STUDIES OF LABORATORY-INDUCED JUVENILE HORMONE MIMIC RESISTANCE IN ORDERS OF THREE SPECIES OF INSECTS

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ABSTRACT

STUDIES OF LABORATORY-INDUCED JUVENILE HORMONE MIMIC RESISTANCE IN SPECIES OF THREE ORDERS OF INSECTS

by

Donald H. DeVries

Susceptibility test and selection methods were developed for juvenile hormone mimics in *Oncopeltus fasciatus* and *Tribolium confusum*. A tolerance to one of the compounds was achieved in *Oncopeltus* and to 4 of the 5 compounds in *Tribolium*. The after-effects of methoprene were studied in *Tribolium* revealing that reproduction is inhibited in the abnormal adults. The mechanism of methoprene resistance was studied in a strain of *Culex pipiens pipiens* by means of cross resistance and synergist studies.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LITERATURE REVIEW

The problem of insecticide resistance in pest insects has reached a point where frequent development of resistance occurs. Resistance to the organochlorines is widespread, while resistance to the organophosphorus and carbamate insecticides is found in 82 and 17 species respectively (Brown, 1973). It is evident that there is an urgent need for a new group of chemical insecticides to cope with the problems of insect control. Williams (1967) stated that there was a need for new pesticides because the present ones are too broad and long lasting, plus the increased development of resistance to them. He proposed that a group of compounds known as juvenile hormone (JH) mimics fulfilled these requirements. He stated that JH is selective for insects and "insects will not find it easy to evolve a resistance or an insensitivity to their own hormone without automatically committing suicide". Plapp and Vinson (1973) believed the advantages of JH mimics were their selectivity, high activity, and the difficulty of insects becoming resistant to their own hormone. They later envisaged that disruption of adult development is one of the ways that JH compounds may act as control agents (Vinson and Plapp, 1974).

In contrast to these views Schneiderman (1972) believed that resistance to exogenously applied Insect Growth Regulators (IGR's) would develop. Selection with any insecticide, hormonally based or not, will still act to concentrate resistant mutants that are present in low

frequencies in the original population. Plapp and Vinson (1973) agreed that since different families of insects differ in their response to JH it is possible that differences may also occur within a species. Since variations in penetration, metabolism, excretion, and tissue sensitivity occur between susceptible and resistant strains of other insecticides, these differences could affect hormone sensitivity as well. One would expect a rapid metabolic degradation of applied JH because insects need a means of eliminating their own natural JH (Vinson and Plapp, 1974).

Developed Resistance to JH Mimics:

There have been reports that strains of insects resistant to other insecticides have a cross-tolerance to JH mimics. Cerf and Georghiou (1972) discovered cross-resistance to methoprene in several of their insecticide-selected strains of *Musca domestica*, notably the fenthion-R and dimethoate-R strains and a strain selected with the carbamate AC-5727. Dyte (1972) reported that a strain of *Tribolium castaneum* resistant to organochlorines, organophosphates (OP) and carbamates was also resistant to Cecropia JH I. Benskin and Vinson (1973) found that an OP-resistant strain of *Heliothis virescens* was cross-tolerant to Cecropia JH-II and a methylenedioxyphenyl type of JH mimic; this strain also showed an abnormally high microsomal oxidase activity. A strain of *M. domestica* with a resistance to DDT, deriving from an increased microsomal oxidase activity, had a 30-fold cross resistance to methoprene (Plapp and Vinson, 1973). However, another DDT-resistant strain with normal microsomal oxidase levels showed only a 2-5-fold cross-resistance to methoprene. They also demonstrated that strains resistant to dieldrin and parathion showed a slight 2-fold tolerance to methoprene.

Amos *et al.* (1974), screening JH mimics for activity on *Tribolium castaneum*, found some slight cross-tolerance in a malathion resistant strain. Hrdy (1974) found a slight cross-tolerance to hydroprene in a malathion resistant strain of the aphid *Therioaphis maculata*. Cerf and Georghiou (1974) showed a moderate to high cross tolerance to the chitin synthesis inhibitor diflubenzurone (Dimilin) in a DDT-resistant, carbamate-resistant, and 5 OP-resistant strains of *M. domestica*. Yu (1975) demonstrated that there was increased microsomal-oxidase activity against JH analogs in a strain of *M. domestica* resistant to carbamate, cyclodiene, and DDT. These experimentally induced cross-resistances imply that increased levels of resistance may develop when insect pests are exposed to JH mimics in control programs (Plapp and Vinson, 1973).

With respect to the induction of resistance to JH mimics by their selection pressure on normal strains of insects, Schaefer and Wilder (1973) reported that 20 generations of selection with methoprene on larvae of *Culex quinquefasciatus* did not induce any reduction in their susceptibility. However, Georghiou *et al.* (1974) found that selection of *Culex tarsalis* larvae with methoprene for 14 generations produced a 50-fold resistance. They also selected a highly OP-resistant field strain of *M. domestica* with methoprene and produced a 100-fold resistance (Georghiou pers. comm.). Brown and Brown (1974) reported that 8 generations of selection with methoprene on a strain of *Culex pipiens pipiens* produced a 14-fold resistance. On the other hand Hsieh *et al.* (1974) observed no significant change in the susceptibility of *C. p. quinquefasciatus* after 20 generations of selection with the JH mimic Mon-0585 [2,6-di-t,butyl-4-(a,a-dimethyl benzyl)phenol].

Mode of Action of JH Mimics:

One of the most important features of the JH mimic is their differential activity on representatives of various groups of insects. With respect to taxonomic grouping Slama (1971) has pointed out that there is almost no difference between species of the same genus in their response to JH mimics, rather slight variations at the generic level, and large differences at the family or higher taxonomic levels. Slama also states that some JH mimics have a relatively wide spectrum of activity and act on many unrelated species while others specifically act on representatives of a single family.

Studies have been performed for the activity of JH and JH mimics on various species of the order Hemipter. Riddiford (1972) reported that low doses (0.1-10.0 mg per insect) of JH applied to the early embryo or to the female of *Pyrrhocoris apterus* blocked embryonic development at the embryonic-larvae transition. Wigglesworth (1969) reported that a dose of 2.3 µg/g Cecropia JH was required to produce supernumerary sixth instar nymphs in *Rhodnius prolixus*. Farnesyl methyl ether was about one fourth as active, requiring 6.9 µg/g. 5 µg of methyl farnesoate-10, 11-epoxide also exerted high JH activity on *Rhodnius* (White, 1972). Patterson (1973), working with *R. prolixus*, reported that several farnesol-derived JH mimics produced increased effects when applied over 4 continuous days rather than an equal amount applied on one day. He proposed that these serial applications would allow a more accurate assessment of JH activity, since it produces a much more constant titre of the JH mimic.

Bowers (1969) found that methylenedioxyphenyl derivatives were active in preventing metamorphosis in the large milkweed bug *Oncopeltus*

fasciatus. Riddiford (1970) reported the inhibition of metamorphosis in *O. fasciatus* nymphs which had been treated with JH as eggs. Brieger (1971) described the morphogenetic effects of mimics in *Oncopeltus*; these include adult curled wings low doses, adult-nymphal intermediates at moderate doses, and supernumerary sixth instar nymphs at higher doses. He claimed that the major component for activity of methyl benzoate derivatives was the terpenoid side chain, and stressed the importance of an electronegative group, such as an ester, ether or hydroxyl, at one end of the molecule. Bagley and Bauernfiend (1972) reported that the Romanuk JH mimic (ethyl trans-7, 11-dichloro-3, 7-11-trimethyl-2-dodecenoate) produced supernumerary nymphs of *O. fasciatus* only at 0.01 µg/bug whereas the developed JH mimic RO-20-3600 became active at 10 µg/bug. The terpenoid-aromatic hybrid compounds showed greater activity in *Oncopeltus* than in *Tenebrio* and that their activity was increased in *Oncopeltus* by saturation of the double bonds (Jacobson *et al.*, 1972). The unepoxidized compounds were generally more effective on *Tenebrio* than on *Oncopeltus*; however, epoxidation usually increased the activity on both species. The deuterated synthetic Cecropia JH, methyl-10, 11-epoxy-7-ethyl-3, 11-dimethyltrideca-2, 6-dienoate, was active at 1 µg/insect on *O. fasciatus* (Brown, 1973). Brown and Monroe (1972) reported that a dose of 5 µg of 10, 11-epoxy farnesenic acid methyl ester per bug would produce abnormalities in nearly all *O. fasciatus* molting to the adult stage. When citric acid was applied along with the JH mimic only 58% of the adults were normal indicating that the acid was protecting the insect from the JH mimic. Bryon *et al.* (1974) found that hydroprene topically applied at 1.6 µg per bug was effective in producing supernumerary sixth instar nymphs in *O. fasciatus*.

No synergism was found when molting hormone was added, in fact there was slight antaganism with 1.5-3 μg ecdysterone applied before the hydroprene application. Much work has also been done on the activity of JH mimics on Tenebrionid beetles especially the species *Tenebrio molitor*, *Tribolium confusum*, and *T. castaneum*. Thomas and Batnager-Thomas (1968) working with *T. castaneum* found a critical period at late fourth instar early fifth beyond which the JH mimic MTDD (methyl 3, 7, 11-trimethyl-7, 11-dichloro-2-dodecenoate) is not effective. None of the first or third instar larvae could pupate or become adults at 100 ppm which was the lowest concentration used.

Morphological abnormalities produced in *T. castaneum* by JH mimics include aberrations of the tarsi, legs reduced to unchitinized stumps, lack of differentiation of the antennal club, crumpled and divergent wings and elytra, retention of pupal skin on the rear of adults, and pupa-adult intermediates (Amos *et al.*, 1974; Williams and Amos, 1974). Williams and Amos (1974) also reported that there was no difference between the sexes in the occurrence of deformities and their development. They mentioned that hydroprene and methoprene prolonged the developmental period and that 24% of the morphologically normal and 64% of the deformed F_1 adults died within a week of emergence. They questioned whether the morphologically deformed adults would be sterile or their reproductive capacity reduced. Bhatnager-Thomas (1973) supported this possibility by reporting that in *T. castaneum* treated with MTDD the number of eggs laid and the percent hatch was inversely correlated with the dose. Bowers (1969) and Pallos *et al.* (1972) described the effects of JH mimics of *T. molitor* as being retention of pupal urogomphi and gin traps on the adult, and pupal-adult intermediates.

Schwarz *et al.* (1970) and Pallos *et al.* (1971) both reported that the epoxidized forms of JH mimics had the highest activity on *T. molitor*. The epoxide JH mimic R-20458 arrested adult development of *T. molitor* at 10 ppm and exerted its toxicity through contact and/or ingestion (Pallos *et al.*, 1971).

Metwally *et al.* (1972) working with the khapra beetle (*Trogoderma granarium*) reported the formation of pupal-adult intermediates when the pupae were treated with JH mimics within 48 hours of pupation. Later administration of reduced amounts gave apparently normal adults but exerted an adverse affect on their reproductive capacity, so that the number of eggs laid and the percent hatch decreased. Dissection of the females showed abnormal ovaries. They also demonstrated that groups of beetles exposed to JH vapors developed at a slower rate than the controls.

Methoprene and hydroprene were the most effective of 15 JH-mimics tested against *Tribolium confusum* (Strong and Dickman, 1973). Neither compound affected reproductively mature adults, nor were ovicidal effects observed; however at 10 ppm both compounds inhibited the embryos from growing into fertile F_1 progeny thus successive generations were prevented.

Action of JH Mimics on Diptera:

It is possible that JH mimics may best be suited as insect growth inhibitors against Dipteran insects. Plapp and Vinson (1973) reported that methoprene was 600 times more toxic than DDT to a DDT normal strain of *M. domestica*. Schwarz *et al.* (1974b) found that arylterpenoid JH mimics showed very high activity on the stable fly (*Stomoxys calcitrans*), the house fly (*M. domestica*), the face fly (*M. autumnalis*) and the horn fly (*Haematobia irritans*) but very low activity on the large milkweed

bug (*Oncopeltus fasciatus*). Harris *et al.* (1973) fed RO-20-3600 and methoprene to cattle and seeded the resulting manure with eggs of horn flies and stable slies. They found that methoprene at 0.7 mg/animal/day completely inhibited the development of horn flies in the manure, while stable flies were inhibited by concentrations of 100 mg/animal/day.

Certain JH-mimics are particularly effective against Culicidae. One of them, methoprene in its commercial form of Altosid (ZR-515) has been licensed in California for use against the larvae of irrigation-water mosquitoes *Aedes* and *Culex*. In contrast to the low doses (ppb range) required with this JH mimic, Ittycheriah *et al.* (1974) reported that the Cecropia JH itself was only 46% effective on *Culex tarsalis* larvae at 25 ppm. Hoppe *et al.* (1974) found that field applications of R-20458 and methoprene at 0.3 ppm against *Culex pipiens pipiens* larvae achieved 99% and 86% inhibition of development respectively. Spielman and Williams (1966) reported that fourth-instar larvae of *Aedes aegypti* treated with a JH mimic were all able to pupate but none were able to emerge as adults. The effects of JH mimics on mosquitoes treated as larvae include the following: untanned larvae, larval-pupal intermediates, dead pharate pupae, untanned pupal-pharate adult intermediates, and adults failing to completely emerge (Hsieh and Steelman, 1974; Ittycheriah *et al.*, 1974; Wells *et al.*, 1975). Georghiou and Lin (1974) concluded that the effect of JH mimics is dose-dependent, with high doses resulting in undeveloped pupae and lower doses giving incompletely developed adults.

Speilman and Williams (1966) noticed that mosquito larvae were the most sensitive to JH when they were in the fourth instar. Schaefer and

Wilder (1972) reported that 0.1 ppm methoprene was more active on fourth-instar larvae than on second or third and that the percentage mortality increased as the fourth-instars became older; however, 0.1 ppm methoprene was not active against pupae. Georghiou and Lin (1974) and Brown and Brown (1974) both pointed out that the only period for the effect of methoprene to be observed is 20-24 hours before pupation. Schaefer and Wilder (1973) using field tests with methoprene and RO-20-3600 again showed that the late fourth-instar larvae of *Aedes nigromaculis* were more susceptible than the third instar. A slow release formulation of methoprene, however, offered several days of residual activity and controlled second, third and fourth stage larvae of *A. nigromaculis* (Schaefer and Wilder 1973), *A. taeniorhynchus*, and *Culex nigripalpus* (Rathburn and Boike, 1975). The chitin synthesis inhibitor diflubenzurone (Dimilin) has been reported to be more effective on larvae of *A. taeniorhynchus* and *C. nigripalpus* in the third than in the fourth instar larvae (Rathburn and Boike, 1975).

Georghiou and Lin (1974) noted that the after effects of methoprene treatment of *C. tarsalis* include a decrease in adult longevity and fecundity. If *C. tarsalis* was treated in the egg stage, as Ittycheriah *et al.* (1974) did with 100 ppm CRD-9499 (10, 11-epoxy-N-ethyl-3, 7, 11-trimethyl-2, 6-dodecadienamide) more than 70% of the individuals became apparently normal adults, however, there was a decrease in hatchability of the eggs that they laid. It was hypothesized that inhibition of metamorphosis in larvae of treated eggs was due to the high titres of endogenous JH resulting from initial treatment of the eggs.

Metabolism of Cecropia JH:

Early *in vivo* studies with several species of insects have demonstrated that the main metabolism of Cecropia-JH involves ester hydrolysis and epoxide hydration (Fig. 1) (Slade and Zibitt, 1972; Azami and Riddiford, 1973; Patterson, 1973; Weirich and Wren, 1973; Erley *et al.*, 1975). The products of this metabolism are the JH-diol, the JH-acid, and the acid-diol. Ajami and Riddiford (1973) reported the *in vitro* production of the JH acid, and the JH acid-diol, from the incubation of Cecropia JH with homogenates of several species of insects. The homogenates of *Drosophila melanogaster* produced, in addition traces of the JH bisepoxide and JH-tetrol, which they speculated might be produced by Mixed Function Oxidase attack upon the 6, 7 double bond to produce the bisepoxide followed by subsequent hydration to form the tetrol. Hooper (in press) reported the production of JH-acid and some JH-acid diol from incubation of Cecropia JH with *Culex pipiens* homogenates. This Cecropia JH metabolism was inhibited with paraoxon but not with TOCP. Slade and Zibbit (1972) and Erley *et al.* (1975) reported that epoxide hydrolysis is restricted to the fat bodies whereas esterase activity can occur in several organs and tissues. Whitmore *et al.* (1972) demonstrated that the Cecropia JH induces *Hyalophora gloveri* to produce additional esterases to hydrolyze it to the inactive acid. Sanburg *et al.* (1975) reported that the haemolymph of *Manduca sexta* contains a JH carrier protein which protects the hormone from general esterase degradation; however, this protein did not prevent the degradation by JH specific esterases. They found the final-instar larva contained much higher levels of JH esterase than the fourth-instar larvae. These higher levels of JH esterase are used to decrease the JH titre by hydrolyzing

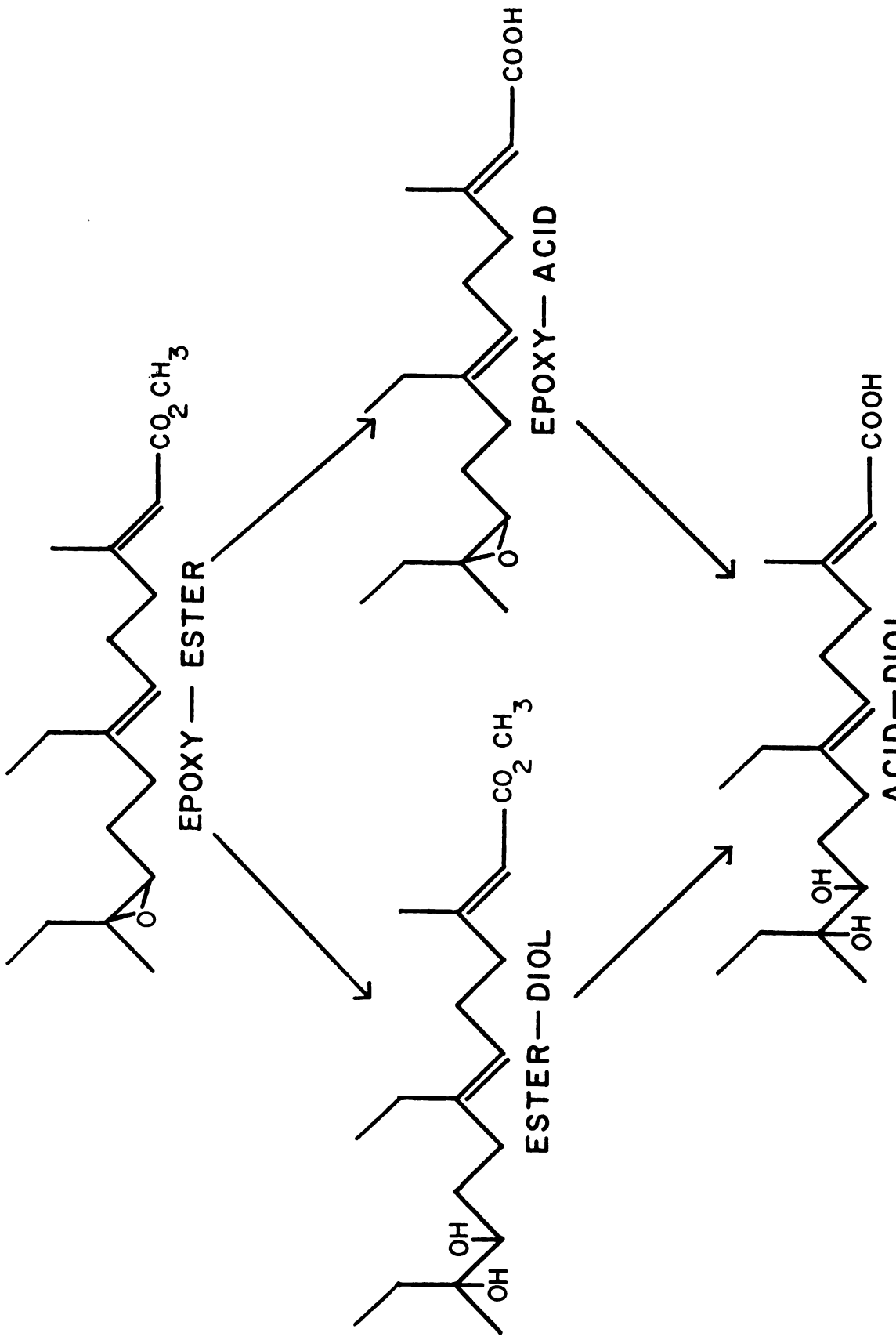


Figure 1. Metabolic Degradation of Cecropia JH.

the endogenous JH so pupation may occur. Erley *et al.* (1975) also mentioned the presence of a JH-protein carrier in the blood of *Locusta migratoria*. Upon injection of radiolabelled Cecropia JH 50% of the label disappeared within 90 minutes and was excreted by way of the Malphigian tubules. They reported that some of the compound was incorporated into the ovaries and 67% was metabolized into polar products. Slade and Zibbit (1972) reported that *Sarcophaga* injected with Cecropia JH can metabolize it to the ester-diol and conjugate it. Slade and Wilkinson (1974) demonstrated that Cecropia JH can undergo metabolism to the acid-diol and form a sulfate conjugate which is excreted. The fate of Cecropia JH therefore appears to be ester hydrolysis and epoxide hydration followed by conjugation and excretion (Ajami and Riddiford, 1973).

Metabolism of JH Mimics:

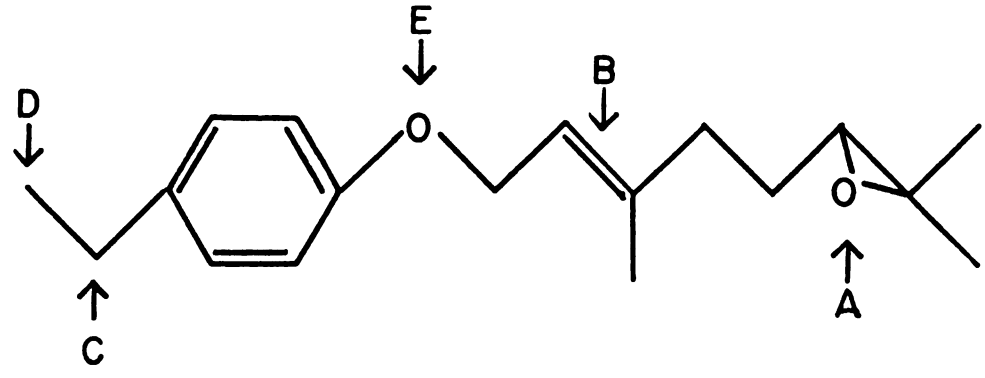
An important factor in the JH activity of farnesol derivatives is the relative ease with which they can be metabolized (Wigglesworth, 1969). Farnesyl methyl ether is much more active than farnesol and is much less readily broken down. Patterson (1973) claims that the effect of a JH mimic is dependent upon three factors: innate JH activity of the compound, rate of penetration, and rate of degradation and/or excretion. He reports that the ether is the most persistent of the JH mimics he tested on *Rhodnius prolixus*; the compound with a terminal epoxide was least persistent while the ester compounds were intermediate in persistence.

The metabolism of methyl farnesoate derivatives in *Schistocerca* and *Rhodnius* closely resembled the metabolism of Cecropia JH (White, 1972). The products of ester hydrolysis and epoxide hydration yielded

the acid-diol, epoxide-acid, and methyl farnesoate diol, and then subsequently conjugated with glucosides.

The ethyl epoxide R-20458 (Fig. 2) may be metabolized by mammals and algae, or photodegraded by 6, 7 hydroxylation, 2, 3-epoxidation and hydroxylation with or without cyclization, oxidation of the ethyl side chain (to a greater extent at the α- and β-oxidation of the ethyl moiety, epoxidation of the 2, 3 double bond, hydroxylation of the 6, 7 epoxide, and some ether linkage cleavage both *in vitro* and *in vivo*. Gill *et al.* (1974) also reported that conjugation is important in ethyl-epoxide metabolism in rats, mice and insects.

Terriere and Yu (1973) demonstrated that Cecropia JH would increase O-demethylation of p-nitro-anisole 100% in house flies and that the JH analogs deriven from farnesenic acid would induce the microsomal oxidases of adult house flies. Mayer *et al.* (1973) reported that the inhibition of rat liver microsomal metabolism of aniline by RO-20-3600 and R-20458 was a combination of competitive and noncompetitive inhibition. This competitive and noncompetitive inhibition suggests these JH mimics were acting as alternate substrates for aniline hydroxylase and these induced microsomal enzymes in the same way as piperonyl butoxide. Yu and Terriere (1971), and Terriere and Yu (1973), showed that methoprene could stimulate heptachlor epoxidase activity, but it had no effect on O-demethylation. They were the first to show evidence for the metabolism of methoprene by incubating methoprene in the presence of microsomes and NADPH, which resulted in the decrease of the peaks for *cis* and *trans* methoprene in the Gas-Liquid chromatogram. The microsomes from a high-oxidase strain of house flies were most active in the metabolism of methoprene, this provided evidence for the



A Hydroxylation

B Epoxidation—Hydroxylation

C,D Oxidation

E Ether Cleavage

Figure 2. Sites of degradation of R-20458 (From Hoffman *et al.* 1973).

oxidative metabolism of methoprene (Terriere and Yu, 1973).

Weirich and Wren (1973) found that Cecropia JH and hydroprene can be cleaved by esterases in the haemolymph of *Manduca sexta*, while methoprene was practically unaffected by these esterases. Yu and Terriere (1975) reported that hydroprene, triprene and kinoprene, but not methoprene, were hydrolyzed by esterases of house flies. It was suggested that these esterases can utilize methyl and ethyl esters but not isopropyl esters and that the esterolytic site of the enzyme is inaccessible to the carboxy isopropyl group (Weirich and Wren,1973; Yu and Terriere, 1975). Hooper (in press) found that *in vitro* homogenates of *Culex pipiens* larvae which could degrade ethyl, propyl, isopropyl and butyl esters of propionic acids failed to degrade methoprene or hydroprene. Yu and Terriere (1975) found high JH analog esterase activity in the egg and adult, with a sudden decrease at pupation; however, as the esterase activity fell at pupation the microsomal oxidase activity increased. There was no metabolism of the *cis-trans* isomer of hydroprene or either isomer of methoprene by esterases, but the microsomal oxidases attacked both isomers of both compounds. They reported that hydroprene metabolism was increased 88% when the house flies were fed Cecropia JH, phenobarbitol or dieldrin. Feeding piperonyl butoxide to the adult flies inhibited the microsomal JHA oxidase metabolism of methoprene, hydroprene and kinoprene.

Pawson *et al.* (1972) reported that 95% of Cecropia JH and RO-20-3600 decomposed within 24 hours as a result of exposure to ultraviolet light. Schaefer and Wilder (1972) found that methoprene and hydroprene undergo degradation due to sunlight and that methoprene is unstable at temperatures above 24°C. Schaefer and Dupras (1973)

reported that only 13% of methoprene applied to water remained after four hours and after eight hours only 2% remained. When acetonitrile was added along with the methoprene applied to water there was no apparent loss of methoprene after 48 hours.

Degradation and Fate of Methoprene:

More recent studies of methoprene penetration and metabolism indicate seven pathways or mechanisms (Fig. 3), namely a) oxidative-demethylation, b) hydrolytic cleavage, c) isomerization of the 2-ene double bond, d) oxidative scission of carbon-carbon bonds, e) anabolism of the metabolic products into structural components, f) conjugation into excretable products and g) reduced uptake.

(a). Oxidative-demethylation occurs when the 11-methoxy group of methoprene (I) is removed to form the hydroxy ester (II). This process has been demonstrated in alfalfa and rice (Quistad *et al.*,1974b), in *Culex* and *Musca* (Quistad *et al.*, 1975b), in *Tenebrio* and *Oncopeltus* (Solomon and Metcalf, 1974), and in pond water by microorganisms (Schooley *et al.*, 1975). As the name implies, this process is oxidative in nature and Terriere and Yu (1973) claim it depends on the mixed function oxidase system. Yu and Terriere (1975) also reported that O-demethylation could occur in kinoprene and triprene.

(b). Hydrolytic cleavage occurs when the isopropyl ester of methoprene (I) or of the hydroxy ester (II) is cleaved with the addition of HOH, leaving the acid group of the methoxy acid (IV) or the hydroxy acid (III) respectively. This type of cleavage is catalyzed by esterase enzymes and has been demonstrated for juvenile hormone analogs by Weirich and Wren (1973). Hydrolytic cleavage of methoprene has been found in alfalfa and rice (Quistad *et al.*, 1974b), in mosquitoes and

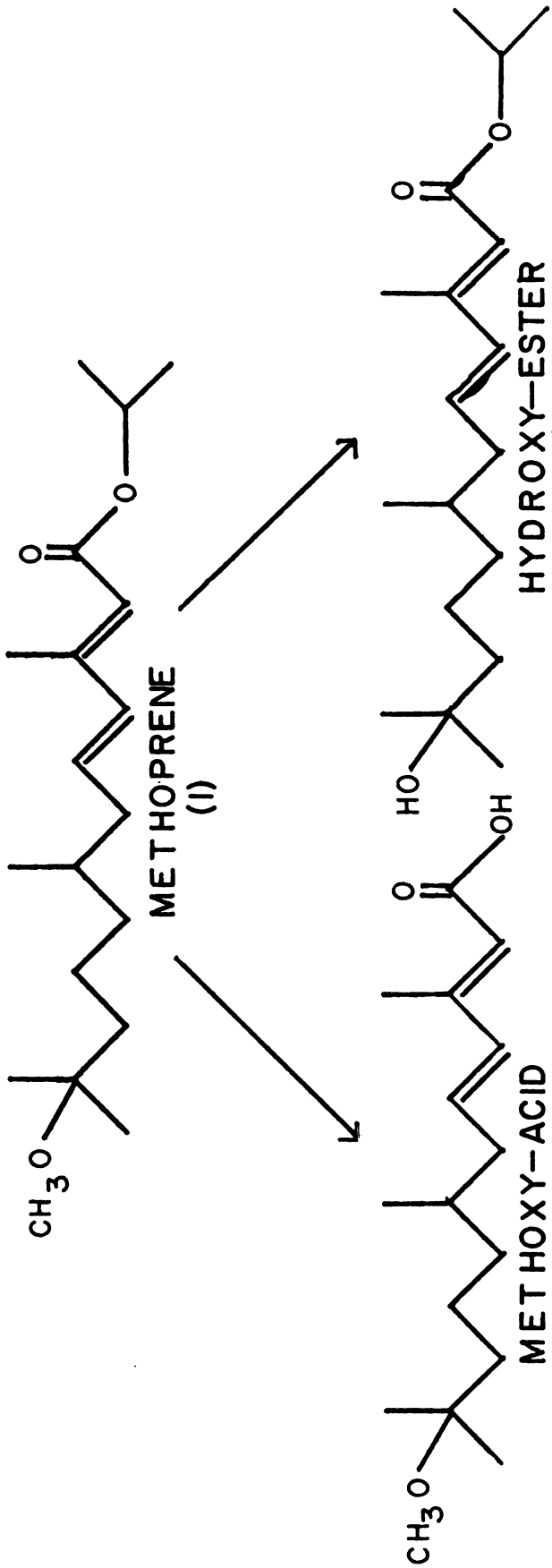


Figure 3. Metabolic degradation of methoprene (From Quistad *et al.* 1975b).

house flies (Quistad *et al.*, 1975b), in pond water (Schooley *et al.*, 1975), and in *Tenebrio* and *Oncopeltus* (Solomon and Metcalf, 1974) as the cleavage of I to IV and of II to III for each of the examples.

(c). There are two double bonds in the compound methoprene at the number 2 and number 4 carbons. The 2E isomer is the most active form of the compound while the 2Z isomer being at least 10-100 times less active (Quistad *et al.*, 1974b, 1975a, 1975b; Schooley *et al.*, 1975; Weirich and Wren, 1973). The 4-ene double bond is relatively stable and will not isomerize (Quistad *et al.*, 1974b, 1975a). The 2-ene double bond is quite susceptible to isomerization, and in aqueous solutions the 2E form is converted to the 2Z form until a 1:1 ratio exists. This type of isomerization is initiated in sterile water when exposed to light (Quistad *et al.*, 1975a; Schooley *et al.*, 1975; Schaefer and Dupras, 1973). Quistad *et al.* (1975b) have shown that larvae of the mosquitoes *Aedes aegypti* and *C. p. quinquefasciatus* and the house fly *M. domestica* can effect biological isomerization at the 2-ene bond of methoprene and its metabolites even when held in the dark. They hypothesized that this isomerization to the less active 2Z isomer constitutes a major mode of detoxification. The mosquitoes and flies can isomerize the 2E to 2Z, but not the 2Z to 2E.

(d). The 4-ene bond of methoprene is subject to oxidative scission which results in a splitting of the compound. This occurs in alfalfa and rice where a major pathway of degradation is the splitting of I to V (Quistad *et al.*, 1974a). This process must occur in pond water because the major product of pond water metabolism of methoprene is VI (Schooley *et al.*, 1975). The shorter chained metabolites have also been found in house flies and mosquitoes (Quistad *et al.*, 1975b). Quistad

et al. (1974a) reported that in a Hereford steer fed radiolabelled methoprene the methoprene undergoes a single alpha oxidation and two beta oxidations which produce acetate molecules. These acetate molecules are incorporated into acetyl-CoA and used to make cholesterol. They also reported that considerable amounts of radiolabelled $^{14}CO_2$ was evolved presumably as Kreb cycle oxidation of acetate. It is conceivable that this type of oxidation could occur in insects.

(e). The possibility arises that once the organism has degraded the methoprene into different metabolites the metabolites may be further broken down and incorporated into structural compounds. The synthesis of cholesterol from the acetate released as a methoprene metabolite is an example of this (Quistad *et al.*, 1974a). By means of radiolabelled methoprene Quistad *et al.* (1974b) have found evidence for the incorporation of secondary metabolites in the tissues of alfalfa and rice plants, since hydrolysis of the structural material yields radioactive compound III, glucose and cellobiose. Solomon and Metcalf (1974) suggested that some of the metabolites may be incorporated into the carbon pool of *Tenebrio* and *Oncopeltus*.

(f). Slade and Wilkinson (1974) have observed that Cecropia JH can undergo metabolism to the acid-diol which can form a sulfate conjugate and be excreted. Excretable conjugates have also been reported for methoprene; however, it is not yet known in what form they were (Quistad *et al.*, 1975b).

(g). Non-metabolic factors such as selective uptake may contribute to the regulation of sensitivity to methoprene (Quistad *et al.*, 1975b). Brown (pers. comm.) has found that the methoprene uptake of larvae of a 13-fold methoprene-resistant strain of *C. p. pipiens* is 25% lower

than that of its methoprene-susceptible counterpart. If uptake of a compound is reduced, then the organism has less of the compound to inactivate and excrete and thus it is less susceptible.

Quistad *et al.* (1975b) working with larvae of *A. aegypti* demonstrated that the initial half-life of methoprene in water was 5-8 hours, and found that the larvae rapidly converted it into polar metabolites and excreted it into the water. As the larvae increased in age they metabolized the methoprene into more polar products. The fourth-instar larvae were more susceptible than younger larvae, for although they metabolized more, there was 2-3 times as much methoprene in their bodies to be metabolized. Methoprene is less toxic to *C. p. quinquefasciatus* because this species seems to metabolize and inactivate it faster, with the hydroxy ester (II) as the predominant metabolite. This would seem to indicate that O-demethylation is the predominant pathway in *Culex*. In *M. domestica* the hydroxy acid (III) is the most abundant metabolite, which would indicate that both O-demethylation and hydrolytic cleavage are important. Studies with insecticide synergists have helped to establish these pathways. Piperonyl butoxide (PB) is a known inhibitor of mixed function oxidases and hence would inhibit O-demethylation. Tri-o-cresyl phosphate (TOCP) is a known inhibitor of carboxyesterases and hence would inhibit the hycrolytic cleavage. PB had no effect on *Culex* (Quistad *et al.*, 1975b) even though one would expect that it would because the primary pathway of *Culex* is the O-demethylation to II (Solomon and Metcalf, 1974). PB also slightly increased the activity of methoprene in *T. molitor* by blocking the O-demethylation of I to II but in *Oncopeltus* it sharply decreased the activity of methoprene by blocking of the O-demethylation of I to II (Solomon and Metcalf, 1974).

The hydroxy ester II was found to be extremely active in *Oncopeltus*, indicating that O-demethylation is an activation process for methoprene in this hemipteran. By blocking the O-demethylation step in *Oncopeltus* the activity of methoprene is greatly reduced. TOCP caused a 5-fold increase in larval mortality of *Culex* but had no effect on *Musca* (Quistad *et al.*, 1975b). TOCP also slightly increased the activity in *Tenebrio* and *Oncopeltus* and caused an increase in II by blocking the hydrolytic cleavage of I to IV and II to III (Solomon and Metcalf, 1974).

These findings indicate that JH mimics are broken down into less biologically active compounds and that biological degradation provides the mechanisms whereby organisms may survive a lethal dose. The pathways and mechanisms discussed above indicate several ways JH mimics can be inactivated. If an insect is basically proficient or has developed a proficiency in any one of these mechanisms the compound will not be active against it. This detoxicative ability is the basis for resistance of the organism to the toxicant. This evidence indicates that the potential exists for insects to develop resistance to JH mimics through enhanced degradation of the compound by one or more of the pathways mentioned.

Part I. Effect of Selection Pressure from Juvenile Hormone Mimics on *Oncopeltus fasciatus* and *Tribolium confusum*

INTRODUCTION

The members of a population of insects will differ in their susceptibility to a given insecticide. Thus the application of a given dose of this insecticide will vary in the effect that it has on individuals of the population. Those individuals which are more susceptible to the insecticide will die, while those which are less susceptible will survive. Consequently as insecticide application may select for those individuals which are less susceptible, and these are the individuals which are then able to mate and contribute their alleles to the gene pool.

A program of laboratory selection to probe the possible development of resistance to an insecticide involves 3 basic procedures. The first is to assess the susceptibility of a sample of each generation in order to determine the dose needed to kill 60% of the individuals. The second is to expose the members of each successive generation to a level of the pesticide which will kill approximately 60% of the individuals and thus leave approximately 40% of the less susceptible insects remaining to produce the next generation. The third is to compare the susceptibility level of the selected strain to that of an unselected strain in order to monitor for resistance. In the present investigation, laboratory selections were undertaken to determine whether *T. confusum* and *O. fasciatus* could

develop resistance to JH mimics applied as insecticides to successive generations.

The induction of resistance to JH mimics has been reported for 3 species of the Diptera. Georghiou *et al.* (1974) produced a 50-fold resistance to methoprene in a strain of *Culex tarsalis* selected with the JH mimic. They also pressured a highly OP-resistant field strain of *Musca domestica* with methoprene and produced a 1000-fold resistance to methoprene (Georghiou pers. comm). Brown and Brown (1974) reported the induction of a 14-fold resistance to methoprene in a strain of *Culex pipiens pipiens* selected for 8 generations. The objective of this study was to investigate by means of laboratory selection the potential of two species, the confused flour beetle *Tribolium confusum* Jacquelin duVal (Coleoptera) and the large milkweed bug *Oncopeltus fasciatus* (Dallas) (Hemiptera), to develop resistance to JH mimics.

JH mimics are ineffective as adulticides. Thus susceptibility test methods were developed for the larvae of *Tribolium* and the nymphs of *Oncopeltus*. In view of their mode of action these compounds must be applied to the insect before the onset of metamorphosis (Thomas and Bhatnager-Thomas, 1968; Williams, 1967).

MATERIALS AND METHODS

Insects:

A strain of the confused flour beetle, *Tribolium confusum*, was obtained by combining insects from Michigan, Kansas and California. The compound strain was reared in screened one-gallon glass jars on a diet of whole-wheat stone-ground flour and yeast (12:1). The culture was maintained at

30° ± 1°C and 65 ± 5 percent relative humidity on a 16:8 (day:night) photoperiod.

The strain of the large milkweed bug, *Oncopeltus fasciatus*, was obtained by combining insects from Michigan, North Carolina and California and crossing with a sunflower-adapted strain of Michigan origin. The culture was reared in covered one-gallon glass jars on a diet of sunflower seeds. Water was provided in cotton-plugged vials. The culture was maintained at 27° ± 1°C and 50 ± 5 percent relative humidity with a 14:10 (day:night) photoperiod.

Experimental Chemicals:

The insect growth regulators used in these investigations included the following compounds:

- methoprene (isopropyl 11-methoxy-3,7,11-trimethyl-2,4-dodecadienoate) 94.8% (85% *trans*) Zoecon Corporation.
- hydroprene (ethyl 3,7,11-trimethyl-2,4-dodecadienoate) 86.5% Zoecon Corporation.
- kinoprene (prop-2-ynyl,3,7,11-trimethyl-2,4-dodecadienoate) 86.5% Zoecon Corporation
- RO-20-3600 (6,7-epoxy-3,7-dimethyl-1-[3,4(methylenedioxy)]phenoxy-2-nonene) technical grade Hofman-LaRoche Company.
- R-20458 (1(4-ethylphenoxy)-6,7-epoxy-3,7-dimethyl-2-octene) 69% Stauffer Chemical Company.
- CG-13353 (4(4'benzylphenoxy)-3-methyl-2-buteonic acid ethyl ester) 90.0% Ciba-Geigy Corporation.
- diflubenzurone (Dimilin) (1-(4 chlorophenyl)-3-(2,6-diflurobenzoyl)-urea) 95% Thompson-Hayward Company.

Experimental Design:

The susceptibility test for *Oncopeltus* consisted of topical application of the JH mimic in 1 µl acetone to the abdomen of CO_2 anesthetized fifth instar nymphs less than 24 hours after the moult. The treated nymphs were held in Sealright^R containers with sunflower seeds and cotton stoppered vials of water at 27° ± 1°C and 50 ± 5% relative humidity. After 10 days the emerged adults were examined for abnormalities which were then scored as "mortalities". The abnormalities acceptable as equivalent to mortality responses in order to obtain the ED_{50} were the following: moulting to a supernumerary nymph, failure to shed the exuvia, grossly distorted wings, and complete loss of abdominal color pattern. Topical applications were done with a calibrated microapplicator (ISCO Model M, Lincoln, Nebraska) fitted with a 250 µl Hamilton syringe. The above procedures were completed in triplicate, 15 insects per replicate, for 4 doses and a control.

The test method developed for assessing the susceptibility levels for *Tribolium* was as follows. The JH mimic in a solution of dichloromethane was added to a round-bottom flask containing 20 g of whole wheat flour. The dichloromethane was evaporated with a rotavapor (Buchi, Switzerland) incorporating the JH mimic into the flour. This procedure was performed with each of 4 concentrations, in addition to a control. The 20 g of treated flour was divided into 3 screened 25 x 60 mm scintillation counting vials to yield 3 replicates for each concentration. Lots of 25 two-week-old larvae were added to the flour which was then held at 30° ± 1°C and 65 ± 5% relative humidity for 3 weeks, at which point the emerging adults were examined for abnormalities. These abnormalities were incomplete emergence from the pupa, pupa-adult intermediates,

persistence of exuvia on the abdomen, or the presence of urogomphi persisting from the pupal into the adult stage (Figs. 11b and 12). The susceptibility test investigating the effect of diflubenzurone (Dimilin, a chitin synthesis inhibitor) employed actual larval mortality as the criterion of effect.

Laboratory selection of *Oncopeltus* was performed on a given generation by spraying approximately 1000 first-day fifth-instar nymphs with an ethanolic solution of the JH mimic at a concentration determined to exert about 60 percent selection pressure. The Potter spray tower (Burckardt Mfg. Co., Rickmansworth, England) was employed to spray 5 ml solution at 20 cm pressure on to lots of 75 CO_2 antethetized nymphs, 90 seconds being allowed for the spray to settle on them. The nymphs were then transferred to glass one-gallon jars (ca. 150 nymphs/jar) with sunflower seeds and cotton stoppered vials of water and held for 10 days at 27° ± 1°C and 50 ± 5% relative humidity. Souffle cups containing cottonwool were placed in the jars for oviposition; and 3 of the approximately 13 lots of nymphs sprayed were scored for abnormalities to determine the selection pressure that had been applied.

Laboratory selection of *Tribolium* was applied to approximately 800 two-week-old larvae by treating with an approximate concentration of whole-wheat flour contained in a screened glass one-gallon wide-mouth jar. The flour was treated by the addition of the JH mimic in a solution of dichloromethane (which was evaporated away in a stream of nitrogen) at a concentration in the flour that had been determined to exert about 60 percent selection pressure. The larvae were held at 30° ± 1°C and 65 ± 5% relative humidity for 3 weeks until emergence, at which time 3 replicates of 25 adults were examined for abnormalities to determine the selection pressure that had been applied.

Selection with the IGR diflubenzurone followed the above procedure, except that the selection pressure was gauged not by abnormalities and mortality, but by the volume of *Tribolium* that survived. Lots of larvae from the unselected colony placed on untreated flour were employed as a control or check for the lots exposed to the selective concentration, the same volume of stock larvae being inoculated into the untreated flour as that of the larvae of the strain under selection inoculated into the treated flour. At the end of 3 weeks the adults were sieved from the flour and the resulting difference in the two volumes of the two strains of adults was used to determine the selection pressure. This method was used because the larvae disintegrated soon after dying and made it impossible to determine the percentage mortality from counting the dead.

The after-effects of larval treatment with JH mimics to adult *Tribolium* were investigated by the following 3 procedures. The first test determined the percentage reduction in fecundity and viability as a function of the concentrations applied to the larvae. At the conclusion of the usual susceptibility test, 20 adults were randomly selected from each vial to yield 3 replicates for each of the 4 concentrations and the control. The adults were placed in covered waxed paper cups containing ca. 20 g of whole wheat flour and held at 30° ± 1°C and 65 ± 5% relative humidity for five days to allow sufficient time for oviposition. They were then sieved away; the flour in which eggs would have been laid was held for another 2 weeks, after which the larvae produced in each cup were counted and compared to the number of larvae in the control group. The second test assessed the mating ability of the adults which had been treated in the larval stage. Pupae from the generation selected with methoprene were sexed and isolated in individual screened scintillation vials containing ca. 10 g of treated flour and held at 30° ± 1°C and

65 ± 5% relative humidity until they emerged and could be examined for abnormalities. Pupae from the unselected strain were similarly isolated for use and held in untreated flour. Single pair matings were made between the abnormal males and untreated females, between abnormal females and untreated males, and between untreated males and untreated females. The pairs were held for 5 days to allow for mating and oviposition, after which period the adults were sieved away and the flour in which the eggs would have been laid was held for 2 weeks, when the larvae in each vial were counted. This procedure was replicated by utilizing 3 generations, but it could not indicate whether some of the matings which were not producing larvae were laying infertile eggs or no eggs at all. Therefore in the third investigation, the adults were placed in finely sieved flour so that after the adults were removed the flour could be sifted and the eggs counted and the number recorded. The eggs were replaced and held for 2 weeks, the larvae in each vial were counted so that the eggs could be checked for viability.

RESULTS

Selection of the bug *Oncopeltus fasciatus* with the JH mimic kinoprene decreased the susceptibility level from an original EC_{50} of 0.65 µg/bug for the parental generation to an EC_{50} of 2.04 µg/bug for the F_9 generation. During this period, the susceptibility level of the unselected stock strain scarcely changed, the original EC_{50} being 0.65 µg/bug and the EC_{50} at the F_9 being 0.73 µg/bug. Thus comparing the EC_{50}'s at the F_9 indicates that a resistance ratio of 2.8-fold has been reached in the selected strain (Fig. 4).

Selection of *Tribolium confusum* larvae with methoprene increased the tolerance level from an original EC_{50} of 1.2 ppm in the parental generation to an EC_{50} of 4.3 ppm in the F_{11} generation. During the same number of generations the EC_{50} of the unselected strain fell from 1.2 ppm to 0.6 ppm, and thus the resistance ratio for the selected strain at the F_{11} generation was 6.7-fold (Fig. 5). Selection with the JHmimic hydroprene for 11 generations decreased the susceptibility from the parental EC_{50} of 1.04 ppm to a level of 7.05 ppm at the F_{11} generation. Since the EC_{50} of the unselected strain increased, from 1.04 ppm to 1.63 ppm, the resistance ratio for the selected strain at the F_{11} generation was 4.3-fold (Fig. 7). Selection of Tribolium with R-20458 for 11 generations increased the tolerance from the parental EC_{50} of 0.037 ppm to an EC_{50} of 0.060 ppm for the F_{11} generation. The susceptibility of the unselected strain fluctuated, but after 11 generations the EC_{50} of 0.037 ppm remained the same as that of the parental generation, resulting in a resistance ratio of 1.6-fold for the resistant strain at the F_{11} generation (Fig. 7). Selection with the JH mimic RO-20-3600 for 5 generations failed to increase the EC_{50} significantly, but EC_{50} changing from the parental to the F_5 level of 0.12 ppm in both the selected and unselected strains and thus the resistance ratio was 1.0 (Fig. 8). Selection with the chitin synthesis inhibitor diflubenzurone (Dimilin) for 8 generations increased the EC_{50} from 0.94 ppm to 2.05 ppm for the F_8. The EC_{50} of the unselected strain having changed from 0.94 ppm for the parental strain to 0.85 ppm for the F_8 generation. The resultant resistance ratio achieved in the selected strain was 2.4 (Fig. 9).

The statistical significance of the differences observed between selected and unselected strains could be estimated from the computer program LGOPROBIT, based on the method of Finney (1952) which calculates

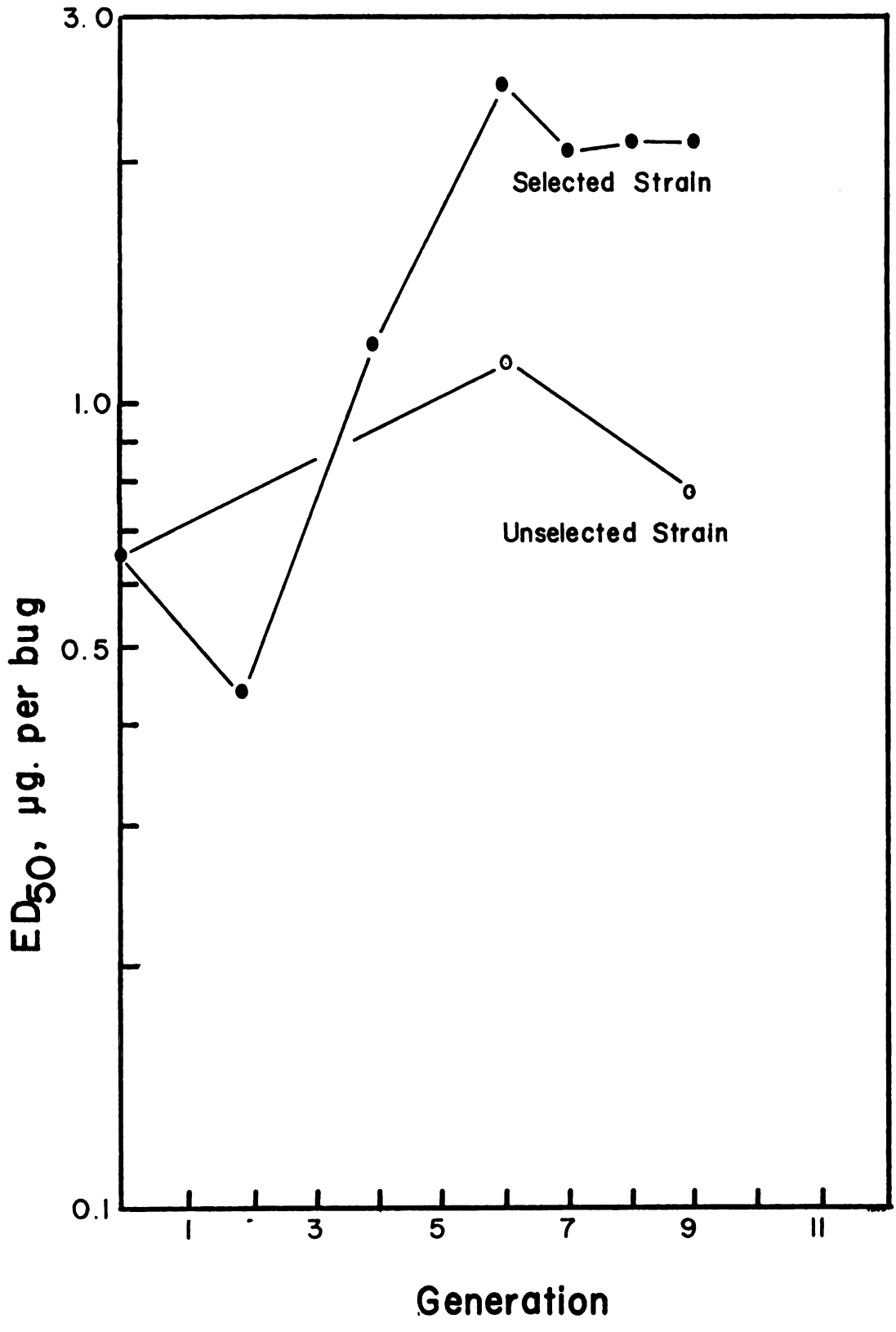


Figure 4. Susceptibility levels to kinoprene of successive generations of *Oncopeltus fasciatus* selected with kinoprene.

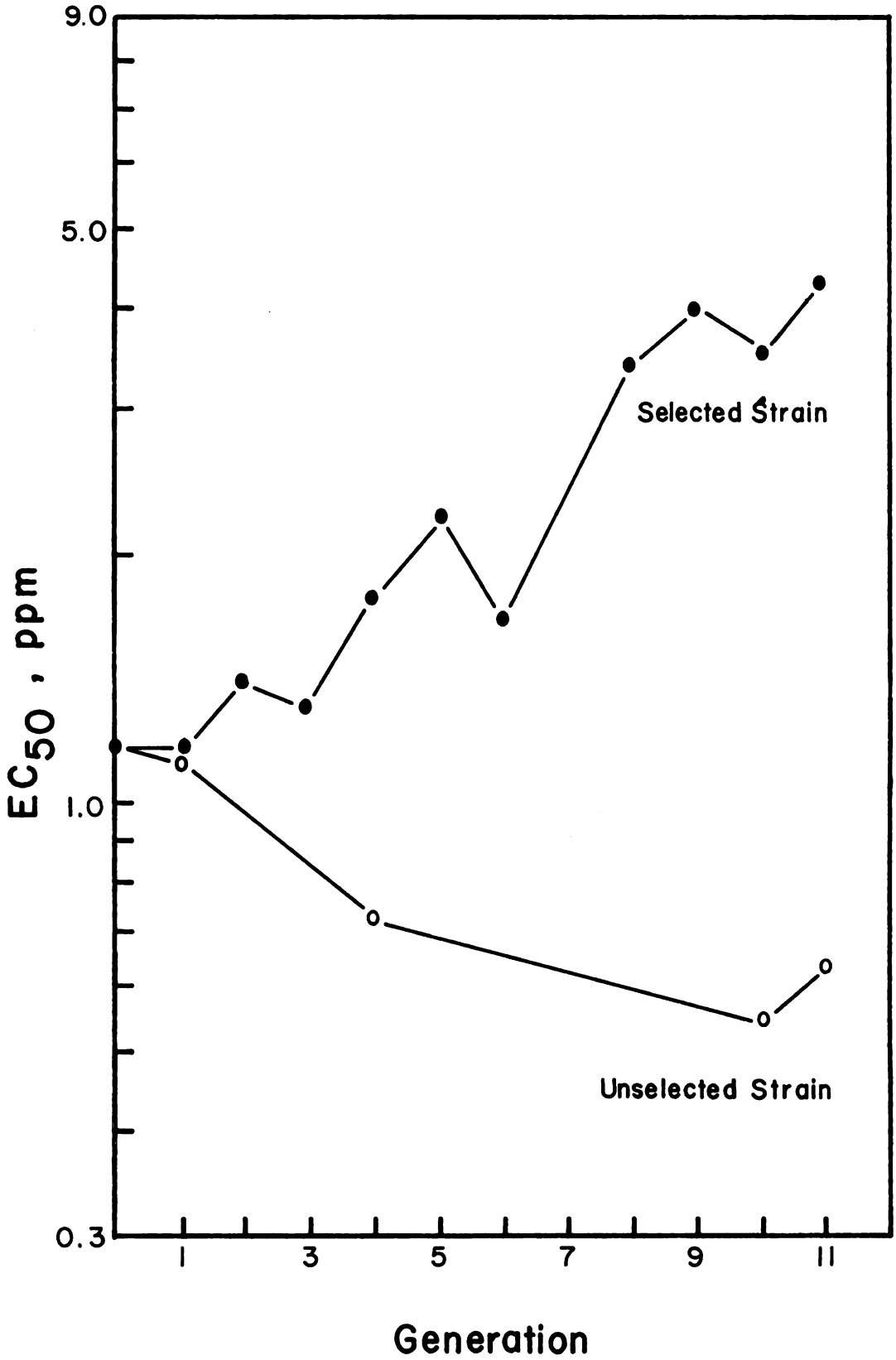


Figure 5. Susceptibility levels to methoprene of successive generations of *Tribolium confusum* selected with methoprene.

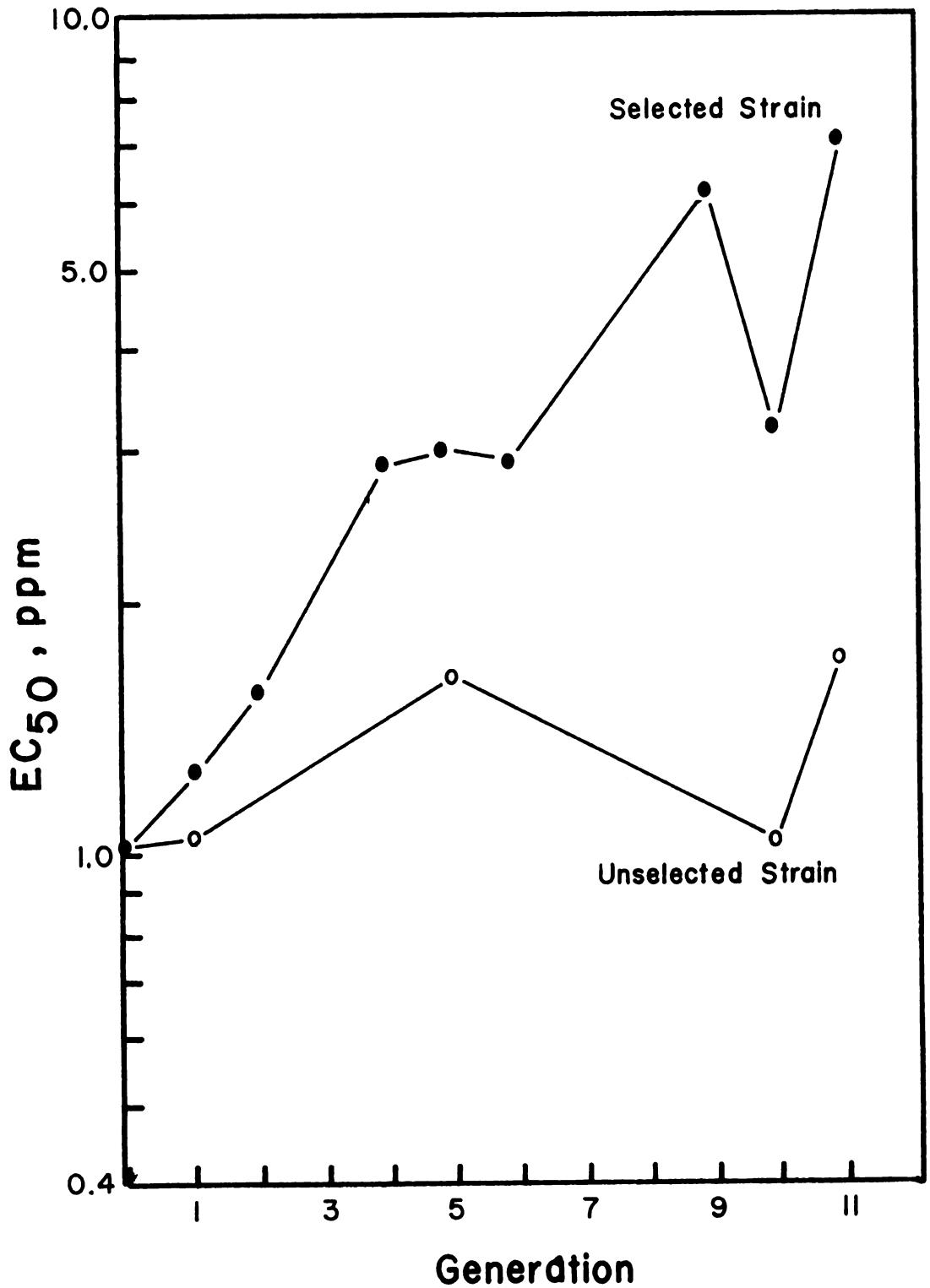


Figure 6. Susceptibility levels to hydroprene of successive generations of *Tribolium confusum* selected with hydroprene.

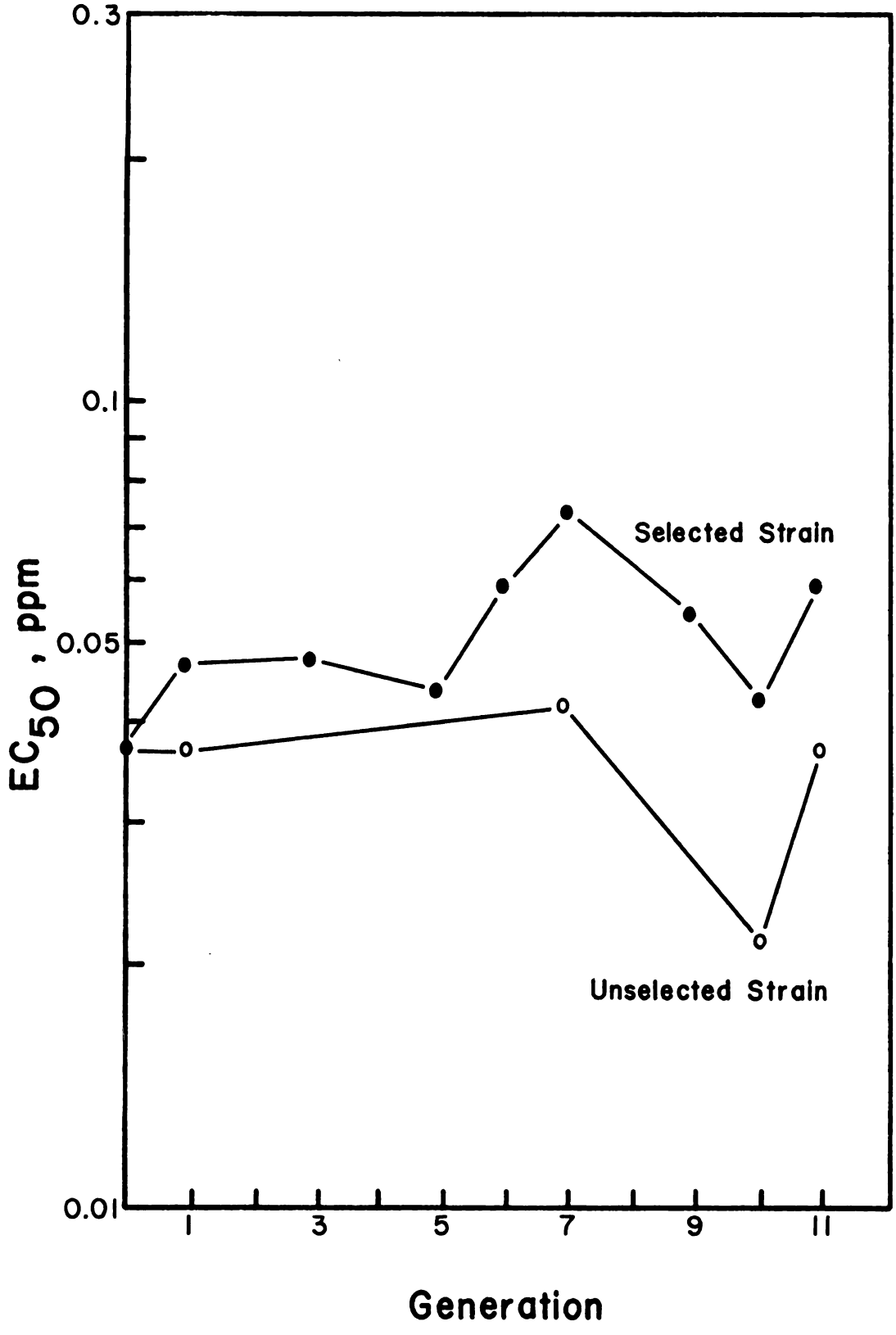


Figure 7. Susceptibility levels to R-20458 of successive generations of *Tribolium confusum* selected with R-20458.

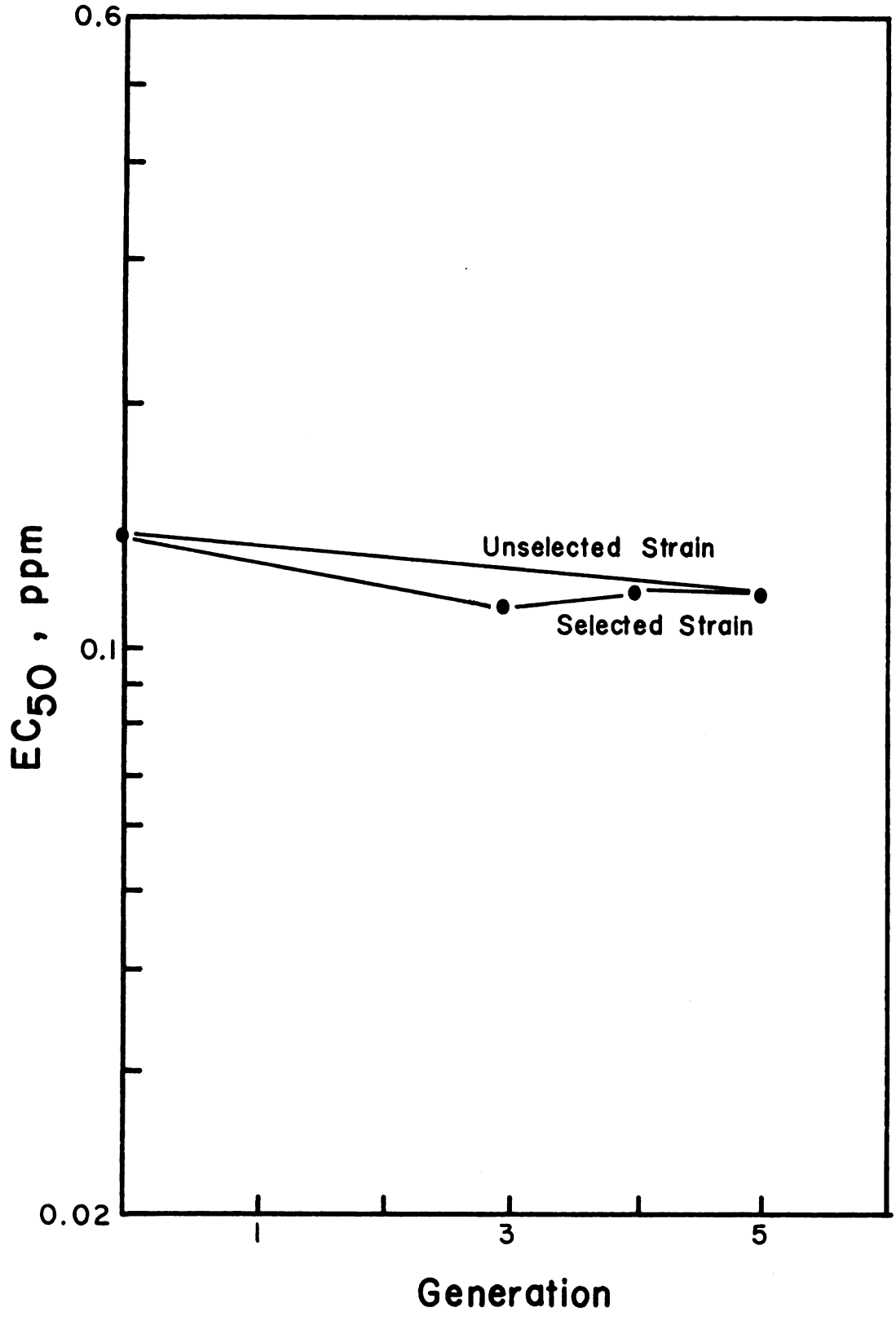


Figure 8. Susceptibility levels to RO-20-3600 of successive generations of *Tribolium confusum* selected with RO-20-3600.

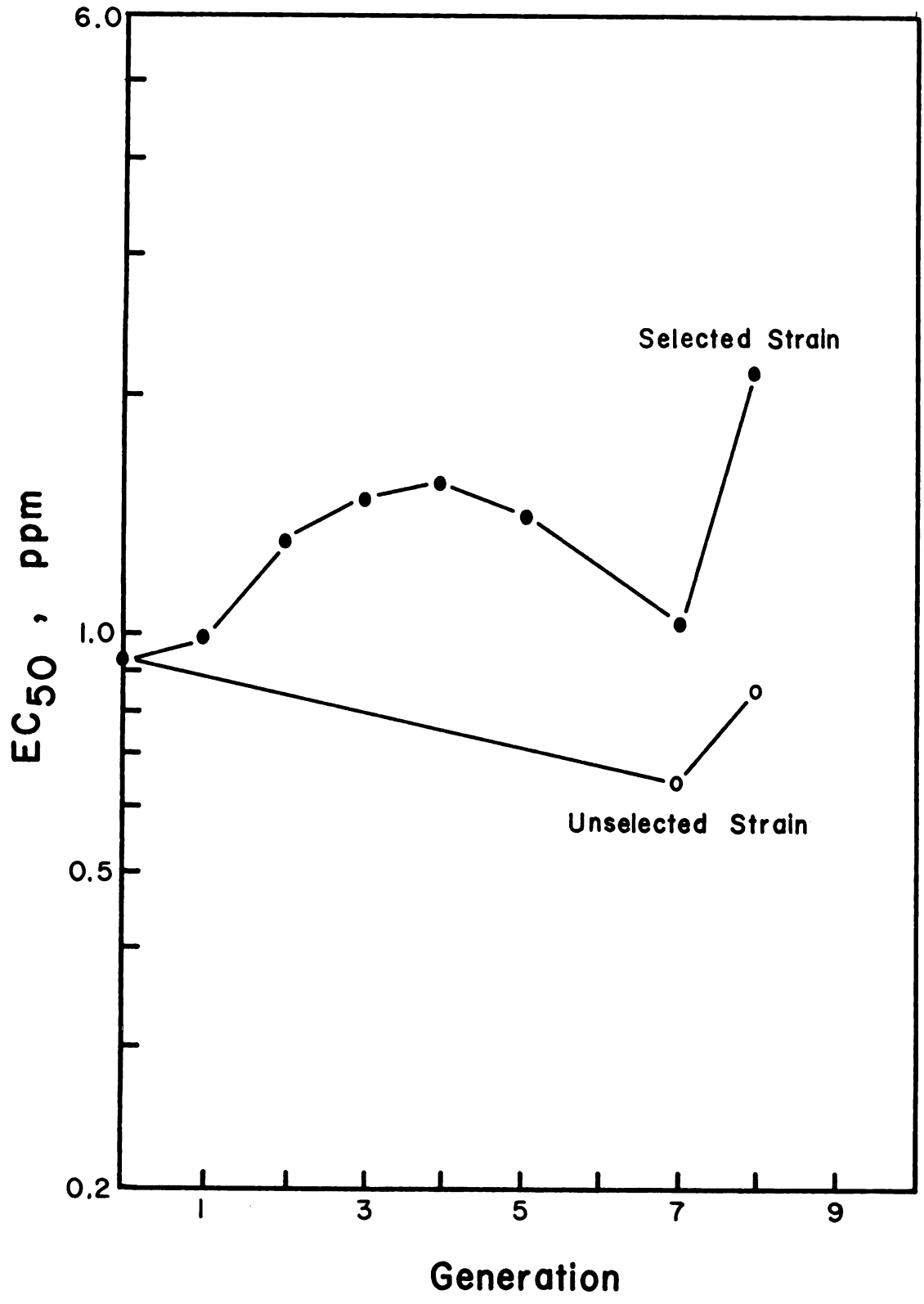


Figure 9. Susceptibility levels to diflubenzurone of successive generations of *Tribolium confusum* selected with diflubenzurone.

not only the EC_{50} and EC_{95}, but also their 95% confidence limits. All of the instances where changes occurred, namely the selection of *Oncopeltus* with kinoprene and all the selections of *Tribolium* except that with RO-20-3600, showed EC_{50} and EC_{95} levels that were significantly different from the unselected strain at the 0.95 confidence limit.

The effect of kinoprene and CG-13353 on *Tribolium* was almost negligible, the EC_{50} values being 77 and 950 ppm respectively. Neither compound was utilized as a selective agent.

The presence of urogomphi on adult *Tribolium* treated as larvae with methoprene was found to be highly correlated (r-.99) with a reduction in their reproductive potential (Fig. 10). The greater the percentage of adults which were classified as abnormal, the fewer the number of larvae that were able to produce.

The results of the single-pair matings (Table 1) show that the proportion of matings between abnormal females and normal males which resulted in the production of larvae is significantly lower than the proportion of control matings, *i.e.* between normal males and normal females that produced larvae. The number of matings between normal females and abnormal males producing larvae was not significantly different from those given by the reciprical cross nor indeed from the control group.

In the investigation of egg fertility in the single-pair matings, 85.7% of the crosses between abnormal males and normal females produced eggs. However, only 28.6% of the crosses produced larvae, which indicates that in fully two-thirds of the productive crosses the eggs were infertile. Of the crosses between the normal males and the abnormal females, 50% produced eggs but only 30% produced larvae, indicating that in 40% of the productive crosses the eggs were infertile. At the same time, 100% of the crosses between normal males and normal females produced eggs and all of these crosses resulted in larvae.

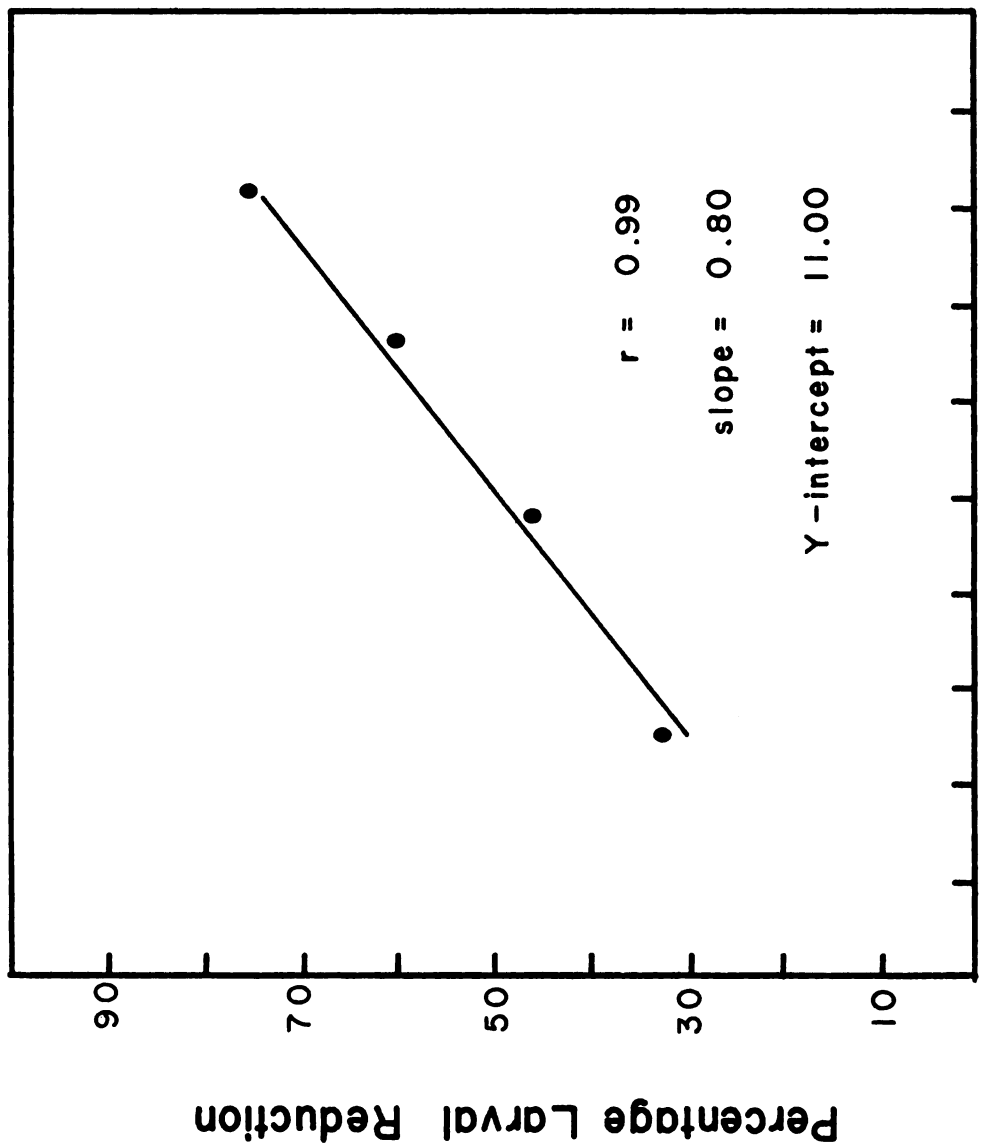


Figure 10. Relationship between percentage of *Tribolium confusum* adults with urogomphi and percentage of larval reduction.

Table 1. Effect of urogomphi abnormalities on larval production in single-pair matings of *Tribolium confusum*.

Type of cross	% of crosses producing larvae	Mean *
normal ♂	0.	
X	22.	28
abnormal ♀	62.	
normal ♀	31.	
X	58.	53.
abnormal ♂	70.	
normal ♀	100.	
X	75.	92.
normal ♂	100.	

* Means with the same letter are not significantly different from each other at $P < .05$ as analyzed by the Student-Newman-Keuls (SNK) test.

DISCUSSION

Laboratory induced resistance to JH mimics has been reported for 3 species of the Diptera (Georghiou *et al.*, 1974; Brown and Brown, 1974), but it has not been reported for a species of any other order. From the results of this study it appears that insects other than Diptera have the capability of developing resistance to JH mimics.

Selection of the large milkweed bug, *Oncopeltus fasciatus*, resulted in a 2.8-fold tolerance to kinoprene after only 9 generations. T.M. Brown (unpublished data) has found that 2 strains of the same species; one selected with methoprene for 11 generations and one with R-20458 for 14 generations have developed a 3.6-fold and 3.5-fold tolerance respectively. Although the 2.8-fold resistance to kinoprene is most accurately labeled as a tolerance because of its low level, the ED_{50} and ED_{95} of the F_9 generation was significantly greater than those of the unselected strain.

Selection of the confused flour beetle, *Tribolium confusum*, with several JH mimic, or with the chitin synthesis inhibitor diflubenzurone, succeeded in producing various levels of tolerance to these compounds. The esters methoprene and hydroprene produced the highest levels of tolerance at 6.7-fold and 4.3-fold respectively after 11 generations of laboratory selection. The compound R-20458 produced a moderate tolerance of only 1.6-fold after 11 generations of laboratory selection. It is interesting to note that T.M. Brown (unpublished data), working with the mosquito *C. p. pipiens*, also found the greatest and fastest resistance with methoprene and hydroprene and that selection with the compound R-20458 also produced a less resistant strain. The strain of *Tribolium* selected with RO-20-3600 failed to show any decrease in susceptibility

as a result of 5 generations of laboratory selection, a stage of selection at which the methoprene-selected strain showed a 3.0-fold tolerance and the hydroprene selected strain showed a 1.9-fold tolerance. The 2.0-fold tolerance to diflubenzurone is of interest because at this time there have been no reports of resistance to this compound, although T.M. Brown (unpublished data) has produced a 4.7-fold resistance in *C. p. pipiens* after 11 generations of laboratory selection.

The discovery of the presence of pupal urogomphi on the abdomens of the treated adult beetles led to the hypothesis that these structures may lead to either a decrease in mating or perhaps may have a sterility effect on the adults. The first investigation led to the finding that the greater the number of adults which had the urogomphal abnormality the fewer the number of larvae they could produce. This fewer number of larvae could have resulted from either of 2 possibilities, — either the females with the urogomphal abnormalities could be producing no larvae at all, or all of the females were producing a reduced number of larvae. The single-pair matings indicate that the females which are affected by the presence of urogomphi are not producing any larvae at all. If the presence of the urogomphi acts as a physical barrier to the mating process, then the urogomphi would be the direct cause for the reduction in reproduction. Metwally *et al.* (1972) reported that administration of JH mimics resulted in reduced amounts of egg production and fertility in *Trogoderma granarium*. Dissection of the females revealed abnormal ovaries. It is possible that the presence of urogomphi is merely an external indication of internal morphological abnormalities of the reproductive system. In this case the presence of the urogomphi would not be the cause of the reduction in reproduction but would only be correlated

with the reduction. In the test for egg fertility 50% of the abnormal females failed to lay any eggs at all, which indicates the possibility of internal morphological abnormalities. Of the 50% of the abnormal females which did lay eggs, 40% had lain eggs which were infertile. In this case no internal morphological abnormalities had occurred because the females were able to lay the eggs, but since the eggs were infertile there must have been a blockage of the fertilization. This blockage may have been the result of the physical presence of the urogomphi or internal abnormalities affecting sperm transfer and storage. It is possible that the presence of the urogomphi acts as a physical barrier to the mating process such that the male is unable to pass the sperm on to the female. Nevertheless in 30% of the crosses between abnormal females and normal males fertile eggs were produced even though the females displayed the urogomphal abnormality. It seems that the presence of the urogomphi may indicate internal abnormalities in some of the adults and in others they may act as a physical barrier to mating in the absence of internal abnormalities. In still other females the presence of the urogomphi have no effect in preventing reproduction. There seems to be a continuum in the effect of the presence of urogomphi: in some of the females the presence of urogomphi has no affect on reproduction; in others the affect is more severe where the urogomphi are present but the females are still able to lay eggs, however, the eggs are infertile; in still other females the affect is most severe where the presence of urogomphi is accompanied by complete sterility.

In the fertility tests of the abnormal males when mated with normal females, 29% of the crosses produced larvae. This is approximately the same percentage as the crosses with the abnormal females and normal males.

Although 86% of the normal females were able to produce eggs, 67% of these crosses were infertile. From this data it is impossible to tell whether the abnormal males were sterile or it was only the physical presence of the urogomphi which prevented mating.

In the test for egg production and fertility there was no ultimate difference between the abnormal males and abnormal females. However, the series of tests for larval production indicated that the abnormal male beetles were affected less than the abnormal females because 53% of the crosses between abnormal males and normal females were still able to produce larvae and these matings were not statistically different from the controls. Sokoloff (1966) states that urogomphi appearing in geneti mutants of *T. castaneum* adult females appear to be larger than those appearing in adult males. He reported that particularly in the mutant females the urogomphi often entrap caked flour, probably contaminated with faeces, with the result that a mass of interfering matter accumulated between these appendages. This phenomenon was noted in the urogomphi persisting in the females after JH mimic treatment. Whereas the vaginal opening of the normal female is accessible to the male (Fig. 11a), that of the abnormal female is blocked by the urogomphi and their accumulations of fecal material (Fig. 11b). The urogomphi of the male do not seem to form as great a barrier to its penis as it does to the female vagina, and thus in the mating process the male genitalia are able to by-pass the urogomphi, and is able to fertilize a normal female (Fig. 12). On the other hand, the normal male is not able to penetrate the urogomphi and accumulated fecal material in some of the abnormal females, so the overall fertility of the females is decreased.

The criterion for the susceptibility testing of *T. confusum* to JH

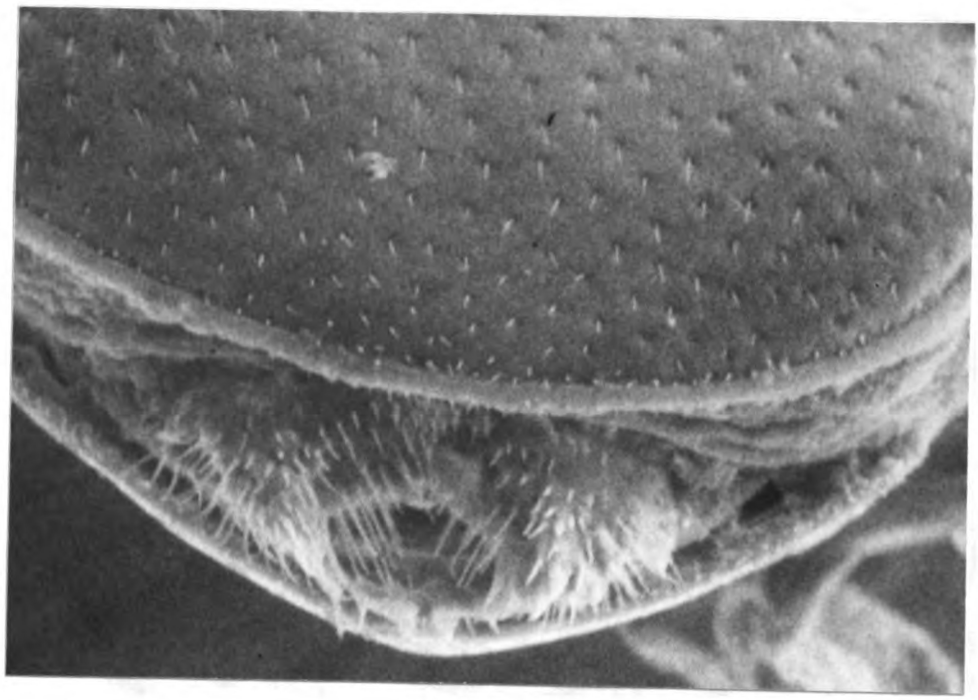


FIGURE 11a

Exposed genitalia of normal female adult *Tribolium*.

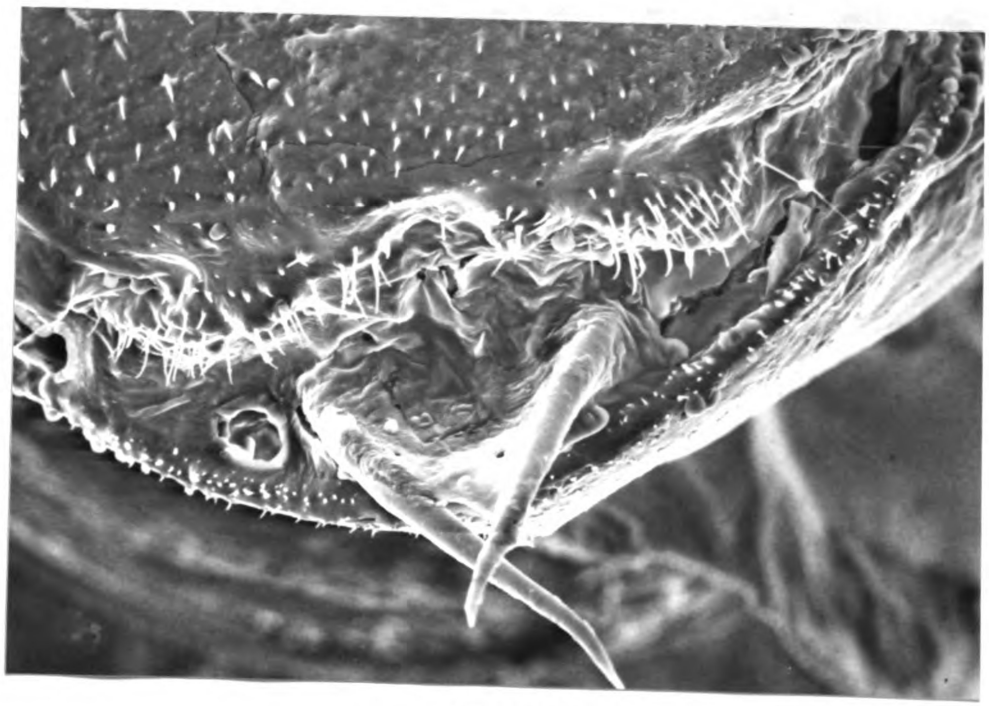


FIGURE 11b

Needle-shaped urogomphi in adult female *Tribolium* treated with methoprene in the larval stage. Note the caked flour and fecal material obstructing the opening to the vagina.

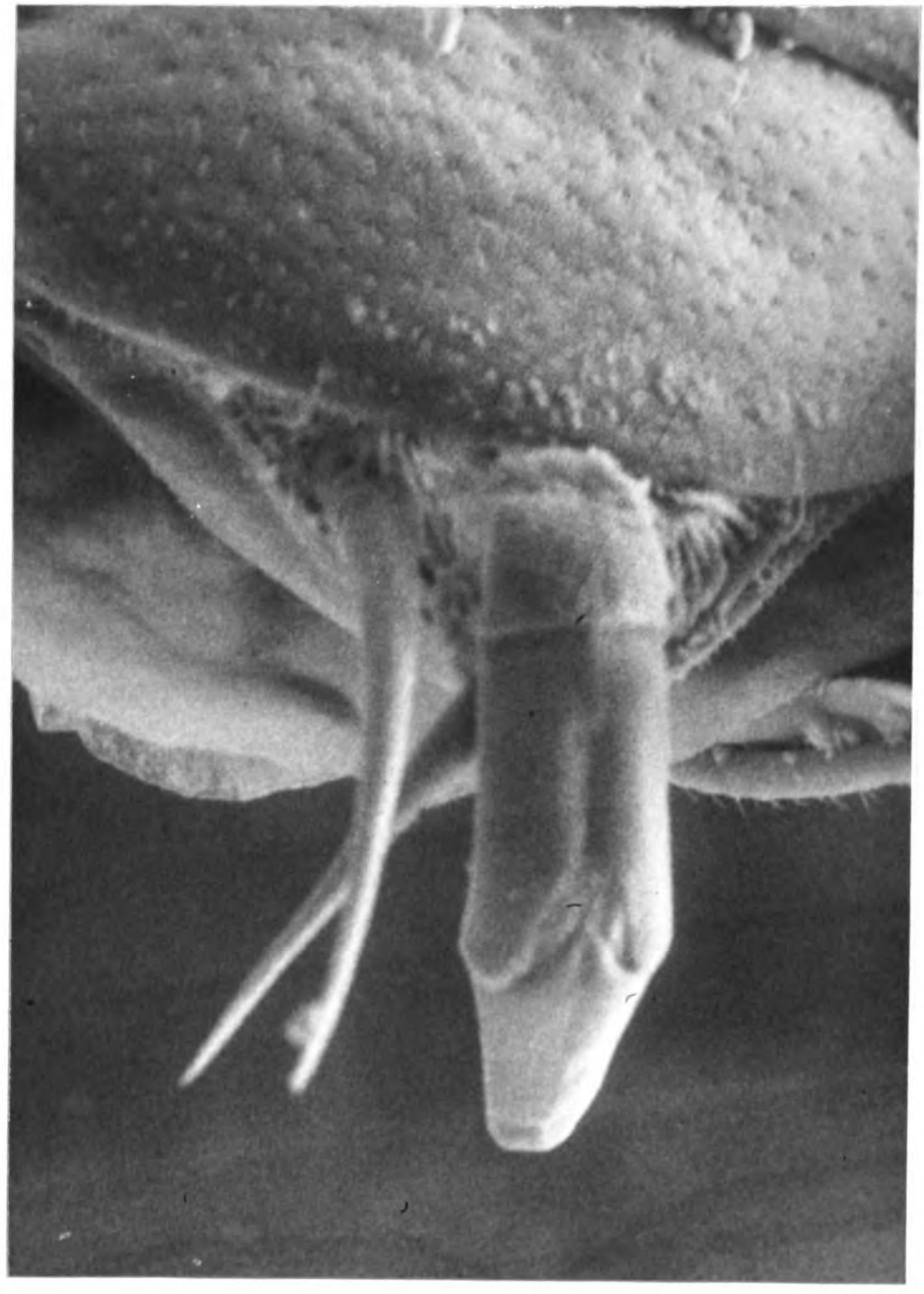


FIGURE 12

Needle-shaped urogomphi in adult male *Tribolium* treated with methoprene in the larval stage. Note how the penis is not obstructed by the urogomphi.

mimics was based on the presence of urogomphi in the emerged adults. The results of these single-pair matings show that even though the adults are still alive after treatment, reproduction is inhibited. It seems then that the presence of the urogomphal abnormality is a legitimate criterion to use to score the adult not as dead but as noneffective. If the urogomphal abnormality inhibits reproduction, then the effect of selection is the same as if the adults had been killed because they are unable to pass their genes on to the next generation.

No attempt was made to investigate the mechanism of JH mimic resistance in either *Oncopeltus* or *Tribolium*. It seems probable that an increase in metabolism of the compound is not the mechanism of resistance because of the low levels of tolerance that the strains exhibited. Typically a biochemical mechanism of resistance is able to produce much higher levels of resistance. It may be that the selected strains possess some physiological process which allows them to survive a somewhat higher concentration of the test compounds. These physiological mechanisms are storage, excretion, follicular development, fecundity, sex ratio, length of developmental period, and vigor (Georghiou 1972). As was described earlier, a reduction in mating and fecundity is one of the effects of JH mimics on *Tribolium*. Table 1 shows that 28.2% of the abnormal female beetles were still able to produce larvae. A possible mechanism of tolerance may be an increase in the ability of the abnormal females to mate and lay fertile eggs. The length of the developmental period also plays a role in the effect of JH mimics on *Tribolium*. The susceptibility levels for the selected and unselected strains increased during the F_{10} generations and this increase in susceptibility coincided with an increase in the larval development time. It was suspected that this increase in

larval development time was due to a decrease in the temperature of the rearing chamber. When the larvae of the next generation were reared at a more stable temperature both the developmental time and the susceptibility returned to previous levels. It was hypothesized that as the developmental time increased, the larvae had more time to ingest and to remain in contact with the insecticide so that they accumulated more of the compound and as a result became more susceptible. Conversely, if the larval developmental time decreased, the larvae may accumulate less compound and become less susceptible.

Conventional insecticides, in general, do not display any effects on adults which are able to survive larval treatment. In contrast, JH mimic insecticides do exhibit effects on those adults which survived treatment as larvae or as nymphs. It has been already mentioned that the egg production and fertility was decreased in abnormal female adult *Tribolium* which were treated as larvae. It was also noted that egg production and fertility of *Oncopeltus* was reduced as a result of nymphal treatment with JH mimics. Additional effects include impairment of flight and of general locomotion. These after effects of the JH mimics may help to decrease the rate at which successive generations of the insects develop resistance to the compounds. The after effects may reduce the competitiveness in the field of the adult insects which survive larval treatment in comparison to an untreated insect. The untreated insect would be able to out-compete the treated adults for mates, and when they mated the untreated insects would be able to produce a greater number of offspring. This lack of competitiveness of the treated adults would be the result of decreases in locomotion, mating ability, egg production, and egg fertility. These factors would act to dilute the resistant genes by way of decreasing the

number of the resistant adults which are able to mate and produce fertile eggs. As a result, although the individuals which survived the treatment would have been selected for resistance during the larval stage, they would be selected for susceptibility during the adult stage if they suffered from after-effects of the compound. This negative selection during the adult stage could impede the progression of resistance. On the other hand, insects which are able to survive the insecticide without suffering from any after-effects would be able to compete with untreated insects for reproductive purposes, and thus would be selected for at both the larval and adult stages in which case the rate at which resistance was established would increase.

Part II. Investigations of the Mechanisms of Resistance in a Laboratory Induced Methoprene-Resistant Strain of *Culex pipiens pipiens*.

INTRODUCTION

Insect resistance to insecticides is due to one or more of the following 3 mechanisms: i) reduced uptake of the compound, ii) increased metabolic degradation of the compound, and iii) decreased sensitivity of the active site to the compound. Apperson and Georghiou (1975) reported that decreased penetration was a factor contributing to the OP-resistance of a strain of *Culex tarsalis*. Quistad *et al.* (1975b) mentioned that selective cuticular penetration may contribute to the sensitivity to methoprene. They also described the metabolism of methoprene in larvae of the mosquito *Aedes aegypti*.

A strain of the northern house mosquito, *Culex pipiens pipiens* L., was available to investigate the physiological or biochemical mechanisms of this resistance. It was necessary to perform preliminary studies of a toxicological nature, including cross-resistance to other JH mimics, cross-resistance to conventional insecticides, and the effect of various synergists on the methoprene-resistance. In addition, an attempt was made to increase the resistance by means of single-brood selections for several generations without exposing the line to methoprene.

MATERIALS AND METHODS

Insects:

These consist of a susceptible strain of the northern house mosquito *Culex pipiens pipiens* obtained from the field at Belding, Michigan in 1973. A resistant strain was produced from the Belding stock in which laboratory selection with the JH mimic methoprene for 9 generations had increased the EC_{50} by 11.9 times, namely from an original 0.004 ppm to a level of 0.046 ppm methoprene (Brown and Brown, 1974). The resistance ratio was 14-fold in the F_8 generation of the resistant strain and between 11- and 19-fold in the generations employed in the present investigations.

Experimental Chemicals:

The JH mimics used in these investigations included the following compounds:

- methoprene (isopropyl 11-methoxy-3,7,11-trimethyl-2,4-dodecadienoate) 94.8% (85% *trans*) Zoecon Corporation.
- hydroprene (ethyl, 3,7,11-trimethyl-2,4-dodecadienoate) 63.1% (75% *trans*) Zoecon Corporation.
- triprene (ethyl 11-methoxy-3,7,11-trimethyldodeca-2,4-dienethiolate) 82.7% Zoecon Corporation.
- R-20458 (1-(4-ethylphenoxy)-6,7-epoxy-3,7-dimethyl-2-octene) 69% Stauffer Chemical Company.
- RO-20-3600 (6,7-epoxy-3,7-dimethyl-1-[3,4(methylenedioxy)]phenoxy-2-nonene) technical grade Hofman-LaRoche Company.
- CG-13353 (4(4'benzylphenoxy)-3-methyl-2-butenoic acid ethyl ester) 90.0% Ciba-Geigy Corporation.

•USDA AI3-36093 (2 ethoxy-9(p-isopropyl phenyl)-2,6-dimethylnonane) unknown purity USDA Laboratory, Beltsville, Maryland.

•diflubenzurone (Dimilin) (1-(4 chlorophenyl)-3-(2,6-diflurobenzoyl)-urea) 95% Thompson-Hayward Company.

The conventional insecticides used in these studies included the following chemicals:

•Abate 99% National Environmental Research Center, Research Triangle, North Carolina.

•pp'-DDT 99% National Environmental Research Center, Research Triangle Park, North Carolina.

•dieldrin 99% Shell Chemical Company.

•carbaryl 99.5% Union Carbide Corporation

•malathion 95.2% American Cyanamid Company.

•parathion 94% Analabs Incorporated.

The synergists employed in the investigations included the following compounds:

•tri-o-cresyl phosphate (TOCP) practical grade Eastman Kodak.

•triphenyl phosphate (TPP) 98% Aldrich Chemical Company.

•piperonyl butoxide (PB) 80% ICN Pharmaceuticals.

•sesamex unknown purity unknown origin.

•SKF-525A unknown purity unknown origin.

•MGK-264 unknown purity unknown origin.

•Lilly-18947 unknown purity Eli Lilly

Experimental Design:

The method employed to test the susceptibility of *Culex* to the JH mimics was that of Brown and Brown (1974). Susceptibility levels to the

JH mimics were expressed as the EC_{50}, a type of LC_{50} based on the effective concentration that inhibits 50% of the emergence to normal adults. Samples of 25 late fourth-instar larvae were exposed in quadruplicate to 4 concentrations of an ethanolic solution of the JH mimic, and a control, in 250 ml deionized water for a period of 6 hours. After removal to clean water for a subsequent period fo 24 hours, dead and incapacitated larvae were scored, and the pupae formed were removed to be scored for those that failed to emerge.

The susceptibility tests were performed on the methoprene-resistant (R) strain and the unselected stock (S) strain simultaneously so that the resistance of the R-strain can be expressed not only by the EC_{50} in ppm but also as a resistance ratio, obtained by dividing the R-strain EC_{50} by the S-strain EC_{50}. Cross-resistance levels for the R strain were found by comparing the resistance ratio for methoprene with the resistance ratios for the other JH mimics and conventional insecticides.

A modification of the susceptibility test was developed for susceptibility testing the response of *Culex* to the Cecropia JH; in this case the hormone dissolved in 0.5 µl acetone was topically applied to the abdomen of late fourth instar larvae resting on filter paper. The treated larvae were held for 2 hours on moist filter paper inside covered petri dishes, before being transferred to 600-ml glass beakers containing 250 ml of distilled water. After this 24-hour holding period, any pupae that had formed were removed and held at 27° ± 1°C and 50 ± 5% relative humidity until emergence, when they were scored according to the method of Brown and Brown (1974). At this time the larvae remaining in the glass beakers were examined and a count was made to determine the actual number of dead larvae and pupae to complete

the comparison between the R and S strains. The above procedure was performed in triplicate with 25 larvae per replicate exposed to 4 concentrations of the Cecropia JH, plus a control, for both the R and S strains.

For determining the cross-resistance of the methoprene-resistant strain to conventional insecticides, the susceptibility tests were performed by the World Health Organization standard method for mosquito larvae (WHO 1970). Lots of 25 early fourth instar larvae were exposed in quadruplicate to 4 concentrations of the insecticide, plus a control, and held for 24 hours. After the 24-hour exposure the number of dead larvae were recorded and the resulting LC_{50} calculated. The tests were performed on the R and S strains simultaneously and the resistance ratio thus obtained for the given insecticide was compared to that for methoprene.

In studies on the effect of synergists, a mixture of the synergist and methoprene (10:1) was added to distilled water, 4 concentrations each in quadruplicate being employed, and the highest concentration of the synergist alone was added to water to serve as a control. The remainder of the test used the method of Brown and Brown (1974) for susceptibility testing of *Culex* to JH mimics. The test was performed on the R and S strains simultaneously in order to assess the effect of the synergist on the resistance ratio.

An attempt was made to raise the resistance level of the R strain by selecting the most tolerant among the single broods (from single egg-rafts) in each of 3 successive generations. These single-brood selections were started with the F_{20} generation and proceeded for the 2 generations following, the broods being tested for susceptibility

but the stem-line being continued without exposure to methoprene. Each single brood (egg-raft of approximately 200 eggs) was tested by taking from it a sample of 30 larvae, divided into 2 replicates of 15 larvae each, and exposing them to a single diagnostic concentration. From the F_{20} generation, 14 broods were assessed, and the most tolerant 5 of them were taken to produce the next generation. This gave 6 broods in the F_{21} of which 2 were taken to continue the line, giving 24 broods in the F_{22} generation, of which five were taken to give the F_{23} (the third filial generation resulting from the third selection). The results were finally computed for each generation in terms of the average percent mortality of all the broods in that generation.

RESULTS

The cross-resistance levels of the methoprene-resistant strain as compared to the normal unselected strain to various JH mimics, conventional insecticides, and the chitin synthesis inhibitor diflubenzurone (Dimilin) were tested on various generations between the F_7 and F_{22}, when the resistance ratio to methoprene was approximately 14-fold.

The EC_{50} and EC_{95} of the JH mimics and the LC_{50} and LC_{95} of the conventional insecticides were calculated by means of the computer program LOGPROBIT. This program utilizes the Finney method of probit analysis (Finney, 1952). The program also calculates the 0.95 confidence limit associated with each EC_{50} and EC_{95}. These confidence limits were used to determine if a significant difference existed between the susceptibility of the selected and unselected strains.

The highest cross-resistance ratios (Table 2), amounting to 14-42-fold, were shown to hydroprene, triprene and CIBA-Geigy-13353, which are ethyl esters or thioesters (Fig. 13). Moderate cross-tolerance ratios (4-10-fold) were shown to the other JH mimics and a 2-fold cross-tolerance was found for diflubenzurone. The methoprene-resistant strain showed a significant increase in EC_{50} to all JH mimics tested and an increase in EC_{95} to all JH compounds except RO-20-3600.

There was negligible cross-tolerance of the methoprene-selected strain to the conventional insecticides (Table 2) except that a 2-fold resistance ratio was shown to dieldrin. The methoprene-resistant strain did not show a significantly higher LC_{50} or LC_{95} with the compounds malathion or carbaryl. The compounds Abate and dieldrin did produce a significantly higher LC_{50} and LC_{95} in the methoprene-resistant strain as compared to the unselected strains. DDT produced a significantly higher LC_{50} but not an LC_{95} in the methoprene-resistant strain. It is interesting to note that parathion produced a significant decrease in the LC_{50} and LC_{95} in the methoprene-resistant strain as compared to the unselected strain.

It proved impossible to obtain a cross-resistance ratio to the natural Cecropia JH; a preparation of 16 intermixed isomers (USDA-A13-33792) had no effect on *Culex* larvae in water, not even at a concentration of 16 ppm. When the Cecropia JH was topically applied from acetone directly to the larvae, the EC_{50} and EC_{95} were significantly lower in the methoprene-resistant strain than in the unselected strain.

Certain compounds known to inhibit detoxication processes were added to methoprene in a 10:1 ratio (synergist:methoprene) to see whether they would act synergistically and lower the EC_{50} of the

Table 2. Cross-resistance of the methoprene-resistant strain to other JH mimics, diflubenzurone and conventional insecticides.

Insecticide	R-Strain Gen'n	EC_{50}, S ppb	EC_{50}, R ppb	EC_{95}, S ppb	EC_{95}, R ppb	Resistance Ratio (EC_{50})
USDA-A13-36093	F_{19}	1.8	17.4 *	5.0	70.4 *	9.7
RO-20-3600	F_{19}	23.4	92.7 *	830.6	1082.7 NS	4.0
hydroprene	F_{18}	20.7	405.8 *	268.9	53938.8 *	19.6
triprene	F_{18}	4.3	58.5 *	55.2	474.3 *	13.7
CIBA-Geigy-13353	F_{20}	35.4	1470.7 *	270.6	3830.9 *	41.6
Cecropia JH @ USDA-A13-33792	F_{19}	27.2	5.8 #	123.4	52.0 #	0.2
diflubenzurone	F_{18}	8.0	21.7 *	26.2	181.3 *	2.7
parathion	F_{20}	4.4	2.7 #	7.0	4.9 #	0.6
malathion	F_{22}	37.7	36.5 NS	64.9	61.9 NS	1.0
Abate	F_{22}	0.5	0.6 *	0.9	1.0 *	1.1
carbaryl	F_{22}	0.5	0.4 NS	0.9	1.0 NS	0.9
DDT	F_{22}	7.3	10.0 *	29.0	23.5 NS	1.4
dieldrin	F_{21}	1.5	3.2 *	2.5	6.9 *	2.1

* Significantly above EC_{50} or EC_{95} confidence limit

NS Not significant at 0.95 confidence limit

\# Significantly below EC_{50} or EC_{95}

@ Compound topically applied to larvae

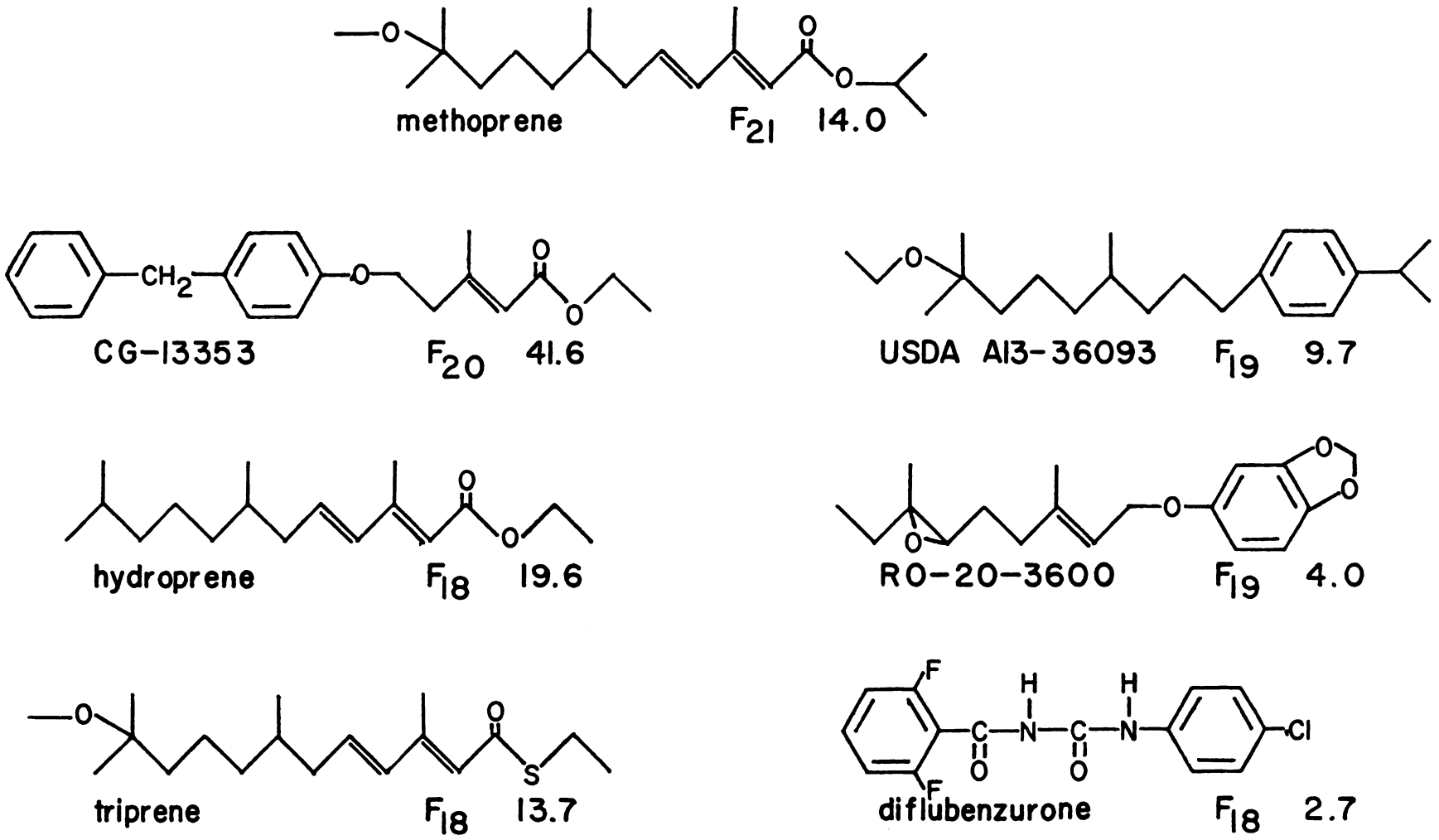


Figure 13. Molecular structure of JH mimics (including generations and cross resistance ratio of the methoprene-resistant strain of *Culex pipiens*).

methoprene-resistant strain. The results obtained on the F_{23} and F_{24} generations of the methoprene-resistant strain show that the resistance ratio of the F_{23} generation had fallen from 14.0 to 5.3, indicating that the synergist studies were performed on larvae which had lost much of their previous resistance (Table 3). Regardless, the microsomal oxidase inhibitor piperonyl butoxide succeeded in lowering the resistance ratio from 5.3 to 2.6 when the larvae were pre-exposed to the synergist 24 hours prior to treatment. The EC_{50} of the R-strain was still significantly greater than the EC_{50} of the S-strain, however; the EC_{95} of the R-strain was not significantly greater than that of the S-strain (CI = .95).

The results of the single-brood selections for tolerance are given in terms of the average percent mortality of all of the broods in each generation (Table 4). The single-brood selection for tolerance level did not succeed in increasing the tolerance of the larvae to methoprene.

DISCUSSION

Weirich and Wren (1973) reported that esterases in the haemolymph of *Manduca sexta* could hydrolyze the Cecropia-JH and hydroprene but not methoprene. Yu and Terriere (1975) found that hydroprene and triprene, but not methoprene, could be hydrolyzed by esterases of house flies. It was suggested that insect esterases can utilize methyl and ethyl esters, but not isopropyl esters, and that the esterolytic site of the enzyme is inaccessible to the carboxy isopropyl group (Weirich and Wren, 1973; Yu and Terriere, 1975). However, Quistad *et al.* (1975b) reported

Table 3. Effect of inhibitors, acting as synergists, on the resistance ratio of the methoprene-resistant strain of *Culex pipiens*.

Methoprene + synergist	R-Strain Gen'n	EC_{50}, S ppb	EC_{50}, R ppb	EC_{95}, S ppb	EC_{95}, R ppb	Resistance Ratio (EC_{50})
methoprene	F_{23}	3.3	17.8 *	19.6	107.0 *	5.3
methoprene + SKF-525A	F_{23}	5.2	29.2 *	20.9	189.5 *	5.6
methoprene + MGK-264	F_{23}	4.8	23.9 *	21.9	105.7 *	4.9
methoprene + Sesamex	F_{23}	3.3	18.9 *	25.5	105.4 *	5.7
methoprene + Lilly-18947	F_{23}	5.0	21.2 *	18.8	88.6 *	4.3
methoprene + TPP	F_{23}	4.9	28.1 *	17.7	87.6 *	5.7
methoprene + Piperonyl butoxide	F_{23}	5.2	32.0 *	20.9	144.4 *	6.1
@ methoprene + TOCP	F_{24}	6.2	39.1 *	28.0	195.1 *	6.3
@ methoprene + Piperonyl butoxide	F_{24}	5.4	13.9 *	53.0	98.2 NS	2.6

* Significantly above EC_{50} or EC_{95} at 0.95 confidence limit

NS Not Significant

@ Larvae pre-exposed to synergist 24 hours prior to treatment

Table 4. Percent mortality of single-brood selections of *Culex pipiens pipiens* larvae.

Generation	No. of broods	Dose (ppm)	Percent Mortality
F_{20} (Original)	14	.096	76.1
	5 out of the 14 broods selected for next generation		
1st Filial	6	.096	81.2
	2 out of the 6 broods selected for next generation		
2nd Filial	24	.080	85.7
	5 out of the 24 broods selected for next generation		
3rd Filial		0.64	84.6

that house flies (*Musca domestica*) and mosquitoes (*Aedes aegypti* and *Culex pipiens quinquefasciatus*) were able to hydrolyze the isopropyl ester of both methoprene and the hydroxy ester metabolite of methoprene. Hooper (1976) found that *in vitro* homogenates of both the methoprene-resistant strain and the unselected strain hydrolyzed ethyl, propyl, isopropyl and butyl esters of propionic acid. However, these same *in vitro* preparations failed to degrade either hydroprene or methoprene. The compounds reported in this study which had the highest cross-resistance ratios were either esters (CG-13353 and hydroprene) or thioesters (triprene). This cross-resistance indicates that there may have been an esterase acting in the methoprene-resistant strain which was also capable of hydrolyzing the esters of CG-13353, hydroprene and triprene.

Tri-o-cresyl phosphate (TOCP) is a known inhibitor of carboxy-esterases and hence should inhibit the hydrolytic cleavage of the isopropyl ester of methoprene. Exposure of the mosquito larvae to TOCP 24 hours prior to treatment and to TOCP plus methoprene during the treatment failed to increase their susceptibility to methoprene.

Soloman and Metcalf (1974) reported that O-demethylation was the primary pathway of methoprene metabolism in *Culex*. Piperonyl butoxide (PB) is a known inhibitor of mixed function oxidases and should inhibit the O-demethylation of methoprene. Quistad *et al.* (1975b) found that PB did not increase the susceptibility of *Culex* to methoprene. This investigation indicates that PB significantly increased the susceptibility of the methoprene-resistant strain when the larvae were treated with the synergist 24 hours prior to the treatment of methoprene plus PB.

When the *in vivo* metabolism of methoprene was compared between the

methoprene-resistant and susceptible strains. T.M. Brown (unpublished data) found no significant difference between the 2 strains. He did find that the R-strain accumulated 23% less methoprene than the S-strain when the larvae were exposed to labeled methoprene for 24 hours. This accumulation difference carried over to the conventional insecticide dieldrin where the R-strain accumulated 22% less of the insecticide than the S-strain. There was not a significant difference between the 2 strains in the accumulation of the insecticide malathion. These results are supported by the observed cross-resistance to these 2 conventional insecticides. There was a 2.1-fold cross-resistance ratio to dieldrin and there was no cross-resistance to malathion. T.M. Brown (unpublished data) investigated the possibility of a difference in rate of ingestion between the 2 strains and found that there was a significantly lower rate of movement of the mouthbrushes in the R-strain as compared to the S-strain. This lowered rate of brushing could indicate a lowered rate of ingestion for the R-strain.

It appears as if the 14-fold resistance exhibited by the F_7-F_{22} generations may have been due to the additive affects of an esterase capable of hydrolyzing the isopropyl ester, an increased O-demethylation, and a decrease in accumulation of the JH mimic methoprene. In view of the failure of TOCP to increase the susceptibility of the R-strain and the lack of a significant difference between strains for *in vitro* and *in vivo* metabolism of methoprene, it is probable that an increased level of an esterase and an increased level of O-demethylation are not the major factors for the increased resistance of the R-strain. It is suggested that the majority of the 14-fold resistance was due to a decrease in accumulation of methoprene in the R-strain. This decrease in accumulation may be the result of decreased penetration across the cuticle or perhaps

a decrease in ingestion of the compound.

The results of the single-brood selections for methoprene tolerance seemed to indicate that there was insufficient genetic variability in resistance characteristics for this type of selection for resistance to be effective. Further selection of the R-strain has increased the resistance ratio from 5.3-fold for the F_{23} generation to a level of 46.5-fold for the F_{31} generation (T.M. Brown, unpublished data), which seems to refute the idea that there was insufficient genetic variability in the R-strain. It may be that the small numbers of broods taken did not contain enough genetic variability for selection for resistance to be effective. An alternative explanation may be that some of the variability among broods may have been the result of age variability as well as tolerance variability, and as a consequence some of the broods may have been labeled as tolerant when in reality their tolerance was simply a result of their age at the time the susceptibility test was performed. This mislabeling would have resulted in the use of nontolerant broods for further selection in which case their susceptible genes would have diluted the resistance allelles and thus prevented the resistance level from increasing.

SUMMARY AND CONCLUSIONS

1. The presence of urogomphi in *Tribolium* completely inhibits reproduction in a portion of the abnormal adults rather than decreasing the amount of reproduction in all of the adults.

2. The inhibition of reproduction may be the result of internal abnormalities of the reproductive system in combination with the physical barrier of the urogomphi.

3. The presence of urogomphi in the adults affects the abnormal female beetles more than it affects the abnormal males.

4. The male genetalia may be able to by-pass its own urogomphi to fertilize females. The presence of urogomphi and accumulated fecal material blocks the vaginal opening of some of the abnormal females preventing fertilization.

5. A significant resistance was induced to kinoprene in *Oncopeltus* and to 4 of the 5 JH mimics applied to *Tribolium*.

6. Insects other than Diptera are able to develop resistance to JH mimics when subjected to laboratory selection.

7. The speed at which this resistance is developed is slower for *Oncopeltus* and *Tribolium* than it is for the Dipteran species.

8. This decrease in the speed at which resistance is achieved may be due to the after-effects which these compounds have on the adults.

9. These after-effects may play an important role in the field by decreasing the competitiveness of the adults which survive larval treatment and thus decrease the rate at which resistance is established.

10. The cross-resistance studies with *Culex* indicate that an esterase may be involved with the methoprene resistance.

11. The synergist studies with *Culex* indicate that the mixed function oxidases may contribute to the resistance.

12. Single-brood selections failed to increase the tolerance of *Culex* larvae to methoprene.

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