DEVELOPMENT OF DDT RESISTANCE IN CERTAIN MAYFLIES IN NEW BRUNSWICK

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Abstract

Populations of the mayfly *Heptagenia hebe* McDunnough in areas of New Brunswick air-sprayed with DDT for budworm control for eight successive years, when tested as nymphs taken from the streams, showed LC_{50} levels 3 times as high as those in untreated areas. Populations surviving an airspray in 1965 proved to be 12 to 40 times as DDT-resistant as the normal. Pre-spray populations of *Stenonema fuscum* (Clemens) showed a 5-fold resistance, and post-spray populations of *Stenonema interpunctatum* (Say) showed a 10-fold DDT resistance when those from treated areas were compared with those from untreated. The DDT-resistant nymphs of *H. hebe* detoxified DDT to DDE 15 times faster than the normal nymphs; DDE was also the metabolite in *S. interpunctatum*. In both these species the DDT-resistant nymphs absorbed roughly twice as much DDT as the normal.

Introduction

Since 1952, DDT in oil solution has been aerially applied over large acreages of spruce–balsam forest in northern and central New Brunswick to control the

spruce budworm (Macdonald 1965). By 1962, populations of this insect in the Miramichi drainage system had developed up to 10-fold resistance to DDT (Randall 1965). By 1957 it was noted that stream populations of mayfly nymphs in sprayed areas in northern New Brunswick recovered their numbers quite rapidly, suggesting the possibility that they had become resistant (Webb *et al.* 1959). However when an unidentified species of *Nemoura* stonefly was tested in 1959 (Sprague 1967), no difference in susceptibility level was found between those from sprayed and unsprayed streams.

The present study, conducted in central New Brunswick in 1965 and 1966, investigated the possibility that DDT resistance had developed in several species of mayflies. This region had been sprayed annually since 1956 with the sole exception of 1959. Collections were made in the Renous River and Rocky Brook in the treated area and were tested in comparison with those taken from the Pollet River and New River in southern New Brunswick, well away from the treated area (Fig. 1). The susceptibility levels of the insects were assessed by standard tests. In those species which proved to have developed DDT resistance, the physiological mechanism of this resistance was investigated by chemical and radiochemical means.

Methods

The area around the sampling station on Rocky Brook had been treated with full coverage of spray for 4 years and partial coverage for 6 years, while the area on the Renous River had been treated with full coverage for 4 years and partial coverage for 5 years, as follows:

1956 and 1957	General area sprayed with DDT.
1958	Headwaters sprayed with DDT.
1959	Entire province left unsprayed.
1960	General area sprayed with DDT.
1961	Upstream areas sprayed with DDT.
1962	Downstream areas, including collection stations, sprayed with DDT.
1963	Short sections sprayed with DDT.
1964 and 1965	General area sprayed with DDT, but phosphamidon used along stream banks.
1966	Rocky Brook area sprayed as in 1965, but Renous River area not sprayed.

The level of susceptibility of mayfly nymphs to DDT was determined by a method based on the tentative WHO method for blackfly larvae (World Health Organization 1963). The mayfly nymphs were collected by picking stones one by one from the stream bottom and plunging them several times in a pail of water until all the nymphs on the stone had been removed; an hour or two of collecting usually yielded enough nymphs from which a selection could be made for a test. The field tests in 1965 were performed in Pakalene plastic pails each containing 1500 ml stream water into which 10 to 15 nymphs were introduced. The appropriate amount of DDT in ethanol was then added to each pail to give a logarithmic series of concentrations. The mayflies were exposed to the DDT for 30 minutes, then transferred to pails of clean water and left for 24 hours. These recovery pails were set in the collecting stream itself, anchored with rocks, and aerated with aquarium airstones and an electric air pump. The tests made in 1966

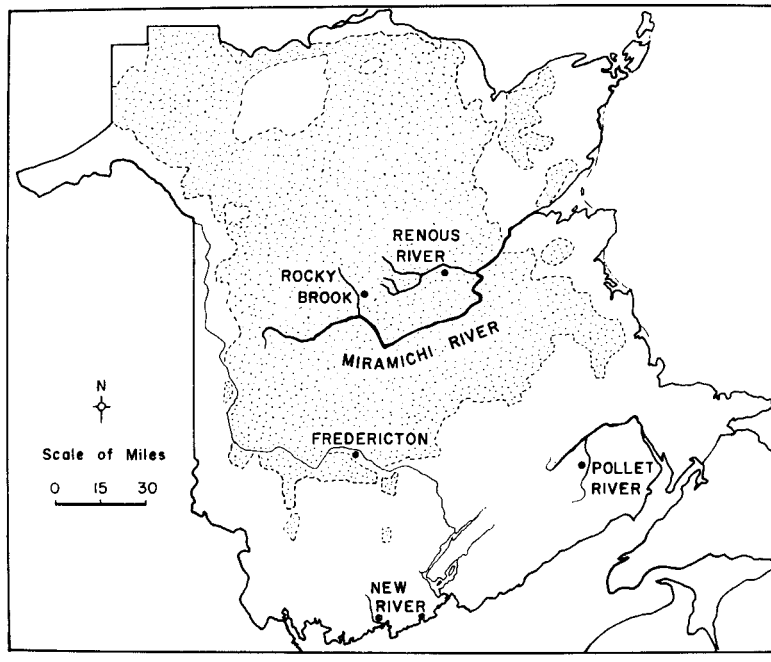

FIG. 1. Location of mayfly collecting sites in New Brunswick; stippled areas have been sprayed at least once. Adapted from Macdonald (1965).

were performed after transporting the nymphs to the Forest Biology Laboratory, Fredericton, where 16-oz glass ice-box jars each containing 250 ml water were employed; 10–15 nymphs were added as before and then the DDT in ethanol was introduced to give the desired concentration. At the end of the 30-minute exposure period the nymphs were removed to distilled water in other jars and these were placed in a cool room with an airstone in each jar.

At the end of the recovery period the nymphs were categorized into live, moribund, and dead, those in the middle category being unable to right themselves and swim but still able to flutter gills or twitch a leg. In calculating the percentage mortality, the moribund were classed along with the dead. The points were plotted on logarithmic-probability paper and the regression lines fitted by the probit analysis method of Finney (1952) in all cases, except for the results from Rocky Brook which were so erratic that they were fitted by eye.

The metabolism of DDT was studied by exposing nymphs to 0.025 p.p.m. DDT in 250 ml distilled water in ice-box jars for 14 hours. The nymphs were then rinsed, ground with sand and anhydrous sodium sulphate, and extracted with carbon tetrachloride for 4 hours in a micro-Soxhlet apparatus. The extracts were evaporated and taken up in *n*-hexane, and an aliquot of 1.5 μl was injected into an electron-capture gas chromatography apparatus.[1] The gas chromatogram was obtained by connecting the ECGC apparatus to a Sargent Model-SR recorder.

The uptake and metabolism of radioactive DDT was studied by exposing nymphs to 0.2 p.p.m. C^{14}-DDT for 18.5 hours, and then grinding them with sand

[1]The ECGC instrument was a Wilkens Model-680 aerograph pestilyzer, fitted with a 60 X ⅛ in. helical Pyrex column containing 5% of the fluorinated silicone QF-1 on 60/80 mesh Chromosorb W support. The gas chromatography was carried out at an oven temperature of 180° C, the carrier being N at 35 ml/min. The electron-capture detector cell contained a 250-mc titanium tritide foil as an ionization source.

and sodium sulphate. For assessment of uptake, the ground tissues were extracted with petroleum ether which was then transferred to a 6 in. × 0.5 in. chromatographic column of Woelm alumina;[2] the absorbed C^{14}-DDT was then eluted with carbon tetrachloride, taken up in petroleum ether and mixed with POPOP scintillation fluid (Hayes 1963) and measured in a Packard Tri-Carb liquid scintillation counter.

For separation of C^{14}-DDE, the ground tissues were extracted for 3.5 hours with carbon tetrachloride in a micro-Soxhlet apparatus and a sample submitted to paper chromatography by the dimethyl formamide method of Robbins and coworkers (vide Abedi *et al.* 1963). The chromatograms were developed with silver-nitrate chromogenic agent and scanned with a gas-flow counter connected to a Tracerlab SC-79 ratemeter and a Texas Recti-Riter recording instrument.

Results

It became apparent that the mayfly nymphs were characterized by considerable heterogeneity in response to DDT, so that the dosage–mortality regression lines showed a gradual slope, making the fiducial intervals of the LC_{50} values relatively large. When full-grown nymphs of *Iron fraudator* Traver in Rocky Brook and Renous River were tested they showed an LC_{50} in the neighbourhood of 0.4 p.p.m. DDT; when the nymphs in the Pollet River were tested they also showed an LC_{50} of approximately 0.4 p.p.m., but no specific comparison could be made with those from the sprayed areas because those from the Pollet River were of the species *Iron humeralis* Morgan. The species *Ephemerella lata* Morgan from the Pollet River showed a range of LC_{50} levels in the neighbourhood of 1 p.p.m., but did not occur in the treated stations. *Ephemerella rotunda* Morgan occurred in both areas but unfortunately it showed too variable a response to enable a comparison to be made.

The small mayfly *Heptagenia hebe* McDunnough was common in all streams and thus allowed a full investigation. A sample taken from Renous River in the treated area on 1 July 1966, a year in which the Renous drainage basin was not sprayed, showed an LC_{50} level of 0.33 p.p.m. DDT (Fig. 2) as compared with the LC_{50} level of 0.09 p.p.m. found for an intact normal population at Pollet River on 21 June of that year. A sample from Rocky Brook in the treated area on 15 June, within 1 week after the spraying of its drainage area, revealed that the surviving nymphs showed an LC_{50} of 7.7 p.p.m. DDT. Thus the population carrying over from one year to the next was 3 times as DDT-resistant as normal, while a sample of individuals that had survived a spray shortly before being collected showed a level 70 times as resistant as normal.

The tests performed on *H. hebe* in the treated area in 1965 were restricted to the survivors of an airspray applied early in June of that year. Tests conducted between 23 and 25 June clearly show (Fig. 3) the difference between them and the population from the untreated area. The LC_{50} levels of three tests performed at Renous River were respectively 1.9, 2.1, and 3.0 p.p.m. DDT, as compared with values of 0.15, 0.16, 0.20, and 0.22 p.p.m. for the normal population at Pollet River obtained 2 weeks later. The dosage–mortality results obtained at Rocky Brook in the sprayed area in mid-July were erratic but indicated that the LC_{50} was at least 10 p.p.m., as compared with the values of 0.17, 0.18, 0.29, and 0.4 p.p.m. for the normal population tested at New River in mid-August. Thus the Renous

[2]Unlike the Alcoa alumina employed by Sternberg and Kearns (1952), the Woelm alumina did not allow any significant amount of the DDE produced to be eluted in petroleum ether.

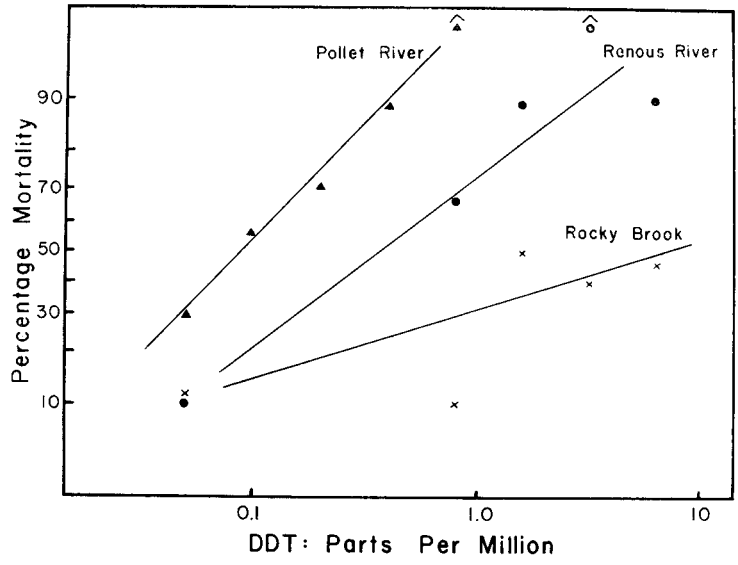

FIG. 2. Dosage–mortality relationships to DDT of nymphs of *Heptagenia hebe* McDunnough tested in 1966. × Rocky Brook 15–VI; ▲ Pollet River 21–VI; ●Renous River I–VII.

River population was 12 times as resistant as the Pollet River population, while the resistance ratio between the Rocky Brook and New River populations was approximately 40.

When the dosage–mortality figures for these tests, performed on nymphs shortly before their midsummer emergence, are tabulated for each month (Table I), it is seen that all of the individuals remaining in the Renous River following the June airspray can survive a concentration (1.6 p.p.m. DDT) that kills all of the Pollet River population. Similarly the surviving sample from Rocky Brook shows zero kill at a concentration (0.8 p.p.m. DDT) which kills all of the normal New River population. It is noteworthy that nymphs that had not emerged by the end of June, the peak emergence time for this species, showed a much lower susceptibility when tested in July.

To find out whether the developed resistance to DDT extended to other insecticides, samples of *H. hebe* from Rocky Brook and Pollet River were tested with three concentrations of dieldrin and the cumulative mortality was observed at successive time intervals; when the LT_{33} or LT_{50} values in hours were determined, they were as follows:

	Rocky Brook	Pollet River
LT_{33} at 0.00125 p.p.m. dieldrin	23.2	21.4
LT_{50} at 0.005 p.p.m. dieldrin	13.1	15.0

Thus there is no significant difference between the two populations with respect to their tolerance to dieldrin.

The quiet water in the Renous River is populated with the large mayfly species *Stenonema fuscum* (Clemens). A single test performed in May 1965, before the spray of that year, showed an LC_{50} of approximately 0.6 p.p.m. DDT; whereas the LC_{50} for the same species in the Pollet River, based on average figures for three tests in June 1965 (Table II), was approximately 0.12 p.p.m. DDT. The

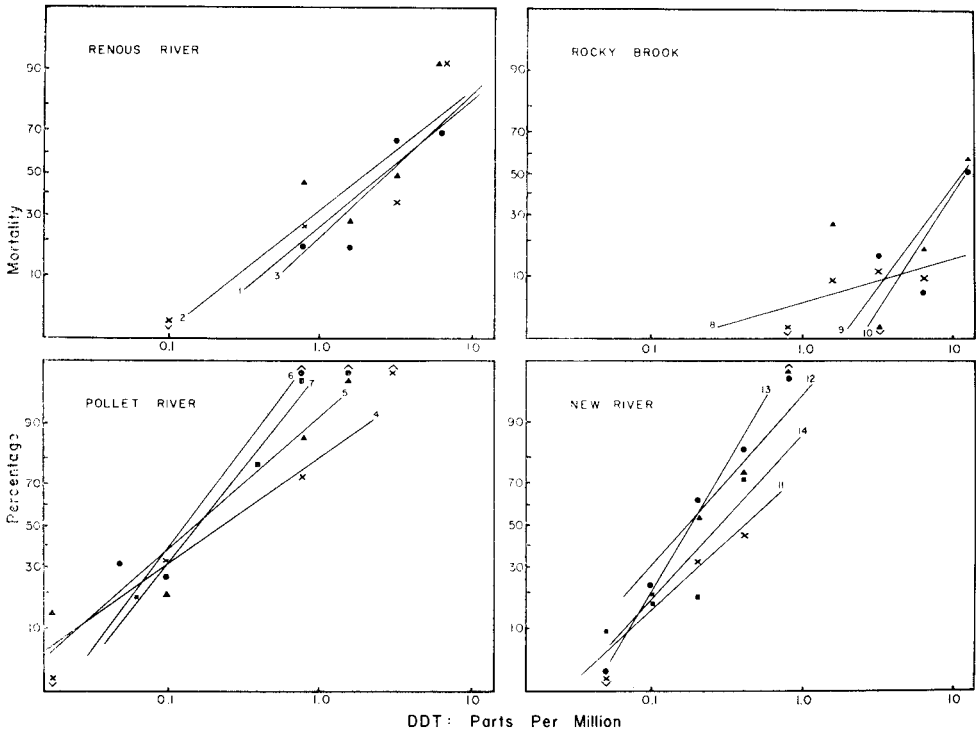

FIG. 3. Dosage–mortality relationships to DDT of post-spray populations of *Heptagenia hebe* McDunnough tested in 1965.

Renous River: × (1) 23– VI; ▲ (2) 24– VI; ● (3) 25– VI.
Pollet River: × (4) 29– VI; ▲ (5) 5– VII; ● (6) 6– VII; ■ (7) 13– VII.
Rocky Brook: × (8) 15– VII; ▲ (9) 16– VII; ● (10) 17– VII.
New River: × (11) 12–VIII; ▲ (12) 13–VIII; ● (13) 14–VIII; ■ (14) 15–VIII.

same water in Rocky Brook is inhabited by *Stenonema interpunctatum* (Say); when tested on 21 June 1965 about 1 week after the airspray, it showed an LC_{50} of approximately 2 p.p.m. DDT, as compared with about 0.2 p.p.m. for a normal population, tested on 13 June 1966 from the untreated Rockwell Stream located about 10 miles east of Fredericton. A similar resistance was found at Rocky Brook in August 1965, but not in June 1966 before another spray was applied. Thus the population of *S. fuscum* at Renous River was about 5 times as DDT-resistant as the normal population in Pollet River, and the sample of surviving individuals of *S. interpunctatum* at Rocky Brook was about 10 times as DDT-resistant as a normal population of this species.

In the study of DDT metabolism by *H. hebe*, a group of 55 nymphs from Rocky Brook in the treated area and a group of 30 nymphs from Pollet River in the untreated area were exposed for 14 hours to DDT and the extracts analyzed by gas chromatography. The chromatograms showed a principal metabolite with a retention time of 3 minutes 48 seconds, identical with that of DDE. This metabolite is particularly conspicuous in the sample from Rocky Brook in the treated area (Fig. 4). Co-chromatography proved that this peak was DDE. Two minor peaks, No. 2 and No. 1 (Fig. 4), showed the same retention times as

TABLE I

Per cent mortalities in samples of *H. hebe* from untreated areas and treated areas; results averaged for each month for both years together

Concn. (p.p.m. DDT)	Untreated areas			Treated areas			
	Pollet River		New River	Renous River		Rocky Brook	
	June	July	August	June	July	June	July
0.8	87	93	100	30	—	10	0
1.6	—	100	—	23	0	50	18
3.2	100	—	—	51	0	20	9
6.4	—	—	—	84	5	45	11

DDD and DOHDT.[3] A third small peak proved to be DDT itself, as confirmed by co-chromatography.

Whereas the extract from the Pollet River nymphs contained less DDE than DDT, the Rocky Brook sample contained much more DDE than the DDT remaining. When the amounts are calculated by triangulation, and the DDE compared with the DDT on a molar basis, it is found that the Rocky Brook nymphs have converted 6 times as much DDT as they have left unconverted, whereas the Pollet River nymphs have converted only 0.3 times as much as that left unconverted, viz.:

	μg DDT	μg DDE	μg DDT converted	Conversion ratio
Rocky Brook	0.46	2.47	2.75	6.0
Pollet River	0.73	0.22	0.25	0.3

Since the amount of DDT to which the nymphs were exposed in 250 ml at 0.025 p.p.m. was 6.25 μg, it may be seen that the 55 nymphs of the resistant population not only absorbed 3.21 μg of it but also succeeded in converting 2.75 μg of it to the non-toxic metabolite DDE. By contrast the 30 nymphs tested from the normal population, which were slightly larger than those of the resistant population tested, absorbed only 0.98 μg of the 6.25-μg dose and detoxified only 0.25 μg

[3]2,2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane.

TABLE II

Per cent mortalities in samples of *Stenonema* spp. from untreated areas and treated areas; results averaged for each month where more than one test was performed

Concn. (p.p.m. DDT)	*Stenonema fuscum*			*Stenonema interpunctatum*			
	Pollet R.		Renous R.	Rockwell Stream	Rocky Brook		
	June 1965	June 1966	May 1965 pre-spray	June 1966	June 1965 post-spray	Aug. 1965	June 1966 pre-spray
0.1	43	100	0	8	—	—	—
0.4	—	100	22	100	—	—	
0.8	92	100	—	100	—	—	
1.6	—	—	—	—	45	7	100
3.2	100	—	100		72	57	100
6.4	100	—	—	—	—	87	100
12.8	—	—	—	—	—	100	100

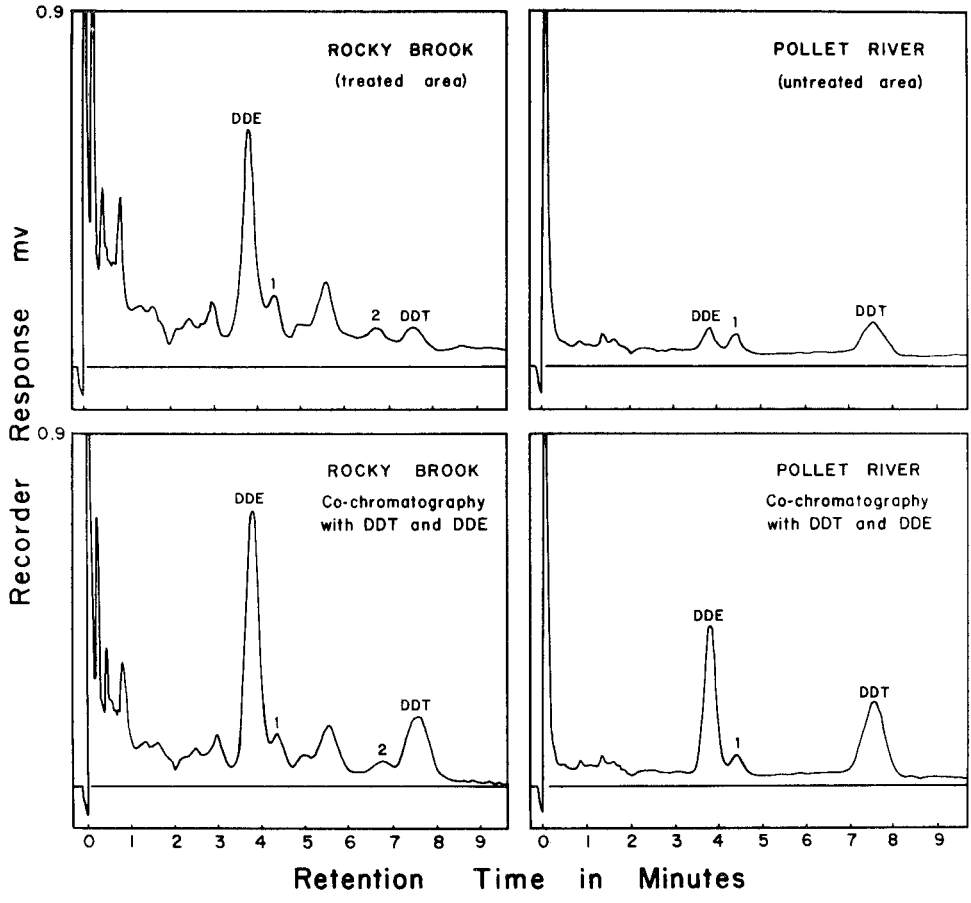

FIG. 4. Gas chromatograms of tissue extracts of *Heptagenia hebe* McDunnough collected from the sprayed Rocky Brook and the unsprayed Pollet River and exposed to 0.025 p.p.m. DDT in 250 ml water for 14 hours. Injection in hexane directly from extraction residue.

of it. It is noteworthy that the resistant strain absorbed nearly 3 times as much DDT as the resistant strain.

The greater absorptive powers of the resistant strain for DDT were confirmed by experiments with C^{14}-labelled DDT. Here the nymphs were exposed to 0.2 p.p.m. in 250 ml water, making a total dose of 50 μg C^{14}-DDT. The results with *H. hebe* (Table III) show that the Rocky Brook strain from the treated area took up 1.6 times as much radioactive DDT as the normal Pollet River strain. This interstrain difference was not as great as that found at the lower dosage in the previous experiment. In fact the amount of DDT absorbed by 30–35 large nymphs of the resistant strain from the 50-μg dose, namely only 7–10 μg in the 18.5-hour period, was proportionally lower than the 2.75 μg absorbed by 55 small nymphs in 14 hours from the lower dose of 6.25 μg in the previous experiment.

The results with *S. interpunctatum* (Table III) show that the Rocky Brook resistant strain took up 2.6 times as much as the susceptible sample of nymphs from Rockwell Stream. The group of 25 nymphs of this large species succeeded

TABLE III

Uptake of C^{14}-DDT by mayfly nymphs exposed to 0.2 p.p.m. for 18.5 hours in 250 ml water (total dose = 50 μg DDT). Radioactive material recovered by absorption from alumina chromatographic columns

Nymphs/sample	Alumina packing of column, Woelm : Fisher	C^{14}-DDT in mayflies (μg)	
		Treated area*	Untreated area†
Heptagenia hebe			
30	100: 0	6.40	3.78
35	85:15	9.90	6.70
Stenonema interpunctatum			
12	100: 0	5.05	2.40
25	85:15	21.68	7.10‡

*Rocky Brook.
†Pollet River for *H. hebe*; Rockwell Stream for *S. interpunctatum*.
‡For untreated area, 39 nymphs; figure corrected to 25 nymphs.

in taking nearly 22 μg out of the 50-μg dose of DDT in the 18.5-hour exposure period. Another sample of 12 nymphs of *S. interpunctatum* from Rocky Brook, exposed to 0.2 p.p.m. C^{14}-DDT for 18.5 hours, was tested for metabolites by paper chromatography of the extract. The chromatogram showed two peaks which, when compared with an approximately 1:2 mixture of DDE and DDT, had R_f values as follows:

	DDE	DDT
DDE–DDT mixture	0.39	0.65
Rocky Brook extract	0.39	0.63

confirming that the main metabolite in this species was DDE. From the area under the peaks it could be roughly calculated that with this small number of nymphs exposed to this higher dose of DDT the DDE/DDT conversion ratio was 0.35.

Discussion

The results with nymphs of *H. hebe* collected from the treated area in the Miramichi drainage system show that the pre-spray population in 1966 was 3 times as resistant to DDT as a normal population, whereas a post-spray population tested in 1965 was 12–40 times as resistant. This reduction in resistance between the 1965 parents and the 1966 offspring might have resulted partly from dilution by surrounding untreated populations, and possibly from a lower biotic potential of the resistant plus-variants as observed in *Aedes aegypti* (L.) by Abedi and Brown (1960). The increase in resistance from pre-spray samples to post-spray samples would have been due to the pruning effect of the spray; just what proportion of the nymphs were removed by the spray was not determined, but from superficial appearances it could well have been about two-thirds, in which case a rise in resistance ratio from 3 for the pre-spray up to more than 10 for the post-spray samples would be reasonably expected. Certainly the surviving nymphs are highly resistant, because after surviving the DDT dose in the stream they go on to show high LC_{50} levels in the subsequent tests; moreover they show 100% survival at doses which kill 100% of the normal population. It is clear that *H. hebe* is capable of developing a high level of DDT resistance, and that the level attained in the population is sufficient to allow considerable survival from airsprays

of DDT. The results with the two species of *Stenonema* indicate that a similar change has taken place in this genus.

Thus the result of a decade of aerial spraying with DDT in the Miramichi drainage area is an approximately 10-fold increase in the LC_{50} of the *H. hebe* and *Stenonema* mayfly nymphs, compared with an approximately 10-fold increase of the LC_{50} in the spruce budworm against which the spray has been applied (Randall 1965). Similar changes have been observed in aquatic insects elsewhere: on the North Shore of the St. Lawrence River a decade of aerial spraying for blackfly control has led to a 10-fold increase of DDT tolerance in the larvae of *Simulium venustum* Say in the Petit Bras River,[4] and similar blackfly control programs near Tokyo, Japan, have induced a 13-fold resistance to DDT in *Simulium aokii* (Takahashi) (Suzuki *et al.* 1963). In Florida, only 4 years of treatment of salt-marsh mosquitoes with DDT induced more than 10-fold resistance in *Aedes taeniorhynchus* (Wiedemann) (Deonier and Gilbert 1950).

This development of DDT resistance involves mayflies that are common in New Brunswick streams; the LC_{50} levels for *H. hebe* and *Stenonema* have risen from the normal 0.1 to 0.3 p.p.m. to values in excess of 1 p.p.m. DDT. Among other species, the LC_{50} of 1 p.p.m. found for *E. lata* and the survival of individuals of other species at similar concentrations of DDT indicated that the DDT tolerance was a normal characteristic for this species; this was also noted by Hitchcock (1965) for *Ephemerella subvaria* McDunnough and *Ephemerella aurivilli* (Bengtsson) in Connecticut. This author suggests that the observed survival of mayflies in the heavily-treated agricultural areas of California could imply that they had developed insecticide resistance. In the species of *Iron* tested in New Brunswick, no evidence for development of resistance in *I. fraudator* could be found, since it showed the same LC_{50} of 0.4 p.p.m. in the treated area as did *I. humeralis* in the untreated areas.

The finding that the DDT-resistant nymphs of *H. hebe* from Rocky Brook were not cross-resistant to dieldrin is consistent with the findings of previous workers that cyclodiene resistance is completely distinct from DDT resistance in insects generally. This lack of cross-resistance to dieldrin indicates that the DDT resistance in *H. hebe* is not merely a general vigour tolerance, but is a case of specific resistance to DDT.

This specificity is confirmed by the chemical assessments which showed that the DDT resistance in these mayfly nymphs involved the dehydrochlorination of DDT at a rate more than 15 times higher than normal. The situation is similar to that in mosquito larvae in which DDT-resistant strains of *A. aegypti* produce up to 10 times as much DDE as susceptible strains (Abedi *et al.* 1963). The DDE production in *H. hebe* appeared to be considerably greater than in the resistant *A. aegypti* for which figures were given by these authors. Evidently dehydrochlorination to DDE is an important defence mechanism in resistant mayfly nymphs.

In *H. hebe* and *S. interpunctatum*, the resistant nymphs absorbed considerably more DDT than the normal susceptible nymphs. This characteristic was not simply a consequence of the resistant nymphs surviving for a longer period, because in most of the tests the susceptible nymphs were not paralyzed any faster than the resistant ones. Increased absorption of DDT, however, could not

[4]Oral communication by A. S. West to Committee on Entomological Research, Defence Research Board of Canada, 24 November 1966.

possibly be a defence mechanism because it operates in the wrong direction. This absorption is considerable, since 55 nymphs of the resistant *H. hebe* could absorb as much as 44% of the 0.025-p.p.m. dose of DDT in 250 ml water overnight. A similar amount of absorption (44%) was achieved by 25 resistant *S. interpunctatum* nymphs from a 0.2-p.p.m. dose in 250 ml water. Extraction of the DDT from the water in some of the jars after the overnight exposure showed that less than 10% of the dose remained in the water, presumably because it had been lost through co-distillation with the water (Bowman *et al.* 1959). It is clear that the resistant mayflies in the New Brunswick streams are powerful concentrators of DDT, converting most of it to DDE.

Acknowledgments

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