SOME PHYSIOLOGICAL ASPECTS OF THE INSECTICIDAL ACTION OF DIFLUBENZURON, AN INHIBITOR OF CHITIN SYNTHESIS

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SOME PHYSIOLOGICAL ASPECTS OF THE INSECTICIDAL ACTION OF DIFLUBENZURON, AN INHIBITOR OF CHITIN SYNTHESIS.

Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
Dr. H.C. van der Plas,
hoogleraar in de organische scheikunde,
in het openbaar te verdedigen
op vrijdag 3 oktober 1980 des namiddags te vier uur
in de aula van de Landbouwhogeschool te Wageningen.

STELLINGEN

1.
Het effekt van diflubenzuron dat resulteert in het niet uitkomen van de eieren na behandeling van adulte vrouwelijke insekten kan beter omschreven worden als ovicide dan als steriliserend.

Dit proefschrift.

2.
De mening van Neville dat chitine geen bijdrage levert aan de hardheid van de insektencuticula is met betrekking tot de dekschilden van de Coloradokever onjuist.

Neville, A.C. in "Biology of the Arthropod Cuticle", blz. 366. Springer-Verlag, Berlin (1975).

Dit proefschrift.

3.
Diflubenzuron kan met meer recht worden gerubriceerd als een I.D.I. (Insect Development Inhibitor) dan als een I.G.R. (Insect Growth Regulator).

4.
Het is aan te bevelen om voor de duur van de periode tussen de registratie verlening en de afloop van de oktrooibescherming voor een pesticide een minimum tijd vast te stellen. Een voorwaarde hierbij zou kunnen zijn dat de registratie tijdig na indiening van het oktrooi wordt aangevraagd.

5.
In verslagen over de biologische aktiviteit van een pesticide is de omschrijving van de daarbij gebruikte formulering vaak onvoldoende. Tevens geeft het ontbreken van een internationale standaardisatie voor de benaming van formuleringen aanleiding tot verwarring.

6.

Bij ecotoxicologisch onderzoek wordt de keuze van organismen en methoden in de meeste gevallen meer bepaald door respectievelijk de mate van toegankelijkheid van hun biotoop voor de mens en de eenvoud van de methoden dan door de biologische relevantie.

7.

Bij het bepalen van de faunistische waarde van natuurterreinen wordt tot nu toe te weinig aandacht geschonken aan insekten.

8.

De mening van Menn dat onder andere de huidige kennis van de fundamentele levensprocessen bij insekten op korte termijn een belangrijke bijdrage kan leveren aan de ontwikkeling van selektieve insekticiden is te optimistisch.

Menn, J.J., J. Agric. Food Chem. 28, 2-8 (1980).

9.

Van de uitgebreide genormaliseerde methode van functievergelijking wordt ten onrechte gesteld dat zij in principe een objektieve waardering van alle functies is

"Principes van functiewaardering volgens de uitgebreide genormaliseerde methode van functievergelijking", Uitgave van het organisatiebureau van de algemene werkgeversvereniging, Haarlem (ongedateerd).

A.C. Grosscurt

Some physiological aspects of the insecticidal action of diflubenzuron, an inhibitor of chitin synthesis

Wageningen, 3 oktober 1980.

CONTENTS

VOORWOORD

Bij het verschijnen van dit proefschrift wil ik allen bedanken die aan het tot stand komen ervan hebben bijgedragen.

Mijn ouders wil ik graag noemen omdat zij het mij mogelijk hebben gemaakt aan de Landbouwhogeschool te studeren.

Prof. Dr. J. de Wilde ben ik zeer dankbaar voor zijn bereidheid om als promotor op te treden. Zijn belangstelling en suggesties hebben een grote invloed gehad op het verloop van het onderzoek.

De publikaties welke dit proefschrift bevat zijn tot stand gekomen tijdens mijn dienstverband met Philips-Duphar B.V. De leiding van de R & D Crop Protection Division, in het bijzonder Dr. W. Maas, wil ik bedanken voor de gelegenheid die geboden is om deze serie publikaties af te ronden.

Verder gaat mijn dank uit naar Ir. J.D. Bijloo, Dr. D.H. Deul, de heer M.J. Gijswijt en Dr. Ir. C.W. Raven voor hun waardevolle adviezen gedurende diverse fasen van het onderzoek en naar de medewerkers van de Entomologische groep van het Agrobiologisch Laboratorium "Boekesteyn" voor hun stimulerende samenwerking. Bij de uitvoering van het experimentele werk is met name grote steun ondervonden van de heren B.J. van der Kolk en A. Stoker.

De plezierige kontakten met de heer J. Tipker van de Biochemische afdeling van de Crop Protection Division van Philips-Duphar B.V. en met Prof. Dr. S.O. Andersen van het August Krogh Instituut van de Universiteit van Kopenhagen resulteerden in een gezamenlijke publikatie met elk van hen. De heer Tipker voerde de berekeningen uit welke in artikel IV zijn weergegeven, terwijl Dr. Andersen de ketocatecholen bepaalde zoals vermeld in artikel VII.

Het aan dit proefschrift verbonden typewerk werd accuraat verzorgd door mevrouw Y.A. Beerthuizen-Zwaartman.

Tenslotte wil ik Trijnie bedanken voor haar steun tijdens de periode waarin aan dit proefschrift is gewerkt.

INTRODUCTION

A list of insecticides in use in the nineteenth century would include as major substances a.o. sulphur, fluorides, lead arsenate and mineral oils. Furthermore various botanicals were used, of which nicotine and Pyrethrum were the oldest, and Derris, Quassia and Sabadilla were much later incorporated in Western agriculture.

The first four decades of the twentieth century can be regarded as a prelude to the subsequent explosive development of organic chemical insecticides. During this period many of the crude materials as mentioned above began to undergo refinement and attention was paid to standardization of the quality of the active ingredients. Furthermore, scientists started to explore the relationship between physical-chemical properties and biological activity.

In the period around World War II chemical synthesis of organic compounds resulted in many insecticides such as the chlorinated hydrocarbons, the organophosphates, and, from the late 1950's onwards, also the carbamates. In 1973 Elliott et al. described a new group of synthetic pyrethroids which, by virtue of a better photostability as well as biological activity, are in practice more potent insecticides than the natural and earlier synthetic pyrethroids. Since 1973 many new representatives of this group of pyrethroids have been synthetized and already some of them are commercially available. A review of the use of pyrethroids in insect control and expectations about their future is given by Elliott et al. (1978).

Electrophysiological investigations indicate that the major target sites of the chlorinated hydrocarbon DDT, and of pyrethroids are the ionic channels of the nerve membrane (Narahashi and Lund (1980), Van de Bercken and Vijverberg (1980)). Organophosphates and carbamates on the other hand appear to interfere with the enzyme acetyl-cholinesterase.

Based upon their mode of action, the selectivity of these insecticides with respect to vertebrates is mainly quantitative. This may be a disadvantage of these compounds because this type of selectivity can be lost when a compound tends to persist or to accumulate in the environment and in animal tissues. As indicated already, the before mentioned synthetic organic insecticides interfere with biochemical processes common to both insects and vertebrates. However, the sensitivity of vertebrates is often lower because of differences in a.o.

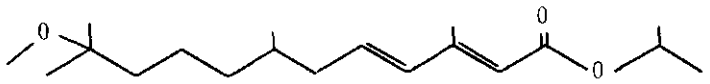
penetration and detoxification or activation. In some of these insecticides, especially in the carbamate group, quantitative selectivity can also be found between insect species, but generally the difference in susceptibility between target and non-target species is low. It is often observed that the killing of the natural enemies of a certain pest insect resulted in an explosive increase in the population of the target insect some time after the insecticide treatment. Broad spectrum insecticides therefore can often lead to an undesired intensification of the use of insecticides as each chemical treatment necessitates the next one. Concomitantly, an additional drawback of the excessive use of broad spectrum insecticides is their tendency to induce considerable resistance.

A new class of insecticides, is composed of control agents that interfere with physiological processes that are mainly specific to arthropods. At present this category includes insect growth regulators (IGR's) and insect development inhibitors (IDI's). In addition to their higher selectivity with respect to vertebrates these compounds may, by their particular mode of action, also be able to overcome serious resistance problems inherent in the earlier mentioned class of synthetic organic insecticides.

However the IGR's and IDI's known at this moment also reveal drawbacks. A general aspect is their rather slow insecticidal activity and the absence of knock-down. This slow action also means that a proper practical judgement of these compounds often requires a longer time lapse between treatment and evaluation. Until recently, the standard insecticidal screening techniques of most industrial companies active in new pesticide research and development were only designed for observing short-term effects. Their evaluation period generally did not last much longer than about 3 days, which now proves to be too short to detect many of the specific aspects of IGR's and IDI's. Present experience with this type of research shows that evaluation of long-term effects often involves complicated logistics and increasing costs. As a matter of fact, IGR's and IDI's are often active at one particular stage or at a few stages during the lifespan of a susceptible insect. In addition in most cases they also show a rather narrow spectrum of insecticidal activity. From an economical point of view therefore these properties often render it unfeasible for a chemical industry to develop such pesticides. Therefore, to ensure the development of selective pesticides, cooperation between governments or large international organizations, and chemical companies should be given more attention (Bijloo, 1973).

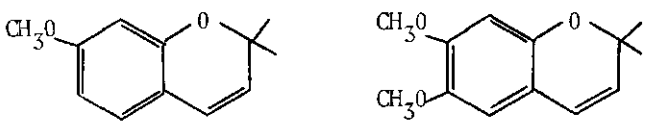
Examples of insecticides with a high level of qualitative selectivity are the compounds with a juvenile hormone-type activity. These compounds became

commercially available in the early 1970's. A review of the entomological aspects of these compounds is given by Staal (1975). The first juvenile hormone analog which was registered for use was methoprene (isopropyl (2E, 4E)-11--methoxy-3,7,11-trimethyl-2,4-dodecadienoate). The trade mark of this compound is Altosid®.



Structural formula of methoprene

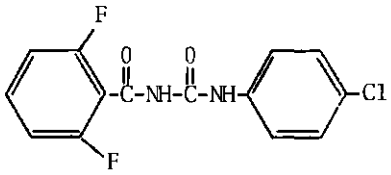
Another example of compounds interfering with the insect hormone balance are the so called precocenes. They were first described by Bowers et al. (1976). These precocenes induce a.o. precocious metamorphosis and sterilization. So far, the practical importance of the precocenes seems to be very limited.



precocene 1 precocene 2

Structural formulae of precocene 1 and 2

Also belonging to the latter class of insecticides are the benzoylureas. These compounds interfere with cuticle formation, probably by inhibiting chitin synthesis (Post and Vincent (1973), Post et al. (1974), Deul et al. (1978), Hajjar and Casida (1979), Van Eck (1979)). In this group of chemicals diflubenzuron (1-(4-chlorophenyl)-3-(2,6-difluorobenzoyl)urea) is still the only compound registered for practical use. It is sold under the trade mark Dimilin®.

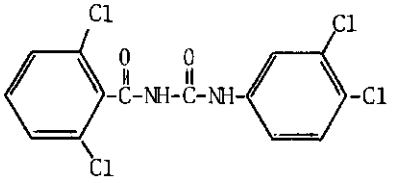


Structural formula of diflubenzuron

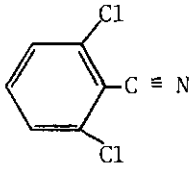
Based upon its mode of action, it is our opinion that diflubenzuron can best be described as an IDI: In contrast to IGR's such as juvenile hormone analogs, diflubenzuron does not regulate but rather inhibits a vital process, viz. normal cuticle deposition.

We should be aware of many other approaches available in modern pest control that are not based on killing insects. Alternative pest control chemicals encompass materials such as pheromones, attractants, repellents and anti-feeding compounds.

The first benzoylurea, which was discovered by Philips-Duphar B.V. as a potent insecticide, was coded Du 19.111 (1-(2,6-dichlorobenzoyl)-3-(3,4-dichlorophenyl)urea). This compound was synthetized in the course of a research programme that centered around the herbicide dichlobenil.



Du 19.111



dichlobenil

Structural formulae of Du 19.111 and dichlobenil

In the biological screening, in which evaluation took place after 5 days, no herbicidal or other phytotoxic effects of Du 19.111 were observed but, instead, larvae of several insect species started to show abnormal symptoms. Mortality, generally, was connected with larval moult. After synthesis of many hundreds of related structures and evaluation of their insecticidal activity, diflubenzuron was chosen for further development. The choice was based upon factors such as level of activity, behaviour in the environment and cost price. Optimisation of

the insecticidal activity was guided by studying quantitative structure-activity relationships.

Histological observations of larvae treated with diflubenzuron revealed a blocking of the formation of the cuticle (Mulder and Gijswijt, 1973). Additional biochemical studies resulted in a number of hypotheses about the primary mode of action of diflubenzuron in insects. As mentioned before, the most plausible hypothesis still appears to be that the compound inhibits chitin synthesis. Explanations of the mode of action of diflubenzuron based on activation of chitinase, phenoloxidase, or on effects on ecdyson-metabolizing enzymes have probably to be considered as secondary. These hypotheses are reviewed in more detail by Verloop and Ferrell (1977).

As already mentioned above, diflubenzuron was first discovered as a larvicide active upon ingestion. Following more detailed research a contact activity on larvae was also found in a few species. After the introduction of diflubenzuron it was also found that the compound could prevent hatching of eggs either by direct application to the eggs or by treatment of the female insects.

The insect species which can be controlled by diflubenzuron in practice mainly belong to the Diptera, Lepidoptera and Coleoptera. Occasionally, susceptible species belong to other orders, e.g. *Psylla piri*, the pear sucker (Homoptera); *Neodiprion sertifer*, the European pine sawfly (Hymenoptera); and *Phyllocoptruta oleivora*, the citrus rust mite (Acarina). Practical field use of diflubenzuron as an insecticide takes place at rates ranging from 20 g a.i./ha up to about 300 g a.i./ha.

As the compound does not penetrate into leaves and, furthermore, often reveals no, or only a low level of, contact activity, many sucking insects like spider mites and aphids, and many hidden feeders, e.g. bollworms, budworms and stem borers cannot be adequately controlled although it is known that larvae of many species belonging to these groups are intrinsically susceptible. Another aspect contributing to the selectivity of diflubenzuron is that the compound is active as a larvicide and as an ovicide but not as an adulticide. Even in those cases in which an ovicidal effect is obtained by adult treatment, the adults themselves survive the treatment. The above-mentioned aspects are the main reason why the impact of diflubenzuron on the non-target insect population is quite reduced. The compound therefore is increasingly becoming an important tool in integrated pest management programs.

In addition, the side effects of diflubenzuron on the environment are confined by a rapid degradation in soil and water and a low toxicity towards mam-

mals, birds and fish (Maas et al. 1980).

When we started our work on benzoylureas in 1975, the larvicidal activities of two representatives from this chemical group, viz. diflubenzuron and Du 19.111 (1-(2,6-dichlorobenzoyl)-3-(3,4-dichlorophenyl)urea), had already been studied rather in detail. However, only little attention had been paid to effects of these compounds on eggs and on adult insects, whereas the experimental results on the development of resistance proved rather conflicting. We therefore decided to focus our experiments on the above-mentioned topics. In most trials diflubenzuron was used because it turned out to be the most important representative of the benzoylureas.

In general, in the literature up to 1975 the prevention of egg hatch by female treatment was indicated as chemosterilization. The aim of a number of our experiments was to investigate whether prevention of egg hatch is indeed caused by chemically induced sterility or whether it can be explained by the interference of diflubenzuron with chitin synthesis. Furthermore, from a practical point of view, we became interested to learn which factors could influence the ovicidal activity after female treatment or after direct application to eggs.

When a new insecticidal compound is introduced into the market, it is very important to monitor its properties with respect to resistance and cross-resistance. This was studied by means of laboratory experiments in which the housefly appeared to be a very convenient test insect.

Optimisation of the insecticidal activity of benzoylureas was guided by calculation of structure-activity relationships. From some preliminary trials we had obtained the impression that the structural requirements of benzoylurea analogs in order to obtain optimum activity were different when tested as an ovicide, as compared to a larvicide. Therefore it became of interest to investigate this phenomenon and furthermore to assess whether correlations could be found by means of calculated physical-chemical parameters between these distinct biological activities. While we assumed that penetration through the gut wall of insects could be an important barrier for certain benzoylurea compounds, we also planned to distinguish in our experiments between ovicidal activity after ingestion by the adult and after injection into the hemolymph.

Chitin synthesis does not only occur in insect embryos and larvae but also a.o. during a restricted period after adult emergence in the cuticle of adult insects. Treatment with diflubenzuron during this period does not result in adult mortality. However, upon feeding diflubenzuron to newly hatched adults of the Colorado potato beetle we observed that the elytra remained soft. For this

reason, the elytra of the Colorado potato beetle appeared to us an interesting tool to study effects of diflubenzuron on the structure, and on some chemical and mechanical properties of the cuticle of adult insects.

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I OVICIDAL EFFECTS OF DIFLUBENZURON ON THE HOUSEFLY *(MUSCA DOMESTICA).*

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SUMMARY

Diflubenzuron can cause ovicidal effects on *Musca domestica* by topical application to, or after oral uptake by, gravid females. In all cases the larvae in the eggs develop normally and lie apparently fully grown in the eggs at the moment that normal eclosion would occur. The compound has no contact activity on the eggs of *Musca domestica*. After topical application to females or after discontinuation of oral feeding of diflubenzuron to both sexes, the percentage of egg eclosion increases with a rate, depending on the concentration of diflubenzuron in the previous treatment. Injection of 1 µg diflubenzuron per female totally prevents eclosion of eggs laid two or more hours later.

Oral uptake of 1000 ppm of the compound by both sexes influences neither the number of eggs produced per female nor the percentage of unfertilized eggs. Also injection of males with 5 µg diflubenzuron, one day before copulation with untreated females, has no effect on egg eclosion. Therefore it is concluded that males play no role in the ovicidal effects of diflubenzuron.

The ovicidal effects of diflubenzuron can be explained by the known mode of action of diflubenzuron as a compound interfering with chitin synthesis.

INTRODUCTION

Diflubenzuron is the common name for 1-(4-chlorophenyl)-3-(2,6-difluorobenzoyl)urea [1], a representative of a new class of insecticides, developed by the Research Laboratories of Philips-Duphar B.V. in The Netherlands.

Initially, diflubenzuron was suggested to be only a stomach poison (Van Daalen et al.; 1972, Wellinga et al., 1973a, b; and Mulder and Gijswijt, 1973). Histological examinations revealed that in various larvae the endocuticular deposition during an instar was disturbed after ingestion of diflubenzuron. After moulting, only epicuticular tissue could be found. The synthesis of chitin, one of the main components of the cuticle, which is responsible for its rigidity, is blocked by diflubenzuron. As a result the newly formed cuticle is very delicate and cannot withstand muscular traction during moulting. This explains why affected larvae succeed only partly or not at all in casting their exuviae (Mulder and Gijswijt, 1973).

However, the suggestion that diflubenzuron had only larvicidal activity proved to be too limited. In our laboratory the first observations of the ovicidal effects of diflubenzuron were made in 1973 (Swennen, unpublished).

With several insect species, ovicidal effects were demonstrable in laboratory experiments, after the dipping of 0-1 day old eggs in a flowable formulation of diflubenzuron. An ovicidal effect of 100% was assessed with *Pieris brassicae* at 3 ppm, with *Leptinotarsa decemlineata* at 300 ppm, while with *Delia brassicae* only a slight effect was assessed with a concentration of 1000 ppm. Ascher and Nemny (1974) found diflubenzuron to be very toxic to eggs of *Spodoptera littoralis* in laboratory dipping experiments, 100% control was obtained with 0.25 ppm active ingredient of a liquid formulation. Holst (1974) sprayed diflubenzuron in a concentration of 500 ppm on eggs of *Epilachna varivestis*. This treatment resulted in a significant reduction of egg eclosion. With *Tribolium castaneum* Carter (1975) found ovicidal effects of 100% after 14 days continuous exposure of eggs to flour containing 1 ppm diflubenzuron.

Besides an ovicidal effect by contact activity, diflubenzuron can also prevent egg eclosion after oral uptake by the female. This phenomenon has been reported for several insect species.

1 Code names PH 60-40, TH 6040, Du 112.307, ENT-29054, OMS 1804, PDD 6040 I. Trade name DIMILIN ®

Taft and Hopkins (1975) undertook field trials on cotton with a sprayable invert sugar bait which contained diflubenzuron in a dosage of 280 g active ingredient/ha. The field population of *Anthonomus grandis* was suppressed to the extent of 98% by the ovicidal activity of diflubenzuron after uptake by the adult insects. Leuschner (1974) found that egg eclosion of *Eurydema oleraceum* decreased in successive batches of eggs after spraying of the host plants with concentrations ranging from 250 to 1000 ppm. Holst (1974) found complete prevention of egg eclosion of *Epilachna varivestis*, fed with plants spraid with 250 ppm. Holst (1974), Taft and Hopkins (1975), and Moore and Taft (1975) used the word "sterility" for the egg mortality they found.

The aim of our experiments was to establish whether these ovicidal effects could be explained by the mode of action of diflubenzuron as a compound interfering with chitin synthesis, as described by Mulder and Gijswijt (1973) and Post, De Jong and Vincent (1974), and furthermore to ascertain whether other mechanisms were involved in this process.

MATERIALS

The susceptible strain of *Musca domestica* used in these tests has been obtained from T.N.O., Utrecht (the present L.I.O. Wageningen) in 1960 and reared ever since at "Boekesteyn".
In feeding trials and in injection trials a 20% flowable formulation of diflubenzuron was used. The particle size of the active ingredient was $\leq 5\ \mu$.
For topical application a solution of the technical material in acetone was used.
All experiments were conducted at a day/night cycle of 18:6 hrs and a temperature and relative humidity of 29°C, 35-45%, and 19°C, 45-55% respectively.

METHODS

Feeding trials

The flies were fed with cubes of sugar and a suspension containing one part of full cream milk powder in two parts of water. The milk was offered in a cotton wool pad, and changed twice a day.

In the treatments, diflubenzuron was added while stirring to the milk suspension up to the desired concentration. The dry weights of the milk in both treated and untreated suspensions were the same. Care was taken that the milk used for the oviposition medium was sour, to stimulate oviposition by the flies.

Eggs were collected daily from the cotton wool pads. All feeding treatments started on the day of adult eclosion. The percentage of hatching eggs was determined with randomly selected eggs. They were placed in a Petri dish containing moistened black filter paper and kept at 24°C and a relative humidity of 100%. The number of viable and non-viable eggs was counted after one day.

Injection trials

The flies were anaesthetized with CO_2 and injected in the dorso-lateral side of the thorax with a hand-made glass injection-needle. The contents was calibrated with a "Microcaps®" pipette of 1 or 5 µl. In most cases the percentage egg eclosion was corrected with Abbott's formula (Abbott, 1925).

Trials with topical application

The acetonic solution of diflubenzuron was dispersed as 1-µl drops from an "Agla" micrometer syringe on the dorsal side of the thorax or the ventral side of the abdomen. The houseflies were anaesthetized with CO_2.

RESULTS

1. Contact activity on eggs

No egg mortality by diflubenzuron was found when eggs of *Musca domestica*, between 0 and 12 hours old, were dipped into a 1000-ppm flowable suspension for 90 minutes.

It was mentioned under "methods", that in the experiments where the flies were fed with diflubenzuron, the eggs were deposited on an oviposition medium containing diflubenzuron. For that reason we had to find out whether diflubenzuron had any contact activity under these conditions.

Table 1. Eclosion of *Musca domestica* eggs, 0-1 h old, in an oviposition medium either untreated or treated with diflubenzuron

Oviposition medium	total number of eggs	average percentage non-hatching eggs, variability between brackets
Untreated	6207	18 (14-27)
Treated with 1000 ppm diflubenzuron	6889	14 (11-17)

Average of 3 experiments, 6 replicates of at least 300 eggs each.

From Table 1 it is clear that in our experiments diflubenzuron shows no contact activity on the eggs of *Musca domestica*.

2. Continuous feeding of diflubenzuron to both sexes

At concentrations of 100 ppm or higher, egg eclosion is prevented (table 2).

Table 2. Inhibition of egg hatching by diflubenzuron, when fed to both sexes.

Concentration in ppm	% non-hatching eggs (correction with Abbott's formula), variability between brackets		
	days of treatment		
	4	6	8
100	100	100	100
30	99 (99-100)	98 (94-100)	90 (69-100)
10	75 (44-96)	69 (39-85)	67 (39-81)
3	28 (0-66)	21 (0-32)	23 (19-27)
1	16 (0-32)	14 (0-36)	22 (21-23)

The data in the table represent the average of 3 experiments, each with 3 replicates of 10 males and 10 females per concentration.

The ovicidal effects of diflubenzuron are reversible. Table 2 shows the influence of diflubenzuron on the percentage non-hatching eggs. When afterwards the flies were fed with untreated food, egg eclosion increased, depending on the concentration of diflubenzuron in the previous treatment.

Table 3. Results of termination of feeding of diflubenzuron to both sexes (data corrected with Abbott's formula).

treated food		untreated food		
concentration in ppm till day 8	% non-hatching eggs on day 8	% non-hatching eggs on day 9	10	13
1000	100	100	99	48
300	100	100	90	63
100	100	93	55	13
30	90	72	35	7
10	67	38	15	0
3	23	17	15	15
1	22	9	6	9

Average of 3 experiments, each with 3 replicates of 10 males and 10 females per concentration.

3. Topical application to females

In previous experiments it had been found that diflubenzuron influenced egg eclosion after being fed to females.

In Table 4 results are given of topical application of an acetonic solution of diflubenzuron to the thorax or the abdomen. For these experiments, 3-day-old female adults were used.

Though there is a possibility that females ingest the compound after body contact, it is very likely that an acetonic solution of diflubenzuron can penetrate the female adult cuticle.

Table 4. Influence of topical application of diflubenzuron to adult females on egg hatching.

application of 1 µg	% non-hatching eggs (correction with Abbott's formula), variability between brackets.			
	days after treatment			
	1	2	3	4
dorsal side of thorax	71 (67-74)	44 (32-58)	36 (33-39)	0
ventral side of abdomen	88 (85-90)	77 (65-86)	23 (6-52)	0

Experiments each with 3 replicates of 10 untreated males and 10 treated females. Results of application on the thorax are an average of 3 experiments, those on the abdomen are an average of 4 experiments.

4. Mode of action

If in these experiments eggs do not hatch, there may be several reasons for this:

1. the egg has not been fertilized;
2. the embryo has died during its development;
3. the embryo has developed into a larva which is unable to leave the egg.

These three possibilities were investigated.

Possibility 1. The egg has not been fertilized

Even 7 days of feeding with a high dosage of the compound did not influence fertilization (Table 5). Eggs were reported as unfertilized when they did not show any sign of embryonic development.

Table 5. Influence on fertilization of adult feeding with diflubenzuron

treatment	% unfertilized eggs, variability between brackets			
	days of treatment			
	4	5	6	7
check	8 (5-10)	22 (14-34)	13 (4-24)	18 (11-25)
1000 ppm diflubenzuron	9 (5-15)	3 (0-5)	11 (5-21)	18 (8-27)

The results represent the average of 3 trials, each with 1 replicate of 125 males and 125 females on days 4, 5 and 6, and of 2 trials on day 7. According to Student's t-test no significant difference was found at the 0.05 level.

Table 5 leads to the conclusion that diflubenzuron has no influence on the percentage unfertilized eggs.

In Table 6 results are given of the percentage of unfertilized eggs upon injection of males with diflubenzuron. The males were injected one day after adult eclosion. They were allowed to mate one day after injection, with untreated females of the same age. In these experiments, too, no influence on fertilization was found.

Table 6. Influence on fertilization of injecting males with diflubenzuron

treatment	% unfertilized eggs, variability between brackets				
	days after injection				
	2	3	4	5	6
check	6 (2-9)	6 (2-11)	15 (2-25)	15 (10-24)	18 (1-43)
5 µg/male	10 (4-16)	15 (11-18)	19 (1-43)	14 (0-31)	31 (11-60)

Average of 3 trials, each with 2 replicates of 10 treated males and 10 untreated females. According to Student's t-test no significant differences was found at the 0.05 level.

Possibility 2. The embryo has died during its development.

Figures 1, 2, and 3 show that larvae in the eggs of flies that are fed with diflubenzuron develop normally. They lie apparently fully grown inside the egg at the moment normal eclosion would occur.

Additionally to the experiments in which both sexes were fed with diflubenz-

uron, males were injected with 5 µg of diflubenzuron/male. Of these experiments, the influence on fertilization was listed in Table 6. Table 7 gives the influence on egg mortality after the males were allowed to mate, 1 day after injection, with untreated females of the same age. Even with this extremely high dosage no difference was found between mortality of eggs from treated and those from untreated flies (egg mortality is defined as the percentage non-hatching eggs, with correction for the percentage of unfertilized eggs).

Table 7. Influence on egg mortality of injecting males with diflubenzuron

	% egg mortality, variability between brackets				
treatment	days after treatment				
	2	3	4	5	6
check	3 (2-3)	6 (0-12)	7 (4-12)	3 (1-6)	2 (0-6)
5 µg/male	6 (2-10)	9 (6-15)	4 (2-8)	5 (1-10)	5 (2-8)

Average of 3 trials, each with 2 replicates of 10 treated males and 10 untreated females. According to Student's t-test no significant difference was found at the 0.05 level.

Possibility 3. The embryo has developed into a larva which is unable to leave the egg.

Observations showed that the fully grown larvae did move inside the egg. Sometimes the larva can split the egg wall but is unable to leave the egg.

These phenomena can be explained in the same way as those described for the "free-living" larvae (Mulder and Gijswijt, 1973). In larvae, diflubenzuron disturbs the formation of chitin in the cuticle, and this is most likely followed by an incapacity to use their muscles. This may explain why apparently fully grown larvae are unable to leave the eggs.

On considering these 3 possibilities we can conclude that diflubenzuron causes effects as described under possibility 3.

5. Influence of diflubenzuron on the fecundity

We define fecundity as the number of eggs produced per female, and fertility as the percentage of normally hatching eggs.

In previous experiments we saw that diflubenzuron influenced egg fertility after being fed to both sexes.

The fecundity however was not influenced by the feeding of diflubenzuron. In Table 8 the results are listed of a treatment of both sexes with 1000 ppm during 7 days.

Table 8. Influence of diflubenzuron on the fecundity upon feeding both sexes with 1000 ppm diflubenzuron in the food.

days of treatment	number of eggs per female per day, variability between brackets	
	check	fed with 1000 ppm
3	79 (65-104)	73 (48-92)
4	48 (12-66)	39 (14-66)
5	31 (22-44)	44 (31-60)
6	52 (28-74)	40 (28-54)
7	48 (28-72)	53 (46-58)

Average of 3 trials, each with 1 replicate of 125 males and 125 females. According to Student's t-test no significant difference was found at the 0.05 level.

6. Quickness of action after contamination of females

From several observations it was clear that diflubenzuron could cause ovicidal effects on eggs of *Musca domestica* laid very soon after oral uptake of the compound by the females.

In four trials diflubenzuron was added to the food of 4-day-old flies in a concentration of 1000 ppm. The flies had been fed with untreated food previously. After 18 hrs the eggs were collected and subsequently the percentage of non-hatching eggs was assessed (Table 9).

Table 9. Percentage of non-hatching eggs after feeding of diflubenzuron for 18 hrs to 4-day-old adult flies.

	total number of eggs	average percentage non-hatching eggs, variability between brackets
check	1395	15 (9-21)
with 1000 ppm diflubenzuron	1598	100

Average of 4 trials, each with 1 replicate of 200 males and 200 females.

Feeding of 1000 ppm during 18 hrs resulted in a total prevention of egg

eclosion. However, in this experiment it is uncertain after short periods whether the flies have taken up the compound and if they did, what they have ingested.

Therefore in the next experiments female houseflies were injected with the compound. We used 4-day-old females. In the first eggs, collected 2 hrs after applications, egg eclosion was fully prevented already (Table 10).

Table 10. Mortality of eggs, collected after injection of females of *Musca domestica*.

	Percentage egg mortality			
	Hours after injection when eggs were collected, number of eggs between brackets			
	2	3	4½	6
check	8	-	6	9
	(423)		(95)	(442)
1 µg/female	100	100	100	100
	(398)	(29)	(134)	(372)

Average of 3 trials, each with 50 treated females and 50 untreated males.

DISCUSSION

Some authors use the word "sterility" for the egg mortality caused by diflubenzuron. They include Holst (1974), Taft and Hopkins (1975), and Moore and Taft (1975).

According to the definition of Labrecque (1968), chemosterilants are chemicals that either

- Cause sexual sterility by preventing the development of sperm or ovum; or
- Cause the death of the sperm or ovum after it has been produced; or
- So severely injure the genetic make-up of the sperm and ovum that fertilization, if accomplished, will not result in viable progeny.

In our experiments we found no indications that diflubenzuron satisfies the above mentioned definition:

- No influence on fertilization was found after treatment with diflubenzuron fed to both sexes or injected into males;

- It can be concluded that diflubenzuron neither prevents the development of sperm nor causes the death of the sperm after it has been produced.
- Pictures 1, 2, and 3 show that, after treatment with diflubenzuron, apparently normal development of the embryo takes place.

However, prevention of egg hatch can be explained by the known mode of action of diflubenzuron. When chitin synthesis is blocked, the larva probably cannot use its muscles to free itself from the egg wall. An injury of the genetic make-up of the sperm or ovum that would cause the same effects after fertilization is very unlikely, as the phenomena are the same as those described for "free-living" larvae.

In view of these considerations, we believe that diflubenzuron cannot be listed as a chemosterilant according to the definition of Labrecque (1968). We therefore think it correct to call the prevention of egg eclosion an ovicidal activity of diflubenzuron.

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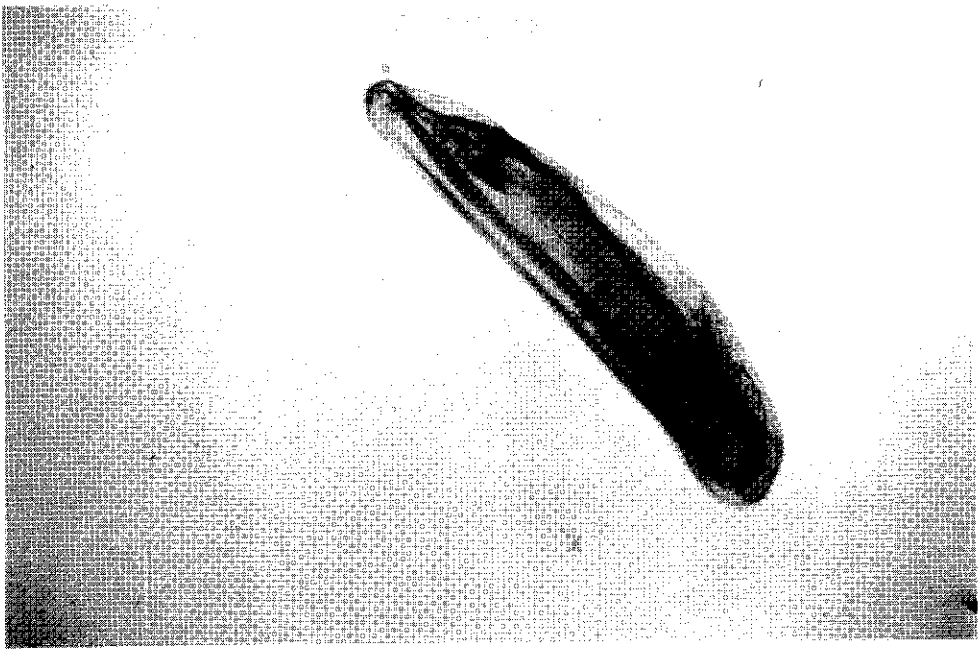

Figure 1. General view
Eggs of *Musca domestica* after feeding adults with 1000 ppm diflubenzuron. After 24 hours at 24°C and a relative humidity of 100% the eggs were dehydrated in ethyl alcohol and cleared in methyl benzoate.

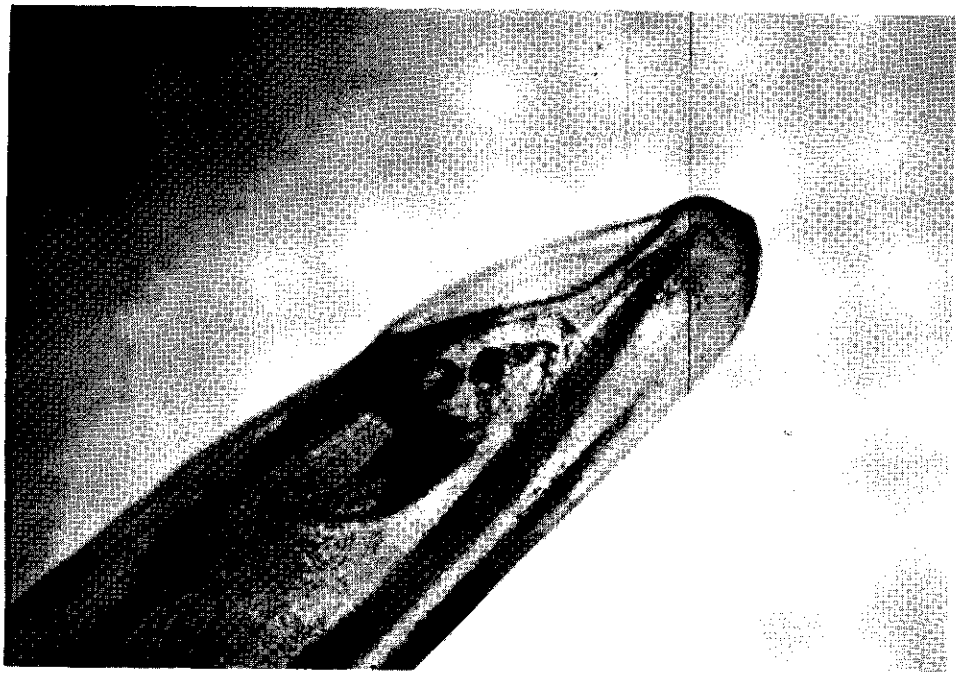

Figure 2. Detail of the anterior part.

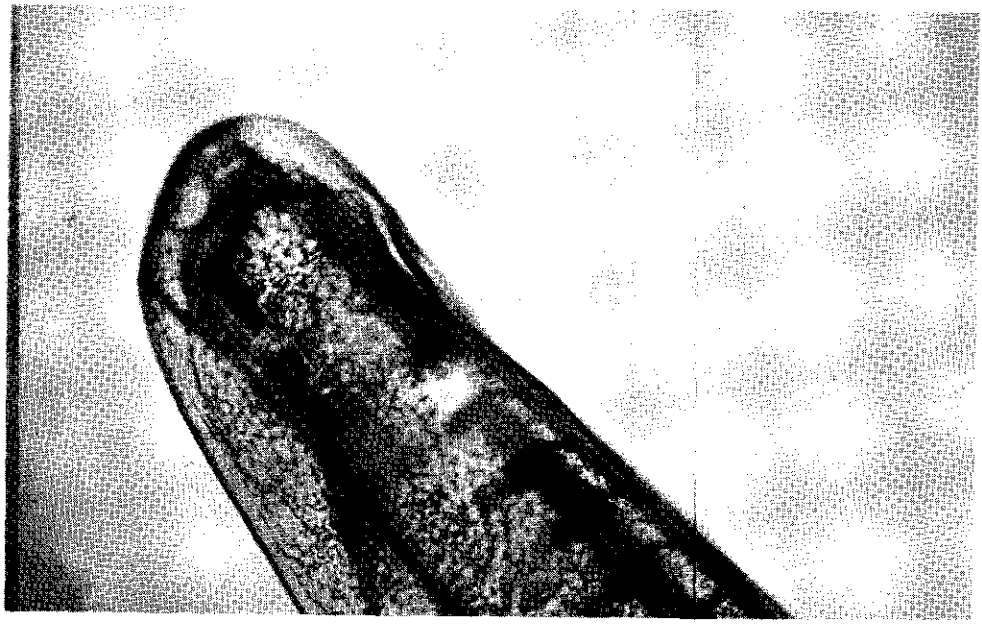

Figure 3. Detail of the posterior part.

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II MODE OF ACTION OF DIFLUBENZURON AS AN OVICIDE AND SOME FACTORS INFLUENCING ITS POTENCY.

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SUMMARY

Electron microscopical observations of eggs of *Leptinotarsa decemlineata* treated with diflubenzuron through the female showed that the developing embryo formed an amorphous cuticular region instead of a normal lamellate cuticle. Treated embryos probably cannot use their muscles to leave the egg by virtue of a lack of rigidness in the cuticle. These symptoms are comparable with those studied in larvae and provide evidence that the ovicidal activity of diflubenzuron is caused also by its interference with chitin synthesis.

Effects on fertilization, fecundity, and the role of males are discussed. In the case of direct contact of diflubenzuron with susceptible eggs, activity increases with smaller particle size and higher relative humidity of the air, and in some species is also affected by surfactants. Younger eggs are generally more sensitive than older eggs.

Inhibition of egg hatch after treating female adults can be caused by oral uptake or by contact. After excretion of the compound, normal egg hatch is resumed. The rate of this reversibility depends on the dose received and, as reported in literature, can depend on the age of the adult. Ovicidal effects after treating female adults by contact are greatly dependent on the formulation of the compound.

INTRODUCTION

Diflubenzuron is the common name for 1-(4-chlorophenyl)-3-(2,6-difluorobenzoyl)-urea[1]. Initially diflubenzuron was thought to be a stomach poison with larvicidal activity only (Mulder and Gijswijt, 1973), but has since proved to prevent egg hatch and to possess contact activity. In a previous paper we concluded that diflubenzuron was not a true chemosterilant but that the prevention of egg hatch could be attributed to an interference with chitin synthesis (Grosscurt, 1976). In the present paper, histological evidence is adduced in favour of that conclusion. Furthermore the effects of diflubenzuron on fertilization and fecundity and the role of males in the ovicidal effects are discussed.

During our experiments it became obvious that many factors influenced the ovicidal activity of diflubenzuron. In the second part of this paper these factors are evaluated and summarized with previously published data.

MODE OF ACTION OF DIFLUBENZURON

Histology

Ovicidal effects of diflubenzuron can be obtained either by topical application to the eggs or by treating female insects. In either case the symptoms are similar.

The larva in the egg develops fully but is unable to leave the egg, though it sometimes ruptures the egg wall. For *Musca domestica* photographs of this phenomenon were presented by Grosscurt (1976). In accordance with our knowledge concerning the mode of action of diflubenzuron and the related compound Du 19.111 (1-(2,6-dichlorobenzoyl)-3-(3,4-dichlorophenyl)-urea) in larvae (Mulder and Gijswijt, 1973; Post and Vincent, 1973; Post, De Jong, and Vincent, 1974) we assumed that the formation of cuticular chitin in the embryo was blocked by diflubenzuron. This was likely to prevent the larva using its muscles to leave the egg. To check this hypothesis, electron microscopic preparations were made of embryos of *Leptinotarsa decemlineata*, after the adults had been given 1000 ppm diflubenzuron in the food for 3 days. The tissue was fixed and stained by the method of Akster and Smit (1975), first with saturated uranyl acetate and then with a lead

1 Code names PH 60-40, TH 6040, Du 112.307, ENT-29054, OMS 1804, PDD 6040 I. Trade name DIMILIN®

solution. Micrographs were made with a Philips EM 201C electron microscope by Miss E.H. Velzing and Drs. W.A. Smit, Zoological Laboratory, University of Amsterdam, The Netherlands.

The embryos had been fixed a few hours before hatching. Embryos in the eggs, both from treated and from untreated females could be seen moving inside the egg at that moment. In diflubenzuron-contaminated eggs we saw instead of the normal patterns of lamellate cuticle deposition the formation of an amorphous cuticular region. Globular structures were vaguely discernible. They were possibly similar to the globules which in treated larvae could be observed under a light microscope (Mulder and Gijswijt, 1973). The microvilli of the epidermal surface of larvae in diflubenzuron-treated eggs had a more irregular shape than microvilli in normal eggs.

All cells were not equally affected, suggesting an individual response of the epidermal cells to diflubenzuron. In untreated embryos, dark spots consisting of dense, granular material occurred in the tips of the microvilli. These findings are similar to those of Delachambre (1970) in a study of epicuticle formation in adult *Tenebrio molitor*.

In diflubenzuron-treated embryos the dark spots in the tips of the microvilli were less clear. For unknown reasons the contrast in the affected tissue was less than in normal tissue. The micrographs made it clear that the ovicidal effects of diflubenzuron were caused by interference with chitin synthesis in the developing embryo. After exposure to diflubenzuron, the larva probably could not use its muscles to leave the egg because now they were attached to a cuticle which lacked the normal structure and strength.

Effects on fertilization

In *Musca domestica* we found no effect of diflubenzuron on fertilization after 7 days of feeding with 1000 ppm diflubenzuron in the food (Grosscurt, 1976).

Effects on fecundity

We shall define fecundity as the number of eggs produced per female. Findings concerning the effects of diflubenzuron on fecundity are not unanimous, but a decrease in fecundity has been reported in some insect species including *Epilachna varivestis* (Holst, 1974), *Eurydema oleraceum* (Leuschner, 1974), and *Dacus oleae* (Fytizas, 1976).

With *Epilachna varivestis*, Holst found that the reduction in fecundity after

three days' oral administration of diflubenzuron was 65% on treatment of 3-day-old adults and 37% on treatment of 11-day-old adults. In *Dacus oleae* a reduction of fecundity was found after dipping the last larval instar into a suspension of diflubenzuron in water (Fytizas, 1976). However with *Tribolium castaneum* (Carter, 1975), *Anthonomus grandis* (Moore and Taft, 1975), *Culex tarsalis* (Arias and Mulla, 1975), *Stomoxys calcitrans* and *Musca domestica* (Wright and Spates, 1976), *Musca domestica* (Grosscurt, 1976) and *Pectinophora gossypiella* (Flint and Smith, 1977) no effects of diflubenzuron on fecundity were observed. The maximum exposure to the compound was 7 days at 3.0 ppm for *Tribolium castaneum*. With *Anthonomus grandis* the weevils were dipped into a 0.1% acetone solution of diflubenzuron; with *Pectinophora gossypiella* a maximum of 200 ppm diflubenzuron was fed for 6 days, and with *Musca domestica* the compound was offered 7 days at a concentration of 1000 ppm in the food. Wright and Spates (1976) give no information about their methods with *Stomoxys calcitrans* and *Musca domestica*. With *Culex tarsalis* 4th-instar larvae were treated 48 hours with 0.4 ppm diflubenzuron.

The role of males in the ovicidal phenomenon

Moore and Taft (1975) found a reduced hatch of eggs from untreated females of *Anthonomus grandis* that had been mated with males fed with diflubenzuron.

McGregor and Kramer (1976) concluded from feeding experiments that the ovicidal effects of diflubenzuron in *Sitophilus granarius* could be attributed to an effect on the females and to a lesser extent to the males. In experiments with *Stomoxys calcitrans*, Wright and Spates (1976) found that the egg hatch was reduced by diflubenzuron after dipping males into aqueous suspensions or after topical application of an acetone solution.

In these experiments, the techniques used might have allowed direct uptake by the female insects as well as transfer from the male to the female by body contact in the cage or during copulation, as also suggested by Moore and Taft (1975). To avoid this possibility, we injected males of *Musca domestica* (Grosscurt, 1976) and males of *Leptinotarsa decemlineata* with 5 µg of diflubenzuron and allowed them to mate with untreated females. In these experiments we found no influence on fertilization and egg mortality after the males had been treated with diflubenzuron. We therefore concluded that in *Musca domestica* and in *Leptinotarsa decemlineata* diflubenzuron neither prevented the development of sperm nor caused the death of sperm after it had been produced.

FACTORS INFLUENCING THE DIRECT CONTACT ACTIVITY OF DIFLUBENZURON ON EGGS

The formulation of the compound

The formulation of diflubenzuron has two important aspects: the particle size and the presence of surfactants.

With larvae, the effect of the particle size of diflubenzuron was published first by Mulder and Gijswijt (1973). Ascher and Nemny (1974) studied the effect of particle size with eggs of *Spodoptera littoralis*. They found that the LC_{100} of dilutions of the liquid formulation was ten times lower than the LC_{100} of aqueous suspensions of the dispersible powder.

Depending on the insect species and the host plant, surfactants can play an important role to obtain good covering of the eggs. For example eggs of *Leptinotarsa decemlineata* on potato leaves can be sufficiently covered without a surfactant, but in the case of eggs of *Pieris brassicae* on cabbage leaves addition of a surfactant to the spray liquid is absolutely necessary.

The age of the eggs

The ovicidal activity of diflubenzuron by direct contact with the eggs decreases with increasing age of the eggs. With *Spodoptera littoralis*, Ascher and Nemny (1974) found 0-1 day old and 1-2 day old eggs to be equally susceptible, whereas 2-3 day old eggs were much less susceptible.

Similar results were obtained in our laboratory with eggs of *Pieris brassicae* (Table 1).

Table 1. The effect of various ages of eggs of *Pieris brassicae* on the ovicidal effect of a 0.3 ppm flowable of diflubenzuron in water containing 250 ppm Citowett as a surfactant and sprayed till run-off.

age in days	mean % nonhatching eggs	number of experiments
0-1	97	2
1-2	95	4
2-3	82	3
3-4	54	4

Average temperature 25°C, relative humidity 100%. Data have been corrected for mortality in the control treatments (Abbott, 1925). The mean percentage of nonhatching eggs denotes the average value of the experiments in which 170-300 eggs per treatment were used.

Table 1 indicates that younger eggs are more susceptible to diflubenzuron than older eggs. With the parameter free trend-test of Page (1963) this appeared to be significant at P = 0.06 (two-sided).

Miura et al. (1976) reported that younger eggs of *Culex pipiens quinquefasciatus* were more sensitive than older ones. This was only true, however, of the percentage of abnormal hatch and not of the percentage of unhatched eggs.

With *Culex pipiens fatigans*, *Anopheles gambiae*, and *Anopheles quadrimaculatus*, Busvine et al. (1976) found 12-hour-old eggs to be somewhat less susceptible than 8-hour-old eggs.

Experiments with *Epilachna varivestis* (Holst, 1974), however, gave results which were contrary to those mentioned above: eggs of between 0 and 12 hours were less susceptible than older eggs.

The relative humidity

The relative humidity greatly affects egg hatch after treatment with diflubenzuron (Table 2).

Table 2. The effect of the relative humidity on the mean percentage of nonhatching eggs after spraying eggs of *Leptinotarsa decemlineata* aged between 0 and 24 hours till run-off with suspensions of diflubenzuron made from the wettable powder.

% relative humidity	mean % nonhatching eggs					
	concentrations in ppm					
	300	100	30	10	3	0
30	70	56	59	31	40	21
50	85	59	44	33	39	32
80	96	78	66	47	53	24
100	99	97	100	97	76	21

Average temperature 25°C. The mean percentage of nonhatching eggs denotes the average value of 3 experiments in which 35-84 eggs per treatment were used.

Table 2 indicates that the mortality caused by diflubenzuron increases with increasing relative humidity. By means of the parameter free trend-test of Page (1963) this increase was found to be significant at P = 0.001 (two-sided).

FACTORS INFLUENCING THE OVICIDAL ACTIVITY OF DIFLUBENZURON APPLIED TO ADULT FEMALE INSECTS

Oral uptake of the compound

The formulation

Just as the formulation, including particle size, has an important bearing on the ovicidal activity of diflubenzuron by contact activity, so it is likely to be of importance, too, after oral uptake by adults. However, no information is available yet about this topic.

The concentration

In *Eurydema oleraceum*, the ovicidal activity increased from one egg batch to the next when the food was sprayed with 250 ppm. In the fifth egg batch eclosion was fully blocked. With the 1000 ppm treatment, egg eclosion was blocked almost completely in the second egg batch (Leuschner, 1974).

When diflubenzuron treatment is discontinued, egg eclosion in newly laid egg batches can increase again. In *Musca domestica* the rate of this increase depends on the concentration of diflubenzuron in the previous treatment (Grosscurt, 1976).

The age of the adult

The effects on egg hatch after feeding of diflubenzuron to adults aged 3 or 11 days was studied with *Epilachna varivestis* (Holst, 1974). The adults were fed on a diet containing diflubenzuron for 3 days. After this treatment the ovicidal effects of diflubenzuron decreased much more quickly in the older adults than they did in the younger ones.

Treatment of the adults by contact with the compound

The formulation

When adults of *Musca domestica* were treated topically with an acetone solution of diflubenzuron, recrystallization of the compound on the integument was observed after evaporation of the acetone.

After application of 1 µg diflubenzuron in acetone to adult females the effect on egg hatch lasted for four days after treatment (Grosscurt, 1976). Though egg hatch returned to normal after this period, diflubenzuron was still

present as crystals on the outside of the integument. Apparently diflubenzuron can penetrate the integument of *Musca domestica* as an acetone solution, but the diflubenzuron crystals formed after evaporation of the acetone are incapable of penetrating. In that case the rate of evaporation of acetone, is of great importance to the effect of the solution of diflubenzuron.

After topical application of diflubenzuron, ovicidal effects were obtained with *Musca domestica* (Grosscurt, 1976), *Hylemya brassicae* (Van de Veire and Delcour, 1976) and *Haematobia irritans* (Wright and Harris, 1976).

Adults can also be treated with diflubenzuron by contact with a treated surface. Uptake of the compound in this case might be by penetration through the cuticle and, in some cases orally.

The ovicidal activity during the first four days of egg laying was assessed after putting houseflies in cages treated with an aqueous suspension of diflubenzuron (formulated as a w.p.). After one month, more flies were introduced and the ovicidal activity was assessed in the same way.

At a dose of 1.0 g a.i./m^2 the initial activity and the activity after one month were both 100%. At a dose of 0.1 g a.i./m^2 the initial activity was 88% and after one month it was 57%. This experiment was performed in duplicate for each concentration, always with a cage having an inner surface of 0.5 m^2 and containing 400 flies of both sexes. The temperature was 28°C and the relative humidity 50%.

Wright and Harris (1976) released *Stomoxys calcitrans* and *Haematobia irritans* into holding cages attached to the winter coat of a Hereford steer which had been sprayed with a 1% aqueous suspension of diflubenzuron (formulated as a w.p.). After 4 days of continuous exposure, starting just after spraying, egg hatch of *S. calcitrans* was inhibited only 83%. With *H. irritans* however, complete prevention of egg hatch was obtained for 5 weeks, and some effects on hatchability (data not shown) were apparent for another 6 weeks.

Flint and Smith (1977) exposed adults of *Pectinophora gossypiella* to a surface treated with diflubenzuron (0.02 g/cm^2). Egg hatch in several batches had decreased to 0% after 6 days exposure.

The residue of a suspension has a particle size equal to that of the original formulation, but the residue of a solution always has an unknown particle size on recrystallization of the compound after evaporation of the solvent. This factor might account for the difference in residual activity of an acetone solution applied to the adults of *Musca domestica* and the activity by contact with a spray suspension made from the w.p.

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III DIFLUBENZURON: SOME ASPECTS OF ITS OVICIDAL AND LARVICIDAL MODE OF ACTION AND AN EVALUATION OF ITS PRACTICAL POSSIBILITIES.

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Diflubenzuron, 1-(4-chlorophenyl)-3-(2,6-difluorobenzoyl)urea, possesses larvicidal and ovicidal activities. On larvae it acts mainly as a stomach poison, yet it sometimes exhibits important contact activity. Though all instars can be controlled, older instars are generally less susceptible than younger ones. Histological inspections of *Leptinotarsa decemlineata* larvae revealed that after ending exposure to the compound, distortions in newly deposited cuticular layers decreased gradually. Ovicidal effects resulted from direct contact of diflubenzuron with eggs or from contamination of females by contact or feeding. Electron microscopic observations of embryos of *Leptinotarsa decemlineata*, contaminated via the female, also showed disturbed cuticule formation, suggesting a similar activity of the compound in larvae and in eggs.

Spraying the eggs of *Leucoptera scitella* shows the compound to be mainly ovidical at a rate of 100 mg litre^{-1}, whilst with lower concentrations (10 and 1 mg litre^{-1}) the young larval instar will be killed. The levels of cross-resistance to diflubenzuron as a larvicide are low and the compound can be used effectively in the field against populations that are highly resistant to conventional insecticides. Laboratory and field results, based on larvicidal and ovicidal activities of diflubenzuron, are discussed in respect of species belonging to the Diptera, Lepidoptera, Coleoptera, Acarina (*Phyllocoptruta oleivora*) and Hemiptera (*Eurydema oleraceum* and *Psylla piri*).

1. Introduction

Diflubenzuron is the common name for 1-(4-chlorophenyl)-3-(2,6-difluorobenzoyl)urea, the active ingredient of the insecticide Dimilin. In addition to the larvicidal activity by oral uptake as described by Mulder and Gijswijt,[1] diflubenzuron in some cases exhibits contact activity against larvae. Furthermore, diflubenzuron is ovicidal on contact with eggs or on contamination of females of several insect species. Previous studies of this mode of ovicidal action gave evidence that this effect was also caused by an interference with chitin synthesis, similar to the described larvicidal activity.[1,2] This ovicidal activity means an interesting extension of its insecticidal spectrum.

This paper does not deal with environmental aspects. A review of the fate of diflubenzuron in soils, plants and animals is given by Verloop and Ferrell.[3]

2. Effects on larval development

Abnormal larval development was the first observed insecticidal effect of this benzoylurea. In most species the effects of diflubenzuron become visible at the next moult, when the larvae are incapable of casting the exuviae. In some species, e.g. *Mamestra brassicae* (cabbage moth), the larvae are able to ecdyse but remain pale and immobile, and eventually die. When larvae of *Leptinotarsa decemlineata* (Colorado potato beetle) are treated with diflubenzuron immediately after moulting

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they show effects long before their next moult is due. The normal wrinkles in the larval skin disappear and they become balloon-shaped. Normal locomotion is seriously interfered with.

Histological examinations of diflubenzuron-contaminated larvae of *Pieris brassicae* (large white butterfly) and *Leptinotarsa decemlineata* revealed blocking of the formation of the endocuticular tissue, long before effects were visible externally. Endocuticular thickness remained constant with fourth instar larvae of *Pieris brassicae* after treatment with diflubenzuron, whereas in untreated larvae the thickness increased until shortly before moulting.[1] With *Leptinotarsa decemlineata* the blocking of endocuticular growth probably results in an integument which is too weak to maintain the normal appearance with increasing internal pressure during larval growth.

Biochemical studies with diflubenzuron and the related 1-(2,6-dichlorobenzoyl)-3-(3,4-dichlorophenyl)urea (Du 19.111) gave evidence that the blocking of endocuticular growth was caused by interference with chitin synthesis.[2,4,5]

Mulder and Gijswijt[1] found diflubenzuron to have no contact activity on third instar larvae of *Pieris brassicae* when applied topically in a concentration of 100 μg per larva or sprayed as a suspension (1000 mg litre^{-1}). This lack of contact activity was also found in other species. Holst[6] reported that topical application of even 2 μl of a solution (1000 mg litre^{-1} acetone) to larvae of *Epilachna varivestis* (Mexican bean beetle) had only minor effects. Contact activity was absent[7] in third, fourth, fifth and sixth instar larvae of *Choristoneura fumiferana* (spruce budworm) after topical application (20 μl) of the highest concentration (50 g litre^{-1}). In testing the contact activity on *Heliothis virescens* (tobacco budworm), Plapp[8] coated the inner surfaces of rearing vials with a solution of diflubenzuron in acetone (which was allowed to evaporate). Using this method the concentration (LC_{50}) killing 50% of the insects was 2844 μg diflubenzuron per vial.

In some species the eggs, larvae, and adults are susceptible to contact activity of diflubenzuron: Cerf and Georghiou[9] applied diflubenzuron, dissolved in tetrahydrofuran, topically to white prepupae of *Musca domestica* (house fly). They found doses killing 50% (ED_{50}) and 95% (ED_{95}) of the insects to be 0.265 and 70 μg per prepupa respectively. Topical application of a solution of diflubenzuron in methanol to white pharate pupae of *M. domestica* and *Stomoxys calcitrans* (stable fly) by Wright,[10] however, was totally ineffective in inhibiting adult eclosion at 10 μg per pupa.

Ascher and Nemny[11] applied diflubenzuron in acetone topically on larvae of *Spodoptera littoralis* (Egyptian cotton leafworm) finding ED_{50} values of 0.04 and 0.066 μg per larva for larvae of 100 and 200 mg respectively. The corresponding ED_{50} in feeding larvae was 0.5–0.6 μg per larva,[12] suggesting that the compound is more toxic to *S. littoralis* by topical application than by ingestion. When dipping last instar larvae of *Dacus oleae* (olive fruit fly) in a solution of 500 mg diflubenzuron litre^{-1}, Fytizas[13] found 61.4% mortality of the pupae.

In general all larval instars can be controlled by diflubenzuron. In most insect species older instars are less susceptible than younger ones. This was found by comparing second and fourth stage larvae of *Lambdina fiscellaria*[14] (hemlock looper), intermediate and fourth stage larvae of *Hypera postica*[15] (alfalfa weevil), third and fourth instar larvae of *Culex nigripalpus*, and third and fourth instar larvae of *Aedes taeniorhynchus*[16] (black salt-marsh mosquito). With *Yponomeuta* spp., L_1 to L_3 were the most susceptible instars and L_4 was considerably more susceptible than L_5.[17] Comparison of the LC_{50} values of several instars of *Culex pipiens fatigans* and *Anopheles gambiae* did not reveal any great differences.[18] Retnakaran and Smith,[7] on the other hand, found that the fifth and sixth instars of *Choristoneura fumiferana* were more sensitive than the third or the fourth instar to diflubenzuron in the diet, the fifth instar being the most sensitive.

As discussed earlier, treated larvae can usually feed normally until the next moult, so that some crop damage can still occur depending on the life habit of the species and the moment of application. From this point of view applications on the young larval instars are preferable. An additional advantage is that the younger larval instars are generally more sensitive than the older ones. In asynchronous populations, diflubenzuron has advantages over growth regulators such as juvenile hormone (JH) mimics, which do not affect younger larvae. This can play a role in mosquito control for example.[19] This difference in the mode of action between diflubenzuron and JH mimics also plays a role in univoltine species, e.g. *Lambdina fiscellaria*, where diflubenzuron, in contrast to JH mimics, can prevent the current year's defoliation by the pest.[14]

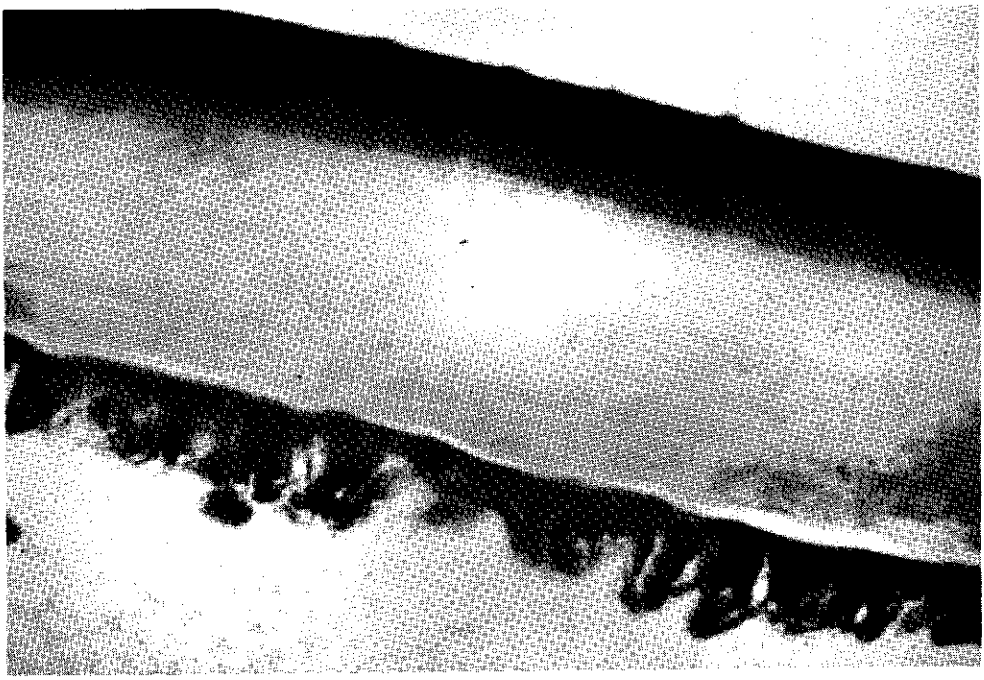

Figure 1. Transverse section of the cuticle of a 3-day-old fourth instar larva of *Leptinotarsa decemlineata* (Azan staining after fixation in Carnoy).

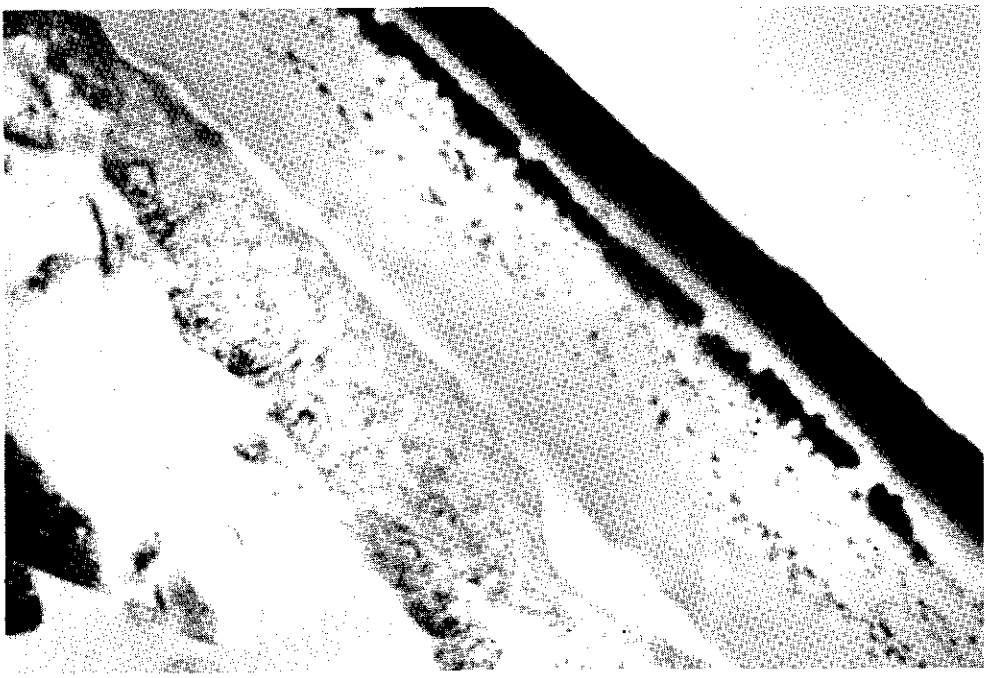

Figure 2. Transverse section of the cuticle of a 3-day-old fourth instar larva of *Leptinotarsa decemlineata* (Azan staining after fixation in Carnoy) after injection of 1 μg diflubenzuron within 17 h after moulting (for explanation see section 2).

As death normally occurs during or shortly after a moult it is generally advisable to feed larvae as continuously as possible until at least the next moult, to avoid recovery from intoxication. Ascher and Nemny[12] obtained evidence with *Spodoptera littoralis* that a longer period of feeding diflubenzuron yielded a greater mortality at the next moult. The same effect was found by Lacey and Mulla[20] with *Simulium vittatum* (a blackfly). To study the phenomena after a restricted period of exposure by histological methods, we imitated this effect by injection of 17-hour-old fourth instar *Leptinotarsa decemlineata* larvae with diflubenzuron (1 μg). The larvae were stained after 75 h and the sections were coloured with 'Azan' tissue stain. The same procedure was followed with untreated larvae of the same age. Figure 1 represents an untreated larva with normal endocuticular layers. In the endocuticular layers of the treated larva (Figure 2), distortions are visible as globules of apparently coagulated material, similar to those described for *Pieris brassicae*.[1] Under one layer of very severe distortions, normal layers of endocuticular tissue alternate with partly disturbed layers, suggesting some periodicity in the interference of diflubenzuron with chitin synthesis. The globular distortions in the inner layers no longer form a continuous chain and the size and number of the globules decrease gradually. Though the epidermal cells can regain their function in chitin synthesis the quickness of this process is not uniform, indicating a degree of variability on the response of the epidermal cells to the chemical.

The resumption of chitin synthesis after termination of a diflubenzuron treatment can explain the lower level of final mortality after short exposures to the compound. As a consequence of such a phenomenon one would expect higher mortalities when this short exposure takes place later during the development of an instar.

3. Ovicidal effects

Ovicidal effects of diflubenzuron can be obtained either by topical application to the eggs or by contamination of gravid female insects. In either case the phenomena are similar and result in non-emergence. The larva in the egg develops fully but is unable to leave the egg, though it sometimes ruptures the egg wall.[21] At marginal dosages the egg hatches sometimes and mortality can occur in the first larval instar.

Electron microscopic inspection of contaminated embryos of *Leptinotarsa decemlineata*, where the gravid females had ingested potato leaves sprayed at 1000 mg a.i. litre^{-1} showed interference with cuticle formation in the embryo, resulting in an amorphous cuticular region instead of the normal lamellate cuticle deposition patterns.[22] These phenomena are comparable to those described in larvae by Mulder and Gijswijt.[1] In the normal embryo (Figure 3) patterns of lamellate cuticle deposition are visible. In the contaminated embryo (Figure 4) these have been replaced by an amorphous cuticular region with globular structures. Microvilli in the contaminated embryo have a more irregular shape and the plasma membrane plaques (the dark spots in their tips) are less clear than in the microvilli of the normal embryo.

The direct contact activity of diflubenzuron on eggs is species dependent and influenced by many factors. In general, younger eggs are more susceptible than older ones. Also the effect of diflubenzuron increases with smaller particle size and a higher relative humidity.[22] *Leucoptera scitella*, a leaf miner on apple, deposits its eggs on the underside of the leaves. Table 1 shows that after spraying of the eggs the concentration of diflubenzuron determines whether the effect is ovicidal or whether the young larvae are killed. The time of larval mortality is correlated with the size of the mines.

The effects of diflubenzuron on eggs through the female can be influenced by the age of the adult, the concentration and the formulation. These factors are summarised by Grosscurt.[22] The spectra of activity by direct contact activity and by contamination of females are not always identical. No contact activity was found with eggs of *Musca domestica*, not even after dipping for 90 min in a flowable suspension in water (1000 mg litre^{-1}). Egg eclosion was fully prevented after the females had ingested food containing 100 mg a.i. kg^{-1}.

4. Cross-resistance and resistance development

Cross-resistance levels to diflubenzuron are based on the ratio of the LC_{50} for the strain resistant to another insecticide and the LC_{50} for the susceptible strain. Cross-resistance to diflubenzuron was first

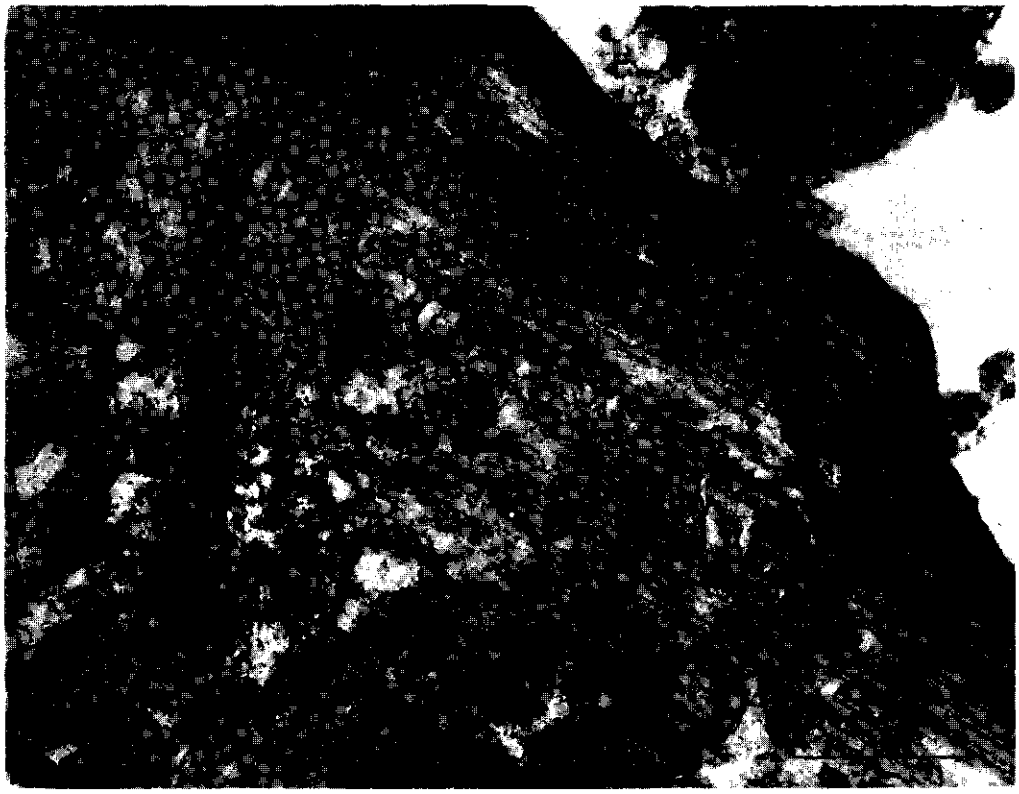

Figure 3. Transverse section of an embryo of *Leptinotarsa decemlineata* just prior to egg hatch. Meaning of indications: cl, chitin layer; mv, microvilli; →, arrow indicating the plasma membrane plaques in the microvilli.

found by Cerf and Georghiou[9] with organophosphorus-, carbamate- and organochlorine-resistant strains of *Musca domestica* on topical application to white prepupae of a diflubenzuron solution in tetrahydrofuran. The levels of cross-resistance in these experiments, which ranged from ten-fold to considerably higher, give an exaggerated view; the pupal stage of the susceptible strain is relatively insusceptible to topical application (only 87% mortality at 10 μg diflubenzuron per prepupa), resulting in a very flat dosage–mortality curve and furthermore topical application of a solution of diflubenzuron gives no certainty of the quantity that penetrates, especially at the concentrations used.

Rongsriyam and Busvine[23] assessed cross-resistance levels in larvicide tests of DDT-resistant mosquito strains, obtained from several sources and colonised in the laboratory. The following levels were found: for *Culex pipiens fatigans*, 3.2; for *Aedes aegypti*, 2.1; for *Anopheles gambiae*, 2.9; for *Anopheles quadrimaculatus*, 2.5. Georghiou *et al.*[24] found a cross-resistance level of 1.5 in larvae of an organophosphorus-multiresistant field strain of *Culex pipiens quinquefasciatus*. Dame *et al.*[25] in laboratory experiments found a cross-resistance level of 0.7 in larvae of a DDT-resistant strain of *Anopheles quadrimaculatus* and a level of 1.0 in malathion-resistant larvae of *Aedes taeniorhynchus*. Carter[26] found a remarkable reverse susceptibility in a strain of *Tribolium castaneum* (red flour beetle) showing malthion-specific resistance; the LC_{50} of the malathion-resistant strain was 0.15 mg litre^{-1} and the LC_{50} of the susceptible strain was 0.36 mg litre^{-1}, resulting in a cross-resistance level of 0.4. Wardlow *et al.*[27] found, in contrast to these observations, an activity of diflubenzuron on larvae, pupae and adults of *Trialeurodes vaporariorum* (greenhouse whitefly) after leaf treatment.

The mortalities of larvae, pupae and adults in a susceptible and in a DDT/malathion-resistant strain at the test concentration of 250 mg a.i. litre^{-1} differed only slightly. Schaefer *et al.*[28] found an effective dosage of 28 g a.i. ha^{-1} using a w.p. formulation of diflubenzuron in aerial trials against mixed populations of organophosphorus-resistant strains of *Aedes nigromaculis* and *Aedes melari-*

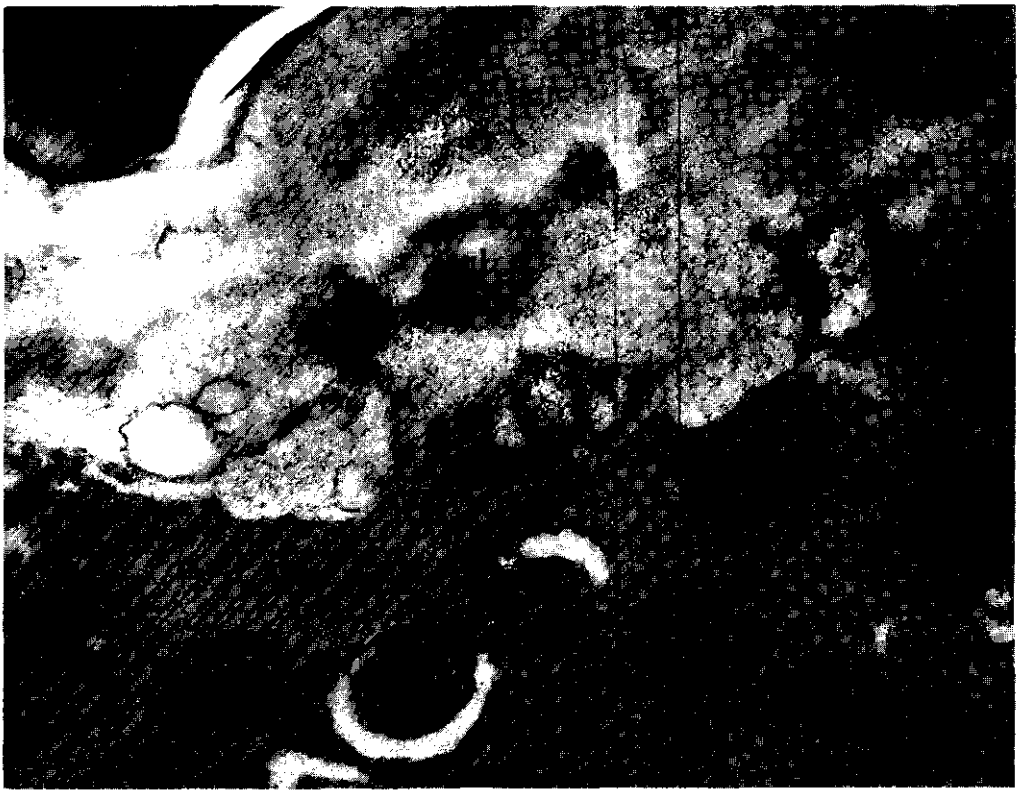

Figure 4. Transverse section of an embryo of *Leptinotarsa decemlineata* just prior to egg hatch. The egg had been contaminated by feeding the female diflubenzuron treated food. Meaning of indications: cl, chitin layers; mv, microvilli; gl, globule; acr, amorphous cuticular region; ⇢, arrow indicating the plasma membrane plaques in the microvilli.

Table 1. Effect of diflubenzuron concentration on *Leucoptera scitella* eggs (0 to 24 h old) on apple foliage sprayed with a wettable powder formulation

Diflubenzuron concentration (mg a.i. litre $^{-1}$)	Eggs failing to hatch[a] (%)	Mine diameter[a] (mm)
0	14	2.0–5.5
1	22	⩽ 1.5[b]
10	47	⩽ 1[b]
100	91	⩽ 1[b]

[a] The leaves were maintained with an 18 h day (24°C, r.h. 60 to 70%) and 6 h night (19°C, r.h. 80 to 90%). The result was assessed 14 days after spraying and the figures represent the mean of two experiments, using from 40 to 170 eggs for each concentration.

[b] All larvae dead.

mon. Diflubenzuron also provided a means of controlling an organophosphorus-resistant strain of *Culex tarsalis*, a vector of encephalitis, in California.

Up to now I have discussed the cross-resistance to diflubenzuron. However, resistance can also be built up by selecting with an insecticide. In selection experiments with diflubenzuron, Brown[29] found a five- to eight-fold resistance after selecting larvae of *Culex pipiens* for ten generations. He selected a resistance to an unspecified degree in a normal strain of *Tribolium confusum*. Nothing is known about the importance of selection towards increasing resistance in the field. The mechanisms of resistance still cannot be defined. Plapp[30] has proposed that increased oxidative degradation of the chemical is the resistance mechanism.

5. Synergism and antagonism

Synergism between diflubenzuron and chlordimeform in a resistant population of *Heliothis virescens* (tobacco budworm) was studied by Plapp.[8] He coated the inner surface of test vials by adding solutions of the compounds in acetone (which was allowed to evaporate) and placed larvae in each vial. With this method, inappropriate for a compound like diflubenzuron, he found a 17-fold synergism by chlordimeform. The synergism level was based on the ratio of the LC_{50} for diflubenzuron and the LC_{50} for the 1:1 combination by weight of diflubenzuron + chlordimeform. In seed treatment of Lima beans with solutions containing combinations in acetone of diflubenzuron and piperonyl butoxide (0.5%), Vea[31] found a synergistic ratio of 1.1 after the addition of the latter. Combinations of *Bacillus thuringiensis* and diflubenzuron, offered in an artificial diet to *Choristoneura fumiferana* (spruce budworm), were studied by Boucias.[32] An antagonistic response was found when *Bacillus thuringiensis*, in a concentration of 212 or 53 IU ml^{-1}, was added to a diet containing 50 mg diflubenzuron kg^{-1}. This effect is probably due to gut paralysis, preventing the larvae from ingesting an effective dosage of diflubenzuron.

6. Practical results obtained with diflubenzuron

6.1. Diptera

6.1.1. Anthomyiidae (Anthonomid flies)

In laboratory experiments with *Hylemya platura* (seed corn maggot), seeds were immersed in a solution of diflubenzuron in acetone for 1 h, the solution decanted, the seeds well aired and then covered with sand, after which 3-day-old larvae were placed on top of the sand. With this method Vea *et al.*[31] found an LC_{50} of 16 mg a.i. $litre^{-1}$ solution. In field experiments seeds were slurry treated. Even dosages of 2.2 g a.i. kg^{-1} seed gave insufficient protection against *Hylemya platura.*

Seed treatment with the w.p. formulation against larvae of *Hylemya cilicrura* in the laboratory by Van de Veire *et al.*[33] was effective at rates of 0.25 and 0.5 g a.i. kg^{-1} seed with low infestations but the rate had to be raised to 2 g a.i. kg^{-1} of seed with higher infestations. The practical possibilities for this purpose seem to be very limited. Van de Veire and Delcour[34] found ovicidal effects by topical application of diflubenzuron to female *Hylemya brassicae* (cabbage root fly).

6.1.2. Chironomidae (midges)

Excellent inhibition of emergence of *Chironomus* spp., *Tanytarsus* spp. and *Procladius* spp. at 110 g a.i. ha^{-1} surface area of lakes in California was found by Mulla *et al.*[35] However, once *Labrundinia maculata* had become established in the lakes, two treatments with 280 g a.i. ha^{-1} surface area with the w.p. formulation did not effectively control this midge.

Using a granular formulation at a rate of 110 g a.i. ha^{-1} surface area the residual activity against *Chironomus* spp., *Tanytarsus* spp. and *Procladius* spp. lasted 4 to 5 and 3 to 4 weeks at water depths of < 1 m and 1 to 2 m respectively (Vea *et al.*[31]).

6.1.3. Culicidae (mosquitoes)

Laboratory experiments on culicidae revealed the outstanding activity of diflubenzuron against all larval stages of many species. No generalisation concerning the susceptibility of genera can be made, while the susceptibilities of species belonging to one genus vary widely and overlap those of species from other genera. A summary of the results of several authors is given in Table 2.

Schaefer *et al.*[28] found feasible results in trials with aerial spraying against *Culex tarsalis*, *Aedes melanimon* and *Aedes nigromaculis* using the w.p. formulation at 34 to 56 g a.i. ha^{-1}. Field trials on *C. tarsalis* with solutions of diflubenzuron in acetone[19] on *Aedes taeniorhynchus* with a granular formulation containing the w.p.[16] and aerial application of the w.p. formulation[25] on *Culex nigripalpus*, *C. salinarius* and *A. taeniorhynchus* gave good control at concentrations ranging from 22 to 28 g a.i. ha^{-1}. Steelman *et al.*[38] found effective control of *Psorophora columbiae* in rice fields at 28 g a.i. ha^{-1}.

Table 2. Summary of LC_{90} values of diflubenzuron in laboratory experiments against larvae of various mosquito species. Results expressed as inhibition of adult emergence

Species	LC_{90} (μg litre^{-1})		Formulation	Reference
	Third instar[a]	Fourth instar[a]		
Aedes *spp.*				
A. aegypti	0.706	—	5% e.c.	37
A. melanimon	—	4–10	25% w.p.	28
A. nigromaculis	—	1–4	25% w.p.	28
A. sollicitans	0.036	—	5% e.c.	37
A. taeniorhynchus	—	1–4	25% w.p.	28
	0.69	2.05	25% w.p.	16
	0.045	—	5% e.c.	37
A. triseriatus	0.718	—	5% e.c.	37
Anopheles *spp.*				
An. albimanus	—	1	Acetone solution	19
An. quadrimaculatus	0.086	—	5% e.c.	37
	—	4	Acetone solution	25
Culex *spp.*				
C. nitripalpus	0.16	0.80	25% w.p.	16
C. pipiens quinquifasciatus	—	1.3	Acetone solution	19
	0.064	—	5% e.c.	37
C. salinarius	0.121	—	5% e.c.	37
C. tarsalis	1.049	—	5% e.c.	37
Culiseta inornata	1.639	—	5% e.c.	37
Psorophora *spp.*				
P. confinnis	3.833	—	5% e.c.	37
P. ferox	0.072	—	5% e.c.	37
P. varipes	0.069	—	5% e.c.	37

[a] Instar at beginning of experiments.

6.1.4. Muscidae

Mulder and Swennen[39] found complete control of larvae of *Musca domestica* for at least 3 months in chicken manure treated with 5 ml of a suspension of diflubenzuron (1 mg litre^{-1}) per 500 ml manure. Jakob[40] found complete control of larvae of a dimethoate-resistant strain of *M. domestica* by mixing a solution of diflubenzuron in acetone through the breeding medium to a concentration of 5 mg kg^{-1}. On topical application of the compound to the larval breeding medium, in laboratory experiments[10] the w.p. formulation (10 mg a.i. m^{-2}) gave 99% control of *M. domestica* and 100% control of *Stomoxys calcitrans* (stable fly). Diflubenzuron (0.5 g m^{-2}) gave 90% control of *M. domestica*[10] in field experiments in a cattle feedlot and at a waste water treatment plant. Campbell and Wright[41] proved the efficacy of diflubenzuron for controlling *S. calcitrans* in feedlot breeding areas.

Another method to control fly larvae in the faeces is by oral intake of diflubenzuron mixed into poultry or cattle feed or into mineral blocks. In this application of diflubenzuron as a food additive, *S. calcitrans* seems to be more susceptible than *M. domestica.* Miller *et al.*[42] fed diflubenzuron to chickens finding complete control of *M. domestica* at rates between 6.2 and 12.5 mg kg^{-1} food and Hayakawa[43] found complete control of *S. calcitrans* even with the lowest test concentration of 1 mg kg^{-1} diet using diflubenzuron as a food additive for laying hens.

In laboratory experiments an ovicidal effect through the female insect has been reported for *M. domestica*,[21,22,44] *Haemotobia irritans* (horn fly)[44,45] and *S. calcitrans.*[45] This ovidical effect can be obtained by topical treatment or by feeding of adult females, or after contact of adults with a diflubenzuron-treated surface.

Field tests with *H. irritans* were conducted by Kunz *et al.*[46] Treatment of range cattle with a spray (10 g a.i. litre^{-1}) resulted in an elimination of adult emergence for 4 weeks.

6.1.5. Mycetophilidae (fungus gnats) and Phoridae (humpbacked flies)

These two families contain species of which the larval instars live in mushroom compost, mycelium and the sporophores. In Great Britain diflubenzuron is registered under the Pesticides Safety Precautions Scheme for application in case mixing and for postcasing drench at a rate of 30 mg a.i. litre^{-1}. In this use species belonging to the Mycetophilidae (*Sciara* spp.) seem to be more susceptible than species belonging to the Phoridae.

6.1.6. Simuliidae (black flies)

Lacey and Mulla,[20] using field-collected *Simulium vittatum*, found almost complete inhibition of egg hatch after 1 h exposure of eggs (aged 24–48 h) to a diflubenzuron w.p. suspension (1 mg a.i. litre^{-1}). Older eggs were less sensitive. The LC_{95} values for larvae exposed to the w.p. formulation for 15 min, 60 min and 24 h were 0.7, 0.2 and 0.03 mg a.i. litre^{-1}, respectively.

6.2. Lepidoptera

6.2.1. Arctiidae (tiger moths)

Szanto and Varjas[47] found promising results on third and fourth larval instars of *Hyphantria cunea* (fall webworm) in field experiments with concentrations of 10 and 100 mg a.i. litre^{-1}.

6.2.2. Gelechiidae (gelechiid moths)

When administered in the larval diet,[48] diflubenzuron w.p. (1.0 mg a.i. kg^{-1}) reduced the emergence of *Pectinophora gossypiella* (pink bollworm) by 64%. No control was obtained in a field evaluation on cotton with 2.2 kg a.i. ha^{-1}. This failure was probably due to the hidden feeding of larvae in the cotton bolls.[49] Ovicidal effects by contamination of adult females were also observed by Flint and Smith.[48]

6.2.3. Geometridae (geometrid moths)

In laboratory experiments with *Lambdina* f. *lugubrosa* (western hemlock looper) by Sahota and Shepherd,[14] the lowest concentration (5 mg a.i. litre^{-1}) of the w.p. formulation gave 100% control of second instars and 95% control of fourth instar larvae. Field experiments by Pree[50] using the liquid formulation indicated that 165 mg a.i. litre^{-1} effectively controlled larvae of *Operophtera brumata* (winter moth).

6.2.4. Gracillariidae (leaf blotch miners)

As discussed in section 3, diflubenzuron had good contact activity on eggs of *Leucoptera scitella*. Other leaf miners which can be controlled in practice are *Lithocolletis blancardella* and *Stigmella malella* (apple pygmy moth) with spray concentrations from 125 to 200 mg a.i. litre^{-1}.

6.2.5. Lymantriidae (tussock moths)

Retnakaran *et al.*[51] obtained spectacular results against *Malacosoma disstria* (forest tent caterpillar) in Canada by spraying trees with 10 g a.i. litre^{-1} suspension using a mist blower (rate per ha was not mentioned). Grannett and Dunbar[52] found an LC_{50} of 13 μg a.i. kg^{-1} diet for third instar larvae of *Lymantria dispar*, syn. *Porthetria dispar* (gypsy moth) by mixing a solution of diflubenzuron in acetone with the diet and allowing the solvent to evaporate. Good results were obtained in the field with a spray concentration of 47 mg a.i. litre^{-1}, a concentration considerably lower than with conventional insecticides.

Leaf treatment with the w.p. formulation (10 μg a.i. litre^{-1}) resulted in complete mortality[53] of *Lymantria dispar* and *L. monacha* (nun moth). Aerial application with the w.p. formulation on *L. dispar* (75 g a.i. ha^{-1}) and *L. monacha* (7.5 g a.i. ha^{-1}) also yielded complete control.[54]

Neisses *et al.*[55] found significant reductions in larval populations of *Orgyia pseudotsugata* (Douglas-fir tussock moth) at 280 g a.i. ha^{-1}. In the Netherlands diflubenzuron was granted a registration against *Euproctis chrysorrhoea* (brown-tail moth) at a spray-rate of 30 mg a.i. litre^{-1}. Elings and Dieperink[67] even obtained good results with a spray of 7.5 mg a.i. litre^{-1}

6.2.6. Lyonetiidae

Excellent control was found at a rate of 220 g a.i. ha^{-1} by Flint *et al.*[49] in field experiments on *Bucculatrix thurberiella* (cotton leaf perforator).

6.2.7. Noctuidae (owlet moths)

Wolfenbarger *et al.*[56] incorporated diflubenzuron into an artificial diet in laboratory experiments with *Heliothis virescens* (tobacco budworm) finding an LC_{50} for neonatal larvae of 13 mg a.i. kg^{-1} diet.

In field-cage tests on cotton plants treated with diflubenzuron (280 g a.i. ha^{-1}) in 10% cottonseed oil in water, the numbers of medium and large larvae were reduced by 90% but damage to the squares in the treated plots was not significantly reduced. Taft and Hopkins[57] found that a bait formulation (280 g a.i. ha^{-1}) did not effectively control *Heliothis* spp.

Janes[58] found inadequate control of a mixed population of *Heliothis zea* (corn earworm) and the more abundant species *Spodoptera frugiperda* (fall armyworm) in sweet corn in Florida with 220 g a.i. ha^{-1}.

Ovicidal effects by contact activity of diflubenzuron to *Spodoptera littoralis* (Egyptian cotton leafworm) were found by Ascher and Nemny.[59] The LC_{100} for the w.p. formulation was as low as 2.5 mg a.i. $litre^{-1}$. The same authors also found a surprisingly high contact activity on larvae of *S. littoralis* of a solution of diflubenzuron in acetone. Comparison of the LD_{50} values revealed this contact activity to be even higher than the activity by oral administration.[11,12]

Anticarsia gemmatalis (velvetbean caterpillar) on soybeans in Brazil was controlled very well by foliar application of the w.p. formulation (75 g a.i. ha^{-1}).[60] *Ceramica picta* (zebra caterpillar) has been controlled effectively at 560 g a.i. ha^{-1}.[61] Henzell *et al.*[62] found complete control of fourth instar larvae of *Pseudaletia separata* (army caterpillar) by foliar application of the w.p. formulation (10 mg a.i. $litre^{-1}$). Flint *et al.*[49] controlled *Trichoplusia ni* (cabbage looper) with the w.p. formulation (2.2 kg a.i. ha^{-1}).

6.2.8. Olethreutidae (olethreutid moths)

Diflubenzuron gives very good results against *Laspeyresia pomonella* (codling moth). Audemard *et al.*[63,64] working in French apple orchards, where integrated chemical-biological control was in use, obtained good control with rates of 40 g a.i. ha^{-1}. Under similar conditions Gruys[65,66] obtained very good control of *L. pomonella* with one or two sprays at 200 mg a.i. $litre^{-1}$.

6.2.9. Pieridae (whites)

In small-scale field experiments by Mulder and Swennen[39] a spray (1 mg a.i. $litre^{-1}$) gave complete control of larvae of *Pieris brassicae*. In field experiments[67] complete control was obtained after 6 days with 180 g a.i. ha^{-1} and after 14 days with 18 g a.i. ha^{-1}.

6.2.10. Pyralidae (pyralid moths)

In experiments of Reinert,[68] larvae of the tropical sod worm, *Herpetogramma phacopteralis*, on Bermuda grass could be controlled for 2 weeks with the w.p. formulation (340 g a.i. ha^{-1}).

6.2.11. Thaumetopoeidae (processionary caterpillars)

In France, diflubenzuron has been registered against *Thaumetopoea pityocampa* at a concentration of 150 g a.i. ha^{-1}. A great deal of field work on *T. pityocampa* was done by Ribrioux and Dolbeau.[69]

6.2.12. Tortricidae (leafrollers)

In greenhouse experiments,[7] sixth instar larvae of *Choristoneura fumiferana* (spruce budworm) on balsam fir trees were completely controlled by liquid formulation equivalent to 190 g a.i. ha^{-1}. Field tests with diflubenzuron have not shown encouraging results until now; one of the main problems appears to be the delivery of material to bud areas, to which feeding is confined (Internal report Thompson-Hayward Chemical Company, USA, 1976). Many leafrollers are of importance in orchards. Of this group *Adoxophyes orana* (summer fruit tortrix), *Archips rosana* (rose tortrix moth) and *Pandemis heparana* show a low susceptibility to diflubenzuron in the field, whilst *Spilonota*

6.4. Other orders of arthropods

Though the main orders on which diflubenzuron is active have been mentioned, some other orders deserve a mention from the practical point of view.

In the Orthoptera, activity was found against the migratory locust, *Schistocerca gregaria*. In laboratory experiments spraying of the food with a solution of diflubenzuron in acetone and evaluation after 14 days showed an LC_{95} of 100 mg a.i. $litre^{-1}$. No practical control of Orthoptera is known.

In the Hemiptera and the suborder of the Heteroptera an interesting activity in the laboratory was found by Leuschner[75] against the cabbage bug, *Eurydema oleraceum* (Pentatomidae, stink bugs). When sprayed as an acetone solution on the leaves, diflubenzuron resulted in complete control of second, third and fifth instar nymphs at rates of 100 to 400 mg a.i. $litre^{-1}$, depending on the instar. Ovicidal effects were observed after offering of contaminated food to adults.

In the suborder of the Homoptera no activity was found against the bean aphid, *Aphis fabae* (Aphididae, aphids) by Mulder and Gijswijt.[1] The pear sucker, *Psylla piri* (Psyllidae, psyllids), however, can be controlled effectively in practice with concentrations of 300 mg a.i. $litre^{-1}$ when sprayed at egg hatch.

In the Hymenoptera, activity was found against the European pine sawfly, *Neodiprion sertifer* (Diprionidae, conifer sawflies). Aerial applications of the w.p. formulation (75 g a.i. ha^{-1}) gave complete control of *N. sertifer*.[49]

In the Acarina no effects of diflubenzuron were found on the carmine spider mite, *Tetranychus cinnabarinus* (Tetranychidae, spider mites) by Mulder and Gijswijt[1] or on the mold mite, *Tyrophagus putrescentiae* (Acaridae, acarid mites) by Lipa and Chmielewski.[76] With the citrus rust mite *Phyllocoptruta oleivora* (Eriophyidae, eriophyid mites), good results were obtained, however, on nymphal stages in field experiments at a rate of 350 g a.i. ha^{-1}, and 2–3 replicates per year (Internal report Thompson–Hayward Chemical Company, USA, 1976).

7. Summary of practical results with diflubenzuron

In the Diptera, diflubenzuron reveals an outstanding activity against larval stages of many species belonging to the Culicidae (mosquitoes), at rates of 22 to 56 g a.i. ha^{-1}.

The control of Chironomidae (midges) requires higher dosages. For larvae of *Chironomus* spp., *Tanytarsus* spp. and *Procladius* spp. 110 g a.i. ha^{-1} was effective. However, this rather high dosage appears to be too low for *Labrundinia maculata*.

In the family of the Muscidae, larvae of *Musca domestica* (house fly) and *Stomoxys calcitrans* (stable fly) can be controlled in feedlot breeding areas and manure at a rate of 0.5 g a.i. m^{-2}. Another method to control these larvae in the manure is by oral intake of diflubenzuron (rates of 6.2 and 1 mg a.i. kg^{-1} food, respectively). Ovicidal effects by contamination of female insects can be used in the field against *Haematobia irritans* (horn fly) by spraying range cattle. In the laboratory this phenomenon also has been reported for other species of the Muscidae.

Against larvae of fungus gnats (Mycetophilidae) and humpbacked flies (Phoridae) living in mushroom compost, mycelium or sporophores, diflubenzuron can be used in a drench of 30 mg a.i. $litre^{-1}$.

Preliminary results with *Simulium vittatum* (black fly) belonging to the Simuliidae, indicate that diflubenzuron has a potential as a larvicide with simultaneous ovicidal activity.

Practical possibilities to control Anthomyiidae [*Hylemya platura* (seed corn maggot)]. *H. brassicae* (cabbage root fly) and *H. cilicrura* seem to be limited.

ocellana (eye-spotted bud moth), *Hedya nubiferana* and *Pammene rhediella* (fruitlet mining tortrix) can be adequately controlled by diflubenzuron. The reason for this difference is not clear. Excepting possible differences in behaviour in the field, several closely related species show big differences in susceptibility when fed on an artificial diet containing diflubenzuron. In preliminary experiments Van der Molen[70] compared in this way fourth instar larvae of *Archips podana*, *Archips rosana*, and *Adoxophyes orana* and found LC_{50} values of 0.85, 200 and 120 mg a.i. kg^{-1} diet respectively.

6.2.13. Yponomeutidae (small ermine moths)

In laboratory experiments an extremely high susceptibility (LC_{100} 10 μg $litre^{-1}$) of *Yponomeuta evonymellus* to diflubenzuron was found by feeding twigs dipped in a suspension of the compound. In field experiments[17] complete control was obtained on first to third instar larvae at 60 μg $litre^{-1}$ on fourth instar larvae at 0.6 mg $litre^{-1}$ and on fifth instar larvae at 60 mg $litre^{-1}$.

6.3. Coleoptera

6.3.1. Chrysomelidae (leaf beetles)

In small scale field experiments Mulder and Swennen[39] obtained 98% control with larvae of *Leptinotarsa decemlineata* (Colorado potato beetle) using the liquid formulation of diflubenzuron (100 mg a.i. $litre^{-1}$).

6.3.2. Coccinellidae (ladybird beetles)

Direct application of a solution of diflubenzuron in aqueous acetone (500 mg a.i. $litre^{-1}$) on eggs of *Epilachna varivestis* (Mexican bean beetle) gave 100% control of all egg ages; ovicidal effects could also be obtained by contamination of the female. Feeding of first to fourth instar larvae with leaves sprayed with a solution of diflubenzuron in acetone (50 mg a.i. $litre^{-1}$) gave almost complete control whilst 100 mg a.i. $litre^{-1}$ gave full control in laboratory experiments.[6]

6.3.3. Curculionidae (weevils)

In small-plot tests, Neal[15] tested the activity of diflubenzuron on *Hypera postica* (alfalfa weevil). Though the larvae were collected from the treated plots 1 or 2 days later and reared on untreated food until adult emergence, high mortality was obtained with the e.c. formulation (1.1 kg a.i. ha^{-1}). Ovicidal activity by contamination of the female can be used in practice to control *Anthonomus grandis* (cotton boll weevil). By using a sprayable invert sugar bait containing diflubenzuron, Taft and Hopkins[57] and Moore and Taft[71] found 98% reduction in adult emergence from the infested squares after 14 applications with 280 g a.i. ha^{-1}. This dosage and the number of applications may be reduced. Ganyard *et al.*[72] obtained better than 99% reduction after 12 applications with 140 g a.i. ha^{-1} using the w.p. formulation in cottonseed oil. Similar effects were obtained in field tests with *Diaprepes abbreviatus* (a sugar cane rootstalk borer weevil) on citrus. Aerial application of 699 g a.i. ha^{-1} was effective for at least 26 days in significantly reducing the reproduction.[73] Egg hatch of *Graphognathus leucoloma* (white-fringed weevil) was reduced by 95% when adults were fed leaves of white clover dipped in a suspension of the w.p. formulation (10 mg a.i. $litre^{-1}$).[62] Carter [26] achieved good control of stored product beetles on spraying wheat with the e.c. formulation (2 mg a.i. $litre^{-1}$) and hence subsequent infestation with adults. Progeny of a resistant strain of *Sitophilus oryzae* (rice weevil) and a susceptible strain of *S. granarius* (granary weevil) were reduced by 91.5 and 97.5%, respectively. Using the same techniques but the wettable powder (0.25 mg a.i. $litre^{-1}$) McGregor and Kramer[74] obtained a reduction in progeny of 96% in *S. oryzae* and 95% in *S.*

In the Lepidoptera, diflubenzuron can be used in dosages ranging from 7.5 up to 200 g a.i. ha^{-1} to control the following species, belonging to many economically important families: *Anticarsia gemmatalis* (velvet bean caterpillar), *Bucculatrix thurberiella* (cotton leaf perforator), *Euproctis chrysorrhoea* (brown-tail moth), *Hedya nubiferana* (leafroller), *Hyphantria cunea* (fall webworm), *Lambdina* f. *lugubrosa* (western hemlock looper), *Laspeyresia pomonella* (codling moth), *Lymantria dispar* (gypsy moth), *L. monacha* (nun moth), *Malacosoma disstria* (forest tent caterpillar), *Operophtera brumata* (winter moth), *Orgyia pseudotsugata* (Douglas-fir tussock moth), *Pammene rhediella* (fruitlet mining tortrix), *Pieris brassicae* (large cabbage white), *Spilonota ocellana* (eye-spotted bud moth), *Thaumetopoea pityocampa* (processionary caterpillar) and *Yponomeuta evonymellus* (small ermine moth).

Economically important species which cannot be controlled at test concentrations of 220 to 280 g a.i. ha^{-1} are *Adoxophyes orana* (summer fruit tortrix), *Archips rosana* (rose tortrix moth), *Heliothis virescens* (tobacco budworm), *H. zea* (corn earworm), *Pandemis heparana* (leafroller), *Pectinophora gossypiella* (pink bollworm) and *Spodoptera frugiperda* (fall armyworm). For control of *Trichoplusia ni* (cabbage looper), a dosage of 2.2 kg ha^{-1} is needed.

With some of the before mentioned species, lack of larval control is probably due to their feeding in sites inaccessible to treatment. This is the case for larvae of species belonging to the Gracillaridae [*Leucoptera scitella*, *Stigmella malella* (apple pygmy moth) and *Lithocolletis blancardella*] which feed inside the leaves. However these species can be controlled by the ovicidal activity of diflubenzuron (sprays of 125 to 200 mg a.i. $litre^{-1}$).

In the Coleoptera, promising laboratory results are obtained with *Epilachna varivestis* (Mexican bean beetle), belonging to the Coccinellidae.

In the Curculionidae, the ovicidal activity by contamination of females can be used successfully in the field to control *Anthonomis grandis* (cotton boll weevil) and *Diaprepes abbreviatus* (sugar cane rootstalk borer weevil).

Diflubenzuron can be used by spraying as a suspension on the feeding substrate against a great number of stored product beetles, belonging to several families.

Target insect species from orders outside the Diptera, Lepidoptera and Coleoptera can be controlled incidentally by diflubenzuron in the field. Amongst these are the pear sucker, *Psylla piri* (Hemiptera) and the European pine sawfly (*Neodipiron sertifer* (Hymenoptera).

In the Acarina, rather surprisingly, activity is found on the citrus rust mite, *Phyllocoptruta oleivora*.

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IV OVICIDAL AND LARVIDAL STRUCTURE-ACTIVITY RELATIONSHIPS OF BENZOYLUREAS ON THE HOUSEFLY *(MUSCA DOMESTICA).*

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SUMMARY

Benzoylureas are insectidal compounds interfering with chitin synthesis. A number of benzoylureas, with variations in lipophilic properties, were tested for their larvicidal and ovicidal activities on *Musca domestica*. The larvicidal activities were tested by mixing of the compounds with the larval medium. The ovicidal activities were assessed either by feeding or by injection of female adults and resulted in nonemergence of the eggs, though the embryos seemed to be fully developed. A significant correlation ($p = 0.02$) was found between ovicidal activity after adult feeding and ovicidal activity after injection.

Correlation of the activities by means of a physical chemical parameter revealed that ovicidal activity after injection was the basic activity in both ovicidal activity by adult feeding as well as in larvicidal activity by feeding. However, in either case this activity was coupled to a lipophilic parameter (expressed as π in the octanol/water system). Optimum π values for ovicidal activity after adult feeding or for the larvicidal activity were 1.0 and 1.5, respectively. It is likely that at increasing lipophilicity of the compounds above the optimum values, the gut wall becomes an important barrier but that the activity at the target site is not affected. At decreasing lipophilicity the activity at the target site probably also decreases.

INTRODUCTION

Diflubenzuron is the common name for 1-(4-chlorophenyl)-3-(2,6-difluorobenzoyl)urea [1].

Diflubenzuron was discovered as a larvicide. The mode of action of diflubenzuron and its related compound Du 19.111 (1-(2,6-dichlorobenzoyl)-3-(3,4-dichlorophenyl)urea) was studied in larvae of insects by Mulder and Gijswijt (1), Post and Vincent (2), Post et al. (3) and Deul et al. (4). Evidence was given that both compounds interfered with chitin synthesis.

Diflubenzuron can also cause ovicidal effects by contact activity on eggs or by treatment of females. After application of diflubenzuron, embryos can be observed which are unable to hatch although they are apparently fully developed (5). Electron microscopic observations of embryos of *Leptinotarsa decemlineata* (Colorado potato beetle), after feeding female adults with diflubenzuron, showed a disturbance of cuticle formation, suggesting a similar mode of action of the compound in larvae and in embryos (6).

Contact activity on eggs is species dependent (6, 7). In our laboratory strain of *Musca domestica* this contact activity is completely absent. Ovicidal effects by contamination of females can result from topical treatment with an acetonic solution, from contact of females with a treated surface, and from feeding or injection of the compound (5, 6).

Verloop and Ferrell (8) correlated biological activities of benzoylureas with physical-chemical parameters. They demonstrated that lipophilic, electronic and steric effects influence the larvicidal activity against larvae of *Pieris brassicae*.

Instead of correlation of biological activities with physical-chemical parameters we intended to correlate the distinct biological activities on several stages of an insect. Our aim therefore was to look for any correlation between larvicidal and ovicidal activities of the test compounds in *M. domestica*, and furthermore for the relation between the level of these activities and the degree of lipophilicity of the test compounds.

In the study reported here, we used twenty-five diflubenzuron related benzoylureas with a large variation in lipophilic properties.

1 Code names: PH 60-40, TH 6040, Du 112.307, ENT-29054, OMS 1804, PDD 6040 I. Registered trade mark of Philips-Duphar B.V.: DIMILIN

MATERIALS AND METHODS

Chemicals

For both incorporation into the larval medium and injection and feeding experiments with *M. domestica*, all technical materials were milled to a particle size of ≤ 4 µm, with addition of Polyfon H and Tween 80.

Rearing and bio-assays

Larvicidal tests

In larvicidal tests, the compounds were thoroughly mixed with the larval medium before this solidified. This larval medium consisted of a mixture of 10 g yeast powder, 10 g full cream milk powder and 2 g agar in 100 ml water. For each test compound the different concentrations were obtained by mixing the weighed quantity of the chemical with 100 ml of the larval medium, which was then divided over 3 plastic cups. Each cup was infested with twenty 1-day-old larvae. The medium was covered with a layer of vermiculite. The cups were kept at a temperature of 24°C until adult emergence.

Ovicidal tests by injection of females

Female flies of 3- to 4-days old were anaesthetized with CO_2 and injected in the dorso-lateral side of the thorax with a hand-made glass injection needle. All compounds were injected in a quantity of 5 µg (1 µl of 5000 mg/l). We used 3 replicates per treatment. Each replicate consisted of 10 injected females and 10 untreated males in a cage.

The flies were fed with cubes of sugar and a suspension containing one part of full cream milk powder in two parts of water. The milk suspension was offered by means of a soaked cotton wool pad. Eggs which were deposited on this cotton wool pad were collected daily. At least 100 eggs per replicate were smoothed on black filter paper in a Petri dish and kept at 24°C and a relative humidity of about 100%. After one day the percentage of hatched eggs was determined and corrected for the percentage unfertilized eggs. Adults were kept under a day/night cycle of 18/6 h at a temperature and relative humidity of respectively 28°C, 60-70% and 19°C, 80-90%.

Ovicidal tests by feeding of adults

From adult eclosion 10 females and 10 males per cage were fed with cubes of

sugar and a milk suspension containing the test compound. The test compound was added under stirring to the milk suspension up to a concentration of 50 mg/l. We used 3 replicates per treatment. Four days after adult eclosion the treatment was finished and untreated milk was offered again. Ovicidal effects were assessed as described for the injection tests. Environmental conditions were also similar to those described for the injection tests.

All data were corrected for natural mortality according to Abbott (9).

Handling of data for calculations

For calculations of the equations and of the correlation-coefficients we used several methods to handle the experimental results:

Ovicidal activity by injection or feeding of adult *M. domestica* was measured during 5 days after treatment. The prevention of egg hatch on each day was expressed by using +, ± and - symbols, indicating 90-100%, 50-90%, and < 50% prevention of egg hatch, respectively. For calculations (equation 1), the ovicidal activity was expressed as the total number of days at which a 90-100% ovicidal effect was observed. Days with only a 50-90% effect were counted as half. In Table 1 the experimental ovicidal activity is expressed in the same way.

Larvicidal activity at a certain test concentration was also expressed by using +, ± and - symbols, now indicating 90-100%, 50-90% and < 50% larval mortality, respectively. We used the test concentrations of 100, 30, 10, 3, 1 and 0.3 mg a.i./l. In the results (Table 1) the experimental larvicidal activity is expressed as the lowest test concentration with a ± effect. When two successive ± effects were obtained, the average value of the two test concentrations was used (for example compound 8).

The larvicidal activities which are calculated with equation 2, using the ovidal activities after injection are expressed as the negative logarithm of the lowest test concentration with a ± effect. In Table 1 these negative logarithms are recalculated to concentrations in mg a.i./l in order to allow an easy comparison with the experimental figures.

RESULTS

Table 1 shows the larvicidal and ovicidal activities on *M. domestica*. The compounds show variations in both the benzoyl and the phenyl group. They are

arranged according to increasing lipophilic character. The π-values, in the octanol/water system, are related to the unsubstituted compounds; they have been taken from Hansch and Leo (10) or were calculated according to Nijs and Rekker (11).

The activities from Table 1 were used in a quantitative structure-activity relationship (QSAR) study, with the Hansch method (12). A matrix of correlation coefficients is given in Table 2. The low correlation coefficients in this Table illustrate the mutual independence of the parameters used. Only the ovicidal activities show a significant but low correlation ($p = 0.02$). In equation (1) the ovicidal activities were related to each other in combination with the hydrophobic constant π. Although the dependent variable is not quite normally distributed by a relatively high percentage of zero values, a regression analysis still appears to be possible.

			t
(1)	ovicidal activity after adult feeding (days) =	+ 0.33 ovicidal activity after injection (days)	4.73
		+ 0.26 π	1.94
		- 0.12 π^2	2.40
		+ 0.04	

$n = 24 \quad r = 0.753 \quad s = 0.509 \quad F = 8.73 \quad (p < 0.001)$

In this equation, n is the number of compounds, r is the correlation coefficient, s is the standard error of the estimate, and F indicates the significance of the correlation. The t-value indicates the importance of the factor for the regression (higher values indicate a more important contribution). In equation (1) compound 22 was excluded because it proved to be an outlier (the difference between calculated and experimental values more than twice the standard error). The optimal π-value of the ovicidal activity after adult feeding is about 1, which implies that very lipophilic or hydrophilic compounds have only a weak activity after adult feeding in spite of a good activity after injection. The latter type of ovicidal activity can be considered as a kind of basic activity. A dummy variable for the presence of a 2,6-F_2 group did not contribute significantly to the equation. The correlation obtained in equation (1) is significant but not very high. However, the rather rough measure used to express the ovicidal test data seriously hinders the development of a regression equation

with a lower standard deviation.

In equation (2) the larvicidal activity is taken as the dependent variable. Again the ovicidal activity after injection can be considered as a basic activity.

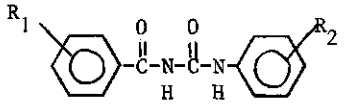
			t
(2)	larvicidal activity (-log conc.) =	+ 0.22 ovicidal activity after injection (days)	3.43
		+ 0.62 π	3.29
		- 0.21 π^2	4.25
		+ 0.85 D	3.55
		- 1.32	

n = 23 r = 0.847 s = 0.480 F = 11.41 ($p < 0.0003$)

In equation (2) compounds 5 and 6 have been omitted because of their undefined biological activity. Considering again the quality of the biological data, the equation shows a good correlation. The optimal π-value for the larvicidal activity in combination with a good ovicidal activity is about 1.5.

The D parameter in equation (2) is a dummy variable for the presence of a 2,6-F_2 group. D is equal to 1 when this group is present, otherwise D is 0. Hence 2,6-F_2 compounds are much more active than the other derivatives. As the dummy variable is not important in equation (1) it can be concluded that the 2,6-F_2 derivatives are relatively more potent in the larvicidal than in the ovicidal tests.

Table 1. Ovicidal and larvicidal activities of substituted 1-phenyl-3-benzoylureas on *Musca domestica*



compound	R_1	R_2	π	D[a]	ovicidal activity after injection[b] (days)	ovicidal activity after feeding, experimental[b] (days)	ovicidal activity after feeding, calculated[c] (days)	larvicidal activity, experimental[d] (mg a.i./l)	larvicidal activity, calculated[e] (mg a.i./l)
1	2,6-F_2	4-$SO_2C_2H_5$	-0.89	1	0	0	-0.3	30	15
2	2,6-Cl_2	4-SO_2CH_3	-0.19	0	0	0.5	0	30	32
3	2,6-F_2	4-NO_2	0.00	1	3.5	0.5	1.2	0.3	0.5
4	2-Cl	4-CN	0.17	0	2	0	0.7	3	6
5	2,6-F_2	2-OH, 4-Cl	0.35[f]	1	0	0.5	0.1	> 100[g]	2
6	2,6-F_2	3-OH, 4-Cl	0.35[f]	1	0	0	0.1	> 100[g]	2
7	2,6-Cl_2	4-$SO_2N(CH_3)_2$	0.70	0	0	0.5	0.2	100	11
8	2,6-Cl_2	4-CN	0.91	0	1	0	0.5	2	5
9	2,6-F_2	4-Cl	1.02	1	3.5	1.5	1.3	0.3	0.2
10	2,6-Cl_2	4-NO_2	1.20	0	2.5	1	1	3	2
11	2,6-F_2	4-C_2H_5	1.28	1	0	0	0.2	0.3	1
12	2,6-F_2	4-CF_3	1.37	1	4.5	2	1.7	0.1	0.1
13	2,6-Cl_2	4-F	1.62	0	0	0	0.2	2	7
14	2,6-F_2	4-C_3H_7	1.78	1	1.5	0	0.6	0.3	0.5
15	2-Cl	4-CF_3	1.83	0	5	2.5	1.8	1	0.6
16	2,6-Cl_2	4-Cl	2.22	0	1.5	0.5	0.5	3	4
17	2-Cl	3,4-Cl_2	2.22	0	1	0.5	0.4	1	5
18	2,6-F_2	4-t-C_4H_9	2.26	1	1	0	0.3	3	0.8
19	2-Br	3,4-Cl_2	2.46	0	2.5	0	0.8	3	3
20	2,6-Cl_2	3,4-Cl_2	2.96	0	1.5	0	0.3	10	8
21	2-Cl	4-OCH_2-⬡-Cl	3.14	0	5	1.5	1.3	10	2
22	2-Cl	4-SCH_2-⬡-NO_2	3.28	0	0	1.5[g]	-0.4	100	23
23	2,6-Cl_2	4-t-C_4H_9	3.46	0	2.5	1.5	0.4	10	8
24	2,6-Cl_2	4-⬡-Br	4.22	0	5	0.5	0.5	10	28
25	2-Cl	4-SCH_2-⬡-Cl	4.30	0	5	0	0.7	10	30

a. Dummy variable for the presence of a 2,6-F_2 group.
b. Activities are expressed as the total number of days during which ovicidal activity was observed. For details see "Handling of data for calculations".
c. Calculated by means of equation 1, activities are expressed as mentioned under b.
d. Activities are expressed as the lowest test concentration with 50-90% mortality. For details see "Handling of data for calculations".
e. Calculated by means of equation 2, and expressed in this table as the concentration in mg a.i./l.
f. Calculated as an undissociated OH group.
g. These points are not included in derivation of final equations because of either undefined biological activities (compounds 5 and 6 in equation 2) or a difference between calculated and experimental value which is more than 2 times the standard error (compound 22 in equation 1).

Table 2. Matrix of correlation coefficients of ovicidal and larvicidal activities in *Musca domestica*

	ovicidal activity after injection	ovicidal activity after feeding	larvicidal activity
ovicidal activity after injection	1.000	0.521	0.305
ovicidal activity after feeding		1.000	0.119
larvicidal activity			1.000

DISCUSSION

In both larvicidal and ovicidal tests after adult feeding, the test compound enters the insect body by absorption from the alimentary tract through the gut wall. After arrival in the hemolymph, the compound is then most probably distributed throughout the insect body.

In the injection experiments, the barrier of the gut wall was avoided. However, in order for ovicidal activity to occur in adults, still some other barriers are present upon arrival in the hemolymph. For example, the compounds also have to be transported into the egg in order to be able to disturb cuticle formation in the developing embryo (5, 6).

The availability of the compounds might also be influenced by their metabolism. However, we see no reason why there should be a difference in metabolism after either feeding or injection. This is the reason that we do not focus our discussion on metabolism but rather on transport of the compounds.

Shah et al. (13) studied the relations between increasing lipophilicity and gut wall penetration in isolated guts of *Manduca sexta* (tobacco hornworm) and *Blaberus cranifer* (cockroach) with dimethoate and some dialkoxy analogues. They found that penetration through the gut diminished at increasing lipophilicity of the chemicals. Furthermore, penetration was found to be species dependent.

Generally a parabolic relationship is found between lipophilicity and penetration. Therefore the conclusion of Shah et al. (13) is probably only valid when the optimum value of the lipophilicity has been passed.

A similar relation between lipophilicity and gut wall penetration of the benzoylureas in *M. domestica* can explain the decrease in both ovicidal activity after adult feeding and larvicidal activity with lipophilicities above the optimum values of $\pi = 1$ or 1.5, respectively (equation 1 and 2). This is best demonstrated by the highly lipophilic compounds 21, 24 and 25. These compounds show good ovicidal activity after adult injection, when the gut wall barrier has been avoided, but by contrast they show a low or even absent activity after adult feeding. It is remarkable that at increasing lipophilicity of the compounds only the gut wall should become an important barrier but that transport into the egg is apparently much less influenced.

Transport of diflubenzuron into eggs after adult injection occurs very quickly, as in eggs collected 2 hours after injection eclosion was already fully prevented (5). In the experiments described in this paper, the earliest assessment of the ovicidal effect was made 1 day after injection. After this period even the highly lipophilic compounds 21, 23, 24, and 25 already caused a complete block of egg eclosion.

At decreasing lipophilicity we observed not only low activities in the larvicidal test and the ovicidal test after adult feeding but also in the ovicidal test after injection. Though penetration decreases as the lipophilicity decreases to below the optimum values, it is also possible that the activity at the target site is too low to allow the gut barrier to play an important role.

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V LARVICIDAL AND OVICIDAL RESISTANCE TO DIFLUBENZURON IN THE HOUSEFLY *(MUSCA DOMESTICA).*

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SUMMARY

Upon selection with diflubenzuron incorporated into the larval culture medium, development of both larvicidal and ovicidal resistance was studied in a susceptible laboratory strain (S) and a multi-resistant strain (Nic) of the housefly. In addition to changes in larvicidal and ovicidal activity, selection also resulted in growth retardation, decreased pupal weight, diminished adult fertility and fecundity, and an increase in pupal mortality. These effects led to a collapse in the F32 of strain Nic, in spite of a drastically lowered selection pressure during a number of previous generations when these deleterious effects became apparent. Observations for this study with strain S were discontinued in the F40.

The final assessment of the development of larval resistance in strain Nic was made in the F26, where it showed a 13.6-fold increase based upon LC_{50} values. In the F40 of strain S larval resistance had developed to an about 40-fold level. These resistance levels cannot be considered high.

The ovicidal activity in the F40 of strain S had decreased 32-fold, which factor is comparable with that obtained for the development of resistance to the larvicidal activity. In strain Nic the development of ovicidal resistance initially followed a similar pattern as in strain S. Thereafter resistance increased drastically. In the F26, even the highest test concentration of 3000 mg a.i./l of diflubenzuron showed an ovicidal activity of only 26%.

Factors of cross-resistance to diflubenzuron were assessed in the parental generation of strain Nic and in two field-collected strains (SL and AA). As compared with strain S, larvicidal cross-resistance was absent in strain Nic and strain SL. In strain AA it amounted to a factor of 5. Ovicidal cross-resistance factors were 3.5 for strain Nic, 8.4 for strain SL and 87.5 for strain AA, all factors based on LC_{50} values. The level of ovicidal cross-resistance is probably related to the resistance of adults against some standard insecticides tested.

INTRODUCTION

Diflubenzuron, 1-(4-chlorophenyl)-3-(2,6-difluorobenzoyl)urea, is the active ingredient of the insecticide DIMILIN® . The compound shows larvicidal as

well as ovicidal activities in many insect species, mainly those belonging to the Diptera, Lepidoptera, and Coleoptera. A review of its spectrum of activity has been given by Grosscurt (1978). Studies of the larvicidal mode of action of diflubenzuron and of the structurally related 1-(2,6-dichlorobenzoyl)-3-(3,4-dichlorophenyl)urea (=Du 19.111) provided evidence that these compounds interfered with chitin synthesis and for this reason blocked normal cuticular growth (Mulder and Gijswijt, 1973; Post and Vincent, 1973; Post *et al.*, 1974; Deul *et al.*, 1978).

Diflubenzuron is highly active against larvae of the housefly *(Musca domestica)* (Mulder and Swennen, 1973; Jakob, 1973), yet on eggs no contact activity can be observed. Ovicidal activity with respect to the housefly can only be obtained upon contamination of females either by feeding or direct contact. In this case the embryo inside the egg apparently develops normally but is not capable of normal hatch due to inhibition of chitin formation in the cuticle (Grosscurt, 1976). Adult mortality of the housefly upon diflubenzuron treatment is completely absent.

With an insecticidal compound such as diflubenzuron, with a new mode of action, it is of great interest to explore its properties in connection with cross-resistance and resistance development. Some information can already be obtained from the literature:

In 1974 Cerf and Georhiou were the first to report on cross-resistance to diflubenzuron upon topical application to white prepupae. The strains used were resistant to organophosphorus, carbamate, and organochlorine insecticides. Their cross-resistance factors ranged from 10 to considerably higher. However, as discussed earlier (Grosscurt, 1978), the method used in these experiments gives an exaggerated view.

Keiding *et al.* (1977) and Arevad and Keiding (1978) found cross-resistance factors of diflubenzuron for larvicidal activity, by treatment of the breeding medium, ranging from 1.0 to 2.7. They used larvae of multiresistant houseflies collected from several farms in Denmark and compared them with the susceptible reference strain from the World Health Organization (W.H.O.-S.R.S.). However, cross-resistance proved to be "considerable" with respect to the ovicidal activity by direct contact or feeding of adult females, Low cross-resistance factors in larvicidal feeding tests were also found by Rupes *et al.* (1977) with an organophosphorus-resistant strain. Oppenoorth and Van der Pas (1977) observed somewhat higher factors (ranging from 3–10) in larvicidal tests with strains resistant to malathion, trichlorphon, diazinon and some other organophosphates when compared with the susceptible W.H.O.-S.R.S. strain. In the experiments by Oppenoorth and Van der Pas, cross-resistance was also quite obvious with respect to the ovicidal effect upon topical application of females.

Experiments to study the development of resistance to diflubenzuron in houseflies were made by Keiding *et al.* (1977) and Oppenoorth and Van der Pas (1977). Keiding *et al.* (1977) attempted resistance selection with diflubenzuron with a multiresistant strain by mixing the compound with the larval medium.

After 6 generations, no indications of any resistance development were obtained. Moreover, there was also no tendency for a significant increase of tolerance to the larvicidal effect of diflubenzuron in flies from Danish farms where experimental treatment of breeding places with diflubenzuron was the main method of fly control in 1976–1977. Oppenoorth and Van der Pas (1977) used a mixture of resistant laboratory strains for selection with the compound in the larval medium. After 10 generations, larval resistance, based on LC_{50} values, increased to about 50-fold as compared with the susceptible W.H.O.-S.R.S. strain. However, this factor varied from 5 to 15 as compared with the parental strains.

As a result of the rather conflicting data concerning the development of resistance against diflubenzuron we decided to study the resistance development and cross-resistance to diflubenzuron, both as a larvicide and as an ovicide, using susceptible and resistant strains of *Musca domestica*.

MATERIALS AND METHODS

Chemicals

Diflubenzuron was used as a flowable formulation with a particle size of $\leq 1\mu$. Dimethoate [0,0-dimethyl-S-(N-methylcarbamoylmethyl)phosphorodithioate] was formulated as a 20% e.c., and trichlorphon [0,0-dimethyl (2,2,2-trichloro-1-hydroxyethyl)phosphonate] as a 30% w.p. Parasect, which is a combination product (1 : 1, w/w) of dimethoate and fenitrothion [0,0-dimethyl 0-(3-methyl-4-nitrophenyl)phosphorothioate] was formulated as a 15% e.c.

Strains of the housefly (Musca domestica)

a. Laboratory strains

In our experiments we used 2 laboratory strains. Strain S was a susceptible strain obtained in 1960 from T.N.O., Utrecht, and reared ever since at the Agrobiological Laboratory "Boekesteyn". Strain Nic was resistant to diazinon and other organophosphates and showed a high oxidation capacity for several insecticidal compounds (Oppenoorth and Houx, 1968; El Bashir and Oppenoorth, 1969). This strain was obtained in 1977 from the laboratory for Research on Insecticides (Wageningen) by courtesy of Dr. F.J. Oppenoorth.

b. Field collected strains

In 1978 a housefly strain (strain SL) was collected at Slagharen (Netherlands) on a farm where in recent years mainly Parasect and trichlorphon were used. In 1979 another strain (strain AA) was collected at Aalten (Netherlands) on a farm where recently mainly trichlorphon had been used. Neither on the two farms nor in their vicinity had diflubenzuron ever been applied. Of these field collected strains the 1st and 2nd generations, reared in the laboratory, were used for our experiments.

Rearing and selection of flies

The larval breeding medium consisted of 20 g agar, 100 g yeast powder and

100 g full cream milk powder in 1 liter of tap water. For preparation of the medium, the agar was added to ½ liter water which was then boiled with stirring. The other ingredients and, when required for selection, the calculated amount of diflubenzuron were added to the remaining ½ liter water. Thereafter both parts of the medium were combined, thoroughly mixed, and poured out into plastic jars of 3000 cm^3. The jars used for breeding and/or selection were each filled with 400 ml medium, resulting in a layer of about 3 cm thickness. After the introduction of about 1500–2000 eggs per jar, the medium was covered with vermiculite and kept at 31° until pupation. Pupae were sieved from the vermiculite and transferred into cages for adult eclosion. Adult flies were fed with cubes of sugar and a suspension containing one part of full cream milk powder in two parts of water. The milk was offered via a cotton wool pad. Adults were maintained on a regime of an 18-h day (29°, r.h. 35–45%) and a 6-h night (19°, r.h. 45–55%). In a number of generations selection in the larval breeding medium was achieved at two concentrations. Survivors of both treatments were reunited for further selection.

Testing of larval susceptibility

Testing of larval susceptibility was done by mixing of the required amount of diflubenzuron with 100 ml of the breeding medium. The mixture was then distributed over three plastic cups. Each cup was provided with 20 newly hatched larvae. The medium was then covered with vermiculite. Effects of the compound were calculated from the average percentage inhibition of adult eclosion, the average result of 6 cups without diflubenzuron serving as a control.

Testing of ovicidal effects of diflubenzuron

Ovicidal effects were measured by feeding of adults with diflubenzuron which had been thoroughly mixed with the milk suspension. Eggs were randomly collected on the 3rd and 4th day of the treatment. The percentage of hatched eggs was determined by means of between 200 and 300 eggs put into a Petri dish on moistened black filter paper (r.h. about 100%, temp. 24°). The viable and non-viable eggs were counted after completion of eclosion. The results were corrected for the percentage of unfertilized eggs; these showed no development of the embryo whatsoever, an effect which was not caused by diflubenzuron (Grosscurt, 1976). For each concentration 3 replicates were used.

Table 1. Insecticidal susceptibility in various strains of the housefly.

Strain	LC_{50} (LC_{90}) values in mg a.i./l				
	diflubenzuron		dimethoate	trichlorphon	Parasect
	larvicide	ovicide		adulticide	
S	0.10 (0.56)	5.6 (16.1)	8.2 (18.2)	0.79 (2.15)	0.11 (0.21)
Nic	0.11 (0.46)	19.5 (44.5)	29.5 (72.0)	2.95 (6.2)	0.71 (1.1)
SL	0.08 (0.57)	47 (420)	–	47 –	0.53 (0.8)
AA	0.50 (1.7)	490 (1300)	83 (150)	21 (49)	–

Insecticidal activity on adult flies of some standard insecticides

Tests on contact activity of the standard insecticides were performed by treating the bottom of a petri dish (9 cm diameter) with 1 ml of an acetonic solution of the test compound. Solutions of dimethoate and Parasect were made directly in acetone. With trichlorphon the stock solution of 3000 mg/l was made by dissolution of the compound in water. Dilutions were made with acetone. After the above treatment, the Petri dishes remained open for 10 minutes in order to allow vaporization of the acetone. Subsequently they were placed upside down and provided with 10 flies each. The age of the flies was 3–5 days. To prevent mortality due to dryness a small piece of moistened filter paper (diameter 2.5 cm) was stuck on the untreated inner side of the lid. Evaluations were made after 20 h. For each concentration 3 replicates were used.

RESULTS

A. Cross-resistance to diflubenzuron

Strain Nic

To characterize the strains, the contact activity on adults was assessed with some widely used insecticides. The results (table 1) indicate that, based on LC_{50}

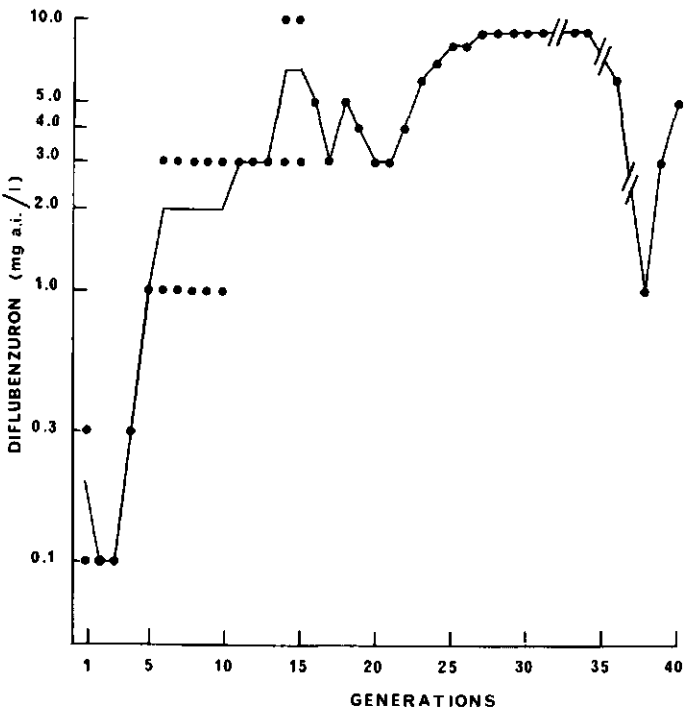

Fig. 1. Selection dosages of diflubenzuron in mg a.i./l larval breeding medium, in successive generations of strain S. Where 2 selection dosages have been used, the line connecting the selection dosages for the different generations has been drawn through the average values. // means deferring of selection.

values, strain Nic is nearly 4 times less susceptible to dimethoate and trichlorphon and 6.5 times less susceptible to Parasect, than Strain S. On the basis of LC_{90} values these ratios range from 3–5. In spite of this difference in susceptibility of the adults to a number of insecticides, the larval susceptibilities to diflubenzuron were about the same for strain S and strain Nic. However, the ovicidal activity via the female on strain S was 3.5 fold higher than on strain Nic, when based on LC_{50} values and 2.8-fold higher when based on LC_{90} values (table 1).

Strain SL

On the particular farm where strain SL had been collected, mainly Parasect and trichlorphon had been used and therefore the susceptibility of this strain to these insecticides was tested. From table 1 it is clear that the adults of strain SL

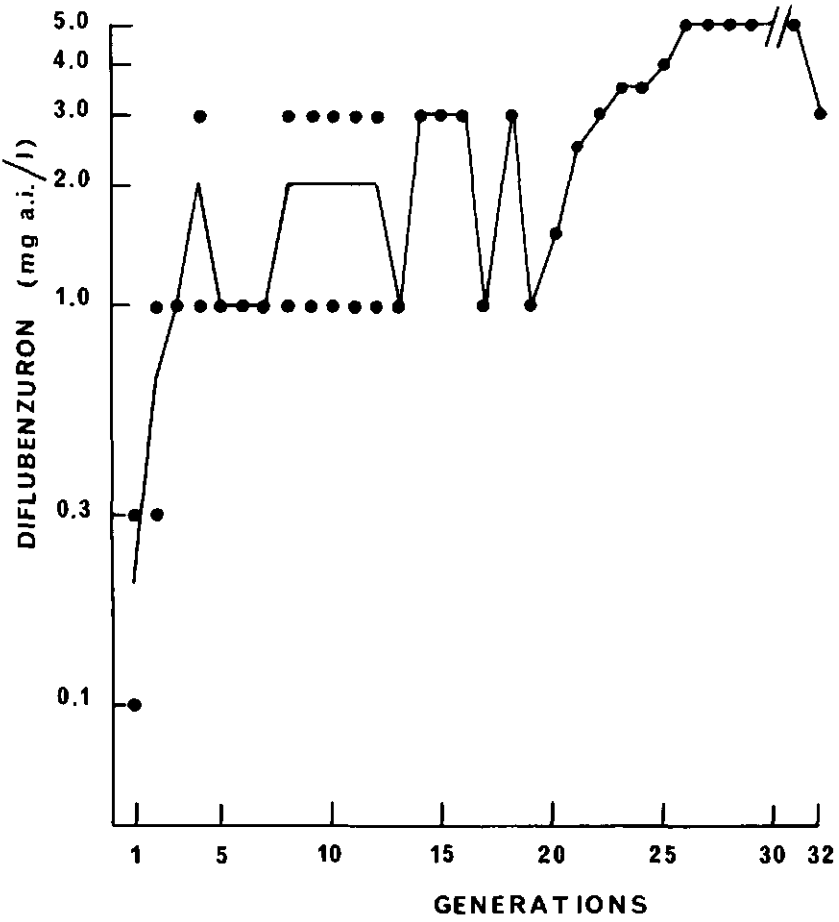

Fig. 2. Selection dosages of diflubenzuron in mg a.i./l larval breeding medium, in successive generations of strain Nic. Where 2 selection dosages have been used, the line connecting the selection dosages for the different generations has been drawn through the average values. // means deferring of selection.

were mainly resistant to trichlorphon. As compared with strain S this resulted in a 59-fold increase in resistance at the LC_{50} level. For Parasect the resistance ratio was only about 5-fold.

Test of diflubenzuron as a larvicide showed cross-resistance to be absent. However, when the ovicidal activity of diflubenzuron was tested an LC_{50} was found of 47 mg a.i./l, resulting in a cross-resistance factor of 8.4. At the LC_{90} level a cross-resistance by a factor 26 was obtained, indicating a considerable flattening at higher rates of the dose-response curve.

Strain AA

In strain AA the adults showed resistance factors to dimethoate and trichlorphon of 10 and 27 respectively. The larvicidal cross-resistance to diflubenzuron in this strain was a 5-fold, at the LC_{50} level, and a 3-fold, at the LC_{90} level, of that obtained with strain S. The ovicidal cross-resistance factor however, was 87.5-fold (table 1).

B. Development of resistance to diflubenzuron in two laboratory strains (S and Nic)

Selection with diflubenzuron in strain S and strain Nic

Selection for resistance by treatment with diflubenzuron in the larval medium

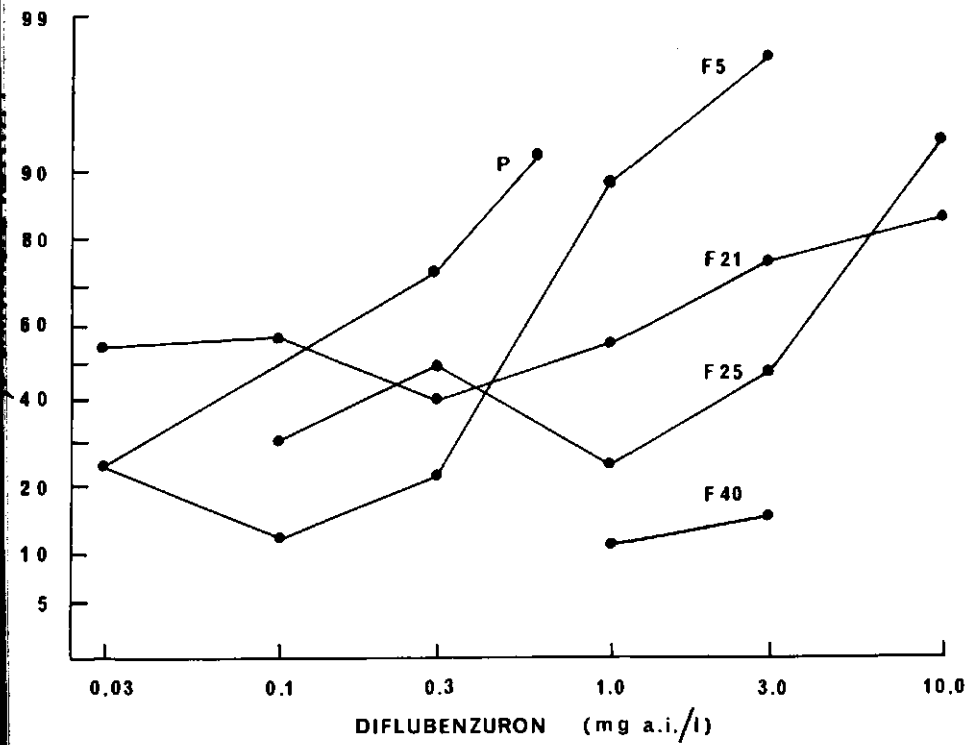

Fig. 3. Larvicidal activity of diflubenzuron in various generations of strain S upon selection in the larval medium.

was started in both strains at concentrations of 0.1 and 0.3 mg a.i./l larval breeding medium.

Figs. 1 and 2 illustrate the course of the selection dosages. In some generations two selection dosages were used to avoid a threatening collapse of the strain. In these cases, the line connecting the selection dosages for the different generations has been drawn through the average values.

After about 25 generations the selection dosages of diflubenzuron for strain S could gradually be increased to 9 mg a.i./l in the larval breeding medium, and for strain Nic to 5 mg a.i./l medium. At these levels, however, it proved necessary in a number of generations of both strains to defer selection and sometimes to decrease the concentration in the larval breeding medium.

The concomittant larvicidal resistance levels induced in each strain are presented in figs. 3 and 4. Starting with a more or less linear dose-mortality relationship in both parental generations, the dose-mortality curves first became discontinuous upon resistance selection. At lower dosages the curves appear rather flat, whilst at higher dosage rates the curves become much steeper. The slopes of these steep portions of the lines are roughly identical with those of the parental generations. This type of dose-mortality curves indicates that the housefly populations under study are mixtures of intermediately resistant and resistant individuals.

On comparing the development of resistance within strain S and strain Nic we see that in the F26, the last generation for assessment of larval susceptibility in

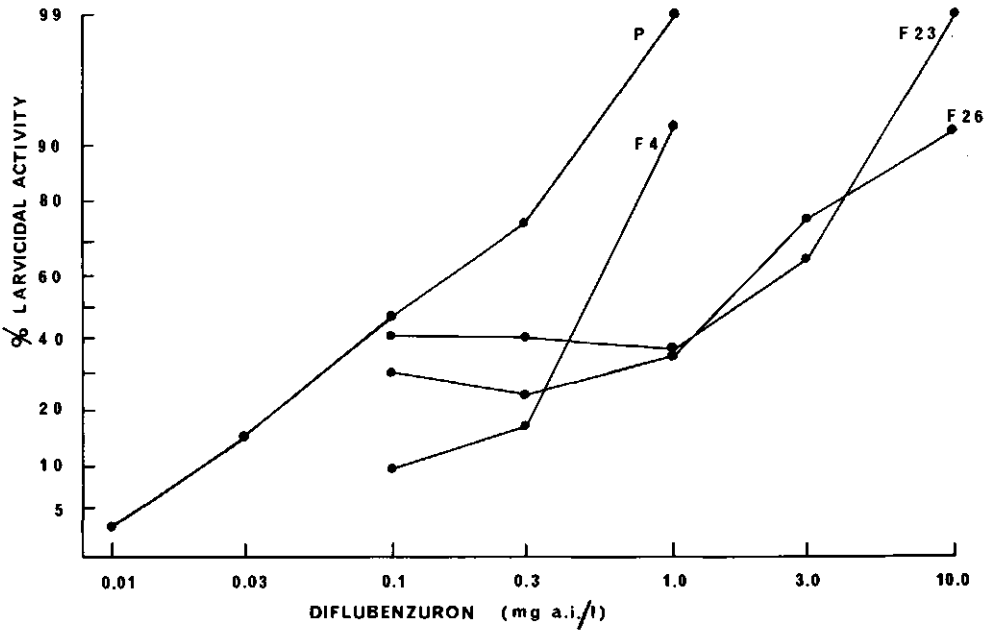

Fig. 4. Larvicidal activity of diflubenzuron in various generations of strain Nic upon selection in the larval medium.

strain Nic, the LC_{50} value of diflubenzuron is 1.5 mg a.i./l larval medium, resulting in a 13.6-fold decrease in susceptibility. In the more or less comparable F25 of strain S the larvicidal LC_{50} value is 3.2 mg a.i./l, resulting in a 32-fold decrease in susceptibility. Based upon LC_{50} values it therefore appears that the resistance development in strain Nic shows a tendency to lag behind the resistance development in strain S. Based upon LC_{90} values the decrease in susceptibility in the F25 of strain S and the F26 of strain Nic is 15.3- and 17.6-fold, respectively.

With strain S, observations were continued until the F40. However, in that generation an exact LC_{50} value could not be determined. The test concentrations of 1.0 and 3.0 mg a.i./l gave 11 and 15% control respectively, whilst 0.3 mg a.i./l gave no control and 10.0 mg a.i./l provided 100% larval control. For this reason, the LC_{50} in the F40 may turn out to be situated somewhere around 4 mg a.i./l. Based upon LC_{50} values this would than result in an approximately 40-fold decrease in susceptibility.

The ovicidal resistance levels during selection are illustrated in figs. 5 and 6. The picture concerning the development of ovicidal resistance in the two strains

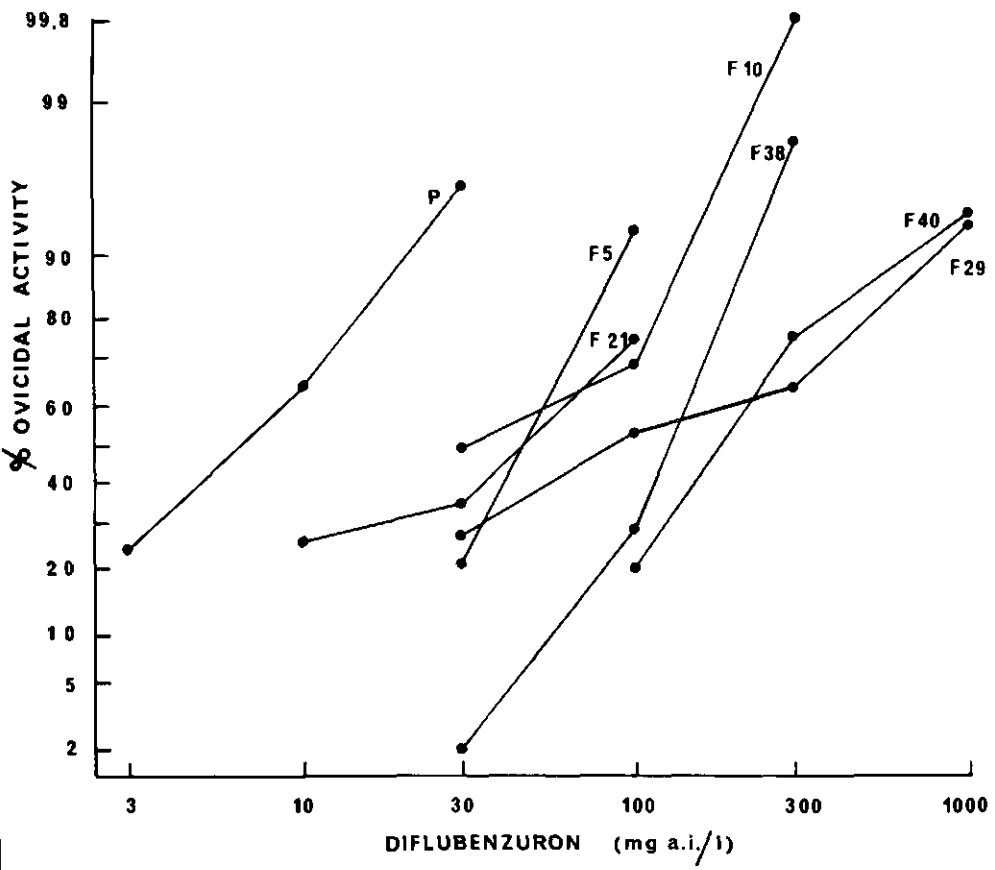

Fig. 5. Ovicidal activity of diflubenzuron by feeding adults in various generations of strain S upon selection in the larval medium.

is different. In strain S, resistance gradually increased. In the F40 of strain S the susceptibility to diflubenzuron, determined as the ovicidal activity after treatment of adult flies, had decreased 32-fold on the basis of LC_{50} values. The build-up of this level of ovicidal resistance is quite comparable to that for larvicidal resistance (fig. 3).

In strain Nic the development of ovicidal resistance initially followed a pattern similar to that in strain S. However, in the F21 and F26 the resistance had increased drastically. As a result, the highest test concentration of 1000 mg a.i./l in the F21 of strain Nic showed an ovicidal activity of only 24%. In the F26, even the highest test concentration of 3000 mg a.i./l also yielded an ovicidal activity of only 26%. For this reason the level of resistance development based upon LC_{50} values could not be established in the F26. However, it seems very probable that this level is at least 150-fold.

Additionally to larvicidal and ovicidal effects of diflubenzuron, growth retardation, a decrease in pupal weight and in adult fertility and fecundity, and an increase in pupal mortality were observed upon prolonged selection. The growth retardation concerns 1–2 days related to a normal developmental period under our conditions of about 8 days from egg to adult.

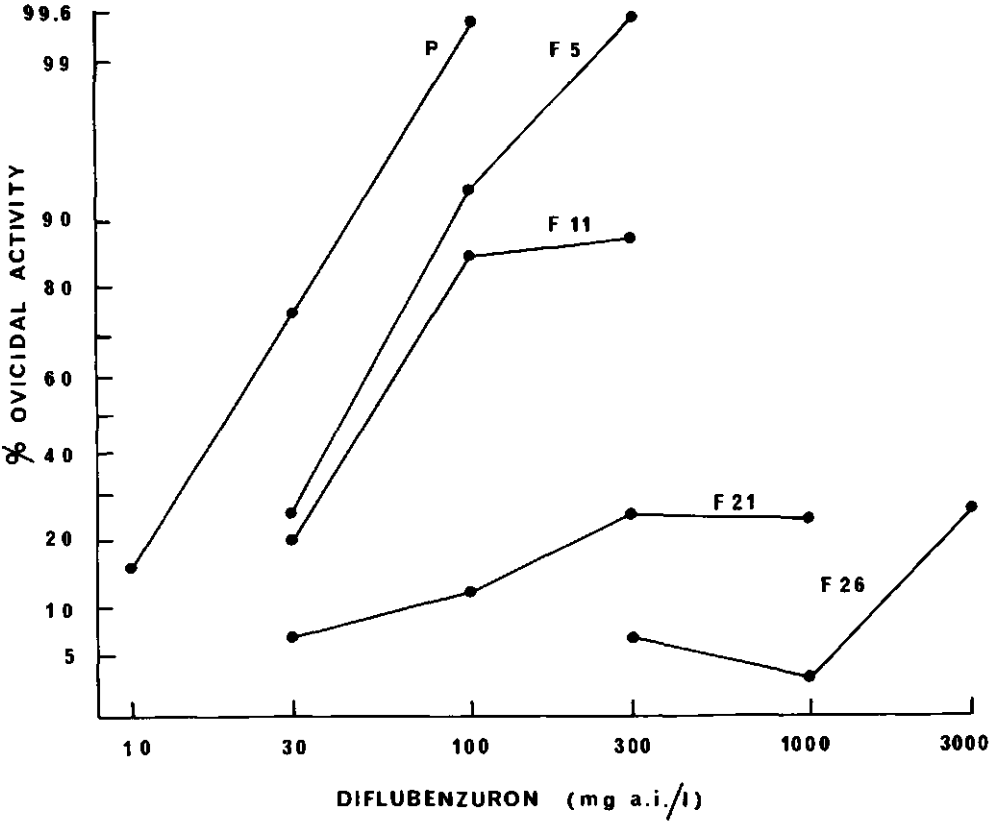

Fig. 6. Ovicidal activity of diflubenzuron by feeding adults in various generations of strain Nic upon selection in the larval medium.

In table 2, effects on pupae are demonstrated for the susceptible strain S during the first 8 generations of selection. Similar effects were observed in the strain Nic. Pupal weight and pupal mortality are strongly dependent upon the concentration of diflubenzuron in the larval medium. With increasing concentrations of diflubenzuron the shape of the pupae also becomes thinner and more oblong. From these pupae a high percentage of adults originate with morphogenetic abnormalities, such as deformed and nonfunctional wings. Table 2 also illustrates that the pupal weight increases again when the selection pressure in the F2 and F3 is compared with the same level in the F1 (0.1 mg a.i./l).

With progressive selection pressure this flexibility in pupal weight becomes less apparent, as illustrated in the F6, F7, and F8. As a result of this phenomenon, in some cases we were forced to rear during one generation on untreated medium to avoid a collapse of the strains. This brief intermittence of insecticidal pressure almost immediately resulted in a strong increase in the average pupal weight.

With progressing selection we also observed a marked drop in adult fecundity and fertility, and an increase in larval mortality. Combined, these factors resulted in a strong decrease in reproductive potential of adults in both strains after about 30 generations of selection. The following example of a drop in reproductive potential upon prolonged selection can be given for strain Nic: In the F26 of this strain (fig. 2), selection with diflubenzuron had achieved a level of 5 mg a.i./l larval medium. However upon further selection at this dosage, pupal weight gradually decreased to 9 mg in the F28, whilst only about 10% of the infested eggs resulted in pupae. As a consequence in the F30 no selection was made, immediately resulting in many pupae and a pupal weight of 19.5 mg. Resumed selection at 5 mg a.i./l in the F31 resulted again in few pupae with an average weight of only 10.5 mg. In the F32, selection was therefore lowered to 3 mg a.i./l. Despite this, pupal weight only increased to 10.9 mg and of the few pupae only 8 adults emerged. Due to this very small number of flies, work with strain Nic was stopped after the F32. These observed phenomena all point to a serious weakening of the Nic strain by a decrease in reproductive potential. It seems likely that also the level of resistance from the F30 onward will show a strong tendency to drop.

e 2. Effects on pupal weight and pupal mortality during 8 generations of selection with diflubenzuron in a susceptible ekesteyn" strain (strain S).

	Generation															
	P	F1		F2	F3	F4	F5		F6		F7		F8	
centration of benzuron in the l medium a.i./l)	0	0.1	0.3	0.1	0.1	0.3	1.0	3.0	1.0	3.0	1.0	3.0	1.0	3.0
ber of pupae/jar	1699	1353	466	1480	911	933	425	4	1150	16	1023	321	840	920
l weight (mg)	23.0	13.0	11.0	21.0	19.4	17.3	11.9	10.0	13.0	12.5	12.6	10.0	14.2	11.6
l mortality (%)	5	40	80	80	41	17	47	100	41	87	22	78	37	56

With strain S the level of 9 mg a.i./l of the larval medium could also not be maintained over a prolonged period. Even deferment of selection in the F32, F35, and F37 could not avoid the need for a gradual lowering of the selection pressure to 5 mg a.i./l in the F40 (fig. 1).

Regression of resistance in the diflubenzuron-resistant strain S

Starting with flies of the F31 of strain S, regression of larvicidal and ovicidal resistance was studied by breeding successive generations on untreated larval medium. The generations thus obtained are indicated as F31–1, F31–2 etc. The larvicidal and ovicidal resistance levels of the F25 and F29, respectively, are presented as approximations of the resistance levels of the F31, due to lack of flies at this generation for this particular experiment. The changes in levels of resistance within following generations are shown in table 3.

As expected, the level of resistance drops with successive generations without selection. This regression seems to take place more quickly with respect to the larvicidal resistance than to the ovicidal resistance.

Table 3. Regression of larvicidal and ovicidal resistance, starting with the diflubenzuron-selected F31 of strain S *

Generation	LC_{50} (LC_{90}) values of diflubenzuron in mg a.i./l in the larval medium or the food of the adults			
	larvicide		ovicide	
P	0.10	(0.56)	5.6	(16.1)
F25	3.2	(8.3)	–	
F29	–		85	(810)
F31-5	–		66	(150)
F31-7	–		44	(134)
F31-8	0.95	(2.8)	–	
F31-12	0.27	(0.8)	30	(58)

* successive generations are indicated as F31-1, F31-2 etc.

DISCUSSION

Cross-resistance to diflubenzuron

Additionally to a resistant laboratory strain (Nic), two field-collected resistant strains (SL and AA) were compared with our susceptible laboratory strain (S). In spite of the several multiresistance levels of the adults no significant differences in the larvicidal effect of diflubenzuron upon feeding were found between strain S and the resistant strains Nic and SL (table 1). In strain AA a 5-fold lower larval susceptibility was found at the LC_{50} level.

Oppenoorth and Van der Pas (1977) compared strain Nic with the susceptible W.H.O.-S.R.S. strain and found strain Nic to have a ten-fold higher resistance upon feeding on diflubenzuron in the larval breeding medium. This might indicate that strain W.H.O.-S.R.S. was particularly susceptible as compared with our strain S. Rupes *et al.* (1977) found low cross-resistance with larvae of

several organophosphate resistant strains of the housefly when compared in feeding experiments with the W.H.O.-S.R.S. strain (resistance factors are not mentioned). However, when they applied diflubenzuron topically to larvae 24 h before pupation, a "considerable" cross-resistance was found. The latter observation agrees with the results of Cerf and Georghiou (1974) who found, with the same method, cross-resistance factors ranging from 10 to considerably higher in organophosphorus, carbamate and organochlorine resistant strains. However, as discussed earlier (Grosscurt, 1978), the levels of cross-resistance upon topical application to prepupae give an exaggerated view: The prepupal stage is relatively insusceptible to topical application, resulting in very flat dose-mortality curves, and furthermore topical application of a solution of diflubenzuron gives no certainty of the quantity that actually penetrates, especially at the concentrations used.

Though in our experiments little or even no larvicidal cross-resistance was found upon treatment with diflubenzuron of the larval medium, more severe cross-resistance was found when diflubenzuron was tested as an ovicide by oral administration. The lower ovicidal activities of diflubenzuron in these resistant strains are probably related to a higher resistance of adults against the other insecticides tested (table 1). This might indicate that the pesticide metabolizing mechanisms which bring about the ovicidal resistance to diflubenzuron are in this case nonspecific.

Arevad and Keiding (1978) in Denmark used two susceptible laboratory strains of *Musca domestica* (17f and WHO-I) and two multiresistant field strains. With diflubenzuron mixed with their food the strains 17f and WHO-I showed LC_{50} values for ovicidal activity of about 100 mg a.i./l, whilst in our strain S this LC_{50} value was as low as 5.6 mg a.i./l. Furthermore their multi-resistant field strains showed LC_{50} values of about 10.000 mg a.i./l, whilst our Dutch field strains SL and AA showed LC_{50} values of only 47 and 490 mg a.i./l, respectively. Though they possess a considerable resistance, the two Dutch field strains are therefore far less resistant to the ovicidal effect of diflubenzuron than the two Danish field strains.

Summarizing the larvicidal and ovicidal resistance results obtained by feeding diflubenzuron we find only low or no larvicidal cross-resistance. This is in contrast to the much more severe occurrence of ovicidal cross-resistance, especially in field strains.

Development of larvicidal and ovicidal resistance

Characterization of the parental generations of strain S and strain Nic showed about the same larval susceptibility to diflubenzuron and a 3.5-fold lower ovicidal susceptibility of strain Nic, on the basis of the LC_{50} values. Furthermore adults of strain Nic proved to be more resistant to dimethoate, trichlorphon, and Parasect (table 1).

Both in strain Nic and in strain S the respective 13.6-fold and approximately 40-fold resistance factors at the LC_{50} level for larvicidal activity after respectively 26 and 40 generations cannot be considered high when compared with

other insecticides. However, the methods of induction of resistance as well as of testing larvicidal or adulticidal activity often differ considerably. An indication of the resistance levels of diflubenzuron and the juvenoid Methoprene, the latter used in a recent study of Georghiou *et al.* (1978), can be obtained by comparing the selection concentrations in the larval medium and the pupation medium, respectively. By doing so for diflubenzuron we find, for example, a 25-fold increase of the concentration in the larval medium of the F40 of strain S. For Methoprene the increase in the concentration in the pupation medium of the corresponding generations in the two test strains was about 100- and 130-fold. Furthermore, in the case of Methoprene, selection concentrations could be strongly increased in the successive generations, whilst with diflubenzuron a low threshold concentration is obvious (figs. 1 and 2).

The increase in resistance in the strains S and Nic was comparable to the increase in a mixture of resistant strains, including strain Nic, in an experiment by Oppenoorth and Van der Pas (1977). On comparison with strain W.H.O.-S.R.S. they found a 45-fold increase in the LC_{50} value in the F10. However, Arevad and Keiding (1977) found a total lack of resistance development in a multiresistant strain after selecting with diflubenzuron for 6 generations.

Selection in the larval medium of the housefly strains also effectuated development of resistance against the ovicidal effect of diflubenzuron. In strain S the larvicidal and ovicidal resistance ratios are comparable. However upon selection in the larval medium of strain Nic, the ovicidal resistance in this strain increased to a very high level. In the F26 of strain Nic even the highest test concentration of 3000 mg a.i./l of diflubenzuron resulted in only 26% mortality. As mentioned above, the parental generation of strain Nic only showed a 3.5-fold ovicidal cross-resistance when compared with strain S. The phenomena occurring upon larval selection with diflubenzuron indicate that additionally to a larvicidal resistance development, also an ovicidal resistance development takes place. Yet, it seems that the larvicidal and ovicidal levels of resistance are not linked to each other.

Other phenomena observed during selection with diflubenzuron

As selection progressed, also a marked decrease in the reproductive potential was observed as a result of an increased pupal mortality and a decreased fecundity and fertility. Consequently the selection pressure sometimes had to be lowered drastically. When this intermittence in selection pressure dit not take place in time, as had obviously been the case with strain Nic, a complete collapse of the strain occurred. Similar phenomena were observed also by Brown *et al.* (1978) during selection with diflubenzuron using the mosquito *Culex pipiens pipiens*. They obtained a 7-fold increase in the level of resistance in the F5, but this was followed by a decrease of the level of resistance and a total reproductive failure of the mosquito strain in the F11.

In our strain S a collapse was avoided by periodical deferring of selection in several generations and lowering of the selection dosage.

In practice this drop in reproductive potential at sublethal concentrations

might well impair the competition of the treated flies with untreated ones, and in this way prevent rapid resistance development in a population.

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VI EFFECTS OF DIFLUBENZURON ON MECHANICAL PENETRABILITY, CHITIN FORMATION, AND STRUCTURE OF THE ELYTRA OF *LEPTINOTARSA DECEMLINEATA.*

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Abstract—In untreated adults of *Leptinotarsa decemlineata* the mechanical penetrability of the elytra decreases until about 10 days after adult emergence. At any time during this period, this change in penetrability can be blocked by administering diflubenzuron. The blocking (by diflubenzuron) of the process by which the penetrability decreases shows identical kinetics as the inhibition of chitin formation by this compound.

Histological observations of elytra revealed several types of mesocuticles. Treatment with diflubenzuron causes characteristic distortions in each of them.

INTRODUCTION

DIFLUBENZURON is the common name for 1-(4-chlorophenyl)-3-(2,6-difluorobenzoyl)urea*. This compound was discovered as a larvicide. Its larvicidal mode of action or that of the related compound Du 19.111 [1-(2,6-dichlorobenzoyl)-3-(3,4-dichlorophenyl)urea] was studied by MULDER and GIJSWIJT (1973), POST and VINCENT (1973), POST *et al.* (1974) and DEUL *et al.* (1978). Evidence was given that diflubenzuron and Du 19.111 interfered with chitin synthesis in larvae.

However it is well known that chitin synthesis also occurs after emergence in adult insects. Effects of diflubenzuron on adult locusts were described by HUNTER and VINCENT (1974) and by KER (1977). On handling, we observed that the elytra of *Leptinotarsa decemlineata* (Colorado potato beetle) remained weak after feeding diflubenzuron to newly-hatched adults.

When we started our experiments we asked the following questions:

(1) What is the precise effect of diflubenzuron on the mechanical properties of the elytra?

(2) Can this observed weakness of the elytra be explained by the known mode of action of diflubenzuron, being a compound interfering with chitin synthesis, as studied previously in larvae?

(3) What histological changes occur in the elytra after treatment with diflubenzuron?

As no information was available on the histology of elytra of *L. decemlineata* we also studied the structure of untreated elytra.

MATERIALS AND METHODS

Larvae and adults of *L. decemlineata* were reared on potato plants at 24°C ± 1°C in 16 hr fluorescent light (TLF 40W/33) and 8 hr darkness per day.

The mechanical penetrability was measured as the resistance of the elytra to being punctured. To measure the resistance, a blunt needle (tip 0.3 mm diameter) was placed on the underside (concave side) of the elytron opposite the middle of one of the black stripes in the central region. The elytron itself was placed on a ring with a diameter of 3 mm. The needle was connected to a cantilever (Triple beam balance). At the start of the experiment at equilibrium the cantilever had a beaker with 2 l. of water at one end and a proper counterweight at the other. Thereafter, the pressure of the needle on the elytron was increased as a result of a constant outflow of water from the beaker. At the moment that the needle penetrated the elytron, the flow of water was stopped. The quantity of water lost was used as an arbitrary measure of the resistance of the elytron to being punctured. Only right side elytra were used. All material was tested in fresh condition at room temperature.

With this method of measuring the mechanical penetrability, the elytron first becomes deformed locally, followed by the intrusion of the needle. These two stages cannot be separated in the method used.

Diflubenzuron was used as a 1000 ppm aqueous suspension with a particle size of ≤ 1 μm. In the treatments, the potato plants were sprayed until run off and used after drying.

As a measure of chitin synthesis elytra were cut into pieces and boiled in 30% KOH for 15 min. Next they were washed six times with water, twice with 96% alcohol, and twice with ether. The elytra were weighed after drying.

Alternatively to measure chitin synthesis, 3-day-old adults were injected with 5 μl radioactive glucose (20 nCi/μl), or with 5 μl of a mixture containing 100 nCi glucose and 1 μg diflubenzuron. D-(6-^{14}C)-glucose (3.0 Ci/mole) was used as the radioactive label. After injection, adults were fed untreated potato leaves. The next day elytra were cut into pieces and boiled in 30% KOH for 15 min. The remaining part of the beetles was cut open and treated in the same way. The assay of labelled products as a measure of chitin synthesis was performed as described by POST *et al.* (1974).

* Code names: PH 60–40, TH 6040, Du 112.307, ENT-29054, OMS 1804, PDD 6040I. Registered trade mark: DIMILIN.

For histological observations elytra were fixed in Bouins fluid, cut at 6 μm and stained with Mallory's triple stain. With this stain the endocuticle is stained blue, the mesocuticle red and the exocuticle remains unstained. Observations were done using a light microscope.

All results were analysed using Student's *t*-test.

RESULTS

Mechanical penetrability of the elytra

In a preliminary experiment the penetrability of elytra of untreated, fully-grown males and females was compared. No statistically significant difference was found in the penetrability. ($P \geq 0.05$, $n = 20$). Therefore males and females were used at random in all further experiments.

Figure 1 illustrates the effect of contamination of adults with diflubenzuron on postemergence penetrability of elytra in two experiments. Under our experimental conditions the resistance to being punctured increases linearly during the first 10 days after adult eclosion.

After 10 days the resistance does not increase. For example, in normally-fed adults no significant increase in resistance was found in elytra of 36-day-old, as compared with those of 11-day-old adults. ($P \geq 0.05$, $n = 10$).

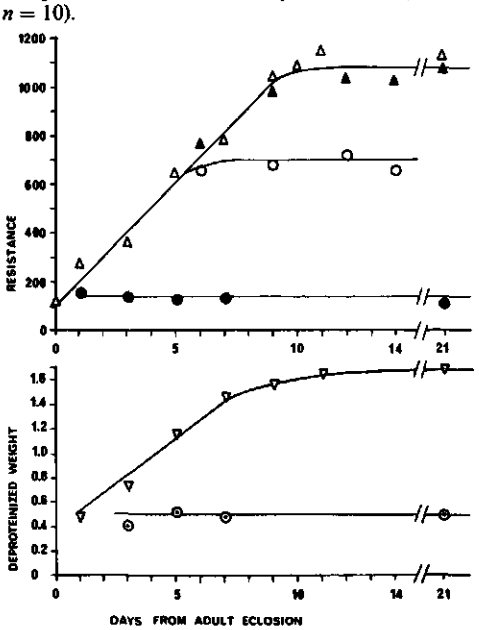


Fig. 1. Decrease of penetrability with time, expressed as the increase in resistance of elytra to being punctured (arbitrary units) and dry weight (mg) of deproteinized elytra of control and diflubenzuron treated adults of *L. decemlineata* (average of 10 right-sided elytra per treatment). The average variation coefficient of all treatments was 17%. Resistance measurements: △–△: Untreated (experiment 1). ▲–▲: Untreated (experiment 2). ○–○: Treated with diflubenzuron from day 5 onwards (experiment 1). ●–●: Treated with diflubenzuron from eclosion onwards (experiment 2). Assessment of the dry weight of deproteinized elytra: ▽–▽: Untreated. ⊙–⊙: Treated with diflubenzuron from eclosion onwards.

Figure 1 further illustrates that an increase in resistance to being punctured can be prevented fully by feeding diflubenzuron from adult emergence onward. When diflubenzuron treatment is started some days after emergence the increase in resistance is promptly and completely blocked.

Furthermore (not shown in Fig. 1) we observed that the inhibition could be abolished by the provision of untreated food. In that case, the increase in resistance resumed and after a short lag phase a similar rate of increase was obtained as in the controls. However, also in this case the process by which resistance increases appeared to be completed at 10 days postemergence. For example, feeding of treated food on the first day after eclosion, followed by 10 days with untreated food, resulted in a resistance of 645 arbitraty units (a.u.) whilst the control resistance at that time was 1127 a.u., and the resistance after permanent treatment was 128 a.u. The fact that the maximal level of resistance is reached after about 10 days in both controls and treated adults indicates that the control mechanism, by which resistance to being punctured increases, was unaffected by diflubenzuron.

Sufficient feeding appears to be a prerequisite for proper resistance development of the elytra as it did not occur in starved adults. In diflubenzuron treatments feeding behaviour seemed not to be influenced by the compound. In contrast with the diflubenzuron-treated adults, starved adults did not produce eggs.

Assessment of the dry weight of deproteinized elytra

Figure 1 also illustrates that in control beetles the increase in dry weight of deproteinized elytra increases linearly until about 8 days from adult eclosion. After 8 days, the dry weight does not increase much. Furthermore it can be seen that diflubenzuron treatment causes the dry weight increase to cease fully. In starved adults (not shown in Fig. 1) no increase in dry weight of deproteinized elytra occurred.

Incorporation of labelled glucose into the chitin fractions

Table 1 represents the interference of diflubenzuron with chitin formation in the elytra and in the remaining part of the beetles. From Table 1 it appears that in the control beetles, during the 4th day after emergence, one-third of the total chitin synthesis takes place in the elytra. One day after injection of 3-day-old adults with 1 μg diflubenzuron, chitin synthesis in both elytra and the remaining parts of the body appears to be inhibited to almost the same extent, namely 81 and 74% respectively.

Histology

Figure 2 shows schematically the upperside of an elytron of *L. decemlineata* with alternating black and yellow stripes. A large number of pits is also indicated. These pits are usually near to the border between the stripes. Rather shallow pits can be found at corresponding spots on the underside of the elytron.

Upon making a transverse section through the elytron, we observed that the black and yellow stripes consist of hard and compact material. The underside of the elytron is transparent and relatively tough if opposite a black stripe. Opposite a yellow stripe the

Table 1. Effects of diflubenzuron on incorporation of radioactivity from glucose into 3-day-old adults of *Leptinotarsa decemlineata*. Beetles were killed 1 day after treatment

Treatment	[^{14}C]-glucose incorporation* and percentage inhibition			
	Elytra		Remaining part of the beetles	
	ng	% Inhibition	ng	% Inhibition
100 nCi glucose	193(±44)	0	399(±59)	0
100 nCi glucose + 1 μg diflubenzuron	36(±12)	81	105(±41)	74

* Expressed as ng glucose per 10 adults, S.D. between brackets.

underside is white and consists of an irregular network of fibres. The inside of an elytron is hollow, containing cells and haemolymph. This cavity also contains pillars which connect the upper and underside. The location of these pillars completely coincides with that of the pits.

Hence, the inter-connections are not ridges which extend along the border of the stripes of an elytron but can fully be compared to pillars.

Ten days after adult emergence, transverse sections were made of elytra from control and treated adults. A schematic interpretation of the layers in elytra of untreated adults is given in Fig. 3.

In a transverse section we can distinguish differences in structure under the black and yellow stripes. Occasionally they are separated by a pillar and alternately succeed each other.

The structure under a black stripe consists of a black upper exocuticle, an orange–yellow upper and lower mesocuticle, and an amber lower exocuticle. The structures under a yellow stripe differ from those under a black stripe by virtue of the amber upper exocuticle and the lower mesocuticle being stained red. The latter is also thicker than the lower mesocuticle under a black stripe. Both upper mesocuticles show a vaguely layered structure. In the lower mesocuticles layering is more pronounced. Only under the yellow stripes are the layers in the lower mesocuticle alternately lamellated. The layers in the lower mesocuticle under a yellow stripe have a thickness of about 3–4 μm. The corresponding layers under the black stripes have a thickness of about 1.5–3 μm. In the fully developed elytra the number of layers is 9 or 10.

Features common to structures under yellow and black stripes are:

(1) The upper exocuticle is thicker than the lower exocuticle.

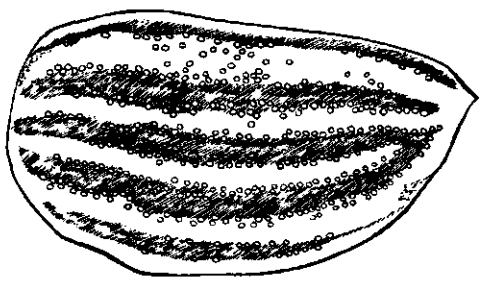
(2) The upper epidermal cells are smaller than the lower ones.

(3) Both the upper and the lower cuticles contain pore canals.

(4) With Mallory's triple stain no blue-coloured layers in the cuticle could be distinguished.

In diflubenzuron-treated elytra the structures are drastically changed. Under a black stripe we observe a thin band of the original orange–yellow upper mesocuticle (b in Fig. 3) which was already deposited before the onset of feeding. As a result of the treatment a thicker, amorphous yellow zone becomes visible below it. Instead of the orange–yellow lower mesocuticle (c in Fig. 3), upon treatment with diflubenzuron a compact yellow zone is deposited in which the layers can now be distinguished only vaguely. As a result of diflubenzuron, under a yellow stripe a thicker amorphous orange–red zone is deposited under the thin original orange–yellow upper mesocuticle (f in Fig. 3). In the lower mesocuticle (g in Fig. 3) treatment results in the formation of irregularly-stained, loosely connected layers, instead of the alternately lamellated, red layers.

Pore canals can also be found in affected layers.



0.1 cm

Fig. 2. Upperside (convex side) of a right-sided elytron of *L. decemlineata*. The dots schematically indicate the position of the pits. Black and yellow stripes are also indicated.

Pillars show a black or yellow centre, depending on their position either under a black or a yellow stripe. It is formed by the inward bending of the upper exocuticle closely above the lower exocuticle. Around this central column layers of upper mesocuticle are found (b or f in Fig. 3). These layers penetrate as wedges into the lower mesocuticle. Treatment with diflubenzuron affects the mesocuticles in the pillars in the same way as described for the upper mesocuticles under the black and yellow stripes.

As we saw before in starved adults, hardening and increase in dry weight of deproteinized elytra are also fully absent. Histological observations after 10 days of such elytra revealed that they consist only of a normal upper and lower exocuticle. All other structures are so reduced in size that they become indistinguishable.

DISCUSSION

Mechanical penetrability of elytra

Insect cuticles consist basically of a network of chitin and protein. The chitin forms a structural framework around which the protein molecules arrange themselves. During postemergence growth the following mechanisms might influence the penetrability of elytra: overall changes in thickness, interactions between protein chains, protein tanning and interactions between protein and chitin.

The results of the measurements of the penetrability of elytra (Fig. 1) demonstrate that diflubenzuron can fully block the process by which the resistance to being punctured increases.

In this paper the residue after KOH treatment is designated as 'deproteinized elytra'. As it will mainly consist of chitin, it can be used as an indication of the chitin content. The increase in dry weight of deproteinized elytra follows a pattern similar to that found in legs and wings of *Schistocera gregaria* by CANDY and KILBY (1962). The diflubenzuron-induced blocking of growth corresponds with the decrease in incorporation of radioactivity from glucose by diflubenzuron (Table 1). Both the resistance of elytra to being punctured and the dry weight of deproteinized elytra increase linearly after adult eclosion. In the case of the resistance this continues for about 10 days, and the dry weight of deproteinized elytra continues to increase for about 8 days after adult eclosion. The beetles used for the assessment of the dry weight of deproteinized elytra are from the same group as those used in experiment 1 for the measurement of resistance. It is therefore unlikely that this difference in bending point can be attributed to differences in beetles. It might support the idea that still another process is involved in the increase in resistance. In that case this other process would still contribute to the increase in resistance for a short time after chitin deposition has finished. However, during the first 8 days chitin deposition is seemingly a prerequisite for a contribution of this other process to the increase in resistance.

Table 1 shows that by injecting of both diflubenzuron and radioactive glucose into 3-day-old adult beetles an 81% decrease in chitin formation in the elytra occurs within 1 day. In this experiment we did not observe a 100% inhibition of chitin formation within 1 day because this inhibition is not instantaneous. We may therefore conclude from Table 1 that in the elytra, diflubenzuron prevents chitin synthesis in an analogous way to that found in larvae of *Pieris brassicae* by DEUL *et al.* (1978) and with a related compound (Du 19.111) by POST *et al.* (1974). This indication of inhibition of chitin synthesis is supported by the observation that the dry weight in deproteinized elytra from continuously-treated insects does not increase (Fig. 1).

In our experiments with elytra of *L. decemlineata* the similarity of the diflubenzuron-effect on both the increase in resistance and the chitin formation is obvious. However, we still can not completely rule out the possibility that diflubenzuron affects either incorporation of cuticular protein or tanning. With *P. brassicae*, POST *et al.* (1974) found no indications of an effect of Du 19.111 (a diflubenzuron-related compound) on the incorporation of tyrosine and proline. HUNTER and VINCENT (1974) and KER (1977),

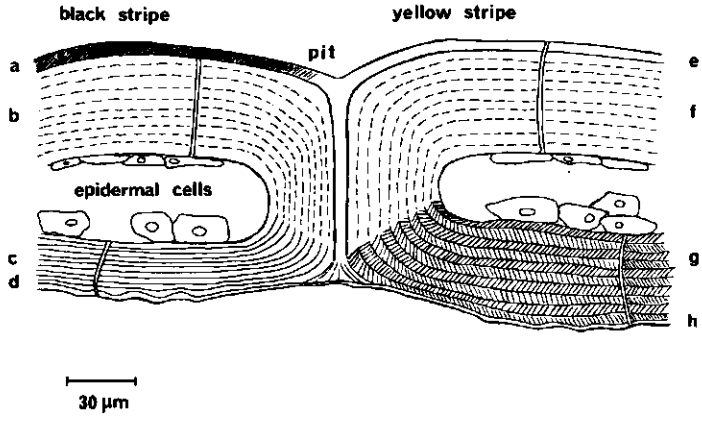


Fig. 3. Schematic representation of the layers in the elytra of untreated adults of *L. decemlineata*. (a) Upper exocuticle (stains black); (b) upper mesocuticle (stains orange–yellow; (c) lower mesocuticle (stains orange–yellow); (d) lower exocuticle (stains amber); (e) upper exocuticle (stains amber); (f) upper mesocuticle (stains orange–yellow); (g) lower mesocuticle (stains red); (h) lower exocuticle (stains amber).

with locust species found no effects of diflubenzuron on either protein content or the amino acid composition. As regards the tanning, HUNTER and VINCENT (1974) concluded from their experiments that tanning was not influenced by diflubenzuron. From histological observations KER (1977) came to the same conclusion. In these insects we may conclude therefore that diflubenzuron inhibits chitin synthesis but that there is as yet no evidence of any other direct effect on the cuticle.

We assume that these findings are also valid for the elytra of diflubenzuron-treated adults of *L. decemlineata*. The plausibility of our hypothesis is supported by the observation that the mesocuticle continues to grow during diflubenzuron treatment. Therefore, since the synthesis of chitin is arrested (Table 1), this can only be due to protein synthesis.

Histology

In accordance with RICHARDS (1967), the terms exo-, meso-, and endocuticle, used in this paper, are based on the histological criteria visible with Mallory's triple stain. Using this method the fully-developed cuticle consists of an exocuticle which is refractory to staining and shows an amber, black or brown colour, a mesocuticle staining red and an endocuticle staining blue. KRZELJ (1969) also used Mallory's stain. However, he called the unstained layers epicuticle, the carmine red layers exocuticle, and the blue layers endocuticle. In elytra of two species also belonging to the Chrysomelidae (*Timarcha tenebricosa* and *Chrysomela sanguinoleta*) he similarly observed no endocuticle. The relatively large red-staining proportion of the elytra agrees with the observations of RICHARDS (1967) that structures known to be elastic usually seem to be or to contain structures staining red with Mallory's stain.

As far as we know, no literature is available on the structure of elytra of *L. decemlineata*. In our opinion they show some resemblance to the elytra of *Tenebrio molitor*, as described by HUNDERTMARK (1935). However, the elytra of *T. molitor* can be described in terms of only one basic structure, whereas in the elytra of *L. decemlineata* we can distinguish two basic structures, viz. below the black and the yellow stripes.

In diflubenzuron-treated elytra chitin synthesis is arrested (Fig. 1, Table 1) but in spite of that, the thickness of the mesocuticle still increases. In normal elytra, the structures under the black and the yellow stripes differed in their lower mesocuticles but had seemingly similar upper mesocuticles. Yet, these upper mesocuticles reacted differently to diflubenzuron treatment. We can therefore conclude that the upper and lower mesocuticles under both the black and yellow stripes differ in structure. The observation that the thickness of the cuticle continues to increase even after treatment is in agreement with HUNTER and VINCENT (1974) and KER (1977). It indicates that protein synthesis is not affected by diflubenzuron.

On the contrary, in larval cuticles of *Pieris brassicae* (MULDER and GIJSWIJT, 1973) and *L. decemlineata* (GROSSCURT, 1978) no distinct cuticular growth takes place after diflubenzuron treatment. Using the same fixation methods apparently only globular masses can be observed after the treatment. Therefore the statement of HUNTER and VINCENT (1974) that *P. brassicae* larval cuticle apparently does not grow after treatment with diflubenzuron, referring to MULDER and GIJSWIJT (1973), is not warrented. An explanation for their statement might be that the thickness as measured by MULDER and GIJSWIJT (1973) related to the undamaged layers and neglected protein deposition in the globules. POST *et al.* (1974) did indeed find amino acid incorporation in endocuticular tissue of *P. brassicae* after diflubenzuron treatment. The differences in effects of diflubenzuron on the cuticles of elytra of *L. decemlineata* on one hand and larval cuticles on the other are presumably caused by differences in the stabilisation of protein in the affected tissue.

Summarizing the histological changes we observed an increase in thickness of diflubenzuron-treated elytra. However this did not coincide with any measurable changes in penetrability. This rules out the possibility that decrease in penetrability is due to thickness increase. From the properties of diflubenzuron as an inhibitor of chitin synthesis we may conclude that effects on penetrability are due to interference of diflubenzuron with chitin-protein bonding in the elytra. However, a direct effect on tanning cannot completely be ruled out. From the literature there are no data available on this matter with respect to *L. decemlineata*. With locust species, HUNTER and VINCENT (1974) and KER (1977) also found no effect of diflubenzuron on tanning.

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VII EFFECTS OF DIFLUBENZURON ON SOME CHEMICAL AND MECHANICAL PROPERTIES OF THE ELYTRA OF *LEPTINOTARSA DECEMLINEATA*

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SUMMARY

Effects of diflubenzuron on elytra of *Leptinotarsa decemlineata* were measured during the first 11 days after adult emergence. The parameters studied were mechanical penetrability, amounts of chitin and protein, and the yield of ketocatechols.

The amount of protein in the elytra was the only parameter which was unaffected by the treatment. Chitin synthesis and change in mechanical penetrability were strongly affected by diflubenzuron: both parameters were inhibited by 50% at a concentration between 3 and 10 mg a.i./l. The yield of ketocatechols, a parameter which has been related to the degree of tanning, was inhibited by 50% at a diflubenzuron concentration between 300 and 1000 mg a.i./l. The results could indicate that the interference of diflubenzuron with the tanning process may be of a secondary nature.

INTRODUCTION

In a previous paper (Grosscurt, 1978a) it was shown that after adult emergence of *Leptinotarsa decemlineata* (Colorado potato beetle), chitin synthesis in the elytra did occur until about 10 days after emergence. Concomitantly, an increase in resistance to mechanical penetrability of the elytra was observed. Both chitin synthesis and increase in resistance of the elytra could be blocked by administering diflubenzuron*. Despite a complete blockage of chitin synthesis, the mesocuticle continued to grow throughout diflubenzuron treatment, and we

* Diflubenzuron is the common name for 1-(4-chlorophenyl)-3-(2,6-difluorobenzoyl) urea. Code names: PH 60–40, TH 6040, Du 112.307, ENT-29054, OMS 1804, PDD 6040 I. Registered trade mark of Philips-Duphar B.V. of diflubenzuron formulations: DIMILIN®

assumed that protein synthesis was not arrested. However, in the above mentioned study no quantitative or qualitative observations were made on actual protein synthesis or tanning.

Tanning or sclerotization in the insect cuticle is a process during which the cuticular proteins are rendered less soluble, and the overall structure gradually becomes harder. A widely accepted model for this process is that tanning is due to the introduction of covalent cross-links between the protein molecules. Two models have been suggested for the formation of cross-links: The model proposed by Pryor (1940) is based on the cross-linking of cuticular proteins by ortho-quinones. Andersen and Barrett (1971) suggested a model which in many respects resembles the scheme of quinone tanning, but bonds are assumed to be formed between the proteins and the aliphatic side chain adjacent to the aromatic ring in N-acetyl-dopamine. This pathway is called β-sclerotization (for details see review by Andersen, 1979). By acid hydrolysis of cuticles, the aromatic residues are assumed to be released from the proteins in the form of ketocatechols.

Another model for cuticular tanning, to which litle attention has been paid so far, was recently rediscussed by Vincent and Hillerton (1979). In their opinion dehydration is the all-important factor in stabilizing insect cuticle. The dehydration process may be achieved by introduction of quinones which selectively occupy strongly hydrated groups on structural proteins. Some sort of relation also can be assumed in this model between tanning and incorporation of aromatic material.

The effects of diflubenzuron or the related benzoylurea Du 19.111 (1-(2,6-dichlorobenzoyl)-3-(3,4-dichlorophenyl)urea) on protein deposition in cuticle have been reported for several insect species, and it appears that while protein synthesis and secretion are not affected, the ordered deposition of protein in the cuticle may be disturbed to varying extents. Histology reveals drastic disturbances in soft, non-sclerotized cuticle, where cuticular material appears as separate droplets and not in organized layers (Mulder and Gijswijt, 1973; Grosscurt, 1978b). According to Hunter and Vincent (1974) and Ker (1977) in the cuticles of adult locusts neither protein deposition nor tanning seems to be affected. However, the authors did not present data as far as the effect on tanning is concerned. The data published so far have led to the conclusion that diflubenzuron and the related benzoylurea Du 19.111 primarily inhibit chitin synthesis and that there is as yet no evidence of any other primary effect on the cuticle (Post *et al.*, 1974; Deul *et al.*, 1978; Gijswijt *et al.*, 1979).

The aim of the present study is to determine any effects of diflubenzuron on depostion of protein and on the degree of cuticular sclerotization in the elytra of *L. decemlineata*, in order to decide whether the changes in mechanical penetrability are caused only by the reduction in chitin content or whether the

VII EFFECTS OF DIFLUBENZURON ON SOME CHEMICAL AND MECHANICAL PROPERTIES OF THE ELYTRA OF *LEPTINOTARSA DECEMLINEATA*

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SUMMARY

Effects of diflubenzuron on elytra of *Leptinotarsa decemlineata* were measured during the first 11 days after adult emergence. The parameters studied were mechanical penetrability, amounts of chitin and protein, and the yield of ketocatechols.

The amount of protein in the elytra was the only parameter which was unaffected by the treatment. Chitin synthesis and change in mechanical penetrability were strongly affected by diflubenzuron: both parameters were inhibited by 50% at a concentration between 3 and 10 mg a.i./l. The yield of ketocatechols, a parameter which has been related to the degree of tanning, was inhibited by 50% at a diflubenzuron concentration between 300 and 1000 mg a.i./l. The results could indicate that the interference of diflubenzuron with the tanning process may be of a secondary nature.

INTRODUCTION

In a previous paper (Grosscurt, 1978a) it was shown that after adult emergence of *Leptinotarsa decemlineata* (Colorado potato beetle), chitin synthesis in the elytra did occur until about 10 days after emergence. Concomitantly, an increase in resistance to mechanical penetrability of the elytra was observed. Both chitin synthesis and increase in resistance of the elytra could be blocked by administering diflubenzuron*. Despite a complete blockage of chitin synthesis, the mesocuticle continued to grow throughout diflubenzuron treatment, and we

* Diflubenzuron is the common name for 1-(4-chlorophenyl)-3-(2,6-difluorobenzoyl) urea. Code names: PH 60–40, TH 6040, Du 112.307, ENT-29054, OMS 1804, PDD 6040 I. Registered trade mark of Philips-Duphar B.V. of diflubenzuron formulations: DIMILIN®

assumed that protein synthesis was not arrested. However, in the above mentioned study no quantitative or qualitative observations were made on actual protein synthesis or tanning.

Tanning or sclerotization in the insect cuticle is a process during which the cuticular proteins are rendered less soluble, and the overall structure gradually becomes harder. A widely accepted model for this process is that tanning is due to the introduction of covalent cross-links between the protein molecules. Two models have been suggested for the formation of cross-links: The model proposed by Pryor (1940) is based on the cross-linking of cuticular proteins by ortho-quinones. Andersen and Barrett (1971) suggested a model which in many respects resembles the scheme of quinone tanning, but bonds are assumed to be formed between the proteins and the aliphatic side chain adjacent to the aromatic ring in N-acetyl-dopamine. This pathway is called β-sclerotization (for details see review by Andersen, 1979). By acid hydrolysis of cuticles, the aromatic residues are assumed to be released from the proteins in the form of ketocatechols.

Another model for cuticular tanning, to which litle attention has been paid so far, was recently rediscussed by Vincent and Hillerton (1979). In their opinion dehydration is the all-important factor in stabilizing insect cuticle. The dehydration process may be achieved by introduction of quinones which selectively occupy strongly hydrated groups on structural proteins. Some sort of relation also can be assumed in this model between tanning and incorporation of aromatic material.

The effects of diflubenzuron or the related benzoylurea Du 19.111 (1-(2,6-dichlorobenzoyl)-3-(3,4-dichlorophenyl)urea) on protein deposition in cuticle have been reported for several insect species, and it appears that while protein synthesis and secretion are not affected, the ordered deposition of protein in the cuticle may be disturbed to varying extents. Histology reveals drastic disturbances in soft, non-sclerotized cuticle, where cuticular material appears as separate droplets and not in organized layers (Mulder and Gijswijt, 1973; Grosscurt, 1978b). According to Hunter and Vincent (1974) and Ker (1977) in the cuticles of adult locusts neither protein deposition nor tanning seems to be affected. However, the authors did not present data as far as the effect on tanning is concerned. The data published so far have led to the conclusion that diflubenzuron and the related benzoylurea Du 19.111 primarily inhibit chitin synthesis and that there is as yet no evidence of any other primary effect on the cuticle (Post *et al.*, 1974; Deul *et al.*, 1978; Gijswijt *et al.*, 1979).

The aim of the present study is to determine any effects of diflubenzuron on depostion of protein and on the degree of cuticular sclerotization in the elytra of *L. decemlineata*, in order to decide whether the changes in mechanical penetrability are caused only by the reduction in chitin content or whether the stabilized protein matrix is also important in this respect.

MATERIALS AND METHODS

Larvae and adults of *L. decemlineata* were reared on potato plants at

24°C ± 1°C in 16 hr fluorescent light (TLF 40 W/33) and 8 hr darkness per day. The freshly emerged beetles were collected daily from the rearing cages, in which no food was supplied. From the first day they were fed either untreated or diflubenzuron treated potato plants.

Diflubenzuron was applied as an aqueous suspension with a particle size of ≤ 1 μm. In the treatments, the potato plants were sprayed with the suspension until run off. They were used after drying.

Mechanical penetrability of the elytra was assessed as described by Grosscurt (1978a). For chemical analysis of the elytra they were dried to constant weight at 100°C, and hydrolyzed by boiling in 100 ml 1 M HCl for 5 hr. The residue was washed in water, redried and weighed. The supernatant was concentrated and fractioned on a column of Bio-Gel P-2, and the amount of ketocatechols was determined according to Andersen and Barrett (1971). Alternatively hydrolysis was done by boiling in 30% KOH for 15 min. Next they were washed six times with water, twice with 96% alcohol, and twice with ether.

In this paper the residue obtained after treatment of the elytra with acid is called "chitin". Though this residue will consist mainly of chitin, small amounts of epicuticular material and melanin may still be left (the black stripes were clearly visible after hydrolysis). The material solubilized by acid hydrolysis is called "protein", but it also contains small amounts of phenolic material such as ketocatechols.

In this paper we assessed the dosages of diflubenzuron needed for 50% decrease in mechanical resistance, chitin content and ketocatechol content. The dosages are calculated using the values of the parameters which are halfway between the base levels obtained on elytra from recently emerged (0–1 day old), untreated, starved animals and the values from untreated, mature (11 days old), and normally fed animals.

RESULTS

Fig. 1 illustrates the effects of feeding Colorado potato beetles with potato plants, sprayed with 1000 mg a.i./l diflubenzuron, for various lengths of time after eclosion. It is clear that the diflubenzuron treatment blocks the normal increase in mechanical resistance of the elytra and the increase in chitin content, determined as the residue left after hydrolysis in 1 M HCl. In table 1 a comparision is given of the assessments of the residues by boiling in either 1 M HCL or in 30% KOH.

The amounts of protein removed by hydrolysis of elytra of both treated and untreated beetles are variable, but no indication of any influence exerted by the diflubenzuron treatment is observed. The yield of ketocatechols has been partly affected at 1000 mg a.i./l diflubenzuron: at 3, 5, 7, and 10 days after adult eclosion the level of ketocatechols in elytra of treated beetles was, respectively, 54%, 50%, 41%, and 45% of the controls.

From the results presented in fig. 1 it is difficult to decide whether the blocking of the increase in mechanical resistance of the elytra is due to the inhibition of chitin synthesis, to the roughly 50% lower yield of ketocatechols,

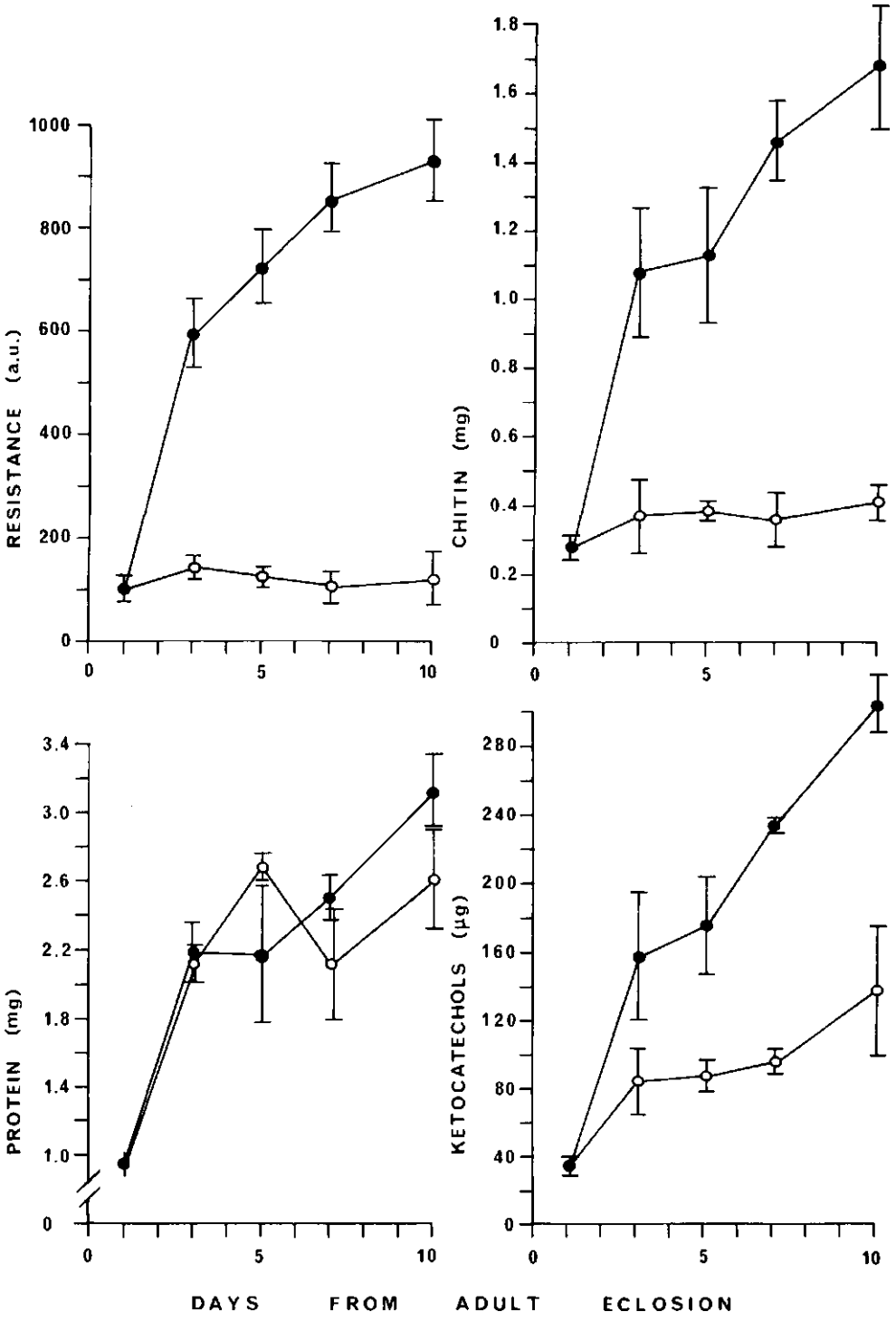

Fig. 1. Effects of diflubenzuron with time on mechanical resistance to penetrability, chitin and protein levels, and the yield of ketocatechols in elytra of *L. decemlineata*. Beetles were treated from adult eclosion onwards. Each point is the mean ± S.D. of 2–6 elytra. The beetles used for assessment of resistance in the elytra belong to another group than those used for assessment of the other parameters. Closed dots: untreated. Open dots: adults fed with potato plants treated with 1000 mg a.i./l of diflubenzuron from adult eclosion onwards.

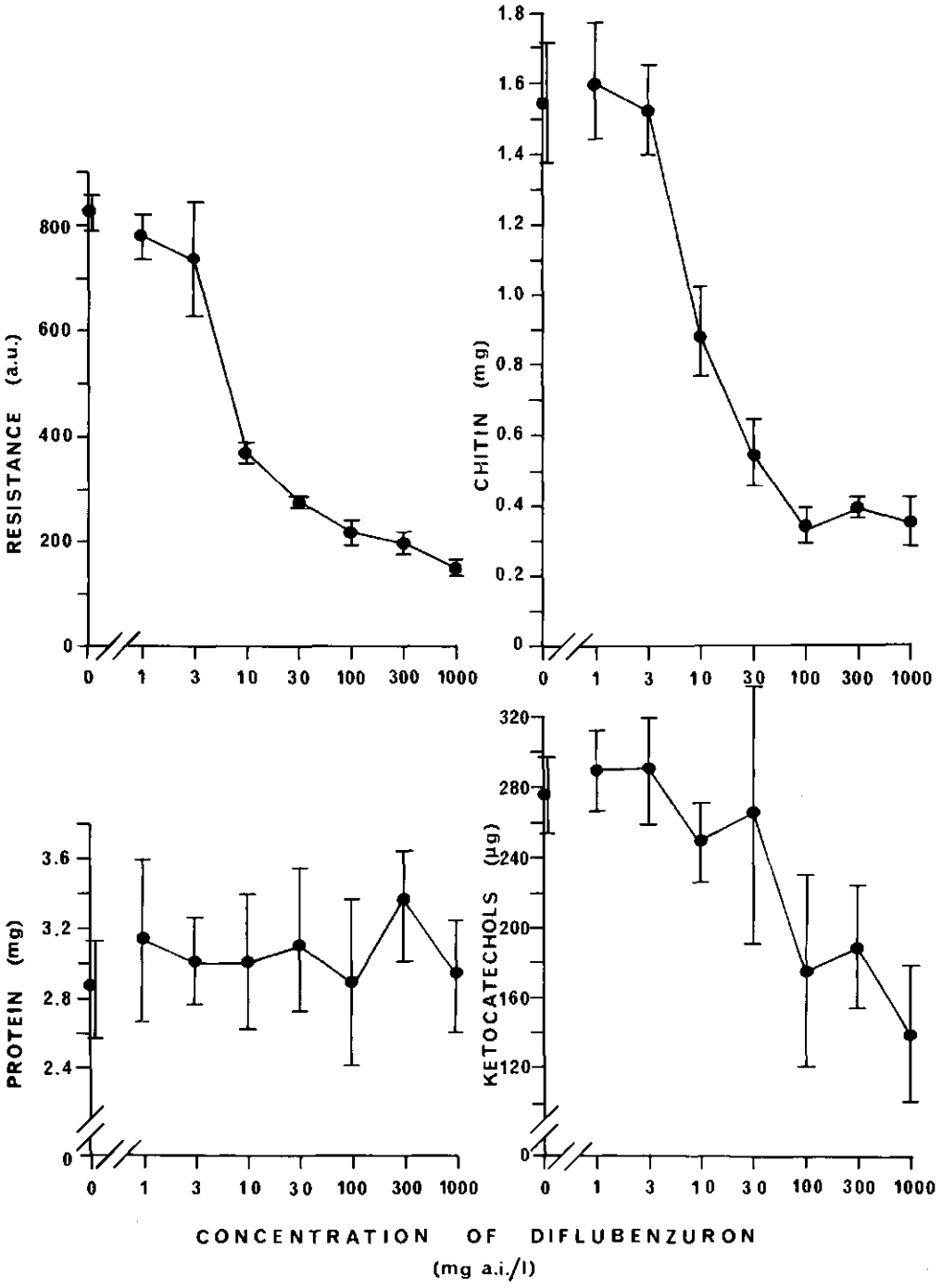

Fig. 2. Effects of several concentrations of diflubenzuron on resistance to mechanical penetrability, chitin and protein levels and yield of ketocatechols in elytra of 11 day-old adults of *L. decemlineata*. Beetles were treated from adult eclosion onwards. Each point is the mean ± S.D. of 6 elytra. All parameters were assessed using the same elytra.

Table 1. Effects of diflubenzuron on the average dry weight (mg) of residues of elytra from 11-day-old adults of *Leptinotarsa decemlineata* *

Diflubenzuron treatment (mg a.i./l)	average dry weight (mg) of residues obtained by hydrolysis in	
	1 M HCl (n = 6)	30% KOH (n = 10)
0	1.5	2.0
1	1.6	1.8
3	1.5	1.7
10	0.9	0.9
30	0.5	0.5
100	0.3	0.4
300	0.4	0.4
1000	0.4	0.2

* Beetles were fed with the required concentration of diflubenzuron from adult eclosion onwards.

or to both. We repeated the experiment therefore but this time varied the concentration of diflubenzuron in order to determine which parameter was the most sensitive. Concomitantly, the resistance to mechanical puncture and the protein content of the elytra were also assessed.

The results (fig. 2) confirm that the amount of protein is not affected at the concentrations of diflubenzuron used. By interpolation, the dosages needed for 50% decrease in the value of both the mechanical resistance and the chitin content are found between 3 and 10 mg a.i./l. For the yield of ketocatechols this value is found between 300 and 1000 mg a.i./l (table 2). We conclude that the susceptibility for diflubenzuron of the mechanical resistance and the chitin

Table 2. Average values (n = 10) of several parameters from untreated elytra of *Leptinotarsa decemlineata*

	mechanical resistance (a.u.)	chitin (mg)	ketocatechols (μg)
0–1 day old, starved adults	128	0.31	45
11 days old, normally fed adults	826	1.55	275
interpolated value at which 50% of the total increase is obtained	477	0.93	160
corresponding dosage (mg a.i./l) needed for 50% decrease in value (obtained from fig. 2)	between 3 and 10	between 3 and 10	between 300 and 1000

content of the elytra are of the same order of magnitude, but that it differs significantly for the yield of ketocatechols.

DISCUSSION

The mechanical resistance of the elytra has been determined as the force needed to penetrate the cuticle with a blunt needle (0.3 mm tip diameter). While this cuticular property cannot be related to the stiffness of the material, it will presumably be related to its breaking strength and may be a useful indicator of how effective the cuticle will be as a shield protecting the insect from predators.

The "chitin" content has been determined as the residue left alter hydrolysis in 1 M HCl as well as the residue after boiling in 30% KOH. Despite the contrast in pH's between the two extraction methods, results (table 1) nevertheless indicate a great similarity in efficiency of the extraction. The "protein" content is determined as the weight loss during acid hydrolysis. It can be asumed that this method will slightly overestimate the amounts of protein, since some other materials may also be degraded by this treatment. The phenolic material released as ketocatechols amounts to about 10% of the acid-solubilized material, and the "protein" content has not been corrected for this contribution, since it is less than the standard deviation.

The results presented in Fig. 1 show that the increase in mechanical resistance and chitin contents can be fully blocked, when adults of the Colorado potato beetle are fed potato plants sprayed with a concentration of 1000 mg a.i./l of diflubenzuron. The results confirm those presented in a previous paper (Grosscurt, 1978a).

With regard to the protein content of the elytra, the results obtained with 1000 mg a.i./l of diflubenzuron in the food of adults show no significant effect during the treatment up to 10 days after adult eclosion (fig. 1). The conclusion is confirmed by the results obtained with various concentrations of diflubenzuron and measured 11 days after eclosion (fig. 2). Protein extractability, and thus probably synthesis and deposition, have apparently not been quantitatively disturbed.

Table 2 and fig. 2 clearly demonstrate that the mechanical penetrability of the elytra of the Colorado potato beetle is correlated to the chitin content. The protein content is not influenced by the diflubenzuron treatment (fig. 2), and the yield of ketocatechols is affected for 50% at very high dosages. The differences in sensitivity between mechanical resistance and chitin on one hand and ketocatechols on the other may indicate the secondary nature of the effect of diflubenzuron on the latter.

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SUMMARY

Diflubenzuron is the common name for 1-(4-chlorophenyl)-3-(2,6-difluorobenzoyl)urea, the active ingredient of the insecticide Dimilin.

Diflubenzuron was discovered in 1971 as a larvicide. Evidence was provided by several authors that the larvicidal effect of this compound was caused by its interference with chitin synthesis. Chitin is one of the main components of the insect cuticle. As a result, treatment of larvae with diflubenzuron prevents normal growth of the cuticle in many cases. In most susceptible insect species this effect expresses itself as an incapacity of larvae to moult.

During the development of diflubenzuron it was discovered that in some species the compound additionally showed an interesting ovicidal activity, caused either by treatment of females (papers I and II), or by direct contact with eggs (paper II).

In paper I is shown that in the housefly *(Musca domestica)* diflubenzuron can evoke ovicidal effects by topical application to adult females, by oral uptake or by injection of the compound. It is remarkable, however, that in all cases of ovicidal activity the embryo inside the egg appears to develop normally. However, the apparently full-grown embryo turned out to be unable to hatch.

Treatment with diflubenzuron did not influence the fecundity of the females. The compound also had no effect on fertilization of the eggs.

In order to study the role of male insects in the ovicidal phenomenon, adult males of the housefly (paper I) or of the Colorado potato beetle, *Leptinotarsa decemlineata* (paper II) were injected with 5 μg of diflubenzuron before they were allowed to mate with untreated females. In this type of experiment no effect on fertilization or on egg mortality was observed. It was therefore concluded that diflubenzuron does not exert its ovicidal activity through the males of these two insect species.

Based upon the above mentioned experiments it was concluded that the activity of diflubenzuron on eggs of insects could best be described as an ovicidal effect of the compound rather than a type of chemosterilization.

To finally prove that chemosterilization plays no role whatsoever in the mode of action of diflubenzuron, embryos from eggs laid by treated females of the Colorado potato beetle were examined under the electron microscope (paper III, figures 3 and 4). It was found that instead of the normal lamellate cuticle

deposition patterns that could be observed in untreated embryos, the diflubenzuron treatment resulted in an amorphous cuticle. Again, these observations suggest an identical mode of action of diflubenzuron on larvae and embryos. As a result, by virtue of a lack of rigidity in the embryonic cuticle, treated embryos cannot use their muscles in the process of egg hatching.

We also found that after termination of adult treatment with diflubenzuron, the percentage of egg eclosion again increases. The degree of this reversibility depends a.o. on the concentration of diflubenzuron during the previous treatment (paper I).

Many factors appeared to influence the ovicidal activity of diflubenzuron in the case of direct contact activity on susceptible eggs. From the literature there was already evidence that the particle size of the active ingredient plays an important role. In paper II is shown that the susceptibility of eggs of the large cabbage white *(Pieris brassicae)* decreased with increasing age. With eggs of the Colorado potato beetle we also observed that egg mortality increased with increasing relative humidity. The addition of surfactants to the spray liquid also proved to be necessary sometimes to obtain a sufficient coverage of the eggs

With eggs of the leaf miner *Leucoptera scitella* we found that the concentration of diflubenzuron in the spray liquid influenced the moment mortality occurred. Generally, at rates of diflubenzuron between 100 and 1 mg a.i./l, mortality occurred either in the egg stage or in the early larval stages. However, at lower concentrations the contributions of the ovicidal mortality to the overall activity of diflubenzuron decreased.

Several aspects of the larvicidal activity of diflubenzuron such as feeding and contact activity, larval behaviour after treatment, and differences in susceptibility are described in paper III. This article also deals with the resumption of normal cuticle deposition in larvae of the Colorado potato beetle, after termination of diflubenzuron treatment, which is illustrated with histological observations.

In paper IV a number of benzoylureas, with variations in lipophilic properties were tested for their larvicidal and ovicidal activities on the housefly. In larvicidal tests the compounds were incorporated into the larval culture medium. Ovicidal activities were assessed on female adults either by feeding or by injection.

Correlation of biological activities by means of a physical chemical para-

meter revealed that the ovicidal activity after injection was the basic activity in both the ovicidal activity by adult feeding and in the larvicidal activity. However, in either case this activity was coupled to a lipophilic parameter, which was expressed as π in the octanol/water system. Optimum π values for the ovicidal activity after adult feeding and for the larvicidal activity were 1.0 and 1.5, respectively.

It is likely that at increasing lipophilicity of the compounds above the optimum π values, the gut wall becomes an important barrier. Though penetration also decreases when the lipophilicity decreases to below the optimum π value, it is possible that the activity at the target site is too low to allow the gut barrier to play an important role.

The properties of diflubenzuron in connection with cross-resistance and with the development of resistance in the housefly are described in paper V. Upon selection with diflubenzuron, incorporated into the larval culture medium, development of both larvicidal and ovicidal resistance was studied in a susceptible laboratory strain (S) and in a multiresistant strain (Nic). Due to the decrease in reproductive potential upon progressive selection, the breeding of strain Nic had to be determined in the F32. Observations for this study with strain S were discontinued in the F40.

The larval resistance against diflubenzuron in both strains increased slowly. The final assessment of larval resistance in strain Nic, which was made in the F26, showed a 13.6-fold decrease in susceptibility when based on LC_{50} values. In the F40 of strain S this factor was 40-fold.

With respect to the egg resistance against diflubenzuron in the F40 of strain S we measured a similar factor as compared to the larval resistance development. However, in strain Nic the ovicidal resistance proved to increase so rapidly that in the adult feeding experiments in the F26 even the highest test concentration of 3000 mg a.i./l of diflubenzuron in the milk only resulted in an ovicidal activity of 26%.

In two field-collected strains of the housefly, cross-resistance factors for the larvicidal effect were either absent or low (viz. 5). The ovicidal cross-resistance factors for the two field strains were found to be 8.4 and 87.5 respectively. Furthermore, the level of ovicidal cross-resistance could probably be related to the resistance of the adults to some standard insecticides tested.

Chitin synthesis in the integument not only occurs in embryos and larvae,

but also in adult insects during a restricted period after adult emergence. On feeding diflubenzuron to newly hatched adults of the Colorado potato beetle we observed that the elytra remained weak (paper VI). For that end the stiffness of the elytra was measured mechanically as the resistance of the elytra to being punctured. In untreated beetles the mechanical penetrability of the elytra decreases up to about 10 days after adult emergence. At any time during this period we found that this decrease in penetrability could be blocked by administering diflubenzuron to the adults. Also of interest is the fact that concomitant with the effect of diflubenzuron on the penetrability of the elytra, a strong inhibition of chitin formation could be measured.

Histological observations of elytra of the Colorado potato beetle revealed several types of mesocuticles. Treatment with diflubenzuron induces characteristic distortions in each of them. In the elytra of treated beetles the thickness of the cuticle, surprisingly, continued to increase. We explained this observation as an indication that protein deposition was not affected by diflubenzuron (paper VI).

Quantative data in addition to the histological observations are presented in paper VII. From the experimental data we furthermore concluded that the level of ketocatechols, which parameter can be related to the degree of tanning of the structural proteins, is only partially inhibited by diflubenzuron. However, we observed that, upon feeding adults with diflubenzuron, inhibition of chitin synthesis took place at lower concentrations than its effect on the level of ketocatechols. This might indicate that the interference of diflubenzuron with the tanning process is only of a secondary nature.

Based upon experiences with both larvicidal and ovicidal applications, the practical possibilities of diflubenzuron for insect pest control are evaluated in paper III, section 6.

SAMENVATTING

Diflubenzuron (1-(4-chlorophenyl)-3-(2,6-difluorobenzoyl)ureum is de werkzame stof van het insekticide Dimilin.

In 1971 werd in de laboratoria van Philips-Duphar ontdekt dat diflubenzuron een insekticide werking bezit tegen larven van vele soorten insekten, voornamelijk behorende tot de Lepidoptera, Coleoptera en Diptera. Uit verschillende onderzoekingen is gebleken dat deze larvicide werking berust op een verstoring van de chitine synthese.

Chitine is een van de belangrijkste bestanddelen van de cuticula van insekten. Een verstoring van de opbouw van chitine beïnvloedt de normale groei van de cuticula. Een van de meest opvallende symptomen die hiermede gepaard gaat is het verschijnsel dat de larven niet meer in staat zijn om nog normaal te vervellen of te verpoppen.

Gedurende de ontwikkelingsfase van diflubenzuron werd tevens gevonden dat deze verbinding in sommige gevallen een interessante ovicide werking bezit. Deze ovicide werking kan optreden bij behandeling van de adulte wijfjes (artikelen I en II), of door direkte kontaktwerking van diflubenzuron op de eieren (artikel II).

In artikel I is aangetoond dat een ovicide werking door behandeling van wijfjes van de kamervlieg *(Musca domestica)* optreedt na uitwendige behandeling, na orale toediening of na injektie van de wijfjes. De verschijnselen bij de ovicide werking door middel van behandeling van wijfjes en bij de ovicide werking door direkt kontakt van diflubenzuron met de eieren zijn identiek. In beide gevallen ontwikkelt het embryo zich schijnbaar normaal, maar het is niet in staat om het ei te verlaten. De behandeling van de wijfjes met diflubenzuron heeft geen invloed op de bevruchting van de eieren of op de vruchtbaarheid van de wijfjes.

Om na te gaan of bij de behandeling met diflubenzuron ook de mannetjes van de kamervlieg een rol spelen bij het veroorzaken van een ovicide effekt werden adulte mannetjes van de kamervlieg geïnjiceerd met 5 µg diflubenzuron, waarna paring met onbehandelde wijfjes plaatsvond. In deze experimenten werd noch een effekt op de bevruchting, noch op de eisterfte waargenomen. De conclusie werd daarom getrokken dat diflubenzuron, toegediend via de mannetjes, geen effekt heeft op het uitkomen van de eieren. Dezelfde conclusie kan getrokken worden uit overeenkomstige experimenten met de Colorado kever *(Leptinotarsa decemlineata)*,

welke zijn vermeld in artikel II.

Voornoemde experimenten leidden tot de conclusie dat de werking van diflubenzuron op eieren van insekten het best kan worden beschreven als een ovicide werking en niet als een effekt van chemosterilisatie, welke benaming soms in de literatuur wordt aangetroffen. Het uiteindelijke bewijs dat het niet uitkomen van eieren na behandeling van adulte wijfjes met diflubenzuron niet berust op chemosterilisatie, werd geleverd met behulp van elektronen microscropische waarnemingen bij embryos van de Colorado kever (artikel III, figuren 3 en 4). Na behandeling van wijfjes met diflubenzuron bleek dat in de embryos de normale gelaagde opbouw van de cuticula vervangen was door een amorfe zone, hetgeen eveneens duidt op een verstoring van de opbouw van de cuticula. Deze waarnemingen suggereren een identiek werkingsmechanisme van diflubenzuron bij larven en bij embryos. Als resultaat van de behandeling met diflubenzuron is de stevigheid van de embryonale cuticula waarschijnlijk te gering om nog te kunnen funktioneren als exoskelet. Dit kan tevens verklaren waarom de embryos het ei niet kunnen verlaten.

Na beëindiging van de behandeling van de wijfjes met diflubenzuron neemt het percentage eieren dat normaal uitkomt langzamerhand weer toe. De snelheid van deze toename hangt af van o.a de concentratie gedurende de voorafgaande behandeling (artikel I).

Een aantal faktoren bleek tevens van invloed te zijn op de ovicide aktiviteit door direkte kontaktwerking. Uit de literatuur was reeds bekend dat de deeltjesgrootte van de werkzame stof een rol speelt. Andere belangrijke faktoren bleken de leeftijd van de eieren en de relatieve vochtigheid van de omgeving te zijn. Voor een goede bevochtiging van de eieren is het bovendien in sommige gevallen nodig om bevochtigers aan de spuitvloeistof toe te voegen (artikel II). De voornoemde relatie tussen leeftijd van de eieren en de aktiviteit van diflubenzuron werd onderzocht bij eieren van het koolwitje *(Pieris brassicae)*. Hierbij bleek dat bij toenemende leeftijd van de eieren de aktiviteit van het insekticide afnam. De invloed van de relatieve vochtigheid werd onderzocht bij eieren van de Colorado kever. De ovicide werking van diflubenzuron bij dit insekt was positief gecorreleerd met de relatieve vochtigheid.

Bij de eieren van de bladmineerder *Leucoptera scitella* werd gevonden dat de concentratie van diflubenzuron invloed heeft op het moment waarop doding optreedt (artikel III). Na bespuiting van de eieren met concentraties tussen 100 en 1 mg a.i./l vindt sterfte plaats in het ei stadium of in de eerste larvale stadia. Naarmate de concentratie van diflubenzuron daalde nam echter de bijdrage van de

eisterfte aan de totale aktiviteit af.

Verschillende aspekten met betrekking tot de larvicide aktiviteit van diflubenzuron na orale opname of door kontaktwerking, verschijnselen na intoxicatie en verschillen in gevoeligheid, zijn beschreven in artikel III. In dit artikel wordt tevens aan de hand van histologische preparaten geïllustreerd dat de verstoring van de afzetting van de cuticula bij larven van de Colorado kever reversibel kan zijn. Na een eenmalige behandeling met een sublethale dosis diflubenzuron zien we dat in de cuticula welke afgezet is na de behandeling de verstoringen geleidelijk minder worden en tenslotte geheel verdwijnen.

In artikel IV zijn van een serie van 25 benzoylureum-derivaten, die verschillen in lipofiele eigenschappen, de larvicide- en ovicide aktiviteiten op de kamervlieg vermeld. De ovicide aktiviteiten zijn bepaald door voeding of door injektie van adulte wijfjes, de larvicide aktiviteiten uitsluitend door voeding.

Deze verschillende biologische aktiviteiten konden onderling gecorreleerd worden met behulp van een fysisch-chemische parameter voor de lipofiliteit, welke uitgedrukt was als π in een octanol/water systeem. Hierbij bleek dat zowel de ovicide aktiviteit door voeding van adulte wijfjes als de larvicide aktiviteit voornamelijk bepaald worden door de ovicide aktiviteit na injektie.

De optimale π waarden voor de ovicide aktiviteit na voeding en voor de larvicide aktiviteit waren respektievelijk 1,0 en 1,5. Het is waarschijnlijk dat voor verbindingen die een lipofiliteit bezitten welke belangrijk groter is dan de optimale waarde, de darmwand een belangrijke barrière wordt. Ofschoon de penetratie door de darmwand ook afneemt als de lipofiliteit onder de optimale π waarde daalt is het mogelijk dat de aktiviteit op het aangrijpingspunt van het insekticide zo laag wordt dat de penetratie door de darmwand geen belangrijke rol meer speelt.

Bij een insekticide met een geheel nieuw werkingsmechanisme is het van belang de eigenschappen te kennen met betrekking tot de ontwikkeling van resistentie en het optreden van cross-resistentie. In artikel V zijn waarnemingen hieromtrent beschreven bij een gevoelige laboratoriumstam (S) en een multi-resistente stam (Nic) van de kamervlieg. Selektie met diflubenzuron vond plaats door het insekticide te mengen door de kunstmatige voedingsbodem van de larven. Tijdens de selektie werd zowel de ontwikkeling van resistentie tegen het larvicide effekt als die tegen het ovicide effekt bestudeerd. De ovicide werking werd bepaald door middel van orale toediening van diflubenzuron aan de wijfjes.

Als gevolg van een vermindering van het reproduktie vermogen van de vliegen, welke optrad na langdurige selektie, moest de kweek van stam Nic worden afgebroken in de F32. Met stam S werden de waarnemingen voor dit onderzoek vervolgd tot aan de F40.

De larvicide resistentie van beide stammen bleek slechts langzaam toe te nemen. De laatste bepaling van de larvicide resistentie in stam Nic werd gedaan in de F26 en vertoonde een 13.6-voudige daling van de gevoeligheid, indien gebaseerd op de LC_{50} waarden. In de F40 van stam S was deze faktor ongeveer 40.

De ontwikkeling van ovicide resistentie tot aan de F40 van stam S was overeenkomstig aan de ontwikkeling van de larvicide resistentie. Daarentegen ontwikkelde de ovicide resistentie in stam Nic zich veel sneller. In de F26 van deze stam bleek de hoogste test concentratie van diflubenzuron, namelijk 3000 mg a.i./l in de melk waarmee de adulte wijfjes werden gevoerd, nog slechts te resulteren in een ovicide aktiviteit van 26%. Hoewel een resistentie faktor niet kon worden bepaald in de F26 van stam Nic kan wel geconcludeerd worden dat deze tenminste 150 moet zijn.

Naast de resistentie ontwikkeling werd tevens aandacht geschonken aan de cross-resistentie ten opzichte van diflubenzuron. Een cross-resistentie faktor is gebaseerd op de verhouding van de LC_{50} waarde van een stam welke resistent is tegen een of meerdere insekticiden (en nog nooit behandeld is geweest met diflubenzuron), en de LC_{50} waarde voor de gevoelige stam. In twee veldstammen van de kamervlieg, welke resistent waren tegen dimethoaat en trichloorfon, was de cross-resistentie faktor voor het larvicide effekt van diflubenzuron laag of afwezig. De ovicide cross-resistentie faktor bleek echter veel groter te zijn, in een van de stammen was deze zelfs 87.5. Het niveau van de ovicide cross-resistentie kan waarschijnlijk gerelateerd worden aan het resistentie niveau van de adulten tegen standaard insekticiden.

Chitine synthese in het integument vindt niet uitsluitend plaats bij embryos en larven. Ook bij imagines vindt chitine synthese plaats gedurende een beperkte periode na het vervellen tot het adulte stadium. Gedurende deze periode vindt tevens o.a. het uitharden van de dekschilden plaats. Na voeren van diflubenzuron aan adulte Colorado kevers, vanaf het ontpoppen, namen we waar dat de dekschilden zacht bleven. Om voor het uitharden een maat te hebben werd de weerstand van de dekschilden gemeten tegen doordringen met een naald. Bij dekschilden van de onbehandelde Colorado kevers bleek de weerstand tegen mechanische doordringbaarheid onder laboratorium condities tot gedurende ongeveer 10 dagen na het ontpoppen toe

te nemen. Deze toename in de weerstand van de dekschilden kon op elk tijdstip gedurende deze periode geblokkeerd worden door de adulten te voeren met diflubenzuron. Tegelijk met deze blokkering werd een remming van de chitine synthese gemeten.

Uit histologisch onderzoek bleek dat de dekschilden van de Colorado kever verschillende typen mesocuticula bevatten. Behandeling met diflubenzuron induceert karakteristieke verstoringen in elk van deze typen mesocuticula. Het is opvallend dat na behandeling met diflubenzuron gedurende de periode van 10 dagen na het ontpoppen, de cuticula nog steeds in dikte toeneemt. Deze waarnemingen zijn geïnterpreteerd als een indikatie dat de afzetting van proteïnen in dit geval niet beïnvloed is door de behandeling (artikel VI).

Kwantitatieve gegevens ter ondersteuning van deze conclusie over de afzetting van proteïnen zijn gepresenteerd in artikel VII. In dit artikel is tevens beschreven dat het gehalte aan ketocatecholen, welke parameter gerelateerd kan worden aan de mate van looiing van de strukturele eiwitten, slechts in geringe mate verlaagd is ten gevolge van de behandeling met diflubenzuron. De remming van de chitine synthese treedt bovendien op bij veel lagere concentraties dan de verlaging van het gehalte aan ketocatecholen. Deze laatste waarneming zou er daarom op kunnen duiden dat het effekt van diflubenzuron op de looiing van secundaire aard is.

Een evaluatie van de mogelijkheden van diflubenzuron voor de praktijk, gebaseerd op zowel larvicide als ovicide toepassingen wordt gegeven in artikel III, sektie 6.

CURRICULUM VITAE

Arnoldus Cornelis Grosscurt werd op 15 april 1947 te Amsterdam geboren. Na de ULO te hebben doorlopen behaalde hij in 1965 het diploma HBS-B aan het Christelijk Lyceum aan de Moreelsestraat te Amsterdam.
In juni 1972 studeerde hij af aan de Landbouwhogeschool te Wageningen in de richting Tuinbouwplantenteelt, met als bijvakken Entomologie, Fytopathologie en Plantenfysiologie.
Van september 1972 tot maart 1974 werd de militaire dienstplicht vervuld.
Sedert 1 maart 1974 is hij werkzaam bij Philips-Duphar B.V., als groepschef Entomologie op het Agrobiologisch Laboratorium Boekesteyn te 's-Graveland.