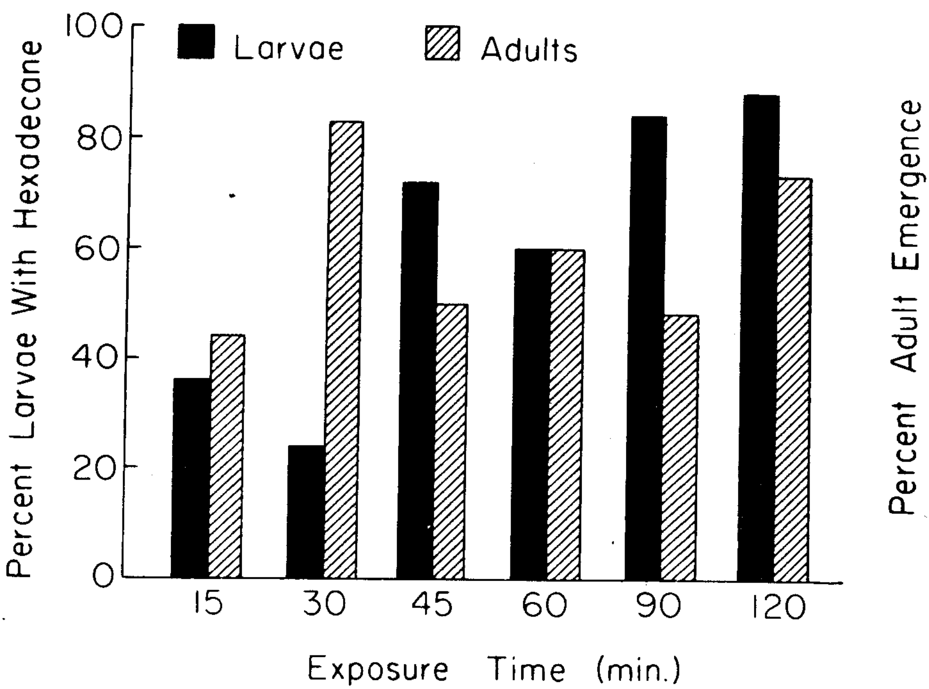


Fig. 1. Relationship between the presence of hexadecane in the tracheal system of *Cx. quinquefasciatus* larvae and the completion of development. Percent adult emergence represents the percent of only those larvae containing dye in their tracheae.

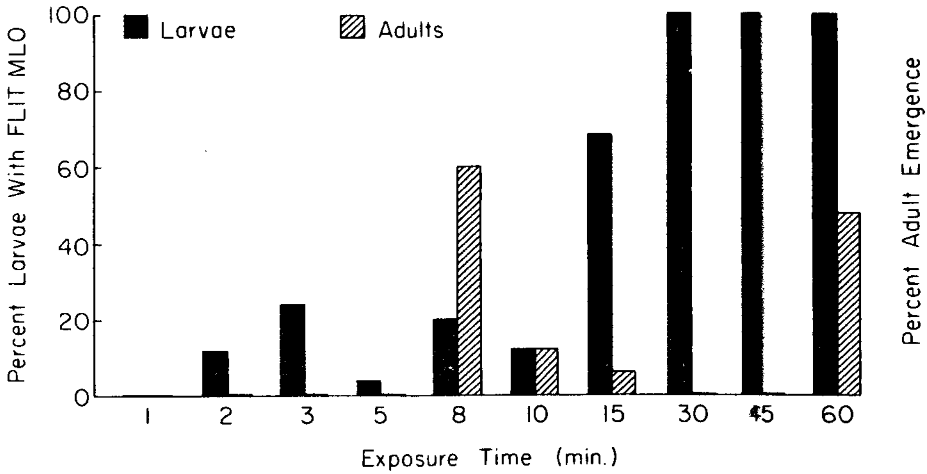
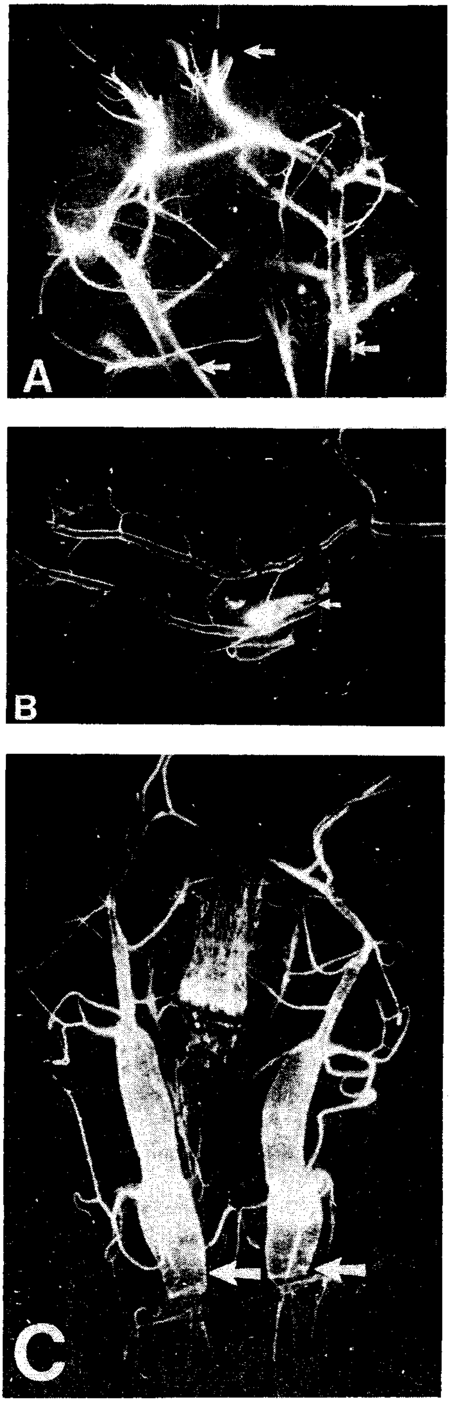


Fig. 2. Relationship of the presence of FLIT MLO in the tracheal system of *Cx. quinquefasciatus* larvae and the completion of development. Percent adult emergence represents the percent of only those larvae containing dye in their tracheae.

a function of the viscosity of a petroleum hydrocarbon as claimed by Reid and Ganapathipillai (1951).

Sufficient Oil Red A could not be incorporated into the FLIT MLO so that the hydrocarbon could be seen beyond the tracheal trunks when the normal application rate of 2 μl was used. However, when the fluorescein label was used, all ramifications of the respiratory system were observed including those in the thorax which is the thickest part of the larva (Fig. 3A). The tracheoles which comprise the peripheral portion of the tracheal system can just be seen as the smallest diameter tubules. The small quantity of petroleum derivative which enters at a given surfacing moves along the tracheal walls as readily observed in the main trunks (Fig. 3B), and rarely are these main tracheae filled completely when recommended dosages are used. This technique also demonstrated that both hydrocarbons traveled throughout this system in *Cx. quinquefasciatus* larvae approximately twice as rapidly as in *Ae. aegypti* specimens. Further, with any given number of surfacings, the FLIT MLO moved farther into the tracheal system of *Cx. quinquefasciatus* as compared with *Ae. aegypti* larvae. Thus, there was a slower speed of entry of both petroleum derivatives in larvae of the latter species, which is probably a function of its larger tracheal system. Whereas both species of larvae exhibited a "siphon-biting" phenomenon, this activity was twice as frequent in the *Ae. aegypti* larvae. That this may represent a mechanism of cleaning hydrocarbon from the siphon was suggested by the frequent occurrence of fluorescence in the gut (Fig. 3B).

Even though the main tracheae collapsed and remained flattened when small amounts of petroleum derivatives first entered the siphon, these materials continued to move anteriorly with time and subsequent surfacings (Fig. 3C). Once they spread throughout the respiratory system, further exposure was accompanied by entry and forward movement of a much more intense fluorescence than that already present in the petroleum hydrocarbon distributed throughout the tracheal system. This finding is interpreted as representing the continuing entry of the hydrocarbons. However, we cannot be absolutely certain that some separation of



the fluorescein and separate entry of it do not occur. A specimen which had been exposed to a surface film of unlabelled FLIT MLO for 2 hr and then immediately treated with the fluorescein-marked hydrocarbon for a period of 4 hr is shown in Fig. 3A.

In summary, we have found fluorescence microscopy of petroleum derivatives to be much more sensitive and generally superior to the usual dyes and stains employed in studies of the respiratory system of mosquito larvae. It is especially useful in observing the dynamics of the tracheal system-hydrocarbon interaction in the living specimen. Its further application and refinement is expected to be useful in arriving at a clearer understanding of mechanisms of action of existing and future control agents derived from petroleum.

ACKNOWLEDGMENT. We thank Mr. G. V. Chambers of the Exxon Research & Engineering Co., Baytown Petroleum Research Laboratory, Baytown, Texas, for supplying the petroleum derivatives used in this study.

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Fig. 3. Fluorescing FLIT MLO in the tracheal system of live 3rd instar larvae. (A) Tracheal network in the posterior head capsule (top arrow) and thorax of *C. quinquefasciatus* (X200). The diameter of the smallest branch which can be seen in the photo is approximately 1 μ. The anterior portions of the main abdominal tracheae can also be seen (bottom arrows). (B) The two thick, main tracheal trunks with connecting and lateral tracheae in the abdomen of *C. quinquefasciatus* (X100). The siphon at the posterior end is at the extreme right. Note the fluorescing hydrocarbon in the posterior midgut (arrow). (C) Main tracheal system of the posterior head capsule, thorax and anterior abdomen of *A. aegypti*. Note the fluorescing hydrocarbon in the flattened, ribbon-like tracheal trunks (arrows).

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