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The Insecticidal Mode of Action of Asimicin and a  
standardized extract, F020, isolated from *Asimina triloba*  
(Annonaceae).

THESIS SUBMITTED  
TO THE SCHOOL OF GRADUATE STUDIES  
AS PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE  
DEGREE OF MASTER OF SCIENCE

By Mark Andrew Lewis

at the  
UNIVERSITY OF OTTAWA

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**I dedicate this thesis to Mary-Ann, my loving wife, and to my three beautiful children Benjamin, Samuel and Kailey.**

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## Abstract

The natural plant toxin asimicin and the standardized extract F020 from *Asimina triloba* (Annonaceae) were examined for their insecticidal properties using six insect species, encompassing four orders. The inhibition of growth, the antifeedant effect, the food assimilation efficiency, the juvenile hormone properties of, and the effect on whole body respiration and *in vitro* mitochondrial respiration were examined. Growth of *Ostrinia nubilalis* and *Manduca sexta* larvae was inhibited at 100 and 500  $\mu\text{g F020} \cdot \text{g diet}^{-1}$ , respectively and mortality was significantly higher than controls at levels as low as 500 and 1  $\mu\text{g F020} \cdot \text{g diet}^{-1}$ , respectively. Antifeedant properties were observed for F020 when incorporated into artificial diets or when applied to corn leaf surfaces at a level of 26.7  $\mu\text{g} \cdot \text{cm}^{-2}$  for *O. nubilalis*. The antifeedant effect was separated from the toxic effect through a novel approach comparing the results of nutritional indices for those insects which had been topically treated with F020, to those insects exposed to it in their diet. F020 was established as being toxic to *O. nubilalis*. There was no reduction in digestibility due to exposure to F020. This observation was supported by histological examinations of the midguts of last instar larvae reared on F020 laden diet. The hypothesis that F020 and asimicin possess properties that mimic those of insect juvenile hormone could not be supported. Toxicity and growth reduction from exposure to F020 were apparent for *Oncopeltus fasciatus* (Hemiptera) and *Tenebrio molitor* (Coleoptera). *Tenebrio molitor* was significantly affected at doses as high as 250  $\mu\text{g F020} \cdot \mu\text{l}^{-1}$  and at 20  $\mu\text{g asimicin} \cdot \mu\text{l}^{-1}$ . F020 was also not effective at changing the rate of tanning in the puparium of *Sarcophaga bullata*, although a mottled appearance occurred in some.

Examination of the effect of F020 on the respiratory rate of third instar *O. nubilalis* larvae demonstrated that F020 is an effective respiratory inhibitor. *In vitro* examination of isolated midgut mitochondria from *O. nubilalis* showed that both F020 and asimicin were very effective at inhibiting complex I, the NADH-ubiquinone site of the electron transport chain. The  $\text{EC}_{50}$  values for inhibition of state 3 respiration by asimicin and F020 were 0.546 nmoles  $\cdot \text{mg protein}^{-1}$  and 1.656  $\mu\text{g} \cdot \text{mg protein}^{-1}$ , respectively.

## Résumé

La toxine d'origine végétale asimicine et l'extrait standard F020 d'*Asimina triloba* (Annonaceae) ont été étudiés pour leurs propriétés insecticides chez six espèces d'insectes réparties dans quatre ordres. L'inhibition de la croissance, l'effet antiappétant, les indices nutritionnels, les propriétés d'hormones juvéniles ainsi que l'effet sur la respiration de l'insecte entier et sur la respiration mitochondriale *in vitro* ont été examinés. La croissance a été inhibée à 100 et 500  $\mu\text{g}$  de F020  $\cdot$  g de nourriture<sup>-1</sup> chez les chenilles d'*Ostrinia nubilalis* et de *Manduca sexta* respectivement et la mortalité a été significativement plus grande que les témoins à des niveaux aussi faibles que 500 et 1  $\mu\text{g}$  de F020  $\cdot$  g de nourriture<sup>-1</sup> respectivement. Des propriétés antiappétantes ont été observées pour le F020 lorsque celui-ci était incorporé à de la nourriture préparée ainsi que lorsqu'il était appliqué directement sur la surface d'une feuille de maïs à 26.7  $\mu\text{g} \cdot \text{cm}^{-2}$  pour *O. nubilalis*. L'effet antiappétant a été séparé de l'effet toxique à l'aide d'une nouvelle approche qui compare les indices nutritionnels pour les insectes qui ont été traités localement avec le F020 et pour ceux qui ont été exposés au produit dans leur nourriture. Ainsi, le F020 s'est avéré être toxique pour *O. nubilalis*. Il n'y a pas eu de réduction de la digestibilité due à l'exposition au F020, un résultat qui est supporté par des examens histologiques des estomacs moyens des chenilles de dernier stade élevées sur de la nourriture traitée au F020.

L'hypothèse selon laquelle le F020 et l'asimicine possèdent des propriétés qui miment celles des hormones juvéniles des insectes n'a pu être supportée. La toxicité et la réduction de la croissance due à l'exposition au F020 ont été observés pour *Oncopeltus fasciatus* (Hemiptera) et *Tenebrio molitor* (Coleoptera). *Tenebrio molitor* a été significativement affecté à des doses aussi fortes que 250  $\mu\text{g}$  F020  $\cdot \mu\text{l}^{-1}$  et 20  $\mu\text{g}$  d'asimicine  $\cdot \mu\text{l}^{-1}$ . Le F020 n'a pas changé le taux de coloration des puparium de *Sarcophaga bullata*, même si certaines coloration anormales ont été notées.

L'étude du taux de respiration pour les chenilles de troisième stade d'*O. nubilalis* traitées avec du F020, a montré que le F020 est un inhibiteur efficace de la respiration. L'examen *in vitro* des mitochondries isolées des estomacs d'*O. nubilalis* a montré que le F020 et l'asimicine sont très efficaces pour inhiber le complexe I, le site NADH-

ubiquinone de la chaîne de transport d'électrons. Les valeurs de  $EC_{50}$  pour l'asimicine et pour le F020 sont de  $0.546 \text{ nmoles} \cdot \text{mg protein}^{-1}$  et de  $1.656 \mu\text{g} \cdot \text{mg protéine}^{-1}$  respectivement.

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# **Chapter I**

## **General Introduction**

### **Hypothesis :**

Acetogenins of pawpaw (*Asimina triloba*) are able to induce discernible adverse effects on the behaviour and physiology of insects which are exposed to them.

### **Objectives to establish the validity of the hypothesis through the study of:**

- 1) the effects of dietary acetogenins on insect feeding, growth and food assimilation efficiency
- 2) the possible insect hormonal activities of acetogenins
- 3) the effect of acetogenins on insect respiration.

## **1.1 Background**

As primary producers plants have an essential role in supplying the animal world with their nutrient requirements. The immense number of herbivores in the animal world constitutes a threat of serious damage to all given plants. Unlike typical predator-prey relationships, plants are unable to move from a given location, and as a result must deal with an oncoming herbivore through other means. The physical defence strategies of plants can be effective and may involve tough fibrous coverings such as bark or thick cuticles, lignification of leaves and stems, sticky exudates, trichomes or spines. Chemical defense through the production of certain secondary plant compounds may also be effective. Although by definition, secondary substances are not involved in the essential metabolic processes in a plant, they can, in many instances, fulfil some defensive role for the plant. Of the thousands of known plant secondary metabolites, some 18 000 are known to be involved in plant-animal interactions (Harborne, 1988). Some of the major classes of secondary compounds that have been linked to plant-animal

interactions include the nitrogenous compounds such as alkaloids, amines, and non-protein amino acids; the terpenoids, including monoterpenes, sesquiterpenes, limonoids and carotenoids; as well as the phenolics which include compounds such as the flavonoids and quinones (Harborne, 1988).

Plants exhibiting insecticidal properties have been known for thousands of years. However, only very recently, over the past fifty years, have many significant reports been published that have examined and confirmed the toxic principles of plants to insects. More than 2000 plant species, of various plant families and genera are reported to be insecticidal (Adityachaudhury *et al.*, 1985). Among the many plants which exhibit insecticidal properties, several have been utilized for hundreds of years (Matsui and Yamamoto, 1971), for example, *Chrysanthemum cinerariaefolium* (Fam: Asteraceae) from which pyrethrin is extracted; members of the genera *Lonchocarpus*, *Tephrosia*, *Mundulea* and especially *Derris* (Fam: Fabaceae) from which rotenoids are obtained; and *Nicotiana* species (Fam: Solanaceae) from which nicotinoids are obtained (Matsui and Yamamoto, 1971; Adityachaudhury, 1985).

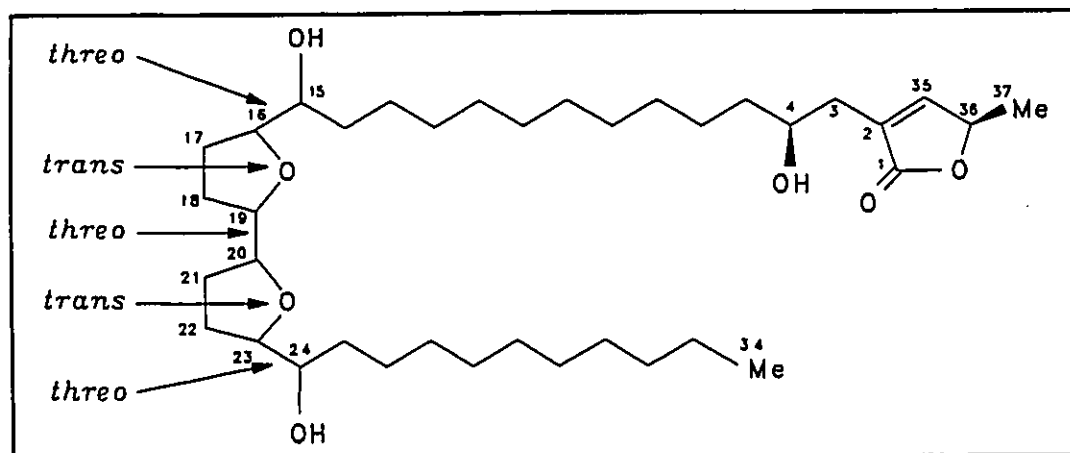
The plant family Annonaceae, comprised of approximately 120 genera, is known for its large array of biochemical compounds including lipids, amino acids, proteins, polyphenols, essential oils, terpenes, aromatic compounds, carotenes, cyanogenic glycosides, non-alkaloidal nitrogen heterocycles, as well as a wide variety of alkaloids (Leboeuf *et al.*, 1982). These identified compounds represent investigations of only approximately 7% of the more than 2000 species and 33% of the genera which comprise the Annonaceae family (Leboeuf *et al.*, 1982).

Many species from this family have also been utilized as traditional medicines in the treatment of various ailments. For example, the seeds of *Annona muricata* (L.), have been found to be useful as an emetic, an astringent and as a fish poison. The bark and fruit of *A. reticulata* (L.), have been described as having astringent, as well as antihelminthic and antidysenteric properties, respectively. Preparations from seeds, fruit or leaves of the custard apple, *A. squamosa* have been reported to have insecticidal and piscicidal properties, and to possess abortifacient properties (Chopra *et al.*, 1956). The bark of *Annona senegalensis*, has been used to treat cancer and convulsions in children (Adesogan and Durodola, 1976). Some other insecticidal properties of this family of

plants include the induction of mortality and paralysis in the insects *Macrosiphoniella sanborni* and *Aphis fabae* through exposure to alcoholic extracts from the seeds, stem, leaves and roots of *A. reticulata* (Tattersfield and Potter, 1940). However, for the most part the actual chemistry attributed to the above claims is unknown. Nonetheless, some biological activity has been associated with particular compounds from species of the Annonaceae. For example antimicrobial properties were identified for the flavanone uvafzelin isolated from *Uvaria afzelii* (Scot Elliot) (Hufford *et al.*, 1980a); antitumour activity was associated with some diterpenes from *Annona senegalensis* (Pers) (Adesogan and Durodola, 1976); as well, antitumour and antibacterial activity was attributed to liriodenine from *Liriodendron tulipifera* (Hufford *et al.*, 1980b).

More recently potent antibiotic activity has been associated with a newly recognized group of compounds from the family Annonaceae, the linear acetogenins. Structurally the linear acetogenins are similar, exhibiting one or two tetrahydrofuranoid rings, a methylated  $\gamma$ -lactone, and a long unbranched aliphatic region which may be either hydroxylated, acetoxyated or ketonized (Rupprecht *et al.*, 1990). They are believed to be biologically synthesised through the polyketide pathway via a linear combination of 2 or 3 carbon units analogous to the pathway for fatty acid biosynthesis (Rupprecht *et al.*, 1990). The first isolated structure of this kind was, uvaricin, from the roots of *Uvaria acuminata* Oliv. (Jolad *et al.*, 1982). Uvaricin displayed potent antitumour activity against 3PS lymphocyte leukaemia in mice (157% test/control at 1.4 mg·kg<sup>-1</sup>). Since then, other structurally similar acetogenins have also shown potent biological activity including rollinone, rollinacin and isorollinacin, all cytotoxic to 9PS murine lymphocyte leukaemia (Dabrah and Sneden, 1984a,b). Cherimoline and dihydrocherimoline are antimicrobial, toxic to brine shrimp, and are cytotoxic (Cortes *et al.*, 1984). Rolliniastatin, exhibits cytotoxicity (Pettit *et al.*, 1987), squamosin elicits both cytotoxic and pesticidal activity (Fujimoto *et al.*, 1988); bullatacin exhibits strong brine shrimp lethality, cytotoxicity and pesticidal activity; bullatacinone also displays brine shrimp lethality and cytotoxicity (Hui *et al.*, 1989; Li *et al.*, 1990), annonin I and annonin VI are both cytotoxic (Born *et al.*, 1990); 4-hydroxy-25-desoxyneorollinacin exhibits cytotoxic activity (Abreo and Sneden, 1989) and neoannonin elicits strong pesticidal activity (Kawazu *et al.*, 1989).

Asimicin (Figure 1.1), isolated from the bark and seeds of the pawpaw, *Asimina triloba* (L.) Dunal, has been found to be toxic to brine shrimp, to possess insecticidal and insect antifeedant activity, antimalarial activity and immunosuppressant activities (Rupprecht *et al.*, 1986, 1990; Mikolajczak *et al.*, 1988; Alkofahi *et al.*, 1989; McLaughlin *et al.*, 1988). Promising insecticidal activity of asimicin has been exhibited against the Mexican bean beetle, *Epilachna varivestis*, (70% mortality at 10 ppm, 100% at 50 ppm), the melon aphid, *Aphis gossypii* (20% mortality at 100 ppm and 100% at 500 ppm), mosquito larvae, *Aedes aegypti* (100% mortality at 1 ppm), the nematode, *Caenorhabditis elegans* (100% mortality at 0.1 ppm) blowfly larvae, *Calliphora vicina* (100% mortality at 1000 ppm) and the striped cucumber beetle, *Acalymma vittatum* (50% mortality at 0.5% solution) (Mikolajczak, *et al.*, 1988; Alkofahi *et al.*, 1989).



**Figure 1.1** Chemical structure of asimicin.

Asimicin has also been extracted from *Annona cherimola* Mill. seeds (Rios *et al.*, 1989) and from the seeds of *Rollinia mucosa* (Jacq.), (Pettit *et al.*, 1989) and *Annona squamosa* (L.) (Born *et al.*, 1990).

Because of the limited amounts of the pure material, the extract from *A. triloba*, identified as F020, was used in place of asimicin during experimental procedures carried out in this study. To derive F020, ground bark of freeze dried *A. triloba* was initially extracted with 95% ethanol. The extract was evaporated and partitioned between dichloromethane and water. The dichloromethane solubles were dried and further partitioned between hexane and 90% methanol. The methanol was then evaporated and

the resulting thickened syrup was labelled as F020 (Mikolajczak *et al.* 1988; Alkofahi *et al.*, 1989). The resulting F020 is an enriched mixture of acetogenins including asimicin. The toxicity of F020 is very comparable to that of pure asimicin. The LC<sub>50</sub> for F020 was very similar to that of pure asimicin. When tested with brine shrimp values were 0.04 and 0.03 ppm, respectively. F020 also exhibited insecticidal activity comparable to that of asimicin. F020 induced mortality to the melon aphid, *Aphis gossypii* (30% mortality at 200 ppm); the mosquito larvae, *Aedes aegypti* (100% mortality at 10 ppm and 80% at 1 ppm); the blowfly, *Colliphora vicina* (100% mortality at 1000 ppm ); the nematode, *Caenorhabditis elegans* (100% mortality at 10 ppm); the two spotted spider mite, *Tetranychus urticae* (Koch) (60% at 5000 ppm); and the mexican bean beetle, *Epilachna varivestis*, (60% mortality at 100 ppm) (Mikolajczak *et al.*, 1988; Alkofahi *et al.*, 1989).

At the biochemical level, the elucidation of the mode of action of a particular plant toxins towards a given insect can be of considerable importance in the light of developing new pest control products. Knowing which metabolic pathway or enzyme system is being affected is vital to the evaluation of toxicity and non-target effects and may allow modification to ensure target specificity. Studies examining the toxic mode of action of the linear acetogenins, including asimicin and the plant extract F020, are lacking. Such an examination is followed through in this study with the elucidation of the mechanism of action to be the NADH-reductase site 1 of the mitochondrial electron transport chain.

### **1.1.1 The Pawpaw**

Members of the Annonaceae are found throughout the world. Of the 120 genera, and approximately 2100 species, 51 genera and about 950 species are confined to Asia and Australia; in Africa and Madagascar, there are 40 genera with about 450 species (Takhtajan, 1969). On the North American continent there are 38 genera and approximately 740 species (Takhtajan, 1969) including the pawpaw or *Asimina triloba* (Hosie, 1969). *Asimina triloba* is the only member of the family which can be found in

Canada, specifically located near the north shores of Lake Erie in southern Ontario (Hosie, 1969; Kral, 1960; Hutchinson, 1964). *Asimina triloba* (Figure 1.2) can be found as a tropical or subtropical tree or shrub. It is a highly shade tolerant tree and can be found sparsely in rich, moist, better drained soils of deciduous forests in river valleys near streams and on low slopes (Hosie, 1969). As a tree, *A. triloba* has a short slender trunk with a broad, high crown formed by straight spreading side branches alternating up the trunk. The wood of *A. triloba* is soft and very aromatic when freshly cut. Its bark is reddish brown in colour and has slightly raised pale lenticels, it is smooth except on very old trees when it becomes slightly furrowed. Its leaves are membranous, found on short stalks, are thin, light green on the upper surface and paler and lacking pubescence on the underside.

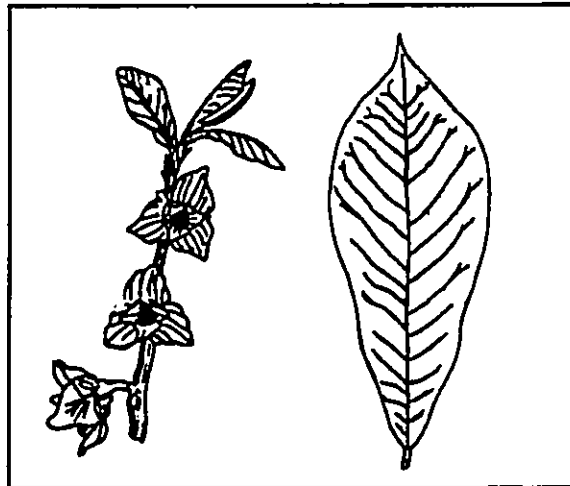


Figure 1.2 A young flowering branch and a typical mature leaf of *Asimina triloba*.

Mature leaves measure 10-30 cm long by 5-10 cm wide, broadest near the top then tapering to the base. The flowers characteristically appear before or during leaf emergence on the wood of the previous season and are said to have a rather repugnant odour. Stamens and pistils are found within the same reddish purple flowers (Kral, 1960; Hosie 1969). The fruit is a round to oblong pulpy, edible berry, whose quality has been improved through breeding programs (Zimmerman, 1941). Its seeds are brown and vary in size depending on the number within the fruit, the average size matching that of the common kidney bean. The seed coat is tough except near the point of attachment (Kral, 1960).

There are very few insect species which feed on the pawpaw, and those that do all are lepidopteran and are considered specialist feeders of the pawpaw, although some will feed on other plants. For example the zebra swallowtail, *Eurytides marcellus* (Cramer) is found most commonly on *A. triloba* but will also feed on other *Asimina* species (Opler and Krisek, 1984); the greater wax moth, *Omphelocera munroei* (Martin)

will feed on pawpaw fruit (Covell, 1984); the pawpaw sphinx, *Dolba hyloeus* (Drury), is a small dark hawkmoth which commonly feeds on *A. triloba* but will also feed on the possum haw, *Ilex decidua* (Walt.), the inkberry, *Ilex glabra*, and the littleleaf sweetfern, *Myrica aspleniifolia* (L.) (Hodges, 1971).

### 1.1.2 The European Corn borer

The European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Pyralidae) (Figure 1.3), is a pest of great economic importance in regions of Europe and North America. In Canada, *O. nubilalis* is a major pest of corn in the eastern and central provinces and to a lesser extent in Manitoba and Saskatchewan. The natural historic distribution of *O. nubilalis* is in Europe, the Mediterranean region of Africa, and in certain areas of southwestern Asia (Hudon and LeRoux, 1986). It was accidentally introduced into North America, in and around Massachusetts, U.S.A. (Vinal, 1917) and has since spread southward and westward being found in more than 40 states and in the eastern and central provinces of Canada. It has since moved into the Canadian prairies, being found in many parts of Manitoba and Saskatchewan and recently, as far west as Grassy Lake Alberta (Liu, 1983). At present the European corn borer populations in North America consist of three ecotypes (Showers *et al.*, 1975). The northern type, a univoltine strain, found in Minnesota and Quebec; the central type, a bivoltine strain, found in Iowa, Nebraska and Ohio; and the southern type, multivoltine, found in Alabama, Georgia and Missouri.

The European corn borer is polyphagous and is known to attack just about every wild and cultivated plant that has stems that provide enough space for the larvae to enter (Hudon and LeRoux, 1986). The eggs of *O. nubilalis* are white and deposited in overlapping masses, typically on the underside of the leaves, averaging 18 eggs/mass.. Egg development is complete within 5 to 7 days at a temperature of 17-20°C (Hudon and LeRoux, 1986). The emerging larvae feed on the leaves, in the tassels, beneath the husks and within the ears and stalks, in short, they can damage all of the aerial parts of a given corn plant. Once stalk damage has occurred, further economic damage may

occur through lodging. The developing larvae will go through 5 instars requiring on average, 22.8 days (at a temperature of 25°C) (Matteson and Decker, 1965). The fifth instar larvae are slightly pinkish with brown spots with several narrow brown lines extending lengthwise. The insect will overwinter in diapause in the final larval stage and pupate in early spring. The onset of pupation is characterized by a change in the behaviour of the larvae as noted by sluggishness, spinning of the web, and a motionless attitude which lasts for approximately two days. This is followed by subsequent ecdysis via an anterior\posterior dorsal suture. This insect has been successfully reared in our laboratory for several generations (see Appendix A for rearing conditions) and provides a suitable representation for the bioassays, which will test the toxicity and properties of F020 and asimicin.

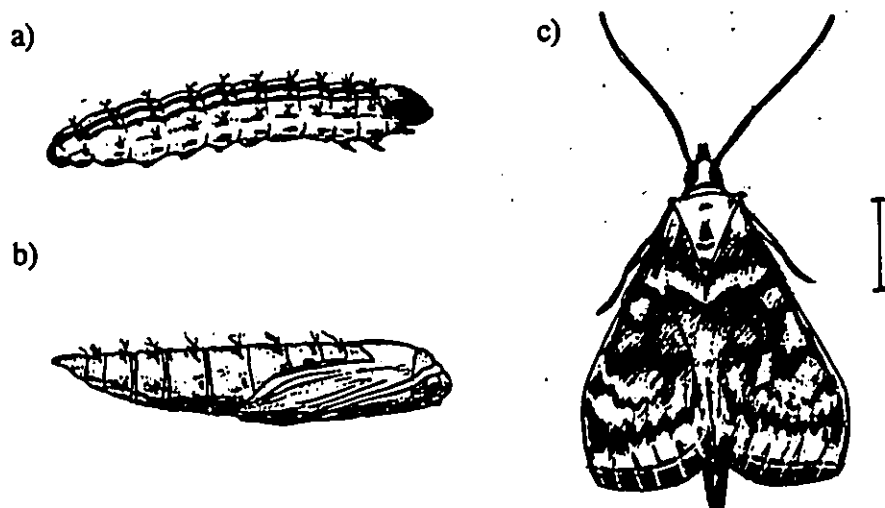
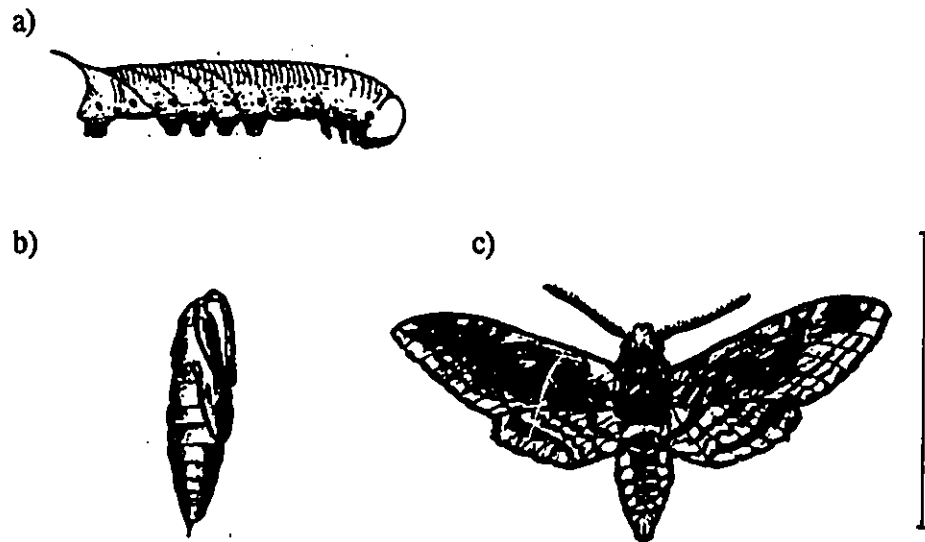


Figure 1.3 The European cornborer, *Ostrinia nubilalis*. a) Fifth instar larva; b) Pupa; c) Adult.

### 1.1.3 The Tobacco Hornworm

The tobacco hornworm, *Manduca sexta* (L.) (Lepidoptera: Sphingidae) (Figure 1.4) overwinters as a pupa and emerges in early summer. Females mate only once, attracting a male with a sex pheromone. Eggs are deposited individually, about 100 to 300 per female, on the underside of a host leaf. Oviposition usually beginning on the third night after adult eclosion. The pale green eggs are smooth and spherical (about 1

mm in dia.), becoming lighter as the hatching date approaches (approx. 3-8 days from the time of oviposition). The emerged larvae are oligophagous and will eat a large number of solanaceous plants including tomato, potato, eggplant, pepper and tobacco. The larvae eat the leaves, leaving only the larger veins. A larva passes through five and occasionally six instars within about 3 weeks during which time it may consume up to 3000 cm<sup>2</sup> of foliage with 90% of this occurring in the final larval instar. When the larva matures the dorsal aorta (heart) becomes visible along the dorsal midline. At this time the larva stops eating, and tunnels into the soil (10-20 cm) or suitable medium for pupation. Before the actual larval to pupal moult, the larva enters a stage called the prepupa where it becomes inactive, shrinks in size and its colour fades slightly. Pupation occurs approximately six days after entering the ground. Under natural conditions, pupae formed in early or mid summer will emerge as adults within a few weeks, but pupae formed in late summer will emerge the following spring. Generally 2-3 generations occur per year in the northern United States (Wilson *et al.* 1980; C.B.S.C. 1982). Like *O. nubilalis*, this insect has been successfully reared in our laboratory (See Appendix A) for several generations and because of its oligophagous nature, is a suitable species for testing the toxic effects of F020.



**Figure 1.4** The tobacco hornworm, *Manduca sexta*. a) Fifth instar larva; b) Pupa; c) Adult.

### 1.1.4 The Large Milkweed Bug

The large milkweed bug, *Oncopeltus fasciatus*, (Hemiptera: Lygaeidae) (Figure 1.5) ranges from Massachusetts, westward to the Rocky Mountains, southward to Florida, Texas, California, Mexico, and Brazil (Blatchley, 1926). It will feed on several species of milkweeds (*Asclepias*). Strains have been developed that will feed and develop completely on sunflower seeds, cashews and almonds, the sunflower strain being the most successful (Gordon and Gordon, 1971). The sexes can be easily distinguished in the fifth (final) instar. The females have two black spots on the ventral side of the two posterior abdominal segments, the male has a black spot only on the posterior segment (Feir, 1974). Oviposition occurs 1-15 days after mating and peaks around 20 days (Lawson and Ball, 1969), the onset of which may be influenced by photoperiod, population density, and temperature (Dingle, 1968). Under natural conditions, eggs are laid in the crevices between milkweed pods and turn deep orange as eclosion approaches (Feir, 1974). In this study, *O. fasciatus* was successfully used to assess the insect juvenile hormone properties of F020 and asimicin.

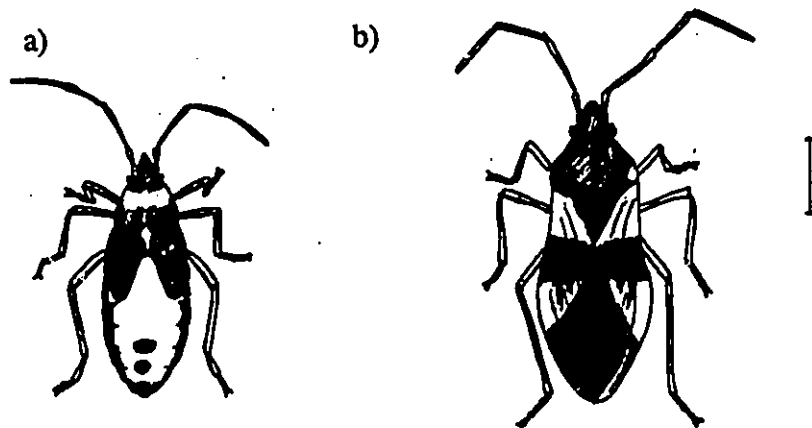


Figure 1.5 The milkweed bug, *Oncopeltus fasciatus*. a) Fifth instar nymph and b) adult.

### 1.1.5 The Yellow Mealworm

The yellow mealworm, *Tenebrio molitor* (L.) (Coleoptera: Tenebrionidae) (Figure 1.6) is found worldwide, being quite abundant in the northern United States. These yellow to light-brown beetle larvae are the largest species known to attack grains and cereal products. The larvae are smooth, cylindrical and when mature are over 25 mm long. They are seldom found in homes but occur frequently in neglected grains and milled products that accumulate in dark corners or in places where livestock feed is stored. It overwinters in the larval stage, with pupation occurring in late spring and adults appearing in early summer. Adults are approximately 13-16 mm long, shiny and dark brown to nearly black and will mate shortly after emergence. Females will oviposit approximately 500 bean shaped white eggs covered with a sticky secretion into the grain or cereal. Hatching occurs in two weeks time with larval development continuing throughout the summer under normal conditions (Cotton, 1950; Davidson and Lyon 1979; White 1983; Papp, 1984).

These insects have been successfully utilized on many occasions to assay for juvenile hormone activity of a particular compound(s) or substance (Bowers 1968, 1969; Jacobson *et al.*, 1975a). For this reason they were chosen as appropriate insects for a bioassay of F020 for juvenile hormone properties.

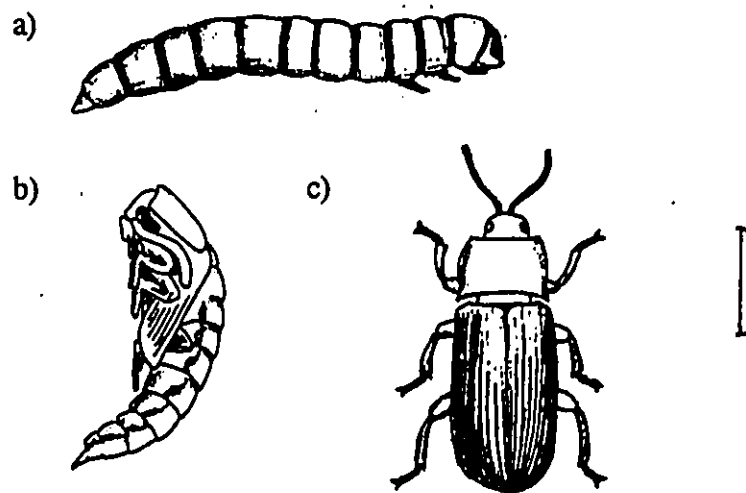


Figure 1.6 The yellow mealworm, *Tenebrio molitor*. a) larva; b) pupa; c) adult.

### 1.1.6 The Flesh Fly

The flesh fly, *Sarcophaga bullata* (Parker), (Diptera: Sarcophagidae) (Figure 1.7) resembles the common house fly in appearance and will feed on carrion, dung, and other similar materials under natural environmental conditions (Borror and White, 1970). One week to ten days post emergence is enough time for adult flies to mate and for females to oviposit. Larvae will emerge from the eggs within a few hours and develop rapidly over a period of 4-5 days. Late in the third and final instar when the larvae cease to feed, they enter into the 'wandering stage', which lasts approximately two days. In the 'wandering stage', the larvae leave the food source and seek a suitable drier medium in which to form the puparium (Žďárek 1980, 1985). Near the end of the wandering stage the larvae enter into what is known as the 'red spiracle stage', in which the larvae show precocious tanning in the posterior region around the spiracles. This stage occurs approximately 3-4 hours before the larvae undergo muscular contraction and the onset of puparium formation. Near the end of the 'red spiracle stage' the larvae become torpid, which signals the beginning of the 'period of immobilization'. The first morphological change is recognised shortly afterwards (minutes) by the anterior retraction stage where the three anterior segments are waved about over a period of 5 minutes, and are then finally retracted over an additional 5 minutes. Within the next 20 minutes, the remainder of the larva contracts, identified as the 'period of contraction', and in the following 10-15 minutes the cuticle shrinks and the surface becomes smooth. At this stage, before tanning begins, the insect is known as a 'white puparium'. Tanning usually proceeds in the subsequent 15 to 30 minutes as a slight yellowing of the cuticle, turns orange in 30 to 60 minutes and brown in 1 to 2 h, finally to become black shortly thereafter (Žďárek and Fraenkel, 1972; Žďárek, 1980, 1985). *Sarcophaga bullata* has been well studied in regards to its puparium formation and a bioassay using this insect has been successfully developed to test the ability of compounds to inhibit or hasten the tanning process (Žďárek and Fraenkel 1969; Žďárek *et al.*, 1980, 1985). For this reason *S. bullata* is used in this study to test the effect of F020 on the tanning process.

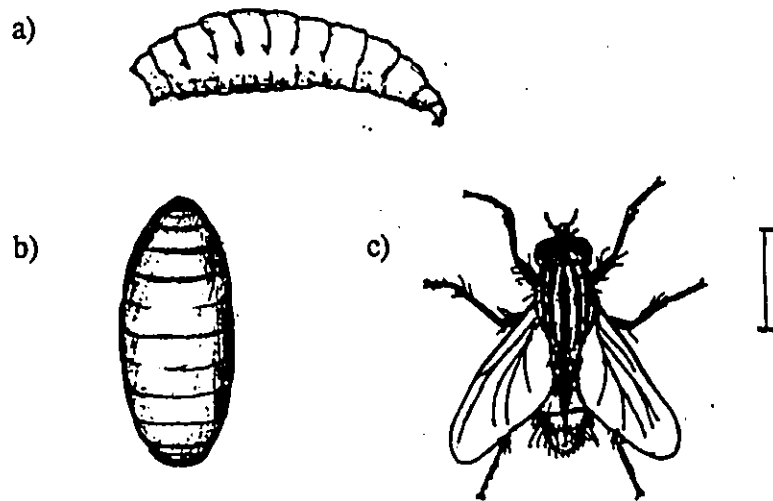


Figure 1.7 The flesh fly, *Sarcophaga bullata*. a) Larva; b) Puparium; c) Adult.

### 1.1.7 The Zebra Swallowtail

The zebra swallowtail, *Eurytides marcellus* (Cramer), also known as *Graphium marcellus* (Cramer), (Lepidoptera: Papilionidae) is the only species of the kite swallowtails (Leptocircini) native to North America. Adults have zebra-striped wings with long tails (Figure 1.3). This butterfly has, characteristically, two different seasonal forms, early spring and summer. The early spring form is smaller and lighter than the summer form and has a tail only half as long. All known hosts plants of this species belong to the genus *Asimina* (Annonaceae), although it is mostly found on the pawpaw or *Asimina triloba*, except in the southernmost regions where it will feed on *Asimina longifolia*, *A. parviflora*, *A. pygmaea*, *A. reticulata*, and *A. speciosa* (Opler and Krisek, 1984).

The zebra swallowtail ranges westward to southern New England across the southern portion of the Great Lakes to southern Minnesota, south to the Gulf Coast and down to southern Florida. Females are usually mated during their first day of flight. Mated females will flutter from plant to plant and lay single eggs on low leaves on young pawpaw. The larvae develop through 5 instars. In the first three instars the larvae are

completely black, in the fourth instar the larvae are black with some light green or white lateral stripes. The fifth instar is about 25 mm long and typically is leafy green in colour with a distinct black, yellow-edged lateral broad stripe on the third thoracic segment. The remaining segments have five alternating lateral stripes of yellow and black. The larvae will pupate in a suspended chrysalis which may either be leafy green or 'dead leaf' brown and is smooth, short, and stout (Opler and Krisek 1984).



Figure 1.8 The Zebra Swallowtail, *Eurytides marcellus*, adult.

## Chapter II.

### The Effect of Dietary F020 on Insect Growth and Development

#### 2.1 Introduction

Recently, ethanolic extracts from members of the family Annonaceae have been found to elicit potent biological activity, including cytotoxicity (Jolad *et al.*, 1982; Dabrah and Sneden, 1984ab; Cortes *et al.*, 1990; Pettit *et al.*, 1987; Fujimoto *et al.*, 1988; Li *et al.*, 1990; Hui *et al.*, 1989; Abreo and Sneden, 1989), antimicrobial activity (Cortes *et al.*, 1990), brine shrimp lethality (Li *et al.*, 1990; Hui *et al.*, 1989) and pesticidal activity (Kawazu *et al.*, 1989; Hui *et al.*, 1989). The active compounds responsible for the above activities all belong to a common structural class of linear acetogenins. All have two tetrahydrofuranoid rings, a  $\gamma$ -lactone, and long unbranched aliphatic regions, that are variously hydroxylated, acetoxyated, or ketonized. The linear acetogenin, asimicin, extracted from the pawpaw, *Asimina triloba*, is also of the same structural class, having two adjacent tetrahydrofuranoid rings. The acute pesticidal properties of asimicin have been examined against a variety of insects and arachnids including the two-spotted spider mite, *Tetranychus urticae* (Koch), the melon aphid, *Aphis gossypii* (Glover), yellow fever mosquito larvae, *Aedes aegypti* (L.), the blowfly larvae, *Colliphora vicina* (Meig), as well as the Mexican bean beetle, *Epilachna varivestis*, and the striped cucumber beetle, *Acalymma vittatum* (F.) (Mikolajczak, *et al.*, 1988; Alkofahi *et al.*, 1989). Except for work with *A. vittatum*, these studies examined the acute lethal effects from exposure to asimicin or the purified bark extract (F020) containing asimicin (Alkofahi *et al.*, 1989). Treatments involved either ingestion or external contact with the compounds at concentrations between 0.1 to 5000 ppm ( $\text{mg} \cdot \text{L}^{-1}$ ). The southern armyworm, *Spodoptera eridania* (Cramer) (Lepidoptera) was

similarly examined in an acute study, where F020 was applied to squash leaves and was presented to the larvae over a 72-h period (Mikolajczak, *et al.*, 1988; Alkofahi *et al.*, 1989); however, no evidence of mortality was observed under these conditions. The potential antifeedant properties of asimicin were also examined through two-choice and no-choice leaf disc bioassays using *A. vittatum* (Mikolajczak, *et al.*, 1988; Alkofahi *et al.*, 1989). In both the two-choice and the no-choice bioassay, cantaloupe leaf disks were dipped in 0.5% (w/v) solutions of asimicin, which were completely effective at inhibiting leaf consumption (0%) by *A. vittatum* and a significant reduction in consumption was also observed from the two-choice assay at the 0.1% solution. In the process of unravelling the mechanism of action of F020 and asimicin this study examines the lethality and sublethal effects from exposure to long term levels of dietary F020 to the polyphagous lepidopteran, *Ostrinia nubilalis* (Hübner) and to the oligophagous lepidopteran, *Manduca sexta* (L.). From this chronic examination, the treated larvae were observed to have reduced rates of growth, and food consumption, increased levels of mortality, reduced pupal weights, and for *O. nubilalis*, a reduction in adult fecundity. These results could be attributed to lower food consumption and therefore the antifeedant properties of F020 were examined using a no-choice leaf-disk bioassay. Differentiation between an apparent toxic effect and an antifeedant effect of a compound can be an arduous task, especially when simply changing an insect's diet e.g. from host plant to a nutritionally adequate artificial diet, may cause the insect to abstain from feeding for extended periods of time (Jermy *et al.*, 1968; Ma, 1976; Hanson, 1976). One method utilized in an effort to distinguish between toxicity and antifeedant effects of a compound consists of comparing the relative growth rate of treated larvae with the relative growth rate of untreated larvae which have been given restricted amounts of food. In this case, a lower slope from a regression of the relative growth rate against the relative consumption rate for the treated group compared to the control group indicates that some level of toxicity can be attributed to the compound in addition to any antifeedant effect (Blau *et al.*, 1978; Miller and Feeny, 1983; Berenbaum and Feeny, 1981).

The novel approach utilized in this study involves comparing the food utilization efficiency in larvae that were topically treated with F020 with those which were presented with levels in their diet. Through this investigation it is evident that F020 has

properties which are toxic to *O. nubilalis*.

## 2.2 Materials and Methods

### 2.2.1 Growth and Development Studies

#### a) *Ostrinia nubilalis*:

Neonate *Ostrinia nubilalis* larvae were reared on an agar-based meridic diet (Appendix A) with F020 extract incorporated at 0, 10, 100, 500, 1000, 5000 and 10000  $\mu\text{g} \cdot \text{g}^{-1}$  fresh diet. Larvae were reared at 25°C, 85% R.H. and 16:8 L:D regime. Diet was changed and weight was measured every 2-3 days throughout the growth period. Observations of mortality, behaviour and moulting difficulty were also recorded. Larvae were collectively reared on diet cubes initially, but after day 9 were placed in individual 6 ml glass vials, at which time the number of larvae being observed was reduced to thirty or the total number remaining if lower (the recorded cumulative percent mortality is based on the original population size - see Appendix B for sample calculation).

No measurements were taken once the developing larvae reached the prepupal stage, which was characterised by an obvious disinterest in the diet, by the movement to the top of the vial, and the spinning of silk. Pupae were collected, weighed and placed on a cotton bed in 6 ml glass vials stoppered with a cotton plug, and placed under the above controlled environment conditions until emergence.

Once *O. nubilalis* adults emerged, they were sexed and placed into 11.5 x 11.5 x 15 cm screened wooden cages, according to the level of dietary F020 to which they had been exposed. Each cage contained a vertical hanging strip of waxed paper 1.5 x 3 cm for oviposition and a 20% sucrose solution supplied through a cotton wick. All deposited eggs and egg masses were counted and numbers of non-viable eggs were recorded.

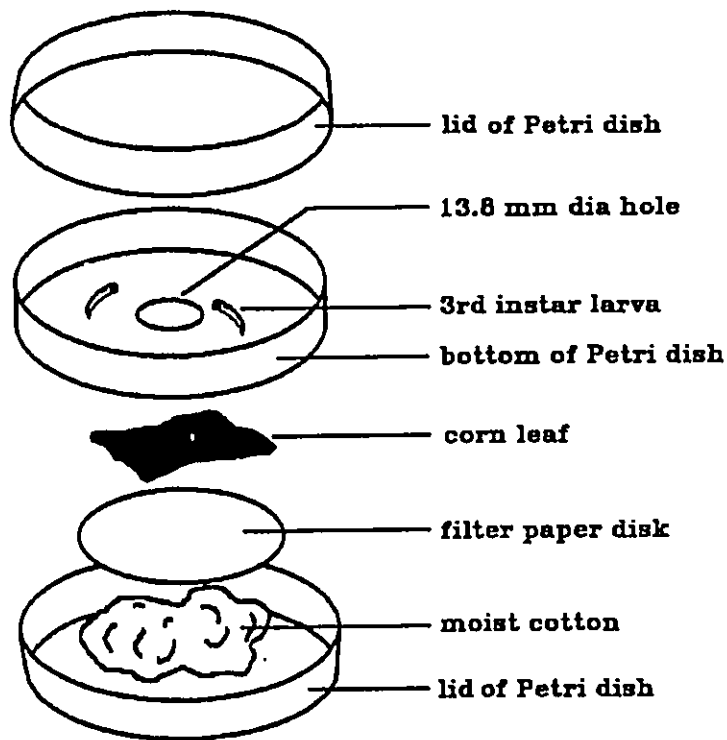
#### b) *Manduca sexta*:

*Manduca sexta* neonate larvae were reared on meridic diets laden with F020 extract at 0, 1, 10, 100 and 500  $\mu\text{g} \cdot \text{g}^{-1}$  fresh diet. Larvae were allowed to develop in a controlled environment chamber at 25°C, and a 16:8 h L:D regime (relative humidity could not be controlled). Observations including mortality, behaviour and moulting

difficulty were recorded. Neonates were reared collectively on diet cubes until day three, at which time they were transferred to individual cells of Falcon 24 cell tissue culture plates and sample size of each treatment was reduced to thirty or to the total number remaining, if lower (for sample calculation of the cumulative percent mortality see Appendix B). As the larvae outgrew the culture plates they were transferred to larger culture plates to accommodate. Once late fourth instar was reached, the larvae were transferred to individual 28 ml plastic vials plugged with foam (38 x 38 mm). They remained there until they displayed prepupal behaviour (cessation of feeding and appearance of the dorsal blood vessel). The vials were then cleaned and filled 1/3 with vermiculite in which the larvae pupated. Pupae were kept in the dark at 20°C. *Manduca sexta* adults were allowed to emerge from the vials, were sexed and then destroyed.

### 2.2.2 Antifeedant Properties

Third instar *Ostrinia nubilalis* larvae were starved for 24 hrs, individually weighed (weight range between 4 and 9 mg) and placed in pairs into Falcon 5 cm dia. Petri dishes which had a circular hole (149.6 sq mm) cut into the bottom (Figure 2.1) to expose a section of the third leaf of a 3 week old sweet corn seedling (Illini Gold) grown under greenhouse conditions. Treatments consisting of F020 in acetone or acetone alone were applied over the exposed leaf surface using a Hamilton syringe. F020 was applied at 5.33, 13.3 or 26.7  $\mu\text{g} \cdot \text{cm}^{-2}$ . All leaves received a total treatment volume of 10  $\mu\text{l}$ . Larvae were allowed 24 hours to feed under controlled environmental conditions (25°C, 16:8 h L:D photoperiod and 85% R.H.). Larvae were removed and the area consumed was estimated by placing the leaf over 1-mm-spaced grid lines under a low power dissecting microscope.



**Figure 2.1.** Exploded diagram of the apparatus used for the no-choice antifeedant bioassay.

## 2.2.3 Nutritional Indices

### 2.2.3.1 Ingested F020

Thirty third instar larvae, between 4 and 8 mg, were placed in individual 6 ml glass vials stoppered with a plastic plug. Each vial contained a portion of meridic diet laden with F020 at 500, 1000 or 5000  $\mu\text{g} \cdot \text{g}^{-1}$  fresh diet. The mass of diet was slightly more than that eaten over 72 hours under normal conditions (to avoid error in estimating food ingested - Schmidt and Reese, 1986). The F020 was initially dissolved in 95% ethanol, not exceeding 1% of the fresh diet weight. Control diets contained an equal quantity of 95% ethanol. Vials were placed in a controlled environment chamber (25°C, 16:8 h L:D and 85% R.H.) for 72 hours and subsequently removed. The nutritional indices, C.I., E.C.I., A.D. and E.C.D were then measured according to Waldbauer, 1968 (Appendix C).

The initial dry weight of each larva and the initial dry weight of the diet were

estimated from the dry weight of 75 larvae and 100 diet cubes of the same fresh weight range. The amount of frass was determined by subtracting the empty vial weight from the vial containing the frass. Because frass weights were small at the higher dosage levels, the weight of the silk, exuviae and dust was estimated from that produced by 60 control larvae within the same weight range as the experimental larvae. An estimate of the silk, dust and exuviae was determined by subtracting the empty vial from that which housed the larvae for 24 h. The 24 hour increase in vial weight was tripled for the 72 hour nutritional index calculations.

#### **2.2.3.2 Topical application of F020**

Third instar *O. nubilalis* larvae (4-8 mg) were treated topically with F020 in acetone at 12.5, 25, 50, 100, 200 and 400  $\mu\text{g} \cdot \mu\text{l}^{-1}$  ( $n=20$  at each concentration) and then placed in 6 ml glass vials stoppered with plastic plugs. All larvae received a total treatment volume of 1  $\mu\text{l}$  applied to the total dorsal surface except for the head capsule. Control larvae received an equal volume of acetone alone. The amount of food supplied to each, the environmental conditions, and the time permitted for feeding were identical to those of the larvae which ingested F020. The nutritional indices were measured in an identical manner (see Appendix C).

#### **2.2.4 Histological Examination**

Midguts from fifth instar *Ostrinia nubilalis* and *Manduca sexta* were prepared and examined for physical damage after having been reared under the conditions described above (see section 2.2.1). Preparation of midguts involved dissection and fixation in alcoholic Bouin fixative (Appendix D). The tissue was then rinsed three times and immersed and refrigerated for three weeks in absolute ethanol to allow tissue to dehydrate. Dehydration was subsequently followed by embedding in Parawax. Transverse sections of the tissue were taken using a microtome set at 8  $\mu\text{m}$ . Sections were stained with Heidenhain iron-haematoxylin and counter stained with eosin (Appendix D).

## 2.3 Results

### 2.3.1 Growth and Development

The mortality of *O. nubilalis* larvae reared on artificial diets treated with F020 at 500 and 1000  $\mu\text{g}\cdot\text{g}^{-1}$  diet (Table 2.1) and at 1, 10, and 500  $\mu\text{g}\cdot\text{g}^{-1}$  diet for *M. sexta*, increased significantly (Table 2.4). The larvae appeared to be most sensitive to F020 during the neonate stage for both species (Figure 2.2 and 2.4).

Figure 2.3 shows the decreased relative rate of growth for *O. nubilalis* corresponding with an increased level of dietary F020. The reduced rate of growth is less apparent for *M. sexta* (Figure 2.5) and only at 500  $\mu\text{g}\cdot\text{g}^{-1}$  diet does it appear to affect larval weight. However, the duration of the larval stage was longer for those treated with F020 at 500 for *O. nubilalis* and 1000  $\mu\text{g}\cdot\text{g}^{-1}$  diet and at 500  $\mu\text{g}\cdot\text{g}^{-1}$  diet for *M. sexta*. The increase in the number of days as larvae (time between hatching and pupal formation) was significant and correlated with increases in dietary F020 ( $P < 0.0001$  and  $P = 0.001$  for *O. nubilalis* and *M. sexta* respectively)(Table 2.1 and 2.4). This finding is supported by the significant decrease in the final fifth instar larval weight for *O. nubilalis* (Table 2.1) ( $P = 0.018$ ) and the significant decrease in the male pupal weights ( $P < 0.0001$ ) and female pupal weights ( $P < 0.0001$ ) for *O. nubilalis* (Table 2.2 and 2.5). The effective concentration for 50 percent of the control weight on day twenty was determined to be 167.8  $\mu\text{g F020}\cdot\text{g}^{-1}$  of diet (Figure 2.6).

The percent of larvae that displayed moulting difficulty increased directly with an increase in dietary F020 for both species (Table 2.1 and 2.4). Moulting difficulty was displayed as a difficulty in casting the old body cuticle or the head capsule from the newly emerging larvae. In one case, at 1000  $\mu\text{g}\cdot\text{g}^{-1}$ , complete failure of the head capsule to split along the ecdysial suture was observed, preventing completion of the moult. All pupae and adults were morphologically normal and there was no significant evidence of any difficulty occurring during adult eclosion. (see 'adults emerging (%)', Table 2.2 and 2.5).

The average number of eggs per female for *O. nubilalis*, and the total number of viable eggs, were significantly lower at 1000  $\mu\text{g}\cdot\text{g}^{-1}$  (Table 2.3) than the control values ( $P < 0.05$ ).

**Table 2.1** The effects of dietary F020 on developmental parameters for *Ostrinia nubilalis*.

F020 treatment ( $\mu\text{g} \cdot \text{g}^{-1}$ diet FW)	final fifth instar larval wt. ( $\text{g} \pm \text{s.e.m.}$ ) †	larval mortality (%)	no. of days as larvae ( $\pm \text{s.e.m.}$ ) ‡	larval moulting inhibition (%)
0	92.3 $\pm$ 2.50 a (n=25)	22.8	25.4 $\pm$ 0.66 a (n=25)	0
10	93.7 $\pm$ 4.07 a (n=25)	53.3	25.1 $\pm$ 0.54 ab (n=25)	0
100	80.0 $\pm$ 3.76 ab (n=23)	37.0	25.0 $\pm$ 0.74 ab (n=23)	0
500	78.8 $\pm$ 6.53 ab (n=17)	*75.7	30.9 $\pm$ 0.98 c (n=18)	3.3
1000	72.7 $\pm$ 3.93 b (n=14)	*71.7	36.7 $\pm$ 1.98 c (n=17)	6.6

† Means followed by the same letters indicate no significant difference ( $P < 0.05$ ) using Tukey's multiple range test.

\* Significantly different from the controls ( $P < 0.05$ ) as measured by 2 x 2 contingency tables using Chi-square test.

‡ Means followed by the same letters indicate no significant difference as compared using distribution free multiple comparisons based on Kruskal-Wallis rank sums ( $P < 0.05$ ).

**Table 2.2** The effects of dietary F020 on pupal parameters for *Ostrinia nubilalis*.

F020 treatment ( $\mu\text{g} \cdot \text{g}^{-1}$ diet FW)	No. of days as pupae		Adults emerging (%)	Pupal wt., (mg)	
	F	M		F	M
0	8.1 a (0.14) n=17	9.0 a (0.24) n=9	96.3	86.5 ab (2.59) n=17	71.2 a (1.65) n=9
10	8.2 a (0.17) n=13	9.6 ab (0.16) n=10	92.0	90.5 a (3.90) n=13	69.7 a (1.18) n=11
100	7.6 a (0.63) n=6	9.1 abc (0.20) n=11	95.7	73.6 ab (3.21) n=6	56.2 b (2.94) n=12
500	7.8 a (0.31) n=6	8.6 acd (0.20) n=11	94.4	83.6 ab (7.75) n=6	53.5 b (3.01) n=12
1000	7.9 a (0.14) n=7	8.1 d (0.23) n=8	93.8	58.7 c (4.05) n=7	55.7 b (2.74) n=9

† Means followed by the same letters indicate no significant difference as compared using distribution free multiple comparisons based on Kruskal-Wallis rank sums ( $P < 0.05$ ).

‡ Means followed by the same letter indicates no significant difference ( $P < 0.05$ ) using Tukey's multiple range test. Values in parenthesis are standard errors of the means.

n=sample size, F=female, M=male.

**Table 2.3.** The effects of larvae exposed to dietary F020 on adult fertility parameters for *Ostrinia nubilalis*.

F020 treatment ( $\mu\text{g} \cdot \text{g}^{-1}$ diet FW)	Sex ratio F/total	Ave. no.eggs per female †	Percentage viable eggs
0	0.65	346.1	62.71
10	0.56	231.6	63.07
100	0.59	298.2	55.46
500	0.35	279.3	63.13
1000	0.50	93.1	*21.77

† Obtained by placing all emerging adults of one treatment into a mating chamber.

\* Significantly different from the controls ( $P < 0.05$ ), as measured by contingency tables using the Chi-square test. F=number of females.

**Table 2.4.** The effects of F020 on developmental parameters of *Manduca sexta*.

F020 treatment ( $\mu\text{g} \cdot \text{g}^{-1}$ diet FW)	Final fifth instar larval wt. ( $\text{g} \pm \text{s.e.m.}$ ) †	Larval mortality (%)	No. of days as larvae ( $\pm \text{s.e.m.}$ ) ‡	Larval molting inhibition (%)
0	$8.52 \pm 0.435$ a n=24	33.3	$32.3 \pm 0.30$ a n=17	3.3
1	$8.72 \pm 0.377$ a n=13	*63.3	$33.8 \pm 0.63$ ab n=10	10.0
10	$8.28 \pm 0.593$ a n=12	*66.6	$33.9 \pm 0.67$ bc n=10	6.6
100	$7.71 \pm 0.367$ a n=15	43.3	$35.2 \pm 0.78$ cd n=15	10.0
500	$9.25 \pm 0.408$ a n=9	*76.6	$38.91 \pm 2.35$ d n=11	20.0

† Means followed by the same letters within the same column indicate no significant difference ( $P < 0.05$ ) using Tukey's multiple range test.

\* Indicates significantly different from the controls ( $P < 0.05$ ) as measured by 2 x 2 contingency tables using Chi-square test.

‡ Means followed by the same letters indicate no significant difference as compared using distribution free multiple comparisons based on Kruskal-Wallis rank sums ( $P < 0.05$ ).

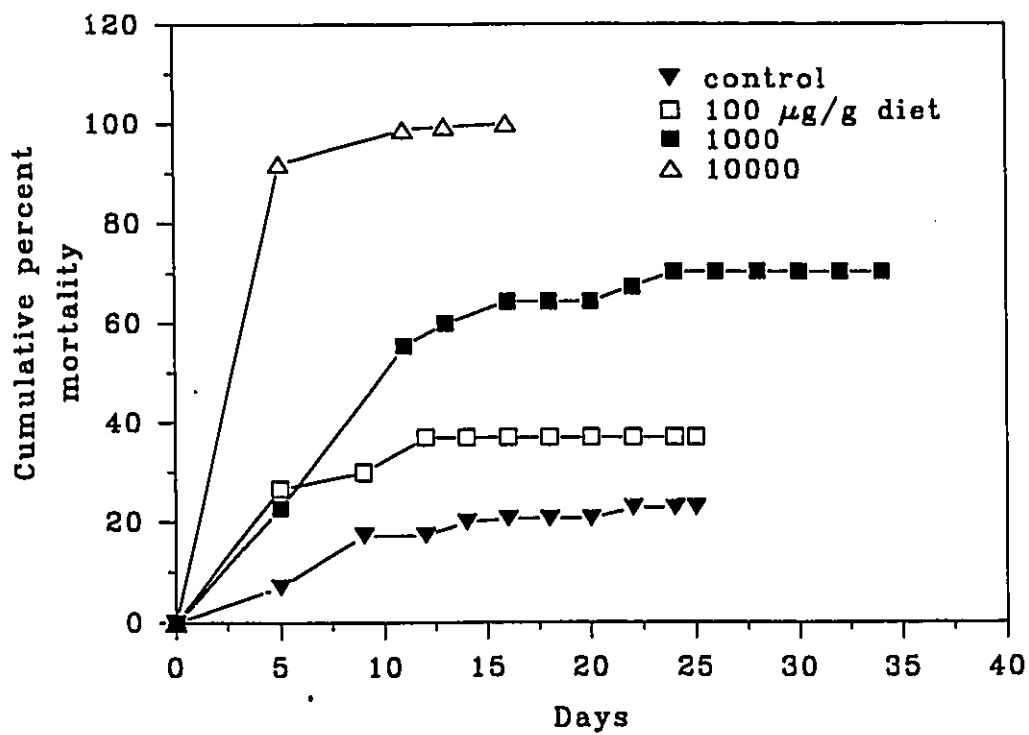
Table 2.5. The effects of F020 on pupal parameters for *Manduca sexta*.

F020 treatment ( $\mu\text{g} \cdot \text{g}^{-1}$ diet FW)	No. of days as pupae (s.e.m.) ‡		Adults emerging (%)	Pupal wt., mg (s.e.m.) †	
	F	M		F	M
0	29.0 a (0.44) n=9	27.4 a (0.46) n=8	77.3	5.00 a (0.170) n=9	4.37 a (83.3) n=8
1	28.5 ab (0.29) n=4	29.3 ab (0.25) n=4	61.5	4.48 a (0.280) n=4	4.31 a (0.124) n=4
10	28.3 abc (0.75) n=4	29.0 bc (0.0) n=3	58.3	4.53 a (0.458) n=4	4.27 a (0.124) n=2
100	27.6 abc (0.50) n=8	27.3 ab (0.42) n=6	93.3	4.02 a (0.255) n=8	3.75 a (0.124) n=6
500	34.0 d (1.53) n=3	30.5 bc (1.19) n=4	63.6	4.77 a (0.526) n=3	4.50 a (0.343) n=4

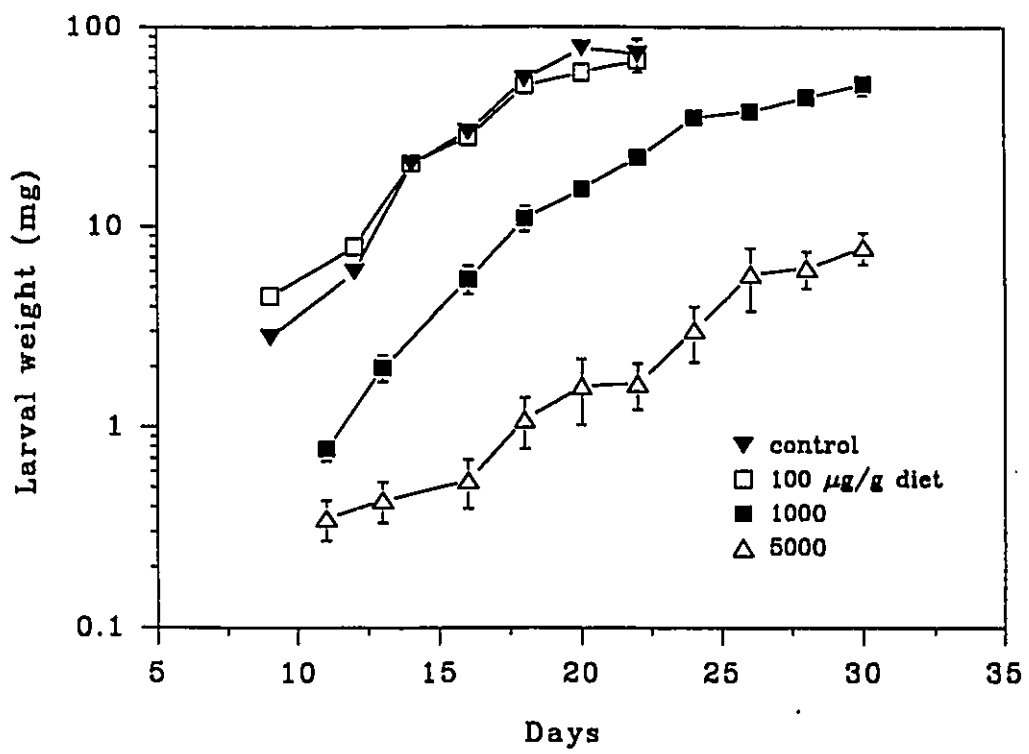
† Means followed by the same letter indicates no significant difference ( $P < 0.05$ ) using Tukey's multiple range test. Values in parenthesis are standard errors of the mean.

‡ Means followed by the same letter indicates no significant difference as compared using distribution free multiple comparisons based on Kruskal-Wallis rank sums ( $P < 0.05$ ). n=sample size, F=female, M=male.

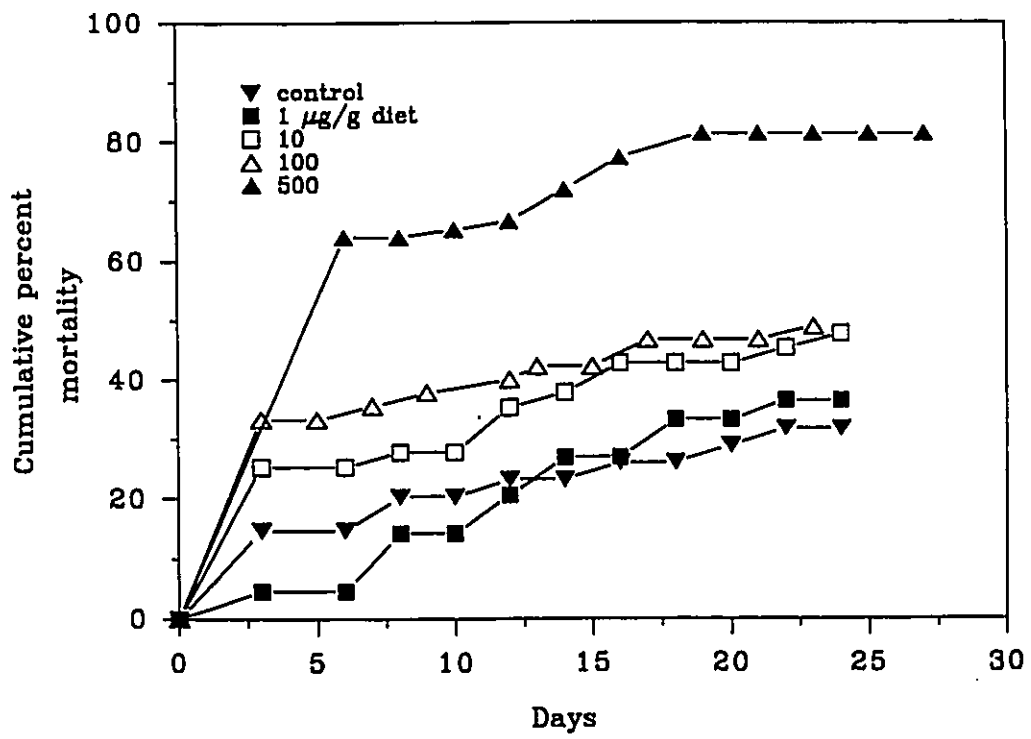
**Figure 2.2** The cumulative percent mortality for *Ostrinia nubilalis* reared on meridic diets laden with F020 at 100, 1000, and 10000  $\mu\text{g} \cdot \text{g}^{-1}$  diet. Larvae were reared from eclosion from the egg to pupation in 10 ml glass vials under controlled environmental conditions.



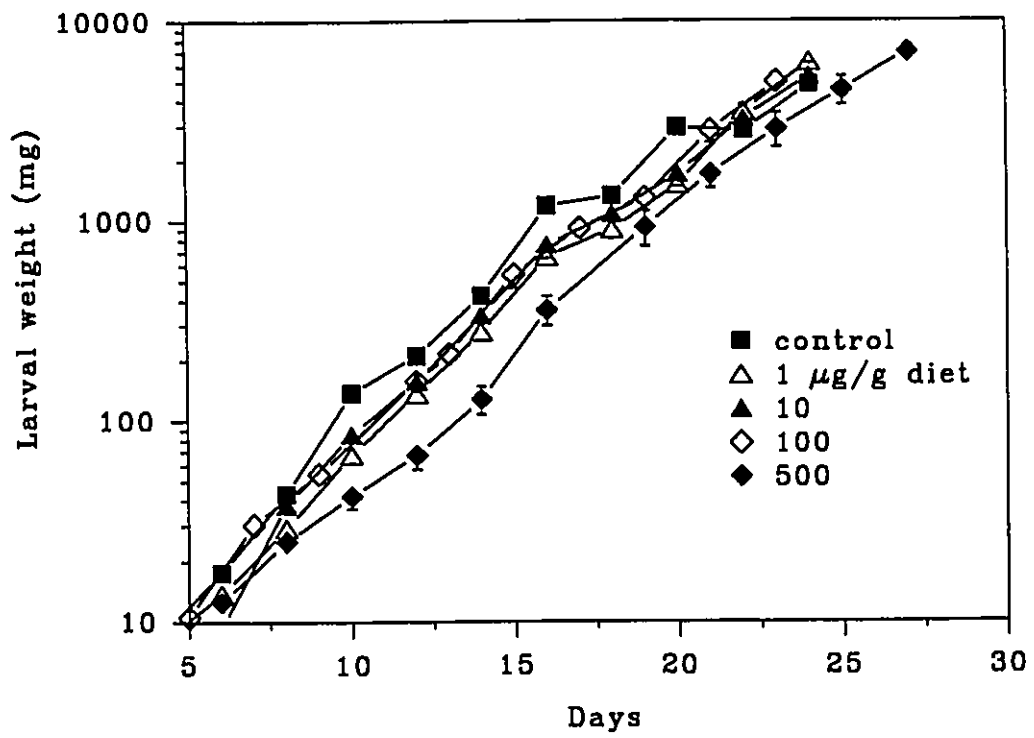
**Figure 2.3** The growth of *Ostrinia nubilalis* reared on artificial meridic diets laden with F020 at 100, 1000, and 5000  $\mu\text{g} \cdot \text{g}^{-1}$  diet. Larvae were reared from eclosion from the egg to pupation, in 10 ml glass vials under controlled environmental conditions. Vertical error bars = s.e.m.



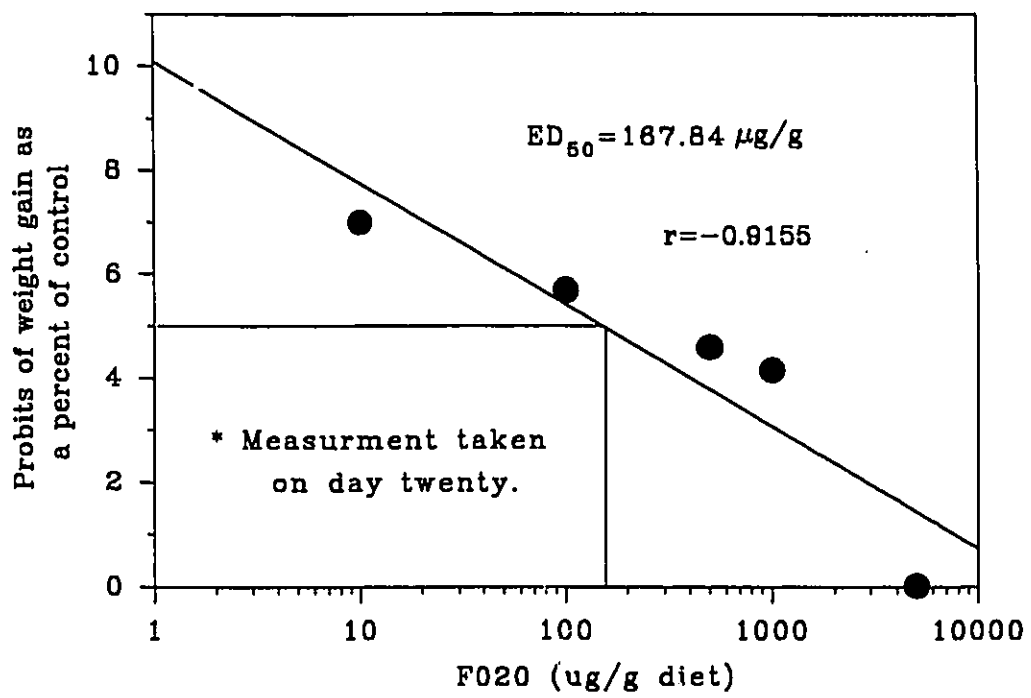
**Figure 2.4** The cumulative percent mortality for *Manduca sexta* reared on an artificial meridic diet laden with F020 at 1, 10, 100 and 500  $\mu\text{g} \cdot \text{g}^{-1}$  fresh diet.



**Figure 2.5** The growth of *Manduca sexta* reared on artificial meridic diet laden with F020 at 1, 10, 100 and 500  $\mu\text{g}\cdot\text{g}^{-1}$  fresh diet. Vertical error bars = s.e.m.



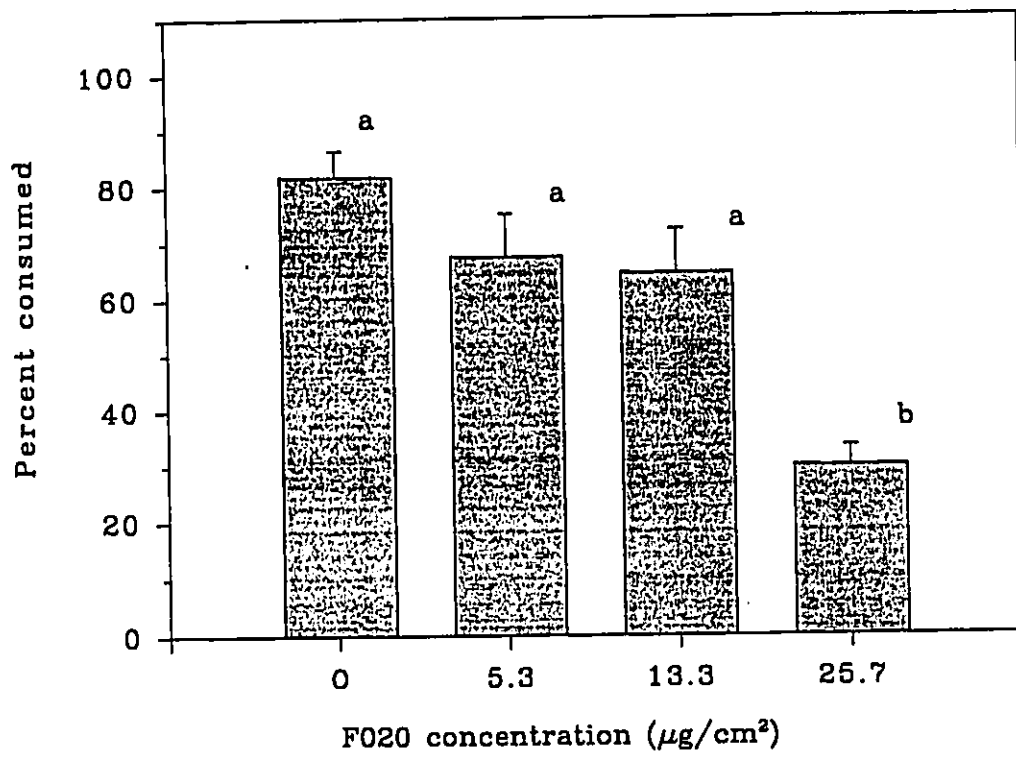
**Figure 2.6** A probit plot of the effect of F020 in the diet, on the weight of *Ostrinia nubilalis* larvae on the twentieth day of development. Probits are taken from weight as a percent of the control values.



### **2.3.2 Antifeedant examination**

The feeding preference study revealed a significantly lower percent of consumption of the leaf area for the leaves treated with  $25.7 \mu\text{g} \cdot \text{cm}^{-2}$  as compared to the control group (Figure 2.7). At the lower concentrations,  $5.33$  and  $13.3 \mu\text{g} \cdot \text{cm}^{-2}$ , a decrease in consumption (not significant) was also observed in comparison to the control group (Figure 2.7).

**Figure 2.7** Feeding response of third instar *Ostrinia nubilalis* larvae in a no-choice feeding preference bioassay. F020 in acetone was topically applied to the exposed young 'Illini gold' corn leaf surface using a microsyringe.



### 2.3.3 Nutritional Indices

The amount of food ingested and the consumption index (C.I.) decreased significantly ( $P < 0.0001$ ) as the concentration of dietary F020 increased (Table 2.6 and 2.7). This phenomenon was also evident ( $P < 0.0001$ ) in the group of larvae that was topically treated with F020 (Table 2.8 and 2.9). Weight gained by each insect was negatively correlated with the concentration of F020, whether in the diet ( $P < 0.0001$ ) (Table 2.6) or whether topically applied ( $P < 0.0001$ ) (Table 2.8).

There was no significant difference in the percentage moisture in feces for all groups under both treatments (at  $P \leq 0.05$ ) except for those larvae that were exposed to diets with F020 at  $5000 \mu\text{g} \cdot \text{g}^{-1}$ , where a decrease in percentage moisture of close to 18% was observed ( $P=0.0004$ ) (Table 2.6). No significant difference in the percent moisture was observed in the feces of those larvae topically treated with F020 (Table 2.8).

The efficiency of conversion of ingested food (E.C.I.) is significantly different between groups for those larvae having ingested the F020 ( $P < 0.0001$ ) and those larvae topically treated with F020 ( $P < 0.0001$ ) (Table 2.7 and 2.9). The approximate digestibility (A.D.) was not significantly different between groups ( $P > 0.05$ ) for both the group having ingested F020 and the group topically treated with F020 (Table 2.7 and Table 2.9).

Ingestion or topical treatment with F020 are correlated with significant decreases in the efficiency of conversion of digested food (E.C.D.) ( $P < 0.0001$  and  $P=0.004$  for dietary and topical treatment, respectively) and efficiency of conversion of ingested food (ECI) ( $P < 0.0001$  and  $P=0.001$  for dietary and topical treatments, respectively) (Table 2.7 and 2.9).

**Table 2.6** The effects of 72-h exposure to dietary F020 on third instar *Ostrinia nubilalis* larvae.

F020 in diet ( $\mu\text{g} \cdot \text{g}^{-1}$ FW)	Food ingested (mg)	Weight gained (mg)	% moisture in frass
0	5.21 a (0.60) n=15	0.67 a (0.06) n=15	81.71 a (1.78) n=15
500	2.56 b (0.26) n=19	0.23 b (0.07) n=19	78.79 a (3.88) n=17
1000	2.85 b (0.34) n=14	0.36 ab (0.14) n=14	82.47 a (2.55) n=12
5000	0.90 c (0.09) n=19	-0.11 c (0.06) n=19	63.93 b (3.91) n=19

Means followed by the same letter within the same column are not significantly different at  $P < 0.05$ , as measured by Tukey's multiple range test.

(s.e.m.), n=sample size.

FW = freshweight.

**Table 2.7** Nutritional indices of *Ostrinia nubilalis* third instar larvae reared on diets laden with F020.

F020 in diet ( $\mu\text{g} \cdot \text{g}^{-1}$ )	C.I.	E.C.I	A.D.	E.C.D
0	1.21 a (0.180) n=15	15.47 a (2.31) n=15	55.8 a (3.94) n=14	27.4 a (4.49) n=14
500	0.75 b (0.037) n=17	9.2 a (2.33) n=15	61.3 a (4.43) n=16	16.93 a (4.45) n=15
1000	0.74 b (0.087) n=14	8.6 a (2.89) n=13	65.1 a (4.96) n=13	15.9 a (4.06) n=13
5000	0.32 c (0.030) n=18	-21.3 b (7.95) n=18	73.1 a (5.54) n=12	-26.0 b (9.10) n=18

C.I. = consumption index, E.C.I. = efficiency of conversion of ingested food, A.D. = approximate digestibility, E.C.D. = efficiency of conversion of digested food. Means followed by the same letter within the same column are not significantly different at  $P < 0.05$ , as measured by Tukey's multiple range test. (s.e.m.), n=sample size.

**Table 2.8** The effects of topical application of F020 in acetone to third instar *Ostrinia nubilalis* larvae.

F020 applied ( $\mu\text{g} \cdot \mu\text{l}^{-1}$ )	Food ingested (mg)	Weight gained (mg)	% moisture in frass
0	3.90 a (0.38) n=20	0.604 a (0.109) n=20	75.62 a (5.07) n=19
12.5	3.41 ab (0.33) n=20	0.424 ab (0.086) n=20	80.99 a (1.96) n=19
25	2.47 abc (0.41) n=20	0.138 bc (0.104) n=20	77.53 a (2.43) n=19
50	2.05 bc (0.37) n=20	0.127 bcd (0.098) n=20	69.36 a (4.86) n=20
100	1.64 cd (0.31) n=20	0.003 cd (0.073) n=20	73.01 a (3.59) n=20
200	1.41 cd (0.37) n=20	-0.067 cd (0.087) n=20	69.48 a (3.30) n=20
400	0.53 d (0.17) n=20	-0.249 d (0.043) n=20	57.31 a (10.03) n=20

Means followed by the same letter within the same column are not significantly different at  $P < 0.05$  as measured by Tukey's multiple range test. (s.e.m.), n=sample size.

**Table 2.9** Nutritional indices for third instar *Ostrinia nubilalis* topically treated with F020 extract in acetone.

F020 applied ( $\mu\text{g} \cdot \mu\text{l}^{-1}$ )	C.I.	E.C.I	A.D.	E.C.D
0	0.89 a (0.075) n=20	9.85 a (3.80) n=20	62.12 a (3.31) n=19	20.13 a (5.54) n=19
12.5	0.83 a (0.058) n=20	6.75 a (4.31) n=20	60.64 a (3.39) n=19	18.24 a (4.23) n=19
25	0.53 ab (0.168) n=20	-34.74 abc (21.21) n=19	60.09 a (4.14) n=15	13.55 ab (6.92) n=15
50	0.57 ab (0.084) n=20	-12.35 ab (9.14) n=18	40.97 a (4.65) n=16	-33.99 ab (35.85) n=16
100	0.50 ab (0.080) n=20	-30.29 abc (11.70) n=20	45.68 a (3.64) n=17	-80.80 ab (40.84) n=17
200	0.37 b (0.083) n=20	-54.99 bc (18.26) n=18	44.17 a (5.34) n=15	-106.19 ab (58.31) n=15
400	0.19 b (0.056) n=20	-72.81 c (17.43) n=16	67.62 a (19.36) n=15	-142.87 b (64.90) n=15

C.I. = consumption index, E.C.I. = efficiency of conversion of ingested food, A.D. = approximate digestibility, E.C.D. = efficiency of conversion of digested food.

Means followed by the same letter within the same column are not significantly different at  $P < 0.05$  as measured by Tukey's multiple range test.

(s.e.m.), n=sample size.

#### **2.3.4 Histological examination**

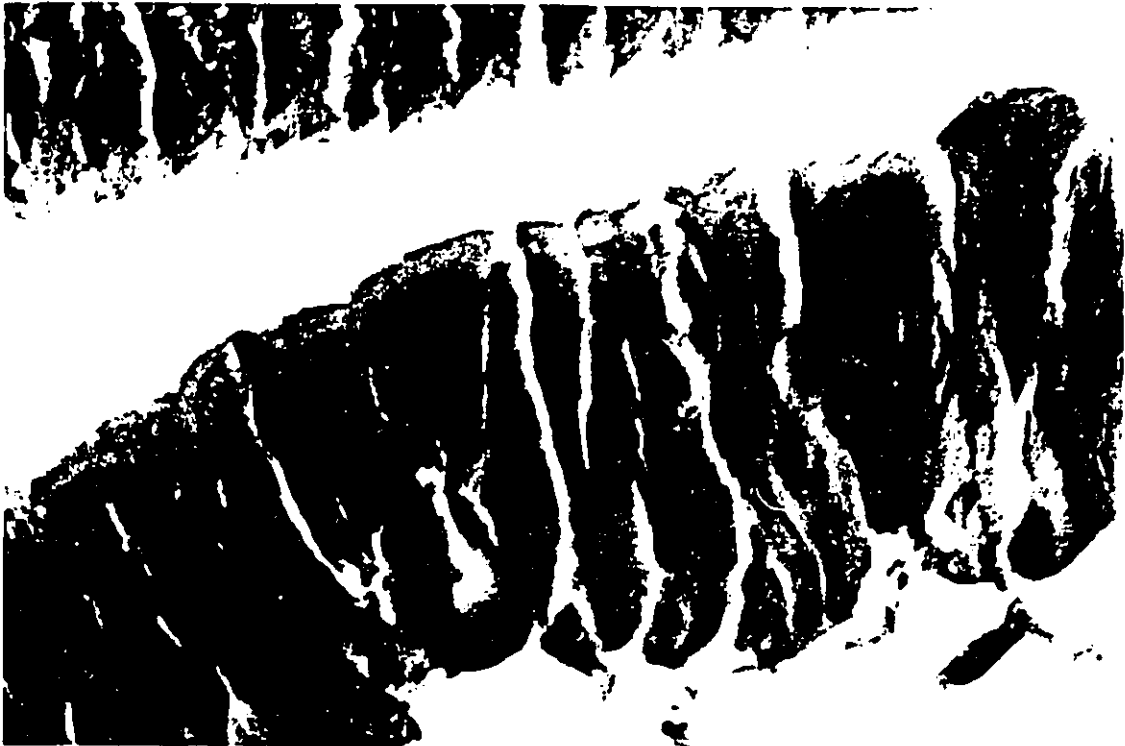
Histological examinations of the guts did not appear to reveal any cellular and/or tissue modifications which could be directly attributed to the ingestion of F020. Typical transverse sections of the midguts of last instar *O. nubilalis* and *M. sexta* larvae, subsequent to being reared on diets laden with F020, are portrayed below.

**Figure 2.8** Transverse sections of the midgut of *Ostrinia nubilalis*. Control larva (A) and larva reared on dietary F020 at  $1000 \mu\text{g} \cdot \text{g}^{-1}$  (B)

A



B

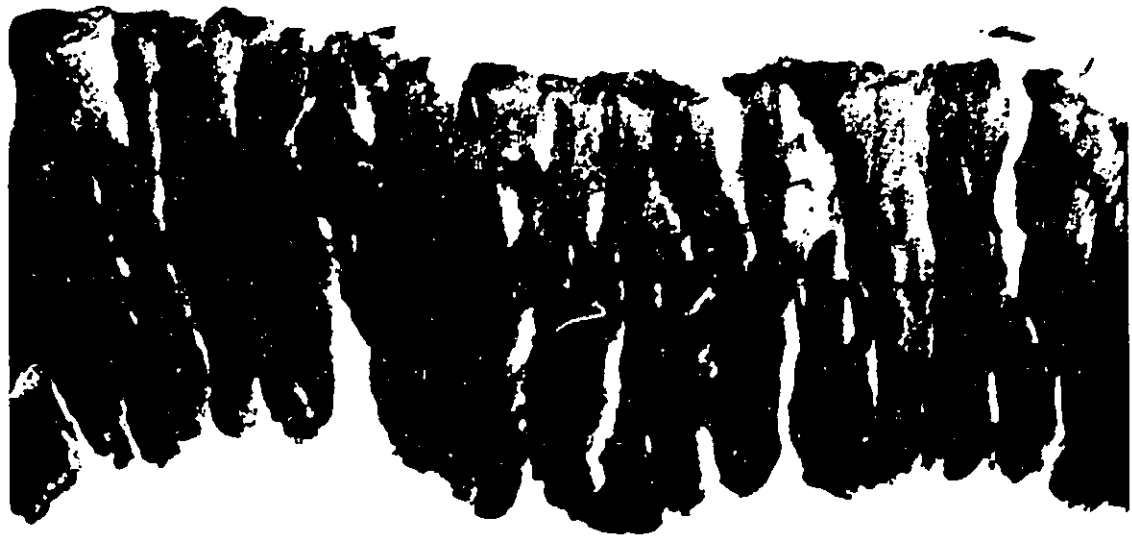


**Figure 2.9** Transverse sections of the midgut of *Manduca sexta* Control larva (A) and larva reared on dietary F020 at  $500 \mu\text{g} \cdot \text{g}^{-1}$  (B).

A



B



## 2.4 Discussion

The observed reductions in the rate of growth, final larval weight, and pupal weights, as well as the increase in percent mortality for both *O. nubilalis* and *M. sexta*, support previously reported insecticidal properties attributed to F020 and asimicin (Mikolajczak, *et al.*, 1988; Alkofahi *et al.*, 1989). These modifications could be brought about by an inhibition of one or a number of vital systems within the insect directly or indirectly related to levels of exposure to F020 or asimicin.

The insects exposed to concentrations of dietary F020 consumed less diet than the control larvae in the chronic exposure study. A similar observation was noted in the food utilization study. The metabolic cost of consumption of F020 becomes the important question. Is it that F020 in the diet deters the larvae from feeding and therefore the resulting lack of adequate nutrition and food energy explains the observed reduction in growth; or is it the result of evidence of certain toxic properties attributed to F020? Because a similar reduction in consumption occurred for those larvae exposed to dietary levels of F020 as well as for those larvae which were topically treated indicates that the substance has toxic properties causing the larvae to become adversely affected, i.e. becoming sick and therefore not feeding. If the decrease in consumption had only occurred in those larvae exposed to dietary F020, then the observed adverse effects could be solely attributable to an antifeedant effect. Consumption was significantly reduced from the control levels regardless of the mechanism of exposure which does suggest a toxic effect but it also makes it difficult to discern which effects are a direct result of toxicity and which are a result of the antifeedant effect. The results of the topical exposure suggests that the reduced growth is due to sickness manifesting itself as a reduction in food intake; however the results from those larvae exposed to dietary levels of F020 suggests that the reduced growth is due to the antifeedant affect or induced sickness. Inadequate nutrition and supply of food energy can be a very powerful impediment to the development of immature insects and adult fitness. Many of the effects of inadequate nutrition observed in other studies can easily be compared to the effects observed for *O. nubilalis* and *M. sexta* in this study. For example, variation in growth, fecundity and survival of *Heliothis virescens* (F.) occurred as a result of being

reared on diets of soybean, cotton or artificial diet (Nadgauda and Pitre, 1983), each diet being of different nutritional quality. Similar to the results obtained in this study, an examination of the nutritional quality between the plant species, *Beta vulgaris* (L.), *Amaranthus retroflexus* (L.), and *Chenopodium album* (L.) and their effect on the development and fecundity of the beet armyworm, *Spodoptera exigua* (Hübner), revealed that the low nitrogen was significant in increasing the development time and decreasing the fecundity ( $P < 0.05$ ) (Al-Zubaidi and Capinera, 1984). The larvae of the moth *Samea multiplicalis* (Lepidoptera: Pyralidae) exhibited a significantly lower number of mature oöcytes ( $P < 0.01$ ) from one-day-old adult females when larvae were reared on plants with a low nitrogen content (Taylor, 1985). A restriction in available diet to fifth and sixth instar larvae of the armyworm, *Pseudaletia unipunctata* (Haw.), resulted in an increase in the number of days spent as a given instar. When fed at particularly low levels (every 4 or 5 days) approximately 45% of the larvae had an extra moult and were smaller than those fed on surplus daily diet (Mukerji and Guppy, 1970). The number of eggs produced per female also decreased in relation to the available food.

The overall effect of F020 on the changes in the food utilization efficiencies, CI, AD, ECI and ECD, were the same whether F020 was incorporated directly into the diet or applied to the insect, and are suggestive of a high metabolic cost from an exposure to F020. The decrease in the volume of food consumed by *O. nubilalis*, would suggest that the nutritional requirements are not being met and therefore providing an explanation for the decrease in the ECD and ECI. The decrease in the ECD and ECI accompanied by the lack of significant change in the AD implies that the food being consumed is digested to the same extent but that less energy is available for growth. The effect cannot be completely attributed to the toxic effect from F020 as, again, inadequate nutrition can effect the indices in a similar manner. In an examination of the utilization of a variety of food plants of different nutritional quality by the polyphagous lepidopteran, *Prodenia eridania* (Cramer), revealed that not all plants were utilized with the same efficiency. The AD varied from 36.28 to 72.58%, E.C.D. varied from 16.8 to 56.81%, and E.C.I. varied from 8.38 to 38.05% (Soo Hoo and Fraenkel, 1966). Nitrogen, an important element for normal insect function and growth, was correlated with changes in the food utilization efficiency of the beet armyworm, *Spodoptera exigua*

(Hübner) when exposed to the different nitrogen levels of its host plants. The AD, ECD, and ECI values were strongly correlated with changes in nitrogen (r-values ranging from 0.84 to 0.96) (Al-Zubaidi and Capinera, 1984).

Restricting the volume of diet available, i.e. partial starvation, can also change the measured nutritional indices in a similar manner to that which was observed in this study. A restriction in the volume of diet available for fifth and sixth instar larvae of the armyworm, *Psuedaletia unipunctata* (Haw.), was strongly correlated with a decrease in the AD, ECI and ECD (Mukerji and Guppy, 1970). Similarly, the cherry scallop moth, *Calocalpe undulata*, deprived of its food, leaves of black cherry (*Prunus serotina* (L.)), for periods of 4 or 8 hours displayed significant decreases ( $P < 0.05$ ) in its rate of growth, quantity of ingested material, in addition to a decrease in faecal production in those larvae deprived of food for 8 hours. Coinciding with this were significant decreases ( $P < 0.05$ ) in (1) the gross efficiency of growth (ECI), from 11.4 to 10.0, and (2) the net efficiency of growth (ECD), from 39.0 to 30.1. As well there was an insignificant increase ( $P > 0.10$ ) in the assimilation efficiency (AD) (Schroeder, 1976) similar to that observed in the present examination for *O. nubilalis*.

The finding in this and other studies establishes that when there is a decrease in the consumption of food there is a corresponding decrease in the in ECD and ECI, as well as a lack of change in AD. Although the nutritional quality of the diet in these studies may have been satisfactory, the forced reduction in consumption limited the available nutrients and thereby changed the nutritional indices. It therefore cannot be ruled out that the changes observed in the nutritional indices in this study may have occurred because of the effect of partial starvation and are not solely attributable to a toxic effect.

Because the ECD and ECI decrease while the AD remains stable implies that F020 is not acting as a digestibility reducer, especially since the results were the same regardless of whether or not the F020 was treated topically or incorporated into the diet. In many cases, the oral administration of sublethal concentrations of toxic substances can rupture or induce swelling in the columnar and goblet cells of the insect's midgut wall. For example, damage to midgut occurred following exposure of tannins to *Papilio polyxenes* (Steinly and Berenbaum, 1985), *Locusta migratoria* and other members of the

Acrididae (Bernays 1978; Bernays *et al.*, 1980), as well as cyanide to *Spodoptera eridania* (Brattsten *et al.*, 1983). In the event of physical damage to the gut the ingestion of a compound may avail itself through increases in percent moisture in the feces (Champagne *et al.*, 1986; Iyengar *et al.*, 1987). A corollary of this, in support of lack of gut damage, is that the percentage moisture in the feces either did not change or decreased with increasing concentration of dietary F020. In addition, the histological examination of the midguts of *O. nubilalis* and *M. sexta* did not reveal evidence of any physical damage subsequent to the ingestion of F020.

The calculation of the ECD and the ECI takes into consideration the mass of food which is converted into body matter. Essentially the mass of food reflects the energy which is required or expended for growth. From this it follows that  $1 - \text{ECD}$ , or  $1 - \text{ECI}$  would reflect the fraction of energy utilized for other things such as respiration, circulation, digestion, immunity, excretion, moulting, movement, detoxification etc. For example, if the ECD is 10% and represents the fraction of energy going towards growth, than 90% of the food energy is going towards other things. It cannot be said with certainty, however, that the reason for the decrease in the ECD or ECI is because more energy is being prescribed for things such as detoxification or respiration since the fraction of energy used is simply a reflection of the quantity of the food consumed. As the quantity of food consumed decreases so also does the energy budgeted towards other processes. Unfortunately it is impossible to determine specifically where if any excess energy demands are occurring. It is likely that both inadequate nutrition, food energy, and toxicity are playing a role in the observed changes in the nutritional indices and that the decreases in growth are attributable to higher maintenance costs associated with detoxification and inadequate nutrition. The higher maintenance costs could be manifested as reduced rates of metabolism or respiration, reduced activity or increased enzymatic activity. Separation of a toxic effect from the effect from inadequate nutrition appears to be difficult if not impossible at the gross morphological and behavioral level, as inadequate nutrition or periods of starvation are linked to reduced activity (Jalees and Murad, 1974), changes in respiration rate (Jalees and Murad, 1974; Schroeder, 1973, 1976; Singhal and Vats, 1976; Newell *et al.*, 1974) and changes in enzymatic activity (Hori, 1970; Ishaaya and Swirsk, 1970; Ishaaya *et al.* 1971). Nonetheless, there

is supportive evidence that F020 is toxic to larvae of *O. nubilalis* and *M. sexta*, namely the high level of mortality and decreases in growth and food assimilation.

# Chapter III

## The effect of F020 and asimicin on moulting

### 3.1 Introduction

The insect juvenile hormones (JH) are long chain fatty acids produced in the corpora allata and are necessary to maintain the proper functioning of the "larval genes" and to modulate what ecdysones can do and, to prevent the degeneration of the prothoracic gland (Eizinga, 1987). Many plants are able to produce the hormone or a mimic of it (Jacobson *et al.*, 1975a; Jaipal *et al.*, 1983), and thus they have the potential to alter the development of the insect which is exposed to them. Examples of plants and plant compounds which possess this juvenilizing property include the mimic juvadecene isolated from New Zealand's *Macropiper excelsum* (Forst, f) (Nishida *et al.*, 1983), echinolone, isolated from *Echinaceae angustifolia* roots (Jacobson *et al.*, 1975b), and the true hormone, juvenile hormone III, isolated from the Malaysian plant, *Cyperus iria* (Toong *et al.*, 1988). Topical treatment of an early final instar larva or nymph with JH or a JH mimic typically results in a supernumerary moult (Bowers, 1968; 1969). The effect of these compounds may also be manifested through retention of certain juvenile characteristics, i.e. gin traps, juvenile coloration and wing deformity rather than perfect metamorphosis (Bowers, 1968; 1969; Jacobson *et al.*, 1975ab; Toong *et al.*, 1988; Das and Gupta, 1974). Juvenile hormone mimics have also been observed to interfere with egg viability and to induce mortality in fifth instar *Oncopeltus fasciatus* nymphs (Brieger, 1971; Toong *et al.*, 1988).

Insect growth regulators (IGR) such as juvenile hormone influence nutrient assimilation. When the IGR's, methoprene, diflubenzeron, and triprene, were applied to mulberry leaves and fed to *Bombyx mori* (L.) the efficiency of food utilization, rate of larval growth and fecundity were altered (Gaaboub *et al.*, 1985). The quantity of food consumed decreased as the IGR concentration increased. The approximate digestibility

seemed to change only slightly and the fecundity of surviving females decreased significantly in direct response to increasing methoprene and diflubenzeron levels (Gaaboub *et al.*, 1985). The above observations were not unlike those reported in this thesis for *O. nubilalis* larvae reared on artificial diets laden with the asimicin-containing extract from the pawpaw, F020. These F020 exposed larvae had lowered consumption rates, decreased growth rates, a decrease in fecundity in surviving females, as well as an inhibition in moulting and an increase in the percent mortality (see Chapter II). Based on these observations F020 and asimicin are suitable candidates for examination as juvenile hormone analogues.

In addition, the chemical structure of asimicin shares enough chemical characteristics with the IGR's to suggest that it may be a juvenile hormone analogue. Historically, a wide variety of chemical structures exist which are able to induce growth regulating effects in insects (Bowers, 1968; 1969; Brieger, 1971; Retnakaran, 1973; 1974; Slama *et al.*, 1974; Nishida *et al.*, 1983). In an examination of the structure activity relationships of hundreds of juvenoids Romañuk (1981) found that straight chain aliphatics possessing 14-16 C atoms were the most active structures, and C:O ratios from 6-8 with high lipophilicity are associated with activity. Brieger (1971) similarly found, upon analysis of juvenile hormone mimics to *O. fasciatus*, that for effective activity, the mimic must be terpenoid in nature, unsaturated and at least one end of the fatty acid chain should be electronegative. Examination of the chemical structure of asimicin (Figure 1.1) show it to have a long straight chain aliphatic region of 15 carbons from the lactone ring to the first tetrahydrofuranoid ring (C2-C16), as well as an additional straight chain aliphatic region of 12 carbons at the terminal end (C24-C34), giving it substantial lipophilicity. It has a C:O ratio slightly lower than optimum of 5.3. Although it is not terpenoid in nature, nor is the carbon chain unsaturated; it is polar at both ends of the chain and unsaturated in the lactone ring. Along with its biological activities enough of its structural properties suggest strong potential for juvenile hormone activity.

The classic *Calliphora* ligation experiments of Fraenkel (1934, 1935) revealed that the hormonal factors responsible for the tanning of the cuticle are present several hours before tanning occurs. Larvae ligated before an apparent critical period were not

observed to tan in the posterior region. Several years following these experiments, the key hormone involved in the tanning process was recognised as the steroid, ecdysone (Huber and Hoppe, 1965). In the last instar larvae, a stage known as the early redspiracle stage begins long after the 'critical period' where enough ecdysone is present to initiate pupation. Ligation of the larvae after this critical period results in both the anterior and posterior parts tanning, with a usual delay in tanning in the posterior part by about 2 hours (Žďárek and Fraenkel, 1972; Fraenkel, 1935; Žďárek, 1980). The delay in tanning is due to the absence of a blood borne factor which is produced or released from the anterior part (Žďárek, 1980). The tanning activity was attributed to a *puparium tanning factor* (PTF), a proteinaceous molecule with at least 12 subunit of approximately 26 000 daltons each (Sivasubramanian *et al.*, 1974). PTF activity has been demonstrated in the pars intercerebralis of the brain of larvae and adults and in that part of the ring gland which contains the corpora cardiaca (Žďárek, 1985). It is assumed, however, that the PTF is stored in the peripheral nerve endings in all segments of the larva. The activity of PTF can easily be tested in larvae of *S. bullata* ligated 3-4 hrs before pupariation. Material to be tested is injected into the posterior part and the PTF activity is evaluated by comparing the time of tanning between the anterior part (A) and the posterior part (P). A typical P/A ratio is about 1.5, with very active materials inducing P/A ratios near 0.5. Because of the presence of abnormal coloration in the adults of some F020 treated *O. fasciatus* nymphs, the affect of F020 was examined for its effect on tanning in *Sarcophaga bullata*.

## 3.2 Materials and Methods

### 3.2.1 Juvenile hormone properties

Zero to twenty hour fifth instar nymphs of *Oncopeltus fasciatus* and 0-20 hour pupae of *Tenebrio molitor* were topically treated, using a Hamilton syringe, on the dorsal surface of the last 4 abdominal segments. Treatments in an acetone solution consisted of  $1 \mu\text{g} \cdot \mu\text{l}^{-1}$  JH III; 12.5, 25, 50 and  $100 \mu\text{g} \cdot \mu\text{l}^{-1}$  F020 and 10, 20 and  $40 \mu\text{g} \cdot \mu\text{l}^{-1}$  asimicin for *O. fasciatus* and for *T. molitor*  $10 \mu\text{g} \cdot \mu\text{l}^{-1}$  JH III, and 100 and  $250 \mu\text{g} \cdot \mu\text{l}^{-1}$  of F020. Control group insects received either no treatment or  $1 \mu\text{l}$  of acetone only. *Oncopeltus fasciatus* were placed in 40 ml plastic 'pill bottles', with up to 30 insects per vial. Vials contained moist cotton, ample sunflower seeds and paper towel as a crawling surface. *Tenebrio molitor* pupae were placed in groups of three into 6 ml plastic 'Solo' cups with enough bran meal as a food source and served as a medium in which the pupae were able to submerge.

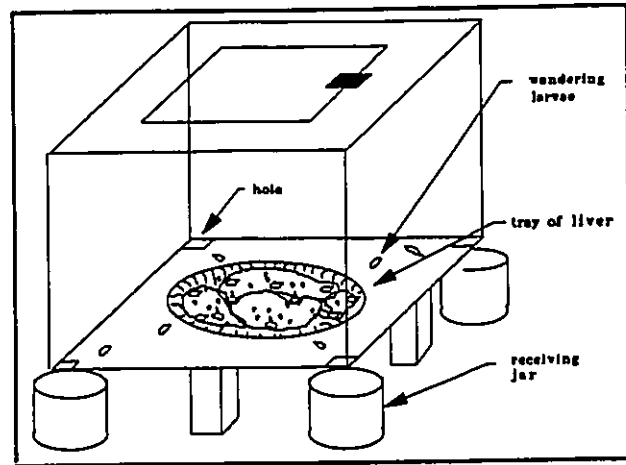
Both insects were reared in a controlled environment at  $21.5^\circ\text{C}$ , 50 % R.H. and a 16:8 hour light:dark regime. Observations of mortality, behaviour and adult malformations were measured and recorded for the two species. Adults which emerged were rated on their juvenile characteristics according to Jacobson *et al.* (1975ab). For *O. fasciatus* a scale of 0 to 3 was used; 0 = normal adult; 1 = retention of nymphal coloration in abdomen; 2 = adult with reduced wings and nymphal coloration; and 3 = supernumary nymph. Similarly emerging *T. molitor* adults were measured on a scale of 0 to 4; 0 = normal adult; 1 = small gin traps or retention of urogomphi without genitalia; 2 = well developed gin traps or intermediate genitalia or both; 3 = well developed gin traps on each abdominal segment, nearly pupal genitalia and patches of pupal cuticle on abdomen; and 4 = virtually a second pupa.

### 3.2.2 Influence on Tanning

Eggs from adults of the flesh fly, *Sarcophaga bullata* were laid onto small pieces (2-3 g) of fresh pork liver which was removed, and subsequently placed into a large aluminum foil tray (20 cm dia.), and kept in a plexiglass enclosure (Figure 3.1).

Emerging larvae were kept at room temperature (approx. 21°C) and allowed to feed on ample supplies of pork liver, placed onto moistened cedar wood shavings. Insects which reached the wandering stage left the liver and crawled along the bottom of the cage (press board) into the corners of the cage and drop through the holes into small receiving jars, about one third full of vermiculite (Figure 3.1). Wandering larvae were collected each morning, removed from the vermiculite, and placed into 500 ml Erlenmyer flasks (approximately 50 larvae per flask). Six ml of distilled water were added to each flask to keep the larvae moist, thereby delaying pupation (Žďárek and Fraenkel, 1971). The flasks were stoppered with 'Solo' cups and two glass tubes (3 mm inner dia.) were inserted through the stopper. The tubes provided the fresh air intake vent (longer tube) and the exhaust flue (shorter tube) (Figure 3.2). The flask was cleaned and water was replaced daily.

After 2-5 days in the water the larvae were removed and placed into trays of vermiculite for 16 to 20 h at room temperature (approx. 21°C) allowing the larvae to dry out and proceed with puparium formation. The larvae which had reached the early red-spiracle stage were then collected for the ligation experiment. Early red-spiracle larvae were placed onto ice for 10 to 15 minutes to immobilize and were then individually ligated (if required) with fine polyester thread posterior to the 6th segment. Immediately following ligation the appropriate test substance (5μl) was injected into the hoemocoel of

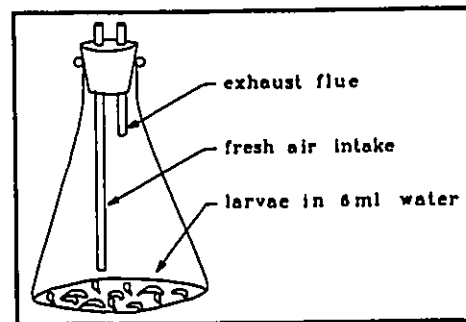


**Figure 3.1** Apparatus for the collection of wandering *Sarcophaga bullata* larvae.

The flask was cleaned and water was replaced daily.

After 2-5 days in the water the larvae were removed and placed into trays of vermiculite for 16 to 20 h at room temperature

(approx. 21°C) allowing the larvae to dry out and proceed with puparium formation. The larvae which had reached the early red-spiracle stage were then collected for the ligation experiment. Early red-spiracle larvae were placed onto ice for 10 to 15 minutes to immobilize and were then individually ligated (if required) with fine polyester thread posterior to the 6th segment. Immediately following ligation the appropriate test substance (5μl) was injected into the hoemocoel of



**Figure 3.2** Apparatus used to synchronize wandering larvae and delay pupation.

the posterior end of each larva using a finely drawn glass pipette. Substances to be injected consisted of Mazola corn oil, haemolymph from light-brown puparium, and F020 in Mazola corn oil between 0.28 and 6.80  $\mu\text{g} \cdot \text{ml}^{-1}$ . F020 concentrations in corn oil were estimated using spectrophotometric analysis (see Appendix G). Injected larvae were placed into individual tissue culture cell plates and kept on ice. Once thirty larvae from each treatment group were completed they were removed from the ice, placed under artificial light at 25°C and 80% RH, at which point the time was recorded. The larvae were monitored every 20 minutes for up to 8 hrs noting the time of the first sign of tanning in the anterior and posterior ends. Larvae were considered to have 'tanned late' if tanning occurred between 8 and 18-h, and considered not to have tanned if it did not tan within this period.

### 3.3 Results

Very few of the treated insects possessed juvenile characteristics in their subsequent molt (Tables 3.1 and 3.3, see also Figures F.1 and F.2, Appendix F). The percent mortality was significantly increased with respect to the topical application of F020 to *O. fasciatus* nymphs; however, the percentage mortality was not significantly affected in the case of the pupae *T. molitor* pupae. In fact the control *T. molitor* pupae had a greater percentage mortality than the treated pupae. All treatment groups of *Oncopeltus fasciatus* nymphs required a longer time to develop under when compared to the means of the acetone treated control group ( $P < 0.05$ , Kruskal Wallis analysis)(Table 3.2). There was no significant effect on the number of days required before the final molt for the F020 treated pupae of *T. molitor* ( $P < 0.05$ ) (Table 3.3). The mean weight of emerged adult *O. fasciatus* appeared to reduced by the topical treatment of F020 as a weak negative trend between level of treatment and weight of both male ( $r = -0.418$ ) and female ( $r = -0.281$ ) emerged adults was apparent, although Kruskal Wallis analysis of variance was not significant.

A large proportion (from 35 to 65%) of F020 treated nymphs of *O. fasciatus* had difficulty in shedding their exuviae at the time of nymphal-adult molt, in particular from the legs and wings. Asimicin treated insects similarly had difficulty shedding their exuviae (approximately 50 % of all survivors), in some cases with a fatal result.

For a small number of treated *T. molitor* and *O. fasciatus* the emerged adults retained pupal or nymphal coloration in their abdomens. A mottled pattern of tanned and untanned cuticle was observed in some *T. molitor* adults and for *O. fasciatus*, some of the darker pigmentation normally present on the abdomen of adults did not develop.

There was no detectable effect of F020 on the tanning process of *Sarcophaga* cuticle (Table 3.4). Although there were some statistically significant differences between means with respect to the number of minutes required for the anterior and posterior parts to tan, examination of the P/A ratio for all groups treated with F020 and corn oil alone, revealed a statistically homogeneous group ( $P < 0.05$ , Tukey's multiple range test). A notably higher number of individuals tanned late, never tanned at all ( $> 18$  hrs), or displayed a mottled tan in the posterior end as a result of treatment,

particularly at the two highest concentrations tested (3.26 and 6.80  $\mu\text{g F020} \cdot \text{ml}^{-1}$ ) (Table 3.5).

**Table 3.1** The juvenilizing effect of a topical application of asimicin and F020 to *Oncopeltus fasciatus*.

Treatment	No. insects treated	No. insects				Percent mortality
		JH rating †				
		0	1	2	3	
none	30	28	0	0	0	6.6
acetone	30	27	0	0	0	10.0
JH III ( $1\mu\text{g} \cdot \mu\text{l}^{-1}$ )	30	11	1	12	3	10.0
<b>Asimicin</b>						
$10\mu\text{g} \cdot \mu\text{l}^{-1}$	12	8	0	0	0	33.3
20	10	4	0	0	0	60 *
40	10	5	0	0	1	40 *
<b>F020</b>						
$12.5\mu\text{g} \cdot \mu\text{l}^{-1}$	30	22	1	0	0	26.7 *
25	30	20	2	0	1	40.0 *
50	30	18	0	0	0	36.6 *
100	30	4	2	1	1	70.0 *

† Juvenile hormone (JH) rating indicating the extent of juvenile characteristics in the emerged adult: 0=normal adult; 1=retention of nymphal coloration in abdomen; 2=adult with reduced wings nymphal; 3=supernumary nymph.

\* Significantly different from the acetone-treated control as measured by 2x2 contingency tables ( $P < 0.05$ ).

**Table 3.2** The effect of the topical treatment of asimicin and F020 on the growth of *Oncopeltus fasciatus*.

Treatment	Average time required for final moult (days) †	Average weight of adult (mg) ‡	
		F	M
none	13.3 a	54.3±2.67 (11)	42.5±1.15 (13)
acetone	12.9 a	52.3±1.66 (7)	42.2±2.23 (5)
JH III (1µg·µl <sup>-1</sup> )	11.3 b	50.0±1.95 (11)	39.6±1.04 (4)
<b>Asimicin</b>			
10 µg·µl <sup>-1</sup>	18.3 c	51.4 (6)	43.0 (2)
20	21.5 c	48.1 (2)	31.7 (2)
40	24.6 c	36.5 (2)	34.9 (2)
<b>F020</b>			
12.5µg·µl <sup>-1</sup>	18.8 c	48.6±1.81 (13)	37.5±1.68 (7)
25	21.9 d	46.7±4.16 (9)	38.5±1.66 (9)
50	22.4 d	45.7±2.32 (11)	36.1±1.32 (7)
100	18.9 c	43.1±0.93 (4)	34.6±0.70 (3)

† mean values followed by the same letter are not significantly different from each other as compared using distribution free multiple comparisons based on Kruskal-Wallis rank sums ( $P < 0.05$ ).

‡ none of the mean weights was significantly different among the controls within the same column nor between the acetone treated control group and the asimicin and F020 treated group ( $P < 0.05$ ) as compared using distribution free multiple comparisons based on Kruskal-Wallis rank sums.

F=female, M=male, Number of insects (n) in parenthesis.

**Table 3.3** The juvenizing effect of a topical application of asimicin and F020 to *Tenebrio molitor*.

Treatment	No. insects treated	No. of insects JH rating ‡					percent mortality	time for final moult (days) †
		0	1	2	3	4		
none	30	22	0	0	0	0	26.7	10.77 ±0.7 a
acetone	36	31	0	0	0	0	3.1	11.06 ±0.9 a
<b>JH III</b> 10 µg · µl <sup>-1</sup>	30	0	0	0	0	28	6.6	10.68 ±0.7 a
<b>F020</b>								
100 µg · µl <sup>-1</sup>	36	32	0	0	0	0	11.1	10.90 ±0.8 a
250	30	28	1	0	1	0	0	10.80 ±1.2 a

† Means followed by the same letter are not significantly different as compared using distribution free multiple comparisons based on Kruskal-Wallis rank sums ( $P < 0.05$ ).

‡ JH rating indicates the extent of juvenile characteristics:

0=normal adult; 1=small gin traps or retention of urogomphi without genitalia; 2=well developed gin traps or intermediate genitalia or both; 3=well developed gin traps on each abdominal segment and nearly pupal genitalia and patches of pupal cuticle on abdomen; 4=virtually a second pupa.

**Table 3.4** The effect of F020 on the time of the first sign of tanning in ligated *Sarcophaga bullata*.

Treatment	time for A	time for P	P/A ratio
ligation only	211.0±14.44 ab (26)	272.7±21.72 bc (23)	1.28±0.043 ab (23)
corn oil	198.2±15.14 a (26)	268.39±22.86 b (23)	1.44±0.103 bc (22)
hemolymph	184.7±9.97 a (25)	165.3±7.91 a (28)	0.94±0.433 a (24)
F020 in corn oil:(µg·µl <sup>-1</sup> )			
0.284	236.5±18.67 abc (20)	364.4±20.71 bcd (14)	1.91±0.180 c (12)
0.320	214.7±11.87 ab (27)	320.7±20.39 bcd (16)	1.67±0.140 bc (16)
0.686	263.9±21.97 abc (20)	366.9±21.36 cd (16)	1.72±0.103 bc (13)
1.352	298.5±19.49 bc (18)	364.3±28.58 bcd (15)	1.49±0.254 abc (9)
3.259	312.1±27.06 c (21)	451.5±53.54 d (6)	2.14±0.502 c (4)
6.804	288.2±26.56 bc (25)	419.6±78.31 bcd (4)	2.07±0.316 bc (4)

Time is measured in minutes.

Values are means ± s.e.m. for those larvae which tanned within 8 hours. Sample size in parenthesis (n). A = anterior and P = posterior.

Means followed by the same letter are not significantly different from each other within the same column (P<0.05) as measured by Tukey's multiple range test.

All treatment solutions were injected at a volume of 5µl.

**Table 3.5** The effect of F020 on the time of the first sign of tanning in ligated *Sarcophaga bullata*.

Treatment	tanned late (8-18 h)		never tanned (> 18 h)		mottled tan	
	A	P	A	P	A	P
*none	5	NA	0	NA	0	NA
ligation only	4	5	0	2	0	0
corn oil	1	1	0	1	2	0
pupal haemolymph	1	1	4	1	0	0
F020 in corn oil: ( $\mu\text{g} \cdot \text{ml}^{-1}$ )						
0.284	1	8	9	7	1	4
0.320	2	8	1	6	0	1
0.686	2	8	7	4	2	4
1.352	4	9	0	7	8	5
3.259	0	12	8	12	0	12
6.804	3	9	2	13	0	9

A=anterior end, P=posterior end. All ligations were on or after the sixth segment. All treatment solutions were injected at a volume of 5  $\mu\text{l}$ .

\* tanning in whole body was compared with the tanning in anterior end of other treatment groups; NA=not applicable

### 3.4 Discussion

Asimicin and F020 do not mimic juvenile hormone, at least not juvenile hormone III. Juvenile hormone III is naturally active in most insects other than the Lepidoptera which instead utilize juvenile hormone 0, I, II and 4-Me I (Schooley *et al.*, 1984). To evaluate the effectiveness of a juvenoid, an 'Inhibition dose-50 Morphological' (ID-50 morph) or the amount of juvenoid required to change treated insects into half adults and half nymphs is often used (Slama *et al.*, 1974). An ID-50 morph value of 0.1  $\mu\text{g}$  or less is taken as highly active, 0.1 to 10  $\mu\text{g}$  as moderately active and 10 or greater as slightly active or inactive (Slama *et al.*, 1974). Given this rating scale, it would appear that neither F020 nor asimicin are effective juvenile hormone mimics since asimicin topically applied at 40  $\mu\text{g} \cdot \mu\text{l}^{-1}$  induced juvenile characteristics after the final molt in only one *O. fasciatus* nymph out of the ten tested, and only two *T. molitor* adults out of thirty appeared to have some pupal characteristics following a topical application of 250  $\mu\text{g} \cdot \mu\text{l}^{-1}$ . Possibly more significant than the lack of juvenile characteristics observed is the high level of mortality reported for *O. fasciatus* nymphs and the difficulty that many of the emerging adults had in escaping from their exuviae. The application of asimicin increased the percentage mortality approximately 3 to 6 times over that of the control insects and F020 induced mortality up to 7 times that of the controls at a dose of 100  $\mu\text{g} \cdot \mu\text{l}^{-1}$ . The high mortality and difficulty shedding the exuviae could indicate toxic rather than juvenilizing properties of F020 or asimicin. Similar effects were reported when the juvenile hormone activity of some common Indian plants was analyzed using the fifth instar nymphs of the cotton stainer, *Dysdercus koenigii* (Fabr.) (Jaipal *et al.* 1983). Topical application of crude extracts of *Melia azadirach* (L.), *Azadirachta indica* (A.) Juss, *Eucalyptus naundina* (Smith), *Lantana camara* (L.) and *Cassia fistula* (L.) prevented some nymphs from completely shedding their exuviae (i.e. sticking to wings and legs), and increased the fifth instar period by up to 15 days. As in *O. fasciatus* nymphs and *T. molitor* pupae, colour was lacking from the abdomen of some of the treated insects. It is possible that some or all of the observed aberrations reported in the above study are not the result of juvenizing properties but manifested by a direct or indirect consequence of the presence of toxic or synergistically toxic compounds

present in the plant extracts. For example, two of the active plants, *M. azedarach* and *A. indica* contain the well known insecticidal compound azadiractin, which has also been found to provoke an antifeedant response, affect growth and interfere with oviposition and fecundity (Schoonhoven, 1980). Interference in moulting by azadiractin is believed to be the result of an alteration in the titre of the structurally similar insect moulting hormone, ecdysone, rather than juvenile hormone (Schoonhoven, 1980).

An examination of the moulting process reveals a complex event both physically and biochemically which, as a result, is susceptible to inhibition at any number of stages, or physical and chemical sites. During the insect moulting process, the old cuticle separates from the epidermis (apolysis) and the newly created space is filled with a fluid ultimately containing activated digestive enzymes. These enzymes digest the endo- and some of the exocuticle, the basic subunits of which are then reabsorbed. Then, using the products of the old cuticle, the insect rebuilds a new, larger cuticle underneath the older one which is subsequently cast off (ecdysis). The new cuticle differentiates and undergoes sclerotization and tanning. Most of the exposed surface areas of the body are involved in the molt including the integumental linings of the tracheae, stomodaeum and proctodaeum. At the time of eclosion the unsclerotized insect is susceptible to physical damage but also large amounts of water loss (up to 6 times normal) through its newly formed cuticle (Elzinga, 1987). The moulting fluid, which helps to provide some lubrication during eclosion, may evaporate quickly under drier conditions and such evaporation before the completion of eclosion can result in an inability to escape, in whole or in part, from the old cuticle and has been known to lead to the death of the emerging insect (Slama *et al.*, 1974; Elzinga, 1987). The whole process is very energy demanding requiring sustained muscular contraction and associated increased oxygen demands and general metabolism (Reynolds, 1980). For example, approximately 33% of the lipid stored by the penultimate instar of the female house cricket, *Acheta domestica* (L.) is metabolized during the molt to the final instar (Woodring *et al.*, 1977) and a sudden increase in oxygen consumption (30%) was measured at ecdysis in *Locusta* (Roussel, 1963). It is likely that those insects which are moribund, due to the prior energy demands such as detoxification or inhibition of other metabolic processes, may

take longer to molt and therefore have difficulty moulting simply because elapsed time has allowed for the evaporation of the moulting fluids.

The inability of insects to cast off old cuticle during ecdysis represents a common side effect associated with juvenoid action (Slama *et al.*, 1974). There are three main types of ecdysial failures, these being 1) where ecdysis proceeds normally but the old cuticle remains fixed to the body at the tips of the legs, abdomen or wings; 2) where ecdysial behaviour is correct, the new cuticle is formed and the old is properly digested; however, the insect remains within the old exuvium; and 3) complete absence of ecdysial behaviour although the new cuticle is formed and the old is ready to be shed (Slama *et al.*, 1974). The ecdysial failures observed with *O. fasciatus* adults are that of type 1. The occurrence of type 1 as well as type 2 failures is believed not to be due to disturbances in the regulation or timing of the ecdysial process, but due to differences in the sizes of formed body parts and tends to occur in those individuals with maximum deviations from both the old and the new developmental stages (Slama *et al.*, 1974). Also because ecdysial failures were never observed in juvenoid treated young larval instars, it appears that juvenoids do not have a direct effect on inducing type 1 or 2 failures, but that these failures result from other developmental abnormalities. Because the type 1 failure was evident in some of the F020 and asimicin treated individuals it could be that the compounds are interfering with morphological development in the wing tips and legs and that other more subtle juvenile characteristics developed in these regions which then hindered a successful ecdysis.

In many species of holometabolous insects the application of a juvenoid to the last larval instar may never be followed by supernumary larval moult no matter when the juvenoid is applied, although the same juvenoid may be highly active on the pupal-adult transformations in the same species. The usual effect on the last larval instars of these insects is a delay in pupation or puparium formation. However, in the majority of other species the application of juvenoids causes a shortening of the intermoult period (Slama *et al.*, 1974). This is supported in our study by the significant decrease in the intermoult period following juvenile hormone III application to *O. fasciatus* fifth instar nymphs (Table 3.2). The *O. fasciatus* nymphs treated with asimicin or F020 all showed significant increases in the number of days required during the intermoult period in an

apparent, although statistically insignificant, dose dependent manner, with the exception of F020 applied at  $100 \mu\text{g} \cdot \mu\text{l}^{-1}$ . Apart from the lack of significant morphological change associated with the treatments this delay in the adult moult also suggests a lack of juvenizing properties associated with these compounds.

Juvenile hormone has been linked to inhibition of melanization in a number of insects. The effect on the newly formed cuticle of the tobacco hornworm *Manduca sexta* has been well studied. The link between juvenile hormone and cuticle coloration was so definite in *M. sexta* that an ultra sensitive bioassay was developed using this principle for detection of juvenile hormone properties of compounds (Truman *et al.*, 1973; Safranek and Riddiford, 1975). It is apparent that the presence of juvenile hormone acts to prevent the synthesis and/or activation of enzymes responsible for the appearance of premelanin granules (Riddiford and Hori, 1981). The fact that melanization and tanning are so very closely linked in time and space and in their biochemical pathway (i.e. conversion of tyrosine to dopa), means that many of the same enzymes are involved and therefore both processes may be inhibited simultaneously by the application of juvenile hormone. Juvenile hormone is thought to inhibit tyrosinase in the conversion of tyrosine to dopa and diphenoloxydase in the formation of dopaquinone (Riddiford and Hori, 1981). The incidence of lack of cuticular colour patterns in treated *O. fasciatus*, *T. molitor* and F020 injected *S. bullata* may have occurred at a time which was critical to the activation of the tanning or melanization process attributing inhibitory activity with these substances. Asimicin or F020 may, in some way be able to inhibit the biochemical pathway common to tanning and melanization and thereby account for the lack of cuticular coloration in regions of the newly moulted adults in the case of *O. fasciatus* and *T. molitor* and in the puparium of *S. bullata*. The localized nature of the loss of pigmentation in the treated insects may imply that the compounds were affecting localized epidermal cells. The epidermis at the onset of ecdysis undergoes rapid cell division resulting in packed, dense columns of cells (Locke, 1969; 1985). These cells, as well as oenocytes and haemocytes have been linked to the site of synthesis of N-acetyldopamine from tyrosine (Andersen, 1985). It is therefore possible that treatment of the insects with asimicin or F020 is inhibiting metabolic processes at the cellular level. These failing cells may be unable to synthesize the appropriate intermediates in the

tanning or melanization pathway and therefore the light or mottled appearance is associated with these affected cells.

The fact that *O. fasciatus* nymphs treated with F020 or asimicin were sluggish in comparison to the control insects, and also that more days were required between the fifth and final molt, suggests that the insects were being robbed of energy normally available for development and growth. Insufficient available energy, combined with the time restriction during ecdysis, could explain the observed failure to shed the cuticle in treated *O. fasciatus* nymphs. As the reduction in growth of treated *O. nubilalis* and *M. sexta* larvae (see Chapter II) and *O. fasciatus* last instar nymphs might suggest, more evidence is needed examining any inhibitory effect of asimicin or F020 on basal metabolism.

# Chapter IV

## Effect of F020 and Asimicin on respiration

### 4.1 Introduction

Plants from the family Annonaceae have long been regarded as having potent medicinal properties. Bioassays with linear acetogenins from plants of the family Annonaceae have shown that many of these compounds have potent biological activity involving cytotoxicity, antitumour activity, brine shrimp lethality, and pesticidal activity (Jolad *et al.*, 1982; Dabrah and Sneden, 1984a,b; Cortes *et al.*, 1984; Pettit *et al.*, 1987; Fujimoto *et al.*, 1988; Hui *et al.*, 1989; Li *et al.*, 1990; Born *et al.*, 1990; Abreo and Sneden 1989; and Kawazu *et al.*, 1989). The pesticidal properties of asimicin, as well as the ethanolic extract, F020, from the bark of *Asimina triloba*, have been demonstrated with the Mexican bean beetle, *Epilachna varivestis*, (70% mortality at 10 ppm, 100% at 50 ppm), the melon aphid, *Aphis gossypii* (20% mortality at 100 ppm and 100% at 500 ppm), mosquito larvae, *Aedes aegypti* (100% mortality at 1 ppm), the nematode, *Caenorhabditis elegans* (100% mortality at 0.1 ppm), blowfly larvae, *Calliphora vicina* (100% mortality at 1000 ppm) and the striped cucumber beetle, *Acalymma vittatum* (50% mortality at 0.5% solution) (Mikolajczak, *et al.*, 1988; Alkofahi *et al.*, 1989). F020 has been shown significantly to increase mortality and inhibit growth of larvae of the European corn borer, *Ostrinia nubilalis* at 500  $\mu\text{g}\cdot\text{g}^{-1}$ , and significantly to increase mortality of neonates and reduce growth, of larvae of the tobacco hornworm, *Manduca sexta*, at 1  $\mu\text{g}\cdot\text{g}^{-1}$  and at 100  $\mu\text{g}\cdot\text{g}^{-1}$ , respectively (see Chapter II). Nutritional indices (Waldbauer, 1964, 1968; Kogan and Parra, 1981; Kogan, 1986) was made for third instar larvae of *Ostrinia nubilalis*. The results from F020 incorporated into an artificial diet were compared to the results from a topical application of F020 in acetone and parallel findings were described (see Chapter II). This study effectively demonstrated

that F020 significantly reduced the larval growth rate, lowered larval dietary consumption and pointed to the site of toxic activity as post-digestive. Inhibition of growth can suggest that food energy is being lost to other metabolic processes. The lack of energy as a result of exposure to F020 and asimicin is also apparent from the examination of the hormonal properties of F020 and asimicin (chapter III) where moulting difficulty was attributed to a lack of energy and again there was a reduction in rate of growth for the exposed insects. It is hypothesised that the adverse effects observed from the examinations carried out are a result of interference or inhibition of the metabolic processes in these insects.

Londershausen *et al.* (1991) have recently reported electron transport chain inhibition for the linear acetogenins, annonin I, II, and VI, extracted from *Annona squamosa*. The results of the current study help to support this finding through an *in vivo* examination of the effect of asimicin and F020 on larval insect respiration and subsequently the effect on respiration *in vitro* through isolated mitochondria focusing on possible sites of inhibition in the electron transport chain or in oxidative phosphorylation.

## 4.2 Materials and Methods

### 4.2.2 Whole Body Respiration:

Early fourth instar *Ostrinia nubilalis* larvae were removed from the stock colony (Appendix A), weighed and placed in individual vials (6 ml) containing a portion of meridic diet cube which was untreated or laden with F020 at 250, 500, 1000, 2000, or 4000  $\mu\text{g}\cdot\text{g}^{-1}$  diet. Both the untreated and treatment diets contained ethanol (95%) at < 1% (w/w). Each vial was closed with a plastic cap punctured with 4 pin holes from a no. 4 pin. The vials were placed horizontally in a styrofoam tray and set in a controlled environment chamber at 25°C, 85% RH, and 16:8 L:D regime. After 72 hours, the larvae were removed, weighed and placed in pairs in the side arm of Warburg manometer flasks. A fanfolded piece of Whatman # 1 filter paper (12 x 20 mm) was placed in each side arm to provide a crawling surface. Each Warburg flask was equipped with a 15 x 6 mm dia. well holding 0.2 ml of 10% KOH solution and a 24 x 20 mm fanfolded piece of Whatman # 1 filter paper as a wick. Each well was lubricated around its upper perimeter with stopcock grease to prevent overflow of KOH. Each flask was attached to a manometer filled with Brodies manometer fluid (see Appendix H) and dyed with acid fushin. The side arm stopper and manometer were sealed to the flask with stop cock grease and held securely to the flask with a rubber elastic band. This airtight Warburg constant volume respirometer was placed so that the flask was completely submerged in a 25°C stirred water bath. To make maximum use of the graduated scale, the fluid in the manometer was raised to the 30 mm mark with the stopcock open, the stopcock was then closed and the manometer fluid was raised from the reservoir until the fluid on the closed reaction flask side read 150 mm. Fifteen minutes were allowed to pass before the first readings for temperature equilibrium, CO<sub>2</sub> absorption, and for larval-acclimation. A thermobarometer flask with manometer was run at the same time under the same conditions to monitor ambient pressure and temperature changes. The thermobarometer flask contained the same materials as the respiring flasks less the larvae. Four control groups of larvae, falling within the weight ranges of 5-9.99, 10-14.99, 15-19.99 and 20-25.0 mg, were examined for weight and

development effect on respiration. These control weight-range groups were taken directly from the stock culture.

A light cotton plug was placed in the entrance to the manometer tubing to prevent larvae from entering and sealing off the manometer.

Measurements were taken at 30 minute intervals for two hours, at which time the location of larvae was noted. The larvae were then individually placed in culture cells and dried in an oven at 60 °C For 24 hours. The larvae were then removed, allowed to cool for 1-2 hours and the dry weight was recorded with a Mettler semi-micro balance.

#### **4.2.2 Mitochondrial Respiration:**

Thirty late, still feeding, fifth instar larvae were selected from stock colony reared on artificial meridic diet. They were dissected under cold isolation medium (IM). The IM (pH 7.5) was composed of 250 mM sucrose, 1 mM EDTA, 1 mM EGTA, 10 mM HEPES and 1% BSA (essentially fatty acid free) in distilled water (pH was adjusted with NaOH or HCl). The whole larval guts were cleared, removed and rinsed 3 times in ice cold IM, before being placed into approximately 1 ml of cold IM. After 30 guts were collected, they were homogenized with 15 passes of a hand held Pyrex mortar and pestle (2 ml capacity with 0.15 mm clearance). The homogenate was poured into a single centrifuge tube and the pestle was rinsed 3 times with IM. The homogenate was then centrifuged at 2500 g at 4°C for 10 minutes. The supernatant was collected and centrifuged for 10 minutes at 10 000 g at 4°C. The resulting pellet was then resuspended in 2.5 ml of cold IM. One half of one ml of this suspension was drawn off and placed in a second centrifuge tube containing 2 ml of distilled water. The two tubes were then placed in the centrifuge at 10 000 g for an additional 10 minutes at 4°C. The supernatant was discarded from both tubes. The larger pellet (suspended in IM) was resuspended in 2.1 ml of IM, and the smaller pellet, washed in water, was resuspended in 0.5 ml of distilled water and subsequently frozen to -12 °C in an Eppendorf tube for protein analysis at a later date. Resuspension took place by rapid suction and expulsion of the liquid with a Pasteur pipet, avoiding aeration of the sample.

One ml of the mitochondrial suspension was added and stirred with 2 ml of 30°C

reaction medium (pH 7.5) consisting of 250 mM sucrose, 10 mM  $\text{KH}_2\text{PO}_4$ , 10 mM Hepes, 1 mM EDTA, 1 mM EGTA and 1% BSA (essentially fatty acid free). The mixture was allowed to equilibrate for 5 min in the reaction chamber at which time the lucite plunger holding the oxygen electrode, covered with a high sensitivity membrane, was inserted into the sample chamber. Measurements were recorded polarographically on a Yellow Springs Instrument (YSI) biological oxygen monitor (model 5300) which had been previously calibrated with air saturated water. A Canlab Chart recorder set at 100 mvolts and chart speed of  $1 \text{ cm} \cdot \text{min}^{-1}$  was used to record the measurements.

After the recordings stabilized (approx. 1-2 min),  $10 \mu\text{l}$  of a solution of 0.2 mM pyruvate and 5 mM malate were injected into the chamber. Once the initial ADP limited (state 4) respiration rate was at a steady rate for approx. 1 min,  $10 \mu\text{l}$  of a 100  $\mu\text{M}$  solution of ADP was injected into the reaction chamber. The ADP-dependent respiration rate (state 3) was then recorded and designated as the control rate for that sample. Once this state 3 respiration terminated the resulting state 4 was recorded for an additional 2 minutes. At this time,  $10 \mu\text{l}$  of F020 or asimicin dissolved in 95% ethanol was injected. One minute was allowed for further stabilization of the ensuing state 4 respiration followed by a subsequent additional  $10 \mu\text{l}$  of ADP into the reaction chamber. The recording of any evidence of state 3 respiration, designated as the treatment rate, was followed by 2 minutes of state 4 respiration at which point all recordings were terminated for that sample. A second 1 ml sample (stored on ice) was immediately analyzed in an identical manner.

Protein analysis was carried out using a modification of the Biuret method of Gornall *et al.* (1949) using essentially fatty acid free BSA as the standard. Three  $150 \mu\text{l}$  replicates from one sample were mixed with  $100 \mu\text{l}$  of 5% (w/v) deoxycholate and 1.5 ml Biuret reagent and whirlmixed briefly. Absorbance of the solution was measured at 540 nm after 30 min using a Varion-Cary spectrophotometer.

### 4.3 Results

The growth of larvae which were fed diets treated with F020 for three days, was reduced in a dose dependent manner (Figure 4.1). The difference between the dry larval weights of treatment groups is evident (Table 4.1 and Figure 4.1) and shown to be statistically significant by Tukey's multiple range test ( $P < 0.05$ ). As well, Pearson's product-moment correlation analysis between the weight of each pair of larvae and the treatment levels of F020, revealed a strong negative correlation  $\rho = -0.6612$  ( $P < 0.0001$ ).

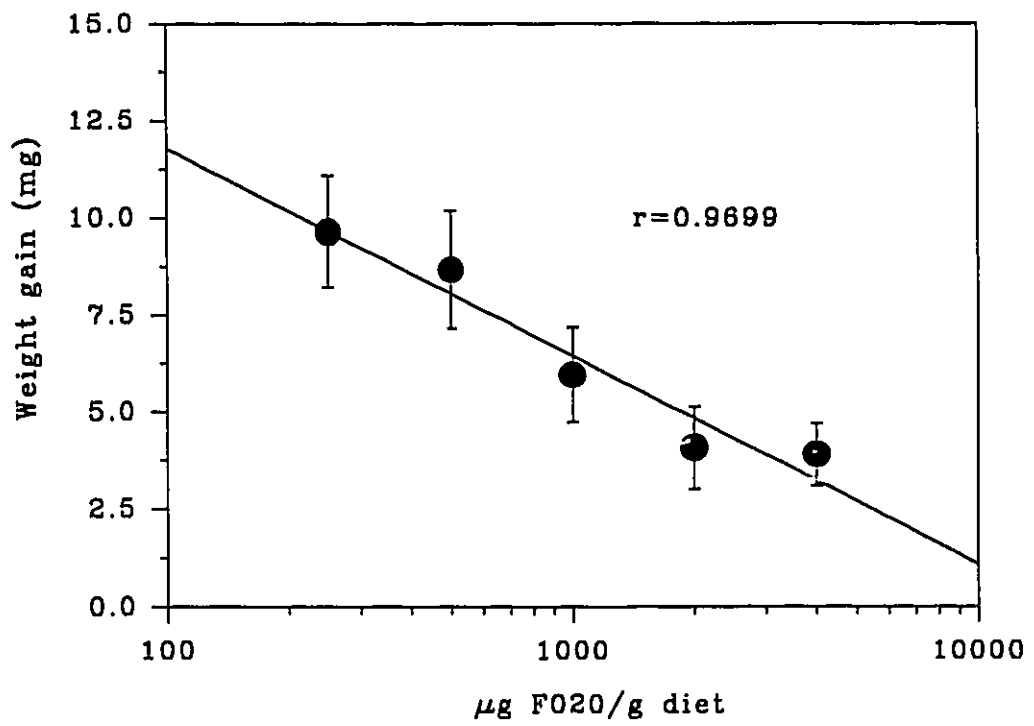
For those larvae exposed to dietary F020 the respiratory rate per mg increased with the concentration of F020 (Figure 4.2). The positive linear relationship between the log of the concentration of dietary F020 and the respiration rate suggests a stimulatory effect of consumption of higher doses of F020 (Figure 4.2). The weight of the larval pair, however, concealed the actual reduction of the respiratory rate occurring following the exposure to F020. Since the exposure to levels of dietary F020 is inversely correlated with growth (Figure 4.1), the larval weight at the highest dose was significantly lower than that found in the low F020 dose range (Table 4.1). For the group of control larvae, the larval pair weight ( $W_{pr}$ , combined weight of the two larvae) was positively correlated with the lower respiration rate ( $\mu\text{mols O}_2 \cdot \text{hr}^{-1}$ ) ( $\rho = 0.8788$ ,  $P < 0.0001$ ). Similarly, a positive correlation was found between the  $W_{pr}$  of the larvae fed on diets laden with F020 and their respective respiration rates ( $\rho = 0.7742$  at  $P < 0.001$ ). However, when a larval weight factor is incorporated into the respiratory rate ( $\mu\text{mol O}_2 \cdot \text{mg}^{-1} \cdot \text{hr}^{-1}$ ), a negative correlation exists between the  $W_{pr}$ , for the range of control larvae examined ( $\rho = -0.5925$  at  $P = 0.0001$ ) and a small positive correlation exists for the treated larvae ( $\rho = 0.3207$ ,  $P = 0.017$ ). In other words, the smaller larvae from the F020 treated group respire at a lower rate than the control group of the same weight range. The respiratory inhibitory effect of F020 is displayed in Figure 4.3 where a reduction in the rate of respiration for smaller larvae coincides with higher levels of exposure to F020, although in this graph the final weights are considered without reference to the actual F020 concentrations. Nonetheless, the weight independent rate ( $K$ ) is higher in the control group (A) than in the treated group (B) showing that the treated group has a lower respiration rate (Figure

**Table 4.1** Effect of consumption of F020 on the final *Ostrinia nubilalis* larval dry weights.

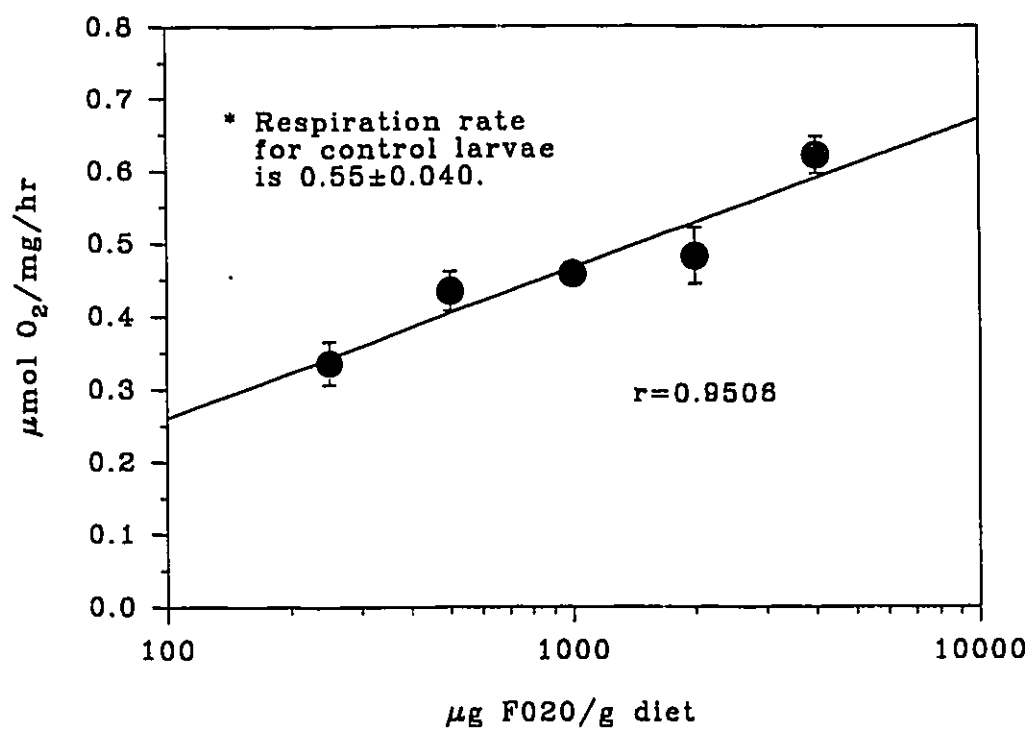
Treatment group	Sample size	mean larval dry wt. (mg)	mean larval dry wt. of pair (mg) †
<b>Control weight range:</b> (mg fresh weight)			
5-9.99	20	1.20±0.043 a	2.62±0.214 a
10-14.99	20	2.33±0.099 b	4.49±0.199 b
15-19.99	20	3.43±0.098 c	6.69±0.321 c
20-25	20	4.25±0.084 d	8.16±0.453 d
<b>F020:</b> (µg · g <sup>-1</sup> diet)			
0	19	4.08±0.140 a	8.13±0.311 a
250	18	3.46±0.266 ab	6.09±0.615 b
500	20	3.09±0.258 bc	6.00±0.607 b
1000	19	2.49±0.226 cd	5.01±0.305 bc
2000	19	2.06±0.184 d	4.19±0.341 bc
4000	20	1.93±0.163 d	3.97±0.387 c

Values are means ±s.e.m. Larvae were exposed to the F020 laden diet for a period of seventy-two hours. Means followed by a different letter indicate a significant difference between treatment groups, as analyzed by Tukey's multiple range test at P < 0.05. † Values are the mean dry weight of the pair of larvae used in each sample flask of the Warburg respirometer. Flasks containing only one larva were disregarded.

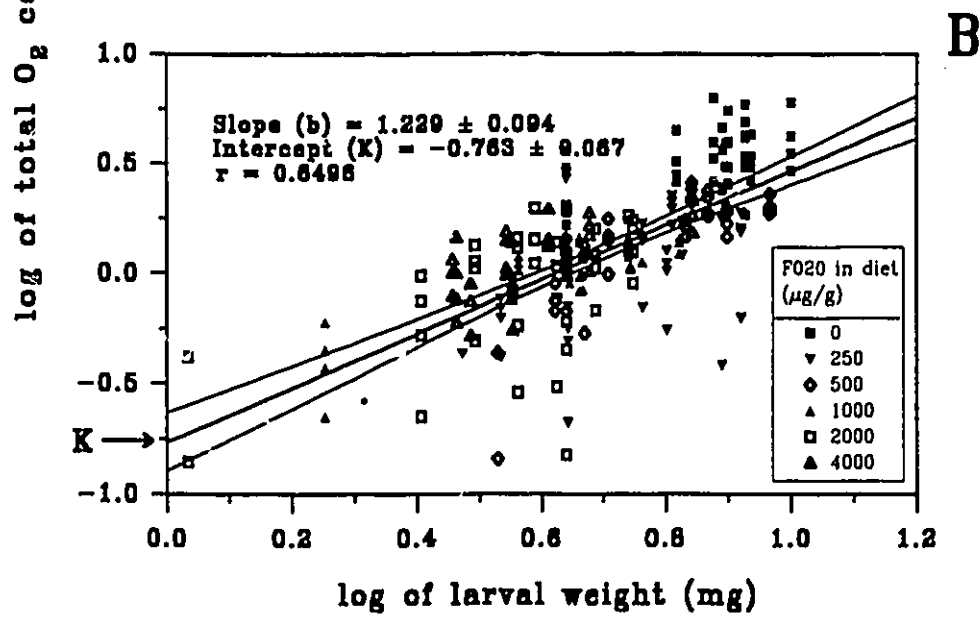
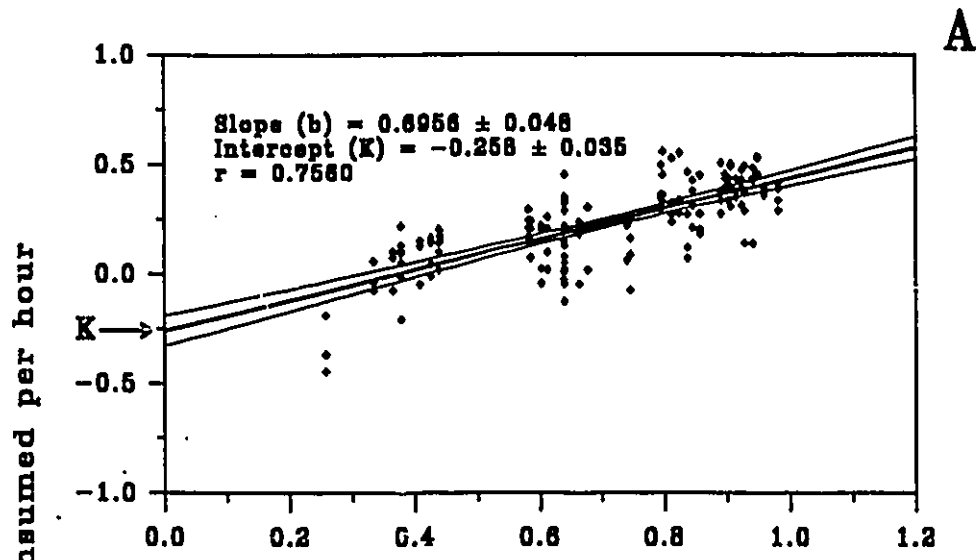
**Figure 4.1** The effect of F020 on the growth of fourth instar *Ostrinia nubilalis* larvae fed on F020 laden diets for a period of 72 hours.



**Figure 4.2** The effect of F020 on the respiratory rate of third instar *Ostrinia nubilalis* larvae, disregarding the size of larvae.



**Figure 4.3** The effect of weight of whole larvae on the rate of respiration. Abscissa values are the combined weight of the pair of larvae in each Warburg flask. Ordinate values represent the log of  $\mu\text{mols}$  of  $\text{O}_2$  consumed per hour for the pair of larvae in each flask of a Warburg apparatus. The control group (A) received meridic diet less the F020 and the treatment larvae (B) received artificial meridic diet laden with F020 at 250, 500, 1000, 2000 and 4000  $\mu\text{g} \cdot \text{g}^{-1}$  diet for a period of 72 hours. All diets, including the control diet, were prepared with less than 1% of 95% ethanol. K = intercept.



4.3). Using statistical analysis to support this graphically derived conclusion, an analysis of covariance (Sokal and Rohlf, 1981) was conducted with larval weight as the covariate. From this analysis, it was apparent that both weight and treatment are significantly affecting respiration,  $P = 0.0002$  and  $P = 0.0000$ , respectively) with the level of F020 being the most significant..

Gut mitochondria isolated from fifth instar *O. nubilalis* showed inhibition of respiratory oxygen consumption when exposed to levels of either F020 or asimicin. For the controls, no treatment and treatment with 95% ethanol, the mean ADP:O ratio (micromoles of ADP added per micro atoms of  $O_2$  utilized) was not significantly different. None of the pre-treatment or 'control' group (Table 4.2) ADP:O ratios was significantly different.

Although the mean values of the respiratory control ratio (RCR), a ratio of the state 3 rate divided by the state 4 rate, are lower following treatment with F020 (Table 4.2), only at a level of  $1.46 \mu\text{g}$  F020, was there any significant difference from the pretreatment values compared using the t-test at  $P \leq 0.05$ . Treatment of the mitochondrial suspension with asimicin, did not show any significant difference in the RCR values from the controls nor between treatment levels (Table 4.2).

The ADP-dependent, or state 3 rate, decreased as the concentration of F020 injected into the chamber increased (Table 4.3). The observed difference in the state 3 respiration rate was significant between treatment groups, as well as between the control and treatment responses among the suspensions treated with F020 at  $< 0.49 \mu\text{g}$  (Table 4.3). Any evidence of an effect of inhibition of the state 3 rate after treatment with asimicin was not as indicative at those levels tested; however, there is an apparent reduction in the state 3 rate compared to the control rates for each treatment (Table 4.3). Addition of F020 at  $> 1.46 \mu\text{g}$  and asimicin at  $> 0.283 \text{ nmols}$  brought about complete inhibition of the state 3 respiration rate using the NADH-linked substrates, pyruvate and malate (not shown). When using the FAD-linked substrate, succinate, neither F020 nor asimicin inhibited the state 3 rate (Table 4.4) at levels of F020 and asimicin injected at greater than 10 times the previous threshold value for maximum inhibition using pyruvate and malate as the substrates. In addition, those preparations sparked with pyruvate and malate which were inhibited at this level could be reactivated by the addition of succinate

into the reaction chamber. The reactivation in preparations inhibited at this level was identical to reactivation of preparations inhibited with rotenone, a well known inhibitor of NADH-ubiquinone oxydoreductase coupling site (complex I) (data not shown).

Asimicin and F020 decreased the rate of respiration (state 3) ( $\text{nmol O}_2 \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$ ). These respiratory values were probitized and are portrayed in Figure 4.4 and 4.5, revealing  $\text{ED}_{50}$ 's of  $1.686 \mu\text{g} \cdot \text{mg}^{-1}$  protein and  $0.546 \text{ nmol} \cdot \text{mg}^{-1}$  protein for F020 and asimicin, respectively.

There is a statistically significant trend showing a decrease in the state 4, ADP limiting respiration rate, as the concentration of F020 injected increases (significant through Tukey's multiple range test at  $P \leq 0.05$  (Table 4.3). This difference in state 4 rate, however, was not significantly different from the control measurements (t-test,  $P=0.05$ ). Treatment with asimicin yielded a similar result in that the state 4 rate showed a reduction from the control rates (significant at  $1.176 \text{ nmol}$ ); however, the reduction in the state 4 rate was not significantly coupled with an increase in the concentration of asimicin in the reaction chamber (Table 4.3).

**Table 4.2** The effect of F020 and asimicin on the ADP:O ratio and the RCR from mitochondria isolated from the guts of fifth instar *Ostrinia nubilalis*.

Treatment	ADP:O ratio		RCR	
	pretreatment	treated	pretreatment	treated
no treatment	3.46±0.16	3.43±0.25	3.36±0.40	2.97±0.26
ethanol (95%)	3.44±0.03	3.36±0.47	3.27±0.18	2.83±0.84
<b>F020:</b>				
<b>(µg)</b>				
0.37	3.24±1.03	3.22±0.63	3.10±0.79	2.74±0.56
0.49	3.06±0.35	4.13±0.50	5.26±0.45	3.69±0.71
0.73	2.52±0.21	4.37±0.77	4.03±0.73	3.51±0.73
0.98	3.91±1.26	>6.0	5.31±1.21	2.47±0.18
1.46	3.55±0.52	>6.0	4.43±0.10	*2.47±0.66
<b>Asimicin:</b>				
<b>(nmol)</b>				
0.141	3.79±0.25	3.48±0.56	3.97±0.89	5.47±1.27
0.188	3.68±0.13	7.32±1.52	3.66±0.09	5.11±0.94
0.283	3.33±0.34	7.89±2.16	3.92±0.35	7.13±1.10
0.376	3.71±0.14	—	3.61±0.28	—
0.564	3.46±0.16	—	3.15±0.19	—

Values given are means ± s.e.m. with n=3 in all cases. The treatment levels indicated represent the final amount in the reaction chamber. F020 was dissolved in 95% ethanol for all treatment groups.

'pretreatment' = ADP-stimulated rate prior to the injection of the treatment.

'treated' = ADP-stimulated rate after the injection of F020 or asimicin.

\* significant difference from the control value using t-test at P < 0.05. No significant difference between treatment groups was found using Tukey's multiple range test at P=0.05.

R.C.R = respiratory control ratio (state 3/state4).

**Table 4.3** The effect of F020 and asimicin on the State 3 and State 4 respiration rate from mitochondria isolated from the midguts of *Ostrinia nubilalis*.

Treatment	State 3		State 4	
	pretreatment	treated	pretreatment	treated
no treatment	86.5±4.31	83.2±3.26	26.2±1.72	28.4±2.48
ethanol (95%)	91.8±5.43	82.5±5.39	29.6±3.71	28.3±4.33
F020: (µg)				
0.37	55.6±20.58	56.8±7.86	17.7±3.23	21.2±1.21
0.49	82.6±23.72	56.6±13.82	16.0±4.69	15.3±1.89
0.73	58.9±3.76	*34.2±5.22	15.8±3.40	11.3±4.05
0.98	72.6±5.95	*26.2±1.52	14.7±2.14	10.8±1.29
1.46	72.0±2.01	*33.5±8.08	16.3±0.78	14.3±2.06
Asimicin: (nmol)				
0.141	81.3±16.98	68.2±11.21	22.0±6.24	14.6±5.61
0.188	111.6±3.23	78.8±5.35	30.6±0.94	*16.1±1.92
0.283	88.8±25.58	65.4±5.34	22.1±5.14	9.8±2.25
0.376	113.4±8.70	CI	32.1±4.72	*13.5±3.24
0.564	87.0±4.65	CI	27.6±0.43	*17.9±1.63

Values given are means of  $\text{nmol O}_2 \cdot \text{min}^{-1} \cdot \text{mg}^{-1} \pm \text{s.e.m.}$  ( $n=3$ ). The treatment levels indicated represent the final amount in the reaction chamber. F020 was dissolved in 95% ethanol for all treatment groups.

'pretreatment' = ADP-stimulated rate prior to the injection of the treatment.

'treated' = ADP-stimulated rate after the injection of F020 or asimicin.

\* significantly different from the control value using t-test at  $P=0.05$ . No significant difference between treatment groups was found using Tukey's multiple range test at  $P < 0.05$ .

CI=Complete inhibition

**Table 4.4** The effect of succinate as a substrate on the inhibitory effect of asimicin or F020 on mitochondrial respiration.

Treatment		State 3 rate		State 4 rate	
		pretreatment	treated	pretreatment	treated
<b>Succinate sparked:</b>					
Asimicin (nmoles)	0.376	97.4±8.64	92.8±5.33	43.1±1.78	35.9±3.04
	48.0	123.4±15.5	113.2±3.62	50.2±3.22	48.3±4.66
F020 (µg)	1.46	96.3±1.07	101.4±6.44	35.5±10.4	31.5±7.36
	15.6	99.3±2.55	135.4±26.5	44.8±2.95	43.5±1.85
<b>Malate and Pyruvate sparked:</b>					
*F020 (µg)	7.81	84.90	CI	24.18	17.99
*rotenone (nmoles)	86	79.73	CI	31.44	17.28

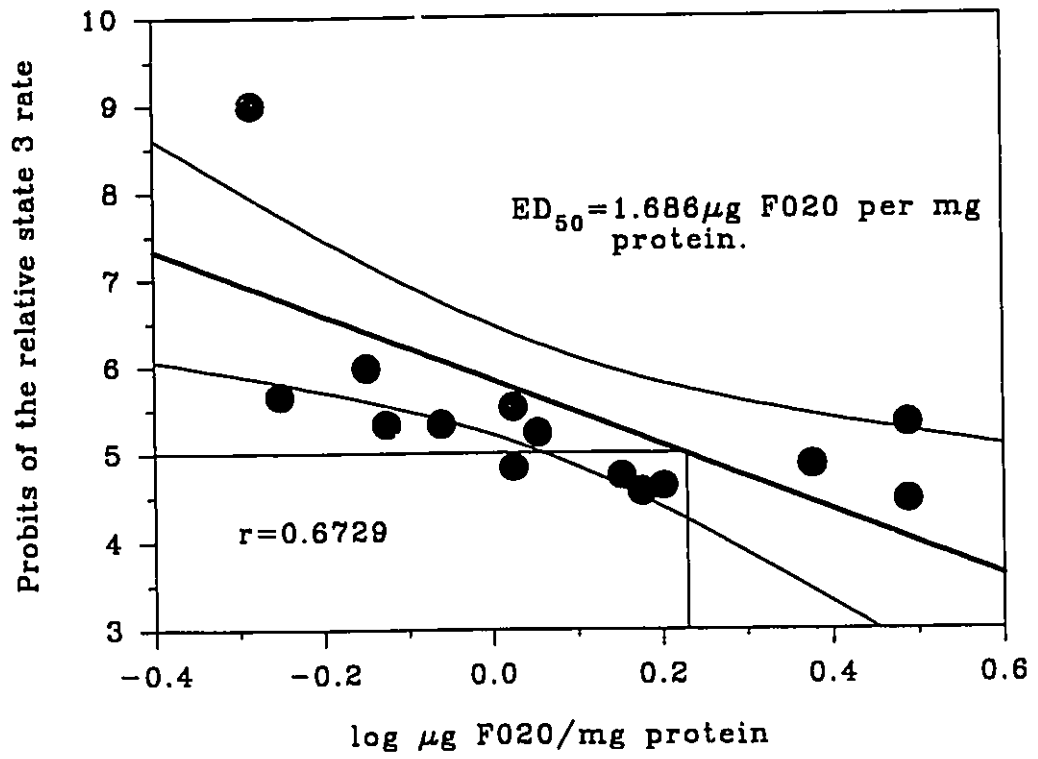
Values given are means of  $\text{nmol O}_2 \cdot \text{min}^{-1} \cdot \text{mg}^{-1} \pm \text{s.e.m.}$ , (n=3, except where marked with \*, where n=1). Concentrations of asimicin, F020, and rotenone represent the final amount in the reaction chamber. 95% ethanol was used as a carrier solvent (0.33% final volume).

'pretreatment' = ADP-stimulated rate prior to the injection of the treatment, and 'treated' =ADP-stimulated rate after the injection of asimicin, F020 or rotenone.

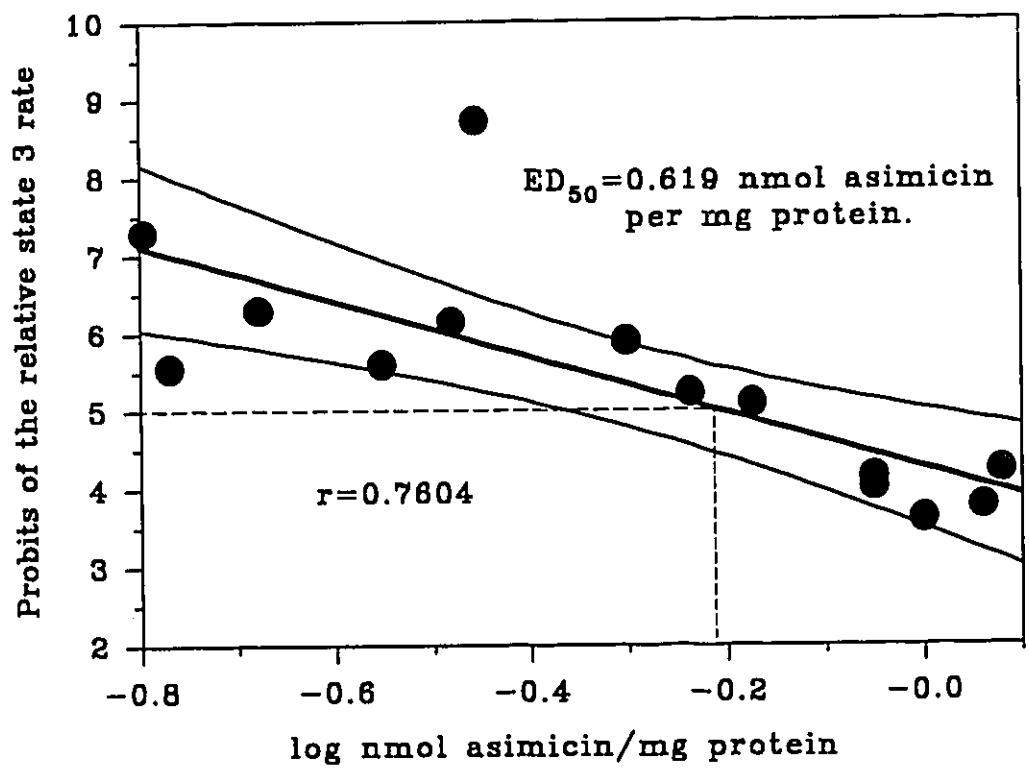
No significant differences were found between treatment groups for state 3 and state 4 rates using Tukey's multiple range test at  $P < 0.05$ .

CI=Complete inhibition

**Figure 4.4** Probit plot of the F020 inhibited state 3 (ADP-dependent) respiratory rate of isolated gut mitochondria from larvae of *Ostrinia nubilalis*. Probits are a reflection of the state 3 rate after injection of F020 in 95% ethanol as a percent of the state 3 rate before injection of F020. All measurements were recorded at 30°C and in a total volume of 3 ml (2 ml of reaction medium and 1 ml isolation medium). Protein was quantified using a modification of the biuret method of Gornall *et al.* (1949).



**Figure 4.5** Probit plot of the asimicin inhibited state 3 (ADP-dependent) respiratory rate of isolated gut mitochondria from larvae of *Ostrinia nubilais*. Probits are a reflection of the state 3 rate after injection of asimicin in 95% ethanol as a percent of the state 3 rate before injection of asimicin. All measurements were recorded at 30°C and in a total volume of 3 ml (2 ml of reaction medium and 1 ml isolation medium). Protein was quantified using a modification of the biuret method of Gornall *et al.* (1949).



## 4.4 Discussion

The influence of the F020 in reducing the growth of the insect is evident through the growth and development, as well as the nutritional indices investigations carried out with *Ostrinia nubilalis* (see Chapter II). A reduction in growth following the consumption of F020 is also evident in the present examination (Figure 4.1). The effect of F020 in reducing larval growth over the three day feeding period demonstrated that the insects were toxicologically affected, and confounded the effect of F020 on the measured whole body respiration. The rate of whole-body respiration, measured on a per mg basis appeared to vary directly with the level of F020 in the diet (Figure 4.2); however, this is inversely correlated with the incidence of weight gain as can be seen when Figure 4.2 and 4.1 are compared. The negative relationship between weight and respiratory rate is a phenomenon which has long been known (von Bertalanffy and Pirozynski, 1951; and Prosser, 1973). The rates of respiration for several species of stone flies (Plecoptera) also varied inversely with body mass (Nelson and Garth, 1984; Knight and Gaufin, 1966; and Modlin and Jayne, 1981). Similar results were obtained for numerous species of Hymenoptera and Coleoptera (Kayser and Heusner, 1964).

In general when large and small animals are compared, it is found that the total metabolism (volume of O<sub>2</sub> per animal) is higher for the larger animals than the smaller, but that the weight specific oxygen consumption (volume of O<sub>2</sub> per mg body weight) is higher in the smaller animals (Prosser, 1973). Metabolism expressed as a power function is a more comparable way to express metabolic rate and is represented by the equation:

$$M = KW^b \text{ or } \text{Log}M = b \text{ log}W + \text{log}K.$$

Where 'M' is the total O<sub>2</sub> consumed per unit time, W is the body weight, and 'b' and 'K' are the slope and y-intercept, respectively (Prosser, 1973). Once this analysis is considered, the respiratory inhibitory effect of F020 is apparent in the log M vs log W plots (Figure 4.3A & B) where the regression line for F020 treated larvae clearly falls below the control group (Figure 4.3A).

For the majority of animals including such animal groups as mammals, birds, reptiles, fish, crustaceans and insects, the value of the slope (b) falls within the range of

0.65 to 0.85 (Prosser, 1973). A slope slightly higher than that obtained for *O. nubilalis* (0.70) is reported for cecropia moth larvae, *Hyalophora cecropia*, at 0.87 (Schroeder and Dunlap, 1970). However, it is important to note that the rate of oxygen consumption is influenced by several internal and external variables including activity, temperature, nutrition, stage in life, season, time of day, as well as previous oxygen experience and genetic background and therefore values for oxygen consumption are dependent on the experimental conditions. Typically the rate of increase in body mass exceeds that of respiratory rate as the animal grows. In other words, under normal circumstances, the slope is usually less than 1.0. In the case of the *O. nubilalis* larvae which had fed on diets laden with F020, the determined slope of 1.229 is markedly above 1.0 (Figure 4.3B), indicating that the rate of respiration was increasing faster than body size. The increase in the slope is credited to larvae of lower weight, since the larvae of a greater weight (20-25 mg) do not have respiratory rates which are notably different from the control larvae in the same weight range (Figure 4.3 A and B). The observed greater slope in the treatment group is evidence of the *in vivo* inhibitory effect of F020 on respiration. From figure 4.3, the reduced rate of respiration is attributed to those larvae of a lower weight which is correlated with higher levels of F020 exposure (Figure 4.1).

The observed inhibition of respiration examined in the *in vivo* study directly supported the findings from the *in vitro* examination. The effect of injection of levels of F020 or asimicin into a suspension of isolated larval midgut mitochondria resulted in inhibition of respiration, apparently within the NADH-ubiquinone reductase site (see Figure 4.6). This was supported by the fact that when using the FAD linked substrate, succinate, neither F020 nor asimicin was able to inhibit the state 3 rate. In addition, those preparations sparked with pyruvate and malate, which were inhibited at this level, could be reactivated by the addition of succinate into the reaction chamber. Preparations inhibited by F020 or asimicin could also be reactivated in an identical manner to those inhibited by rotenone, a well known inhibitor of NADH-ubiquinone oxydoreductase coupling site (complex I). This result gives support to the hypothesis of an identical site of activity. Londershausen *et al.* (1991) have recently reported a similar finding for the linear acetogenins annonin I, II, and VI, extracted from *Annona squamosa*. They investigated the mode of action of these natural products on four different invertebrate

species. An initial reduction in ATP levels (~ 27%) in treated fourth instar larvae of *Plutella xylostella*, lead to further examination of the effect on mitochondrial respiration. Annonins I and VI were able to inhibit NADH cytochrome c

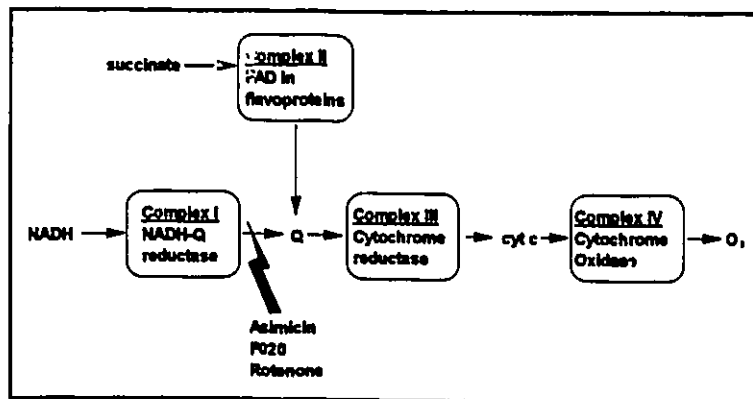


Figure 4.6 Site of inhibition of asimicin and F020 on the electron transport chain.

reductase at IC<sub>50</sub> values between 3 and 8 nmoles · mg protein<sup>-1</sup> from blow fly (*Lucilia cuprina*) muscle submitochondrial particles. The inhibition occurred at a lower dose than that with rotenone (20 nmoles · mg protein<sup>-1</sup>), but in an identical manner. Adenosine triphosphate levels were not measured in our study; however, the general lack of activity in the treated insects in our study (see also Chapters 2 and 3) and in the work by Londershausen *et al.* (1991) as well as the effect observed in rotenone treated insects (Rieske, 1981; Fukami, 1985), correlates well with a decrease in the principal carrier of biochemical energy, ATP.

Preliminary work locating the binding site of annonin I suggests it to be identical to the site used by rotenone, piericidin and ubiquinone (Londershausen *et al.* 1991). Structurally, annonin I and VI are very similar to asimicin, differing only in the stereo specific configurations (e.g. *erythro* as opposed to *threo* between C-23 and C-24, see Figure 1.1) and the position of the hydroxyl group (i.e. on C-4 in asimicin and on C-28 in annonin I). Because of this structural similarity among the three compounds, the binding site and the mode of inhibitory action, are likely to be identical.

Inhibition of the electron transport chain at Complex I site appears to be clearly evident from the results; nonetheless, certain anomalies exist. F020 and asimicin were able to induce high ADP:O ratios (>6.0). The ADP:O ratio used in this context is an expression of the efficiency of oxidative-phosphorylation and is determined by measuring the oxygen consumption from the injection of the known amount of ADP to the release of state 4 respiration. Under normal circumstances, NAD-linked substrates will have

ADP:O ratios which approach 3.0 and FAD linked substrates (succinate) will have ratios approaching 2.0. In this study the very high ratios reported could be the result of incomplete phosphorylation of the added ADP due to inhibition of respiration to a state 4 level during the state 3 measurement. Since the level of ADP is constant for each run an inhibition in the state 3 and therefore the amount of oxygen consumed will result in an increase in the ADP:O ratio

For the F020 treated mitochondria, the state 3 rate had significantly decreased in accordance with treatment levels; however, the state 4 rate did not (Table 4.3), at least not in a statistically significant manner. The state 4 rate is defined as the respiratory rate in which ADP is limited, and gives a good indication of how tightly coupled the mitochondria are. During mitochondrial respiration electrons are passed down the electron transport chain through a series of oxidation reduction reactions to oxygen. In the process, enough free energy is released to allow for the phosphorylation of ADP to ATP. The state 4 rates of asimicin-inhibited mitochondria were significantly lower, but were not completely interrupted, even in the cases where the state 3 rate was completely inhibited (Table 4.4). This apparent anomaly may appear to be a deviation from the inhibitory effect; however, incomplete inhibition of state 4 rate was also observed for rotenone-inhibited mitochondria (Table 4.3). Inhibitions in the state 4 rates, although not complete, did occur in a dose dependent manner in many cases. For example, state 4 rates following treatment of asimicin at 0.141, 0.188, 0.283 and 0.376 nmoles decreased by 66.6, 52.4, 44.3 and 42.2%, respectively. It is important to note that the state 1 rate of oxygen consumption, which is the rate of respiration where both the substrate and ADP is limiting, occurs at a low level prior to and during state 4 respiration. In addition, the oxygen electrode used to measure the oxygen levels, also consumes a certain amount of oxygen during its operation. For these two reasons, complete inhibition of the state 4 rate, as it is measured, is not possible. In addition it is well recognized that in tightly coupled mitochondria the electron transport chain only operates in the presence of ADP so the state 4 rate is often considered to be the result of damaged mitochondria and is often ignored when the mitochondria are not uncoupled.

Although not statistically significant, except for exposure to F020 at 1.46  $\mu\text{g}$ , the RCR's (state 3 rate/state 4 rate) for F020 treated mitochondria appear to decrease relative

to the controls, whereas, those treated with asimicin appear to be elevated relative to the controls (Table 4.2). Since asimicin is a component of F020, one would expect the response from the two treatments to coincide. A decrease in the RCR (i.e. approaches 1) is often an indication of uncoupling i.e. state 4 rate is approaching state 3; however, the opposite is occurring in accordance with the inhibition of the state 3 rate. An increase in the RCR could imply that the state 4 rate was decreasing slightly faster than the state 3 rate. At this time it is not clear as to why the differences appear and, for all intensive purposes, may simply be a reflection of the small sample size.

Because the *in vitro* tests characterize F020 and asimicin as mitochondrial inhibitors strongly implicates the mitochondria as the major target sites *in vivo*. The observed reduction in respiration *in vivo* is in support of this. Nonetheless, it cannot be assumed that mitochondrial respiratory inhibition is the single toxicological property or site of activity of F020 or asimicin *in vivo*. Other effects have been observed subsequent to exposure, such as decreased larval growth and fecundity for adult *O. nubilalis* (Chapter II), delays in moulting for *Oncopeltus fasciatus*, decreased tanning ability for *Sarcophaga bullata* (Chapter III). These observed aberrations may, however, be the direct result of respiratory inhibition. More work in the area of toxicokinetics must be done in order to gain a greater understanding of the complete mechanism of action of F020, asimicin and other related linear acetogenins.

# Chapter V

## 5.0 General Discussion

The exposure of *Ostrinia nubilalis* and *Manduca sexta* to dietary levels of F020 resulted in significant growth inhibition and increased mortality. F020 applied to corn leaf disks or incorporated into artificial diets gave some evidence of an antifeedant effect. However, the reduction in growth and survivorship, observed during the chronic exposure study, cannot solely be attributable to any antifeedant effect but is also due to intoxication as evidenced by the change in the nutritional indices measurements and the observed moribundity and death associated with topical applications to larvae of *O. nubilalis* and nymphs of *Oncopeltus fasciatus*. The biological activity of F020 and asimicin was discovered to be the inhibition of respiration, specifically identified to the complex I site of the electron transport chain. In general, for a compound such as asimicin to exert its activity in an insect, it may face numerous physical and chemical challenges (Brooks, 1976, Welling and Paterson, 1985).

The insect has many adaptive features and abilities to deal with a potential toxin. When an insect encounters a potential food source, it must first be accepted as a food before it is consumed. Determination of the characteristics and ultimately the "decision" to consume the food is a reflection of information received by the numerous contact chemoreceptor sensilla predominately located on the labrum and maxillary palp (Altner and Prillinger, 1980; Zacharuk, 1980; Städler, 1984). The presence of a particular foreign substance such as F020 in a food item may be detected by the insect and subsequently result in the rejection of the otherwise suitable food (Jermy, 1971; Munkata, 1975; Schoonhoven, 1982; Berneys, 1983). If the insect accepts a food which has been tainted with a potential toxin it then will proceed with ingestion. Once ingested, the particular compound is then susceptible to a multitude of potentially damaging digestive enzymes including glycosidases, lipases and proteases. For *O. nubilalis*, midgut protease activity has been attributed to the trypsin proteinases (endoproteinases)

an a chymotrypsin (Houseman and Philogene, 1988). Typical proteolytic enzymes in the alimentary tract of lepidoptera have maximal activity at pH values greater than 9.0 (Applebaum, 1985); However, in *O. nubilalis*, maximal proteinase activity values have been found to occur from 7.5 to greater than 10.0 (Houseman and Philogene, 1988). The gut pH, itself, could also prove to be detrimental to the compound, as it would increase the potential for hydrolysis.

If a particular compound is unaffected by the digestive enzymes toxic activity may be exerted on other aspects of the digestive processes, such as binding or direct interference with the digestive enzymes in competition with the usual substrate. In the realm of plant-insect interactions this type of defence strategy, on the part of the plant, is considered a quantitative defence. These quantitative defences are typified by tannins and resins which are effective through non-specific binding with ingested proteins and digestive enzymes resulting in a reduction in digestive efficiency (Feeny, 1975, 1976; Rhoades and Cates, 1976; Fox, 1981; Berenbaum, 1986). In reference to the plant *Asimina triloba*, which produces asimicin and which was the source of F020, evidence goes against the notion that these compounds show the above. Besides the fact that the plant does not produce asimicin in immense quantities, a characteristic of this type of defence, digestion is not apparently inhibited (see Chapter II) and activity can be attributed to an inhibition of the complex I site in the respiratory chain during oxidative phosphorylation (see Chapter IV). As well, according to the mitochondrial inhibitory *in vitro* assay (see Chapter VI), very small amounts of either asimicin ( $< 1 \text{ nmol} \cdot \text{mg}^{-1}$  protein) or F020 ( $< 3.6 \text{ } \mu\text{g}$  per mg protein) are required for activity.

The potential exists for ingestion of F020 or asimicin to interfere with the proper function of the gut epithelial cells thereby interfering with digestion. However, as noted above, the digestive processes were not evidently affected by the consumption of these substances and cellular or tissue lesions were not apparent upon histological examination of the midgut epithelium (see Chapter II). Perhaps some property or properties of the epithelial cells prevents asimicin from targeting the mitochondria at this location, thereby allowing the active compound to pass through and into the haemocoel.

The gut wall can pose a substantial barrier to the penetration of a potential toxin such as asimicin. Orally administered drugs in vertebrate studies are well known to be

less efficient than the same dose when given intravenously. Intestinal absorption of the drug is exposed to degradation by enzymes in the gut wall and in particular in the liver before it arrives at the systemic circulation (Routledge and Shand, 1979). A similar phenomenon occurs in insects.

A study examining the penetration of the insecticides, dieldrin, DDT, malathion, and carbaryl through the isolated midguts of the cockroach, *Blaberus discoidalis* (Seville) and the tobacco hornworm, *Manduca sexta* (L.) found that penetration and metabolism occurred to varying degrees within the gut (Shah and Guthrie, 1970). The toxic, or potentially toxic, malathion, which penetrated the gut, was comparable to the amount of DDT and dieldrin, between 15 and 26% for *B. discoidalis* and 36 and 42% for *M. sexta*, respectively. For carbaryl, penetration was higher, 31% for *B. discoidalis* and 62% for *M. sexta*. The extent of metabolism of the insecticides also varied between compounds. Malathion was metabolised to the greatest extent (75-80%) whereas 80% or more of carbaryl, DDT, and dieldrin were present in their original state.

In another study, the rate of midgut penetration of dialoxy analogs of dimethoate was determined to be a function of their octanol/water partition coefficient when examined against the adult cockroach, *Blaberus craniifer* (Burm.) and the fifth instar tobacco hornworm larvae, *M. sexta* (Shah, 1972). A lower mineral oil/water partition coefficient of a particular analogue tended to favour penetration, whereas, a higher value tended to result in association with the gut tissue. This conclusion is supported by the finding from the study which examined the penetration rate of carbaryl, malathion, dieldrin, DDT, and nicotine through the isolated midguts of the cockroach, *Blaberus discoidalis* (Seville) and fifth instar larvae of the tobacco hornworm, *M. sexta*. (Shah and Guthrie, 1985). In a similar manner, penetration was found to be negatively correlated with the olive oil/water partition coefficient and correspondingly, a decreased polarity favoured binding to the gut tissue.

As can be expected, differences in penetration rates between each insect were noted in the above studies. This was exceptionally apparent in the study examining the permeability of midguts from locusts, *Schistocerca gregaria*, the milkweed bug, *Oncopeltus fasciatus*, and cockroaches, *Periplaneta americana* to cardiac glycosides, ouabain and digitoxin (Scudder and Meredith, 1982). The solutions containing ouabain

or digitoxin plus 5% sucrose were ingested by *O. fasciatus* and *P. americana*, and exposed to *S. gregaria* via anal injection. Of the three insects only *O. fasciatus* could absorb the compounds even though ouabain and digitoxin have different polarities (polar and non-polar, respectively). For *O. fasciatus*, again the polarity played a significant role in the rate of penetration as digitoxin permeated more rapidly than ouabain. Examination of the structure of asimicin reveals both hydrophilic and hydrophobic regions (see Figure 1.1) a property which would aid in its ability to penetrate into, and through the gut tissue and subsequently aid in its dispersal into the more hydrophilic serosal fluid.

The toxicity of a compound, however, is not necessarily related to its ability to penetrate. The above noted study by Shah (1972) found that although the toxicity of the five dialoxy analogs of dimethoate varied over 40 fold, the penetration rates varied over only 2-fold. Differences in toxicity may be due to the properties of the compound which directly or indirectly prevent or inhibit the compound from being detoxified.

A number of detoxification enzymes exist in the insect which can effectively detoxify a given potential toxin. In general, the enzymes involved in detoxication are able to increase the polarity and thus the water solubility of a foreign compound, thereby allowing it to be more easily eliminated via the excretory system.

There are essentially two types of reactions which take place. Phase 1 reactions involve the introduction of a hydrophilic functional group, typically resulting from an oxidation, reduction or hydrolysis reaction (Dauterman and Hodgson, 1978). The most important oxidase enzyme involved in the phase 1 reaction is the cytochrome P-450 microsomal mixed-function oxidase system which effectively reduces oxygen to water and at the same time oxidizes a given substrate, e.g. a plant secondary substance. The mixed function oxidase system has been shown to occur in the fat body, malpighian tubules and the midgut, with the midgut being the most significant site in the lepidopteran insects (Crankshaw *et al.*, 1979; Brattsten *et al.*, 1980). Phase 2 reactions usually succeed phase 1 reactions and involve a conjugation to an endogenous water-soluble metabolite such as glucose, glutathione, or various amino acids, the products of which are then readily excreted. The enzymes involved in the phase 2 reactions include glutathione transferases, glucosyltransferases, N-acetyltransferases, sulfotransferases and

phosphotransferases (Dauterman and Hodgson, 1978). For a compound to exert its toxic effect it must generally be available at a relatively high level and/or must exert its effect on its target within a relatively short period of time in order to compensate for the detoxication mechanisms present. Further work is required in this area in order to have a greater understanding of the toxicokinetics of asimicin.

Although the insect gut can be a formidable place for a particular xenobiotic evidence exists in the literature demonstrating that the insect cuticle is not an inert region and that degradation of insecticides occurs subsequent to topical application (Oppenoorth, 1967; Singh and Thornhill, 1980). The cuticle, a secretion of the epidermis, is primarily made up of chitin, tanned proteins, lipids, and waxes. The procuticle, is the largest and the first layer to be secreted. It differentiates into an exocuticle (tanned layer) and an endocuticle (untanned layer) and a mesocuticle in between. The layer above the procuticle is the outer layer or epicuticle also made up of several layers. Immediately adjacent to the procuticle is the 'inner epicuticle' made up of tanned lipoproteins, above that is an 'outer epicuticle' made up of highly polymerized lipids and outside that is a water proof 'wax layer' consisting of long chain hydrocarbons and esters of fatty acids and alcohols. In some insects a cement layer may be found over the wax layer. When an insect is exposed to a compound via the cuticle the compound may reach the target site by passing directly through the cuticle or it may enter directly from the outside through the trachea or pore cannals. This latter mode of entry, known as 'lateral transport', is a less well supported theory of contact entry proposed by Gerolt, (1969, 1970, 1975ab). The exact mechanism of entry is still unresolved.

There are many factors which can influence the passage through the cuticle. The hard and rigid cuticle gives support and protection, and at the same time is very important in preventing water loss from the insects body. The procuticular matrix, is a larger, more polar region which also must be traversed, the thickness of which may affect the permeability rate. Typically, potentially toxic compounds which are topically applied are hydrophobic or are non-electrolytes and are thus able to migrate over the cuticle, away from the point of application, thereby reducing the concentration gradient at that point. The rate of penetration into the cuticle, and similarly into the gut, appears to be a function of the lipid solubility of the compound and solvent carrier (Lewis, 1980).

A study examining the rate of penetration of the four insecticides, dimethoate, paraoxon, dieldrin, and DDT through cuticle of the cockroach *Periplaneta americana* found the rate of penetration to be inversely related to the olive oil/water partition coefficient (Olson and O'Brien, 1963). In other words, as with the gut, penetration of more polar compounds was more rapid. If the applied substance is polar or its carrier solvent is polar penetration into the lipophilic epicuticle will be rate limiting; however, polarity would be necessary for the compound to partition into the procuticle (Olson and O'Brien, 1963). Therefore a moderate level of polarity or one with both polar and non-polar regions, as in asimicin, would likely favour complete penetration.

Because of the large amount of protein in the procuticular matrix, there exists a high potential for chemical binding to that protein during penetration. This potential was examined in a study which measured the rate of movement of  $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$ -isomers of hexachlorocyclohexane through *P. americana* cuticle. It was found that the rate of penetration of the  $\alpha$ ,  $\beta$ , and  $\gamma$  isomers was inversely correlated with the determined binding constant on bovine serum albumin, but in contrast was not related to the octanol/water partition coefficient (essentially the same for the three isomers) (Kurihara *et al.*, 1974).

When an insecticide or similar compound passes through the gut or insect cuticle, it will make its way into the circulating haemolymph either free in solution or absorbed by haemolymph proteins or haemocytes and subsequently be distributed throughout the body (Welling and Patterson, 1985). The internal distribution and elimination of a particular xenobiotic varies between organs and tissues depending on the tissue size, as well as its physical and chemical properties. In many cases the rate of elimination is high initially, but rapidly declines with time, not necessarily in a linear fashion (Welling and Paterson (1985). Ingested or topically applied asimicin or F020 has the potential to inhibit mitochondrial oxidative phosphorylation thereby limiting the available ATP, the major source of energy for all biochemical reactions. Because mitochondria are found in all living cells within the insect, there are no restrictions on the potential target sites; however there are a wide variety of cell types and tissue structures which, because of their biochemical nature, would be more or less susceptible to targeted activity. At present it remains speculative as to the tissue or tissues which are the target sites in the

insects exposed to asimicin. Future research is required in this area if we are to arrive at a greater understanding of the target tissue(s) of asimicin activity and the toxicodynamics and kinetics of asimicin within the insect body. Because of the similarity in chemical structure to other linear acetogenins isolated from members of the Annonaceae, it is likely that they would also behave in a similar mode. An understanding of the target tissues of the activity of asimicin and similar biologically active linear acetogenins, may allow these compounds to be safely administered or applied to insects for pest control purposes.

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# Appendices

## Appendix A

### Diet preparation - *Ostrinia nubilalis* Artificial Meridic Diet Composition

(800 g batch):

Distilled Water	692 ml
Agar	14.9 g
Wheat germ	520 g
Dextrose	400 g
Casein	440 g
Cholesterol	32 g
Salt mixture (Wesson)	144 g
Vitamin mixture (Vanderzant Adkisson)	92 g
Ascorbic acid	120 g
Aureomycin	27 g
Sorbic acid	80 g
Methyl Paraben Solution	4.0 ml
Propionic acid	4.6 ml
Formaldehyde	0.38 ml
Fumidil B	0.40 g

Early fourth instar larvae were selected from the stock colony reared in this laboratory on an artificial meridic diet. The laboratory colony of *Ostrinia nubilalis*, originated from both M. Hudon (Agriculture Canada, St. Jean, Quebec) and G. McLecd (Agriculture Canada, London, Ontario). Adults were maintained in the laboratory under a 16h:8h L:D photoperiod at 25°C and 80% relative humidity. Adults were provided with 20 % sucrose solution available through a 3-5 cm cotton wick. Eggs were deposited onto vertical hanging wax paper sheets approximately 12 x 30 mm. The adults had been allowed 2-3 days to oviposit before paper was removed and replaced. The number of adults in the cage remained relatively constant. Wax paper disks containing the egg masses were cut, and collectively placed in the growth containers holding ample diet for complete larval development. The containers remained inside a controlled environment chamber.

**Diet Preparation - *Manduca sexta***  
**Meridic diet composition (~900g)**

<u>Premix (g)</u>	
Wheat germ	50.0
Casein	22.5
Sucrose	20.0
Brewers Yeast	15.0
Wesson's salt mix	7.5
Ascorbic acid	2.0
Sorbic acid	1.5
Methyl-p-hydroxy benzoate	1.5
Cholesterol	0.25
<u>Vitamins (mg)</u>	
Nicotinic acid	5.0
Calcium pantothenate	5.0
riboflavin	2.5
Thiamine HCl	1.65
Pyridosine HCl	1.65
Folic acid	0.10
Biotin	0.10
<u>Others</u>	
Formaldehyde (40%) (ml)	2.5
Raw linseed oil (ml)	2.0
Distilled water (ml)	750.0
Agar (g)	100.0
Streptomycin sulfate (g)	0.5

Eggs from a laboratory colony originating from the Carolina Biological Supply Co. were placed collectively into petri dishes containing artificial diet for the emerging larvae. The dishes were placed in a controlled environment chamber of 25°C, 16:8 L:D regime (relative humidity was not controlled). The larvae were allowed to grow in the dishes for several days before being transferred individually into cells of tissue culture plates. As the larvae grew they were transferred to larger cells, and finally into individual vials where they were allowed to pupate in vermiculite. Pupae were removed from the vials and placed on the floor of a large screened cage under the same environmental conditions to allow for adult emergence. The emerging adults were allowed to mate and females could oviposit onto the surface of a young tobacco plant growing in a small pot within the cage.

## Appendix B

Sample calculation of cumulative percent mortality for *Ostrinia nubilalis* and *Manduca sexta* growth and development study.

original population size: 127 larvae  
total number dead on day 9: 12  
total number remaining alive on day 9: 115

population reduced to 30 larvae.

Assumption: The probability of a larva dying is independent of the population size at the neonate stage.

If, on day 11, one larva dies out of 30 then:

$$1/30 = X/115$$

then  $X = \underline{3.833}$  larvae died.

Since mortality is cumulative,  
 $9 + 3.833 = \underline{12.833}$

Therefore, cumulative percent mortality on day 11 is:  
 $12.833/127 \times 100 = \underline{10.1\%}$

## Appendix C

Formulas for nutritional indices (Waldbauer, 1968)

Consumption index (CI)

$$CI = \frac{\text{weight of food ingested}}{\text{average weight of larvae}}$$

Approximate digestibility (AD)

$$AD = \frac{\text{weight of food ingested} - \text{fecal weight}}{\text{weight of food ingested}} \times 100$$

Efficiency of conversion of ingested food (ECI)

$$ECI = \frac{\text{weight gained}}{\text{weight of food ingested}} \times 100$$

Efficiency of conversion of digested food (ECD)

$$ECD = \frac{\text{weight gained}}{\text{weight of food ingested} - \text{fecal weight}} \times 100$$

Note: The average weight of larvae was determined not from the area below the curve as in Waldbauer (1968) but from the arithmetic mean of the initial and final weights (SooHoo and Fraenkel, 1966)

## Appendix D

### Histology - preparatory reagents

#### Bouin alcoholic fixative:

Picric acid	75.0 ml
Formalin, concentrated	25.0 ml
Glacial acetic acid	5.0 ml

#### Heidenhain iron hematoxylin:

##### A) (Mordant)

Ferric alum, (hemalum) ( $\text{FeNH}_4(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ )	4.0 g
Distilled water	100 ml

##### B)

Hematoxylin	10.0 g
95 % ethanol	100 ml
let stand until deep red in color (4-5 mos.)	

take 4-5 ml of solution B and add to 100 ml distilled water.

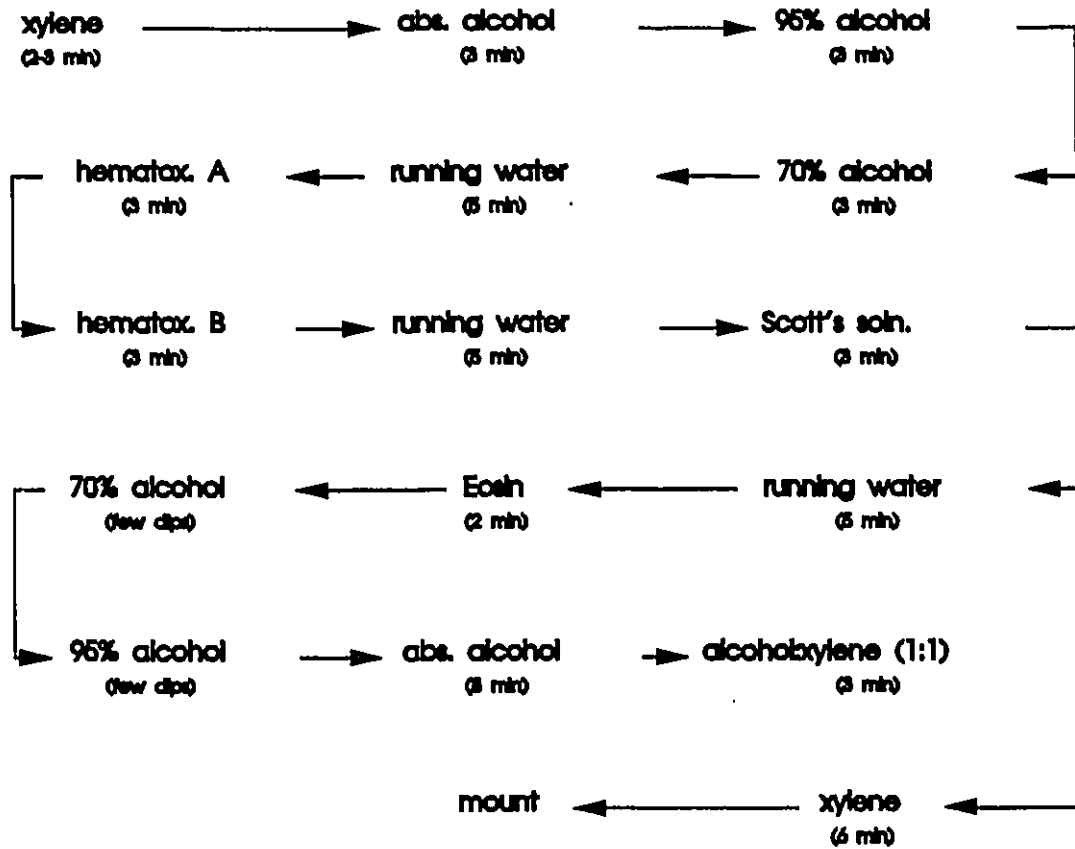
A and B are not mixed.

#### Eosin:

Eosin Y CI 45380	1.0 g
Potassium dichromate	0.5 g
Saturated aqueous picric acid	10.0 ml
Absolute ethyl alcohol	10.0 ml
distilled water	80.0 ml
glacial acetic acid	1 drop

**Staining procedure:**

The following staining procedure was employed:



Cover slips were mounted over sections using Paramount.

## Appendix E

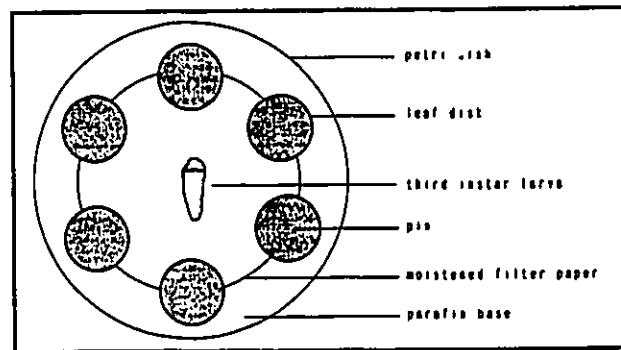
### Field Research at The University of South Florida: The Feeding Preference of *Eurytides marcellus* (Lepidoptera: Papilionidae).

*Eurytides marcellus*, an insect which feeds and develops on *Asimina triloba*, was examined for its ability to be attracted to and to be stimulated to feed by its natural host extract, F020.

**Materials and Methods:** *Eurytides marcellus* larvae were collected as eggs or in early instars on *Asimina reticulata* in the Tampa Bay area during early spring (Feb., 19 - Mar., 5, 1990). Young larvae were reared on *Asimina reticulata*. Eggs and larvae were reared in a controlled environment chamber at 12L:12D, at 26 and 18°C day and night temperatures respectively. Humidity was maintained high by placing moistened 5.5 cm dia. filter paper disks into individual 9.0 cm Petri dishes in which the larvae or eggs were located. The larvae were weighed and if the larval weight fell within the range of 0.1 to 0.25 mg, they were used in the feeding trial experiment.

The feeding trials involved placing one larva into an arena provided with six, 1.5 cm dia leaf disks of *Annona squamosa*, a non-host, but one which is closely related and will be consumed by *G. marcellus* larvae. Within each arena, three different treatments in two replicates were used. Leaf disks were topically treated with an acetone solution of F020. In trial A, F020 was applied at 0, 5.7 and 56.6  $\mu\text{g}/\text{cm}^2$  or 0, 56.6 and 569  $\mu\text{g}/\text{cm}^2$ . Control disks (0  $\mu\text{g}/\text{cm}^2$ )

were treated with acetone only. The disks were arranged in a circular pattern so that no two identical treatments were adjacent, such that the larva approaching one disk would have the other two treatment disks immediately to the right and left of it. The disks were



**Figure E.1.** Arrangement of *Eurytides marcellus* larva and *Annona squamosa* leaf disks in the feeding arena.

suspended above a thin layer of paraffin wax and fixed into position with short pins pierced through the centre of each disk (Figure E.1). The larva was placed in the centre and allowed time to consume approximately, 60% of the total available leaf areas. Twenty-four hours was the maximum time allowed. The area consumed was determined for each leaf disk.

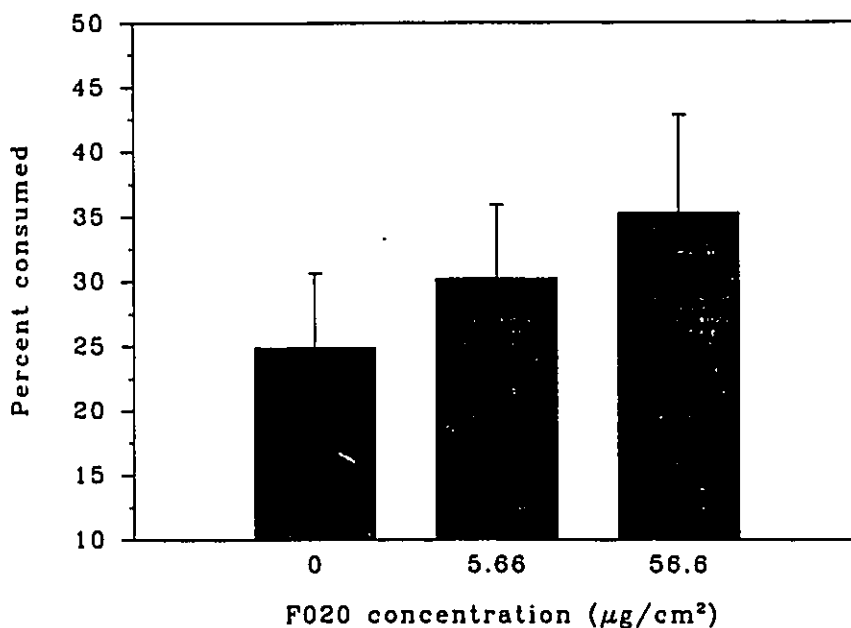
**Results:**

**Table E.1** Feeding study of *Eurytides marcellus* larvae on *Annona squamosa* leaf disks treated with F020.

Trial	No. of larvae	F020 treatment ( $\mu\text{g}/\text{cm}^2$ )	leaf disk area consumed ( $\text{mm}^2$ ) †
A	16	0	$87.88 \pm 80.3$ a
		5.7	$106.63 \pm 79.9$ a
		56.6	$124.31 \pm 106.6$ a
B	15	0	$55.67 \pm 80.0$ a
		56.6	$161.53 \pm 101.6$ b
		565.9	$59.73 \pm 52.05$ a

Mean larval weights for A,  $214.0 \pm 41.0$  mg and B,  $154.0 \pm 49.0$  mg.

† Within each trial, mean areas followed by the same letter are not significantly different, comparisons were made using Tukey's multiple range test ( $P < 0.002$ ).



**Figure E.2.** Effect of topical application of F020 to *Annona squamosa* leaf disk on the feeding preference of *Eurytides marcellus*

## Discussion

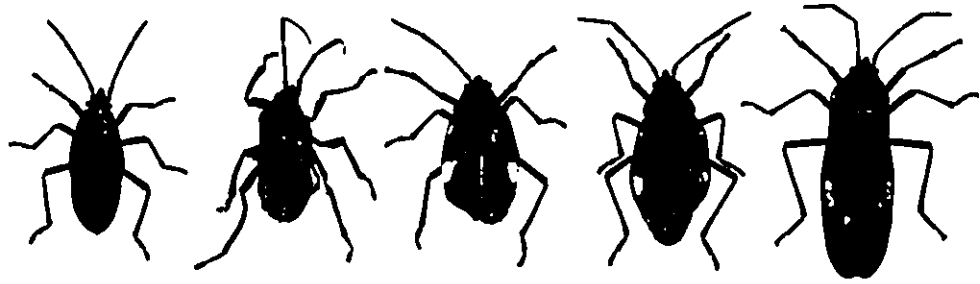
From this feeding preference study using *E. marcellus* larvae it is apparent that F020 does not inhibit feeding, but rather stimulates it. A response which is completely opposite to that observed for *O. nubilalis*, where application of F020 to a leaf disk inhibited feeding (see Figure 2.7, Chapter II). This result is not surprising however when one considers that *E. marcellus* is a specialist feeder on a number of *Asimina* sps., many of which which are known to produce linear acetogenins (Rupprecht *et al.*, 1990). Perhaps the presence of the linear acetogenins in F020 are used by the insect as a recognition of the host. It would be interesting to determine whether *E. marcellus* would feed on a non-host species which had been covered with F020 or whether oviposition is stimulated on those leaves or surfaces covered with F020.

## Appendix F

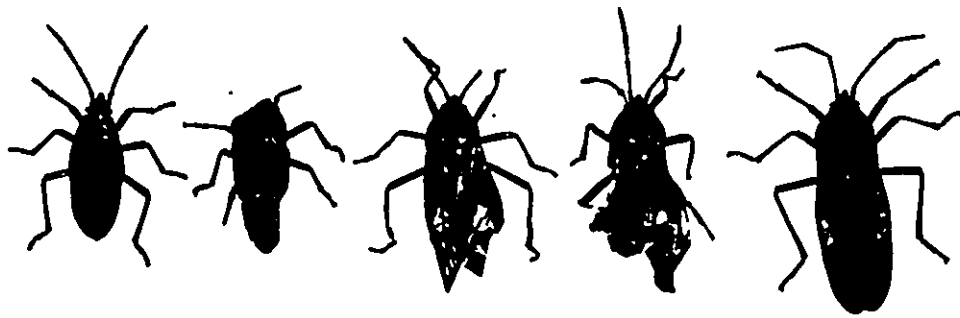
Photographs of juvenile characteristics in *Oncopeltus fasciatus* and *Tenebrio molitor* following treatment with F020 or juvenile hormone III

**Figure F.1** The effect of a topical application of F020 (A) and juvenile hormone III (B) to last instar nymphs of *Oncopeltus fasciatus*. In both (A) and (B) the insect on the far left is a normal late fifth instar nymph and a normal adult is represented on the far right. The three insects in the middle represent intermediate forms. F020 was applied dorsally at  $100 \mu\text{g} \cdot \mu\text{l}^{-1}$  and juvenile hormone III was applied at  $1 \mu\text{g} \cdot \mu\text{l}^{-1}$ .

A

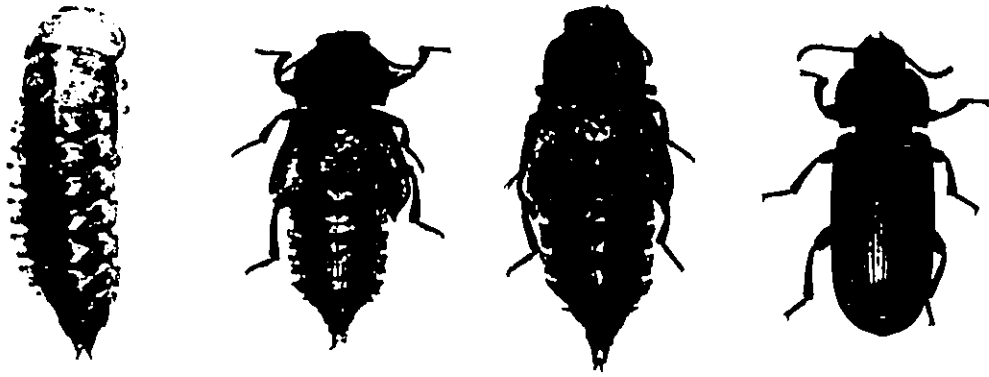


B

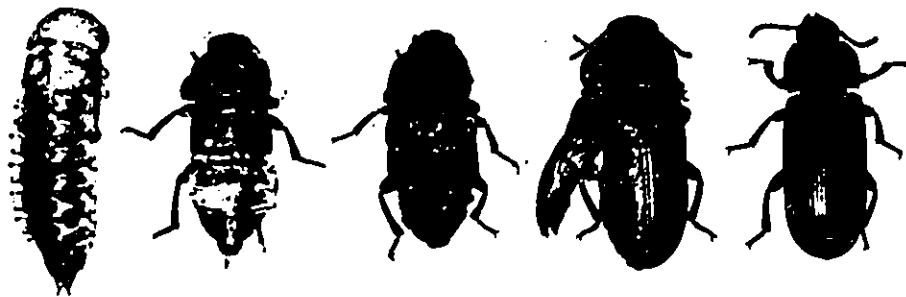


**Figure F.2** The effect of a topical application of F020 (A) and juvenile hormone III (B) to pupa of *Tenebrio molitor*. In both (A) and (B) the insect on the far left is a normal pupa and a normal adult is represented on the far right. The insects in the middle represent intermediate forms. F020 was applied dorsally at  $250 \mu\text{g} \cdot \mu\text{l}^{-1}$  and juvenile hormone III was applied at  $10 \mu\text{g} \cdot \mu\text{l}^{-1}$ .

A



B



## **Appendix G**

### **Determination of the quantity of F020 in corn oil samples:**

A reference standard of F020 made up by dissolving a range of concentrations (0.31 to 12.5 mg/ml) in 95% ethanol. The absorbance was measured spectrophotometrically using a Varian-Cary spectrophotometer at 400 nm. A linear relationship was established for the measured absorbance at this wave length and at the concentrations tested. The correlation coefficient (r) for the standard curve was determined to be 0.996. To estimate the amount of F020 in the corn oil, the absorbance of three samples of corn oil alone were measured against a 95% ethanol blank. The average absorbance of the three samples was subtracted from the absorbance of the measured from the samples with F020 in corn oil. Concentrations of F020 in the corn oil, used in the injections, were determined to range from 0.28 to 6.8  $\mu\text{g}/\mu\text{l}$ .

## Appendix H

### Brodies manometric fluid:

(Umbreit et al., 1964)

23 g NaCl

5 g Sodium Choleate

500 mls H<sub>2</sub>O

100 mg Acid Fuchsin

density of final solution: 1.033 g/ml

P<sub>o</sub> = 10 000 mm at standard pressure (i.e. equals 760 mm Hg)

### Calculations used to determine respiratory rates from Warburg manometer readings:

#### **Determination of the flask constant.**

(From Umbreit *et al.*, 1964)

The flask constant is used to convert observed pressure changes (change in mm of brodies solution in the manometer) to the number of  $\mu$ l of oxygen taken up.

The following symbols are employed:

h=the observed change in the manometer (open side) reading in mm.

x= $\mu$ l gas (0°C, 760 mm Hg pressure).

V<sub>g</sub>=Volume of gas in flask including connection tubes down to the reference point (150 or 250 mm on closed arm of manometer)

V=Volume of fluid in vessel.

P=Initial pressure in vessel of the gas involved in the determination. This is actually the partial pressure of the particular gas in a gas mixture. If this gas mixture contains water vapour, the partial pressure of gas involved in the determination will be less than its partial pressure in the dry condition.

P<sub>o</sub>=Standard pressure, which is 760 mm Hg or 10000 mm Brodies fluid.

T=Temperature bath in degrees Kelvin.

$\alpha$ =The solubility in reaction liquid of gas involved (expressed as ml gas/ml liquid when gas is at a pressure of 760 mm Hg at the temperature T).

R=Vapour pressure of water (or other fluid) at temperature T. The fluid in the flask will exert a vapour pressure (R) in the gas phase and some gas will dissolve in the fluid.

Based on the gas law, if the gas phase in the manometer has a volume of gas (V<sub>g</sub>) at a temperature (T) and at a pressure P - R, then the gas volume can be changed to standard conditions with the formula:

$$PV/T = P'V'/T'$$

Where the prime symbols represent standard conditions. Therefore, in the flask:

$$(P-R)V_g/T = P_o V'/273$$

and therefore at standard conditions:

$$V' = V_g (273/T)(P-R)/P_o$$

Some gas is dissolved in the fluid initially. The amount of gas in the fluid is:

$$V_f \alpha (P-R)/P_o$$

Therefore, the gas at the start was that in the gas phase plus that in the fluid phase or:

$$\text{Gas at start} = V_g (273/T)(P-R)/P_o + V_f \alpha (P-R)/P_o$$

The gas at the end has been changed by an amount x which has resulted in a pressure change of h mm (if gas is taken up h is negative).

x = initial gas - final gas

$$x = \left[ V_g \frac{273}{T} \frac{P-R}{P_o} + V_f \alpha \frac{P-R}{P_o} \right] - \left[ V_g \frac{273}{T} \frac{P-R-h}{P_o} + V_f \alpha \frac{P-R-h}{P_o} \right]$$

$$x = h \left[ \frac{V_g \frac{273}{T} + V_f \alpha}{P_o} \right]$$

$$x = hk$$

where k is the flask constant.

The value of  $\alpha$  used was 0.2822 which is ml of oxygen dissolved per ml of water at one atm. pressure.

$P_o$  was 10000 for Brodies solution derived as follows:

$$P_o = 760 \times \frac{13.60}{1.033} = 10000$$

Where 13.60 is the density of mercury and 1.033 is the density of Brodies solution.

## Appendix I

A list of abbreviations used in this thesis:

$\rho$  = Spearman's rank correlation coefficient.

A = anterior end.

AD = approximate digestibility.

ADP = adenosine diphosphate.

ATP = adenosine triphosphate.

BSA = bovine serum albumin.

C = carbon

CI = consumption index (chapter II) or completely inhibited (chapter IV).

cyt c = cytochrome c

D = dark

EC<sub>50</sub> = the effective concentration representing a 50% reduction of a certain measurable effect.

ECD = efficiency of conversion of digested food into body matter.

ECI = efficiency of conversion of ingested food into body matter.

ED<sub>50</sub> = the effective concentration representing a 50% reduction of a certain measurable effect.

EDTA = ethylenediamine tetraacetic acid

EGTA = ethylene-glycolcol-bis-(aminoethyl) tetraacetic acid.

F = female.

FAD = flavine adenine dinucleotide.

FW = fresh weight.

g = force of gravity (chapter IV)

IM = isolation medium.

JH = juvenile hormone

L = light

M = male.

Me = methane

NA = not applicable.

NADH = nicotinamide adenine dinucleotide.

O = oxygen

P = probability of making a type 1 error or abbreviation of posterior end (chapter III).

ppm = parts per million.

Q = ubiquinone.

r = Pearson's product moment correlation coefficient.

R.H. = relative humidity

RCR = respiratory control ratio (state3/state4).

s.e.m. = standard error of the mean.