THE EFFECTS OF FEEDING EXPERIENCE WITH ANTIFEEDANTS ON LARVAL FEEDING AND ADULT OVIPOSITION BEHAVIOR IN GENERALIST AND SPECIALIST HERBIVORES

By

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Abstract

The overall objective of this thesis was to assess the effects of larval experience with antifeedants upon feeding preference of larvae and oviposition preference of adults in generalist (*Trichoplusia ni*, Lepidoptera: Noctuidae) and specialist herbivores (*Pseudaletia unipuncta*, Lepidoptera: Noctuidae; *Plutella xylostella*, Lepidoptera: Plutellidae; and *Epilachna varivestis*, Coleoptera: Coccinellidae).

Selection of plant extracts and pure allelochemicals used in the experiments was based on their growth inhibiting and antifeedant properties against the test species. Initial screening showed that an extract of *Melia volkensii* (Meliaceae) was the most effective growth inhibitor among all the antifeedants for both *T. ni* and *P. unipuncta*. It also acted as a strong antifeedant to *T. ni*, *P. unipuncta*, and *E. varivestis* (*DC_{50} values* [deterrency concentration causing 50% feeding deterrency compared to the control] = 8.3, 10.5, and 2.3μg/cm² respectively).

The effects of feeding experience with antifeedants on subsequent feeding preference showed that all instars of *T. ni* tested (second, third or fifth) exhibited a decreased feeding deterrent response to most of the antifeedants tested (*M. volkensii*, *M. azedarach* (Meliaceae), *Origanum vulgare* (Lamiaceae) and pure allelochemicals; xanthotoxin, toosendanin and thymol), following prolonged exposure. Cardenolides (digitoxin and cymarin) were the exceptions.
Xanthotoxin, acting as a noxious stimulus, dishabituated (reversed) the decreased antifeedant response to *M. volkensii*.

Feeding responses of specialist insects showed interspecific differences. Neither *P. unipuncta* nor *P. xylostella* showed a significant decrease in feeding deterrent response to *M. volkensii* in either choice or no-choice tests. However, there was a decrease in feeding deterrent response following prolonged exposure to *M. volkensii* by *P. xylostella* in no-choice test. In contrast, both species showed a significant decrease in feeding deterrent response to a pure allelochemical, thymol. *Epilachna varivestis* showed a decrease in feeding deterrent response to *O. vulgare* and thymol following prolonged exposure.

*Trichoplusia ni* larvae also showed a generalization in feeding deterrent response to unrelated antifeedants following prolonged exposure in some instances. There was a significant decrease in feeding deterrent response to *O. vulgare* in larvae with previous exposure to *M. volkensii* extract and *vice versa*.

Further investigation of feeding responses of *T. ni* larvae showed that there was a decrease in feeding deterrent response following prolonged exposure to plant extracts or pure allelochemicals when presented singly, but not to binary mixtures.

Larval feeding experience influenced the oviposition behaviour of the adult moths. Comparison of ODIs (oviposition deterrence indices) of experienced and naïve moths showed that there was a significant decrease in oviposition deterrent response by the experienced moths. The weight of *F*₁ larvae from
experienced female moths on the treated plants suggested that there was a positive correlation between larval growth performance and adult moth choice.
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Preface

 Portions of this thesis have been submitted for publication. The contents of Chapter 7 are published (Journal of Chemical Ecology). The contents of Chapters 2 (Journal of Applied Entomology), and 6 (Chemoeconomy) are in press. The contents of Chapters 3 (Journal of Insect Behavior), 4 (Entomologia Experimentalis et Applicata), and 5 (Journal of Chemical Ecology) are being revised. Dr. Isman supervised the research and edited the papers prior to submission for publication. Dr. Rankin closely monitored the research part of my thesis related to insect behaviour and provided tremendous help in the preparation of the manuscript (Chapter 2 of the thesis). Dr. Gobaszewski (Agricultural University, Poland) and Dr. Kozak (Forestry, UBC) provided help with statistical analysis. Dr. Chapman never hesitated to discuss any research related questions.

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Murray B. Isman
1.0 GENERAL INTRODUCTION

The host ranges of phytophagous insects are determined to a large degree by plant chemistry (Renwick, 2001). Chemicals emanating from a plant may have a negative or positive effect on an approaching insect. The presence or absence of specific volatiles can promote landing, and key compounds at the surface may then signal the suitability of a plant, resulting in acceptance for oviposition or feeding (Huang and Renwick, 1994). The presence of repellents or deterrents usually signals unsuitability of a plant, and the resulting rejection behaviour allows the insect to avoid contact with or ingestion of, toxins that are likely to be encountered in the plant (Renwick, 2001).

Chemicals that inhibit feeding of phytophagous insects may be an integral part of plant defence itself, conferring on it some measure of resistance to insect attack, or they may be applied to the plant in the same way as other agricultural chemicals (Chapman, 1974) and have a potential value in crop protection (Jermy, 1965; Munakata, 1970). Interest in the feeding deterrent properties of plant secondary compounds has arisen both because deterrence is an important mediator of plant-insect interactions and because it is potentially useful for manipulating the behaviour of crop pests (Usher et al., 1988).

The responses of insect larvae to chemical cues from potential host plants may be influenced by dietary experience. Using the imported cabbageworm, *Pieris rapae*, as a model, Renwick and Huang (1995, 1996) have developed good evidence that the gustatory receptors of neonate larvae are initially so malleable that the chemical signature of a novel host may not deter feeding and
successful development if it is the first signature encountered. Feeding behaviour may be affected by various forms of learning, including habituation, sensitization, and classical conditioning (Szentesi and Jermy, 1989).

The extent to which larval learning influences feeding preferences on different hosts varies considerably between insect species. In some species, strong preferences are retained for particular host plant species regardless of experience, whereas in other species larvae show the strongest preferences for the host species on which they have previously fed regardless of innate preferences and in the extreme may refuse to feed on any but the experienced host species (Szentesi and Jermy, 1989; Renwick and Huang, 1995).

Experience may involve neurological changes in the peripheral sensilla or central nervous system of feeding larvae (Bernays and Weiss, 1996) resulting in changes in the host finding or host recognition behaviour of larvae towards different species (Bernays, 1995). In addition, experience has been shown to increase the efficiency with which larvae can digest the hosts' tissue; through changes in the uptake of nutrients or detoxification of defence chemicals present in the host tissue (Lindroth, 1991; Bernays and Weiss, 1996; Huang et al., 1997).

When phytophagous insects lay their eggs on many host species (polyphagy), adult females do not select all hosts equally. Instead, preferences in both pre-alighting (host finding) and post-alighting (host acceptance) foraging behaviour are often shown towards particular host species. Host preferences of ovipositing females show a strong heritable component and are thought to represent the suitability of hosts for larval survival (Singer, 1983; Courtney et al.,
Suitability can depend upon a number of factors such as nutritional quality, host plant secondary chemicals, prevalence of natural enemies or microenvironment (Thompson and Pellmyr, 1991).

Such preferences are not always fixed. Ovipositing females have been shown to change their preferences for different host species during their lifespan. Specifically, experimental work has demonstrated that insect learning can significantly change the host selection behaviour of ovipositing females; previous experience of a host plant can lead to an increased preference for that host species (e.g. Prokopy et al., 1982; Papaj, 1986; Landolt and Molina, 1996; Cunningham et al., 1998). Factors such as innate preferences, changes in preference (learning) and environmental abundance of each host species all interact to determine which host an insect is most likely to alight on (Courtney and Kibota, 1990).

Explanations for the advantages of learning in oviposition are currently based around the concept that by concentrating on a particular host species, the rate at which adults find and utilise hosts will be increased (Papaj and Prokopy, 1989; Schoonhoven et al., 1998). Offspring quality will be determined by the suitability of the host species on which they develop (Cunningham and West, 2001).

The host plant selection behaviour of phytophagous insects provides a variety of related questions at a number of levels. In generalist species, why do
females exhibit preference hierarchies, preferring to lay eggs on some host species rather than others? Given the existence of preference hierarchies, should they change with the experience of individuals (learning)? A large body of theory has addressed these questions, utilizing a range of different approaches, and based upon general foraging models as well as those specifically constructed for phytophagous insects.

Recently, there has been some exploration of the possibility that learning may, in itself, influence the direction of evolutionary change. An example may be given in the case of a female butterfly, which is encountering plants other than its normal host plants for oviposition. A large egg load may result in her decision to oviposit on nonhosts. If the act of oviposition is an unconditioned stimulus for learning, she may then continue to oviposit on the same plant. Such persistent oviposition on a novel or nonpreferred host may increase the likelihood of some offspring accepting the new plant and growing well on it. This could be the first stage of a host shift. The phenomenon involves a physiological state variable (egg load) that alters acceptance levels of a plant, combined with learning, and a resultant increase in selection for larvae with improved ability to utilize a novel host (Bernays and Chapman, 1994).

One might point out that if some small percentage of eggs persistently ends up on “non-hosts” and if neonates hatching from these eggs have a greater chance of finding these plants to be suitable hosts than previously thought, why then are the host ranges of the vast majority of phytophagous insects narrowly constrained to only a few species (Bernays and Graham, 1988)? For the answer
we must reconsider all of the ecological and evolutionary constraints on host range in natural systems.

In natural systems, host range is ecologically constrained by the behavioral, neurophysiological and physiological traits shared by members of a population, by the suite of plant species that have been and are currently within the geographic range of the population, and by the intensity of inter- and intraspecific competition, predation, and parasitism that the population must endure. Changes in host specificity are most likely to occur when one or more of these constraints are relaxed during periods of allopatric (Mayr, 1963) or allochronic (Wood and Keese, 1990) isolation. Oviposition “mistakes” and the malleability of neonate gustatory receptors might very well have played important roles in defining the current host ranges found in natural populations of phytophagous insects.

Not only are the oviposition “mistakes” responsible for the host shifts but close proximity of abundant parent host species and novel plants also creates an ecological opportunity for insects physiologically capable of interpreting the compounds in novel plants as phagostimulants rather than deterrents. For example, proximity has been evoked to explain the seven species of British Lepidoptera that expanded their host range from native moorland plants in several genera (Myrica – Myricaceae; Vaccinium, Erica and Calluna – Ericaceae) to Pinus contorta Douglas that were planted extensively among them (Winter, 1974). Strong et al. (1984) agree that host shifts can occur even without the collapse of the parent host’s population.
It is apparent however, that studies on the basis of food plant selection by phytophagous insects are few in number compared to the number of insect species. Nor is there any clear understanding of the features of inhibitory chemicals, which cause them to produce their aversive responses by insects. Clearly, if feeding inhibitors are to play any major role in crop protection, as is desirable in order to reduce the amounts of synthetic pesticides used, these deficiencies must be reduced through extended and extensive research on all aspects of their role in host-plant selection by insects (Bernays and Chapman, 1994). Basic research on the feeding and oviposition behaviour of insects is therefore required to understand the phenomena responsible for manipulation of insect behaviour.
LITERATURE REVIEW

1.1 INSECT-PLANT INTERACTIONS

One of the most interesting associative relationships that exists in nature is that of insect interaction with the host plants (Spencer, 1988), and has been a focus of attention for a very long time. Insect-plant interaction involves, though is not limited to, the concepts of behaviour, physiology and chemistry. Feeding by herbivorous insects exerts selective pressure on host plants to cause a shift in plant chemistry (change in the makeup and distribution of plant secondary compounds) which in turn exerts pressure on insects to develop better methods of coping with it. Insects have developed several ways (physical or chemical, Spencer, 1988) of striking the chemical barriers put in place by plants. For example, the coccinellid beetle, *Epilachna tredicimnotata*, cuts a circular trench on the leaf surface to stop the mobilization of induced plant toxins prior to feeding (Carroll and Hoffman, 1980).

Many insects are also capable of detoxification of plant chemicals (Berenbaum and Zangerl, 1993). For example Monarch caterpillars (*Danaus plexippus*) are adapted to feed on milkweeds; some of the cardenolides in the milkweeds are sequestered while others are metabolized and excreted through the action of an enzyme carbonyl reductase (Marty and Krieger, 1984).
1.2 PLANT SECONDARY COMPOUNDS

It is probably true that all green plants produce secondary compounds, likely more than 100,000 (Schoonhoven, 1982), in at least some stage of their development (Hartmann, 1996). Secondary metabolites were once thought to be waste products (Muller 1969), but many of these compounds are now known to be biologically active against insect herbivores and pathogens (Rosenthal and Berenbaum, 1992).

Secondary metabolites are also referred to as allelochemicals (semiochemicals mediating intraspecific interactions). Allelochemicals are subdivided into many groups: 1. **Allomones:** When the response is adaptively favourable to the emitter but not the receiver, 2. **Kairomones:** When the response is favourable to the receiver but not the emitter, and 3. **Synomones:** When the response is favourable to both emitter and receiver. Within the allelochemicals it is sometimes useful to refer to chemicals as attractants, repellents, deterrents, or stimulants according to their effects on the behaviour of insects (Bernays and Chapman, 1994). The terms used in this classification were clearly defined by Dethier et al. (1960).

1. **Attractant:** a chemical that causes an insect to make oriented movements towards the source of the stimulus.

2. **Repellent:** a chemical that causes an insect to make oriented movements away from the source.
3. **Feeding or oviposition stimulant:** a chemical that elicits feeding or oviposition. "Feeding stimulant" is synonymous with "phagostimulant".

4. **Feeding or oviposition deterrent:** a chemical that inhibits feeding or oviposition.

The attractants and repellents have an orientation component and they can be detected at some distance from the plant. By contrast, phagostimulants and deterrents have no orientation component. Phagostimulation and deterrence occurs only when an insect touches or bites the plant. Only 3 and 4 will be discussed in the later sections with more emphasis on feeding deterrents.

The literature on plant secondary metabolites is extensive and has been a subject of discussion for a long time (Mann, 1987; Harborne, 1993; Rosenthal and Berenbaum, 1991). The following is merely a brief introduction to the role of secondary metabolites in defence of plants, host selection and deterrence of insect pests.

**1.2.1 ROLE OF PLANT SECONDARY COMPOUNDS IN THE DEFENSE OF PLANTS**

There is compelling evidence that at least some of the secondary compounds are important in the defence of plants against herbivores (Schoonhoven, 1982).
As immobile organisms, plants have necessarily evolved mechanical (Coley 1983), phenological (Aide 1993) and chemical defences (Coley 1983; Rosenthal & Berenbaum 1991).

The study of the protective properties of plants began many years ago in the late 1800's. The goal was to determine if the secondary metabolites played a purely metabolic role or if they were largely defence chemicals, fulfilling an ecological role for the plant. Many experiments yielded evidence suggesting an ecological role for the so-called secondary metabolites. This meant that the chemicals helped to defend the plants from potentially harmful predators (bacteria, viruses, fungi, insects, and other invertebrate organisms) that could kill or significantly damage the plant (Rosenthal and Janzen, 1979).

One of the many defence mechanisms known to plants is the use of volatile chemicals to attract or repel certain species of insects. The volatile substances cue predators of the herbivores in to their existence and provide protection to the plant itself. The volatile chemicals that have been characterized and seem to be the most prevalent are compounds of the terpenoid family and the compound indole, a product of the shikimate pathway. Several agricultural species including corn, cotton, lima beans, and brussel sprouts have been shown to possess the defence pathways responsible for these compounds (Pare and Tumlinson, 1997).

Turlings et al. (1990) found that when damage was inflicted to corn seedlings by beet armyworm (Spodoptera exigua) larvae, volatile compounds of
the terpenoid class and indole were released, attracting an endoparasitic wasp species *Cotesia marginiventris*. Since the terpenoids became less palatable to *S. exigua*, it could be argued that that induced production of plant allelochemicals evolved first as a direct defense against herbivorous insects, and that the attractive function probably evolved secondarily (Turlings and Tumlinson, 1991).

Studies have indicated that feeding of a polyphagous grasshopper (*Zonocerus variegatus*) on cassava (*Manihot esculenta*) leaves resulted in the release of hydrogen cyanide that repelled the insects (Bernays et al., 1977). The leaves normally contain the cyanogenic glycoside, linamarin, which is not deterrent to the grasshopper and, apparently, is not toxic. When the plant is damaged by the bite of a single insect, hydrogen cyanide is enzymatically produced from the glycoside and is probably detected by the insect's antennae (Bernays and Chapman, 2000a).

### 1.2.2 PLANT SECONDARY COMPOUNDS AS DETERRENTS

Insect-plant chemical interactions in nature are usually very subtle. Most plant defensive chemicals discourage insect herbivory, either by deterring feeding and oviposition or by impairing larval growth, rather than by killing insects outright (Isman, 2002).

Antifeedants are described as substances that reduce feeding by an insect (peripherally-mediated or centrally-mediated). They can be found amongst all the major classes of secondary metabolites – alkaloids, phenolics and terpenoids (Frazier, 1986). But it is in the last category that the greatest number and
diversity of antifeedants, and the most potent have been found (Isman, 2002). Specific examples of some of the well documented antifeedants from plants are listed in Table 1.1.

All phytophagous insects that have been examined respond behaviourally to some of these compounds, most of which produce a deterrent response in the insects (Chapman, 2003). Reduction or complete inhibition of feeding has been demonstrated in Acridadae, Hemiptera, Coleoptera, larval Lepidoptera, and larval Hymenoptera (Morgan and Warthen, 1990; Chyb et al., 1995; Powell et al., 1995; Schoonhoven and van Loon, 2002).

Although most antifeedants may likely act by either stimulating specialized deterrent cells (the deterrent cell of Pieris brassicae, is strongly stimulated by strychnine and quinine and that of Manduca sexta and Mamestra brassicae by salicin) or interfering with the perception of a phagostimulatory cell (azadirachtin inhibits the sugar receptor in Spodoptera littoralis, Simmonds and Blaney, 1984) while others may cause erratic bursts of electrical impulses in the nervous system (sugar receptor in S. exempta to 2,4-dihydroxymethyl-3,4-dihydroxy pyrroliidine) preventing the insect from acquiring appropriate information to feed or not to feed (Schoonhoven, 1982).

A phenomenon occasionally seen in insect-plant interactions is the insect herbivore sequestering secondary compounds in order to use these compounds for their own defences (Harborne, 1993).
1.2.3 DETERRENCE AND TOXICITY

There is an ample evidence to show that the host range of a grasshopper species is determined largely by the distribution of plant secondary compounds acting as feeding deterrents (Bernays and Chapman, 2000 b). There is also an extensive literature showing that plant secondary compounds have deleterious effects on insect development, as a result of toxicity (Berenbaum, 1986). However, two phenomena contribute to these deleterious effects: a compound may have adverse effects because it is not acceptable (deterrent) and the insect is essentially deprived of food, or it may have postingestive toxic effects (Bernays and Chapman, 2000 b). According to Berenbaum (1986) deterrence is associated with toxicity under ecological conditions, meaning that either the deterrent itself is toxic or it is associated with a toxin. However, an unequivocal link between deterreny and toxicity has rarely been demonstrated (Bernays and Chapman, 1987). Azadirachtin is one example of a compound possessing both deterrent and toxic properties at the same time. For practical use, compounds with combined behavioural and toxic effects will have greater chances of success than compounds with antifeedant activity alone (Bernays and Chapman, 2000b).
Table 1.1 Some examples of potent insect antifeedants isolated from terrestrial plants (Isman, 2002).

<table>
<thead>
<tr>
<th>Chemical type</th>
<th>Compound</th>
<th>Plant source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoterpene</td>
<td>Thymol</td>
<td><em>Thymus vulgaris</em> (Lamiaceae)</td>
</tr>
<tr>
<td>Sesquiterpene lactone (germacranolide type)</td>
<td>Glaucolide A</td>
<td><em>Vernonia</em> species (Asteraceae)</td>
</tr>
<tr>
<td>Sesquiterpene (drimane type)</td>
<td>Polygodial</td>
<td><em>Polygonum hydropiper</em> (Polygonaceae)</td>
</tr>
<tr>
<td>Diterpene (abietane type)</td>
<td>Abietic acid</td>
<td><em>Pinus</em> species (Pinaceae)</td>
</tr>
<tr>
<td>Diterpene (clerodane type)</td>
<td>Ajugarin I</td>
<td><em>Ajuga remota</em> (Lamiaceae)</td>
</tr>
<tr>
<td>Triterpene (limononoid type)</td>
<td>Azadirachtin</td>
<td><em>Melia indica</em> (Meliaceae)</td>
</tr>
<tr>
<td>Triterpene (cardenolide type)</td>
<td>Digitoxin</td>
<td><em>Digitalis purpurea</em> (Scrophulariaceae)</td>
</tr>
<tr>
<td>Triterpene (ergostane type)</td>
<td>Withanolide E</td>
<td><em>Withania somnifera</em> (Solanaceae)</td>
</tr>
<tr>
<td>Triterpene (spirostane type)</td>
<td>Aginosid</td>
<td><em>Allium porrum</em> (Liliaceae)</td>
</tr>
<tr>
<td>Alkaloid (indole type)</td>
<td>Strychnine</td>
<td><em>Strychnos nuxvomica</em> (Loganiaceae)</td>
</tr>
<tr>
<td>Alkaloid (steroidal glycoside)</td>
<td>Tomatine</td>
<td><em>Lycopersicon esculentum</em> (Solanaceae)</td>
</tr>
<tr>
<td>Phenolic (furnanocoumarin)</td>
<td>Xanthotoxin</td>
<td><em>Pastinaca sativa</em> (Apiaceae)</td>
</tr>
<tr>
<td>Phenolic (lignan)</td>
<td>Podophyllotoxin</td>
<td><em>Podophyllum peltatum</em> (Berberidaceae)</td>
</tr>
<tr>
<td>Phenolic (benzoate ester)</td>
<td>Methyl salicylate</td>
<td><em>Gaultheria procumbens</em> (Ericaceae)</td>
</tr>
</tbody>
</table>
1.2.4 SECONDARY COMPOUNDS AS PHAGOSTIMULANTS

Secondary compounds may also have positive effects on feeding, acting as phagostimulants. In some cases these are widely-occurring compounds that affect insect feeding on a range of different plants. The flavonoid glycoside rutin is an example. It occurs in many plant families and stimulates feeding in polyphagous species, like the larva of *Helicoverpa zea* and the grasshopper, *Schistocerca americana* (Bernays and Chapman, 1994). It is often true that phagostimulatory effects are only observed when the compounds are present in low concentrations.

In many other cases, however, secondary compounds are only found in one or a small number of plant taxa. In these cases, the chemicals can provide indicators or sign stimuli to a monophagous or oligophagous insect that it is on the correct host and so help to define host range. For example, larvae of the butterfly subfamily, *Pierinae* feed almost exclusively on cruciferous plants characterised by the presence of glucosinolates. They also serve as oviposition stimulants for adult females of *Pierinae* and other oligophagous insects that feed on these plants. Iridoid glycosides, which are monoterpenoids, characterize the host plants of the buckeye butterfly, *Junonia coenia*, and the checkerspot butterflies, *Euphydryas* spp., and are phagostimulants and oviposition stimulants for them (Bernays and Chapman, 1994). There are many other examples of insect genera or species in which phagostimulatory effects are produced by chemicals that are characteristic of the host plant.
The principal phagostimulants are nutrients, especially sugars. In general, the same sugars are stimulating for different species, sucrose and fructose generally being the most effective. Pentose sugars are not usually stimulating. The effectiveness of sugars increases with increasing concentration. Despite the importance of proteins nutritionally, there is no evidence that insects can taste protein (Bernays and Chapman, 1994). They can, however, taste some amino acids, although the stimulating power of these compounds is usually low compared with sugars. Consequently, for most insects feeding on most plants, phagostimulatory effects are likely to be dominated by sugars.

In contrast to deterrent cells that respond to deterrent compounds, phagostimulatory cells are usually sensitive to only one type of compound (Chapman, 2003). Gustatory neurons specific to glucosinolates are present in larval Pieris species (Schoonhoven and van Loon, 2002). Given the great number of phytophagous insects and the widespread occurrence of host specificity, the number of examples of insect species with gustatory cells tuned to host chemicals is still small (Bernays and Chapman, 2000 a).

1.2.5 SECONDARY COMPOUNDS IN HOST-PLANT SELECTION

It is now widely recognized that host plant selection by phytophagous insects is largely based on the presence of secondary plant substances. The final response of an insect to accept or reject a particular plant is thought to be mediated by a balance of sensory inputs from these chemical stimuli in the plant (Dethier, 1982; Ma, 1972; Miller and Strickler, 1984; Schoonhoven, 1987;
Bernays and Chapman, 1978; 2000a). However, this balance can be modified by the internal physiological state of an insect (Bernays and Chapman, 1994). A striking example of this is seen in locusts: injection of amino acid solutions into the hemolymph significantly reduces the sensitivity of amino acid receptors on the maxillary palps (Abisgold and Simpson, 1988). Although the sensory basis for feeding deterrents has been well studied, no clear picture of their mode of action has yet emerged. Feeding deterrents may act by stimulating specialized deterrent cells or inhibiting sensilla which normally respond to phagostimulants such as sugars (Blaney et al., 1986; Mitchell and Sutcliffe, 1984; Schoonhoven, 1977, 1982, 1987; Simmonds and Blaney, 1984).

Among polyphagous insects, the balance of phagostimulatory and deterrent inputs is probably the sole determinant of acceptance or rejection of food, as shown most clearly by the work of Simmonds and Blaney (1990), Dethier (1973), Schoonhoven (1987) and demonstrated in simplified models (Blom, 1978; Hiraoa and Arai, 1991; Ma, 1972). Simmonds and Blaney (1990) showed that saps from acceptable and nonacceptable plants produced broadly similar levels of stimulation in the phagostimulatory cells of the galeal sensilla of *Helicoverpa armigera* and *Spodoptera littoralis* but the response of the deterrent cells was markedly higher with unacceptable plants. There is evidence from studies of *Pieris brassicae* suggesting that inputs from deterrent cells are weighted more heavily (2.5 times) in the CNS than are the inputs from phagostimulatory cells (Schoonhoven, 1987). In the polyphagous grasshopper *Schistocerca americana*, L-canavanine is a feeding deterrent that does not stimulate the deterrent cell but
suppresses the response to sucrose cell (Chapman and Ascoli-Christensen, 1991).

Food selection in many specialist species (oligophagous and monophagous) appears to be driven by a chemical or group of chemicals acting as a sign stimulus coupled with a relative lack of deterrent effects in the host plants (Schoonhoven, 1987; Bernays and Chapman, 1994).

1.3 SENSORY PHYSIOLOGY OF AN INSECT

The basic unit of any sensory system is the sense cell, or neuron. In insects, the cell bodies of the sense cells are usually in the epidermis immediately under the cuticle. The dendrite of the sense cell is modified to interact with the environment so that an appropriate stimulus produces a change in the electrical potential across the cell membrane. This transfer of energy from one form (e.g. light or mechanical), to another (electrical), is called transduction (Bernays and Chapman, 1994). The electrical potential created by the stimulation of the sense cell is called a receptor potential. The receptor potential spreads to the cell body and somewhat near the origin of axon, action potentials are produced. An action potential is a very brief change in the membrane potential lasting only 2-3 ms. It moves rapidly along the axon without changing; unlike the receptor potential it is not graded so that the information arriving at the end of the axon in the central nervous system is precisely the same as that which leaves the body of the sense cell.
Two general models for neural coding have been described (Stadler, 1984). Where a high degree of specificity is involved (e.g. pheromone perception), the message is encoded largely by a single class of neural input (labeled lines). Primary neurons involved in pheromone perception are thought to act as labeled lines, but according to Chapman (1988), there is also some evidence for labeled lines in the mouthparts of grasshoppers and locusts responding to specific feeding deterrents.

The second type of neural coding (across-fiber patterning), involves a complex signal from a number of cells with largely overlapping sensitivity. In this case, the decision is made by the central nervous system from the totality of the sensory input. Since most receptor cells respond to some range of compounds, even if the range is limited, it is believed that decision making by phytophagous insects always involves an element of across-fibre patterning (Bernays and Chapman, 1994; Isman, 1992).

A comparison of the activity of chemosensilla during feeding and absolute feeding rate in Pieris brassicae allowed the construction of a hypothetical “feeding center” in the central nervous system (Schoonhoven and Blom, 1988). There is a strong correlation between the consumption and chemosensory cell stimulation; nerve impulses signalling the presence of different phagostimulants (sugars, amino acids, glucosinolates) are algebraically summed in the feeding center (suboesophageal ganglion). However, impulses from the deterrent cells counteract the effects of phagostimulants. Creation of a neural code representative of the stimulus may not always result in a behavioural response
(due to the effects of internal physiological state of an insect such as degree of satiety; tending to inhibit feeding when the gut is full [Abisgold and Simpson, 1988]).

1.3.1 THE SENSE OF TASTE

The sense of taste in insects is more commonly referred to as “contact chemoreception” because it differs in some respects from the common perception of taste in vertebrates. In vertebrates, taste receptors occur in the oral cavity, especially on the tongue. In insects they occur outside the mouth, and are often present on the tarsi, antennae, and other parts of the body such as the ovipositor.

Insect taste receptors are characterized by the possession of a small number (3-10) of sensory neurons within a hair or cone of cuticle with a single relatively large pore at the tip (Chapman, 2003). From the pore, a tube of cuticle-like material extends down inside the hair, and within this tube, the dendrite sheath, are the dendrites of the sensory cells. Commonly there are four or six sense cells associated with a single hair and their dendrites run through the dendrite sheath, ending just inside the pore. The ends of the dendrites are embedded in a mucopolysaccharide through which any stimulating molecule must pass in order to stimulate the dendrite.

The whole structure of hair and its socket, sense cells and supporting cells which surrounding the sense cells is called a sensillum. Each of the sense cells within a sensillum is sensitive to a different range of chemicals. Commonly, one
cell responds to sugars, one to inorganic salts, one to behaviourally deterrent compounds, and one to water or amino acids. With time, however, it has become apparent that individual gustatory cells respond to more than one class of compound. For example, the water cell in the blow fly, *Protophormia terraenovae*, also responds to fructose (Wieczorek and Koppl, 1978); the sugar cell in the red turnip beetle, *Entomoscelis americana*, also responds to sucrose, glucose, several amino acids, and the iridoid glycoside, catalpol (Bernays and Chapman, 2000 a). This led Bernays and Chapman (2001) to suggest that gustatory neurons should be designated according to their function rather than by cells. Thus, in relation to feeding there would be phagostimulatory cells and deterrent cells. Activity of these in response to appropriate stimuli would enhance or reduce feeding, respectively (Chapman, 2003).

A single cell may have more than one type of acceptor site, and this may usually be the case. In the sugar-sensitive cells of flies there are three different kinds of acceptor molecules, presumably proteins that interact with different types of “sugar” molecule. A pyranose site reacts with glucose and arabinose and other pyranose sugars that have six-membered ring. Sucrose also reacts with the pyranose ring. There are furanose sites reacting with furanose sugars such as fructose and galactose (5-membered ring). A third class of site, reacts with carboxylate anions of amino acids. The possibility exists that there is even a fourth type of acceptor site that reacts with molecules having other characteristics (Bernays and Chapman, 1994).
The possession of different acceptor proteins by a single cell has important implications for the information an insect receives. Sugars that interact with the pyranose site, like glucose and arabinose, will compete for the same acceptor sites so that mixtures of the two sugars may be additive in their effects depending on the concentrations. On the other hand, if glucose and fructose are present together their effects will be additive, irrespective of the concentrations, because they react with different acceptor sites (Bernays and Chapman, 1994).

1.3.2 THE SENSE OF SMELL

In most insects all, or nearly all, the olfactory receptors occur on the antennae. However, small numbers are present on the palps in grasshoppers, and on the labial palps of some Lepidoptera (Bernays and Chapman, 1994).

The cuticle of olfactory sensilla is perforated by numerous small pores, ranging from 10-50 nm (nanometer) in diameter in different insects. This is called a multiporous condition. The cuticular structures are either hairlike (a very short sensory hair, or peg, is sunk within a cavity in the cuticle; known as coeloconic sensilla) or flat plates (known as pore plates or plate organs). Plate organs are common in Hymenoptera, Coleoptera and Homoptera. The olfactory sensilla resemble contact sensilla except the dendrite sheath ends at the base of the hair and the dendrites extend up into the cavity of hair, passing close beneath the cuticular pores (Bernays and Chapman, 1994).

The number of sensory cells in each sensillum varies. Sensilla containing nerve cells responding to plant odours often have two or three sensory neurons,
but there may be more. In grasshoppers, there are two morphologically
 distinguishable types of olfactory hair, one with only three nerve cells and one
 with as many as 30. Grasshoppers also have coeloconic sensilla with three
 sense cells. Phytophagous insects can smell a wide range of odors. All the leaf
 feeding insects can smell components of the commonly occurring green leaf
 volatiles such as hexanol and hexenal (Bernays and Chapman, 1994). Because
 of this general sensitivity, all phytophagous insects probably have the capacity to
 smell any plant, whether it is a host or not. The response spectra of individual
 olfactory cells in any insect may vary considerably. In addition to these responses
 to widely occurring plant volatiles, some insects also exhibit sensory responses
 to the odor of compounds that are specific to their host plants (e.g. the onion fly,
 Delia antiqua, exhibits a large antennal response to some of the volatile
 compounds from the onion, and Psila rosae, the carrot fly, to carrot volatiles)
 (Bernays and Chapman, 1994).

 However, it is not always true that insects have receptors for chemicals
 specific to their hosts. For example, cells responding to specific host-plant odours
 have not been found in adult Pieris brassicae or in Colorado potato beetle,
 Leptinotarsa decemlineata (Bernays and Chapman, 1994).

 As with contact chemoreception, it is assumed that the sensitivity of the
 receptors depends on the presence of acceptor sites in the dendrite membranes
 of the sense cells. Cells responding to a range of compounds presumably have a
 number of different types of acceptor sites, while more specific cells have a small
 number of types (Bernays and Chapman, 1994).
The axons from olfactory cells all end in an area of the brain called the antennal lobe, consisting of a series of discrete regions of neuropile, called glomeruli. These are the primary centers where extensive integration of the olfactory sensory input occurs (Bernays and Chapman, 1994).

1.3.3 THE SENSE OF TOUCH

The sense of touch is mediated by hairs, distributed over all parts of the body. When the response of the hair is purely tactile, the hair has no pores, and it is classified as aporous. A single dendrite ends at the base of the hair and it is characterized by the possession of a dense bundle of microtubules, called the tubular body at the tip. In addition to touch, insects also respond to hardness of food by sensilla located in the mandibles, and sometimes in the maxillae (Bernays and Chapman, 1994).

1.3.4 THE SENSE OF SIGHT

The principle organs of vision in larvae and adult hemimetabolous insects (e.g. grasshoppers) are the compound eyes, consisting of large numbers of similar units called ommatidia. In addition to the compound eyes, three simple eyes, or ocelli are also present in some insects (Bernays and Chapman, 1994).

Larval holometabolous insects (butterflies, moths, beetles, and sawflies), do not have compound eyes, but often they have simple eyes called stemmata (consisting of a lens system and a receptor system similar in basic structure to that in compound eyes) (Bernays and Chapman, 1994).
1.4 EXPERIENCE-INDUCED CHANGES IN FEEDING BEHAVIOUR

It has been reported in a number of studies (Jermy et al., 1982, 1986; Szentesi and Bernays, 1984; Gill, 1972; Raffa and Frazier, 1988; Usher et al., 1988; Bomford & Isman 1996) that repeated contact of feeding insects with a deterrent-containing diet resulted in an increase in its acceptance over time. Bernays (1983) reported data of reduced effectiveness of a neem extract sprayed against Zonocercus variegatus when tested after 12 days of treatment, which was attributed to a decreased deterrent response and not to inactivation of chemical components. Subsequently, increased consumption of deterrent-containing food over time was demonstrated in the laboratory with the polyphagous Mamestra brassicae and Schistocerca gregaria, and the oligophagous Pieris brassicae and Locusta migratoria (Jermy et al., 1982, 1986; Szentesi and Bernays, 1984).

In an examination of decreased response to a feeding deterrent, nicotine hydrogen tartrate (NHT), in last instars of S. gregaria (Szentesi and Bernays, 1984), small pieces of nylon tubing were attached to both maxillary palpi of the larvae. For the experimental group, the tubing was filled with NHT solution for given periods of time daily. The control group received distilled water only in the tubes. On the test day, no chemical was applied to the capillaries, but feeding response was measured by the quantity of plant material consumed after applying NHT to the leaves. The insects that had been repeatedly exposed to the chemical consumed more of the treated leaves compared with the control group, despite the fact that direct perception of the chemical by the sensilla on the
maxillary palpi was prevented by the nylon tubing still in place. The experiment showed that there was a decrease in feeding deterrent response to NHT that was mediated centrally.

There are also reports of generalization of decreased feeding deterrent response to unrelated compounds following prolonged exposure to an antifeedant. This type of experience-based response was described as “cross-habituation” by Huang and Renwick (1995). Studies relating to decrease in feeding deterrent response to unrelated compounds are few (e.g. Huang and Renwick, 1995; Glendinning et al., 2001; Glendinning and Gonzalez, 1995), but are considered very important for understanding manipulation of insect feeding behaviour in the design of pest management strategies.

Jermy et al. (1982) also observed differences with species varying slightly in their patterns of showing decreased deterrent responses to different chemicals. For example, the pattern of development of the responses by *M. brassicae* and *P. brassicae* to strychnine was different and the same was true of *P. brassicae* tested on strychnine or quinine.

It is obvious from the few cases known so far that polyphagous species are more likely to show a decreased deterrent response to a single compound in the laboratory than oligophagous species (Jermy et al., 1982).
1.4.1 MECHANISMS UNDERLYING DECREASED RESPONSE TO ANTIFEEDANTS FOLLOWING REPEATED OR PROLONGED EXPOSURE

Different mechanisms may be responsible for the waning of response such as neural changes in the central nervous system, in peripheral sensilla, or both (Bernays and Weiss, 1996). Szentesi and Bernays (1984) showed that decreased response to antifeedants following prolonged exposure resulted from changes in the central nervous system, during palpation and feeding, or from effects which follow ingestion of the deterrent (e.g. induction of a detoxifying enzyme; Szentesi and Bernays, 1984; Bernays and Chapman, 2000 a).

Schoonhoven (1969) found that the increase in acceptability of salicin-containing food in *Manduca sexta* was correlated with a reduction in the activity of deterrent cells responding to this compound and concluded that sensory change was responsible for the increase in amount eaten. Glendinning et al. (2001) however obtained only a marginal decrease in the sensory response to salicin in *M. sexta* despite a major change in acceptance and concluded that the behavioural change was mediated centrally rather than by peripheral changes in the response from taste receptors. Their data for caffeine, on the other hand, largely mirror the earlier results of Schoonhoven with salicin (1969).

1.4.2 IMPORTANCE OF MIXTURES IN PREVENTING DECREASED DETERRENT RESPONSE

Decreasing deterrent response to feeding deterrents following prolonged exposure occurs most readily when a single antifeedant provides a weak
inhibitory stimulus (Jermy et al., 1982; Szentesi & Bernays, 1984). When a complex mixture of substances inhibits feeding, however, a decrease in feeding deterrent response may not occur (Jermy, 1987; Bomford and Isman, 1996). It is assumed that a binary mixture (having two components, A and B) can elicit at least three types of responses in an insect (Bitterman, 1996; Caouvillon and Bitterman, 1982). Two of these correspond to each of the components (A and B) and would be similar to the effects produced when presented separately. These elemental qualities might be diminished or enhanced in intensity via mixture suppression or synergism (Cromarty and Derby, 1997). The third type would be a mixture-unique “synthetic” quality.

1.5 OVIPOSITION BEHAVIOUR OF LEPIDOPTERA

Plant compounds are in general the most important sensory cues mediating oviposition in phytophagous insects. Plant mechanical characteristics such as trichomes may also play an important role in oviposition behaviour (Bernays and Chapman, 1994). Trichomes prevent some insects from ovipositing on plants, but in other cases have a positive value for insects. For example, *Helicoverpa zea* lays more eggs on hairy surfaces, because the ovipositing female is able to hold on to the hairs, (Bernays and Chapman, 1994). Visual characteristics such as color of the plant (Renwick and Radke, 1988; Traynier, 1979) can play a significant role too, but plant compounds seem to be the major element of "search images" and / or "recognition images" of herbivores. Plant compounds may act as oviposition stimulants (Traynier and Truscott, 1991;
Renwick et al., 1992) or deterrents (Sachdev-Gupta et al., 1990; Dimock et al., 1991; Renwick and Radke, 1985). Host plant acceptance by an ovipositing female is mediated by a balance of sensory inputs from both positive and negative stimuli received from these compounds (Dethier, 1982; Huang and Renwick, 1993). The relative balance between these opposing cues is weighted by the internal physiological state of the insect such as egg load (Minkenberg et al., 1992; Jones, 1977).

Ovipositing butterflies typically locate resources by some combination of vision (Traynier, 1984), and olfaction (Feeny et al., 1989), and then complete their assessment of host plant quality by contact chemoreceptors (Feeny et al., 1983), present on the mouthparts, tarsi or ovipositor. Specific recognition occurs only after a female has landed on a plant. In many species the female drums on the leaf with her fore tarsi. This behaviour leads to acceptance of the plant and oviposition, or to its rejection. It seems evident that tarsal drumming involves contact chemoreceptors on the tarsi, which provides information about the chemical composition of the plant, enabling the female to distinguish an acceptable from an unacceptable plant (Bernays and Chapman, 1994). The sensory cues that elicit or inhibit oviposition play a critical role in the survival of most phytophagous insects.

1.5.1 EXPERIENCE-INDUCED CHANGES IN OVIPOSITION BEHAVIOUR

The probability that an insect will feed or oviposit on a particular host individual will depend on the acceptability of the host to the insect (Singer, 1986).
This will be influenced by both innate tendencies and prior experience; a number of such influences may operate synchronously, increasing or decreasing the probability of acceptance (Miller and Strickler, 1984).

For more than a century, entomologists have suggested that feeding and oviposition behaviour of herbivorous insects is not only influenced by experience of the adult insect (Cassidy, 1978; Rausher, 1978; Prokopy et al., 1982; Jaenike, 1982, 1983; Hoffman, 1985) on a specific food, but also by experience obtained during the larval stage with that food.

Experience-induced changes of oviposition preferences have been demonstrated in only a few phytophagous insect species so far.

### 1.6 PROBLEMS ASSOCIATED WITH THE USE OF ANTIFEEDANTS

As crop protectants, antifeedants must meet the same criteria as insecticides, viz, they must show selectivity towards the target pest (and thus be non-toxic to mammals, natural enemies and pollinators), and have sufficient residual action for crop protection (Isman, 2002).

Compared with synthetic insecticides, antifeedants show greater interspecific differences in bioactivity. Azadirachtin can be an excellent example supporting this point. Owing to its outstanding antifeedant activity against the desert locust ($EC_{50} = 0.05$ ppm, concentration causing 50% feeding deterrence compared to control insects) (Isman, 1994), a comparable level of activity was expected against the migratory grasshopper (a major pest of cereal crops in
North America). Surprisingly, this species showed an EC$_{50}$ > 1000 ppm (Champagne et al., 1989). However, the insect growth regulatory (moult disrupting) effects of azadirachtin in the two species were comparable (Isman, 1993). When azadirachtin was added to an artificial diet, there were no significant differences in the degree of growth inhibition among six species of noctuid caterpillars (EC$_{50}$ = 0.12-0.24 ppm) (Isman, 1994). In a leaf-disc choice test employing fourth instar larvae, EC$_{50}$ values varied more than 30-fold, with *Spodoptera litura* the most sensitive, and *Actebia fennica* (black army cutworm) the least. This information is very helpful in understanding that interspecific behavioural responses to allelochemicals appear far more plastic than do physiological effects.

A recent investigation of a series of silphinene sesquiterpenes as antifeedants found profound differences in activity of individual compounds when tested against the cotton leafworm (*Spodoptera littoralis*), the Colorado potato beetle (*Leptinotarsa decemlineata*) and five species of aphids (Gonzalez-Coloma et al., 2002).

Another problem associated with the use of antifeedants is the lack of any appreciable systemic action in plants (with the exception of azadirachtin). As a consequence, within one or two days after application, pest insects may be diverted to feeding preferentially on the growing tissues of plants, rendering them unprotected (Isman, 1994). In simulated field trials, electrostatic spraying of an ajugarin protected the upper parts of mustard plants, *Brassica nigra* (Griffiths et
al., 1991); as the plant grew through the applied antifeedant, the insect fed more avidly on the new leaves.

Another operational problem related to the use of antifeedants is the potential for insects to show a decrease in feeding deterrent response following repeated or continuous exposure.

Decreased effectiveness of feeding deterrents over time is a major concern and a factor that might limit the practical applications of such natural compounds for insect pest control (Isman, 2002).

1.7 POTENTIAL USE OF ANTIFEEDANTS

The idea of using non-toxic antifeedants as crop protectants is an attractive one and has received considerable attention (Bernays, 1983; Jermy, 1990).

The simplest method of using an antifeedant as a crop protectant is to apply it as a water- or oil-based spray similar to an insecticide. There are some examples of antifeedants showing efficacy in the field. Pickett et al. (1997) have shown that application of polygodial or methyl salicylate resulted in reduced aphid populations with concomitant increases in yields of winter wheat (comparable to that achieved with the pyrethroid insecticide cypermethrin).

In order to protect the new growth from insect pests, Griffith et al. (1991) investigated the joint effects of an antifeedant, ajugarin, and the insect growth regulator, teflobenzuron, on the mustard beetle, *Phaedon cochleariaea* and
larvae of the diamondback moth *Plutella xylostella* feeding on mustard plants. Application of the two protectants together resulted in reduction of foliar consumption by 50% and pest mortality was greater than 75%. Currently this approach is being applied to oilseed rape, *Brassica napus*, with the insect growth regulator replaced by various species of fungal pathogen against Chrysomelidae and the other coleopterous pests (Pickett et al., 1995).

Several authors have suggested that the efficacy of a deterrent-based method may be increased if used in combination with another method that attracts the pest to a nonvalued resource in a stimulo-deterrent diversionary strategy (SDDS), sometimes also referred to as the “push-pull” strategy (Miller and Cowles (1990). The “push” component can come from the antifeedant applied to the crop needing protection, while the “pull” component is the attractant applied to an adjacent trap crop. It has been shown that moths of the genus *Heliothis* were deterred from ovipositing on cotton by azadirachtin and stimulated to oviposit on pigeon pea or maize crops (Pyke et al., 1997).

1.8 PROSPECTS OF ANITFEEDANTS FOR COMMERCIAL USE

Given the aforementioned limitations to the use of antifeedants, viz. specificity differences in response to antifeedants, potential decrease in response to antifeedants following repeated or prolonged exposure, and rapid environmental degradation, it is most unlikely that an antifeedant will emerge with sufficient field efficacy to act as a stand-alone crop protectant (Isman, 1994).
However, antifeedants may have utility if used in combination with other pest management strategies, as described above for *Heliothis*.

1.9 THESIS OBJECTIVES

The general objective of this thesis was to assess if feeding experience with a variety of antifeedants could influence the feeding preferences of larvae and oviposition preferences of adult phytophagous insects. Specific objectives were:

1. to screen various plant extracts and pure allelochemicals for bioactivity (antifeedant and growth-inhibiting effects) against larvae of *Trichoplusia ni*, *Plutella xylostella*, *Pseudaletia unipuncta* and *Epilachna varivestis*;

2. to assess whether a variety of antifeedants produce a decrease in the antifeedant or deterrent response of larvae of a generalist herbivore, *T. ni*, following prolonged exposure and if feeding deterrent response is affected by different concentrations of antifeedants or by insect developmental stages;

3. to assess if decrease in antifeedant or deterrent response is the result of habituation in *T. ni* larvae;

4. to assess if larvae of specialist herbivores (*Plutella xylostella*, *Pseudaletia unipuncta*) and adult *Epilachna varivestis* show the same response to antifeedants following prolonged exposure as a generalist herbivore (*T. ni*);
5. to assess if the feeding response of larvae of a generalist herbivore (*T. ni*) to an antifeedant following prolonged exposure is generalized to another unrelated antifeedant;

6. to assess if the decreasing response of larvae to feeding deterrents following prolonged exposure could be prevented by using mixtures of antifeedants as opposed to single antifeedants in a generalist herbivore (*T. ni*); and

7. to assess if feeding experience with the antifeedants changes the subsequent oviposition behaviour of adult moths in a generalist (*T. ni*) and a specialist herbivore (*Plutella xylostella*).

### 1.10 MODEL SYSTEMS

To meet these objectives, the model systems consisted of the following:

#### 1.10.1 Insects

*Trichoplusia ni, Plutella xylostella, Pseudaletia unipuncta and Epilachna varivestis* (*T. ni* is a generalist herbivore, feeding on a number of plant species, and all others are specialists, feeding on a few plant species).

#### 1.10.1.0 Biology of insects tested

#### 1.10.1.1 *Trichoplusia ni* (Lepidoptera: Noctuidae)

Widely distributed, the cabbage looper *Trichoplusia ni* is a generalist and an important pest of cruciferous plants. This species also attacks other crops including lettuce, beets, peas, celery, tomatoes, certain ornamentals and many
weedy plants. The larvae are light green with white or pale yellow stripes and three pairs of prolegs. The adult of the looper has dark brown mottled fore wings, each having a small silvery spot resembling a figure eight near its center; the hind wings are almost uniformly light brown. The moths have a wingspan of slightly more than 4 cm (Davidson and Lyon, 1979).

The loopers overwinter in the pupal stage, the pupae enclosed in flimsy silken cocoons attached to the food plants or to nearby objects. Moths emerge in the spring and deposit dome-shaped, pale green eggs singly on the host-plants, chiefly at night. After hatching, the destructive larval stage reaches full development in two to four weeks; pupation then occurs and in almost 10 days the new adults emerge (Fig. 1.1). Three or more generations are produced each season, depending on the latitude (Davidson and Lyon, 1979).

1.10.1.2 Plutella xylostella (Lepidoptera: Plutellidae)

The diamondback moth, *Plutella xylostella* is a specialist on crucifers worldwide. This insect does not overwinter in Canada in large numbers. Each summer moths immigrate from the United States. The biology and life cycle of this lepidopteran in Canada is summarized in Burgess et al. (1979). Eggs are laid singly on lower surfaces of the leaves and the neonates burrow into the leaf. The later instars (second to fourth) feed mainly on the underside of the leaves. The damage to the foliage is normally not significant. However, the larvae will feed on the flowers and young leaves resulting in reduced yields. In about two weeks they become fully developed. Pupation occurs in silken cocoons attached to the
plants with the moths emerging a week later (Fig. 1.2). Although *P. xylostella* has three generations each year in Canada, only the second (normally the last week of July) is economically important. Pesticides are normally recommended for control (Burgess et al., 1979).

1.10.1.3 *Pseudaletia unipuncta* (Lepidoptera: Noctuidae)

The true armyworm, *Pseudaletia unipuncta* is a specialist on grasses. Wheat, corn, oats, barley, and rye are among its favored food plants. They crawl in large numbers from field to field devouring grasses and grain crops. Outbreaks are more common following cold, wet, spring weather, and damage (Fig. 1.4) may occur from late April to June. It occurs throughout most of the United States east of the Rocky Mountains, and it has also been found in New Mexico, Arizona, California, and Canada.

The buff or sand-colored moth has a wingspan of about 4 cm with a small white dot in the center of each fore wing and somewhat darker margins on the hind wings. The dot is a convenient recognition mark and the basis for the specific name. Adults feed on the nectar of flowers. The full-grown larva is a nearly hairless, smooth, striped caterpillar, about 5 cm long. Its general color is green to brown, and the stripes, one along each side and a broad one down the back, are dark and often nearly black. The head is pale brown with a green tinge and mottled with dark brown (Davidson and Lyon, 1979).

The larvae hibernate in the soil or debris at the surface and complete their growth in the spring. In the latitude of central Ohio pupation takes place the latter
part of April, and about 2 or 3 weeks later adults emerge and lay eggs in large masses (resembling small white beads) at night in the folded blades or under the leaf sheaths of grains or grasses. In 8 to 10 days tiny green neonates hatch from the eggs and begin feeding. After molting several times they become fully grown in about 3 to 4 weeks, then pupate and emerge as adults (Fig. 1.3). There are usually 3 generations per year (Davidson and Lyon, 1979) depending on latitude.

This pest is notorious for attacking a variety of crops (Fig. 1.4). It gets its name from its feeding pattern; a group of thousands feed together and then when food supplies are exhausted, they migrate en masse to new areas.
Fig. 1.1 Life stages of cabbage looper, *Trichoplusia ni*

(insects.tamu.edu/)
Fig. 1.2 Life stages of diamondback moth, *Plutella xylostella*

(www.agf.gov.bc.ca/)
Fig. 1.3 Life stages of armyworm, *Pseudaelia unipuncta* (www.ppdl.purdue.edu/ppdl/images/)
Fig 1.4 Damage caused to maize plant by *Pseudaletia unipuncta*

(www.entm.purdue.edu/)
1.10.1.4 *Epilachna varivestis* (Coleoptera: Coccinellidae)

The Mexican bean beetle (Fig. 1.5a) is a specialist on legumes and commonly attacks various varieties of bush, pole, and lima beans. Although it can reproduce on both cowpeas and soybeans, injury to soybeans is more common and in some regions is serious. The feeding by the larvae and adults, primarily on the lower surface of the leaves, results in skeletonized foliage (Fig. 1.5b).

The adult is yellow, coppery, or bronze, depending on its age, with 16 black spots on the wing covers. It is hemispherical in shape and about 8 mm long. The females deposit yellow eggs in masses of 40 to 60 on the underside of the leaves. Over 1500 eggs may be deposited by a single female, but the average is about 460. The newly hatched spiny larvae are green, gradually becoming yellow as they mature, then changing to the broad yellow pupae that are attached to the plant by the gray-colored, last larval molt skin at the posterior end (Davidson and Lyon, 1979).

Only the adult beetle overwinters, usually among plant debris on the ground. The starting date of beetle activity in the spring is dependent on the prevailing temperature. In the northern end of the range, they are usually noticed feeding on bean foliage in late May and early June. Egg-laying soon follows with hatching taking place in about 7 days. There are 4 larval instars, each approximately 5 to 7 days apart, followed by a pupal period lasting a week. The total developmental period from egg to adult averages about 33 days in
midsummer; this is greatly extended in cooler weather. There are one to four
generations per year, depending on the latitude (Davidson and Lyon, 1979).

1.10.2 Antifeedants: Crude plant extracts and pure allelochemicals

Crude methanolic extract of seeds of *Melia volkensii*, oil of oregano
(*Origanum vulgare*) and bark extract of *M. azedarach* (containing 60-75%
toosendanin) were used.

Pure allelochemicals used were digitoxin, cymarin, thymol, *trans*-anethole,
toosendanin and xanthotoxin.

These compounds chemically represent discrete classes of antifeedants
and the extracts are well known antifeedants of common interest. Although *T. ni*
is primarily known as a pest of cruciferous plants, it also attacks several other
crops including celery, an important source of xanthotoxin. Thymol, xanthotoxin,
digitoxin, cymarin, toosendanin, oregano, *M. volkensii* and *M. azedarach* would
not be expected to occur in the normal host plant range of the test species but it it
could encounter closely-related phenolic (thymol, xanthotoxin) or terpenoid
(digitoxin, toosendanin) allelochemicals among its host plants. There is also a
great likelihood that *T. ni* may encounter oregano, *M. azedarach* and the plants
containing the above mentioned pure allelochemicals.

1.10.2.1 *Melia volkensii*

*Melia volkensii* is a tall (15-25m) woody tree, which grows in semi-arid
areas of Ethiopia, Somalia, Kenya and Tanzania at altitudes between ca 350 and
1,700 meters above sea level. Its large, olive-like yellow ripe fruits are 4-5 cm long and ca 3 cm in diameter and consequently more than four times heavier in weight than the fruits of *Azadirachta indica* or *M. azedarach* (Rembold & Mwangi, 1995) (Fig. 1.6).

*Melia volkensii* extract contains unique, active biochemicals that belong to the tetranortriterpene class of compounds commonly known as limonoids. These limonoids are related to azadiractin, the active principle in seeds of the neem tree, *Azadirachta indica*. The limonoids identified from *M. volkensii* extract include volkensin (20.3%), salannin (13.5%), 1-cinnamoyltrichilinin (1%), melanin (1%), 1-tigloyltrichilinin (1.5%), 1-acetyltrichilinin (1%), ohchinin-3-acetate (6%), and meliacin (1%) (Rajab and Bentley, 1988). *M. volkensii* does not contain azadirachtin but salannin and ohchinin-3-acetate are present in neem seed extracts.

Little is known about the insecticidal activities of individual limonoids in the extract. Volkensin, ohchinin-3-acetate, and salannin reduce the feeding of third instar fall armyworm (*Spodoptera frugiperda*) on corn leaf discs with DC\(_{50}\) (concentration causing 50% feeding deterrence compared with the control) values ranging from 3.5 µg/cm\(^2\) for volkensin to 1.3 µg/cm\(^2\) for salannin (Rajab et al., 1988). In preliminary tests, I found salannin to possess weak growth-inhibiting and antifeedant properties against *T. ni* larvae.
Fig. 1.5a Life stages of bean beetles, *Epilachna varivestis*  
(www.psu.missouri.edu/soydoc/images/insect/)

Fig. 1.5b Damage caused by *E. varivestis* to bean plant  
(http://creatures.ifas.ufl.edu/)
The fruit extract is toxic to a broad range of insects including dipterans, lepidopterans, and coleopterans (Mwangi and Rembold, 1987, 1988). Fruit extracts were first reported to exert insect growth-inhibiting and antifeedant effects (22.4% deterrence at 1 ppm to 95% deterrence at 10 ppm) on the nymphs and adults of the desert locust, *Schistocerca gregaria* (Mwangi, 1982).

The efficacy of *M. volkensii* extract has also been demonstrated in the field. When applied at an ultra low-volume of 0.2 lb/acre to field plots of forage crops infested with the desert locust, *S. gregaria* (Wilps et al., 1993), the control rate ranged from 40-70%, which was similar to treatments containing a comparable concentration of neem seed oil.

*Melia volkensii* extract kills insects through a broad range of actions when applied topically or mixed in the diet (Mwangi, 1982; Mwangi and Rembold, 1988; Wilps et al., 1993). These actions include acute toxicity, antifeedant activity, reduced growth rate and reproduction and insect growth regulating effects (IGR). Chemically, *M. volkensii* extract is very stable. A solution in methanol/water held at room temperature and protected from light has lost only 15% of its toxicity to mosquitoes in eight years (Mwangi et al., 1996).

1.10.2.2 *Origanum vulgare* (Oregano)

*Origanum vulgare* (Lamiaceae), usually known as oregano, is a common culinary herb (*Fig. 1.7*). Within the genus *Origanum*, based on morphological criteria, letswaart (1980) recognised 3 groups, 10 sections, 38 species, 6 subspecies and 17 hybrids. The members of the genus are mainly distributed
along the Mediterranean region while 75% are restricted to the East Mediterranean. Eleven species occur in Greece.

*Origanum* species are characterised by a wide range of volatile compounds. Seventy-five compounds have been identified in the volatile fraction of five *Origanum* species. One of two major biochemically related groups of compounds seems to be present in all the species examined. The first, the aromatic monoterpenes, are represented in oregano mainly by *p*-cymene, thymol and carvacrol (referred to as 'cymyl' compounds). The second group, the thujanes, are represented mainly by sabinene and by *cis*- and *trans*-sabinene hydrates (Skoula et al., 1999). Daferra et al. (2002) reported that the proportion of different compounds in the essential oil of oregano varied according to season, locality and other factors that resulted in different chemotypes. The two different chemotypes in oregano are based on the quantitative differences in carvacrol or thymol concentration. The chromatogram (gas chromatography/mass spectrometry) of oregano oil used in my study shows the presence of *p*-cymene (12.20%), thymol (1.68%), carvacrol (70%) and a trace amount of alpha-terpinene (Bradbury, unpublished data; see Appendix).

Oregano is known for its antifungal (Thompson, 1989), antiviral, antibacterial (Sivropoulou et al., 1996), insecticidal and strong antifeedant properties against a number of insects (Sivropoulou et al., 1996; Karpouhtsis et al., 1998; Isman et al., 2001).
1.10.2.3 *Melia azedarach*

*M. azedarach* (Meliaceae; Fig. 1.10), (syn. *M. toosendan*) is grown in China, Thailand and Japan mainly as an ornamental and medicinal plant. As an avenue tree it can be found in Spain, Greece, Cyprus, Israel, Tunisia, Algeria, India, Australia, New Zealand, the Caribbean, Brazil and Argentina (Ascher et al., 1995). Although *M. azedarach* is originally a native of tropical Asia (Munz and Keck, 1973 cited in Ascher et al., 1995), it is now widely distributed in drier regions of the southern and western U.S.A (Duffield and Jones, 198, cited in Ascher et al., 1995), e.g. Texas, Arizona, south eastern Nevada, and south western Utah. *M. azedarach* contains limonoids closely related to *Azadirachta indica*. Some of the limonoids isolated from the fruits of *M. azedarach* are meliantriol, melianone, melianol (Lavie and Jain, 1967a), meliacin (1-cinnamoyl melianone) and meliacarpin (Lee et al., 1991). Melantriol showed strong antifeedant properties against the desert locust, *S. gregaria* (Kraus et al., 1981).

Seed oil of *M. azedarach* acted as a strong oviposition deterrent for rice gall midge, *Orseolia oryzae*, and a feeding deterrent for oriental armyworm, *Mythimna separata* (Chiu et al., 1984).

1.10.2.4 Digitoxin and cymarin are cardiac glycosides or cardenolides. Digitoxin (Fig. 1.8) is derived from the parent glycoside digitalis and it is now produced commercially for therapeutic purposes from the perennial herb foxglove (*Digitalis lannata*) (Mastenbroek, 1985), or *D. purpurea* (Scrophulariaceae, figwort family). Cymarin is derived from *Apocynum cannabinum* (Apocynaceae, dogbane family) and is also the principal glycoside of pheasant's eye herb (*Adonis microcarpa*).
Cardenolides have been reported to be oviposition deterrents (Renwick et al., 1989; Dimock et al., 1991; Huang and Renwick, 1994) and feeding deterrents (Sachdev-Gupta et al., 1993a,b) to *Pieris rapae* (Lepidoptera: Pieridae).

1.10.2.5 **Furanocoumarins** (e.g. Xanthotoxin, benz-2-pyrone compounds with a furan ring fused at carbon 6, 7 [linear] or 7, 8 [angular]) have been shown to be potent antifeedants for *Spodoptera litura* (Yajima and Munakata, 1979) and *S. exigua* (Berdegue et al., 1997), and widely toxic to generalist insect herbivores (Berenbaum et al., 1991) *(Fig. 1.8).*

1.10.2.6 **Thymol** *(Fig. 1.8)*, a monoterpenoid phenol, a major constituent of garden thyme, *Thymus vulgaris* (Lamiaceae), possesses toxic and antifeedant properties against *S. litura* (Hummelbrunner and Isman, 2001).

1.10.2.7 **Trans-anethole** *(Fig. 1.8)*, a phenylpropanoid, is the main constituent of the essential oil from anise seed (*Pimpinella anisum*: Lamiaceae; *Fig. 1.9*). It is a contact toxin and feeding deterrent to the tobacco cutworm, *Spodoptera litura* (Hummelbrunner and Isman, 2001).

1.10.2.8 **Toosendanin** *(Fig. 1.8)*, another limonoid, from the bark of the tree *M. azedarach* has been used as a botanical insecticide since 1980. It possesses strong growth inhibitory and antifeedant properties against a number of insects (Chiu, 1984, 1985, 1989; Zhang and Chiu, 1983; Chen, et al., 1995).
Fig. 1.7 *Origanum vulgare* (www.tiscali.co.uk/)
Fig. 1.8 Chemical structures of pure allelochemicals
Introduction

Seeds of *Pimpinella anisum*

Fig. 1.9 *Pimpinella anisum* (www.mpiz-koeln.mpg.de/)
Fruits and flowers of *M. azedarach*

Bark of *M. azedarach*

*Fig. 1.10 Melia azedarach* (www.palo.org/)
CHAPTER 2

COMPARATIVE GROWTH INHIBITORY AND ANTIFEEDANT EFFECTS OF PLANT EXTRACTS AND PURE ALLELOCHEMICALS ON FOUR SPECIES OF PHYTOPHAGOUS INSECTS.
2.1 INTRODUCTION

Due to increasing problems (resistance, impacts on non-target organisms) associated with the use of acutely toxic synthetic insecticides, there is a pressing need for the development of safer, alternative crop protectants such as botanical insecticides and antifeedants. Plant secondary compounds have been the subject of thorough investigation for the past thirty years in an effort to discover new sources of botanical insecticides and antifeedants. Among the plant families studied, the Meliaceae (Champagne et al., 1992; Connoly, 1983, Klocke et al., 1989), Rutaceae (Arnason et al., 1987; Klocke and Kubo, 1982), Asteraceae (Arnason et al., 1989; Bowers, 1983), Lamiaceae (Schmutterer and Tervooren, 1980; Hummelbrunner and Isman, 2001), Piperaceae (Miyakado et al., 1989; MacKinnon et al., 1997), Apiaceae (Berenbaum et al., 1991; Yajima and Munakata, 1979; Berdegue et al., 1997, Klocke et al., 1989), and Annonaceae (He et al., 1997; Zhao et al., 1993) are perhaps the most promising (Schoonhoven, 1982; Isman, 1995; Jacobson, 1989). The Meliaceae and Rutaceae have received much attention at least partly owing to the presence of triterpenoids called limonoids (Connolly, 1983). Azadirachtin, a limonoid from seeds of the neem tree (*Melia indica*, Meliaceae) possesses strong antifeedant and growth inhibitory effects against various insect pests (Isman, 1997). *Melia volkensii* contains limonoids related to azadiractin. Crude *M. volkensii* fruit extract is toxic to a broad range of insects including dipterans, lepidopterans, and coleopterans (Mwangi and Rembold, 1987, 1988). Toosendanin present in the bark of the Chinaberry tree, *M. azedarach* (syn. *M. toosendan*) is another
example of a limonoid used commercially for its insecticidal properties (Chen et al., 1995).

The search for plant-derived chemicals that have potential use as crop protectants (insecticides, antifeedants, and growth inhibitors) often begins with the screening of plant extracts. Initially, the test insects are fed the extracts and effects on insect behaviour and development are monitored. Once a promising extract has been discovered, the next step is to find out how it is affecting the insect; what is the mode of action? This kind of information is needed to ensure safety of the non-target organisms (humans, beneficial insects).

The purpose of the present study was to quantitate the growth inhibitory and antifeedant effects of *Melia volkensii*, *M. azedarach* (containing 60-75% toosendanin) and *Origanum vulgare* extracts as well as pure allelochemicals, digitoxin, cymarin, xanthotoxin, thymol, and trans-anethole (cymarin and trans-anethole were tested only for antifeedant effects) against larvae of a generalist herbivore, *Trichoplusia ni*. *Melia volkensii*, *O. vulgare* extracts and thymol were tested for growth inhibitory and antifeedant effects against a specialist, *Pseudaletia unipuncta* but only for antifeedant effects against two other specialists, *Plutella xylostella*, and *Epilachna varivestis*. Chemical structures of the plant extracts and pure allelochemicals used in this study and their natural sources have been described in detail in Chapter 1. *Trichoplusia ni* and *P. xylostella* are serious pests of cruciferous crops. *Pseudaletia unipuncta* attacks corn and cereals, and *E. varivestis* is a pest of legumes.
This study served as the foundation for more detailed investigations of the effects of selected plant extracts and pure allelochemicals on feeding and oviposition behaviours of generalist and specialist herbivores described in the forthcoming chapters.
2.2 MATERIALS AND METHODS

2.2.1 Plant Material: Cabbage plants (*Brassica oleracea*; var. Stonehead) were routinely grown (for bioassays and for diamondback moth colonies) in plastic pots containing a mixture of sandy loam soil and peat moss (4:1) in the greenhouse at the University of British Columbia, Vancouver, BC, Canada. Cabbage plants used in the bioassays were 5-6 weeks old. Corn (*Zea sacharata* or *Z. rugosa*) (var. Hybrid Sweet Corn) and bean (*Phaseolus vulgaris*) (var. Mirado) plants were grown for bioassays with the armyworm, *P. unipuncta*, and Mexican bean beetles, *E. varivestis*, respectively.

2.2.2 Test substances

2.2.2.1 Plant extracts: An essential oil of oregano was provided by EcoSMART Technologies Inc. (Nashville, USA). A refined extract of the seeds of *M. volkensii* (Meliaceae) was obtained from the University of Nairobi, Kenya, and a refined bark extract of *M. azedarach* containing 60-75% toosendanin was provided by Northwest Agricultural University, Yangling, P. R. China.

2.2.2.2 Pure allelochemicals: Xanthotoxin (99%), digitoxin (97%), cymarin (99.8%) and thymol (99.5%) were purchased from Sigma Chemical Co. (St. Louis, USA) and used as obtained. *Trans*-Anethole (>95%), was provided by EcoSMART Technologies Inc. (Nashville, USA).
2.2.3 Test Insects: *Trichoplusia ni, P. unipuncta, P. xylostella,* and *E. varivestis* were obtained from established laboratory colonies maintained for > 50 generations. Colonies of *T. ni* and *P. unipuncta* were reared on artificial diet (No. 9795, Bioserv Inc., Frenchtown, NJ) supplemented with finely ground alfalfa, to improve acceptability, and vitamins (No. 8045, Bioserv Inc.). Colonies of *P. xylostella* and *E. varivestis* were reared on greenhouse-grown cabbage and bean plants, respectively. All colonies were reared at 19-25°C and a 16:8 LD photoperiod.

2.2.4. Chronic larval growth bioassay: In order to assess the effects of plant extracts or compounds on larval growth, test substances were incorporated into artificial diet at different concentrations according to Isman and Rodriguez (1983). Control diets were prepared with the carrier solvent alone. Test diets (20 g) containing plant extracts or pure compounds were prepared as follows. For a diet containing 1000 ppm fresh weight (fwt) of the test substance, 20 mg of the test substance was dissolved in 1 ml of the carrier solvent (MeOH: dichloromethane, 2:1 by volume for digitoxin, cymarin and xanthotoxin and MeOH alone for all others). This was pipetted onto 3.5 g of dry diet contained in a Petri dish. The solvent was allowed to evaporate in a fume hood for one hour. One-half gram of agar was dissolved in 16 ml distilled water and heated to a boil in a microwave oven. Dry diet with the test material was added to it and mixed thoroughly. The diet was allowed to cool by placing in a refrigerator (4°C). After cooling, the diet was cut into 20 pieces that were placed individually into each cell of an injection moulded plastic tray. Each tray contained twenty-two-five cells. Two freshly
eclosed neonate larvae (< 24h old) were introduced into each cell of the plastic tray. The trays were covered with plastic lids and kept in a plastic box lined with moistened paper towels to maintain humidity. The boxes were then placed in a growth chamber at 25°C and a 16:8 LD photoperiod. On day three the number of larvae in each cell was thinned to one larva per cell of the plastic tray. After 10 days, all larvae were individually weighed and mean weights were determined. Mean weight for each treatment group was then expressed as a percentage of the mean weight of the control. Four or five different concentrations (Fig. 2.1) were used for each test substance along with a control to calculate EC$_{50}$ (effective concentration causing 50% growth inhibition relative to controls) for that substance by linear regression analysis. Twenty to twenty-five replicates were set up for each concentration of the test substance. The concentration ranges used for each test substance were chosen based on preliminary assays.

2.2.5 Antifeedant choice bioassay. Antifeedant activity of test substances was assayed by using a leaf disc choice test as follows.

Freshly moulted third instar larvae were selected in the morning at 0900h, and put into clean Petri dishes with no food. The number of larvae taken was always greater than that needed, ensuring an adequate number of active insects for the bioassays. Larvae were starved 4-5 h prior to each bioassay. Fresh leaf discs were cut from greenhouse-grown cabbage ~5 weeks old, using a # 6 (1.2cm diameter, 1.13cm$^2$ area) cork borer. Control leaf discs were painted on each side with 8.0 µl of the carrier solvent and test leaf discs with the same amount of the test substance. One treated and one control disc (after being
dried) were placed in each compartment [(4.2cm X 3.0cm (length X width)] of a plastic assay tray with a small piece of moistened cotton to prevent desiccation. The distance between the two discs was ~ 0.7 cm. After 5h of starvation, one larva was introduced gently into the center of each compartment using forceps and allowed to feed. The trays were covered with plastic lids. The plastic trays with larvae and test discs were put into a clear plastic box [(39 x 27 x 14 cm (length x width x height)] lined with moistened paper towel and the box was placed in an illuminated growth chamber at 26 °C. When ~50% of the control leaf discs had been eaten (normally 3-5h), larvae were removed from the assay trays. The leaf discs were placed on glass plates, and a digital picture was obtained using an IS-500 digital imaging system (Alpha Innotech Corp.). Areas of control and treated leaf discs eaten were measured using Scion Image software.

A feeding deterrence index (FDI) was calculated using the formula: FDI = 100 {(C − T) / (C+T)} where C and T are the control and treated leaf areas consumed by the insect (Isman et al., 1990). The mean feeding deterrence index was calculated for each concentration of the test substance. Four or six different concentrations were used for each test substance (Fig. 2.2) (n = 20-25 insects per concentration of test substance). A regression of the mean feeding deterrence against each treatment level was used to compute a DC50 (concentration causing 50% feeding deterrency) value as shown in Fig. 2.2.
2.2.6 Comparison of EC$_{50}$ and DC$_{50}$ values

For each plant extract and pure allelochemical tested against *T. ni* larvae, EC$_{50}$ values were plotted against DC$_{50}$ values to determine if these indices showed similar proportional trends (Fig. 2.3). From the linear regression, expected DC$_{50}$ values were computed and tested by a chi square ($X^2$) test to determine significant differences from the trend line.

2.2.7 Data analysis

Data analyses were carried out using statistics software (Statistix 7, 2000) on the basis of actual numbers observed (variance of sample means were determined to be homogeneous). Linear or logarithmic regression analysis was applied for all dose-response experimental data to get the line of best fit. Expected DC$_{50}$ values were calculated by substituting EC$_{50}$ values for x in the regression equation (Fig. 2.3) ($Y = bx + a$, where b stands for the slope of the line, x stands for concentration tested, and a stands for the Y-intercept). A chi square ($X^2$) was used to determine significant differences between the observed and expected DC$_{50}$ values at a given EC$_{50}$ value.
2.3 RESULTS

2.3.1 Chronic larval growth bioassay: The plant extracts and pure compounds tested inhibited larval growth of *T. ni* and *P. unipuncta* neonates in a dose-dependent manner when added to artificial diet. EC\textsubscript{50} values (effective concentration inhibiting larval growth by 50% relative to controls, by interpolation) generated by linear regression analysis (Fig. 2.1) after 10 days of feeding, are shown in Table 2.1.

2.3.1.1 Plant extracts- EC\textsubscript{50} values for *M. volkensii*, *M. azedarach* and oregano extracts for *T. ni* were 7.8, 201.7 and 524.3 ppm, respectively (Table 2.1), indicating that *M. volkensii* is ~69 times more potent than oregano. The EC\textsubscript{50} values for *M. volkensii* and oregano in *P. unipuncta* were 12.5 and 783.9 ppm, respectively (Table 2.1), a ~62 fold difference. The lower EC\textsubscript{50} values indicate that *M. volkensii* is very effective in reducing larval growth in both species.

2.3.1.2 Pure allelochemicals- Based on EC\textsubscript{50} values, digitoxin (299.8 ppm) was the least potent and cymarin (157.0 ppm) the most for *T. ni* (Table 2.1).

The EC\textsubscript{50} value for thymol in *P. unipuncta* was 462.9 ppm, about twice that for *T. ni*. In general the EC\textsubscript{50} values for both plant extracts and all allelochemicals tested were higher for *P. unipuncta* compared with *T. ni*. The EC\textsubscript{50} value for *M. volkensii* is ~22-37 times less than that for any of the pure allelochemicals tested against *T. ni* and ~38 times less than that of thymol in *P. unipuncta* (Table 2.1).
Calculation of EC$_{50}$

$y = -1.3276x + 60.34$

$50 - 60.34 = -1.3276x$

$x = 10.34 / 1.3276 = 7.79$

**Fig. 2.1a** Dose response relationships (concentration versus larval weight) for plant extracts against *Trichoplusia ni* in chronic growth bioassays (10 days).
Fig. 2.1b Dose response relationships (concentration versus larval weight) for pure allelochemicals against *Trichoplusia ni* in chronic growth bioassays (10 days).
Fig. 2.1c Dose response relationships (concentration versus larval weight) of plant extracts and a pure allelochemical against *Pseudaletia unipuncta* in chronic growth bioassays (10 days).
Fig. 2.2a Dose response relationships (concentration versus feeding deterrenz) of plant extracts against *Trichoplusia ni* in leaf disc choice bioassays.
Fig. 2.2b Dose response relationships (concentration versus feeding deterreny) of pure allelochemicals against *Trichoplusia ni* in leaf disc choice bioassays.
Fig. 2.2c Dose response relationships (concentration versus feeding deterrency) of plant extracts and a pure allelochemical against *Pseudaletia unipuncta* and *Plutella xylostella* in leaf disc choice bioassays.
Fig. 2.2d Dose response relationships (concentration versus feeding deterrenncy) of plant extracts and a pure allelochemical against *Epilachna varivestis* in leaf disc choice bioassays.
Table 2.1 Growth inhibitory effects of crude plant extracts and pure compounds on neonate *T. ni* and *P. unipuncta* larvae.

<table>
<thead>
<tr>
<th>Antifeedants</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; values (ppm)</th>
<th>Plant extract/ Pure compound</th>
<th>T. ni</th>
<th>r²&lt;sup&gt;c&lt;/sup&gt;</th>
<th>P. unipuncta</th>
<th>r²</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. volkensii</em></td>
<td></td>
<td></td>
<td>7.8</td>
<td>0.85</td>
<td>12.5</td>
<td>0.74</td>
</tr>
<tr>
<td><em>O. vulgare</em></td>
<td></td>
<td></td>
<td>524.3</td>
<td>0.88</td>
<td>783.9</td>
<td>0.86</td>
</tr>
<tr>
<td><em>M. azedarach</em></td>
<td></td>
<td></td>
<td>201.7</td>
<td>0.96</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>digitoxin</td>
<td></td>
<td></td>
<td>299.8</td>
<td>0.96</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>cymarin</td>
<td></td>
<td></td>
<td>157.0</td>
<td>0.85</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>xanthotoxin</td>
<td></td>
<td></td>
<td>253.0</td>
<td>0.7</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>thymol</td>
<td></td>
<td></td>
<td>247.2</td>
<td>0.74</td>
<td>462.9</td>
<td>0.82</td>
</tr>
</tbody>
</table>

<sup>a</sup> Regression lines were calculated from 4-6 points, n = 20-25 for each point (each point represents mean weight of larvae at a particular concentration of test substance relative to mean weight of the control larvae).

<sup>b</sup> EC<sub>50</sub> = effective concentration of the test substance to reduce larval growth by 50% relative to control after 10 days of feeding.

<sup>c</sup> r² coefficient of determination.

<sup>d</sup> not tested due to insufficient number of insects.
Inhibition of larval growth in this bioassay may be due to either behavioural (feeding deterrent) or physiological (post-ingestive) effects (Chen et al., 1995).

**2.3.2 Antifeedant bioassay** - There was a direct relationship between concentration of test substances and feeding deterrenacy. DC$_{50}$ values calculated by using regression from the line of best fit (Fig. 2.2) are shown in Table 2.2.

**2.3.2.1 Plant extracts**: The DC$_{50}$ values for *M. volkensii* and oregano extracts in third instar *T. ni* were 8.3 and 41.1 µg/cm$^2$, respectively, indicating that *M. volkensii* is ~5 times more deterrent than oregano. The same trend was seen with EC$_{50}$ values for these two plant extracts. The results of my experiments indicate that *M. volkensii* is not only a growth inhibitor but also a strong direct feeding deterrent to *T. ni*.

DC$_{50}$ values for *M. volkensii* in *P. unipuncta*, *P. xylostella* and *E. varivestis* were 10.5 and 20.7 and 2.3 µg/cm$^2$, respectively. As a feeding deterrent, *M. volkensii* extract is comparable in potency to *T. ni* and *P. unipuncta*, but *P. xylostella* is much less sensitive. The DC$_{50}$ values for oregano extract in *P. unipuncta*, *P. xylostella* and *E. varivestis* were 29.6, 27.2 and 16.8 µg/cm$^2$, respectively. These results indicate that *E. varivestis* exhibits greater deterrent properties against *M. volkensii* and oregano extracts compared to all three lepidopteran species tested (Table 2.2).

**2.3.2.2 Pure allelochemicals**: Based on DC$_{50}$ values, xanthotoxin was by far the most effective deterrent and *trans*-anethole was the least among the pure allelochemicals tested against *T. ni* (Table 2.2).
Table 2.2 Antifeedant effects of crude plant extracts and pure compounds on third instar *T. ni*, *P. unipuncta*, *P. xylostella*, and adult *E. varivestis*.

<table>
<thead>
<tr>
<th>Antifeedants</th>
<th>DC$_{50}$ μg/cm$^2$</th>
<th>Plant extract/pure compound</th>
<th>T. ni $r^2$</th>
<th>P. unipuncta $r^2$</th>
<th>P. xylostella $r^2$</th>
<th>E. varivestis $r^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. volkensii</em></td>
<td>8.3 0.98</td>
<td>10.5 0.93</td>
<td>20.7 0.98</td>
<td>2.3 0.97</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>O. vulgare</em></td>
<td>41.1 0.79</td>
<td>29.6 0.91</td>
<td>27.2 0.95</td>
<td>16.8 0.92</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. azedarach</em></td>
<td>24.8 0.97</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>digitoxin</td>
<td>18.8 0.94</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cymarin</td>
<td>10.8 0.9</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>xanthotoxin</td>
<td>0.9 0.7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>thymol</td>
<td>37.4 0.86</td>
<td>57.2 0.93</td>
<td>22.8 0.96</td>
<td>10.1 0.95</td>
<td></td>
<td></td>
</tr>
<tr>
<td>trans-anethole</td>
<td>151 0.98</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Regression lines were calculated from 4-5 points, n = 20-25 for each point (each point represents mean feeding deterrence [%] of larvae for a particular concentration of the test substance when given a choice between control and treated leaves).

$^b$ DC$_{50}$ = concentration of the test substance to deter feeding by 50% in leaf disc choice bioassay.

$^c$ $r^2$ coefficient of determination

$^d$ not tested due to insufficient number of insects.
The results of my experiments indicate that thymol was ~6 times more effective as a feeding deterrent to *E. varivestis* than to *P. unipuncta* and ~ 2-2.5 times more effective to *P. xylostella* than to *T. ni* and *P. unipuncta*, respectively (Table 2.2).

2.3.3 Comparison of EC$_{50}$ and DC$_{50}$ values of the plant extracts and pure allelochemicals

The EC$_{50}$ values in the chronic growth bioassays were plotted against DC$_{50}$ values in the leaf disc choice bioassays to look for a relationship between the two using regression to find the line of best fit (Fig. 2.3). Although a straight line was proposed, the regression was not significant (F = 3.768, p = 0.11) ($R^2 = 0.416$, Fig. 2.3) for the three plant extracts and four allelochemicals in *T. ni*. The correlation was also not significant (estimated r value, 0.6447, < 0.754 which was the critical value of r (coefficient of correlation) at df = 5 and alpha level = 0.05).

Comparison of the observed and expected (predicted) DC$_{50}$ values indicated significant differences in thymol and xanthotoxin ($X^2 = 13.6$ and 19.1 respectively, $p < 0.05$) (Table 2.3). The DC$_{50}$ value for thymol is far greater than predicted, whereas the DC$_{50}$ value for xanthotoxin is far less. Therefore, when the analysis was done without the outliers, regression becomes significant (F = 16.23, p = 0.027). The amount of variation accounted for by fitting the regression is double what it was ($R^2 = 0.844$) showing that approximately 84% of the variation in DC$_{50}$ is accounted for by EC$_{50}$. 


Table 2.3 Expected DC$_{50}$ values (using the regression equation) for third instar *Trichoplusia ni* larvae. Chi$^2$ ($X^2$)$^a$ values (generated from differences in expected and observed values [Table 2.2]) followed by asterisks represent significant differences between expected and observed DC$_{50}$ values ($X^2 = 12.6$, df = 6 and alpha = 0.05) at a given EC$_{50}$ value.

<table>
<thead>
<tr>
<th>Antifeedants</th>
<th>Plant extract/ Pure compound</th>
<th>DC$_{50}$ ug/cm$^2$$^b$</th>
<th>$X^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Expected$^c$</td>
<td>observed - expected$^d$</td>
</tr>
<tr>
<td><em>M. volkensii</em></td>
<td>5.8</td>
<td>2.5</td>
<td>1.07</td>
</tr>
<tr>
<td><em>O. vulgare</em></td>
<td>37.82</td>
<td>3.18</td>
<td>0.27</td>
</tr>
<tr>
<td><em>M. azedarach</em></td>
<td>17.84</td>
<td>6.96</td>
<td>2.74</td>
</tr>
<tr>
<td>digitoxin</td>
<td>23.91</td>
<td>-5.11</td>
<td>1.09</td>
</tr>
<tr>
<td>cymarin</td>
<td>15.07</td>
<td>-4.27</td>
<td>1.21</td>
</tr>
<tr>
<td>xanthotoxin</td>
<td>20.99</td>
<td>-20.99</td>
<td>19.13*</td>
</tr>
<tr>
<td>thymol</td>
<td>20.65</td>
<td>16.75</td>
<td>13.6*</td>
</tr>
</tbody>
</table>

$^a$ Chi$^2$ ($X^2$) = (observed – expected)$^2$ / expected.

$^b$ DC$_{50}$ = concentration of the test substance to deter feeding by 50% .

$^c$ expected = DC$_{50}$ value calculated by substituting the EC$_{50}$ value for x (for a given point) in the regression equation shown in Fig. 2.1.

$^d$ observed – expected = the difference of the observed DC$_{50}$ value (Table 2.2) and the expected DC$_{50}$ value.
Fig. 2.3 Plot of EC\textsubscript{50} versus DC\textsubscript{50} values for third instar *T. ni*. Numbers represent the points of interception for the plant extracts or pure allelochemicals.

1 = *M. volkensii*, 2 = cymarin, 3 = *M. azedarach*, 4 = thymol, 5 = xanthotoxin, 6 = digitoxin, and 7 = *O. vulgare*. Although a straight line is proposed, regression is not significant (*F* = 3.76, *p* = 0.11).

The lower R\textsuperscript{2} value (0.416) also indicates a poor relationship between EC\textsubscript{50} and DC\textsubscript{50} values.
2.4. DISCUSSION

The results of my experiments indicate that *M. volkensii* is the most effective growth inhibitor among the plant extracts and pure allelochemicals tested, for both *T. ni* and *P. unipuncta*. It also acted as a strong antifeedant to *T. ni*, *P. unipuncta*, and *E. varivestis* (DC\(_{50}\) values = 8.3, 10.5, and 2.3 \(\mu\)g/cm\(^2\), respectively). The efficacy of *M. volkensii* as a growth inhibitor and feeding deterrent is not unexpected owing to the presence of limonoids related to azadirachtin, the active principle in neem insecticides. Fruit extracts of *M. volkensii* were first reported to exert insect growth-inhibiting and antifeedant effects on nymphs and adults of the desert locust, *Schistocerca gregaria* (Mwangi, 1982). Since *M. volkensii* extract has insecticidal action and biochemistry similar to those of neem, its use as an alternative crop protectant against a number of phytophagous insects has been proposed. *Melia azedarach* was a reasonably effective growth inhibitor and feeding deterrent for *T. ni*. Oregano was not an effective growth inhibitor for *T. ni* or *P. unipuncta*, but a reasonably effective antifeedant for all the test species. The lowest DC\(_{50}\) value for oregano (16.7 \(\mu\)g/cm\(^2\)) was exhibited by *E. varivestis*. Earlier studies have indicated that oregano possesses insecticidal and strong antifeedant properties against a number of insects (Sivropoulou et al., 1996; Karpouhtsis et al., 1998; Isman et al., 2001).

Most of the compounds were quite effective in reducing growth of *T. ni* larvae and demonstrated a good linear dose response in the leaf disc choice bioassay. Cymarin is about twice as effective as digitoxin in reducing larval
growth and feeding of *T. ni*. Cardenolides have been reported to deter feeding (Sachdev-Gupta et al., 1993a,b) and oviposition (Renwick et al., 1989; Dimock et al., 1991; Huang and Renwick, 1994) in *Pieris rapae*. Xanthotoxin was the most effective feeding deterrent of all the test substances against *T. ni* (*DC₅₀* value < 1 μg/cm²). Furanocoumarins (e.g. xanthotoxin) have been shown to be potent antifeedants for *Spodoptera litura* (Yajima and Munakata, 1979) and *S. exigua* (Berdegue et al., 1997). Thymol was not a strong growth inhibitor but reasonably effective antifeedant for *T. ni*. *Epilachna varivestis* had the lowest *DC₅₀* value for thymol (10.1 μg/cm²) making it ~3 times more susceptible than *T. ni* and two times more than *P. xylostella*. I obtained much higher *EC₅₀* values (201.6 ppm) for *M. azedarach* containing 60-75% toosendanin for *T. ni*, than previously reported for *P. saucia* (41.3 ppm) (Chen et al., 1995). One possible explanation may be the use of different test insect. Many studies have shown that even closely related species may exhibit widely different susceptibilities to the same plant extract or pure allelochemical (Isman, 1993). Arnason et al. (1987) reported that gedunin (a limonoid from Spanish cedar, *Cedrela odorata*, Meliaceae) is not very active against *P. saucia* or *S. litura*, but toxic to the European corn borer, *Ostrinia nubilalis* as well as to aphids and earwigs. Although azadirachtin is a potent antifeedant for most phytophagous insects, its potency varies among species too. It has outstanding antifeedant properties against *S. gregaria* but is not a feeding deterrent for the acridid *Melanoplus sanguinipes* (Champagne et al., 1989).
Earlier studies with *M. azedarach* showed that a methanolic extract of seed kernels inhibited feeding by fourth and fifth instars of *Pieris rapae* by 98% in a no-choice test (Chiu, 1985). Toosendanin, a limonoid, present in the bark extract of *M. azedarach* plays an important role as an antifeedant, growth inhibitor, and a stomach poison against a number of insects (Chiu, 1989; Chen et al., 1995). Liao and Chiu (1986) reported that toosendanin was not lethal to the oriental armyworm *Mythimna separata* but acts as a potent antifeedant by inhibiting the mouthpart chemosensilla and also by interfering with the signal transduction process in the central nervous system. Thymol possesses toxic and antifeedant properties against *S. litura* (DC$_{50}$ value = 85.6 μg/cm$^2$) (Hummelbrunner and Isman, 2001). *Trans*-anethole was the only allelochemical with a DC$_{50}$ value above 100 μg/cm$^2$ for *T. ni* (all other test substances have DC$_{50}$ values < 60 μg/cm$^2$). *Trans*-anethole possesses both toxic and antifeedant properties against *S. litura* (DC$_{50}$ value = 103.1 μg/cm$^2$) (Hummelbrunner and Isman, 2001). The toxicity of *trans*-anethole has also been demonstrated against a number of species, including various beetles, weevils, mosquitoes, and moths (Sarac and Tunc 1995 a,b; Kelm et al., 1997; Ho et al., 1997).

In order to assess the potential of these test substances, it was important to compare their bioactivities to those of existing botanical insecticides. The results of my experiments have shown that two of the test materials, *M. volkensii* extract (EC$_{50}$ = 7.8 ppm for *T. ni* and 12.5 ppm for *P. unipuncta*) and cymarin (EC$_{50}$ = 132.0 ppm for *T. ni*) have bioactivities comparable to those of commercial botanicals such as pyrethrum (EC$_{50}$ = 98 ppm), ryania (EC$_{50}$ = 117 ppm) or
rotenone (EC$_{50}$ = 163 ppm) in the *Spodoptera* larval growth bioassay (Isman et al., 1997). However, as with most other extracts and pure allelochemicals, activity cannot be compared to the outstanding activity of azadirachtin, with EC$_{50}$s of 0.19 and 0.24 ppm against *P. saucia* and *S. litura*, respectively (Isman, 1993).

The results of my experiments also indicate that one type of bioassay is not sufficient to assess the bioactivity of a potential crop protectant. There may or may not be a direct relationship between growth inhibition and feeding deterreny (regression was not significant when I included all the seven treatments but became significant by removing the outliers, thymol and xanthotoxin) as indicated in my chronic growth and feeding deterreny bioassays with *T. ni* larvae (Fig. 2.1). The observed DC$_{50}$ value for thymol was significantly higher (37.4 μg/cm$^2$) and lower for xanthotoxin (0.95 μg/cm$^2$) than the predicted values (20.65 and 20.99 μg/cm$^2$, respectively) at their corresponding EC$_{50}$ values (Table 2.3).
2.5 CONCLUSION

The results of my experiments indicate that there are interspecific differences in response to feeding deterrents (Schoonhoven, 1982). Differences not only exist between the generalist (\(T. \ ni\)) and specialists (\(P. \ unipuncta\), \(P. \ xylostella\), and \(E. \ varivestis\)) but also among specialist species in their feeding responses towards the plant extracts and pure allelochemicals tested. \(Epilachna \ varivestis\) was the most sensitive among the insect species tested, with the lowest DC\(_{50}\) values for \(M. \ volkensii\), oregano and thymol (2.3, 16.8, and 10.1 \(\mug/cm^2\), respectively).

The results of my experiments not only emphasize the need to test a battery of bioassay species with candidate compounds if the goal of the research is discovery and development of an insecticide or an alternative crop protectant for the management of phytophagous pests of agriculture and forestry (Isman, 1997), but also suggest the use of more than one bioassay. Results obtained from these bioassays are helpful in understanding the feeding behaviour of an insect. Identifying sources with useful biological activity is only the starting point in the long process of development of a botanical pest management product. Application and success in the field depends upon a number of factors such as ongoing availability of the natural resource, adequate biomass to justify extraction, the feasibility of extraction near the harvest site, and the stability of the extract in storage after preparation (Isman et al., 1997). The growth inhibitory and antifeedant properties exhibited by the test species against a number of test substances in my experiments suggests that some of them may serve as
potential crop protectants and also as models for studying host-plant shifts in nature.

In the field, prolongation of developmental stages (as a result of growth inhibition) and increased search time (as a result of antifeedants) to seek viable food sources, likely expose herbivores to increased mortality as a result of biotic and abiotic factors. Although the concept of using nontoxic antifeedants in crop protection is very attractive, in reality there are potential problems associated with their use. There are many examples of antifeedants loosing efficacy as a result of repeated exposure to insects (Raffa and Frazier, 1988; Bomford and Isman, 1996; Szentesi and Bernays, 1984; Szentesi and Jermy, 1989; Usher et al., 1988; Simmonds and Blaney, 1984; Chapman, 2003; Glendinning et al., 2002 a,b; Held et al., 2001).
CHAPTER 3

DECREASED RESPONSE TO FEEDING DETERRENTS FOLLOWING PROLONGED EXPOSURE IN THE LARVAE OF A GENERALIST HERBIVORE, *Trichoplusia ni* (LEPIDOPTERA: NOCTUIDAE).
3.1 INTRODUCTION

It is now widely recognized that host plant selection by phytophagous insects is largely based on the presence of secondary plant substances. In some cases these substances act as phagostimulants that promote feeding and in others as antifeedants or deterrents that inhibit feeding and, as such are major determinants of host ranges of many phytophagous insects (Bernays and Chapman, 2000; Jermy, 1966).

Repeated exposure to antifeedants in the diet of some insect pests can result in their increased acceptance (Gill, 1972; Jermy et al., 1982; Raffa and Frazier, 1988; Usher et al., 1988; Bomford & Isman, 1996) and thus a loss of efficacy (Szentesi and Jermy, 1989; Bernays, 1983). Decreased effectiveness of feeding deterrents over time is a major concern and a factor that might limit the practical applications of such natural compounds for insect pest control (Isman, 2002).

There are several possible mechanisms for the decrease in efficacy including sensory adaptation, motor fatigue, and habituation. Many investigators have used the term habituation liberally without actually proving that the decrement (decrease in response) represents habituation and not sensory adaptation or motor fatigue. Habituation, perhaps the simplest form of learning, is defined as the waning of a response as a result of repeated or prolonged presentation of a stimulus, which is not due to sensory adaptation or motor fatigue (Carew and Sahley, 1986). Habituation differs from sensory adaptation in
its ability to be terminated or reversed immediately by a novel or noxious stimulus (Thompson and Spencer, 1966).

Different mechanisms may be responsible for the waning of response. Szentesi and Bernays (1984) showed that decreased response to antifeedants following prolonged exposure may result from the effect of mouth part chemosensory information on the central nervous system, during palpation and feeding, or involving persistent synaptic changes in specific neural pathways (Bernays and Chapman, 1994), or from effects which follow ingestion of the deterrent (e.g. induction of a detoxifying enzyme; Szentesi and Bernays, 1984; Bernays and Chapman, 2000; Bernays and Weiss, 1996).

The experiments contained within this Chapter are designed to test whether a variety of antifeedants (plant extracts or pure compounds) would produce a decrease in the antifeedant response following prolonged exposure in a generalist herbivore, *Trichoplusia ni*. *T. ni* was chosen because it is an important polyphagous pest of food, fiber, and ornamental crops throughout the New World (Chapter 1). The selection of antifeedants used was based upon their feeding deterrent and growth inhibiting properties on *T. ni* as shown in the initial screening bioassays (Chapter 2).

The main objectives of the experiments contained within this Chapter were:
1. To determine under what conditions (different concentrations and larval instars) feeding experience with antifeedants changed subsequent feeding preferences of the cabbage looper \((T. \text{ni})\) larvae.

2. To determine if decreased response to antifeedants following prolonged exposure was the result of habituation in \(T. \text{ni} \). If so, it should then be possible to demonstrate dishabituation.

Knowledge of these factors may have consequences for the use of antifeedants in pest management and might also be helpful in understanding host-plant shifts in insects.

3.2 MATERIALS AND METHODS

3.2.1 Plant Material

Cabbage plants \((Brassica oleracea \text{ var. Stonehead})\) were routinely grown for bioassay in the greenhouse as described in Chapter 1.

3.2.2 Chemicals

Digitoxin, cymarin, xanthotoxin, thymol, oil of oregano, refined seed extract of \(Melia \text{volkensii}\) and refined bark extract of \(M. \text{azedarach}\) (containing 60-75% toosendanin) were obtained from sources listed in Chapter 2.
3.2.3 Concentration of antifeedants used for rearing (training)

Concentrations used for rearing insects (see Table 3.1) were chosen based on preliminary experiments so as to avoid toxicity or growth inhibitory effects on the insects.

3.2.4 Concentration of antifeedants used for testing

Concentrations of the plant extracts or compounds used in the experiments were determined by calculating the DC$_{50}$ (concentration that causes 50% deterrence compared to untreated leaf discs) in preliminary experiments using naïve T. ni (Chapter 2). Concentrations used in the experiments were greater than the DC$_{50}$ values (except for xanthotoxin) (Table 3.1).

3.2.5 Test Insects

Trichoplusia ni were obtained from an established laboratory colony as described in Chapter 2.
Table 3.1 Rearing and testing conditions of *Trichoplusia ni* for determining feeding responses to antifeedants (plant extracts and compounds) following prolonged exposure experiments.

<table>
<thead>
<tr>
<th>Antifeedants</th>
<th>Concentration</th>
<th>Instars tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant extract / Compound</td>
<td>mg/cabbage leaf</td>
<td>µg/cm²</td>
</tr>
<tr>
<td><em>M. volkensii</em></td>
<td>0.1</td>
<td>15</td>
</tr>
<tr>
<td><em>M. volkensii</em></td>
<td>0.01</td>
<td>15</td>
</tr>
<tr>
<td>oregano</td>
<td>5</td>
<td>80</td>
</tr>
<tr>
<td>oregano</td>
<td>2</td>
<td>80</td>
</tr>
<tr>
<td>oregano</td>
<td>1</td>
<td>80</td>
</tr>
<tr>
<td><em>M. azedarach</em></td>
<td>0.01</td>
<td>40</td>
</tr>
<tr>
<td>digitoxin*</td>
<td>0.01</td>
<td>40</td>
</tr>
<tr>
<td>cymarin*</td>
<td>0.01</td>
<td>25</td>
</tr>
<tr>
<td>xanthotoxin*</td>
<td>0.01</td>
<td>0.6</td>
</tr>
<tr>
<td>thymol</td>
<td>1.0</td>
<td>40</td>
</tr>
</tbody>
</table>

*carrier solvent MeOH / dichloromethane (2:1 v:v). All others used MeOH alone.
3.2.6 General procedure

3.2.6.1 Training

All experiments included two main groups, experienced and naïve. The experienced groups were reared on cabbage foliage treated with different concentrations of antifeedants (either plant extract or pure compound) for different periods of time. A one-ml solution of the plant extract or compound was applied to a cabbage leaf (approx. 100-110 cm²) using a micropipette (0.5 ml on each side of the leaf). The chamber for training was a transparent plastic cylinder (220 mm X 300 mm) (diameter x length). The cut leaf petiole end was placed into a 100 ml plastic cup filled with water, in the center of the chamber. The cylinder was covered with a clear plastic lid to prevent larvae from escaping (Fig. 3.1). Approximately 20 neonates (<24 hours old) were placed on each cabbage leaf and allowed to feed ad libitum until bioassays were conducted. New leaves were introduced every second day or as needed. The naïve group was reared for the same length of time and under the same conditions on cabbage foliage treated only with MeOH or MeOH / dichloro methane (carrier controls).

3.2.6.2 Testing

After the training, larvae from the experienced and naïve groups were tested in leaf disc choice bioassays (Isman et al., 1990) to determine their feeding responses to the same plant extracts or compounds they were exposed to during training. The leaf disc choice bioassay is described in detail in Chapter 2.
Fig. 3.1 Cylinders containing control and treated leaves for rearing insects. Neonates <24 hours old were introduced onto cabbage leaves treated with antifeedants (plant extracts or pure compounds) for the experienced groups and carrier solvent alone for the naïve group.
I employed a choice test for three reasons. Firstly, in my experience, choice tests are more sensitive than no-choice tests with respect to insect feeding behavior; secondly, whether feeding on a single plant or foraging on several plants, phytophagous insects are almost always exposed to varying concentrations of plant secondary compounds (i.e. they always have a choice); and lastly, in a related study (Chapter 4) using specialist insects, I used no-choice bioassays when there was no effect of prior experience in the choice bioassay. In that study I found that the insects responded in the same manner in both tests.

3.2.7 Response to antifeedants following prolonged exposure experiments

In these experiments I investigated whether there were developmental differences in response to extended exposure to a variety of antifeedants by testing different instars of *T. ni*. I varied the concentrations of antifeedants to further explore their effects. One of the main problems associated with the use of antifeedants is the decreasing response following repeated or prolonged exposure. Therefore, my objective was to determine if developmental differences or variation in the concentration of antifeedants could limit the process of decreased response to antifeedants following exposure.

3.2.7.1 Treatment groups

The experienced groups for the experiment were reared on cabbage foliage treated with *M. volkensii* extract, oregano extract, *M. azedarach* extracts or the pure compounds digitoxin, cymarin, xanthotoxin, or thymol. Altogether,
there were 17 experimental groups exposed to antifeedants as shown in Table 3.1.

For each experimental group there was a naïve control group, which was reared on cabbage foliage treated with carrier solvent only.

Feeding responses of the experienced and their respective naïve groups were measured in leaf disc choice bioassays as indicated in the general procedure.

3.2.8 Dishabituation experiment

Since pilot experiments showed that there was a decrease in feeding deterrent response to many of the antifeedants tested, I wanted to determine whether the decreased response might be the result of habituation. If it was habituation, then the initial response (deterrent response to feeding deterrents tested) should be restored by a novel or noxious stimulus. I chose to see if I could dishabituate third instar T. ni reared on cabbage foliage treated with M. volkensii extract at 0.01 mg/cabbage leaf because it is a potent antifeedant for T. ni (see Results section).

In an attempt to produce dishabituation I tested three different aversive stimuli: CO₂, cold shock, and the pure compound xanthotoxin, which is a strong feeding deterrent. Prior to testing whether these three aversive stimuli would dishabituate antifeedant responses it was important to test the effects of the aversive stimuli alone on feeding. To do this I examined whether these stimuli would alter feeding responses in third instars reared on cabbage leaves treated
with MeOH. On the test day all larvae were removed from the cabbage leaves and placed in Petri dishes where they received the dishabituation treatment. There were four groups of larvae. A control group received no aversive stimulus (baseline). One experimental group was exposed to CO₂ for 5 min, another was cooled in a freezer (-10 °C) for 20 min and one group was introduced onto cabbage foliage treated with a very high concentration (10 μg/cm²) of the strong antifeedant xanthotoxin for 20 min. Following treatment all the groups were then starved for 5 hours. To determine whether the aversive stimuli affected feeding, responses to *M. volkensii* were measured for all the groups in paired leaf disc choice bioassays (Isman et al., 1990).

Because none of the three aversive stimuli affected normal feeding, I was able to use them to test whether one or more of these stimuli would dishabituate the decreased antifeedant response following prolonged exposure to *M. volkensii*. *T. ni* were reared on cabbage foliage treated with *M. volkensii* at 0.01 mg/cabbage leaf to the third instar and then tested for dishabituation. The experimental groups used in the experiment were similar to the groups described above except that they were reared on *M. volkensii*. The groups used were: *M. volkensii* ↔ no exposure, *M. volkensii* ↔ CO₂, *M. volkensii* ↔ cold, and *M. volkensii* ↔ xanthotoxin. In addition there was a fifth experimental group reared on cabbage foliage treated with MeOH only. It served as a baseline. Testing was done as described before.
3.2.9 Data analysis

The area of control and treated leaf discs consumed by the larvae was measured using the Scion Image software and mean feeding deterrence index (%) of each group was calculated as described in Chapter 2. Data were analysed by analysis of variance (ANOVA) (Zar, 1984) using statistics software (Statistix 7, 2000) on the basis of actual numbers observed (variance of the sample means were determined to be homogeneous). Where significant F values were found, Tukey's HSD multiple comparison tests were used to test for significant differences between individual treatments. All experiments were performed twice except for the dishabituation experiment. Because there was no effect of treatments between experiments, data from the two experiments were pooled. The alpha level used in all analyses was 0.05.
3.3 RESULTS

3.3.1 Response to plant extracts

_Melia volkensii:_

A three factor ANOVA (concentration * instar * treatments) on the feeding deterrence indices (FDIs) for three instars (second, third and fifth) of naïve and experienced (reared on _Melia volkensii_ at 0.1 and 0.01 mg/cabbage leaf) larvae of _T. ni_ studied showed that there was a significant main effect of instars (_F_2,588 = 4.64, _p_ = 0.01) with fifth instars showing significantly greater antifeedant response to _M. volkensii_ (Tukey's test, _p_ < 0.05) than the second instars (Tukey's test, _p_ < 0.05). There was no significant main effect of concentration (_F_1,588 = 0.21, _p_ = 0.647). There was a significant main effect of treatments (_F_1,588 = 144.46, _p_ < 0.001) with experienced larvae showing significantly less antifeedant response to _M. volkensii_ (Tukey's test, _p_ < 0.05) (Fig. 3.2) compared with naïve larvae (MeOH). None of the two-way interactions, concentration * instar (_F_2,588 = 0.28, _p_ = 0.754), concentration * treatment (_F_1,588 = 0.17, _p_ = 0.681), instar * treatment (_F_2,588 = 1.69, _p_ = 0.185), or three-way interactions, concentration * instar * treatment (_F_2,588 = 0.53, _p_ = 0.590), were significant. This suggested that the experienced groups (_M. volkensii_ at 0.1 and 0.01 mg/cabbage leaf) of all the instars (second, third and fifth) showed a significant decrease in antifeedant response to _M. volkensii_ (Tukey's test, _p_ < 0.05) compared with the naïve groups (MeOH).
**Origanum vulgare** (oregano)

A three factor ANOVA (concentration * instar * treatments) on the feeding deterrence indices (FDIs) for two instars (second and fifth) of naïve and experienced larvae (reared on oregano at 5, 2, and 1mg/cabbage leaf) of *T. ni* studied, showed that there was a significant main effect of concentration ($F_{2,516} = 9.78, p < 0.0001$). Larvae reared on oregano at 1 mg/cabbage leaf, showed a significantly greater antifeedant response than larvae reared on oregano at 5 or 2 mg/cabbage leaf (Tukey's test, $p < 0.05$). There was no significant main effect of instar ($F_{1,516} = 0.01, p = 0.938$). There was a significant main effect of treatment, ($F_{1,516} = 44.97, p < 0.001$) with experienced larvae showing significantly less antifeedant response to oregano (Tukey's test, $p < 0.05$) (Fig. 3.3) compared with naïve larvae (MeOH). There was a significant concentration * treatment interaction ($F_{2,516} = 12.83, p < 0.001$) with experienced larvae (oregano at 5 and 2 mg/cabbage leaf) showing a significantly greater decrease in antifeedant response to oregano compared with the naïve group with no exposure to oregano (Tukey's test, $p < 0.05$). The antifeedant response to oregano of the experienced second and fifth instars (oregano at 1 mg/cabbage leaf) was the same as naïve larvae. The two-way interactions, concentration * instar ($F_{2,516} = 1.12, p < 0.329$), instar * treatment ($F_{1,516} = 0.88, p < 0.349$) and the three-way interaction, concentration * instar * treatment ($F_{2,516} = 1.13, p = 0.323$), were not significant.

To rule out the possibility that decreasing feeding deterrent response to antifeedants exhibited by the experienced larvae was the result of deprivation of
Feeding responses of a generalist food compared with the naïve larvae, larval weights of the two groups were compared in pilot experiments. Experienced (reared on cabbage leaves treated with *M. volkensii* at 0.01 mg/cabbage leaf) and naive third instar larvae of *T. ni* were weighed and starved (4h) (*n* = 30/group). They were then allowed to feed on untreated cabbage leaves for four hours and weighed. Comparison of the weights of the two groups indicated that there was no difference in the weight gain suggesting that similar amount of cabbage was eaten by the two groups (*t* = 0.469, df = 58, *p* = 0.64).

### 3.3.2 Response to *M. azedarach* and individual compounds

A two-factor ANOVA (antifeedant * treatment) on the feeding deterrency indices for the naïve and experienced (digitoxin, cymarin, xanthotoxin, *M. azedarach* and thymol at 0.01 or 1.0 mg/cabbage leaf) third instars of *T. ni* studied showed that there was a significant main effect of antifeedants (*F*<sub>4, 490</sub> = 4.78, *p* < 0.001) with larvae reared on *M. azedarach* (0.01 mg/cabbage leaf) showing significantly greater decrease in antifeedant response than digitoxin, xanthotoxin or cymarin (Tukey’s test, *p* < 0.05). There was a significant main effect of treatment (*F*<sub>1, 490</sub> = 41.71, *p* = 0.001) with experienced larvae showing a significantly reduced antifeedant response to the antifeedants tested (Tukey’s test, *p* < 0.05) (*Fig. 3.4*) compared with naïve larvae (MeOH). The two-way interaction (antifeedant * treatment) was significant (*F*<sub>4, 490</sub> = 2.86, *p* < .02). There was a significant decrease in the antifeedant response to xanthotoxin, *M. azedarach* and thymol by experienced larvae with previous experience with these compounds compared with naïve larvae (Tukey’s test, *p* < 0.05). Experienced
larvae with previous exposure to digitoxin or cymarin failed to show significant decreases in feeding deterrent response and retained the initial deterrent response to these compounds like naïve larvae (Fig. 3.4).
Feeding responses of a generalist

Feeding responses of T. ni (second, third, and fifth instars) to M. volkensii with previous exposure to M. volkensii (0.1, or 0.01 mg/cabbage leaf). Feeding deterrence means (± SE) followed by asterisks represent significant differences between the experienced and naïve groups (Tukey's test, p < 0.05), under the null hypothesis that both the experienced and the naïve groups were equally deterred by M. volkensii (n = 25, 2 replications).
Feeding responses of a generalist

Fig. 3.3 Feeding responses of *T. ni* (second, and fifth instars) to oregano with previous exposure to oregano (5, 2 or 1 mg/cabbage leaf). Feeding deterrence means (± SE) followed by asterisks represent significant differences between the experienced and naïve groups (Tukey's test, p < 0.05), under the null hypothesis that both the experienced and the naïve groups were equally deterred by oregano (n= 22, 2 replications).
Fig. 3.4 Feeding responses of *T. ni* (third instar) to digitoxin, cymarin, xanthotoxin, *M. azedarach* and thymol with previous exposure to digitoxin, cymarin, xanthotoxin or *M. azedarach* at 0.01 and thymol at 1.0 mg/cabbage leaf. Feeding deterrence means (± SE) followed by asterisks represent significant differences between the experienced and naïve groups (Tukey's test, p < 0.05), under the null hypothesis that both the experienced and the naïve groups were equally deterred by the compounds treatments (n = 25, 2 replications).
3.3.3 Dishabituation experiment

3.3.3.1 Effects of aversive stimuli on feeding response

When the effects of aversive stimuli on feeding were assessed, a one-way analysis of variance (ANOVA) on the FDIs (feeding deterrence indices) of the four groups (reared on cabbage leaves treated with MeOH) did not produce a significant F value for the different groups (group with no exposure to aversive stimuli, group with exposure to CO₂, group with exposure to cold, and the group with exposure to xanthotoxin) \( (F_3, 156 = 0.02, p = 0.996) \). The aversive stimuli had no significant effects on feeding behaviour of \( T. ni \) larvae (Fig. 3. 5) and all groups maintained their initial deterrent response to \( M. volkensii \) like the naïve group with no exposure to aversive stimuli.

3.3.3.2 Effects of aversive stimuli on deterrent response

Because the aversive stimuli had no effects on feeding, they were used to test for dishabituation of the decreased deterrent response following prolonged exposure to \( M. volkensii \). A one-way analysis of variance (ANOVA) on the FDIs (feeding deterrence indexes), produced a significant F value, \( (F_4, 245 = 4.56, P = 0.002) \) for the different groups (\( M. volkensii \) - experienced group with no exposure to aversive stimulus, \( M. volkensii \) - experienced group with exposure to CO₂, \( M. volkensii \) - experienced group exposed to cold, \( M. volkensii \) - experienced group exposed to xanthotoxin, and the naïve group [reared on MeOH]). Comparison of feeding deterrent means of the different groups by Tukey's test, showed that the \( M. volkensii \)-experienced group with no exposure,
showed a significantly reduced antifeedant response to *M. volkensii* (*p* < 0.05), compared with naïve group (reared on MeOH). The *M. volkensii* - experienced group with exposure to CO$_2$ also showed a significantly reduced antifeedant response to *M. volkensii* unlike the naïve group but did not differ significantly from the *M. volkensii*-experienced group with no exposure to aversive stimulus and the *M. volkensii*-experienced group exposed to cold or xanthotoxin. The *M. volkensii* - experienced group exposed to cold did not differ significantly from any of the other groups. The *M. volkensii* - experienced group exposed to xanthotoxin, differed significantly from the *M. volkensii* - experienced group with no exposure to aversive stimulus (Tukey’s test, *p* < 0.05) but not from the naïve group or the other *M. volkensii* - experienced groups exposed to cold or CO$_2$ (Fig. 3. 6).

In order to see whether xanthotoxin had any deleterious effect on the *T. ni* larvae resulting in abnormal behaviour, total leaf area consumed by each group was compared. There was no significant difference in the mean leaf area (control and treated cabbage leaves), consumed by the larvae exposed to CO$_2$ (0.41 cm$^2± 0.02$ (SE)), cold (0.52 cm$^2± 0.04$ (SE)), xanthotoxin (0.53 cm$^2± 0.04$ (SE)) *M. volkensii* - experienced (0.53 cm$^2± 0.03$ (SE)) and naïve larvae with no exposure (0.51 cm$^2± 0.03$ (SE)) (One-way ANOVA; $F_{4,195} = 1.79$, $p = 0.13$).

Comparison of the leaf areas of the control and treated discs of all the groups by one-way ANOVA, showed a significant $F$ value ($F_{9,490} = 9.63$, $p < 0.001$) with control leaf discs eaten significantly greater than treated leaves in naïve group and *M. volkensii*-experienced group exposed to xanthotoxin (p. 111).
Fig. 3.5 Test of the effects of aversive stimuli on the feeding behaviour of third instar *Trichoplusia ni* (reared on cabbage leaf treated with MeOH). Naïve larvae were exposed to CO₂, cold and xanthotoxin. Feeding deterrence means (± SE) followed by the same letter do not differ significantly from the baseline (representing the naïve larvae reared on cabbage leaves treated with MeOH, solid line represents feeding deterrence mean of the naïve larvae, and dotted lines represent ± SE) (n = 40/group).
Fig. 3.6 Test of the effects of aversive stimuli on antifeedant effect of third instar *Trichoplusia ni* larvae (reared on *M. volkensii* at 0.01 mg/cabbage leaf) after exposure to CO$_2$, cold or xanthotoxin. Feeding deterrence means ($\pm$ SE) followed by asterisks in each group are significantly different from baseline (naïve group reared on MeOH-treated leaves with no exposure to aversive stimulus, solid line represents feeding deterrence mean, and dotted lines represent $\pm$ SE) according to the Tukey's test ($p < 0.05$), under the null hypothesis that all the groups were equally deterred by *M. volkensii*. Feeding deterrence means ($\pm$ SE) followed by the same letters do not show significant differences between groups, Tukey's test, $p < 0.05$) (n= 50/group).
3.4 Discussion

3.4.1 Response to antifeedants

The results of the experiments demonstrate that following prolonged exposure there is a large decrease in the feeding deterrent response to antifeedants.

All instars of *T. ni* tested (second, third or fifth) showed a decreased response to most of the antifeedants tested. Experienced larvae were significantly less deterred by *M. volkensii* when compared with naïve groups. The fact that second and third instars also showed a decrease in response to *M. volkensii* shows that these earlier instars are capable of developing a tolerance to the deterrent like later instars. This could be beneficial to the insect as earlier instars are not as mobile as the later instars and have to feed on the plant chosen by the adult female.

For both second and third instars, and at both concentrations of *M. volkensii*, experienced larvae ate significantly more of the treated leaf discs, resulting in mean feeding deterrence indices (FDI) values ranging from –23 to –45% (Fig. 3.2). These appear to represent cases of induced preference. Although I do not know of a mechanism that would lead to induced preference, I would argue that habituation, as a mechanism, has at least facilitated the observed switch from deterrence to preference.

Second and fifth instar *T. ni* larvae with previous exposure to oregano became less deterred by it unless the previous exposure was a very low
concentration (oregano at 1.0 mg/cabbage leaf). It appears that a concentration of oregano extract higher than 1 mg/cabbage leaf is required for a decrease in response to take place following prolonged exposure. This is consistent with the finding of Huang and Renwick (1995) that cross-habituation to *Nasturtium* via erysimoside disappeared at a dose of 0.001 mg/cabbage leaf in larvae of *Pieris rapae*. Each compound may have a unique detection value. The observation that *T. ni* showed a decrease in response to *M. volkensii* when reared on cabbage foliage treated with 0.1 and 0.01 mg/cabbage leaf, much lower concentrations than was effective for oregano, suggests that the sensitivity of an insect varies between stimuli. This is further exemplified by the fact that the deterrent neuron in the medial galeal sensillum of *Pieris brassicae* has a threshold of about $10^{-3}$ M for chlorogenic acid but of $10^{-7}$ M for azadirachtin (Chapman, 2003) and for cucurbitacin by adult *Diabrotica virgifera* (western corn rootworm) less than $10^{-7}$ M.

I also observed a decrease in the antifeedant response in third instar *T. ni* following prolonged exposure to xanthotoxin, *M. azedarach* and thymol. Decreased antifeedant responses following prolonged exposure to a single feeding deterrent have also been reported for other phytophagous insects (Jermy et al., 1982; Szentesi and Bernays, 1984; Bomford and Isman, 1996; Usher et al., 1988; Glendinning et al., 2001 a and b; Glendinning and Gonzalez, 1995). Interestingly, third instar *T. ni* failed to show a decrease in the antifeedant response following prolonged exposure to the cardenolides, digitoxin or cymarin. This finding corroborates earlier results, that larvae of *P. rapae* reared on
Nasturtium or wheat germ diet have different feeding responses to most of the compounds tested, when compared with cabbage-reared larvae, but consistently retained their initial deterrence to digitoxin (Sachdev-Gupta et al., 1993b; Huang and Renwick, 1995). Since a decrease in antifeedant response following prolonged exposure occurred with all the other compounds tested, it is assumed that digitoxin perception differed from that of other compounds tested (Huang and Renwick, 1995), and the same may be true for both digitoxin and cymarin in T. ni. It has been shown that Manduca sexta larvae showed a decrease in response to salicin but not to aristocholic acid after two days of exposure to a diet containing salicin or aristocholic acid (Glendinning et al., 2001a,b). In this case failure to show a decreased response to aristocholic acid was attributed to its toxicity to the insect.

Decreased feeding deterrent response following prolonged exposure may allow an increased diet breadth in generalist insects in situations when a larva falls off its food plant, or completely consumes its host and has to find something else and when the eggs are laid on a non-host (oviposition mistakes). However, future testing in the field is needed to confirm this.

3.4.2 Dishabituation

My results indicated that exposure of naive third instar T. ni to aversive stimuli (CO₂, cold or xanthotoxin) did not affect their normal feeding behaviour and that they consumed the same amount of control leaf discs (MeOH) as the group that received no aversive stimulus. Groups with and without exposure to
Feeding responses of a generalist

aversive stimuli were equally deterred by *M. volkensii* in leaf disc choice bioassays.

When the larvae reared on *M. volkensii* were given the aversive stimuli, there was a change in feeding behavior. With no exposure to aversive stimuli the feeding deterrence indices for third instar *T. ni* larvae differed significantly from the naïve group (reared on MeOH with no exposure to aversive stimuli). However, the feeding deterrence indices of larvae exposed to xanthotoxin differed significantly from the *M. volkensii*-experienced group with no exposure to aversive stimuli, but, not from the naïve group. There was a significant difference in the amount of leaf areas of the control (0.48 cm\(^2\) ± 0.04) and treated discs (0.14 cm\(^2\) ± 0.08) consumed by the naïve group (reared on MeOH with no exposure to aversive stimuli) and the *M. volkensii*-experienced group with exposure to xanthotoxin (Control = 0.45 cm\(^2\) ± 0.04; treated = 0.17 cm\(^2\) ± 0.04) according to a Tukey's test (p < 0.05) unlike the other groups. Thus xanthotoxin exposure restored the feeding deterrent response to *M. volkensii*. Since the deterrent response was restored, it suggests that the decrement in antifeedant response was due to habituation and that xanthotoxin produced dishabituation. Some dishabituation was also shown by the groups exposed to cold and CO\(_2\), but the response did not return to baseline (group reared on MeOH-treated cabbage leaves, with no exposure to aversive stimulus) showing that they were only marginally effective. Longer exposures to CO\(_2\) or cold might have produced significant changes in the feeding behavior of *T. ni*. Xanthotoxin is known to be a strong feeding deterrent to this insect and a very high concentration was
deployed (10 x the DC$_{50}$ value). It is important to note that the total amount of leaf area consumed (control + treated) did not differ between the group exposed to xanthotoxin and the naïve group with no exposure. This rules out the possibility that xanthotoxin had any deleterious effect on T. ni larvae which could have resulted in their abnormal behavior. The result of the dishabituation experiment clearly indicates that the decrease in response following prolonged exposure to M. volkensii can be explained on the basis of habituation.

Although I have observed decreases in response to other antifeedants in this study, I cannot say with confidence that these are the result of habituation because I have yet to demonstrate dishabituation. Further testing of each of the antifeedants using aversive stimuli is necessary before such conclusions can be drawn. Caution should be taken in using the term habituation without demonstrating dishabituation.

To my knowledge this is the first report of dishabituation of feeding deterrence in a generalist herbivore insect. Moreover, there are no previous reports of any method used to dishabituate reduced antifeedant responses in insects.

Decrease in feeding deterrent response might enable the insect to feed normally on plant species that belong to the potential host plant spectrum but are in some degree deterrent to naïve inexperienced individuals (Szentesi and Jermy, 1989). This could be of adaptive value where there is not a strong correlation between deterrence and toxicity of plant phytochemicals (Jermy et al.,
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1982; Jermy, 1987; and Bernays and Chapman, 1994). Unfortunately, a decrease in response to antifeedants following prolonged exposure could have many disadvantages from the pest management point of view. My experiments have clearly indicated that continuous contact of a feeding T. ni larva with a deterrent-containing food caused increased acceptance of that food over time, thereby decreasing efficacy of the deterrent.

My findings that this decrease in the antifeedant response can be explained by habituation may offer some solutions for pest management.

Decreased deterrence resulting from habituation has different implications for pest management than does decreased deterrence resulting from increased tolerance to toxic substances. Compounds to which insects have become habituated can be made effective deterrents again through the process of dishabituation. Future tests should be designed to test the efficacy of this approach.

My experiments have looked at the behavioural effects of deterrent substances on insects. Physiological effects (postingestional effects) have not been taken into account which could prove to be even more important and potentially useful (Isman, 1994). It was shown by Szentesi and Bernays (1984) that repeated exposure of S. gregaria to nicotine hydrogen tartarate resulted in its conversion to the less toxic cotinine, owing to induction of a mixed function oxidase system in the midgut epithelium. In the case of the black swallowtail, Papilio polyxenes, metabolism of the furanocoumarin, xanthotoxin, is
accomplished by the cytochrome P\textsubscript{450} monooxygenase system (Ivie et al., 1983; Bull et al., 1984).

Repeated exposure of an insect to toxic substances may lead to a build-up of a number of mechanisms, which will serve as barriers to toxicity and possibly result in tolerance to other compounds that are normally toxic. Therefore, decreasing response to antifeedants following prolonged exposure as demonstrated in my experiments, combined with the potential detoxicative capacity of insects, suggests that they are capable of exploiting food resources far beyond their normal host plant range. The question then arises, how prevalent is this in nature? Does it lead to rapid host shifts in insects? Partly because of the idiosyncratic taxonomic distribution of most phytochemicals, a diverse diet of plants virtually guarantees ingestion of a diverse array of phytochemicals, with minimal intake of any single class (Berenbaum, 1999). Circumstances such as breakdown of host population, accidental fall from the host plant and oviposition mistakes would permit broadening of diet (Bernays and Weiss, 1996).

The fact that insects are observed to suffer from ingested allelochemicals, fail to perceive conspecific pheromones under certain conditions, and make ovipositional "mistakes" is evidence that there are real limitations on insects for the utilization of chemicals as ecological mediators (Isman, 1992). If we understand those limitations, perhaps, we will be able to manipulate their behaviour to our interests.
CHAPTER 4

FEEDING RESPONSES OF SPECIALIST HERBIVORES TO PLANT EXTRACTS AND PURE ALLELOCHEMICALS: EFFECTS OF PROLONGED EXPOSURE
4.1 Introduction

A number of studies have indicated that previous experience with a food containing an antifeedant may increase the acceptance of that food during subsequent exposure (Schoonhoven, 1969; Gill, 1972; Jermy et al., 1986). Examples include decreasing feeding deterrent response to nicotine hydrogen tartarate in nymphs of *Schistocerca gregaria*, azadirachtin in *Spodoptera* spp. (Simmonds and Blaney, 1984; Bomford and Isman, 1996), salicin and nicotine in *Manduca sexta* (Glendinning et al., 2001) and aristocholic acid in *S. frugiperda* (Raffa and Frazier, 1988). Declining response to feeding deterrents may result from habituation, sensory adaptation or motor fatigue. Habituation is described as the waning of response to a repeatedly presented stimulus over time (Carew and Sahley, 1986), which is not due to sensory adaptation or motor fatigue. Different mechanisms may be responsible for the waning of response. It may result from the effect of mouthpart chemosensory information on the central nervous system, during palpation and feeding (Szentesi and Bernays, 1984) or from effects which follow ingestion of the deterrent (e.g. induction of a detoxifying enzyme; Szentesi and Bernays 1984; Bernays and Chapman, 2000). It may involve persistent synaptic changes in specific neural pathways (Bernays and Chapman, 1994; Szentesi and Jermy, 1989).

Habituation occurs more rapidly when an antifeedant is weak (Jermy et al., 1982; Szentesi and Bernays, 1984) and when the antifeedant is presented as a pure compound rather than a mixture of compounds (Jermy, 1987; Bomford and Isman, 1996).
Habituation can be an important phenomenon, biologically and economically. If the goal is to use antifeedants for crop protection, habituation could result in loss of efficacy of the deterrent against insect pests (Bernays, 1983). This phenomenon may also be important in understanding the food-selection strategies of herbivorous insects. Investigations to this point suggest that polyphagous insects are more likely to show a decrease in feeding deterrent response to antifeedants following prolonged exposure than oligophagous ones (Jermy, 1983; Bernays and Chapman, 1994; Bernays et al., 2000). This may be related to the fact that polyphagous insects are endowed with the ability to detoxify many different plant secondary compounds (Bernays and Chapman, 2000b).

In Chapter 3, I investigated the decreased feeding deterrent response of a generalist herbivore (*Trichoplusia ni*) to antifeedants following prolonged exposure in leaf disc choice tests. The experiments in the present study are designed to look at the decreased feeding deterrent responses of some specialist herbivores, viz. the lepidopterans *Pseudaletia unipuncta* and *Plutella xylostella* and the coleopteran *Epilachna varivestis*, to antifeedants following prolonged exposure based on choice and no-choice tests. I define specialists as those insect herbivores whose host range is limited to one, or at best, two to three plant families.

To my knowledge, there are no published reports of effects of experience with antifeedants on the feeding behaviour of the Coleoptera, or adult insects (e.g., Prokopy and Lewis, 1993) except for one reporting the effects of
Azadirachtin on a polyphagous beetle, *Popillia japonica* (Held et al., 2001). However, antifeedant effects of azadirachtin against *P. japonica* had been previously reported (Ladd et al., 1978). The present study is intended to look at the effect of experience with antifeedants on the feeding behaviour of a coleopteran and two lepidopterans.
4.2 MATERIALS AND METHODS

4.2.1 Plant material

Cabbage (*Brassica oleracea* var. Stonehead), corn (*Zea sacharata* or *Z. rugosa*) (var. Hybrid Sweet Corn) and broad bean plants (*Phaseolus vulgaris*) (var. Mirado) were routinely grown for bioassays in plastic pots as described previously (Chapter 2).

4.2.2 Chemicals

A refined seed extract of *M. volkensii*, oil of oregano, *M. azedarach* (containing 60-75% toosendanin) and thymol were obtained from sources, listed in Chapter 2.

4.2.3 Test insects

*Pseudaletia unipuncta*, *P. xylostella* and *E. varivestis* were obtained from established laboratory colonies as described previously (Chapter 2).

4.2.4 General procedure

Previous experiments looked at the feeding responses of experienced and naïve groups of a generalist herbivore (*T. ni*) using leaf disc choice tests alone (Chapter 3). The present study looks at feeding responses of experienced and naïve specialist herbivores using both choice and no-choice tests (fig. 4.0).
4.2.5 Training

Experienced larvae were reared on a test substance (plant extract or pure allelochemical) sprayed on the host plant and naïve larvae were reared on plants sprayed with MeOH (the carrier solvent) alone (described in detail in Chapter 3) until tested (as the third instar).

4.2.6 Testing

After the training period, experienced and naïve third instars were tested in leaf disc bioassays to determine their feeding deterrent responses to the same plant extract or pure allelochemical they were exposed to during training. The choice assay was conducted as previously described (Chapter 1, Fig. 4.0).

The no-choice leaf disc assay (Fig. 4.0) was carried out in exactly the same manner except that the larvae were provided with only one leaf disc to eat after starvation for four hours. For one set of larvae, the discs were treated with the carrier solvent (MeOH) alone. The other set of larvae was given leaf discs treated with the test substance dissolved in MeOH. Each leaf disc was placed into a randomly assigned assay tray as previously described (Chapter 2). The assay was run for 3-4 hours (until approximately 50% of the control leaf discs were consumed). The leaf area eaten of the controls and treated leaf discs was calculated and used in the equation to calculate a mean feeding deterrence index (%) for each group, $\text{FDI} = 100 \{((C-T) / (C+T))\}$, C is the mean leaf area consumed of the controls and T is the leaf area consumed of the treated leaf disc.
Fig. 4.0 Leaf disc choice and no-choice tests
4.2.7 Feeding responses to plant extract in choice and no choice tests

This experiment was designed to see if the feeding responses of two specialist herbivores to an antifeedant following prolonged exposure were similar to a generalist herbivore as demonstrated in Chapter 2 using the leaf disc choice test. Secondly, I wanted to know if there was a change in feeding response in a no-choice situation. *M. volkensii* was selected as the test substance as it turned out to be the most potent antifeedant (plant extract) for the test species based on previous screening experiments (Chapter 2).

4.2.7.1 Feeding response of *P. unipuncta* following prolonged exposure to *M. volkensii* in and no-choice tests.

Neonate larvae of *P. unipuncta* were reared on corn sprayed with *M. volkensii* (0.01 mg/ml) until tested (n = 47/group).

4.2.7.2 Feeding response of *P. xylostella* following prolonged exposure to *M. volkensii*.

Neonate larvae of *P. xylostella* were reared on cabbage sprayed with *M. volkensii* (0.01 mg/ml) until tested (n = 27/group for both choice and no choice tests).

Both experienced and naïve groups in the above experiments (4.2.7.1 and 4.2.7.2) were tested with *M. volkensii* (15 ug/cm²) in leaf disc choice and no-choice tests.
4.2.8 Feeding responses to plant extract and pure allelochemicals in choice tests

In previous experiments (4.2.7.1 and 4.2.7.2) feeding responses of larvae were measured with previous exposure to *M. volkensii*, a potent antifeedant. The aim of these experiments was to measure feeding responses to a less potent antifeedant, oregano (plant extract), based on the DC$_{50}$ values (concentrations causing 50% reduction in feeding compared to controls in the leaf disc choice test) described in Chapter 2. This was in accordance with the hypothesis proposed earlier that decrease in antifeedant response occurs more rapidly to weaker stimuli (Jermy et al., 1982; Jermy, 1987; Szentesi and Bernays, 1984). Use of pure allelochemical such as thymol, was based on Jermy’s hypothesis (1986), that antifeedants lose efficacy faster when presented as a pure compound than as a mixture.

4.2.8.1. Feeding responses of *P. unipuncta* following prolonged exposure to oregano and thymol.

Neonate larvae of *P. unipuncta* were reared on corn sprayed with oregano (2 mg/ml) (n = 25/group) or thymol (1 mg/ml) (n = 25/group) until tested.

Both experienced and naïve groups were tested with oregano (70 ug/cm$^2$) and thymol (40 ug/cm$^2$), respectively.
4.2.8.2 Feeding responses of P. xylostella following prolonged exposure to oregano, thymol, and M. azedarach.

Neonate larvae of P. xylostella were reared on cabbage sprayed with oregano (2 mg/ml) (n = 28/group), thymol (1 mg/ml) (n = 28/group) or M. azedarach (0.01 mg/ml) (n = 28/group) until tested.

Both experienced and naïve groups were tested with oregano (70 ug/cm²), thymol (40 ug/cm²) and M. azedarach (40 ug/cm²).

4.2.8.3. Feeding responses of adult E. varivestis following prolonged exposure to oregano and thymol.

Newly eclosed first instar larvae of E. varivestis were reared on bean plants sprayed with oregano (2 mg/ml) (n = 47/group) or thymol (1 mg/ml) (n = 47/group) until tested as adults (< 24h after eclosion). The total training period (exposure) lasted for about 8 weeks.

M. volkensii was not used because of its toxic and strong growth inhibiting effects against E. varivestis larvae.

Both experienced and naïve groups were tested with oregano (25 ug/cm²) and thymol (5 ug/cm²).

4.2.9 Data analysis Data were analyzed as described in Chapter 3.
4.3 RESULTS

4.3.1 Feeding response to plant extract in choice and no-choice tests

4.3.1.1 Feeding response of *P. unipuncta* following prolonged exposure to *M. volkensii* in choice and no-choice tests.

A two-way ANOVA (treatment * group) on the feeding deterrence of *P. unipuncta* larvae showed that there was no significant main effect of treatment (choice and no-choice test) ($F_{1,184} = 1.26, p = 0.26$). There was no significant main effect of group (experienced and naïve) ($F_{1,184} = 0.00, p = 0.96$). The interaction (treatment * group) was not significant ($F_{1,184} = 0.85, p < 0.36$). Third instar *P. unipuncta* with previous exposure to *M. volkensii* and the naïve group were equally deterred by *M. volkensii* in both choice and no choice tests (Fig. 4.1).

4.3.1.2 Feeding response of *P. xylostella* following prolonged exposure to *M. volkensii* in choice and no choice tests.

A two-way ANOVA (treatment * group) on the feeding deterrence of *P. xylostella* larvae showed that there was a significant main effect of treatment (choice and no-choice test) ($F_{1,104} = 6.50, p < 0.01$) with larvae showing greater deterrence in the choice test. There was no significant main effect of group (experienced and naïve) ($F_{1,104} = 3.13, p = 0.08$). The interaction (treatment * group) was not significant ($F_{1,104} = 0.00, p < 0.81$). Even though Tukey's test did not show a difference in the deterrence responses of experienced larvae, those in no-choice tests tended to show reduced deterrence, whereas those in choice tests were not deterred (Fig. 4.2).
Fig. 4.1 Feeding response of *P. unipuncta* following prolonged exposure to *M. volkensii* in choice and no choice test (n = 47/group). Feeding deterrence means (± SE) followed by the same letter do not differ significantly (Tukey's test, p < 0.05. Concentration used for testing *M. volkensii* = 15 ug/cm².
Feeding response of P. xylostella following prolonged exposure to *M. volkensii* in choice and no choice tests (n = 27/group). Feeding deterrence means (± SE) followed by the same letter do not differ significantly (Tukey’s test, p < 0.05). Testing concentration for *M. volkensii* = 15 ug/cm².
4.3.2 Feeding responses to plant extract and pure allelochemicals in choice test only.

4.3.2.1 Feeding responses of *P. unipuncta* following prolonged exposure to oregano and thymol in choice tests.

A two-way ANOVA (compound * group) on the feeding deterrence of larvae showed that there was a significant main effect of compound ($F_{1, 96} = 7.90$, $p < 0.05$) with thymol showing more feeding deterrent response than oregano. There was no significant main effect of group (experienced and naïve) ($F_{1, 96} = 0.81$, $p < 0.35$). The interaction (compound * group) was not significant. Third instar *P. unipuncta* larvae did not show a decrease in feeding deterrent response to oregano following prolonged exposure like the naïve group. In contrast, larvae reared on thymol showed a significant decrease in feeding deterrent response to thymol when compared to the naïve larvae (Tukey's test, $p < 0.05$) (Fig. 4.3).

4.3.2.2 Feeding responses of *P. xylostella* following prolonged exposure to oregano, thymol, and *M. azedarach* in choice tests.

A two-way ANOVA (compound * group) on the feeding deterrence of larvae showed that there was a significant main effect of compound ($F_{2, 162} = 29.54$, $p < 0.0001$) with *M. azedarach* showing a greater decrease in feeding deterrent response than thymol or oregano. There was a significant main effect of group (experienced and naïve) ($F_{1, 162} = 26.18$, $p < 0.0001$) with the experienced group showing a greater decrease in feeding deterrent response than the naïve group. The interaction (compound * group) was significant ($F_{1, 162}$
= 10.16, p < 0.0001) with larvae reared on thymol and M. azedarach showing a significantly greater decrease in feeding deterrent response than the naïve groups. Larvae with previous exposure to oregano maintained the feeding deterrent response to oregano and were deterred as much as the naïve larvae (Tukey’s test, p < 0.05) (Fig. 4.4).

4.3.2.3 Feeding responses of adult E. varivestis following prolonged exposure to oregano and thymol in choice tests.

A two-way ANOVA (compound * group) on the feeding deterrence of adult beetles showed that there was a significant main effect of compound (F₁,₁₈₄ = 14.05, p < 0.0002) with thymol showing a greater decrease in feeding deterrent response than oregano. There was a significant main effect of group (experienced and naïve) (F₁,₁₆₂ = 26.18, p < 0.0001) with the experienced group showing a significantly greater decrease in feeding deterrent response than the naïve group. The interaction (compound * group) was significant (F₁,₁₈₄ = 38.26, p < 0.0001) with the experienced beetles reared on oregano and thymol showing a significantly greater decrease in feeding deterrent response to oregano and thymol, respectively than the naïve adults (Tukey’s test, p < 0.05) (Fig. 4.5).
Fig. 4.3 Feeding response of *P. unipuncta* following prolonged exposure to oregano and thymol (n = 25/group) in choice tests. Feeding deterrence means (± SE) followed by the same letters do not differ significantly (Tukey's test, p < 0.05). Testing concentrations for oregano and thymol = 70 and 40 ug/cm², respectively.
Fig. 4.4 Feeding response of _P. xylostella_ following prolonged exposure to oregano (n = 28/group), thymol (n = 28/group), and _M. azedarach_ (n = 28/group) in choice tests. Feeding deterrence means (± SE) followed by the same letters do not differ significantly (Tukey's test, p < 0.05). Testing concentrations for oregano and thymol were the same as _P. unipuncta_; 40 ug/cm² was used for _M. azedarach_.

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Fig. 4.5 Feeding responses of adult E. varivestis following prolonged exposure to oregano and thymol (n = 47/group) in choice test. Feeding deterrence means (± SE) followed by the same letter do not differ significantly (Tukey's test, p < 0.05). Concentrations used for testing with oregano and thymol were 25 ug/cm², and 5 ug/cm², respectively.
4.4 DISCUSSION

The results of the experiments indicate that there were interspecific differences in the feeding deterrent responses of the two lepidopterans and a coleopteran species following prolonged exposure to antifeedants. Neither *P. unipuncta* nor *P. xylostella* showed a significant decrease in feeding deterrent response to *M. volkensii* in either choice or no choice tests following prolonged exposure. However, *P. xylostella* showed a significant decrease in feeding deterrent response to *M. azedarach* following prolonged exposure.

Both species (*P. unipuncta* and *P. xylostella*) showed a significant decrease in feeding deterrent response to a pure allelochemical, thymol. There are reports indicating loss of deterrence to caffeine and nicotine with time in *P. unipuncta* (Usher et al., 1988). A number of studies have indicated a decrease in feeding deterrent response to a single antifeedant following prolonged exposure in phytophagous insects (Bomford and Isman, 1996; Simmonds and Blaney, 1984).

One possible explanation for the lack of decrease in feeding deterrent response to *M. volkensii* in either *P. unipuncta* or *P. xylostella* may be that the plant extract contains numerous compounds that may have synergistic or additive effects. This corroborates an earlier study with neem and its main constituent azadirachtin, showing that *Spodoptera litura* larvae developed a rapid decrease in feeding deterrent response to azadirachtin but not to neem containing a mixture of compounds including azadirachtin (Bomford and Isman,
It also supports Jermy's hypothesis (1987) that mixtures prevent a decrease in feeding deterrent response resulting from repeated exposure to the deterrent.

The results of my experiments have also shown that *E. varivestis* (adults) exhibited a decrease in feeding deterrent response to oregano and thymol. These results do not support Jermy's hypothesis (1987) as a decrease in feeding deterrent response was observed for both the oil of oregano (comprising a mixture of compounds) and the pure allelochemical, thymol. In the case of the Mexican bean beetle, larval training was retained through metamorphosis. The effects of larval training on adults could not be tested with the two lepidopteran species (*P. unipuncta* and *P. xylostella*) as their adults feed on nectar only.

Transfer of information about the larval environment can be carried through to the adult stage by "chemical legacy" (Corbet, 1985). Traces of chemicals from the larval stage in the hemolymph of the insect or on the outside of the pupa might modify the behaviour of the adult. Length of exposure does not seem to be responsible for the observed differences in the decreased feeding deterrent responses of the coleopteran species as both of the lepidopteran species (*P. unipuncta* and *P. xylostella*) exhibited a decreased feeding deterrent response to thymol.

Many studies have shown that even closely related species can exhibit widely different susceptibilities to the same plant extract or pure allelochemical (Isman, 1993). Arnason et al. (1987) have shown that gedunin (a limonoid from Spanish cedar, *Cedrela odorata*, Meliaceae) is not very biologically active against
Peridroma saucia or S. litura, but is toxic to the European corn borer, Ostrinia nubilalis as well as to aphids and earwigs. Although azadirachtin is a potent antifeedant for most phytophagous insects, its potency varies between species. It has outstanding antifeedant properties against Schistocerca gregaria but is not a feeding deterrent for the acridid Melanoplus sanguinipes (Champagne et al., 1989).

The response of the two specialist lepidopteran species tested here differs from that demonstrated by the generalist species (Trichoplusia ni) reported earlier (Chapter 3). The results from Chapter 3 indicated that T. ni, a generalist herbivore, showed a decrease in feeding deterrent response to M. volkensii (habituation) and oregano extracts, following prolonged exposure, in a choice test. Results obtained from the present study show that both P. unipuncta and P. xylostella failed to show a decrease in feeding deterrent response to M. volkensii and oregano following prolonged exposure in choice and no-choice tests. Thus, these results do not support Jermy's hypothesis (Jermy et al., 1982) that a decrease in a feeding deterrent response occurs to weak stimuli (oregano is a weak antifeedant in comparison to M. volkensii) and not to stronger ones.

The lack of significant decrease in feeding deterrent response in the two lepidopteran species (P. xylostella and P. unipuncta) following prolonged exposure to M. volkensii in no-choice tests suggest that in a field situation, it would be unlikely for these insects to accept a new host in the absence of a normal host plant. Field tests will be needed to confirm this with an emphasis on a longer period of behavioural observation for the test insect species than the 4
hour bioassay period in the laboratory. Since there is a decrease (not significant) in the feeding deterrent response of *P. xylostella* following prolonged exposure to *M. volkensii* in no-choice test there is a likelihood that response of the insect towards feeding deterrents might change with the increased starvation time. In contrast, a generalist herbivore (e.g. *T. ni*) may be more likely to accept a new plant outside its normal host range in the absence of a normally acceptable host plant (Chapter 3). Polyphagous insects may be more flexible in host plant selection compared to oligophagous or monophagous insects. Bernays et al. (2000b) reported that taste sensitivity of insect herbivores to deterrents is greater in specialists than in generalists. They found that the specialist noctuid *Heliothis subflexa* was more strongly deterred than the generalist *H. virescens* to a number of plant secondary metabolites tested (caffeine, sinigrin, chlorogenic acid, salicin, linamarin, hordinene and linamarin).

A decrease in feeding deterrent response to thymol but not to either of the plant extracts tested, shown by *P. unipuncta* and *P. xylostella* following prolonged exposure, supports Jermy's hypothesis (Jermy et al., 1982) that insects are more likely to show a decrease in feeding deterrent response to a single antifeedant compound than to a mixture of compounds. This trend was not apparent for *E. varivestis* in the present study. Experienced beetles showed a decrease in feeding deterrent response to oregano (a plant extract) and thymol (a compound) in choice tests. Decrease in feeding deterrent response to azadirachtin (applied to a preferred host plant, *Tilia cordata*) has been reported in
Feeding responses of specialists

a polyphagous beetle (*Popillia japonica*) in no-choice tests (following repeated exposure) and choice tests following prolonged exposure (22 hour) (Held et al., 2001).

The rate and extent of decreased feeding deterrent response following prolonged exposure may also be influenced by the compound itself and the insect species used. Jermy (1993) reported that polyphagous insects should be more inducible than monophagous or oligophagous species. A generalist may already have the enzymatic capability to utilize a new host. Krieger et al. (1971) found higher activity of midgut microsomal oxidase enzymes in polyphagous than in monophagous species of caterpillars.

It may be concluded from the present and previous studies (Chapter 3) that not only are there interspecific differences between generalist and specialist species but also among specialist species, with respect to the plasticity of response to feeding deterrents.
CHAPTER 5

GENERALIZATION OF A FEEDING DETERRENT RESPONSE TO UNRELATED COMPOUNDS IN T. ni LARVAE
5.1 INTRODUCTION

In the previous Chapters I have shown that prolonged feeding experiences can sometimes change feeding preferences of generalist and specialist herbivores. *Trichoplusia ni* larvae showed a decrease in feeding deterrent response to plant extracts (*Melia volkensii, M. azedarach* and *Origanum vulgare*) and pure allelochemicals (thymol and xanthotoxin) following prolonged exposure (Chapter 3). In the case of the extract of *M. volkensii*, decrease in feeding deterrent response was demonstrated to be the result of habituation. A similar decrease in feeding deterrent response was exhibited by the specialist herbivores, *Pseudaletia unipuncta* to thymol and *Plutella xylostella* to thymol and *M. azedarach* (Chapter 4) but not to *M. volkensii*. However, *Epilachna varivestis*, another specialist herbivore, showed a decrease in feeding deterrent response to *M. volkensii* and thymol. This suggests that many phytophagous insects may be capable of changing their feeding preferences based on their feeding experience, the extent to which may vary among different species.

There are other reports of phytophagous insects showing decreased feeding deterrent response to feeding deterrents following repeated exposures. For instance, decreased feeding deterrent response to azadirachtin has been reported for *Spodoptera litura* (Bomford and Isman, 1996), nicotine hydrogen tartrate in *Schistocerca gregaria* (Szentesi and Bernays, 1984), aristocholic acid in *Spodoptera frugiperda*, and to caffeine in *Pseudaletia unipuncta* (Usher et al., 1988). In all of these studies the responses of insects to feeding deterrents were determined after exposing the insect to the same compounds.
This raises the question of whether the same phenomenon occurs with unrelated compounds. This type of experience-based response has been described as “cross-habituation” by Huang and Renwick (1995). Glendinning (1996) and Glendinning et al. (1999, 2001 and 2002) used the term “generalization of a habituated response” for the same phenomenon. Since it is not clear what causes the decrease in feeding deterrent response (for example habituation, sensory adaptation, or motor fatigue) following prolonged exposure, we use the term “generalization of a decreased feeding deterrent response to unrelated compounds” herein.

The main objective of the present Chapter was to assess if decrease in feeding deterrent response to one antifeedant generalizes to another unrelated antifeedant in *T. ni* larvae. This was tested by rearing neonate larvae of *T. ni* on cabbage leaves treated with certain plant extracts or pure allelochemicals to the third instars and then measuring their feeding deterrent responses to unrelated plant extracts or pure allelochemicals in a leaf disc in a leaf disc choice bioassay.

Studies relating to generalization of decreased feeding deterrent response to unrelated compounds are few but are considered very important for understanding insect feeding behaviour in relation to pest management strategies based on insect antifeedants, and in understanding the potential for host-plant shifts and extension of host-plant range in herbivorous insects.
5.2 MATERIALS AND METHODS

5.2.1 Plant Material

Cabbage plants (Brassica oleracea) were routinely grown for bioassays in plastic pots as described previously (Chapter 2).

5.2.2 Chemicals

A refined extract of the seeds of M. volkensii, oil of oregano, thymol, digitoxin, and xanthotoxin were obtained from sources listed in Chapter 2. The chemical structures of the pure allelochemicals are shown in Chapter 1.

5.2.3 Solvents

Methanol (MeOH) or methanol / dichloromethane (2:1, v:v) were used as carriers.

5.2.4 Test Insects

Trichoplusia ni were obtained from an established laboratory colony as described previously (Chapter 2).

5.2.5 General procedure

There was an experienced and a naïve group in each experiment. The experienced group was exposed to the antifeedant from the neonate stage until testing as third instars. The naïve group was reared on cabbage leaves treated with the carrier solvent only (Chapter 3). Feeding responses of experienced and
naive larvae were measured to unrelated plant extracts or pure compounds in leaf disc choice bioassays (Chapter 2).

5.2.6 Reared on a plant extract (M. volkensii or oregano) and tested with both the same and a different extract.

This experiment sought to determine whether a decreased feeding deterrent response following prolonged exposure to a plant extract generalized to an unrelated plant extract. The objective here was to measure the difference in the feeding response of the two experienced groups under similar testing conditions (each tested with the same plant extract and the different plant extract) along with the naïve groups (n = 40/group).

Experienced groups were reared on M. volkensii at 0.01 mg and oregano at 2.5 mg/cabbage leaf until the third instar. Experienced and naïve groups were tested with oregano (80 ug/cm²) and M. volkensii (15 ug/cm²).

5.2.7 Reared on plant extracts (M. volkensii or oregano) and tested with pure allelochemicals (thymol or xanthotoxin).

This experiment sought to determine whether the decreased feeding deterrent response generalized to unrelated pure allelochemicals following prolonged exposure to plant extracts.

The experienced groups were reared on M. volkensii (0.01 mg/cabbage leaf) or oregano (2.5 mg/cabbage leaf) until the third instar. Experienced and
naïve groups were tested with thymol (40 ug/cm²) or xanthotoxin (0.6 ug/cm²) (n = 25/group) in leaf disc choice bioassays.

5.2.8 Reared on pure allelochemicals (digitoxin or thymol) and tested with plant extract (extract of oregano).

This experiment sought to determine whether the decreased feeding deterrent response generalized to an unrelated plant extract following prolonged exposure to pure allelochemicals.

The experienced groups were reared on digitoxin or thymol at 0.01 mg/cabbage leaf and 1.0 mg/cabbage leaf, respectively until the third instar and then tested with oregano (80 ug/cm²) (n = 23/group) in leaf disc choice bioassays.

5.2.9 Reared on a pure allelochemical (digitoxin or xanthotoxin) and tested with another pure allelochemical (thymol).

This experiment sought to determine whether the decreased feeding deterrent response generalized to an unrelated pure allelochemical following prolonged exposure to pure allelochemicals.

The experienced groups were reared on digitoxin or thymol at 0.01 mg/cabbage leaf and 1.0 mg/cabbage leaf, respectively until the third instar and tested with thymol (40 ug/cm²) (n = 25/group) in leaf disc choice bioassays.
5.2.10 Data analysis

Leaf area consumed of the control and the treated leaf discs by the experienced and the naïve groups was calculated using the Scion Image program and mean feeding deterrenacy (%) of each group was calculated and compared as explained in Chapter 2. Data were analysed on the basis of actual numbers observed (variance of the sample means were determined to be homogeneous) by analysis of variance (ANOVA) (Zar, 1984) using statistics software (Statistix7, 2000). Where significant F values were found, Tukey’s HSD test was used to test for significant differences between individual treatments. The alpha level used was 0.05.
5.3 RESULTS

5.3.1 Reared on a plant extract (*M. volkensii* or oregano) and tested with both the same and a different extract.

A two-way ANOVA (compound*group) on the mean feeding deterrence indices of third instar *T. ni* larvae showed that there was no significant main effect of compound ($F_{1,234} = 1.62, p = 0.204$). There was a significant main effect of group ($F_{2,234} = 18.10, p < 0.0001$) with experienced larvae showing a greater decrease in feeding deterrent response than the naïve larvae. There was no significant (compound*group) interaction ($F_{2,234} = 1.39, p = 0.2507$). No significant differences were found between the feeding deterrent responses of larvae reared on *M. volkensii* or oregano and tested with the same and the unrelated extracts, but all differed significantly from their respective naïve groups (Tukey’s test, $p < 0.05$) (Fig. 5.1).

5.3.2 Reared on plant extracts (*M. volkensii* or oregano) and tested with pure allelochemicals (thymol or xanthotoxin).

A two-way ANOVA (compound*group) on the mean feeding deterrence indices of experienced (reared on *M. volkensii* at 0.01 mg and oregano at 2.5 mg/cabbage leaf) larvae to thymol or xanthotoxin, and naïve groups (Fig. 5.2) did not show a significant main effect of compound ($F_{3,192} = 0.37, p = 0.77$).
**Fig. 5.1** Feeding responses of third instar *T. ni* to *M. volkensii* and oregano (represented by bars) following prolonged exposure to *M. volkensii* and oregano. Feeding deterrence means (± SE) followed by the same letter do not differ significantly (Tukey’s test, p < 0.05, n = 40/group).
Fig. 5.2 Feeding responses of experienced (M. volkensii and oregano) and naive third instar T. ni larvae to thymol and xanthotoxin. Mean feeding deterrence indices (represented by bars) (± SE) followed by the same letters do not differ significantly (Tukey's test, p < 0.05, n = 25/group).
There was no significant main effect of group ($F_{1, 192} = 0.39, p = 0.53$) with experienced and naïve larvae showing the same deterrent response. There was no significant main effect of treatment ($F_{3, 192} = 0.65, p = 0.58$) with larvae reared on *M. volkensii* and oregano showing the same deterrent response to thymol and xanthotoxin as the naïve larvae (Fig. 5.2).

5.3.3 Reared on pure allelochemicals (digitoxin or thymol) and tested with a plant extract (extract of oregano).

A one-way ANOVA of the mean feeding deterrence indices of experienced groups (reared on digitoxin and thymol) and the naïve group to oregano did not produce a significant F value ($F_{2, 66} = 0.01, p = 0.99$) (Fig. 5.3). Larvae of *T. ni* did not show a generalization of feeding deterrent response to oregano following prolonged exposure to digitoxin or thymol like the naïve larvae.

5.3.4 Reared on pure allelochemicals (digitoxin or xanthotoxin) and tested with another unrelated pure allelochemical (thymol).

A one-way ANOVA of the mean feeding deterrence indices of experienced groups (reared on digitoxin or xanthotoxin) and naïve group to thymol produced a significant F value ($F_{2, 72} = 4.51, p = 0.01$) (Fig. 5.4).

Comparison of means of feeding deterrence indices of experienced and naïve groups showed that both experienced groups demonstrated a decrease in feeding deterrent response to thymol unlike the naïve group (Tukey's test, $p < 0.05$).
Fig. 5.3 Feeding response of third instar *T. ni* to oregano (represented by bars) following prolonged exposure to digitoxin and thymol. Feeding response of naïve group is shown by solid line (dotted lines represent ± SE). Means (± SE) followed by same letters do not differ significantly (Tukey’s test, *p* < 0.05, *n* = 23/group).
Fig. 5.4 Feeding response of third instar T. ni to thymol (represented by bars) following prolonged exposure to digitoxin and xanthotoxin. Feeding response of naïve group is shown by solid line (dotted lines represent ± SE). Means (± SE) followed by same letters do not differ significantly (Tukey’s test, p < 0.05, n = 25/group).
5.4 DISCUSSION

The results of our experiments indicate that third instar *T. ni* larvae showed a generalization of a decreased feeding deterrent response to antifeedants in some cases. *T. ni* larvae showed a generalization of a decreased feeding deterrent response to a plant extract and a pure allelochemical with previous exposure to an unrelated plant extract and pure allelochemicals respectively.

There was a significant generalization of decreased feeding deterrent response to oregano by larvae with prolonged exposure to *M. volkensii* extract and *vice versa*. No significant differences were found in the feeding deterrent responses of larvae to oregano between the groups following prolonged exposure to *M. volkensii* or oregano. Similarly no significant differences were found in the feeding deterrent responses of larvae to *M. volkensii* following prolonged exposure to either *M. volkensii* or oregano. This generalization of decreased feeding deterrent response to unrelated compounds was not shown by naïve larvae in either case. A similar generalization of decreased feeding deterrent response was also exhibited by *T. ni* larvae to thymol, a pure allelochemical, with previous exposure to the unrelated pure allelochemicals, digitoxin or xanthotoxin unlike the naïve groups.

Generalization of decreased feeding deterrent response to unrelated compounds following prolonged exposure has been reported previously. Huang and Renwick (1995) reported that *Pieris rapae* larvae accepted foliage of...
Tropeolum majus (nasturtium) following exposure to the unrelated feeding deterrents strophanthidin, cymarin, erysimoside, digitoxigenin, cucurbitacin, and rutin. T. majus is not acceptable to P. rapae larvae as a host plant following feeding on cabbage foliage, owing to the presence of chlorogenic acid, a feeding deterrent for P. rapae.

There is a considerable amount of evidence relating a decrease in feeding deterrent response following prolonged exposure, to changes in receptor characteristics, measured by a change in firing characteristics of gustatory sensilla (Blaney et al., 1986). Meijsser (1983) reported that the deterrent receptor in P. rapae showed a somewhat lowered responsiveness to strychinine when reared on cabbage leaves treated with strychinine compared with naïve larvae. Likewise the addition of azadirachtin to the artificial diet of Spodoptera exempta and S. littoralis for a period of two days reduced the sensitivity of deterrent receptors but not the sugar-sensitive receptor (Simmonds and Blaney, 1984). Blaney et al. (1986) reported a decrease in sensitivity of the feeding deterrent receptor in tobacco hornworm, Manduca sexta, larvae exposed to a diet containing salicin for two and a half days. Dietary exposure to salicin not only reduced sensitivity to salicin but simultaneously to caffeine, despite the fact that the latter compound probably acted on a different receptor site on the same cell (Blaney et al., 1986).

A similar mechanism might explain the observed generalization of decreased feeding deterrent response in T. ni to unrelated feeding deterrents following prolonged exposure. However, some central phenomena may also be
involved in this process, apart from changes in receptor characteristics (R.F. Chapman, personal communication).

In my experiments, the generalization of decreased feeding deterrent response observed when larvae were reared on a plant extract and tested with another plant extract or reared on a pure allelochemical and tested with another pure allelochemical, might be explained on the basis that a common transduction pathway is shared by the plant extracts or pure allelochemicals (Glendinning et al., 1999) within the same bittersensitive taste cells. It could be that they elicit excitatory responses that are virtually identical in terms of maximal firing rate and temporal pattern of firing resulting in lack of discrimination by the CNS as was the case with salicin and caffeine in *M. sexta* (Glendinning et al, 1999; 2002). Electrophysiological studies would be needed to confirm that.

My experiments did not show a generalization of a decreased feeding deterrent response when *T. ni* larvae were reared on a plant extract (oregano) and tested with pure allelochemicals (thymol or xanthotoxin) or reared on pure allelochemicals (digitoxin or thymol) and tested with a plant extract (oregano). A possible explanation for the lack of generalization in these instances may be that they do not share common signalling pathways. If pure allelochemicals and plant extracts are sensed by different populations of bitter-sensitive taste cells, they would produce a distinct spatial pattern of activation within the primary projection site of the bitter-sensitive taste cells, the suboesophageal ganglion (SOG). If decreased feeding deterrent response is assumed to be restricted to loci in the SOG that receive input from taste cells responsive to pure allelochemicals, then it
would not be expected to generalize to loci in the SOG that receive input from
taste cells responsive to plant extract (mixtures) or *vice versa* (Glendinning et al., 2002).

Glendinning (1996) and Glendinning et al. (2001, 2002) reported that *M. sexta* larvae showed a decrease in feeding deterrent response to caffeine and salicin after 24hr or 48hr of dietary exposure. This phenomenon of decreased feeding deterrent response was generalized to salicin with previous exposure to caffeine or *vice versa*, but not to aristocholic acid, *Grindelia* extract and *Canna* extract, as indicated by the electrophysiological recording of bitter-sensitive taste cells. An explanation for the limited generalization of decreased feeding deterrent response is that salicin and caffeine stimulate a common signalling pathway within the same taste cells (Glendinning et al., 1999), identified by the excitatory responses that are identical in the maximal firing rate and temporal patterns of firing not seen with aristocholic acid. *Manduca sexta* may not discriminate between caffeine and salicin because they share a common transduction pathway within the same taste cell (Glendinning et al., 2002). Although salicin and aristolochic acid stimulate the same bitter-sensitive taste cells, they do not share the same transduction pathways (Glendinning et al., 1999, 2001, 2002).

Huang and Renwick (1995) have shown that *P. rapae* larvae reared on *T. majus* or wheat germ diet, when compared with cabbage-reared larvae, showed a decrease in feeding deterrent response or no deterrence at all (in the case of wheat germ) to most of the feeding deterents tested (viz. the cardenolides digitoxin, cymarin and erysymoside) (Huang and Renwick, 1995). One
explanation for this behaviour might be that *T. majus* is a marginal host plant for *P. rapae* and any previous exposure to unrelated plant chemicals may affect its acceptance. It has been shown that both *T. majus* and wheat germ diet contain feeding deterrents, the exposure to which might have caused changes in receptor characteristics resulting in the generalization of habituated response to unrelated feeding deterrents (Huang and Renwick, 1995). However, my findings failed to show a generalization of the habituated response to a plant extract with previous exposure to a pure allelochemical. An explanation for the lack of response in this instance may be use of different species.

To my knowledge this is the first report of generalization of a decreased feeding deterrent response to a plant extract following prolonged exposure to an unrelated plant extract. The importance of such a research should be realized by considering the fact that during normal feeding the taste receptors of an insect are exposed to complex mixtures of chemicals, and not the single compound (Bernays and Chapman, 2001).
5.5 CONCLUSION

In conclusion my experiments have shown that *T. ni* larvae are capable of showing a generalization of the decreased feeding deterrent response to unrelated feeding deterrents in some situations only. This property of insects, although limited, can have serious consequences for the design of pest management tactics based on feeding deterrents, as prolonged exposure to one feeding deterrent could lead to tolerance of other unrelated feeding deterrents. Decreased feeding deterrent response to related or unrelated feeding deterrents following prolonged exposure could limit the practical application of such natural compounds for pest control.

Additionally, information obtained from the present and previous studies (Chapters 3 and 4) on the feeding behaviour in larvae of *T. ni* (a generalist) and some specialist herbivores (*Plutella xylostella, Pseudaletia unipuncta*, and *Epilachna varivestis*) suggests that these insects are likely capable of exploiting food resources far beyond their normal host plant range.

That decreased feeding deterrent response to one deterrent compound can generalize to others may have important ecological implications for a polyphagous insect such as *T. ni*. It suggests that a decreased feeding deterrent response to one deterrent compound may facilitate consumption of novel plants containing different deterrent compounds. This phenomenon could be adaptive if the novel deterrent compound occurs at a non-toxic concentration. However, if it occurs at a toxic concentration, then the phenomenon of decreased feeding
deterrent response may prove to be a double-edged sword for insects, particularly in light of the fact that ingestion of some allelochemicals causes toxicity or death after a single meal (e.g. Glendinning and Slansky, 1994). Future studies could evaluate this possibility by including more toxic compounds in the generalization assays.
CHAPTER 6

MITIGATING DECREASED FEEDING DETERRENT RESPONSE TO ANTIFEEDANTS FOLLOWING PROLONGED EXPOSURE IN CABBAGE LOOPER, Trichoplusia ni (LEPIDOPTERA: NOCTUIDAE) BY BINARY MIXTURES OF FEEDING DETERRENTS.
6.1 INTRODUCTION

For the past few decades, research on pest control approaches that are ecologically rational, yet effective, has received high priority worldwide. Innovative developments include the use of natural products such as botanical insecticides and antifeedants to kill or regulate growth and behaviour of insects.

There are a number of examples of phytophagous insects showing a decrease in feeding deterrent response following repeated or continuous exposure (Jermy et al., 1982; Jermy, 1987; Szentesi and Bernays, 1984; Usher et al., 1988). A more detailed description is given in Chapter 3. Modification of the behavioural response in an insect to an initially deterrent compound as a result of repeated contact could result in reduced feeding deterrence and gradually increasing consumption. This could lead to decreased efficacy of the deterrent against insect pests (Bernays, 1983).

Decreasing response to feeding deterents following prolonged exposure occurs most readily when a single antifeedant provides a weak inhibitory stimulus (Jermy et al., 1982; Szentesi & Bernays, 1984). When a complex mixture of substances inhibits feeding, however, a decrease in feeding deterrent response may not occur (Jermy, 1987; Bomford and Isman, 1996).

In the context of host recognition, insects that respond behaviourally and physiologically to "sign" or "token" stimuli (as do many crucifer-feeders to the presence of sinigrin in Brassicaceae, such as Plutella xylostella [Bernays and Chapman, 1994] and Pieris brassicae [Schonhoven, 1967]) exist but have
proved rare (Metcalf et al., 1982). The majority of insects studied instead appear to display a “response spectrum” whereby host acceptability depends not on the presence or absence of a single stimulant or deterrent but upon the total sensory impression derived from an integrated response to multiple plant components (Dethier, 1973). Examples include that of the swallowtail butterfly, *Papilio polyxenes*, and the carrot fly, *Psila rosae* (Bernays and Chapman, 1994) using several compounds to identify their respective hosts (carrot as well as other species of Apiaceae). When presented singly, chlorogenic acid or a flavonoid did not elicit oviposition in *P. polyxenes*; both must be present for egg laying to occur. Similarly in *P. rosae*, a polyacetylene, falcarindiol, was less effective than a fraction from the plant surface (carrot) for inducing oviposition. Both of these examples suggest the importance of mixtures in the selection of host plant by phytophagous insects. During normal feeding, the taste receptors of an insect are exposed to complex mixtures of chemicals, and not the single compound (Bernays and Chapman, 2001).

McKey (1979) argued that selection favors several lines of defense in plants for several reasons. Among these is the fact that chemicals may interact additively or synergistically, thereby increasing the overall effect of a mixture (antifeedant, growth inhibitory or toxic effects). There is reason to believe that combinations of chemicals can be expected to greatly delay the acquisition of resistance in phytophagous insects (Feng et al., 1995; Feeny, 1983).

It has been shown previously that *Trichoplusia ni* (Chapter 3), and *Epilachna varivestis* (Chapter 4) exhibited a decrease in feeding deterrent
response to most of the antifeedants tested (*Melia volkensii*, oregano, *M. azedarach*, thymol, and xanthotoxin in the case of *T. ni* and oregano and thymol in *E. varivestis*). *Pseudoletia unipuncta* and *Plutella xylostella* (Chapter 4) showed a decrease in feeding deterrent response to thymol, a pure allelochemical and also to *M. azedarach* in the case of *P. xylostella*. It was also shown that there was a generalization of feeding deterrent response to unrelated feeding deterrents in *T. ni* (reared on a plant extract and tested with another unrelated plant extract or reared on a pure allelochemical and tested with another pure allelochemical) (Chapter 5).

The experiments contained within this Chapter are designed to investigate the potential for mitigating a decrease in feeding deterrent response following prolonged exposure to antifeedants by using binary mixtures of plant extracts or pure allelochemicals, and to determine the effect of interaction of single antifeedants in a binary mixture.

6.2 MATERIALS AND METHODS

6.2.1 Plant Material

Cabbage plants (*Brassica oleracea*) were routinely grown for bioassays in plastic pots as described previously (Chapter 2).
6.2.2 Chemicals

A refined extract of the seeds of *M. volkensii*, oil of oregano, toosendanin (95%), thymol, digitoxin, and xanthotoxin were obtained from sources listed in Chapter 2.

6.2.3 Solvents

Methanol (MeOH) or Methanol / dichloromethane (2:1, v:v) were used as carriers.

6.2.4 Test insects

*Trichoplusia ni* larvae were obtained from an established laboratory colony as described previously (Chapter 2).

6.2.5 General Procedure

In each experiment, there were experienced and naïve larvae. The experienced groups were reared on cabbage foliage treated with individual or binary mixtures of plant extracts (*M. volkensii* and oregano) or pure allelochemicals.

Concentrations used for rearing were determined in preliminary experiments to ensure that they did not cause any growth inhibition or phytotoxic effects. Plant extracts (*M. volkensii*, 0.01 mg and oregano 2.5 mg) or pure allelochemicals (thymol 1.0mg, and digitoxin or xanthotoxin 0.01 mg) were dissolved in one ml of the carrier solvent. Binary mixtures were made by mixing two plant extracts or pure allelochemicals in a ratio of 1:1; v:v. One ml of the
resulting solutions (individual or binary mixture) was applied to a cabbage leaf (100-110 cm²) by means of a micropipette with 0.5 ml on each side (Chapter 2). Neonate larvae (<24 hours old) were transferred to the cabbage leaves, and allowed to feed *ad libitum* until bioassays were conducted. The naïve groups were reared on cabbage foliage treated with carrier solvent under the same conditions as experienced larvae (method of rearing is described in detail in Chapter 3) until tested.

Third instar *T. ni* from the experienced and naïve groups were then tested for their feeding responses to individual or binary mixtures of feeding deterrents they were exposed to as neonates, in leaf disc choice bioassays (Chapter 2). Concentrations used for testing each plant extract or pure allelochemical were determined in initial screening experiments described in Chapter 2. Concentrations used for *M. volkensii*, oregano and xanthotoxin were 7.5, 40, 0.3 μg/cm², respectively and 20 μg/cm² for thymol, toosendanin, and digitoxin. Binary mixtures were made by mixing two plant extracts or pure allelochemicals in a ratio of 1:1 (v:v). A 20 μl of solution from each mixture was then applied to a leaf disc (1.77 cm²) with 10 μl on each side of the leaf disc.

Areas of the control and treated leaf discs eaten by the insects in leaf disc choice bioassays were measured using Scion Image software. A feeding deterrence index was calculated (Isman et al., 1990) for each group as explained in Chapter 2.
6.2.6 Experiment 1. Feeding response of *T. ni* larvae following prolonged exposure to binary mixtures of plant extracts (*M. volkensii* and oregano).

Previous studies (Chapters 3 and 4) indicated that *T. ni* larvae (n = 30/group) showed a decrease in feeding deterrent response to the plant extracts, *M. volkensii* and oregano, and *E. varivestis* to oregano only. It was also shown that *T. ni* exhibited a generalization of decreased feeding deterrent response to unrelated feeding deterents (Chapter 5). The present study looks at the potential for mitigating the decreased feeding deterrent response by a binary mixture of plant extracts.

6.2.6.1 Training

   Experienced groups were reared on cabbage leaves treated with *M. volkensii*, oregano, or a binary mixture of *M. volkensii* and oregano (described in section 6.2.5) until tested.

   Naïve groups were reared on cabbage leaves treated with MeOH only.

6.2.6.2 Testing

   Feeding responses of experienced and naïve groups were measured in leaf disc choice tests as explained before (section 6.2.5).

6.2.7 Experiment 2. Feeding response of *T. ni* larvae following prolonged exposure to binary mixtures of pure allelochemicals: thymol digitoxin and xanthotoxin.
Previous studies (Chapters 3 and 4) indicated that all test insect species ($T. \text{ni}$, $P. \text{unipuncta}$, $P. \text{xylostella}$ and $E. \text{varivestis}$) showed a decrease in feeding deterrent response to individual allelochemicals ($T. \text{ni}$ to thymol and xanthotoxin; $P. \text{unipuncta}$, $E. \text{varivestis}$ and $P. \text{xylostella}$ to thymol) following prolonged exposure. The present study looks at the potential for mitigating the decrease in feeding deterrent response by binary mixtures of pure allelochemicals.

6.2.7.1 Training

Experienced groups were reared on cabbage leaves treated with thymol, digitoxin, or xanthotoxin and binary mixtures (thymol + digitoxin, thymol + xanthotoxin, or digitoxin + xanthotoxin). Naïve groups were reared on cabbage leaves treated with the carrier solvent only (MeOH and dichloro methane [2:1, v:v]) (n = 40/group).

6.2.7.2 Testing

Testing was done as described before (section 6.2.5).

6.2.8 Experiment 3. Feeding response of $T. \text{ni}$ larvae following prolonged exposure to binary mixtures of pure allelochemicals: thymol, toosendanin and xanthotoxin.

In this experiment, digitoxin was replaced by toosendanin to ensure that decrease in feeding deterrent response was the result of interaction due to a binary mixture and not because of digitoxin alone ($T. \text{ni}$ showed a decrease in
feeding deterrent response following prolonged exposure to all pure allelochemicals tested except for digitoxin as shown in Chapter 3).

6.2.8.1 Training

Experienced groups were the same as in experiment 2, except that digitoxin was replaced by toosendanin (n = 52/group).

Naïve groups were also the same as in experiment 2.

6.2.8.2 Testing

Testing was done as described before (section 6.2.5).

6.2.9 Equation 1: Calculation of expected deterrences from binary mixtures of known dosages.

In order to find out the interaction of individual components in a mixture, expected deterrences of binary mixtures were calculated by the following formula:

\[ E = Oa + Ob(1-Oa) \]

where \( E \) is expected deterrency. \( Oa \) and \( Ob \) are observed deterrences of individual plant extract or pure allelochemicals. This equation is modified from the equation used for calculation of expected mortalities from binary mixtures of known dosages [Trisyono and Whalon, 1999]).

The effects of mixtures were designated as antagonistic, additive or synergistic by analysis using \( X^2 \) comparisons.
6.2.10 Equation 2: Calculation of values for determining additive, synergistic, or antagonistic interaction from binary mixtures (Trisyono and Whalon, 1999).

\[ X^2 = \frac{(O_d - E)^2}{E} \]

where \( O_d \) is observed deterreny from binary mixtures and \( E \) is expected deterreny

\[ X^2 \text{ with } df = 1 \text{ and alpha level } 0.05 \text{ is } 3.84 \]

A pair with \( X^2 \) values greater than 3.84 was determined as synergistic or antagonistic (depending upon the direction), with \( X^2 \) values less than 3.84 representing additive effects.

6.2.11 Data analysis

Leaf area consumed of the control and the treated leaf discs by the experienced and the naïve groups was calculated using the Scion Image program and mean feeding deterrence index (%) of each group was calculated (Chapter 2). Data were analyzed on the basis of actual numbers observed (variance of the sample means were determined to be homogeneous) by analysis of variance (ANOVA) (Zar, 1984) using statistics software (Statistix 7, 2000). Where significant F values were found, Tukey's HSD multiple comparison test was used to test for significant differences between individual treatments.
6.3 RESULTS

6.3.1 Experiment 1. Feeding response following prolonged exposure to a binary mixture of plant extracts, *M. volkensii* and oregano

A two-factor (compound * group) ANOVA on the mean feeding deterreny of third instar *T. ni* showed that there was a significant main effect of compound ($F_{2, 174} = 13.46$, $p < 0.0001$) with both *M. volkensii* and oregano showing less feeding deterrent response than the binary mixture of them. There was a significant main effect of group ($F_{1, 174} = 14.72$, $p < 0.0001$) with experienced larvae showing a greater decrease in feeding deterrent response than the naïve larvae. There was a significant compound * group interaction ($F_{2, 174} = 3.18$, $p < 0.04$) with experienced larvae reared on *M. volkensii* or oregano showing a significantly greater decrease in feeding deterrent response than the naïve larvae or the larvae reared on a binary mixture of *M. volkensii* and oregano (Tukeys’ test, $p < 0.05$) (Fig.6.1).

6.3.2 Experiment 2. Feeding response following prolonged exposure to binary mixtures of pure allelochemicals, thymol, digitoxin and xanthotoxin.

A two-factor ANOVA (compound * group) on the mean feeding deterreny of third instar *T. ni* showed that there was a significant main effect of compound ($F_{5, 612} = 7.57$, $p < 0.0001$) with xanthotoxin and thymol showing less feeding deterrent response (lower feeding deterrence index) than digitoxin or binary mixtures of the compounds. There was a significant main effect of group, ($F_{1, 612} = 18.47$, $p < 0.0001$) with experienced larvae showing significantly less
Fig. 6.1 Feeding responses of third instar *T. ni* to individual and binary mixtures of plant extracts with previous exposure to them as individually or as a binary mixture (*n* = 30/group). Mean feeding deterrence indices (± SE) followed by asterisks indicate significant differences between experienced and naïve groups (Tukey’s test, *p* < 0.05).
deterrent response than the naïve larvae. There was no significant (compound * 
group) interaction ($F_{5,612} = 1.99$, $p = 0.07$) with no differences in feeding deterrent 
responses between experienced (reared on digitoxin or binary mixtures) and 
 naïve larvae (Tukeys’ test, $p < 0.05$) (Fig. 6.2).

6.3.3 Experiment 3. Feeding response following prolonged exposure to 
 binary mixtures of pure allelochemicals, thymol, toosendanin and 
 xanthotoxin.

A two-factor (compound * group) ANOVA on the mean feeding deterrence 
indices of third instar $T. ni$ showed that there was a significant main effect of 
compound ($F_{5,468} = 9.61$, $p < 0.001$) with individual compounds (thymol 
toosendanin or xanthotoxin) showing less feeding deterrent response than binary 
mixtures of these. There was a significant main effect of group ($F_{1,468} = 37.32$, $p$ 
$< 0.0001$) with experienced larvae showing a greater decrease in feeding 
deterrent response than the naïve larvae. There was no significant compound * 
group interaction (Fig. 6.3) with no differences in feeding deterrent responses 
between experienced (reared of binary mixtures) and naïve larvae (Tukeys’ test, 
$p < 0.05$).
Fig. 6.2 Feeding responses of third instar *T. ni* to individual and binary mixtures of pure allelochemicals, thymol, digitoxin and xanthotoxin with previous exposure to them as individual or binary mixtures (*n* = 40/group). Mean feeding deterrence indices (± SE) followed by asterisks indicate significant differences between experienced and naïve groups (Tukeys’ test test, *p* < 0.05).
Feeding responses to mixtures

Fig. 6.3 Feeding responses of third instar *T. ni* to individual and binary mixtures of pure allelochemicals, thymol, toosendanin and xanthotoxin with previous exposure to them as individual or binary mixtures (*n* = 52/group). Mean feeding deterrence indices (± SE) followed by asterisks indicate significant differences between experienced and naïve groups (Tukey’s test, *p* < 0.05).
6.3.4 Effects of binary mixtures on feeding deterrenacy of naïve and experienced *T. ni* larvae and measures of interactions.

The observed feeding deterrent responses of experienced and naïve larvae with binary mixtures were always greater than expected responses (Tables 6.1 and 6.2).

On the basis of $X^2$ values, interactions were characterized as additive in the case of naïve larvae (Table 6.1) and synergistic for experienced larvae (Table 6.2).
## Table 6.1 Effects of binary mixtures on feeding deterrence of naïve *T. ni* larvae and measures of interactions.\(^1\) experiment, \(^2\) additive.

<table>
<thead>
<tr>
<th>Exp(^1)</th>
<th>Plant Extract / Pure compound</th>
<th>Dosage a+b</th>
<th>Feeding Deterrency %</th>
<th>Plant Extract / Pure compound</th>
<th>Binary Mixtures</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Observed a</td>
<td>Observed b</td>
<td>Observed a+b</td>
<td>Expected a+b</td>
</tr>
<tr>
<td>1</td>
<td>Oregano <em>M. volkensii</em></td>
<td>40+7.5</td>
<td>43.2</td>
<td>22.3</td>
<td>66.0</td>
<td>55.9</td>
</tr>
<tr>
<td>2</td>
<td>Thymol Xanthotoxin</td>
<td>20+0.3</td>
<td>36.9</td>
<td>37.3</td>
<td>66.7</td>
<td>60.5</td>
</tr>
<tr>
<td></td>
<td>Thymol Digitoxin</td>
<td>20+20</td>
<td>36.9</td>
<td>35.4</td>
<td>60.8</td>
<td>59.3</td>
</tr>
<tr>
<td></td>
<td>Xanthotoxin Digitoxin</td>
<td>20+0.3</td>
<td>37.3</td>
<td>35.4</td>
<td>64.0</td>
<td>59.5</td>
</tr>
<tr>
<td>3</td>
<td>Thymol Xanthotoxin</td>
<td>20+0.3</td>
<td>35.5</td>
<td>38.8</td>
<td>61.6</td>
<td>60.5</td>
</tr>
<tr>
<td></td>
<td>Thymol Toosendanin</td>
<td>20+20</td>
<td>35.5</td>
<td>35.8</td>
<td>70.9</td>
<td>58.6</td>
</tr>
<tr>
<td></td>
<td>Xanthotoxin Toosendanin</td>
<td>0.3+20</td>
<td>38.8</td>
<td>35.8</td>
<td>66.3</td>
<td>60.8</td>
</tr>
</tbody>
</table>

## Table 6.2 Effects of binary mixtures on feeding deterrence of experienced *T. ni* larvae and measures of interactions.\(^1\) experiment, \(^2\) synergistic.

E is expected deterrency; Oa and Ob are observed deterrences of individual plant extract or pure allelochemicals. \(X^2 = (O_d - E) / E\) where Od is observed deterrency from binary mixtures and E is expected deterrency.
6.4 DISCUSSION

The results of the experiments have clearly demonstrated that third instar *T. ni* larvae show a decrease in feeding deterrent response following prolonged exposure to plant extracts or pure allelochemicals when presented singly but not to binary mixtures. Digitoxin however, proved to be exception (third instar *T. ni* did not show a decrease in feeding deterrent response to either digitoxin or cymarin as shown in Chapter 3).

There was a decrease in feeding deterrent response to *M. volkensii* or oregano in larvae with previous exposure to *M. volkensii* or oregano, respectively. However, larvae with previous exposure to a binary mixture of these did not show a decrease in feeding deterrent response to the mixture. These results corroborate earlier findings with neem and its main constituent azadirachtin (Bomford and Isman, 1996), showing that *Spodoptera litura* developed a rapid decrease in feeding deterrent response to azadirachtin but not to neem containing a mixture of compounds including azadirachtin.

This was further exemplified by other experiments (6.3.2 and 6.3.3) using single or binary mixtures of pure allelochemicals in the present study. Third instar *T. ni* showed a decrease in feeding deterrent response to thymol, toosendanin or xanthotoxin with previous exposure to them as single compounds but not in binary (thymol + toosendanin, thymol + xanthotoxin, thymol + digitoxin, toosendanin + xanthotoxin) mixtures of these suggesting that *T. ni* is less likely to show a decrease in feeding deterrent response to a mixture of antifeedants than
Feeding responses to mixtures

to a single antifeedant. These results also support Jermy's hypothesis (1987) that decrease in feeding deterrent response following prolonged exposure can be prevented by complex mixtures of substances. This is also supported by the finding that extracts of *M. azedarach* containing 60-75% toosendanin showed greater growth inhibition and antifeedant effects against variegated cutworm larvae, *Peridroma saucia*, than toosendanin alone (Chen et al., 1995). This resulted from synergistic affects of various components, since isolated compounds were no more active than toosendanin (Isman et al., 1996). Bhuiyan et al. (2001) showed that mortality of fourth instar *Spodoptera litura* exposed to neem, rotenone, toosendanin, and *Annona squamosa* was significantly enhanced in combination with dillapiol (a major constituent of dill and celery seeds). Other synergistic interactions have been reported for some natural insecticides (Mukerjee et al., 1979), insect growth regulators (Grannet and Hejazi, 1983), insect viruses (Mohammad et al., 1983), pheromones (Pitman et al., 1975) and antifeedants (Moustafa et al., 1980).

One interesting aspect of the present study stems from the fact that although the individual plant extracts or pure allelochemicals had additive effects in the mixture (the observed deterrency values of mixtures were greater than the expected values) as depicted by the feeding deterrency of the naïve larvae (*Table 6.1*), the interaction was synergistic for experienced larvae (*Table 6.2*). This synergistic effect of individual antifeedants might be responsible for the failure of the feeding deterrent response to mixtures to wane following prolonged exposure to mixtures. The effect was greater in a binary mixture of two plant
extracts than to a binary mixture of pure allelochemicals \( (X^2 = 252.9, \textbf{Table 6.2}) \) probably resulting from interactions of numerous compounds in each plant extract.

During feeding on host plants, an insect encounters complex mixtures of nutrients and other plant secondary compounds (Bernays and Chapman, 2001), and there is now ample evidence demonstrating that responses of insect gustatory receptors are greatly affected by interactions between chemicals, including chemicals that may not be stimulating to any of the neurons within a sensillum (Schoonhoven et al., 1992).

Bernays and Chapman (2001) reported that in most of the binary mixtures of nutrients (amino acids) tested, the two compounds were additive in their effects as shown in the electrophysiological responses of taste cells in the polyphagous \textit{Grammia geneura}.

Some examples, however, also suggest that the collective effect or the overall response of an insect may not be reflective of the type of interaction shown by mixtures. Adam and Bernays (1978) demonstrated that mixtures of plant chemicals are more deterrent than single compounds. Although the reduction in feeding by \textit{Locusta migratoria} brought about by mixtures did not exceed the expected reduction (calculated by summing the reduction brought about by the individual components of the mixture), the collective effect was deterrent.
Berenbaum (1985) stated "the search for synergists is hampered by the fact that highly active synergists may themselves lack toxicity". She cited the example of myristicin (a major constituent of parsnip fruit essential oil), which was not toxic to *Heliothis zea* at synergistic dosages.

In the present experiment, no change in the feeding deterrent response by experienced larvae to mixtures corresponds to the synergistic effects of individual antifeedants (Table 6.2).
6.5 CONCLUSION

The data suggests that the combined effect of binary mixtures (plant extracts or pure allelochemicals) is greater than that of pure allelochemicals alone or a plant extract. Synergism or antagonism can occur when sensory neurons respond to the mixture (AB) in a way that exceeds or equals the summed responses to the components (Kang and Caprio, 1991).

Nature provides defense to plants through a mixture of deterents. Schoonhoven (1982) stated that "plants never defend themselves with a monocomponent system" and it is thought that minor components may act as synergists, enhancing the effect of the major constituents through a variety of mechanisms. Plant defense chemicals (or defense chemical combinations) that exhibit more than one mode of action are most suitable for crop protection (Raffa, 1987). Kubo et al. (1984) reported that in *Podocarpus gracilior*, four norditerpenes are both toxic and deterrent, two biflavones lack toxicity but inhibit growth, and one phytoecdysteroid interferes with molting and development; collectively, these constitute a "multichemical defense" against a variety of potential herbivores. Perhaps we have to learn from nature and use combinations of several antifeedants (mixtures), such as are present, for example, in crude neem extracts.

Therefore, it seems logical to use mixture of antifeedants for more durable crop protection rather than any single antifeedant.
CHAPTER 7

LARVAL EXPOSURE TO OVIPOSITION DETERRENTS ALTERS SUBSEQUENT OVIPOSITION BEHAVIOUR IN GENERALIST, *Trichoplusia ni* AND SPECIALIST, *Plutella xylostella* MOTHS.
7.1 INTRODUCTION

Host plant acceptance by phytophagous insects can be conveniently divided into two complex traits; female oviposition preference and larval performance (Thompson, 1988). Oviposition preference refers to the sequence of behavioural traits involved in locating and evaluating a potential host plant that ultimately leads to the decision to oviposit or not. Thompson (1988) used the term oviposition preference as the "hierarchical ordering of plant species by ovipositing females when the plants are presented in equal abundance and availability". It is also defined by the number of eggs laid by a female on each of the plant species, in a choice trial, in which plants of equal mass of several species are offered simultaneously (Wiklund, 1975; Thompson, 1986).

Oviposition preference is especially important in insects such as Lepidoptera because mobile adults must find host plants for their relatively immobile offspring (Tabashnik and Slansky, 1985). Neonates of many species are incapable of locating a new host and are dependent on the host plant location "skills" of their mothers (Feeny et al., 1983). Therefore the site of emergence is of such importance to the larvae of most (but not all) lepidopteran species that the choice of oviposition site by the female is considered in effect a choice of life history for her offspring (Resetarits, 1996), thereby influencing all aspects of larval performance.

The probability that an insect will feed or oviposit on a particular host individual will depend on the acceptability of the host to the insect (Singer, 1986).
This will be influenced by both innate tendencies and prior experience; a number of such influences may operate synchronously, increasing or decreasing the probability of acceptance (Miller and Strickler, 1984).

For more than a century, entomologists suggested that the feeding and oviposition behaviour of herbivorous insects was not only influenced by experience of the adult insect (Cassidy, 1978; Rausher, 1978; Prokopy et al., 1982; Jaenike, 1982, 1983; Hoffman, 1985) on a specific food, but also by experience obtained during the larval stage for that food. This phenomenon was called the "Hopkins Host Selection Principle" (HHSP) (Hopkins, 1917). Several authors claimed to have proven the HHSP in phytophagous insects (Walsh, 1864; cited by Szentesi and Jermy, 1989; Craighead, 1921; Kuznetzov, 1952; Hovanitz and Chang, 1963; Corbet, 1985; Jaenike, 1990; Futuyama et al., 1993; Barron, 2001; Rietdorf and Steidle, 2002), in Drosophila (Thorpe, 1939; Hershberger and Smith, 1967), and in parasitoids (Smith and Cornell, 1979; Thorpe and Jones, 1937). However, a number of studies have failed to show the effects of larval feeding experience upon subsequent oviposition behaviour of the adult (Solarz and Newman, 2001). Jaenike (1983), proposed a "neo-Hopkins host selection principle", suggesting that the "exposure of adult insects to a particular type of host will often, though not always, increase the subsequent acceptability of that host as an oviposition site".

Since there are contradictory reports about the influence of larval feeding experience upon subsequent oviposition behaviour of adult insects, this study aimed to look at the effects of larval feeding experience on subsequent
Feeding experience and oviposition behaviour

Oviposition behaviour of adult moths. *Trichoplusia ni* and *Plutella xylostella* were chosen for the study for several reasons. The cabbage looper, *T. ni*, a generalist, can be an important pest of cruciferous plants. This species also attacks several other crops including lettuce, beet, peas, celery, tomato, certain ornamentals and many weedy plants. The diamondback moth, *Plutella xylostella*, a specialist on plants in the family Brassicaceae, is one of the most serious pests of cole crops worldwide. Both species have short life cycles (egg-adult, 24-33 days for *T. ni* and 10-14 days for *P. xylostella*), and are easily reared in the laboratory. Both lay eggs singly, mostly on the lower surface of leaves.

*Trans*-anethole and *Melia azedarach* were used in the study. *Trans*-anethole, a phenylpropanoid, is the main constituent of the essential oil from anise (*Pimpinella anisum*). It is a contact toxin and a feeding deterrent to the tobacco cutworm, *Spodoptera litura* (Hummelbrunner and Isman, 2001). *M. azedarach* (containing 60-75% toosendanin, a limonoid), has been used as a botanical insecticide since 1980. Toosendanin possesses strong growth inhibitory and antifeedant properties against a number of insects (Chiu, 1989; Zhang and Chiu, 198; Chen et al., 1995).

Selection of test materials used in this study was based on their demonstrated oviposition deterrent properties against the test insects (*T. ni* and *P. xylostella*) (unpublished data; see appendix). They also acted as feeding deterrents against the respective larvae (Chapter 2). Although, *P. anisum* (containing *trans*-anethole) and *M. azedarach* do not constitute the normal host-
Feeding experience and oviposition behaviour

Plant range of *T. ni*, there is a great likelihood that the insect may encounter these and other plants containing closely related compounds.

Using the model system described above, the main objectives of the study were to determine (1) if feeding experiences of the larvae with a volatile (*trans*-anethole) or nonvolatile (*M. azedarach*) oviposition deterrent can change the oviposition preference of subsequent adults; (2) if the response of an ovipositing female is affected by the duration of larval exposure to the deterrent; (3) if there is a correlation between the oviposition choice of the "experienced female" and the growth performance of offspring on a preferred plant (treated cabbage foliage), (4) if a specialist herbivore responds in the same manner as a generalist herbivore. We have measured oviposition preference by recording the distribution of eggs laid by captive females when given a choice between control and treated plants under controlled experimental conditions in the laboratory (Singer, 1986). The present study may be useful in predicting host-plant shifts in nature as a result of experience by phytophagous herbivores.
7.2 Materials and Methods

7.2.1 Plant Material

Cabbage plants (Brassica oleracea var. Stonehead) were routinely grown for bioassays and to maintain the diamondback, P. xylostella moth colony, in plastic pots as described in Chapter 2.

7.2.2 Test chemicals

Trans-anethole (99% purity), was provided by EcoSmart Technologies Inc. (Nashville, USA). Refined bark extracts of Melia azedarach (syn. M. toosendan) containing 60-75% toosendanin were obtained from sources listed in Chapter 2.

7.2.3 Concentrations of the compounds used for larval training (exposure)

Cabbage looper larvae were reared on artificial diet containing 100 ppm of trans-anethole or 50 ppm of M. azedarach. A 10 ppm methanolic solution of M. azedarach was sprayed on cabbage leaves to train diamondback larvae. The concentrations used were neither inhibitory to larval growth nor toxic to the insects; there was no larval mortality attributable to the chemicals during the training period.

7.2.4 Concentrations of compounds used for testing

Concentrations of the chemicals used for testing were based roughly on their OD₅₀ values (concentration of the compound causing 50% oviposition deterrence, compared to controls) determined in preliminary experiments (unpublished data; see appendix). Methanolic solutions of 0.8% trans-anethole
and 0.5% *M. azedarach* were sprayed on cabbage foliage. Use of higher concentrations was avoided due to phytotoxic effects on cabbage foliage.

### 7.2.5 Test Insects

*richoplusia ni* (Lepidoptera: Noctuidae) and *P. xylostella* (Lepidoptera: Plutellidae), were obtained from laboratory colonies as described in Chapter 2. All test diets used in experiments were prepared according to Isman and Rodriguez (1983). Diamondback moths were reared on potted cabbage plants in Plexiglas cages [60 x 60 x 40cm (l x w x h)]. Moths of both species were supplied with a 10% sucrose solution accessed by a cotton wick in a sealed 100 ml plastic container.

### 7.2.6 General procedure

All experiments included two main groups, experienced and naïve. The experienced groups were reared on different concentrations of chemicals added to the diet or sprayed on cabbage plants, until pupation. To exclude the influence of early adult experience, the pupae were removed from the diet or cabbage plants they developed on and placed in plastic containers. Also, as we did not rinse the pupae, the possibility of the presence of chemical on the pupal cuticle modifying adult behaviour cannot be excluded. Pupae of *T. ni* were sexed and kept in separate plastic containers until emergence. Each plastic container had 10 pupae with a 10% sucrose solution accessed by a cotton wick in a sealed 100 ml plastic container for the adult moths. Pairs of moths (one male and one female) were introduced into each cell of the cages with a control and a treated
cabbage leaf. Each leaf (approximately 100-110 cm²) was sprayed with 0.5 ml of MeOH or a methanolic solution of the test chemical on each side. Eggs were counted on each cabbage leaf at intervals described below. Old leaves were replaced by fresh ones after each observation. Two kinds of cages were used in the oviposition experiments (Fig. 7.1a and b). Styrofoam containers used for packaging 4 liter MeOH bottles, were used as oviposition cages for T. ni females. Each styrofoam container provided four cages (cells) (24 cm length, 16.5 cm diameter). A circular window (9 cm diameter) was made on one side of each cell of the cage using a knife, and a plastic mesh (Quick Count, 7 mesh) was glued over it. A single plastic mesh was used to cover the top of the container and was secured by thumbtacks to prevent moths from escaping.

Cages used for diamondback moths, P. xylostella were similar to the T. ni cages with a few modifications (Fig. 1b). Styrofoam containers, used for packaging solvent bottles (18 cm long and 15 cm in diameter) were used. Windows were made in the same way as T. ni cages with the exception that gauze sleeves (25 cm long and 9 cm diameter) were attached to allow moths to be introduced without escaping. Tops of the cages were covered with Petri dishes (14.5 cm diameter) rather than mesh. Cages were placed in growth chambers under the following conditions: temperature ranging from 18-20°C, humidity 50% and 16:8 LD photoperiod.
Fig. 7.0 Cages used for (a) *Trichoplusia ni* and (b) *Plutella xylostella* moths. Each cage consists of four cells with two leaves (control and treated) and sugar solution (c) per cell.
7.2.7 Experiment 1. Oviposition of *T. ni* moths with previous exposure to *trans*-anethole from neonates until pupation.

This experiment was designed to determine if larval experience of *T. ni* with a volatile deterrent compound, *trans*-anethole, changed the oviposition behaviour of the subsequent adults.

7.2.7.1 Training

Neonates <24 h old were introduced onto diet containing 100ppm fwt *trans*-anethole in small styrofoam cups (360 ml), and allowed to feed until pupation. Naïve moths were reared on a diet containing MeOH only.

7.2.7.2 Testing

A pair of moths was introduced into each cell of the cage containing a control (MeOH) and a treated leaf (0.8% *trans*-anethole) (Fig. 7.1c). Plastic mesh was placed immediately over the cages to prevent moths from escaping. Plastic cups containing leaves were removed carefully from each cage and the number of eggs was counted on each leaf after 48h, 96h, 144h, and 192h.

7.2.8 Experiment 2. Oviposition of *T. ni* moths with previous exposure to *trans*-anethole during the last instar only.

This experiment was conducted to determine the effect of the duration of larval experience with the oviposition deterrent on the subsequent ovipositing female.
7.2.8.1 Training

Experimental conditions were the same as above with the exception that freshly molted fifth instars were introduced onto diet containing 100 ppm of trans-anethole and allowed to feed until pupation. Naïve larvae were reared on diet containing MeOH only.

7.2.8.2 Testing

Testing was done in the same manner as experiment 1 and the number of eggs was counted on each leaf as before.

7.2.9 Experiment 3. Oviposition of *T. ni* moths with previous exposure to *M. azedarach* from the third instar until pupation.

This experiment was conducted to determine if larval experience with a nonvolatile oviposition deterrent changed the oviposition behaviour of subsequent moths. I intended to use moths with larval exposure to *M. azedarach* either from neonates or the fifth instar as in the experiments with *trans*-anethole, but virus problems prevented us from doing so.

7.2.9.1 Training

Third instar of *T. ni* were introduced onto a diet containing 50 ppm fwt (fresh weight) *M. azedarach* and allowed to feed until pupation as in the previous experiments. Naïve moths were reared on diet containing MeOH only.
7.2.9.2 Testing

Testing was done in the same manner as the previous experiments except that eggs were counted after 48h and 96h only.

7.2.10 Experiment 4. Performance of the F\textsubscript{1} larvae of *T. ni* (from experienced and naïve moths) on control (MeOH) and treated plants (0.5\% *M. azedarach*).

This experiment was conducted to determine if there was a relationship between the choice of “experienced moths” (exposed to *M. azedarach* as larvae) and the performance of their offspring on preferred plants. Larval weights from the experienced and naïve moths on the control and treated plants were compared.

7.2.10.1 Training

Experienced (50 ppm of *M. azedarach* from third instar until pupation) and naïve moths were released into separate cages containing a control and a treated plant (0.5\% *M. azedarach*) in each cage. Following egg laying (48h), each plant was placed in a separate cage. Eclosing larvae were allowed to feed on plants.

7.2.10.2 Testing

Larvae from experienced and naïve groups were weighed 8 days after eclosion.
7.2.11 Experiment 5. Oviposition of diamondback, *P. xylostella* moths with previous exposure to *M. azedarach* from neonates until pupation.

This experiment was conducted to determine if a specialist herbivore, *P. xylostella* acted in the same way as the generalist herbivore.

7.2.11.1 Training

Cabbage leaves sprayed with a *M. azedarach* solution (10 ppm) were placed in Plexiglas cages with adult diamondback moths. The petioles of the cabbage leaves were immersed in 100 ml plastic containers with water to prevent desiccation. Neonates eclosing from eggs laid on the leaves were allowed to feed on cabbage *ad libitum* until pupation. Pupae were collected and placed in plastic containers (10 / container). Following emergence, six adult moths were introduced into each cell of the cage with the assumption that each group contained at least one female. Sexing pupae is difficult due to their small size. Cages remained covered with Petri dishes and introduction of the moths and leaves was done through the gauze sleeves (Fig. 7.1b). The naïve group was reared on cabbage foliage sprayed with MeOH only.

7.2.11.2 Testing

Eggs were counted at 48h and 96 h on the control and treated leaves for each group, using a binocular microscope.

7.2.12 Comparison of oviposition deterrence indices (ODIs) of the experienced and naive groups.
Both *trans*-anethole and *M. azedarach* were oviposition deterrents for *T. ni* and *P. xylostella* (unpublished data, see appendix). I wanted to see if there was a change in oviposition behaviour of the experienced insects due to prior feeding experience (although the possibility of chemical legacy {Corbet, 1985} affecting the choice of adult moths cannot be excluded). ODI was calculated similar to FDI (Feeding deterrence index) (Isman et al., 1990) using the formula; 

\[ \text{ODI} = 100 \left( \frac{C - T}{C + T} \right) \]

where \(C\) and \(T\) are the number of eggs laid on the control and the treated leaves, respectively.

### 7.2.13 Data analysis

Numbers of eggs laid on the control and treated leaves by the experienced and naive groups were summed for all days. Data were analysed on log-transformed values by analysis of variance (ANOVA) using statistics software (Statistix 7, 2000). Where significant F values were found, Tukey's HSD, multiple comparison tests were conducted to test for significant differences between individual treatments (Zar, 1984). A mean oviposition deterrence index (ODI) was also calculated (as described in the previous section) and compared for experienced and naive groups in each experiment. Two-sample t-tests (on arcsine transformed data) were used to compare the oviposition deterrent responses of the experienced and naive groups in each experiment. All experiments were replicated more than once except for experiment 4. Because there were no effects of treatments between replicates, data from them were pooled. The alpha level used in all analyses was 0.05.
7.3 RESULTS

7.3.1 Experiment 1. Oviposition of *T. ni* moths with previous exposure to *trans*-anethole from neonates until pupation.

A two-factor ANOVA (group*treatment) on the number of eggs laid by experienced and naïve (groups) moths (*n* = 3 replications of 10 pairs of moths) on the control and treated leaves (treatment) showed that there was no significant main effect of group (*F*₁₆ = 1.87, *p* = 0.174). There was a significant main effect of treatment (*F*₁₆ = 14.68, *p* = 0.0002) with moths laying a significantly greater number of eggs on the control leaves than the treated leaves (Tukey’s test, *p* < 0.05). There was no significant group*treatment interaction (*F*₁,₁₁₆ = 2.18, *p* = 0.1422) (Fig. 7.2).

Comparison of the ODIs (oviposition deterrence indices) of the experienced and naïve groups showed that there was a significant decrease in oviposition deterrence to *trans*-anethole by the experienced moths unlike the naïve moths (two sample t-test, df=58, *t* = -6.43, *p*<0.05) (Table 7.1).

7.3.2 Experiment 2. Oviposition of *T. ni* moths with previous exposure to *trans*-anethole during the last instar only (from fifth instar until pupation).

A two-factor ANOVA (group*treatment) on the number of eggs laid by experienced and naïve groups of moths (*n* = 3 replications of 10 pairs of moths) on the control and treated (*trans*-anethole) leaves showed that there was a significant main effect of group (*F*₁,₁₁₆ = 16.02, *p* = 0.0001). Naïve moths laid significantly greater numbers of eggs than the experienced moths (Tukey’s test, *p* < 0.05).
Fig. 7.2 Number of eggs laid by experienced (*trans*-anethole from neonates to pupation) and naïve (MeOH) *T. ni* moths when given a choice between control and treated (*trans*-anethole) cabbage leaves. Bars marked by the same letters do not differ significantly (Tukey's test, p < 0.05, n = 3 replications of 10 pairs of moths).
There was no significant main effect of treatment ($F_{1,116} = 3.21, p = 0.07$) but there was a trend as indicated by the p value (0.07). There was a significant group*treatment interaction ($F_{1,116} = 18.49, p = 0.001$) with naive moths laying significantly greater number of eggs on the control leaves than the treated leaves (Tukey's test, $p < 0.05$) compared with the experienced moths (Fig. 7.3).

Comparison of the ODIs (oviposition deterrence indices) of the experienced and naïve groups showed that there was a significant decrease in oviposition deterrence to trans-anethole by the experienced moths unlike the naïve moths (two sample t-test, $df=58$, $t= -4.87$, $p<0.05$) (Table 7.1).

7.3.3 Experiment 3. Oviposition of *T. ni* moths with previous exposure to *M. azedarach*, from third instar until pupation.

A two-factor ANOVA (group*treatment) on the number of eggs laid by experienced and naïve groups of moths ($n = 4$ replications of 10 pairs of moths) on the control and treated (*M. azedarach*) leaves showed that there was a significant main effect of group ($F_{1,156} = 10.49, p = 0.001$). Experienced moths laid significantly greater numbers of eggs than the naïve moths (Tukey's test, $p < 0.05$). There was a significant main effect of treatment ($F_{1,156} = 5.05, p = 0.026$) with significantly greater numbers of eggs laid on the control leaves than the treated leaves (Tukey's test, $p < 0.05$). There was a significant group*treatment interaction ($F_{1,156} = 9.56, p = 0.002$) with naïve moths laying significantly greater numbers of eggs on the control leaves than the treated leaves (Tukey's test, $p < 0.05$) compared with the experienced moths (Fig. 7.4).
Comparison of the ODIs (oviposition deterrence indices) of the experienced and naïve groups showed that there was a significant decrease in oviposition deterrence to *M. azedarach* by the experienced moths unlike the naïve moths (two sample t-test, df=72, t= -5.10, p<0.05) (Table 7.1).
Fig. 7.3 Number of eggs laid by the experienced (*trans*-anethole in last instar only) and naïve (MeOH) *T. ni* moths when given a choice between control and treated (*trans*-anethole) cabbage leaves. Bars marked by the same letters do not differ significantly (Tukey's test, $p < 0.05$, $n = 3$ replications of 10 pairs of moths).
Fig. 7.4 Number of eggs laid by experienced (*M. azedarach* from third instar to pupation) and naïve (MeOH) *T. ni* moths when given a choice between control and treated (*M. azedarach*) cabbage leaves. Bars marked by the same letters do not differ significantly (Tukey’s test, $p < 0.05$, $n = 4$ replications of 10 pairs of moths).
7.3.4 Experiment 4. Performance of F₁ larvae (from experienced and naïve moths) on control (MeOH) and treated plants (0.5% *M. azedarach*).

A two-factor ANOVA (group*treatment) on the weight of experienced and naïve groups of larvae (n = 50 larvae/group) on the control and treated (*M. azedarach*) plants showed that there was no significant main effect of group (F₁,₁₉₆ = 1.25, p = 0.265). There was a significant main effect of treatment (F₁,₁₉₆ = 41.95, p < 0.0001) with F₁ larvae weighing significantly more on the control plants than the treated plants (Tukey's test, p < 0.05). There was a significant group*treatment interaction (F₁,₁₉₆ = 33.77, p < 0.0001) with naïve larvae weighing significantly more on the control plants than the treated plants (Tukey's test, p < 0.05) compared with the experienced larvae, which showed no difference in weight on the control and treated plants (Fig. 7.5).

7.3.5 Experiment 5. Oviposition of *P. xylostella* moths with previous exposure to *M. azedarach* from neonates until pupation.

A two-factor ANOVA (group*treatment) on the number of eggs laid by experienced and naïve groups of moths (n = 3 replications of 10 units of moths, each unit = 6 moths) on the control and treated (*M. azedarach*) leaves showed that there was a significant main effect of group (F₁,₁₁₆ = 3.87, p = 0.05). Experienced moths laid significantly greater numbers of eggs than the naïve moths (Tukey's test, p < 0.05). There was no significant main effect of treatment (F₁,₁₁₆ = 0.51, p = 0.48). There was a significant group*treatment interaction (F₁,₁₁₆ = 6.12, p = 0.01) with experienced moths laying significantly greater numbers
of eggs on the treated leaves than the control leaves (Tukey's test, $p < 0.05$) compared with the naive moths with no significant difference in the number of eggs on control or treated leaves (Fig. 7.6).

Comparison of the ODIs (oviposition deterrence indices) of the experienced and naïve groups showed that there was a significant decrease in oviposition deterrence to *M. azedarach* by the experienced moths unlike the naïve moths (two sample t-test, $df=58$, $t=-3.47$, $p<0.05$) (Table 7.1).
Fig. 7.5 Weight of $F_1$ larvae resulting from experienced ($M. \text{ azedarach}$ from neonates to pupation) and naïve $T. \text{ ni}$ moths with no exposure to $M. \text{ azedarach}$ on control and treated ($M. \text{ azedarach}$) cabbage plants. Bars marked by the same letters do not differ significantly (Tukey's test, $p < 0.05$, $n = 50$ larvae/group).
Fig. 7.6 Number of eggs laid by experienced (*M. azedarach* from neonates to pupation) and naïve (MeOH) *P. xylostella* when given a choice between control and treated (*M. azedarach*) cabbage leaves. Bars marked by the same letters do not differ significantly (Tukey's test, \( p < 0.05 \), 3 replications of 10 units of moths, each unit = 6 *P. xylostella* moths with the assumption that there is at least one female/unit.
Table 7.1 Oviposition deterrence indices of experienced and naïve *Trichoplusia ni* and *Plutella xylostella* moths. * indicates significant difference between experienced and naïve groups (two-sample t-test, p < 0.05). Experienced moths showed a significant decrease in oviposition deterrent response compared with the naïve moths. Negative values indicate that treated plants are preferred over control plants.
7.4 DISCUSSION

The results demonstrate that feeding experiences of *T. ni* and *P. xylostella* larvae may have influenced the subsequent oviposition behaviour of the adult moths. "Experienced" female moths preferred to lay more eggs on plants treated with a deterrent compound previously experienced as larvae. Although, in most cases there was not a significant difference between the number of eggs laid on the control and treated plants by the experienced moths, naïve *T. ni* moths always laid significantly greater numbers of eggs on the control than the treated plants. However, this trend was not seen in *P. xylostella* moths (Fig. 7.6). Experienced *P. xylostella* moths laid significantly more eggs on the treated than the control leaves. In contrast, naïve *P. xylostella* moths did not discriminate between control and treated leaves, although preliminary experiments (data not shown) strongly suggested that *M. azedarach* was an oviposition deterrent at the concentration used. Higher concentrations were avoided because of phytotoxic effects.

Comparison of ODIs of experienced and naïve moths showed that there was a significant decrease in oviposition deterrent response by the experienced moths (Table 7.1). The fact that experienced moths in all cases exhibit a tendency towards decreasing oviposition deterrent response suggests that larval feeding experience with the oviposition deterrent compounds (or possibly chemical legacy) changed the oviposition choices of adult moths. This was true for both a generalist (*T. ni*) and a specialist (*P. xylostella*) in my study. It appears from my experiments that larval feeding experience or chemical legacy may be
important in shaping the host-selection choice of adult moths. I do not believe that selection played an important role in shaping the behaviour of the “experienced” groups of moths, because there was no appreciable mortality (or even evidence of any sublethal toxicity) of larvae during the training (exposure) period. These results may therefore provide support for the “Hopkins’ (1917) host selection principle”, as has been suggested by others, although I cannot exclude the possibility of chemical legacy. Rietdorf and Steidle (2002) showed that when given the choice between seeds of maize and wheat in an olfactometer, adult granary weevils *Sitophilus granarius* preferred the odour of those plant seeds on which they had developed as larvae.

It is interesting to note that larvae of both the generalist (*T. ni*) and specialist (*P. xylostella*) herbivores used in the present study showed a decrease in feeding deterrent response to *M. azedarach*, following prolonged exposure (Chapters 3 and 4). Therefore, it is not surprising to see the failure of this chemical to deter experienced moths that incurred no deleterious effects upon their growth as larvae. The information is apparently retained through metamorphosis from larva to adult, as is evident by the oviposition choice made by the experienced female moth. One possible explanation for this phenomenon is the storage of information obtained during larval development in the central nervous system (CNS) (Jermy et al., 1968). Alternatively, information about the larval environment can also be carried through to the adult stage by “chemical legacy” (Corbet, 1985). Traces of chemicals from the larval stage in the
hemolymph of the insect or on the outside of the pupa might modify the perception and behaviour of the adults.

In holometabolous insects such as some lepidopterans, the adult female must select a plant for oviposition that maximizes fitness of her offspring. Yet most adult lepidopterans are not equipped to obtain direct information on the quality of the food through feeding. The larva, in turn, while capable of making food choices in an experimental situation, is often, in practice, without the opportunity to choose (Bernays and Chapman, 1994).

Female host plant choice is not only influenced by larval feeding capacities, but also by various ecological factors, such as host plant abundance (Rausher, 1980; Singer et al., 1989), search efficiency (Rausher, 1978; Courtney, 1983; Bernays, 1998) and predator effects (Camara, 1997). It has been shown that *Helicoverpa armigera* moths laid more eggs on the most abundant hosts in fields due to increased encounters (Cunningham et al., 1999). It would be interesting to know how larval feeding experience would affect the choice of an ovipositing female in the natural environment where there can be an interaction of a number of factors. My study has taken into account only the effects of prior larval feeding experience with oviposition deterring compounds upon subsequent oviposition behaviour of the adult moths under controlled experimental conditions. A rigorously controlled laboratory test, although suitable for identifying particular behavioural mechanisms, does little to define the role such mechanisms play in shaping behaviour in the field (Parmesan et al., 1995).
The results of my experiments also show that duration of larval experience did not affect the oviposition behaviour of the adult moths. Other investigators have also sought to determine the developmental stage at which host plant preference is determined (Corbet, 1985; Anderson et al., 1995).

It is believed that if an adult female does not experience any nutritional or noxious effect in relation to where she lays eggs (although, in the present case she had experienced the chemicals as larvae), the adult female may switch from using several hosts to using a single one, depending on plant abundance. She may also tend to lay eggs successively on the same plant type. If this pattern is repeated over generations in a region, larvae may be selected for fitness parameters on this one plant. If this is accompanied by reduced fitness on other plants, differential larval fitness may then place selection pressure on adult behaviour. This dual process may hasten change (Bernays and Chapman, 1994). It would be interesting to see what role experience with the deterrent compound plays in host selection in the natural environment. Host plant acceptance by an ovipositing female is mediated by a balance of sensory inputs from both positive and negative stimuli produced from potential host plants (Dethier, 1982; Huang and Renwick, 1993). The relative balance between these opposing cues can also be influenced by the internal physiological state of the insect, e.g. egg load, as well as other environmental factors (Dimock, et al., 1991).

The results of my experiments also indicated that larvae from experienced female moths weighed almost the same when reared on the control and treated plants, suggesting that there was a positive correlation between larval growth
performance and adult choice. Similar findings have been reported by others (Singer et al., 1994). There may be a flow of information from larvae to the adult moths and then to the $F_1$ larvae (offspring) through the eggs as indicated by the larval performance on the chosen plant. It is possible that this transfer of information has been brought about through chemical trace compounds either within or on the egg (Corbet, 1985; Anderson et al., 1995).

The results of my experiments demonstrate that there was a change in the oviposition behaviour of the ovipositing females that resulted in the relative acceptability of previously unacceptable compounds. A hypothesis to explain the behavioural mechanism that may increase the acceptance of a host with which an individual has experience requires some speculation as to the nature of learning involved (Cunningham et al., 1998). Some butterflies such as Battus philenor (Papaj, 1986) and Pieris rapae (Traynier, 1984) have been shown to learn through classical conditioning. These insects associated a chemical oviposition stimulus with a visual stimulus from the oviposition substrate (leaf shape in B. philenor and color in P. rapae).

Other simple forms of learning could explain the observed change in host preference by an ovipositing female. Habituation, the waning of response to a stimulus upon repeated presentation (Szentesi and Jermy, 1989), could alter the response to oviposition deterrents in the host substrate, decreasing the net "inhibitory" stimuli and thus affecting acceptability and host preference (Cunningham et al., 1998). Although, learning may have been demonstrated in both generalist (T. ni) and the specialist (P. xylostella) herbivores in their host
selection behaviour as a result of prior experience, in my experiments, I cannot establish the category of learning without specific testing. For example, if it is proposed that a decrease in response is a result of habituation, then a novel or noxious stimulus should dishabituate the response (Thompson and Spencer, 1966).

Anderson et al. (1995) showed that females of S. littoralis were no longer deterred by larval frass and potato extracts when they had spent their entire larval development on a potato–based diet, unlike naive females. Three plausible explanations for the loss of deterrence were selection, habituation and induction of preference. An induction of preference (modification of feeding behaviour leading to a change in host plant preference by prior feeding experience) as described by Jermy et al. (1968) for oviposition deterrent compounds has also been shown in the present studies. At this point it is hard to say what the basis for that induction of preference is: selection, learning, chemical legacy or a combination among these.
7.5 CONCLUSION

The response of the ovipositing female may be influenced by prior feeding experience of the larva (irrespective of the duration) or the chemical legacy. To rule out the possibility of chemical legacy, further experiments should be planned (e.g. rinsing of pupae with water or other solvents to remove traces of chemical, or alternatively, administering chemicals to pupae from "naïve" larvae). The question remains whether or not it is possible to completely eliminate any traces of chemicals from the insect between the larval and adult stages. According to Corbet (1985) traces of chemicals obtained during the larval stage in the hemolymph of the insect or on the outside of the pupa might modify the perception and behaviour of the adults. It could be possible to attempt to remove chemicals from the outside of pupae but it may not be possible to eliminate them from the hemolymph.

One interesting aspect of this study is that both of the test chemicals were larval feeding (Chapter 2) as well as oviposition deterrents for T. ni (cabbage looper) and P. xylostella (diamondback moths). Larvae of both species showed a decrease in feeding deterrent response to M. azedarach following prolonged exposure (Chapters 2 and 3). Data from the present study reveals that this decrease in feeding deterrent response may have been maintained in F_1 larvae of T. ni (as indicated by larval weight performance when reared on control and treated plants). This study also suggests a loss of oviposition deterrence as a result of larval experience. The positive correlation between female moth choice and larval growth performance indicates that female moths did not make a host
choice deleterious to their offspring. This may be indicative of field situations when a generalist species like the *T. ni* and specialist species like the *P. xylostella* moth are forced to select a host plant, which is out of their normal host range. This host plant switch can lead to acceptance of the plant as a host by the females in future generations as a result of larval experience (Anderson et al., 1995). This could be an important mechanism for extensions of host range through new feeding and oviposition preferences (Berenbaum and Zangerl, 1991; Bowers et al., 1992). Additional field studies are required to show this.
CHAPTER 8

SUMMARY OF CONCLUSIONS
The overall objective of this thesis was to assess the effects of experience with antifeedants on feeding and oviposition preferences of larvae and subsequent adult moths respectively. I started with the screening of plant extracts and pure allelochemicals for growth-inhibiting and antifeedant effects against three lepidopteran and one coleopteran species (Chapter 2). All the plant extracts and pure allelochemicals showed good growth-inhibiting and antifeedant effects against the insect species tested. The results indicated that *M. volkensii* was the most effective growth inhibitor among the plant extracts and pure allelochemicals tested, for both *T. ni* and *P. unipuncta*. It also acted as a strong antifeedant to *T. ni*, *P. unipuncta*, and *E. varivestis*. Based on DC$_{50}$ values, xanthotoxin was by far the most effective deterrent and trans-anethole was the least among the allelochemicals tested against *T. ni*.

Two of the test materials, *M. volkensii* extract (EC$_{50}$ = 7.8 for *T. ni* and 12.5 ppm for *P. unipuncta*) and cymarin (EC$_{50}$ = 157.0 ppm for *T. ni*) had bioactivities comparable to those of commercial botanicals such as pyrethrum (EC$_{50}$ = 98 ppm), ryania (EC$_{50}$ = 117 ppm) or rotenone (EC$_{50}$ = 163 ppm) in the *Spodoptera* larval growth bioassay.

There may or may not be a correlation between EC$_{50}$ and DC$_{50}$ values indicates that one type of bioassay is not sufficient to assess the full bioactivity of a potential crop protectant. The observed DC$_{50}$ value for thymol was significantly higher (37.4 µg/cm$^2$) and lower for xanthotoxin (0.95 µg/cm$^2$) than the predicted values (20.65 and 20.99 µg/cm$^2$ respectively) at their corresponding EC$_{50}$ values.
There were interspecific differences in response to feeding deterrents. Differences not only existed between the generalist (*T. ni*) and specialists (*P. unipuncta, P. xylostella, and E. varivestis*) but also among specialist species in their feeding responses towards the plant extracts and pure allelochemicals tested.

Since most of the test substances in my experiments have proven to be growth inhibitors and antifeedants against a number of insect species, some of them may serve as potential crop protectants. However, their effects on non-target organisms need be tested. They may also serve as a model system for studying host-plant selection due to their toxic and deterrent properties.

The main focus of chapter 3 was elucidation of the effects of experience with several antifeedants under a variety of conditions (using different antifeedant concentrations and different larval instars) upon feeding behaviour of a generalist herbivore, *T. ni*. All instars of *T. ni* tested (second, third or fifth) showed a decreased feeding deterrent response to most of the antifeedants tested (extracts of *M. volkensii* and *M. azedarach*; oil of *O. vulgare*; and pure allelochemicals: xanthotoxin, toosendanin and thymol), following prolonged exposure. Cardenolides (digitoxin and cymarin) were the exceptions. Response to oregano was affected as a result of previous exposure to different concentrations of oregano unlike *M. volkensii* suggesting that *T. ni* sensitivity varies between stimuli and cannot be generalized. A high concentration of the
particular antifeedant, xanthotoxin, acted as a noxious stimulus, and dishabituated (reversed) the decreased antifeedant response to *M. volkensii*.

Chapter 4 investigated the effects of experience with antifeedants on the feeding responses of some specialist herbivores. The results of my experiments indicated that feeding responses of the two lepidopterans and one coleopteran species varied following prolonged exposure to antifeedants. Neither *P. unipuncta* nor *P. xylostella* showed a significant decrease in feeding deterrent response to *M. volkensii* in either choice or no choice tests. Both species showed a significant decrease in feeding deterrent response to a pure allelochemical (thymol) following prolonged exposure. However, *E. varivestis* showed a decrease in feeding deterrent response to oregano and thymol. The response of the two specialist lepidopteran species tested here differs from that demonstrated by the generalist species in chapter 2.

I conclude from the present (Chapter 4) and previous studies (Chapter 3) that not only are there interspecific differences between generalist and specialist species but also among specialist species.

Chapter 5 investigated the potential of generalization of feeding deterrent response to unrelated compounds. There was a significant generalization of decreased feeding deterrent response to oregano in larvae with previous exposure to *M. volkensii* extract and vice versa. This generalization of feeding deterrent response was not shown by naïve larvae in either case. A similar generalization of decreased feeding deterrent response was also exhibited by *T.*
ni larvae to thymol (pure allelochemical) with previous exposure to the unrelated pure allelochemicals, digitoxin or xanthotoxin unlike the naïve groups. There was a lack of generalization of response to plant extracts following prolonged exposure to a pure allelochemical and vice versa, suggesting that there are some limitations to this phenomenon.

Chapter 6 investigated the effects of experience with binary mixtures on the feeding response of T. ni larvae. There was a decrease in feeding deterrent response to M. volkensii or oregano in larvae with previous exposure to M. volkensii or oregano respectively but not to a binary mixture of these. The larvae also showed a decrease in feeding deterrent response to thymol, toosendanin and xanthotoxin (digitoxin was an exception) with previous exposure to them as single compounds but not to binary mixtures of these suggesting that mixtures can prevent the phenomenon of decreased feeding deterrent response.

Although the individual plant extracts or pure allelochemicals had additive effects in the mixture (the observed deterrence values of mixtures were greater than the expected values) as depicted by the feeding deterrency of the naïve larvae, the interaction was synergistic for experienced larvae. This synergistic effect of individual antifeedants might be responsible for the failure of the deterrent response to mixtures to wane following prolonged exposure to mixtures.

All of the work summarized above was done on the feeding behavior of larvae (trained from neonates <24 h old until tested at a later stage as larva).
Chapter 7 elucidated the effect of feeding experience upon oviposition behavior of the subsequent moths. The results of my experiments demonstrated that feeding experiences of *T. ni* and *P. xylostella* larvae influenced the subsequent oviposition behavior of the adult moths. Comparison of oviposition deterrence indices (ODIs) of experienced and naive moths showed that there was a significant decrease in oviposition deterrent response by the experienced moths. The fact that experienced moths in all cases exhibited a tendency towards decreasing oviposition deterrent response suggests that larval experience is important in shaping the host-selection choice of adult moths. The results of my experiments support the “Hopkins’ (1917) host selection principle” (although the possibility of chemical legacy cannot be excluded). Results also indicated that larvae from experienced female moths weighed almost the same when reared on the control and treated plants, suggesting that there was a positive correlation between larval growth performance and adult choice.

**The Big Picture**

The question that remains is whether effects of antifeedants as crop protectants can be prolonged in the field. Clearly, the problem of decreased feeding deterrent response to antifeedants following prolonged exposure, poses a number of complex questions, and no single approach or hypothesis can hope to answer them all. Therefore, in this thesis I have used a mixture of approaches (different larval instars, different species of herbivores, different concentrations of antifeedants, mixtures) to address this question from different angles.
Although, the results of my experiments have clearly demonstrated that larval feeding experiences not only changed larval feeding preferences but also oviposition preferences of subsequent moths, there are also some limitations to the decreasing feeding deterrent response following prolonged exposure. *T. ni* larvae did not show a decrease in feeding deterrent response to digitoxin and cymarin nor when exposed to a lower concentration of oregano. These findings are very encouraging in a sense that slight changes in the use of antifeedants can be favourable for crop protection. The finding that this decrease in the antifeedant response can be explained by habituation may offer some solutions for pest management. Compounds to which insects have become habituated can be made effective deterrents again through the process of dishabituation as clearly shown in experiment 3.

The results of my experiments suggest that the combined effect of binary mixtures (plant extracts or pure allelochemicals) is greater than that of pure compounds alone or a plant extract. It therefore seems logical to use a mixture of antifeedants for more durable crop protection rather than any single antifeedant. It remains to be seen how far this path will lead, but the results seen to this point appear promising.

In the context of host-plant shifts, induction of preference and performance can lead to genetic sub-structuring of populations because it increases host fidelity and decreases movement to alternative hosts (Via, 1991). Phenotypic plasticity, in general, can promote local adaptation and potentially speciation by
permitting flexible individuals initially to utilize and ultimately to adapt to marginal resources (West-Eberhart, 1989). Substructuring caused by induction of preference, however, is subject to rapid breakdown caused by immigration or other factors that generate gene flow (Via, 1991). Induction of preference and performance may provide benefits similar to genetic specialization, such as a focused ability to find hosts and to deal with their defenses; induction is a more flexible strategy, however, that allows individuals to adapt to varying environments such as changes in host availability (Agrawal et al., 2002).

Where to go from here?

In many ways, the experiments presented here represent the first steps towards understanding insect behavior in response to prolonged exposure to antifeedants under different conditions for the design of pest management tactics. This is clearly a developing area of research and the most important factor will be gaining information on the response of insects in the field.

Some of the information obtained from this thesis has hinted at the existence of limitations in the phenomenon of decreased deterrence response following prolonged exposure. Selection of the antifeedant compounds, biology of the target species and ways of application of antifeedants (concentration, and whether applied singly or in mixtures) are some of the important issues that need to be considered before using an antifeedant for crop protection. The finding that a strong deterrent can dishabituate the decreased feeding deterrent response can make the antifeedants effective again is very encouraging. I believe that this
information will be very helpful in the potential use of antifeedants for crop protection.
REFERENCES


Oregano oil chromatograms

Assays
By HPLC
- a-terpinene: trace
- p-cymene: 11.08%
- thymol: 1.45%
- carvacrol: 70.19%

By GC/MS
- a-terpinene: 2.01%
- p-cymene: 12.20%
- thymol: 1.68%
- carvacrol: 70.00%
Expanded GC/MS chromatogram of oregano oil
Calculation of ODI\textsubscript{50}s for \textit{T. ni} and \textit{P. xylostella} for oviposition experiments (Chapter 7)