Terpenoid antifeedants against insects: a behavioural and sensory study

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Cover by Lindy Messchendorp, 1998
Composition of cabbage, maize and potato leafs

STELLINGEN

1. De vondst van een smaakcel gevoelig voor vraatremmende stoffen in *Leptinotarsa decemlineata* larven verschaft een fysiologische basis voor de hypothese dat de aanwezigheid van vraatremmende stoffen in potentiële voedselplanten beslissend is bij de selectie van voedselplanten door dit insect.
   Jermy T., 1961. On the nature of the oligophagy in *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae). *Acta Zool. Acad. Sci. Hung.* 7: 119-132; Dit proefschrift.

2. Bij onderzoek aan structuur-activiteitsrelaties voor series van vraatremmende stoffen is het essentieel de verschillende mechanismen die kunnen leiden tot vraatremming te onderscheiden en de bioassays hier op aan te passen.
   Dit proefschrift.

3. Het begrip 'Central Inhibitory State' is theoretisch en dient, met name wat betreft de fysiologische oorzaak, nader te worden omschreven om de waarde van dit begrip in het begrijpen van de voedselplantkeuze door fytofage insecten te kunnen inschatten.
   Dethier V.G., R.L Solomon & L.H. Turner, 1968. Central inhibition in the blowfly. *J. Comp. Physiol. Psychol.* 60: 144-150; Jermy T., 1971. Biological background and outlook of the antifeedant approach to insect control. *Acta Phytopath. Hung.* 6: 253-260.

4. Wanneer drimanen als 'warburganal' en 'polygodial' als behorend tot de meest actieve vraatremmende stoffen worden beschouwd is dit niet veelbelovend voor het gebruik van vraatremmende stoffen als vervangers van insecticiden in de gewasbescherming.
   Warthen Jr. J., 1990. Insect feeding deterrents, Part A: Insect feeding deterrents (1976-1980). *In:* E.D. Morgan & N.B. Mandava (eds), CRC Handbook of natural pesticides. Vol VI, Insect attractants and repellents. CRC Press, Boca Raton, Inc., pp. 23-82; Dit proefschrift.

5. Gezien de veelvoud aan afweerstrategieën van planten in de natuur is het niet verwonderlijk dat bij gebruik van uit de natuur afgeleide gewasbeschermingsmaatregelen doorgaans combinaties nodig zijn voor de gewenste bescherming tegen insecten.

6. Over de smaak van insecten valt te twisten.
   Dit proefschrift.

7. Continuïteit in onderzoek is voor veel wetenschappers alleen mogelijk door het regelmatig te onderbreken voor het werven van fondsen.

8. Het toekomstperspectief van wetenschappers in Duitsland is niet veel beter dan in Nederland, en wordt getypeerd door de aspecten 'graue Haare und finanziëlle Fragen'
   Nach A. Jourdan, 1998.

9. Gezien het lage percentage vrouwen in hogere wetenschappelijke functies houdt de creativiteit van veel wetenschappers op zodra het moet komen tot een herverdeling van arbeid en zorg.

10. De onderbelichting van vrouwen-topsport door de media wijst er helaas op dat we nog steeds in een mannenwereld leven.

11. De gemoedstoestand van een 'internet-surfer' wordt vooral bepaald door de snelheid van zijn computer.
    M. de Waal. Maakt internet echt depressief? *De Volkskrant*, 5 september 1998.

12. Bij mensen die tijd hebben om over 'onthaasting' mee te praten valt de stress kennelijk wel mee.

13. De gedachte dat je na de dag van de promotie voldaan achterover kunt leunen houdt je in de periode ervoor overeind.

Stellingen behorende bij het proefschrift 'Antifeedants against insects: a behavioural and sensory study'.

Lindy Messchendorp, Wageningen, 23 oktober 1998.

Voorwoord

Wetenschap is iets wat je over het algemeen niet alleen doet. Dat is bij mij ook zeker niet het geval geweest en daarom wil ik in dit voorwoord nog graag even op een rijtje zetten welke personen hebben bijgedragen aan het tot stand komen van dit boekje en aan wie ik dank verschuldigd ben.

Allereerst heeft dit onderzoek van het begin af aan deel uitgemaakt van een gezamenlijk project met de vakgroep Organische Chemie, genaamd "Vraatremmende stoffen voor insecten: chemie, biologie en toepassing". Op de vakgroep Organische Chemie werden de vraatremmende stoffen gesynthetiseerd, terwijl wij op de vakgroep Entomologie deze stoffen toetsten op hun activiteit. De hoofdrolspelers in dit geheel waren Rinie Bouwman, Edwin klein Gebbinck, Rieta Gols en ikzelf. Hen wil ik bedanken voor de altijd prettige en vriendelijke samenwerking. Rieta, veel van het in dit boekje beschreven werk is door jou uitgevoerd, en ik wil je dan ook speciaal bedanken voor al die jaren directe samenwerking, waarin je het steeds weer klaarspeelde om toetsen nóg efficienter en sneller uit te voeren, en waarin je ook nog jezelf wist te ontplooien in het entomologische onderzoek. Edwin, jouw doorzettingsvermogen, grote inzet en -kunde hebben er voor gezorgd dat we toch nog een groot aantal stoffen hebben kunnen testen waarvan de synthese zeer-veel-staps en bijzonder veel moeilijker was dan aan het begin was ingeschat. Rinie, jij hield het hoofd koel en bezorgde ons vooral in het begin van het projekt veel variaties op de drimaan terpenoïden. De begeleiding van dit onderzoek lag in handen van Ben Jansen, Æde de Groot, André Stork, Joop van Loon en Louis Schoonhoven. Zij hebben mij altijd gesteund en van goede adviezen voorzien, waarvoor mijn hartelijke dank. Joop, jij stond altijd klaar om over het onderzoek te discussiëren en stimuleerde me om me in de interessantere aspecten verder te verdiepen. Louis, jouw ervaring en interesse in dit onderzoeksgebied waren bijzonder hulpzaam, en vaak wist je me nog de betere stukken uit eerdere literatuur aan te reiken. Tot slot was het project gefinancierd door de Stichting voor Technische Wetenschappen, en voorzien van een gebruikerscommissie die, buiten de al genoemde mensen, bestond uit Paul Harrewijn, J. Henfling, A. Kerkenaar, C. Mombers en J. Moskal. De vele vergaderingen waren altijd erg gezellig en er werden vaak goede hints gegeven voor verder onderzoek; mijn hartelijke dank daarvoor.

Naast de directe medewerkers van het project zijn er nog vele mensen zonder wie dit onderzoek niet mogelijk geweest zou zijn. Te noemen zijn de mensen van de insectenkweek, Leo Koopman, André Giddink en Frans van Aggelen, die ons altijd vele rupsen en kevers bezorgden. De medewerkers van Unifarm, die ons steeds van gezonde planten voorzagen. De administratieve medewerkers van de vakgroep, Ans Klunder, Riet Tilanus, Truus de Vries en Ineke Kok, die op elk moment zeer snel van alles organiseerden. Wim Frentz, ik wil je bedanken voor je niet aflatende technische ondersteuning en je altijd goede humeur. I would like to thank the hungarian student Peter Perczel, for his contribution in the development of the biotest for aphids. Hans Smid wil ik bedanken voor de samenwerking in het Coloradokever-smaakzintuig project: je produceerde prachtige foto's waarvan de mooiste uiteindelijk is opgenomen in een zeer mooie publicatie. Ook is

dank verschuldigd aan Gerrit Gort voor de hulp bij de statistische verwerking van de gegevens in hoofdstuk 3. Verder dank ik alle medewerkers van de vakgroep Entomologie, de mensen van Duo-tone voor het helpen bij het realiseren van wilde creatieve ideëen, de bibliotheek, de Gecombineerde diensten, het kantinepersoneel: fijn dat jullie er altijd waren om bij te springen.

De sfeer op de vakgroep Entomologie is altijd erg goed en heeft bijgedragen aan een prettige werkomgeving. Vele gelegenheden worden aangegrepen om gezamenlijk te schaatsen, hardlopen, uitbundig te vieren, enz. enz., wat zorgt voor een vriendschappelijke sfeer, ook buiten het werk. Op het persoonlijke vlak ben ik altijd gesteund door vrienden en familie. Ik wil mijn ouders bedanken, die mij de mogelijkheid hebben gegeven om te studeren. Er zijn vele vrienden die ervoor zorgen dat we genieten van het leven. Voelt jullie vooral aangesproken! Herman, jouw naam kan niet ongenoemd blijven, ook in dit stukje werk is jouw bijdrage onbeschrijfbaar: bedankt!

Contents

1

General introduction

Antifeedants against insects: a condensed story

It is not difficult to guess the meaning of the word 'antifeedant'; a substance that, in some way, stops insects from feeding on plants, without killing them (Ascher, 1970/71). The first antifeedants were identified already in the 1930's (*e.g.* Metzger & Grant, 1932; Guy, 1936). Antifeedants were originally isolated from plants that were known as being unpalatable for many insect species. Antifeedants are also called 'feeding inhibitors' (Jermy, 1966) *or 'feeding deterrents'* (Dethier *et al.*, 1960). During the past decades, many researchers have been working on antifeedants, thereby producing many papers. What makes insect antifeedants so interesting?

Antifeedants can attract researchers' interest for several reasons. Firstly, simply because they were identified in plants. Biologists are interested in the 'why' and 'how' questions of all natural events, originating from a general interest in all 'living things' and the products that are produced in nature. Regarding antifeedants, specific questions can be asked, such as:

- why do they occur in plants? (what is their function?)
- which role do they play in the interaction between insects and the plants on which the insects feed and live?
- how do they exert their effects?
- which role have they been playing in the evolution of insects and plants?

Research emanating from such questions is called 'fundamental research'.

A second reason why probably many researchers are interested in antifeedants is their possible economical value; substances that stop insects from feeding could be used to protect agricultural crops against feeding injury and thereby increase the yield. The interest in alternatives for crop protection is growing, especially because for environmental reasons conventional insecticides are refused more and more. Research emanating from this kind of interest is called 'applied research'.

This thesis describes research on 'terpenoid' antifeedants against several insect species. The interests underlying this study were partly of 'applied' and partly of 'fundamental' nature. We were interested in estimating the potential effectiveness of the antifeedants tested. We compared the feeding inhibiting effects of specific molecular structures. We were also interested in how antifeedants influence the behaviour of insects and how insects perceive antifeedants through their sensory taste system. Answering these questions could help in understanding the role of antifeedants in insect-plant interactions, and in estimating the potential antifeedant efficacy of candidate compounds (Bernays & Weiss, 1996). In this chapter, some aspects of the research field on antifeedants are introduced.

The role of antifeedants in insect-plant biology

When inspecting plants from closeby, very often insects and traces of insect feeding will be met with as well. This is not surprising, when it is realized that by far the highest number of organisms belong to the class of insects, and that about half of all insect species feed on plants (May, 1988). The fact that plants, despite all these herbivorous insects, still grow abundantly worldwide, indicates that they can deal with feeding insects very well. In fact, plants are shown to possess an enormous variety of mechanisms to defend themselves against insects, or even profit from them. On the other hand, insects show a spectacular diversity in the way they are adapted to different plant species, to profit as much as possible from them, or to overcome defence mechanisms.

In the research field of insect-plant interactions all questions are studied concerning the influences that insects and plants exert on each other during their lives. As these can be very diverse, research diverged in many directions. Examples are nutritional value of plants for insects, resistance (or defence mechanisms) of plants against insects, feeding- ovipositing- or mating behaviour of insects on plants, host plant specialisation of insects, sensory recognition of plants by insects, pollination of plants by insects or predators attacking plant feeding insects (for further reading *e.g.* Miller & Miller, 1986; Rosenthal & Berenbaum, 1991; Bernays, 1989-1994; Bernays & Chapman, 1994; Chapman & De Boer, 1995; Schoonhoven *et al.*, 1998).

Antifeedants are generally considered to play an important role in insect-plant interactions, especially in host plant recognition and host plant specialisation of herbivorous insects. In this respect, herbivorous insect species can roughly be classified into three categories *i.e.* monophagous, oligophagous or polyphagous. Monophagous insects feed on only one, or a few related plant species, oligophagous insects feed on a number of plant species, mainly belonging to the same family (the large white butterfly, *Pieris brassicae* and the Colorado potato beetle, *Leptinotarsa decemlineata*, both subject of research in this thesis, fit into this category) and polyphagous insects feed on many different plant species belonging to different families (the green peach aphid, *Myzus persicae* and the cotton aphid, *Aphis gossypii*, both examined in this thesis, belong to this group). The majority of all herbivorous insect species though, are oligophagous and specialize on only a few plant species. How do insects recognize their host plant(s) out of a tremendous offer of different plant species?

In the beginning of this century, it was shown for the first time that the recognition of host plants by herbivorous insects is largely directed by chemical information. It appeared that, when insects encounter plants while searching for food, chemical information obtained from the plant is decisive in either accepting or rejecting it as food plant. Verschaffelt (1910) showed that larvae of *Pieris* species would only feed on plants containing or treated with glucosinolates, chemicals typically occurring in their host plants. Glucosinolates are so-called 'token stimulants' for *Pieris* species in recognizing their host plants. Later, other researchers (*e.g.* Jermy, 1966) found that insects would only feed on plants that contain no or little antifeedants; host plants are recognized by their lack of antifeedants. Since the presence of 'token stimuli' in many host plants could not be demonstrated, it seems plausible that in nature antifeedants play a major role in insects' decisions on whether or not to feed.

Finally, the question remains how so many insect and plant species could evolve during evolution. Several evolutionary theories have been proposed, in which antifeedants play a prominent role. In rough lines, the role of antifeedants can be explained as follows: Most antifeedants belong to the class of secondary plant chemicals. This means that they have no function in the production of primary, vegetative and reproductive parts of plants. Many secondary plant chemicals, among which insect antifeedants, play a role in the defence of plants against natural enemies, such as fungal- and bacterial diseases or herbivores (van Genderen *et al.*, 1996). The presence of secondary compounds in plants is generally believed to increase the fitness of plants, *i.e.* the chance to successfully reproduce, although the production of secondary compounds does have metabolic costs as well (for costs of terpenoid accumulation see *e.g.* Gershenzon (1994)). Because antifeedants are thought to play a major role in host plant selection, it is probable that they also have been playing an important role in the evolution of insect-plant relationships, and especially in the host plant specialisation of insect species. It can be reasoned that plants producing antifeedants exert effects on insects that negatively influence reproduction, and in contrast, positively influence insects that adapt to antifeedants by avoiding them or by developing other mechanisms to overcome the negative effects of antifeedants. Negative effects of antifeedants can be postingestive, toxic effects, or furnishing plants with a to the insect unacceptable taste. These effects cause an increased time spend on food searching, resulting in slower growth and increased vulnerability to predators. Plants producing antifeedants thus provide diversifying pressure on insects, allowing them to form new species. This reasoning could be reversed as well, by proposing that insects provide diversifying pressure on plants, by reducing the fitness of the most vulnerable plants.

How do antifeedants influence the feeding behaviour of herbivorous insects?

The term 'antifeedant' applies to all chemical compounds that inhibit feeding in insects. However, there are different 'mechanisms of action' through which feeding inhibition can be established, divided into two categories:

1) antifeedants that inhibit feeding through sensory perception, *i.e.* compounds having an unpalatable taste to insects.
2) antifeedants that inhibit feeding by postingestive, toxic effects resulting in sick insects without appetite.

During the first decades of antifeedant research, antifeedants were mainly considered to act through sensory perception (*e.g.* Jermy, 1966; Wright, 1967; Chapman, 1974). Later on, it was established that plant compounds can inhibit feeding through postingestive effects as well (*e.g.* Berenbaum, 1986; Mordue & Blackwell, 1993; Frazier & Chyb, 1995; Glendinning, 1996).

Antifeedants can act through one, or both of these types of mechanisms of action. However, for the majority of identified antifeedants, the mechanism(s) of action are not yet elucidated. The mechanism of action of antifeedants varies between insect species.

Taste perception of antifeedants

Many researchers are interested in questions as 'how do insects recognize their host plant(s) with their sensory taste system' and 'how does the sensory taste system respond to antifeedants'. Knowing the answers to these questions could help in understanding how the behaviour of insects is influenced by the chemical composition of the plants, and in understanding the reason why many insects specialize on a few, or only one host plant. During the past decennia, knowledge of the sensory perception of antifeedants has expanded enormously, thanks to the invention of innovative techniques. However, the majority of the research on the sensory taste perception of antifeedants has been restricted to only several insect species, and is dominated by studies on lepidopterans (caterpillars of butterflies), followed by *e.g.* studies on orthopterans (grasshoppers and locusts), coleopterans (beetles), hemipterans and homopterans (*e.g.* aphids). Consequently, the present theories on host plant recognition and host plant specialisation by insects are based on studies of a limited number of insect species.

Figure 1 schematically shows how insects decide whether or not to feed on the basis of information they perceive about the chemical composition of a plant:
After having approached a potential food plant, herbivorous insects mostly start palpating the leaf surface, followed by taking some test bites and eventually feeding. In the case of a non-host plant, or when a plant is treated with antifeedants, initiation of feeding stops at some moment during this process because sensory information on the unpalatable food source is received by the brain (central nervous system), where a rejection response is generated. Which physiological processes occur during the short time between palpating the plant and the rejection response?

Taste organs
For many insect species it is known that their sense of taste is located in conically formed, hair like structures (so-called 'taste hairs': Figure 1), or papilla like structures on the mouthparts. Ablation experiments, in which part of the sensory organs were operationally removed, have given useful information on the sensory organs that are involved in mediating feeding behaviour. For instance in *P. brassicae* larvae, Ma (1972) showed that the sensilla on the maxillary galea and in the mouth cavity are the relevant sensilla in directing feeding behaviour (Figure 2).

Insect species can vary considerably in the amount of chemosensory sensilla they possess. It is known that orthopterans possess numerous chemosensory sensilla on the maxillary palps and galea (between tens and thousands) and that lepidopterous caterpillars in general possess eleven sensilla on the palps and four sensilla on the galea (Chapman, 1995). Furthermore, contact chemoreceptors have been found on the legs and ovipositor of various insects as well. These can play a role in feeding, but can also have other functions, such as regulating oviposition behaviour (*e.g.* Roessingh *et al.*, 1992). In fluid-feeding insects, such as aphids, the chemosensory taste organs are organised quite differently. Aphids pierce their stylets into the plant tissue and feed on phloem sap. It is generally assumed that the most important sensory taste organ is located in the cibarial cavity (Wensler & Filshie, 1969; Tjallingii, 1995). No taste organs have been found on the legs and labium of aphids until now (Tjallingii, 1978), but Pickett

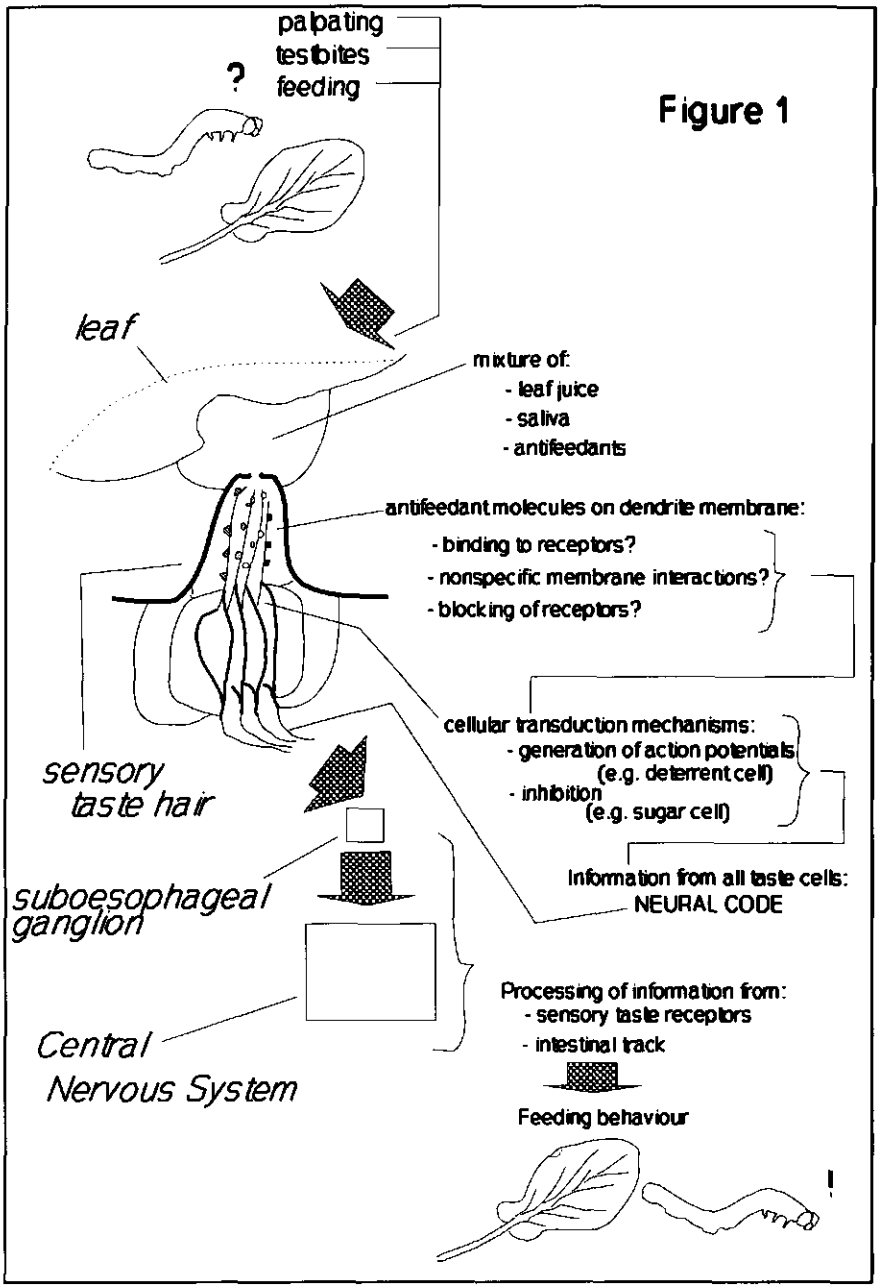

Figure 1. Schematic representation of the neural processes mediating feeding behaviour in herbivorous insects.

et al. (1992) mention that contact chemosensilla on the terminal processes of the antennae may be important in feeding behaviour as well.

Neural coding of antifeedancy

The chemosensory taste hairs contain sensory taste receptor cells of which the dendrites, while feeding, come into contact with plant chemicals (Figure 1). These plant chemicals enter the taste hairs through a small pore at the tip. Upon this, electrical signals ('action potentials') are produced by the sensory taste receptor cells. In many insect species, taste sensilla possess four taste receptor cells, together with one mechano receptor cell (but sensilla with more or fewer receptor cells do also occur). In 1955, Hodgson *et al.* invented a 'tip-recording technique', that made it possible to directly measure the electrical signals through a stimulus solution containing an electrolyte and the plant chemicals under investigation.

By use of the tip-recording technique a sensitivity range of the taste receptor cells in taste hairs was established for several insect species. In most species studied, one of the four cells is sensitive to sugars (the 'sugar cell') and a second to inorganic salts (the 'salt cell'), although the sensitivity range of these cells differs among species. The sensitivity of the remaining two cells varies considerably between species and is tuned to *e.g.* amino acids or deterrents (the 'deterrent cell'). Also cells specifically tuned to 'token stimulants' have been identified. For instance in *P. brassicae* cells sensitive to glucosinolates were found (Schoonhoven, 1967). The latter discovery provided a physiological basis for the concept of host plant recognition through 'token stimulants' by specialist herbivorous insects.

After action potentials are generated in the different sensory taste cells the sensory information is sent to the brain via a sensory nerve. This information, the ensemble of electrical signals from sensory taste cells, is called the 'neural code'. According to Boeckh (1980), three possible neural coding principles can be recognized: 1) Labelled line coding, meaning that the information from one specific receptor cell can elicit specific behaviours, such as initiation of feeding, or a rejection response; 2) Across-fibre pattern coding, meaning that the information to the brain is contained in the 'response pattern' of several receptor cells with different sensitivity spectra and 3) Temporal pattern coding, meaning that information on the stimulus quality is passed on through action potential interval patterns, or adaptation rates. In most cases, chemosensory codes in insects are a combination of these three coding systems (Schoonhoven *et al.*, 1992).

Neural coding of antifeedancy varies considerably among insect species and antifeedants have been shown to affect sensory responses in at least five different ways (Schoonhoven, 1982): 1) stimulation of 'deterrent cells' tuned to diverse plant compounds that deter feeding; 2) stimulation of receptor cells with a broad sensitivity spectrum that includes secondary plant compounds; 3) inhibition of the response of receptor cells that are sensitive to feeding stimulants; 4) changing across-fibre patterns by stimulating some receptor cells and inhibiting others and 5) evoking irregular impulse patterns, often at high frequency (so-called 'bursts').

By using the 'tip-recording technique', researchers measure the neural code of various solutions with antifeedants, plant chemicals, mixtures of chemicals or of plant

juices. In this way, they try to find the neural code for acceptable- and unacceptable food. With such knowledge, one could screen many potential antifeedants by recording their electrophysiological response in a relatively short time, thereby circumventing laborious behavioural tests.

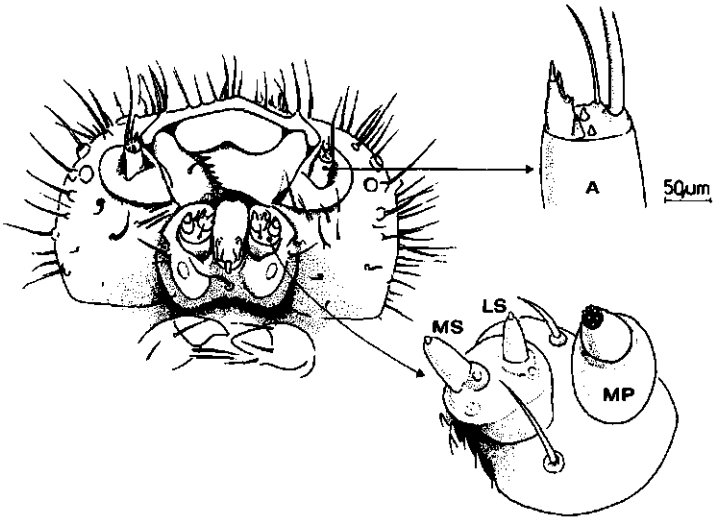

Figure 2. Line drawing of the head of a caterpillar seen from below with enlargements of an antenna (A) and a maxilla. MP = maxillary palp; LS and MS = lateral and medial sensilla styloconica.

Membrane receptors and cellular signal transduction mechanisms

After having entered the insect taste hair through the pore at the tip, antifeedant molecules interact with the dendrite membrane through as yet hardly known mechanisms. Most researchers believe that in insects receptor cell stimulating molecules interact with 'membrane receptors', although to date only two types of presumable receptor proteins binding to sugar have been found in flies (Ozaki *et al.*, 1993). Because, as mentioned above, antifeedants can affect sensory responses in at least five different ways, and because a large number of antifeedants with highly differing molecular structures exist, it is likely that antifeedants can interact with the dendrite membrane of receptor cells through multiple mechanisms. Additionally, from electrophysiological studies it is known that deterrent receptors often exhibit very broad sensitivity spectra (*e.g.* the medial deterrent receptor in *P. brassicae* (Ma, 1969), which suggests that they either possess many different membrane receptors or that different antifeedants interact in different ways with the dendrite membrane. Several mechanisms have been proposed for the interaction of antifeedants and feeding stimulants with the dendrite membrane (*e.g.* Wieczorek, 1976; Ma, 1981; Lam & Frazier, 1987; Fritz *et al.*, 1989; Mullin *et al.*, 1994). The concept of membrane interactions is very important for researchers that

search for highly active insect antifeedants. Different antifeedants interacting in the same way with the dendrite membrane may reveal a relationship between the molecular structure of an antifeedant and it' s feeding deterring activity (a so-called 'structure-activity relationship' (= SAR)), so that prediction of the effectiveness of new compounds would become possible. On the other hand, if indeed many different membrane interaction mechanisms exist, the chances for successfully developing SAR's for antifeedants decreases.

When antifeedant molecules in some way have interacted with the dendrite membrane of receptor cells, cellular transduction mechanisms are initiated. These result in a change in receptor membrane potential, leading to *e.g.* the generation of action potentials in cells sensitive to deterrents or inhibition of the action potential generating mechanisms in cells sensitive to feeding stimulants. On the nature of the cellular taste transduction events in invertebrates very little information is available. However, they are generally assumed to parallel those in vertebrate receptors (for further reading see *e.g.* Brand *et al.*, 1989). Only few articles deal with invertebrate taste transduction mechanisms, *e.g.* on the role of cyclic GMP as 'second messenger' in the excitation of the sugar receptor cell in the fly *Phormia regina* (Amakawa *et al.*, 1990). Recently, more work has been done on invertebrate olfactory transduction mechanisms (*e.g.* Raming *et al.*, 1993; Wegener *et al.*, 1997; Breer, 1997).

Processing of sensory information in the Central Nervous System

Feeding behaviour is ultimately directed by the Central Nervous System (CNS). Here, information from not only the chemical taste organs, but also from other body parts and from environmental factors is processed. Many factors can play a role in the direction of insect feeding behaviour, such as developmental state, degree of satiety, foodplant on which the insect was reared, temperature or light (Lewis & van Emden, 1986). This means that the behavioural response on antifeedants depends not only on its taste or postingestional effects, but also on additional factors, that should be standardized when comparing the response to an array of antifeedants.

There is no restricted area in the insect brain where information from taste receptors is processed, such as does occur for olfactory information (Hildebrand, 1995). Most axons from taste receptor cells directly project to the suboesophageal ganglion (Altman & Kien, 1987), that on its turn is connected to the brain. It is thought that major processing of taste information occurs in the suboesophageal ganglion (Blaney & Simmonds, 1987). A few models have been proposed for the processing of information in the CNS. Schoonhoven & Blom (1988) developed a 'labelled line' processing model on the basis of behavioural and electrophysiological experiments with *P. brassicae* larvae. In this model, action potentials originating from the cells responding to sugars, amino acids and glucosinolates (feeding stimulants) counteract action potentials originating from the deterrent cells, resulting in a positive or negative balance for the proceeding of feeding. However, this model will only work for insects that have taste receptor cells with very specific sensitivities. In many insects with a large number of taste sensilla (such as locusts and grasshoppers) the sensilla appear to be sensitive to a broad range of compounds from different classes *e.g.* sugars and salts. An example is the desert locust *Locusta migratoria*, in which the sensilla on the palps are sensitive to both fructose (a

feeding stimulant) and sodium chloride (a feeding deterrent), but differ in their relative sensitivities to these compounds. Blaney (1975) showed, by using analysis of variance on experimental data on the response of 20 sensilla, that the total output of these 20 sensilla, when stimulated with either fructose or sodium chloride, was distinguishable. This would indicate that the brain integrates complex information from many receptor cells on the quality of the food experienced to more simple information. Recently, with a multiple receptor model on taste discrimination in the blowfly, Nakao *et al.* (1994) showed that the discriminability of an individual taste receptor on the labellum or legs of the blowfly can be improved by the integration of 33-212 receptor cell responses at the central level, which confirms this hypothesis.

Influence of experience on the behavioural response to antifeedants

Many studies have shown that the behavioural response of insects to antifeedants can change after the first moment of exposure. Instances of a declining sensitivity, *i.e.* habituation, as well as an increasing sensitivity, *i.e.* sensitization, are known. Also 'food aversion learning' occurs in some insect species, meaning that the sensitivity to antifeedants increases over repeated exposures, separated by intervals of several hours to several days. The neural mechanisms responsible for these processes are unknown (Szentesi & Jermy, 1990; Bernays, 1995).

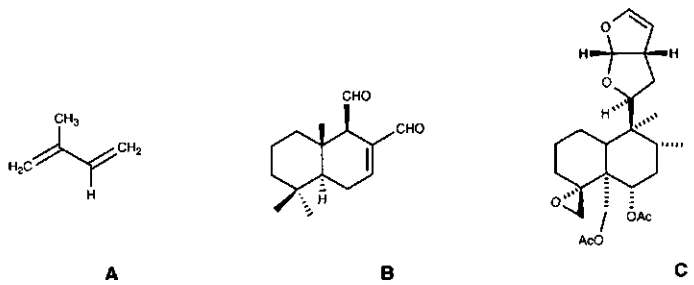

Figure 3. Molecular structures of (A) isoprene unit, (B) polygodial and (C) clerodin.

Origins of terpenoid antifeedants

The antifeedants studied in this work are all 'terpenoid' compounds or related derivatives, which means that their molecular structure is composed of several isoprene units (Figure 3 (A)). Drimanes are sesquiterpenes, composed of 3 isoprene units, and have a bicyclic structure. The first sesquiterpene dialdehyde isolated from plants (from *Polygonum hydropiper*, or 'water-pepper') was polygodial (Figure 3 (B)) (Barnes & Loder, 1962). Kubo *et al.* (1976a) showed that polygodial and other drimane sesquiterpenes exhibited

antifeedant activity against larvae of *Spodoptera littoralis* and *S. exempta*. *Neo*-clerodanes are diterpenes, composed of 4 isoprene units. The first *neo*-clerodane with a fully established structure was clerodin (Figure 3 (C)), isolated from *Clerodendron infortunatum* ('Indian bhat tree') (Harada & Uda, 1978; Rogers *et al.*, 1979; Luteijn, 1982). Kubo *et al.* (1976b) showed that clerodin and other *Neo*-clerodanes exhibited antifeedant activity against larvae of *S. littoralis* and *S. exempta*. A clear introduction to the biosynthesis of secondary plant compounds is given by van Genderen *et al.* (1996).

Practical use of antifeedants in crop protection

I already mentioned people's interest in antifeedants because of their potential use in crop protection. However, until now very few antifeedants have been successfully exploited commercially. Examples are products obtained from the indian neem tree, *Azadirachta indica*, that are highly effective in inhibiting feeding and can be obtained commercially in many countries (Schmutterer, 1995).

In The Netherlands, commercial sale of neem products for crop protection is still not allowed, because Dutch legislation on the use of chemical crop protection agents demands that all product ingredients are known, which can not be guaranteed when natural products are used. This is one of the reasons why organic chemists in the past decennia started to develop synthetic methods to obtain antifeedant compounds. Presently, many antifeedant compounds can be synthesized, such as polygodial, warburganal and other drimanes. All antifeedant compounds tested in the research described in this thesis were synthesized at the Laboratory of Organic Chemistry in Wageningen.

Synthetic antifeedants have some other advantages above natural products when their use in crop protection is aimed: they are readily available and large quantities can be provided; they can be delivered in pure form or in mixtures of which all ingredients are known (so that all ingredients can be tested in toxicity tests, that are required before crop protection agents can be admitted for use); also simple molecules, derived from natural antifeedants ('analogous derivatives'), can be synthesized and tested for their antifeedant effects, thereby possibly creating new, cheap antifeedant products.

Synthetic analogues of natural antifeedants can also be used to study the structure-activity relationship (SAR) of antifeedants in insects, by testing the antifeedant effectiveness of compounds with only slightly different molecular structures, *e.g.* structures differing on only one, or a few substituents. Studying the SAR of antifeedants can provide more insight in the question of how antifeedant molecules interact with the dendrite membrane of sensory taste cells when generating a food rejection response.

Outline of the thesis

In the research described in this thesis the effects of terpenoid antifeedants and related derivatives on the behaviour of several insect species were studied. The SAR of the compounds tested was investigated. Furthermore the perception of the antifeedants by

the different insect species, and long-term effects of the antifeedants were studied.

Aspects that received special attention in the studies were the neural coding of antifeedancy in the different insect species (*i.e.* do they stimulate 'deterrent cells', inhibit cells sensitive to feeding stimulants, evoke irregular activity in all receptor cells or disturb the temporal pattern of firing in receptor cells?) and the occurrence of habituation or sensitization after some time of exposure to the drimanes (*i.e.* do these phenomena occur, and are they related to possible toxicity of the antifeedants?). Knowledge on these aspects could assist in estimating the potential efficacy of the antifeedants against insects, and in enlightening the role of antifeedants in host plant selection.

In chapter 2 research on behavioural and sensory effects of drimane antifeedants on larvae of the large white butterfly *Pieris brassicae* L. (Lepidoptera: Pieridae) is described. *P. brassicae* is an oligophagous insect, of which the caterpillars feed on a number of plant species, mainly belonging to the Cruciferae family (Feltwell, 1982).
The aim of the work was to find a 'neural code' for antifeedancy by drimanes in *P. brassicae*. *P. brassicae* is used as a model insect for studying the sensory taste system in insects. The sensitivity spectra of its different sensory taste cells are known in considerable detail and hypotheses have been proposed on the function of the different taste organs in directing feeding behaviour. This makes *P. brassicae* a suitable insect for studying the perception of feeding deterrents.

In chapter 3, the mechanisms through which drimane antifeedants inhibit feeding in *P. brassicae* larvae are unravelled further. The temporal aspects of the feeding inhibiting effects of several drimanes are studied with aid of detailed, 1 min interval, behavioural observations.

Chapter 4 deals with research on behavioural and toxic effects of drimane antifeedants on larvae of the Colorado potato beetle, *Leptinotarsa decemlineata*. *L. decemlineata* (Say) (Coleoptera: Chrysomelidae) is an oligophagous insect feeding on several solanaceous plants. This insect has a remarkable history, as it suddenly expanded its host range with potato plants around 1840. After this, it spread rapidly throughout the United States and in 1922, also entered Europe. Nowadays the Colorado potato beetle is a major pest of potatoes worldwide, and has evolved resistance to virtually every insecticide used against it (Bishop & Grafius, 1996).

Research on the perception of drimane antifeedants in larvae of the Colorado potato beetle is described in chapter 5. Although the sensory taste system of Colorado potato beetle has been studied extensively, no sensory taste cells specifically tuned to feeding deterrents had been found, so that it was unclear how these insects perceive feeding deterrents.

Chapter 6 presents research on nymphs of two polyphagous aphid species, the green peach aphid *Myzus persicae* (Sulzer) and the cotton aphid *Aphis gossypii* (Glover) (Homoptera: Aphididae). Both species are important pests on many crops throughout the world, especially *A. gossypii*, which rapidly exhibits resistance against many insecticides. Because aphids do not ingest leaf material but feed on plant juices by penetrating plants with their stylets, a different approach is needed to study their feeding behaviour. Research is described on behavioural effects of drimane antifeedants and the putative location of sensory taste organs through which these aphid species perceive antifeedants.

Chapter 7 reports on feeding inhibiting effects of several drimanes on a number of insect species, *i.e.* larvae of *Spodoptera exempta*, *S. exigua*, *Mamestra brassicae*, *P. brassicae* and *L. decemlineata* and nymphs of *Locusta migratoria*.

In chapter 8, the feeding inhibiting effects of a group of analogous derivatives of partial structures of *neo*-clerodanes are examined. Behavioural effects are reported for larvae of *P. brassicae* and *L. decemlineata* and nymphs of *M. persicae*.

Finally, in chapter 9 the main conclusions of the studies are discussed and prospects for future research are given.

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2

Behavioural and sensory responses to drimane antifeedants in *Pieris brassicae* larvae

Abstract

15 Drimane compounds were tested for their feeding inhibiting activity in larvae of *Pieris brassicae* L. (Lepidoptera: Pieridae) when applied to leaf material of the hostplant *Brassica oleracea* L. The antifeedant efficacy of the drimanes was related to their molecular structure in order to identify important functional groups. Of the drimanes tested, those with a lactone group on the B-ring were the most effective feeding inhibitors. Additionally, the sensory responses to 13 of the drimanes were measured. Neural activity was evoked in the deterrent cell in the medial sensillum styloconicum. Also, inhibition of sensory responses to feeding stimulants was found. Results of behavioural and electrophysiological tests were correlated in an attempt to elucidate the sensory code underlying feeding inhibition by drimanes in *Pieris brassicae*. It was concluded that the response of the deterrent cell in the medial sensillum styloconicum contributes significantly to inhibition of feeding behaviour in larvae of *Pieris brassicae*.

Introduction

In many herbivorous insect species the decision to accept or reject a food plant is strongly influenced by the absence or presence of antifeedants in the plant tissue (Jermy, 1966; Bernays & Chapman, 1994). Therefore, great interest is shown in antifeedant compounds that could be applied to plants and give protection against feeding by insects.

Several authors have documented that drimanes, originally isolated from plants *e.g.* from *Polygonum hydropiper* (Barnes & Loder, 1962) inhibit feeding to various degrees in several insects, *e.g. Heliothis* and *Spodoptera* species (Kubo *et al.*, 1976; Ma, 1977; Blaney *et al.*, 1987), aphids (Gibson *et al.*, 1982; Pickett *et al.*, 1987; Asakawa *et al.*, 1988) and *Pieris brassicae* (Schoonhoven & Yan, 1989).

Electrophysiological studies on the effects of drimanes showed that these compounds stimulate 'deterrent cells' to various degrees in several insects, *e.g. Heliothis* and *Spodoptera* species (Blaney *et al.*, 1987) and *P. brassicae* (Schoonhoven & Yan, 1989). When mouthpart sensilla of caterpilars were stimulated with drimanes for one or more min continuously, interference with subsequent responses of receptor cells sensitive to feeding stimulants was found and irregular firing in several neurons occurred, *e.g.* in *Spodoptera exempta* (Ma, 1977), *Manduca sexta* (Frazier, 1986) and *P. brassicae* (Schoonhoven & Yan, 1989).

During the past decades much work has been done on screening potential antifeedant compounds for their effectiveness using time-consuming behavioural assays, which require large quantities of the test compounds. To develop tests that are less time-consuming and that require smaller quantities of test compounds, more insight in the sensory code underlying feeding inhibition in insects will be needed. In the present paper

15 synthetic drimanes were applied to leaf material of *Brassica oleracea*, a hostplant of *P. brassicae*, and tested for their feeding inhibiting activity on fifth instar larvae in a dual choice situation. The sensory responses to 13 drimanes were also measured and correlated with behavioural responses, in an attempt to elucidate the sensory code underlying feeding inhibition by drimane antifeedants in larvae of *P. brassicae*. Also the relation between molecular structure and feeding inhibiting activity was studied in order to identify those functional groups on the drimane structure that were important for inhihbitory activity.

Materials and methods

Insects. Larvae of *P. brassicae* were reared on cabbage plants (*Brassica oleracea* var. gemmifera cv. Titurel) in L16:D8 at 22±3°C and r.h. 40-70%. For the experiments 24-72 h old fifth instar larvae were used.

Antifeedants. The 15 drimanes were synthesized at the Department of Organic Chemistry, Wageningen Agricultural University (Jansen, 1993; C.T. Bouwman, unpubl.) (Figure 1). Because of limited solubility in water the drimanes were first dissolved in ethanol after which distilled water or 5 mM KCl in distilled water was added, to obtain a final ethanol concentration of 2%. For the behavioural tests drimane concentrations of 1 and 5 mM in distilled water were used. A detergent (Tween-80, 2%) was added to promote an even distribution of the solution on the leaf discs. Distilled water with 2% ethanol and 2% Tween-80 served as the control solution. For electrophysiological tests drimanes were dissolved in ethanol and diluted with 5 mM KCl to final drimane concentrations of 0.1 and 0.5 mM, in 2% ethanol. Five mM KCl with 2% ethanol served as control solution.

Dual choice tests on leaf discs. Six cabbage leaf discs (area 3.80 cm^2/disc) were arranged circularly in a glass petri-dish. The upper surface of alternate discs was painted with 10 µl drimane or control solution, after which they were left to dry for 30 minutes. Larvae were placed individually in petri-dishes in a climatic chamber at a temperature of 25 °C, illuminated with 2 fluorescent tubes (36 W) at a distance of 5-50 cm. After three hours of *ad libitum* feeding the remaining disc areas were measured with a leaf-area meter (Hayashi Denko Co. Ltd., Tokyo, Japan). The areas consumed were determined by subtracting the remaining areas from the mean area of 3 reference discs on which no feeding had taken place during the test and that served as shrinkage controls. An antifeedant index (A.I.) was calculated: A.I. = (C-T)/(C+T), (area consumed from control discs (C) minus area consumed from treated discs (T))/(total area consumed), ranging from -1 (feeding stimulation) to +1 (feeding inhibition). Drimanes became available at intervals and were immediately tested after delivery, so that tests were performed over a time span of 1.5 years. Within this period each drimane was tested one to four times. Not all drimanes could be tested repeatedly because of limited availability. Wilcoxon's matched pair signed rank test was used to assess significance. The efficacy of the various antifeedants tested at 5 mM was compared by the nonparametric Kruskal-Wallis

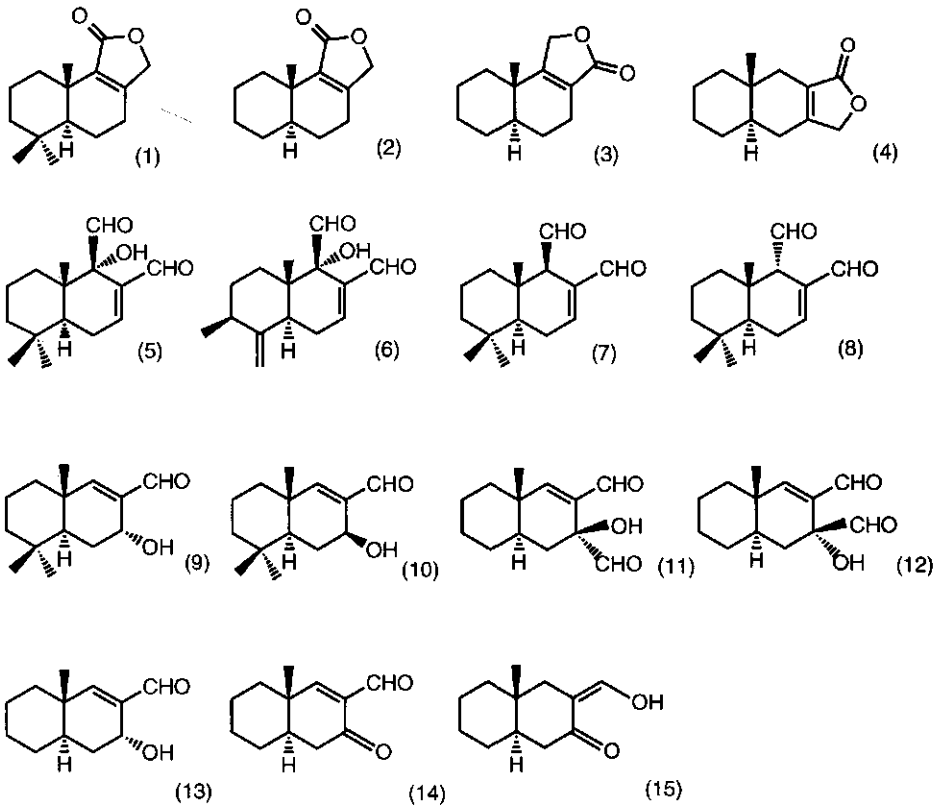

Figure 1. Molecular structures of the 15 drimanes that were tested for their antifeedant activity. Trivial names: (1) isodrimenin, (8) isotadeonal, (6) muzigadial, (9) polygonal, (5) warburganal, (7) polygodial, (10) isopolygonal.

one-way analysis by ranks followed by a multiple comparison procedure (Conover, 1971).

Electrophysiology. The tip recording technique as described by Hodgson *et al.* (1955) and modified by van Loon (1990), was used to record responses to the various stimuli from the sensilla styloconica on the maxillary galea. Larvae were starved for 1 to 3 hours before the experiments in order to improve the signal to noise ratio in the recordings. Isolated insect heads were mounted on a silver wire electrode which was connected to the input probe of an amplifier (Syntech UN-03b, Hilversum, The Netherlands). Stimulus

solutions were provided in glass capillaries (tip diameter *c.* 30 µm) connected to ground to serve as reference electrode. Amplified signals were digitized (DAS 16 Metrabyte Co. AD conversion board) and sampled into a computer (Intel 486 DX) memory at 10 or 16 kHz sampling frequency. Many insects showed an irregular delay (up to 500 ms) in their electrophysiological response to the drimanes. Therefore, impulses during the first 1.5 s (in stead of the usual 1.0 s) of stimulation were counted with the aid of Sapid Tools computer software (Smith *et al.*, 1990). The spikes were sorted visually by the experimenter on the basis of shape and temporal pattern of firing.

The medial and lateral maxillary sensilla styloconica were stimulated with solutions of 13 compounds to investigate the sensory response. Each compound was tested on 1 - 3 days with 3-20 larvae per test.

To test whether drimane antifeedants exerted shortterm effects (within the first 1.5 s of stimulation) on the response of receptor cells sensitive to feeding stimulants, glucose or glucotropaeolin was mixed with a drimane antifeedant. Compound 4 was used after it had been established that it was one of the best antifeedants.

Correlation of behavioural and sensory responses. To investigate a possible relationship between neural input and feeding inhibition, the responses of the deterrent cell to 13 drimanes were correlated with the corresponding antifeedant indices using weighted means of the repeated tests. The results of the behavioural tests using 1 mM and 5 mM drimane solutions were related to the outcome of the electrophysiological tests performed with 0.1 mM and 0.5 mM solutions respectively, because it is assumed that the drimanes applied to the leaf discs would be diluted *c.* 10 times by the leaf contents.

Results

Behavioural response. Five drimanes significantly inhibited feeding at 1 mM (Table 1A). Of the 15 drimanes tested at a concentration of 5 mM, 12 showed significant feeding inhibitory effects (Table 1B). The ranking order of effectiveness is different for the two concentrations tested. The most potent antifeedants were structures with a lactone group on the B-ring (compounds 1, 2, 3 and 4, although 4 had a relatively low effect at 1 mM).

Sensory response to pure antifeedants. The deterrent cell in the medial sensillum styloconicum (Ma, 1972; Blom, 1978; Schoonhoven & Yan, 1989) was excited by stimulation with drimane solutions (Figure 2). This is concluded from the fact that two spike types were recognizable in responses to mixtures of a drimane with either sucrose or glucotropaeolin. The latter compounds are known to evoke monocellular responses from the sugar cell and the glucosinolate cell respectively (Schoonhoven, 1987). The responses of the deterrent cell ranged from *c.* 20 to *c.* 150 impulses / 1.5 sec (Table 2). Compound 6 evoked the strongest response from the deterrent cell at both concentrations (0.1 mM and 0.5 mM). As in the behavioural tests, the ranking order of response intensity from the deterrent cell is somewhat different for the two concentrations tested. The control solution evoked only low and irregular activity. The lateral sensilla styloconica did not respond to the drimanes.

Table 1. Results of the standard dual-choice test on leaf discs. Behavioural responses are expressed as antifeedant index (A.I.) = (C-T)/C+T), C = area eaten from control discs, T = area eaten from treated discs. Results of repeated experiments are given separately, ranking from the highest results on the left to the lowest results on the right: (A) 1 mM treatment, (B) 5 mM treatment.

Compound		A.I. (s.e.)	n	A.I. (s.e.)	n	A.I. (s.e.)	n	A.I. (s.e.)	n	weighted mean
(A) 1.		0.493 (0.13)**	11	0.468 (0.11)**	11	0.229 (0.14)	11			0.397
8.		0.452 (0.08)**	12	0.408 (0.09)**	19	0.204 (0.14)	12			0.363
6.		0.444 (0.12)**	7	0.203 (0.14)	12	0.105 (0.12)	12			0.292
2.		0.305 (0.12)*	19	0.231 (0.11)	18	0.213 (0.08)*	19	0.127 (0.08)*	20	0.213
3.		0.182 (0.14)	17							0.182
12.		0.172 (0.09)	20							0.172
11.		0.150 (0.13)	20							0.150
5.		0.192 (0.13)	11	0.159 (0.11)	12	0.104 (0.11)	19			0.142
9.		0.223 (0.11)	12	0.113 (0.07)*	20	0.089 (0.13)	12			0.136
7.		0.101 (0.10)	20							0.101
4.		0.087 (0.16)	12							0.087
13.		0.069 (0.10)	20							0.069
10.		0.062 (0.10)	12	0.025 (0.19)	12					0.044
14.		0.019 (0.09)	20							0.019
15.		-0.001 (0.06)	19							-0.001
(B) 1.	a	0.881 (0.08)**	11							0.881
2.	a	0.821 (0.08)**	12	0.765 (0.05)**	20	0.763 (0.05)**	20			0.777
4.	b	0.603 (0.06)**	19	0.577 (0.16)**	12					0.593
3.	bc	0.732 (0.09)**	12	0.584 (0.07)**	20	0.444 (0.08)**	20			0.564
7.	bcd	0.616 (0.07)**	20	0.449 (0.12)**	15					0.544
6.	bcd	0.558 (0.10)**	12							0.558
10.	bcd	0.632 (0.11)**	12	0.289 (0.14)	12					0.461
12.	cd	0.483 (0.19)*	12	0.459 (0.09)**	19	0.318 (0.09)**	20			0.409
8.	d	0.452 (0.12)*	12	0.443 (0.07)**	20	0.365 (0.09)**	20			0.415
9.	de	0.482 (0.13)**	12	0.340 (0.08)**	20					0.393
5.	ef	0.374 (0.12)	19	0.265 (0.09)**	20	0.203 (0.09)*	20	0.117 (0.11)	20	0.238
11.	fg	0.269 (0.09)**	20	0.103 (0.12)	20					0.186
14.	fg	0.116 (0.11)	20							0.116
15.	fg	0.139 (0.09)	20	-0.043 (0.14)	20					0.048
13.	g	0.016 (0.08)	20							0.016

s.e. = standard error of mean; n = number of replicates; * $p<0.05$, ** $p<0.01$, Wilcoxon's matched pair signed rank test; multiple comparison: after Conover (1971), based on a Kruskal-Wallis one-way analysis by ranks ($p<0.05$), means not accompanied by identical letters are significantly different.

Inhibition of sensory response to feeding stimulants. Compound 4 mixed with glucotropaeolin depressed the response of the lateral and medial glucosinolate sensitive neurons significantly compared with glucotropaeolin alone (Figure 3A,B). When admixed with sucrose, compound 4 did not significantly inhibit the response of the sugar sensitive neurons, although the responses of the medial sugar cells to the mixture varied consida-

rably: in some individuals the response to sucrose was almost completely inhibited while in others no noticeable decrease occurs (c.v. (coefficient of variation) = *c.* 50%) (Figure 4 A,B). Conversely, the response of the deterrent cell to these mixtures is slightly enhanced (Figures 3C and 4C), which may be ascribed to as yet unknown peripheral interactions.

Correlation of behavioural and sensory responses. Feeding inhibition correlates significantly with the response of the deterrent cell (rho = 0.59, $P < 0.005$, Spearman's rank correlation test: Figure 5).

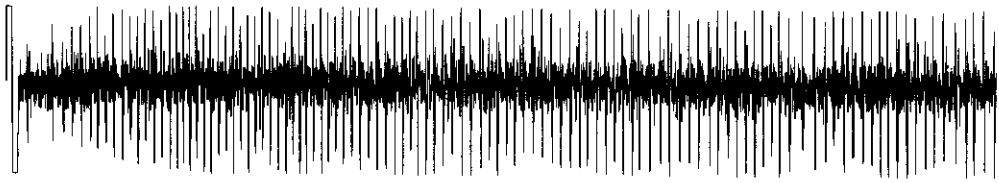

Figure 2. First 1.5 second of the response of the medial deterrent cell to compound 4, concentration 0.5 mM.

Discussion

The drimanes with a lactone group on the B-ring appear to be the most potent antifeedants at 5 mM. It is clear that different substituents on the A-ring can result in varying antifeedant activity (compare for instance compounds 5 and 6 or compounds 9 and 13 at 5 mM), although the absence of the two methyl substituents on C4 does not influence the efficacy of compound 2 when compared to compound 1 at 5 mM. As described by Schoonhoven (1988), chiral differences appear to cause differences in effect on feeding behaviour (compare *e.g.* compounds 2 with 3, or compound 11 with 12 at 5 mM, although not in compounds 7 and 9 compared to compounds 8 or 10 (5 mM), respectively. We note a large variability in outcome of the behavioural tests performed on different days (Table 1), as was reported before (Bernays & Wege, 1987). Since most of the drimanes do not exert a significant feeding inhibiting effect at 1 mM, it is clear that these drimanes are not potent antifeedants for *P. brassicae*.

Because previous research showed that the maxillary sensilla styloconica play an important role in mediating feeding behaviour (Ma, 1972; Blom, 1978), an attempt was made to derive the neural code underlying feeding inhibition by drimane antifeedants in *P. brassicae*. The positive correlation between feeding inhibition and response of the deterrent cell suggests that the latter exerts a direct inhibitory effect on the feeding center of the CNS. However, the strongest feeding inhibitors (compounds 1, 2, 3 and 4), do not evoke the strongest response from the deterrent cell; this suggest that other

Table 2. Sensory responses of the deterrent cell to stimulation with (A) 0.1 mM and (B) 0.5 mM drimane solution, expressed as imp (numbers of impulses in the first 1.5 seconds of stimulation). Results of repeated tests are given separately, ranking from the highest results on the left to the lowest results on the right.

Compound	imp (s.e.)	n	imp (s.e.)	n	imp (s.e.)	n	weighted mean
(A) 6.	137.0 (12.6)	6	137.0 (5.5)	6			137.0
9.	92.0 (15.3)	5					92.0
1.	82.3 (8.7)	20					82.3
2.	77.8 (6.7)	19	75.0 (11.6)	8	42.9 (3.7)	7	69.9
5.	57.3 (6.6)	12	40.6 (4.1)	7			51.2
7.	47.7 (9.2)	13					47.7
8.	39.3 (11.1)	10					39.3
14.	37.0 (14.1)	7					37.0
11.	29.0 (7.0)	8					29.0
10.	25.9 (10.3)	8					25.9
4.	21.4 (3.5)	17					21.4
3.	24.0 (5.8)	15	19.1 (15.4)	15			20.7
15.	17.2 (6.6)	5	4.3 (1.5)	13			7.9
(B) 6.	147.3 (11.5)	8	116.0 (10.9)	6	105.0 (25.9)	3	128.8
2.	126.0 (5.3)	19	120.0 (12.6)	6	81.8 (5.7)	6	116.3
10.	95.5 (9.6)	8					95.5
11.	74.5 (10.6)	8					74.5
5.	88.8 (6.9)	12	46.3 (7.2)	7			73.1
7.	68.5 (7.1)	13					68.5
8.	96.7 (10.4)	7	31.0 (8.2)	6			66.4
14.	65.4 (8.0)	9					65.4
9.	85.6 (8.1)	5	45.0 (10.9)	7			61.9
3.	68.9 (8.2)	15	44.0 (6.0)	7			61.0
1.	60.0 (5.4)	13					60.0
4.	85.5 (8.4)	6	43.8 (4.4)	17			54.7
15.	23.4 (7.4)	5	17.1 (3.0)	13			18.9
control	3.3 (1.1)	10					

s.e. = standard error of mean; n = number of replicates; control: 5 mM KCl, 2% ethanol.

mechanisms (sensory or post-ingestive) are also involved in feeding inhibition.

Luo *et al.* (1995) described a significant correlation between behaviour and responses of the medial deterrent cell for three triterpenoids (azadirachtin, salannin and toosendanin) and a commercial product (Margosan-O). This relationship is comparable to our results presented in Figure 5 except at a 100 times lower concentration. Thus antifeedant compounds of two different classes (sesquiterpenes and triterpenoids) show

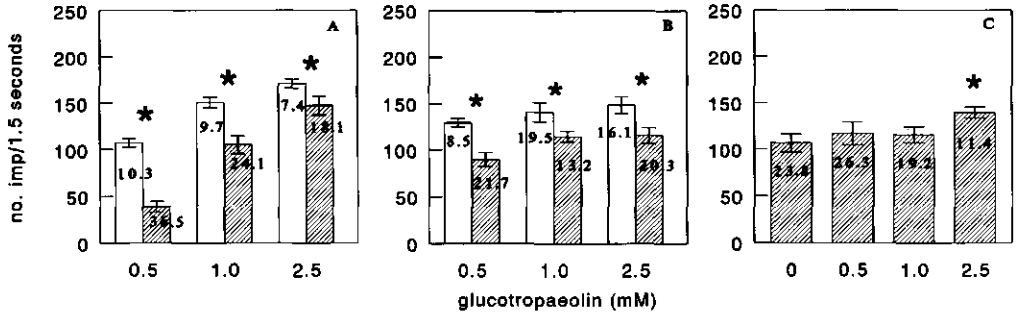

Figure 3. Response of (A) medial and (B) lateral glucosinolate sensitive receptor cells to three concentrations of glucotropaeolin, either pure (white bars) or mixed with compound 4 (1 mM; grey bars), expressed as numbers of impulses in the first 1.5 seconds of stimulation. The response of the medial deterrent cell to compound 4 only (1 mM; first bar) and the response of this cell to the aforementioned mixtures is shown in (C). Numbers in the bar represent c.v.'s (coefficient of variation), error bars indicate the s.e. Each stimulus was tested on 6-7 larvae. Wilcoxon's matched pair signed-ranks test was used assess significance of differences, * $P < 0.05$.

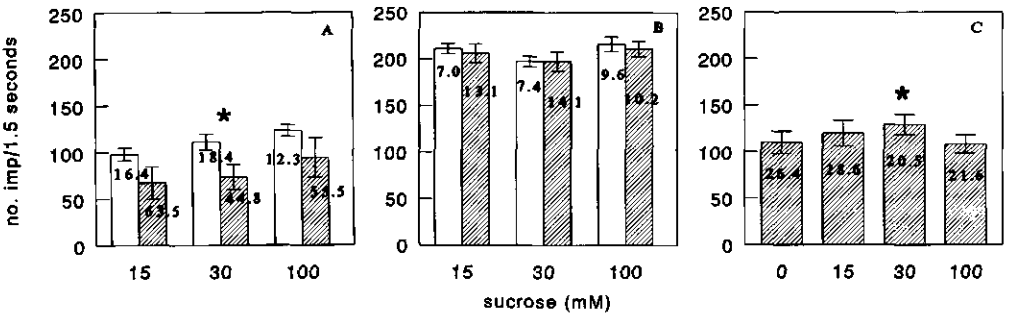

Figure 4. Responses of (A) medial and (B) lateral sugar sensitive receptor cells to three concentrations of sucrose, either pure (white bars) or mixed with compound 4 (1 mM; grey bars), expressed as numbers of impulses in the first 1.5 seconds of stimulation. The response of the medial deterrent cell to compound 4 only (1 mM; first bar) and the response of this cell to the aforementioned mixtures is shown in (C). Numbers in the bar represent c.v.'s (coefficient of variation), error bars indicate the s.e. Each stimulus was tested on 6-7 larvae. Wilcoxon's matched pair signed-ranks test was used to assess significance of differences, * $P < 0.05$.

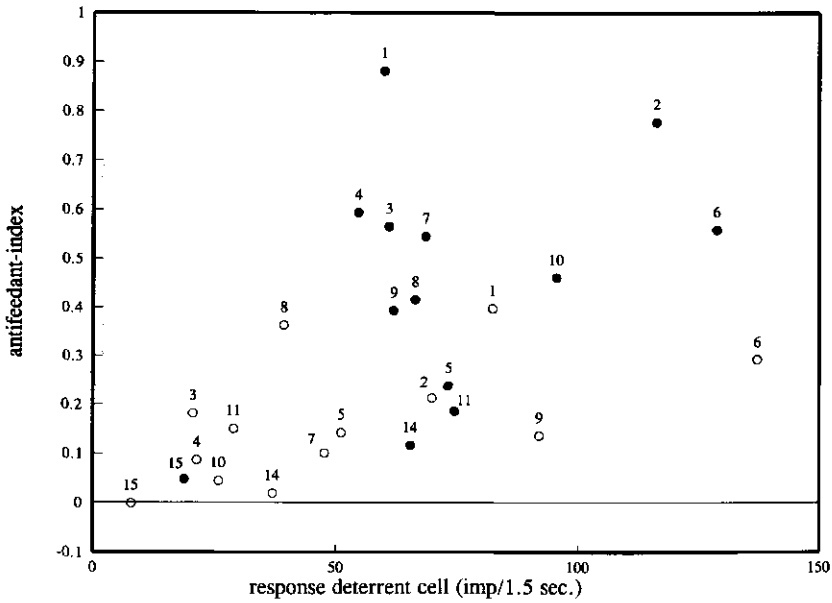

Figure 5. Antifeedant-index ((C-T)/(C+T)) as a function of response of the deterrent cell (weighted means). Numbers indicate the compounds tested. Open circles indicate that 1 mM drimane solutions for the behavioural tests and 0.1 mM for the electrophysiological tests were used, closed circles indicate that 5 mM and 0.5 mM respectively were used. Spearman's rank correlation was used to assess if there is a significant correlation; rho = 0.59, $P < 0.005$.

similar relationships between sensory input and feeding inhibition, supporting the hypothesis that the response of the medial deterrent cell directly causes inhibition of feeding in *P. brassicae.*

Neurons sensitive to feeding stimulants are slightly depressed by at least one of the drimanes tested in the present study (Figures 3 and 4). Until now it is not unequivocally clear if interference with receptor cells sensitive to feeding stimulants contributes to inhibition of feeding. The close correlation between responses of receptor cells sensitive to feeding stimulants and the amount of artificial diet taken up in 24 h found for *P. brassicae* larvae (Blom, 1978) suggests that interference with cells sensitive to feeding stimulants might depress food intake. However, interference with the lateral glucosinolate- and sugar sensitive receptor cells measured for toosendanin (Schoonhoven & Luo, 1994) did not contribute to a closer correlation between sensory response and inhibition of feeding on cabbage leaf discs in *P. brassicae* (Luo *et al.*, 1995).

The large variability found in feeding responses to drimanes is in agreement with the large variability of both the responses of the deterrent cell (c.v. = 11-29%) and the responses of cells sensitive to feeding stimulants to mixtures with compound 4 (c.v. = 10-

64%) (Figures 3 and 4A-C).

We conclude that the response of the deterrent cell significantly contributes to inhibition of feeding behaviour in fifth instar larvae of *P. brassicae*. Highly effective drimane antifeedants could be selected electrophysiologically on the basis of response intensity in the medial deterrent cell. However, the unexpectedly large variability indicates that also other factors (sensory or post-ingestive) could be involved. Therefore, additional tests are needed to determine the mechanism of feeding inhibition.

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3

Behavioural observations of *Pieris brassicae* larvae indicate multiple mechanisms of action of analogous drimane antifeedants

Abstract

We tested 11 analogous synthetic drimane antifeedant compounds for their feeding inhibiting effects on larvae of the large white butterfly *Pieris brassicae* L. (Lepidoptera: Pieridae) in no-choice tests on the host plant *Brassica oleracea* L. Furthermore, we observed larval feeding behaviour in no-choice tests to analyze temporal effects of five drimanes. The results show that the five analogous antifeedants differentially influence feeding behaviour and locomotion activity. Warburganal and polygodial are most likely direct, sensory mediated antifeedants. Habituation to these compounds occurs soon after the onset of the tests (*i.e.* within 0.5-1.5 h). Compound 5 and confertifolin are probably no direct, sensory mediated antifeedants. After 0.5-1.5 h of exposure, these compounds inhibit not only feeding, but also locomotion behaviour, indicating postingestive, toxic effects. Isodrimenin inhibits feeding from the onset of the test and is probably a sensory mediated antifeedant. No habituation occurs to this compound, indicating that isodrimenin is either a very strong antifeedant or that it additionally has postingestive, toxic effects. Topical application of the drimanes on the larval cuticle revealed feeding inhibiting effects, but these could not be related to the occurrence of postingestive feeding inhibiting effects, indicating that this method is inappropriate to show possible postingestive effects of drimanes in *P. brassicae*. In conclusion, the behavioural observations performed in this research indicate that analogous drimanes inhibit feeding by *P. brassicae* larvae through multiple mechanisms of action. The results show that, when developing a structure-activity relationship (SAR) for a series of antifeedants, it is important to distinguish the mode of action which underlies inhibition of feeding.

Introduction

Plants produce a great diversity of secondary chemical compounds, many of which inhibit feeding by herbivorous insects. Particularly well studied feeding inhibitors (socalled 'antifeedants') are *e.g.* the triterpenoid azadirachtin and the sesquiterpene drimanes warburganal and polygodial (reviews are given by *e.g.* Morgan & Warthen, 1990; Warthen, 1990; Prakash & Rao, 1997). Antifeedants are generally considered to play an important role in food-plant selection by herbivorous insects, that avoid plants containing these compounds (Schoonhoven *et al.*, 1998). Many secondary plant compounds that show antifeedant activity are known to exert also other effects and possess insecticidal, molluscicidal, piscicidal or phytotoxic properties (Mabry & Gill, 1979; Jansen, 1993; Schmutterer, 1995). This suggests that these compounds could protect plants from being consumed in multiple ways.

Most of the research on antifeedants has been focussed on their potential use as crop protection agents against insects (Jermy, 1990; Frazier & Chyb, 1995). Over the last decades, chemical synthesis has been accomplished to obtain naturally occurring antifeedants as well as a number of non-natural derivatives. The latter can be as effective as the natural compounds (Messchendorp *et al.*, 1996). In studies on the

feeding inhibiting potency and structure-activity relationship (SAR) of test compounds, mostly either dual-choice or no-choice tests are employed, with the compounds applied to leaf material or artificial feeding substrate (Schoonhoven, 1982). In such tests only the final result is measured by comparing the amount consumed from treated and control substrate and consequently they do not reveal the mechanisms of action of the tested compounds (Bernays & Chapman, 1987; Frazier & Chyb, 1995; Bernays & Weiss, 1996) nor show eventual occurrence of habituation or sensitization (Szentesi & Jermy, 1990; Bernays, 1995). Observation of the insects' behaviour during feeding on a substrate treated with antifeedants (*e.g.* El-Bassiouny, 1991; Glendinning & Slansky, 1994; Bowdan, 1995; Gols *et al.*, 1996) can provide more information on the temporal effects of antifeedants on different behavioural activities and thereby reveal differences in mechanisms of action between the tested compounds.

We tested 11 analogous synthetic drimane antifeedant compounds (Jansen, 1993) for their feeding inhibiting effects on *Pieris brassicae* larvae in no-choice tests on leaf discs of the host plant *Brassica oleracea*. These compounds have been examined previously in behavioural dual-choice tests and electrophysiological tests (Messchendorp *et al.*, 1996), but from earlier work it is known that no-choice- and dual-choice tests can give different results (Blaney *et al.*, 1990; Ortego *et al.*, 1995). We observed the behaviour of the larvae in no-choice tests with five different drimane treatments to study the temporal effects of the drimanes on four behavioural activities, namely palpating, feeding, walking and resting. Furthermore, feeding activity was studied after topical application of the drimanes on the larval cuticle, and after a 1.5 h exposure to confertifolin.

Materials and methods

Insects. Larvae of *P. brassicae* were reared on cabbage plants (*Brassica oleracea* var. gemmifera cv. Cyrus (for the long-term test with compound 3) and cv. Titurel (for the other experiments)) in L16:D8 at 22±3°C and r.h. 40-70%. For the experiments fifth instar larvae were used weighing 175-225 mg.

Chemicals. The 11 drimanes were synthesized at the Department of Organic Chemistry, Wageningen Agricultural University (Jansen, 1993; C.T. Bouwman, unpubl.) (Figure 1). All compounds are racemic. Compounds 1, 3, 6, 7, 8, 9 and 10 also occur in plants (Jansen, 1993). In all tests 5 mM synthetic drimane solutions were used. The compounds were presolubilized in ethanol (>99%) and a detergent (Tween-80), which was used to promote an even distribution of the solution on the leaf discs and, when topically applied on the larval body, to promote absorption of the solution through the cuticle. Distilled water was then added to obtain a final solution of 2% ethanol and 2% Tween-80 (for the no-choice and dual-choice test) and 2% ethanol and 10% Tween-80 (for the topical applications). Distilled water with 2% ethanol and 2 or 10% Tween-80 served as control solution.

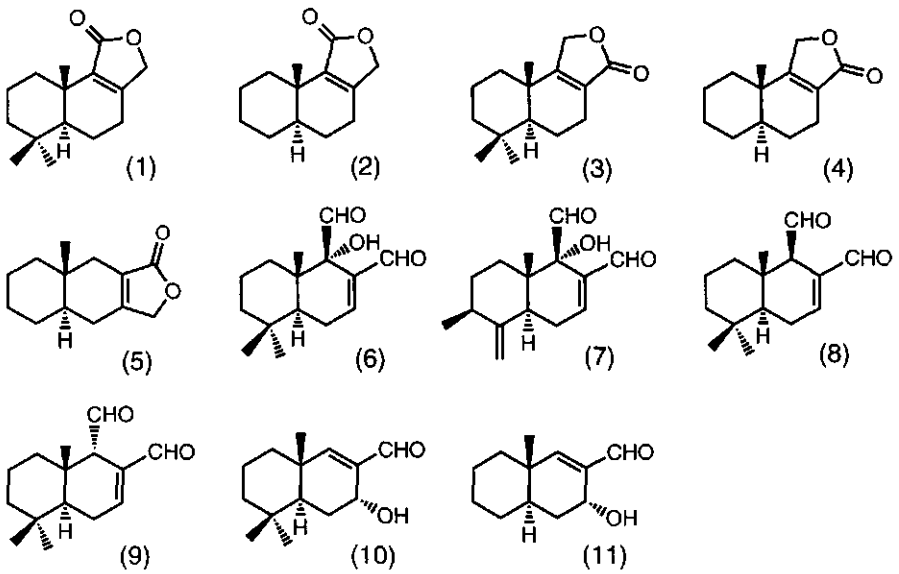

Figure 1. Molecular structures of the 11 drimanes. Trivial names: (1) isodrimenin, (3) confertifolin, (6) warburganal, (7) muzigadial, (8) polygodial, (9) isotadeonal, (10) polygonal.

Dual-choice test. The methods and results of the 3 h dual-choice test were presented in a previous article, except for the results with compound 3 (Messchendorp *et al.*, 1996). Each drimane was tested one to four times, with 7-20 replicates. The results shown in this work are the weighed means of the mean antifeedant indices (A.I.) of the tests performed with each drimane: A.I. = (C-T)/(C+T)*100% (C = area consumed from control leaf discs and T = area consumed from treated leaf discs).

No-choice test. Each drimane was tested with 17-20 replicates. Larvae were placed individually in Petri dishes (9 cm diameter) with moistened filter paper and one leaf disc (diameter 2.2 cm), painted with 10 µl drimane- or control solution on the upper surface. After 1.5 h, the leaf disc was replaced by a new disc with the same treatment. On each test day, the consumption by a control group was assessed as well (n = 18-20). Areas consumed from the treated and control leaf discs were determined using a leaf area meter (Hayashi Denko Co. Ltd., Tokyo, Japan). Consumption was divided by the larval weight, to compensate for possible differences in feeding rate due to differences in weight. The means of the inhibition percentages (I.P.) during the first and the second 1.5 h of the tests were calculated: $I.P.= (C_{(mean\ of\ controls)}-T)/C_{(mean\ of\ controls)} * 100\%$ (C = area eaten/weight of control larva and T = area eaten/weight of treated larva).

Interval observations during the no-choice test. Because some of the drimanes that showed antifeedant activity during the dual-choice test were also active in no-choice tests (compound 1, 2, 3, 4 and 5) while others did not show any antifeedant activity during the no-choice test (compound 7, 8, 9 and 10), we hypothesized that these two groups of drimanes might act via different mechanisms. Rapid habituation might occur to the compounds that do not inhibit feeding during the no-choice test, *e.g.* within the first 0.5 h, or within 1.5 h. To study the influence of the drimanes on the behaviour of *P. brassicae* larvae in more detail, we performed 1 min interval observations on larvae in no-choice tests (as described above) treated with compounds 1, 3, and 5 (compounds that inhibit feeding in the no-choice test) and compounds 6 and 8 (compounds not effective in the no-choice test). Although compound 6 did not significantly inhibit feeding in the dual-choice test we included this compound, because it is known as a potent antifeedant against several insect species (Warthen, 1990). The five compounds were tested according to an incomplete block design: each day, on which one compound was compared with a control group, represented a block. During the 3 h of the no-choice test twenty larvae, ten of which were presented with control discs and ten with drimane treated discs, were observed simultaneously. Four types of larval behaviour were monitored, *i.e.* palpating the leaf disc, feeding, resting and walking. The behaviour of each larva was recorded once a minute. For recording observations The Observer 3.0 software was used (Noldus Information Technology, 1993). To examine whether temporal changes in the behaviour of the larvae occurred, the observation period was split into three intervals, *i.e.* the first 0.5 h (p1), the subsequent 1 h (p2) and the last 1.5 h (p3) of the test. Percentages of time spent on the four behavioural activities were calculated for each time interval. The observations of larvae exposed to compound 8 were terminated 11 min early, because of technical disturbances.

Topical application of the drimanes. Each drimane was tested with 18-20 replicates and effects were compared with a control group monitored on the same day. The larvae were placed individually in Petri dishes (9 cm diameter) with moistened filter paper. A 4 µl droplet of drimane or control solution was topically applied between the pronotum and thorax to avoid contact between the test compound and the gustatory sensilla on the mouthparts. Directly after the application one untreated leaf disc (diameter 2.2 cm) was added. After 1 h and 2 h the leaf discs were replaced by new discs. After 3 h the larvae were allowed to feed on cabbage leafs until 24 h after the start of the experiment. The larvae were weighed just before the test, at 3h and at 24 h. Areas consumed during the first 3 h of the test were determined using a leaf area meter and divided by the larval weights (measured before the test) to compensate for possible differences in feeding rate due to differences in larval weight. Relative weight change during the first 3 h was calculated as weight at 3 h / weight before the test * 100% (r.w.c.(0-3 h)) and relative weight change during the remaining 21 h as weight at 24 h / weight at 3 h * 100% (r.w.c.(3-24 h).

Long-term effects of consumption of leaf discs treated with compound 3. Ten larvae were allowed to feed for 1.5 h on leaf discs treated with compound 3. After 1.5 h the leaf discs were replaced by untreated leaf discs. Also at 3 h and 4.5 h the leaf discs were replaced

by new, untreated leaf discs. At 6 h the leaf discs were replaced by leaf discs treated with compound 3. At the same time a control group was monitored: during the first 1.5 h, 10 larvae were allowed to feed on control leaf discs for limited time periods, in order to mimic consumption of the treated larvae: from 0-45 min they could feed on control leaf discs, from 45-80 min the leaf discs were removed and during 80-90 min they could feed on control leaf discs again (so that the larvae were not starved just before the second 1.5 h). During the next four 1.5 h periods the control larvae were allowed to feed on untreated leaf discs, that were replaced every 1.5 h. Areas consumed during the five 1.5 h periods (i1-i5) were determined using a leaf area meter and divided by the larval weights (measured before the test).

Statistical analysis. To assess significance of differences between treated and control groups, Wilcoxon's matched pair signed rank test was employed for the dual-choice test, and the Mann-Whitney U test for the no-choice, topical application and long-term effects tests. For the statistical analysis of the interval observations the percentages of time spent on the four behavioural activities were arcsine transformed because a large proportion of the percentages fell below 30% (Sokal & Rohlf, 1995). Main effects of block and treatment on the transformed percentages of time spent on the four behavioural activities during p1, p2 and p3 were analyzed using 2-way ANOVA. We subsequently employed a Bonferroni adjusted two-tailed t-test (α=0.05) to analyze possible differences between controls and the various treatments. A two-tailed multiple range test (Tukey-Kramer, α=0.05) was used to analyze possible differences between compounds. Because the Bonferroni- and Tukey adjusted tests are rather conservative in estimating significance levels (Day & Quinn, 1989) we employed a significance threshold of 0.10. For the statistical analyses we used the computer packages Statgraphics Plus 7.0 (no-choice, dual-choice, topical application and long-term effects tests) and SAS 6.11 (interval observations).

Results

Dual-choice and no-choice tests. Table 1 shows the results of the dual-choice and the no-choice tests with the 11 drimanes (for molecular structures see Figure 1). Where repeated, the results of the no-choice tests with the same compound appear to be rather variable, as has been described before for dual-choice tests (Bernays & Wege, 1987; Messchendorp *et al.*, 1996). Only drimane compounds possessing a lactone group inhibit feeding in the no-choice tests. An exception is compound 6, which lacks a lactone group, but inhibits feeding in one of the three no-choice tests during the first 1.5 h and has no significant effect in the dual-choice test. Some compounds (*i.e.* 7, 8, 9 and 10) inhibit feeding in the dual-choice test, but appear ineffective in no-choice tests. In some no-choice tests, significant stimulation of feeding relative to the control group was found (with compounds 7, 9 and 10).

Table 1. Inhibition percentages (I.P.) of the no-choice tests (means ± s.e.) and antifeedant indices (A.I.) of the dual-choice tests (weighed means of 1-4 tests)[a].

Compound	no-choice: I.P. (0-1.5 h)	I.P. (1.5-3 h)	dual-choice: A.I. (3 h)
1	61±10***	39±4***	88**
	56±9**	59±7** @	
2	40±8**	38±8**	78**
	50±7***	57±7***	
3	61±6***	44±4***	56**
	48±5***	60±7*** @	
4	51±6***	43±7***	56**
5	49±6***	65±7***	59**
	-9±9	38±6** @	
6	10±6	-18±5	24
	-1±6	-10±7	
	33±8**	8±4 @	
7	10±5	-22±4*	56**
8	15±8	-20±10	54**
	18±8	13±6 @	
9	-11±6	-41±10***	42*
10	-33±7*	-32±7**	40**
11	-20±10	-21±12	2

IP= $(C_{(\text{mean of controls})}-T)/C_{(\text{mean of controls})}$ * 100%, with C = area consumed/weight of control larvae and T = area consumed/weight of treated larvae
A.I. = (C-T)/(C+T)*100%, with C = area consumed from control leaf discs and T = area consumed from treated leaf discs
[a] antifeedant indices obtained from Messchendorp *et al.* (1996)
@ data from tests during which behavioural observations were performed
Statistics: Wilcoxon's matched pair signed rank test (dual-choice test), Mann-Whitney U (no-choice test); * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Interval observations during the no-choice test. Table 1 gives the I.P.'s of the no-choice tests on which interval observations were performed (tests indicated with @). Figure 2 shows the mean percentages of time spent on palpating, feeding, resting and walking during the three time periods of the tests by groups of larvae that were exposed to one of the five drimanes, and the corresponding control groups. As described earlier (Ma,

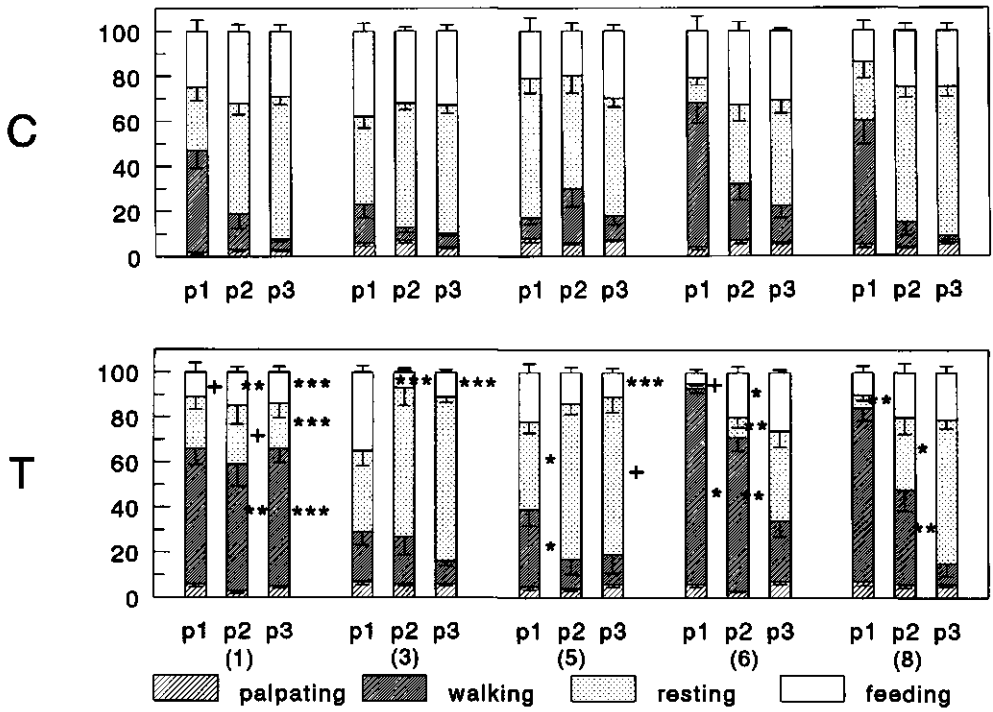

Figure 2. Percentages of time spent on feeding, resting, walking and palpating during p1 (0-30 min), p2 (30-90 min) and p3 (90-180 min) by the groups of larvae that were exposed to the drimanes 1, 3, 5, 6 and 8 (T) and by the corresponding control groups (C) (Means ± s.e.). Significant differences from the control are indicated by asterisks and + signs (Bonferroni adjusted t-test); + $P<0.1$, * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

1972; Schoonhoven, 1979; El-Bassiouny, 1991) we observed that *P. brassicae* larvae on control leaf discs took 'meals' (Simpson, 1995) alternated by periods of rest. In Figure 2 this is reflected in the relatively large percentages of time spent on feeding and resting by the control larvae, especially after the first 0.5 h. Figure 2 and the results of the ANOVA presented in Table 2 show that, especially during the first 0.5 h of the tests, the mean percentage of time spent on the four behavioural activities by the control groups varies between the five experimental days. This might indicate that the larvae need some time to get used to the test environment. Figure 2 clearly shows that all larvae (control and treated) spent relatively little time on palpating the leaf discs. Because the ANOVA (Table 2) reveals no significant effects of the five treatments on palpating, we do not further discuss palpating behaviour. However, it is possible that there are effects on palpating that we could not reveal, because our 1 min interval observation method might be too coarse to detect them.

Larvae exposed to compounds 6 and 8 do not significantly differ in percentage of

Table 2. 2-way ANOVA for main effects of block and treatments on percentage of time spent on feeding, resting, walking and palpating during p1, p2 and p3

		p1		p2		p3	
		F-ratio	Sign. level	*F*-ratio	Sign. level	*F*-ratio	Sign. level
Feeding							
Source of variation	d.f						
Main							
Block	4	3.78	<0.01	3.12	<0.05	1.23	NS
Treatment	5	2.22	NS[a]	11.64	<0.0001	18.01	<0.0001
Residual	90						
Resting							
Source of variation	d.f						
Main							
Block	4	11.95	<0.0001	2.11	NS	2.18	NS
Treatment	5	4.78	<0.001	6.74	<0.0001	9.60	<0.0001
Residual	90						
Walking							
Source of variation	d.f						
Main							
Block	4	13.42	<0.0001	1.88	NS	1.95	NS
Treatment	5	4.69	<0.001	9.39	<0.0001	12.20	<0.0001
Residual	90						
Palpating							
Source of variation	d.f						
Main							
Block	4	2.6	<0.05	4.01	<0.01	4.21	<0.01
Treatment	5	1.81	NS	2.27	NS	1.34	NS
Residual	90						

[a] because P=0.06 the Bonferroni adjusted t-tests and the multiple range test were still performed

Table 3. Multiple range test (Tukey-Kramer, α=0.05)

	p1		p2		p3	
Feeding	1	a	1	ab	1	abc
	3	a	3	a	3	a
	5	a	5	bc	5	ab
	6	a	6	abc	6	bc
	8	a	8	bc	8	bc
Resting	1	a	1	abc	1	a
	3	a	3	bc	3	b
	5	a	5	c	5	b
	6	a	6	a	6	b
	8	a	8	ab	8	b
Walking	1	a	1	b	1	a
	3	a	3	ab	3	b
	5	a	5	a	5	b
	6	a	6	b	6	b
	8	a	8	b	8	b

a, b, c: compounds followed by different letters significantly differ in effect on behaviour

time spent on the four behavioural activities throughout the test (Table 3), indicating that these compounds exert similar temporal influences on behaviour of the larvae. Larvae exposed to 8 prefer control leaf discs in a dual-choice test (Table 1), but percentage of time spent on feeding is not significantly decreased by 8 throughout the no-choice observation test (Figure 2), indicating that in a no-choice situation habituation to this compound probably occurs rapidly (within p1). Figure 2 shows that feeding by larvae exposed to 6 is inhibited during p2, but not during p1. However, without the Bonferroni adjustment we found a significance level of $P<0.05$, which indicates that 6 likely inhibits feeding from the beginning of the test. During p3, no differences in behaviour compared to the control group occur anymore (Figure 2): apparently habituation to 6 occurred.

Larvae exposed to 3 and 5 do not significantly differ in percentage of time spent on the four behavioural activities throughout the test (except for feeding during p2 (Table 3)), indicating similar temporal effects of 3 and 5 on behaviour. Larvae exposed to 3 and 5 initially do not differ in percentage of time spent on feeding compared to the control group (Figure 2). The fact that during p2 and p3 (larvae exposed to 3) and during p3 (larvae exposed to 5) inhibition of feeding does occurs indicates that the feeding inhibiting effect of these drimanes is increasing over time. During p3, while feeding is inhibited, the percentage of time spent on resting by larvae exposed to 5 is increased and the percentage of time spent on walking in larvae exposed to 3 and 5 does not differ compared to the control. This indicates that these larvae are not stimulated to search for better food sources but even are stimulated to rest, despite the fact that starvation occurs.

Larvae exposed to compound 1 are inhibited to feed from the beginning of the test, and no habituation occurs. In these larvae, less feeding compared to the control group is accompanied by less resting and more walking (except during p1) (Figure 2). During p3, larvae exposed to 1 rest significantly less and walk significantly more than all other groups of larvae (Table 3).

Table 4. Consumption/weight (mm^2/mg; means±s.e.) after topical application of drimanes (4 µl of 5 mM drimane, 2 % ethanol, 10 % Tween) and control solution (on day 1-5), and relative weight change (r.w.c.) during the first 3 h and the last 21 h of the tests

	Consumption/weight:			r.w.c.:		
Drimane	0-1 h	1-2 h	2-3 h	(0-3 h)	(3-24 h)	day
1	0.40±0.06*	0.53±0.04	0.65±0.05	107±1	172±4	4
2	0.62±0.05	0.63±0.04	0.63±0.05	106±1	173±4	2
	0.52±0.06	0.65±0.05	0.77±0.03	109±1	186±3	3
3	0.44±0.05	0.39±0.04***	0.70±0.05	104±1**	177±5	4
4	0.54±0.06	0.47±0.04*	0.46±0.05**	105±1**	175±4	3
5	0.53±0.04	0.35±0.05***	0.53±0.04**	107±1*	180±4	3
	0.28±0.06***	0.38±0.06***	0.46±0.04***	103±1	215±5***	1
6	0.58±0.06	0.78±0.05* >c	0.64±0.04	113±1	178±5	3
7	0.38±0.09*	0.55±0.04**	0.47±0.04*	105±1	188±6	2
8	0.61±0.07	0.58±0.03	0.66±0.06	108±1	181±3	1
9	0.89±0.07	0.70±0.06***	1.01±0.05	111±1	171±3	5
10	0.68±0.06**	1.20±0.05* >c	1.01±0.05	113±1	172±4	5
11	0.84±0.05	0.80±0.05**	0.97±0.08	111±2	170±4	5
control	0.65±0.06	0.69±0.05	0.61±0.05	108±1	189±4	1
"	0.66±0.04	0.75±0.05	0.60±0.04	106±1	182±3	2
"	0.60±0.05	0.60±0.03	0.68±0.03	112±2	182±6	3
"	0.56±0.04	0.62±0.04	0.63±0.05	110±1	175±4	4
"	0.93±0.05	1.03±0.05	0.98±0.05	114±1	168±6	5
untreated	0.89±0.08** >c	0.42±0.04***	0.68±0.04	110±1	182±4	1

Statistics: Mann-Whitney U * $P<0.05$; ** $P<0.01$; *** $P<0.001$
>c: consumption/weight is higher than the control

Topical application of the drimanes. After application of compounds 4, 5 and 7 consumption/weight during at least 2 of the first 3 h of the test was lower than for the controls (Table 4). Significantly different relative weight changes (r.w.c.(0-3 h)) occurred in larvae treated with compound 4 and 5 (first test), but not in larvae treated with compound 5 in the second test and in larvae treated with compound 7. After application of compounds 1, 3, 9, 10 and 11 consumption/weight during 1 of the 3 h of the tests was reduced compared to the controls. Of these compounds, only application of compound 3 resulted in a lower relative weight change during the first 3 h. Relative weight changes during the remaining 21 h of the tests (r.w.c.(3-24 h)) were not lower than the controls.

Long-term effects of consumption of leaf discs treated with compound 3. During the first 1.5 h, the mean consumption/weight by the treated larvae was ca. 26% lower than the control, despite the limited feeding time of the control larvae (Figure 3). During i2 and also during i3, when feeding on untreated leaf discs, consumption/weight of the treated larvae was significantly lower compared to the controls. During i4 no significant difference occurred anymore. During i5, when the treated group fed on discs treated with compound 3 again, their consumption/weight significantly differed from the control group, that fed on untreated leaf discs. Consumption/weight by the treated larvae did not significantly differ between i1 and i5.

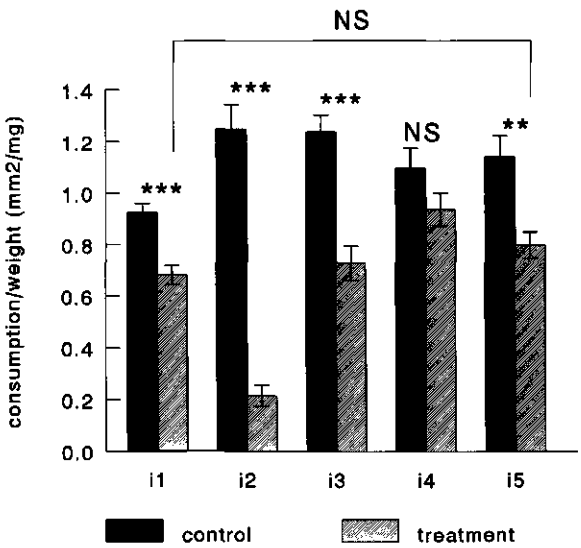

Figure 3. Long-term effects of consumption of leaf discs treated with compound 3 during 1.5 h (i1) on feeding on untreated leaf discs (consumption/weight) (during i2-i4). During i5, treated larvae were exposed to compound 3 treated leaf discs again, while control larvae were exposed to untreated leaf discs. i1-i5 Each lasted 1.5 h. During i1, treated larvae ingested on average 3.5 µl of drimane 16. Statistics: Mann-Whitney U; * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Discussion

The behavioural observations during the no-choice test with *P. brassicae* larvae show that the five drimane antifeedant analogues differentially influence feeding behaviour and locomotion activity (Figure 2, Tables 1-3). Compounds 6 and 8 are probably direct, sensory mediated antifeedants (Schoonhoven, 1982; Frazier & Chyb, 1995; Schoonhoven *et al.*, 1998), to which habituation occurs very fast when applied to the cabbage leaf discs used in these tests (within 0.5-1.5 h). Larvae exposed to compounds 1 and 6 rest less and walk more when feeding is inhibited. The function of this behaviour is probably searching for food, caused by the lack of palatable food sources and subsequent starvation (Barton Browne, 1975; Schoonhoven, 1979). Compounds 3 and 5 are probably no direct, sensory mediated antifeedants, because they do not inhibit feeding in the first 30 min of the tests. However, the possibility that they are sensory mediated antifeedants can not be fully excluded, because it is conceivable that also to these compounds habituation occurs soon after the onset of the test. After 0.5-1.5 h compounds 3 and 5 do inhibit feeding in the larvae, but in addition inhibit locomotion behaviour, probably indicating a postingestive, toxic effect. Because compound 1 inhibits feeding from the beginning of the test it probably inhibits feeding behaviour through direct sensory perception, that might be so strong that no habituation occurs during at least 3 h. It is also possible that compound 1 additionally acts via a postingestive, toxic mechanism of action, as we suggested for compounds 3 and 5. Figure 4 summarizes the difference in temporal influence of the five drimanes on feeding behaviour. The figure shows the mean cumulative time spent on feeding by the different treated and their corresponding control groups, against time (Glendinning & Slansky, 1994; Bernays & Weiss, 1996). The steepness of a line during a certain period represents the mean time spent on feeding by the larvae during that period. During 0-30 min the lines representing larvae exposed to 1, 6 and 8 are rather flat compared to the lines representing larvae exposed to 3 and 5, indicating an initial difference in effect on feeding behaviour between these two groups of compounds. After some time (between 25-40 min), the 3 and 5 curves change direction and become less steep, indicating a decrease in mean time spent on feeding (*i.e.* increased feeding inhibition), whereas the 6 and 8 curves become steeper, indicating increased feeding time (*i.e.* habituation). The 1 curve remains relatively straight throughout the test, indicating stable feeding inhibition.

Theoretically, the delayed feeding inhibiting effects observed during the no-choice feeding tests with compounds 3 and 5 (Figure 2 and Figure 3) could be caused by either postingestive, toxic effects or delayed, sensory effects. Previously, it has been shown that it is difficult to distinguish these two classes of effects by experiments (Bernays & Chapman, 1987; Bernays & Weiss, 1996; Gols *et al.*, 1996) The topical application test was done to examine whether the drimanes inhibit feeding without touching the gustatory sensilla on the mouthparts. The results (Table 4) show that compound 7 inhibits feeding after topical application, although habituation to this compound in a no-choice test occurs within the first 1.5 h (Table 1) and, while it is ingested, no negative effects become apparent during the 3 h of the test. On the other hand, compound 3 inhibits feeding only during the second h after topical application of 4 µl while, after ingestion in the long-term test (Figure 3), inhibition of feeding is still present 3 h after ingestion of on average 3.5 µl

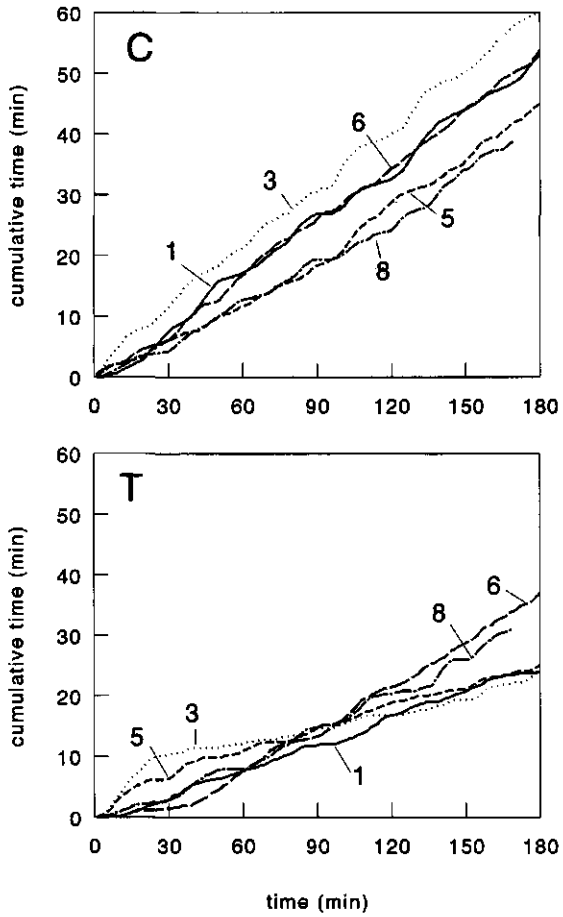

Figure 4. Mean cumulative time spent on feeding by the groups of larvae feeding on leaf discs treated with compound 1, 3, 5, 6 and 8 (T) and the corresponding control groups of larvae (C), against time.

(applied to leaf discs). So, the topical application tests do show toxic, feeding inhibiting effects of the drimanes, but these effects do not relate to feeding inhibiting effects after ingestion in a no-choice situation (Table 1, Figure 3). In conclusion, these results show that topical application of drimanes to *P. brassicae* larvae is an inappropriate method to show possible toxic, feeding inhibiting effects after ingestion of drimanes. Long-term tests, such as performed for compound 3, can only be realized with compounds that do not strongly deter insects from feeding through direct sensory perception. The fact that in the long-term test with compound 3 consumption during the second exposure is as high as during the first exposure shows that in *P. brassicae* larvae no food aversion learning

(Dethier, 1980; Lee & Bernays, 1988) occurs to compound 3.

In previous work by Schoonhoven & Yan (1989) it was shown that compounds 6, 7 and 8 exert delayed, sensory effects after long-term (up to 30 min) electrophysiological stimulation of the maxillary sensilla styloconica in *P. brassicae* larvae; reported effects were 'bursts' (irregular, high frequency firing of multiple neurones) and long-term inhibition (up to 4 h) of sensory neurones sensitive to feeding stimulants. However, in our no-choice tests compounds 6, 7 and 8 do not inhibit feeding, which suggests that the sensory effects after long-term electrophysiological stimulation do not occur while feeding in the no-choice test. Because it is known from video-recordings (van Loon; Messchendorp, unpubl.) that, in contrast to continuous electrophysiological stimulation, during feeding the maxillary sensilla styloconica touch the leaf surface only intermittently, it is possible that the effects found by Schoonhoven & Yan (1989) do not occur during feeding, or only after > 3 h exposure. Also because preliminary long-term (15 min with 2 mM solutions) electrophysiological stimulations with compounds 1, 5 and 9 did not result in the occurrence of 'bursts' or inhibition of the sensory response to sucrose (Messchendorp, unpubl.) it seems doubtful that 'delayed sensory effects' cause the delayed feeding inhibiting effects of compounds 3 and 5. It is more likely that these effects are caused by a postingestive, toxic mode of action of compounds 3 and 5. This hypothesis can be proven by experiments in which these compounds are ingested without touching the gustatory sensory organs on the mouthparts, e.g through microcannulation (Cottee *et al.*, 1988), or through microencapsulation of the compounds before feeding, such as done by Usher *et al.* (1989). However, considering the relatively small size of *P. brassicae* larvae, it will be difficult to perform such experiments.

In a previous study, we found a positive correlation between the number of action potentials fired by the 'deterrent cell' in the medial sensillum styloconicum and the antifeedant index of 15 drimane compounds (Messchendorp *et al.*, 1996). The fairly large variation that was found in the relationship may partly be explained by our current results, which show that drimanes inhibit feeding in *P. brassicae* larvae in different ways, and that besides neural, also postingestive, toxic mechanisms may play a role in inhibition of feeding. The neural mechanisms (on peripheral and/or central level) underlying the habituation found in this work are unknown (Szentesi & Jermy, 1990). We also do not know the probably postingestive, physiological mechanisms that cause the delayed feeding inhibition found in this work (Frazier & Chyb, 1995). Further experiments are needed to elucidate these mechanisms of action.

The fact that drimanes with highly similar molecular structures inhibit feeding through different mechanisms of action indicates that very specific molecular structures are required to induce the different physiological mechanisms resulting in inhibition of feeding behaviour. This study shows that, when developing a structure-activity relationship (SAR) for a series of antifeedants, it is important to distinguish the mode of action which underlies inhibition of feeding. From Table 1 it is clear that compounds possessing a lactone group inhibit feeding in a no-choice test, while compounds lacking such a group do not. Also because some lactones do not inhibit feeding directly after the onset of the no-choice test (*e.g.* 3 and 5), we suggest that the lactone group might be responsible for inducing the above mentioned postingestive feeding inhibiting effect.

Compounds that are known from the literature as potent antifeedants against

many insect species, such as polygodial (8) and warburganal (6) (Warthen, 1990), do not inhibit *P. brassicae* larvae from feeding in no-choice situations. The lactone drimanes do inhibit feeding by *P. brassicae* in a no-choice situation, but not exclusively through sensory mechanisms. Surprisingly we also found that some compounds stimulated feeding in a no-choice test, a phenomenon which seems hard to explain. Field or greenhouse tests are needed to provide final information on the potential use of drimane antifeedants as crop protection agents.

In most plants that contain drimanes as secondary metabolites, several analogous structures occur simultaneously (*e.g.* Barnes & Loder, 1962; Kubo *et al.*, 1976). The present results show that structurally similar compounds can exert different effects on herbivores, *e.g. P. brassicae* larvae, and thereby protect the plant from feeding injury through multiple mechanisms of action.

Acknowledgements

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4

Antifeedant and toxic effects of drimanes on Colorado potato beetle larvae

Abstract

Among the drimane compounds tested, the dialdehydes polygodial and warburganal were the most active as antifeedants against Colorado potato beetle larvae, *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae), in a dual-choice assay with potato, *Solanum tuberosum* L., leaf discs. Lactones were less effective. Direct observations showed that decreased feeding on leaf discs treated with polygodial and warburganal was accompanied by increased locomotory activity. Topical application of these two compounds on the insect's cuticle decreased food intake of untreated leaf discs, indicating that besides deterrent effects, toxic properties of these molecules influence feeding behaviour.

Introduction

The Colorado potato beetle is a major pest insect on solanaceous crops in North America and Europe. Since resistance of these beetles to most insecticides is a serious problem (Forgash, 1985), alternative methods of pest control are needed. Jermy (1966) stated that host specialization of phytophagous insects is associated with an increased sensitivity to feeding inhibitors. Since the Colorado potato beetle is a specialist feeder, it is probably quite sensitive to feeding deterrents. In that case feeding inhibition could be a method of controlling Colorado potato beetle.

The known bioactivity of drimane sesquiterpenes is diverse. Cytotoxicity, regulation of plant growth, antimicrobial, piscicidal and molluscicidal activities are among the documented effects (Kubo & Nakanishi, 1979; Mabry & Gill, 1979; Jansen, 1993). The natural drimane sesquiterpene dialdehydes warburganal and polygodial have been found to inhibit feeding of larvae of some lepidopterans (Kubo & Nakanishi, 1979; Blaney *et al.*, 1987; Messchendorp *et al.*, 1996) and of the aphid *Myzus persicae* (Asakawa *et al.*, 1988).

Commonly used methods of screening antifeedant activity on insects are choice and no-choice assays in which plant material or artificial substrates are treated with the test compound (Schoonhoven, 1982; Jermy, 1990). Food consumption in these tests is quantified for a standardized period. During such bioassays no direct observations of behaviour are performed. No-choice assays in particular do not allow a distinction between sensory/mediated versus post-ingestional feeding inhibition. It has generally been proven to be quite difficult to make this distinction (Szentesi & Bernays, 1984; Bernays, 1991). An empirical approach to this problem is the separate demonstration of deterrency and toxicity, *e.g.* by feeding and topical application respectively. In this paper results are presented on the behaviour of Colorado potato beetle larvae exposed to polygodial, warburganal, muzigadial and related drimane structures in both a no-choice situation, observed directly, and a standard dual-choice assay. In addition, the test

compounds were applied topically to the insect's cuticle to distinguish between antifeedant and possible toxic effects.

Materials and methods

Insects. Colorado potato beetles were reared in the laboratory at 25°C, under a photoperiod of L16:D8 and r.h. 25-50%. Beetles were fed leaves from potato (*Solanum tuberosum* L., cv. Surprise) plants grown in a greenhouse (18°C, L16:D8). In all experiments fourth instar larvae were used. Experiments were conducted in climatic rooms under conditions similar to those in the rearing chamber.

Chemicals. The drimane compounds (Figure 1) were synthesized at the Department of Organic Chemistry, Agricultural University Wageningen (Jansen, 1993; C.T. Bouwman, unpubl.). The drimanes were first dissolved in ethanol to which Tween-80 was added as a detergent. The solutions were diluted with distilled water to a final concentration of 2% (volume) of both ethanol and Tween-80. The control solution consisted of 2% ethanol and 2% Tween-80 in distilled water. Potato leaf discs (1.7 cm diameter) were painted with 10 µl of a test solution (1 or 5 mM) and air dried before use.

Dual-choice bioassays. In the dual-choice test 10 to 20 larvae weighing 90-120 mg were placed individually in glass Petri dishes (9.5 cm diameter) with moistened filter paper and three control and three treated leaf discs placed alternately in a circle. The larvae were allowed to feed for a period of 3 h before the consumed areas of the control and the treated leaf discs were determined using a leaf area meter (Hayashi Denko Co. Ltd., Tokyo, Japan). An antifeedant index (A.I.) was calculated as consumed area of control discs (C) minus consumed area of treated discs (T) divided by the total consumed area (C-T/C+T).

Direct observations on behaviour in a no-choice situation. Twenty larvae (45-80 mg), ten of which were presented with control discs and ten with antifeedant-treated discs (5 mM), were simultaneously observed during 3 h. Larvae were placed individually in Petri dishes (4 cm diameter) with moistened filter paper and one leaf disc. Every minute the behaviour of each beetle was observed. Three behaviourial activities were recorded *i.e.* feeding, resting and exploring. Exploring includes movement on both leaf discs and filter paper. For recording observations and subsequent data processing The Observer 3.0 software was used (Noldus Information Technology, 1993). To enable the distinction of possible behaviourial changes with time, the 3 h observation was divided into three periods: 0-15 minutes (period 1), 16-90 minutes (period 2) and 91-180 minutes (period 3). Since not every larva was in contact with the leaf disc at the start of the observation, period 1 began at first leaf contact. The resulting time overlap with period 2 was neglected as leaf contact occurred within 5 min for 70% of the larvae.

Topical application. To assess if there is any toxic effect of the antifeedant, drimanes were applied topically to the insect's cuticle. Twenty larvae (45-80 mg) were placed

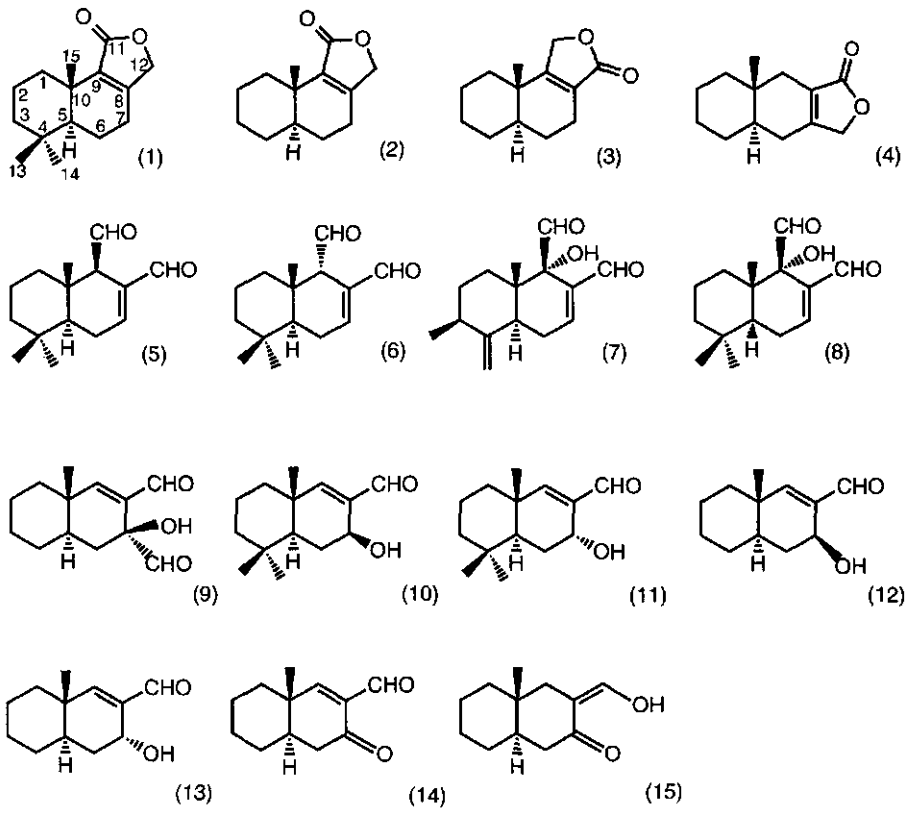

Figure 1. Molecular structures and codes. All compounds are tested as racemic mixtures. (1) Isodrimenin; (5) polygodial; (6) isotadeonal; (7) muzigadial; (8) warburganal; (10) isopolygonal; (11) polygonal.

individually in Petri dishes with moistened filter paper. A 2 µl 5 mM droplet was applied between the pronotum and thorax to avoid contact between the test compound and the gustatory sensilla located on the mouthparts. After the droplet had evaporated, an untreated leaf disc was added which was replaced every hour to reveal possible changes in leaf consumption due to the application of antifeedants. After 3 h the larvae were weighed and consumption during each hour was determined as leaf area removed. Larvae treated with polygodial (Figure 1; compound (5)) and warburganal (8) were fed on untreated leaves until the prepupal stage. The prepupae were then placed together in a plastic box on moistened riversand for pupation. After emergence, adults were weighed and their sex was determined.

Results

Dual-choice bioassays. None of the mono-aldehydes (compounds (10) to (14)) tested, had a significant deterrent effect (Table 1). The lactones, compounds (1) to (4), exerted moderate antifeedant activity (mean A.I. = 0.4-0.7) except isodrimenin (1) which acted as a stronger feeding inhibitor (mean A.I. = 0.87). The dialdehydes were the most potent antifeedants (A.I.>0.7) with the exception of isotadeonal (6), a stereo-isomer of polygodial (5) (A.I. = 0.51) and compound (9) (A.I. = 0.29), which has the second aldehyde group at C7 instead of C9. Of the compounds active at 5 mM, only muzigadial (7) was significantly deterring at 1 mM.

Direct observations on behaviour in a no-choice situation. Larval behaviour was rather variable on both control and treated leaf discs, therefore the test with polygodial (5) was repeated twice to check the reproducibility of the results (Table 2). As to the control groups, replicated experiments differed significantly when the second and third period were compared, whereas for the polygodial (5) treated groups, the replicates showed significant differences mainly during the third period. Differences were noted for all three behaviourial categories. In the three replicates the time spent feeding on polygodial (5) treated leaf discs was reduced by 30-70% compared to the control in all periods (Figure 2). In the second period (16-90 min) reduced feeding from treated leaves is accompanied

Table 1. Antifeedant activity of drimanes in a dual choice test with Colorado potato beetle larvae on potato leaf discs.

Compound number	5 mM A.I.±s.e.[1]		krus[2]	n	1 mM A.I.±s.e.[1]		n
5	0.92±0.02	**	a	19	0.22±0.11		20
1	0.87±0.05	**	ab	12	0.10±0.10		20
7	0.82±0.06	**	ab	20	0.32±0.07	**	20
8	0.79±0.07	**	ab	20	-0.17±0.14		20
2	0.69±0.08	*	bc	20	0.17±0.17		20
6	0.51±0.09	*	cd	19	-0.07±0.09		20
3	0.52±0.08	*	cde	19	0.13±0.12		19
4	0.43±0.12	*	cdef	12	0.26±0.12		12
12	0.22±0.15		def	20			
9	0.29±0.17		defg	12			
10	0.25±0.15		defg	10			
13	0.20±0.10		efg	19			
15	0.13±0.09		fg	12			
14	-0.23±0.19		fg	19			
11	0.04±0.11		g	20			

[1] A.I.(antifeedant index): (C-T)/(C+T). Asterisks indicate significancies: * P<0.05; ** P<0.01; Wilcoxon matched pair signed ranked test.
[2] Kruskal-Wallis one-way analysis of variance on ranks followed by multiple comparison (Conover, 1971). Means followed by the same letter are not significantly different, α=0.05.

by increased exploration. No significant differences were found in resting behaviour between control and treated groups.

Larvae on leaves treated with 5 mM warburganal (8) exhibited similar behaviour as on leaves treated with polygodial (5), *i.e.* reduced consumption and increased exploration compared to the control larvae. Larvae consuming polygodial or warburganal treated discs lost weight after 3 h, whereas the control larvae increased their weight (Table 3).

Compound (4) reduced feeding only during the first 15 minutes after leaf contact (Figure 2). During period 2 and 3 larvae on treated leaves were exploring while larvae on control discs were resting. The amount of time spent feeding was not significantly different in the last two intervals compared to the control.

In an additional experiment (no details reported) larvae were fed on treated leaf discs during the entire final larval stadium. Larvae fed on leaf discs treated with 5 mM polygodial (5) and warburganal (8) did not survive. However, the mortality of a starved control group run in parallel was occurring sooner than in the groups exposed to both compounds. Furthermore, painting leaf discs with polygodial or warburganal caused also some phytotoxic effect. Polygodial induced brown spots and warburganal also caused yellowing of the leaf discs within 24 h after treatment.

Larvae grown on leaf discs treated with compounds (4) or (13) reached the adult stage although in a few beetles wing malformations were observed.

Topical application. Topical application of 5 mM polygodial (5) and warburganal (8) to final instar larvae led to a decrease in consumption of untreated leaf discs (Table 4). This is also reflected in reduced weight gain during the first three hours after application. Only polygodial reduced weight gain significantly compared to the control 24 h after application. Larvae treated with either one of these compounds developed to adults with body weights similar to the controls (Table 5). However, mortality of polygodial and warburganal treated insects was 10 and 25% respectively, whereas no mortality was observed in the control group. Larvae topically treated with 1 mM polygodial fed significantly less in the second hour, but significantly more in the third hour after application. Warburganal at 1 mM had no effect on leaf consumption.

Treatment with compound (2) also led to a reduction in food intake but this reduction was only significant for the third period. Compounds (3) and (4) did not exert serious toxic effects.

Larvae treated with control solution (C) showed a decrease in food intake during the first hour after treatment compared to larvae which were not treated at all (C0). During the second and third hour, the control treated larvae consumed the same amount of food as untreated larvae.

Discussion

In the dual-choice tests the dialdehydes were the most potent deterrents and the lactones had lower activity. For the aphid *Myzus persicae* a similar difference in sensitivity to dialdehydes and lactones was found (Asakawa *et al.*, 1988). Inhibition of feeding

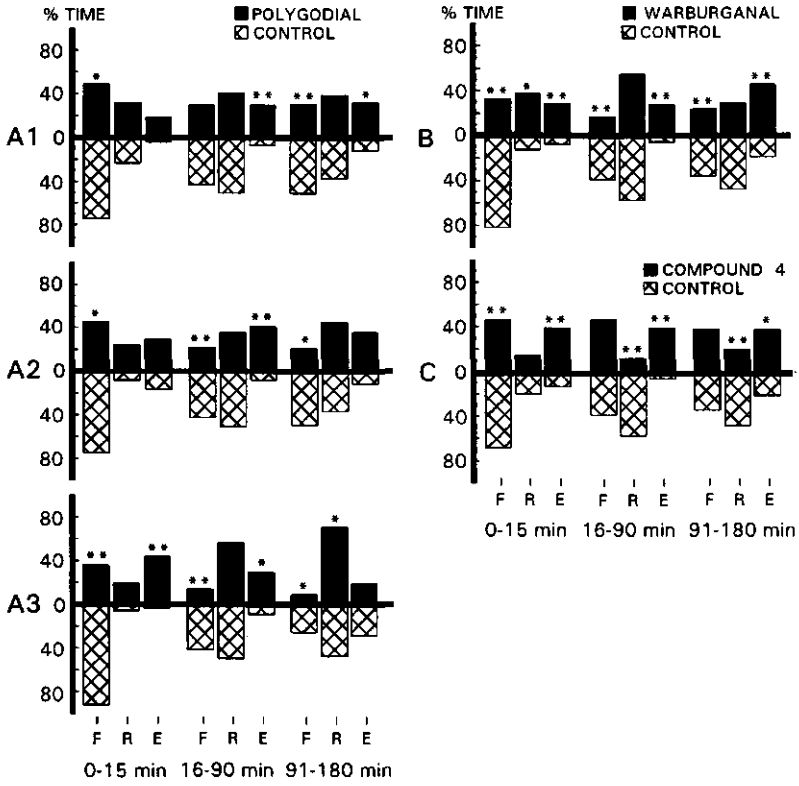

Figure 2. Direct behaviourial observations of Colorado potato beetle larvae on leaf discs treated with polygodial (A1, A2, A3) warburganal (B) and compound 4 (C). Percentages indicate mean time spent on feeding (F), resting (R) and exploring (E), respectively in period 1 (0-15 min), period 2 (16-90 min) and period 3 (91-180 min). Asterisks indicate significant difference between control and treated larvae: *, $P<0.05$; **, $P<0.01$; Mann-Whitney *U* test.

activity by drimanes was also found in some lepidopteran larvae. The dialdehydes warburganal (8) and muzigadial (7) were strong antifeedants against *Spodoptera exempta*; polygodial (5) was a weaker deterrent. The lactones did not have significant effects on feeding behaviour of *S. exempta* (Kubo & Nakanishi, 1979; Blaney *et al.*, 1987). In *Pieris brassicae*, however, the lactones were the most active, polygodial (5) and warburganal were less effective (Messchendorp *et al.*, 1996).

At the molecular level the dialdehydes most active in the present study have a double bond in common between C7 and C8 and a 11-12ß dialdehyde. The importance of this configuration is supported by the lack of activity of compound (9) and lower

Table 2. Comparison of behaviourial observations of Colorado potato beetle larvae on leaf discs treated with polygodial. Data from Figure 2: A1, A2 and A3 are compared in Kruskal-Wallis one-way analysis of variance on ranks followed by multiple comparison (Conover, 1971). Data from control and polygodial groups are analyzed separately. Rows within a column that have a letter in common are not significantly different, α=0.05. Abbreviations: see Figure 2.

Replicates compared	0-15 min			16-90 min			91-180 min		
	F	R	E	F	R	E	F	R	E
Control groups									
A1	a	a	a	a	a	a	a	a	a
A2	ab	a	a	b	b	a	b	a	b
A3	b	a	a	a	a	a	c	a	b
Polygodial groups									
A1	a	a	a	a	a	a	a	a	ab
A2	a	a	ab	ab	a	a	ab	a	a
A3	a	a	b	b	a	a	b	b	b

Table 3. Weight gain or loss (mg±s.e.) during the direct observations of Colorado potato beetle larvae feeding on leaf discs either or not treated with drimane antifeedant (n=10).

Compound (5 mM)	Control	Treated
C0	5.0±1.0	6.0±1.0
(4)	8.7±1.2	6.8±1.0
(5)	4.6±0.3	-0.6±1.2 **
	5.8±1.7	-6.3±2.1 *
	8.5±2.5	-3.2±1.1 *
(8)	7.0±1.1	-1.1±0.8 **

C0 is no treatment at all. Asterisks indicate significant differences from control: * $P<0.05$; ** $P<0.01$; Mann-Whitney *U* test.

activity of isotadeonal (6). The deterrence of the lactones is highest when the lactone group is present at C8/C9. The differences in activity between species indicate that structure-activity relationships cannot be generalized.

Research on the effects of other secondary plant substances on feeding behaviour of Colorado potato beetle revealed deterrence of extracts of the neem tree (Zehnder & Warthen, 1988; Kaethner, 1992) and plants of the sagebrush community (Jermy *et al.*, 1981). Feeding was also inhibited by *Solanum* glycoalkaloids (Schreiber, 1958; Mitchell & Harrison, 1985; Mitchell, 1993) and citrus limonoids (Bentley *et al.*, 1988; Liu *et al.*, 1990;

Table 4. Effects of topical treatment with drimanes on food consumption of Colorado potato beetle larvae.

Compound (5 mM)	Weight gain (mg±s.e.)		Consumption (mm²±s.e.)				
	3 h	24 h	1st h	2nd h	3rd h	total	n
C	8.5±0.7	34.7±2.1	75±8	32±4	20±3	127±11	15
(5)	6.6±1.3	12.0±4.1**	57±9	33±5	24±3	114±10	15
C	8.4±0.7	41.5±1.6	51±7	52±4	37±3	141±10	18
C0	8.9±0.7	39.0±2.5	63±4*	46±4	46±4	155± 6*	20
(3)	8.4±1.0	39.8±2.3	45±3	51±5	36±5	132±10	18
C	9.7±1.0	32.8±4.2	51±4	52±4	46±4	148±7	18
(2)	9.7±0.8	32.7±2.3	45±3	47±3	31±4*	124±6	20
C	8.1±0.6	31.7±1.8	53±5	54±5	43±3	149±7	19
(4)	8.7±0.9	30.8±1.5	52±8	39±4	59±5**	150±11	18
(8)	6.3±1.1*	27.8±3.3	32±5**	33±5**	49±7	114±11**	18
C	8.8±0.6	26.6±2.0	96±5	47±5	43±4	186±7	20
(5)	1.3±0.9**	12.7±1.9**	21±6**	25±5**	23±3**	69±8**	20
(8)	5.9±0.9**	22.7±1.4	47±5**	43±3	32±4**	122±8**	20
C	9.4±0.9		74±6	50±4	41±5	166±9	20
(5)	4.8±1.2**		23±5**	38±6	35±5	96±11**	18
(8)	4.7±0.7**		41±5**	45±4	34±4	121±6**	19
compound (1 mM)							
C	10.9±0.7	35.0±0.9	73±4	63±4	36±5	171±7	20
(5)	9.9±0.6	34.3±1.2	77±5	50±3*	54±5**	181±8	20
(8)	8.6±0.9	34.7±1.2	78±6	57±5	39±6	174±9	20

C is treatment with control solution, C0 is no treatment at all. Asterisks indicate significant differences from control (C): * $P<0.05$; ** $P<0.01$; Mann-Whitney *U* test.

1991). Effective concentrations of the secondary plant compounds tested in these studies were in the same order of magnitude as in the present study *i.e.* about 250 ppm or 5 µg/cm^2.

Among the compounds tested in the behaviourial observations only polygodial (5) and warburganal (8) reduced feeding significantly during the entire 3 h-observation without apparent habituation. Larvae showing feeding inhibition displayed higher locomotory activity. Increased locomotion of Colorado potato beetle caused by deterrents was also noticed by Jermy (1971) and El-Bassiouny (1991). Jermy stated that the deterrent induces an inhibitory state for food intake in the central nervous system which results in increased locomotory activity.

The observed variation in behaviourial responses (Table 2) may be due to the our use of different generations of Colorado potato beetle larvae and potato plants in various

Table 5. Adult body weight (mg±s.e.) of Colorado potato beetle larvae which were during their final larval instar topically treated with polygodial or warburganal.

Compound (5 mM)	Total (n)	Male (n)	Female (n)
control	116±3(20)	107±4(10)	126±4(10)
polygodial	109±4(15)	96±5(6)	118±5(9)
warburganal	114±4(17)	102±5(8)	124±4(9)

No significant differences were found when control and treated beetles were compared, Mann-Whitney *U* test, P=0.05.

experiments (Bernays & Wege, 1987). Topical application of warburganal (8) and polygodial (5) revealed toxic effects. Toxicity induced by topical application could be different from toxicity after ingestion. Unfortunately, the design of the experiments does not allow definite conclusions about the extent to which each mode of action contributes to the observed effects on the larvae. This is also true for the longer term exposure experiment. The mortality observed in this experiment are to be ascribed partly to toxicity but also to starvation, as lower than normal levels of food intake were observed.

Liu *et al.* (1990) made attempts to demonstrate whether feeding reduction was due primarily to inhibitory effects at the host acceptance level, or as a consequence of general toxicity effects by simulating antifeedant activity through controlled starvation. If feeding reduction is also caused by toxicity *e.g.* having a negative effect on assimilation rates, growth rates should be lower at a certain level of consumption. A similar experiment in which the effect of physical and chemical starvation were compared, was done by Higgins & Pedigo (1979). Such experiments could assist in quantifying the relative importance of deterrent and toxic effects.

The phytotoxicity observed at the relatively high doses needed to obtain antifeedancy in laboratory experiments renders these compounds unsuitable as crop protection agents against final instar Colorado potato beetle on potato. Although final instar Colorado potato beetle larvae are known to be the most voracious, earlier instars might be more sensitive which would then lead to an underestimation of their potential efficacy as emerging from our study. However, only field trials can evaluate effects of lower concentrations on population densities. Extended research on isodrimenin (1) and other lactones and muzigadial (7) is necessary to evaluate the promising effect of these compounds.

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The role of an epipharyngeal sensillum in the perception of feeding deterrents by *Leptinotarsa decemlineata* larvae

Abstract

An epipharyngeal taste sensillum in *Leptinotarsa decemlineata* larvae was studied. Electron microscopy showed that the sensillum is innervated by five neurons. Electrophysiological experiments showed that one of these cells responds to water, a second to sucrose and a third to two feeding deterrents that were also effective in a behavioural test. Receptor cells sensitive to feeding deterrents were not previously reported for *L. decemlineata* larvae or adults. The response of the sucrose sensitive cell was strongly inhibited by one of the two feeding deterrents and only slightly by the other feeding deterrent. The relationship between the behavioural and electrophysiological results is discussed in order to elucidate the neural code of feeding deterrents in *L. decemlineata* larvae. We conclude that probably both the response of the deterrent cell and peripheral interactions exerted by feeding deterrents on the sucrose sensitive cell determine the potency of feeding deterrents. The present results provide a physiological basis for the hypothesis that the presence or absence of feeding deterrents in potential food plants is a decisive cue in food plant selection by *L. decemlineata* larvae.

Introduction

The presence or absence of feeding deterrents in potential food plants has been suggested by many authors to be the decisive factor in food plant selection by the Colorado potato beetle, *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae) (Jermy, 1994). This hypothesis is mainly based on the fact that to date no food plant specific 'sign stimuli' to *L. decemlineata* have been identified (Hsiao, 1969), while in contrast, many feeding deterrents to these beetles are known. Examples are several solanaceous alkaloids (Hsiao, 1974), several drimane sesquiterpenes (Caprioli *et al.*, 1987; Gols *et al.*, 1996) that were originally isolated from plants e.g. *Polygonum hydropiper* (Barnes & Loder, 1962), and the glucosinolate sinigrin (Schoonhoven & Jermy, 1977) that can be isolated from several cruciferous plants and protects them from feeding by insects (Chew, 1988). Besides directly inhibiting feeding behaviour in beetles, several feeding deterrents, e.g. drimanes, generate postingestive sub-lethal toxic effects that also result in decreased food intake (Frazier & Chyb, 1995; Gonzalez-Coloma *et al.*, 1995; Ortego *et al.*, 1995; Gols *et al.*, 1996). During the last decades, feeding deterrents received particular interest because of their potential use as insect control agents (Frazier & Chyb, 1995).

Despite the importance of feeding deterrents in food plant selection by *L. decemlineata*, little is known about the sensory perception of such compounds by this beetle. Schoonhoven & Jermy (1977) showed that larvae are immediately deterred from feeding when their mouthparts contact droplets of feeding deterrent solutions. This demonstrates that *L. decemlineata* larvae must use mouthpart sensilla in perceiving feeding deterrents.

Sensilla possibly involved in food selection by L. decemlineata larvae are situated on the maxillary galea (two sensilla; Mitchell & Schoonhoven, 1974) and on the inner surface of the labrum, the epipharynx (*c.* 21 sensilla: Chin, 1950; Figure 2). In electrophysiological experiments the galeal sensilla did not respond to stimulation with feeding deterrents, e.g. solanum alkaloids and strychnine (Mitchell & Schoonhoven, 1974) or drimanes (Messchendorp, unpubl. data). Further, Messchendorp (unpubl. data) found that drimanes do not inhibit the response of galeal sensilla to the feeding stimulant sucrose. Also for *L. decemlineata* adults, the perception of feeding deterrents has not been clarified. Mitchell & Harrison (1985) and Mitchell (1987) did not find evidence of a 'general deterrent receptor'. Mitchell (1987) found that sensory responses to the feeding stimulants sucrose and GABA in an 'α-sensillum' on the maxillary galea were inhibited by some alkaloids. However, no correlation between these effects and feeding inhibition was found. Thus, feeding inhibition by these alkaloids could not be caused by inhibition of the α-sensillum. Mitchell (1988) and van Haeften (1993) mentioned the presence of epipharyngeal sensilla in adult *L. decemlineata* but further functional investigations are not known to us.

In this work the possible function of epipharyngeal sensilla in detecting feeding deterrents by *L. decemlineata* larvae was examined. We measured the behavioural response of the larvae when contacting solutions of the feeding deterrents with their mouthparts. The morphology of one of the epipharyngeal sensilla was studied with electron microscopy. We also measured electrophysiological responses from this epipharyngeal sensillum to two feeding deterrents, a synthetic drimane analogue and sinigrin. Further, sensory responses to the feeding stimulant sucrose and to binary mixtures of the three mentioned compounds were measured. In order to elucidate how feeding deterrents are neurally coded in L. decemlineata larvae, we related the electrophysiological results to the behavioural responses.

Materials and Methods

Insects. L. decemlineata beetles were reared on excised foliage of potato plants (Solanum tuberosum, cultivar Surprise) in L16:D8 at 25°C. In the experiments fourth instar larvae weighing between 110-170 mg were used.

Chemicals. The molecular structures of the synthetic drimane analogue and of sinigrin are given in Figure 1. Sinigrin was obtained from Janssen Chimica, Belgium. The drimane was synthesized at the Department of Organic Chemistry, Wageningen Agricultural University (Jansen, 1993; C.T. Bouwman, unpubl.). It is derived from naturally occurring drimane feeding deterrents (Jansen, 1993) and exhibits significant activity in a standard dual-choice test (Gols *et al.*, 1996). In the test solutions, ethanol was used to improve the solubility of the drimane and Tween-80 was used to lower the surface tension of droplets of solution used in the behavioural 'microsyringe test'. For the behavioural tests the compounds were first dissolved in ethanol and Tween-80. Distilled water was added to obtain a final solution of 2% ethanol and 2% Tween-80. Distilled water with 2% ethanol and 2% Tween-80 was used as control solution. Ethanol was also

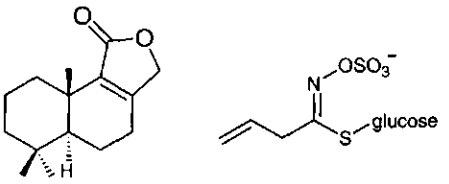

Figure 1. Chemical structures of the feeding deterrents. (A) synthetic sesquiterpene drimane analogue. (B) sinigrin.

used to dissolve the compounds for the electrophysiological tests. A 5 mM KCl solution in distilled water was added to obtain a final solution of 5% ethanol. Distilled water with 5 mM KCl and 5% ethanol was used as control solution.

Electron microscopy. We used electron microscopy (Zeiss EM 109) to determine the number of receptor cells that innervate the epipharyngeal sensillum examined by us (Figure 2). Labrum tissue of 4th instar larvae was immersed for fixation in 2% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.3, for 2 h at 4°C. Postfixation was done in 1% OsO_4 in the same buffer, for 1 h at room temperature. The labrum was trimmed to remove most tissue surrounding the sensillum and embedded in epon. Serial sections of 80 nm were cut and collected on formvar-coated nickel grids and contrasted with 2% uranyl acetate.

Behaviour: the 'microsyringe test'. The experiments were conducted in a climate room at T = 24±1°C and r.h. 60-65%. Before the experiments the larvae were starved for 20 min to enhance their motivation to feed. A 3 cm high glass vial with a diameter of 0.7 cm was filled with moist paper and placed on a white piece of paper. A punched potato leaf disc with a diameter of 1.75 cm was placed on top of the glass vial and fixed to the paper with a glass pin. With use of forceps, the larvae were gently transferred to the middle of the disc. Generally, they started to feed on the leaf disc's edge within 1-3 min. After a larva had been feeding for 1-2 min, a microsyringe was used to carefully put a droplet (1-2 µl) of control solution a few mm away from the chewing mouthparts, so that these would grasp into the droplet within a few seconds. When applied in this way, all the mouthpart sensilla contact the solution while the latter is mixed with leaf substance. Most larvae were not (or only slightly) disturbed upon touching the control solution and continued to feed while ingesting the droplet. After feeding had been resumed for *c.* 1 min, the procedure was repeated with a droplet of feeding deterrent solution. Contacting these solutions in most cases temporarily disturbed feeding and the 'disturbance time' (= period between touching the feeding deterrent droplet and restart of feeding minus period between touching the control droplet and restart of feeding) was measured. Each larva

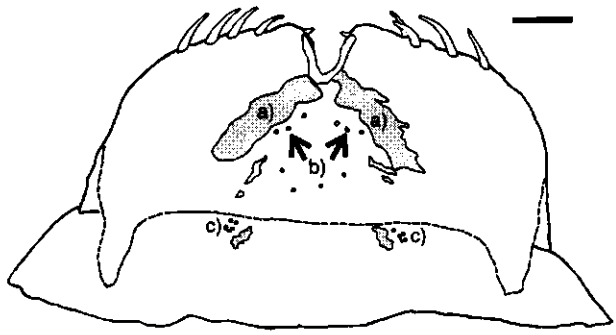

Figure 2. Schematic drawing of the inner surface of the labrum of a 4th instar *L. decemlineata* larva (bar = 0.1 mm). In the majority of insects, two rows of three sensilla occur near two brown pigmented thickened areas (grey, and labeled as a) in figure). The sensilla studied in the electrophysiological experiments are the middle ones of these three sensilla and indicated by arrows. A small percentage of the insects lacks one of the three sensilla or possesses one extra in this area. Furthermore, a group of 4-5 (the number varying among larvae) sensilla occurs on the middle portion of the epipharynx (labeled b)) and two groups of five sensilla are lying laterally of brown pigmented thickened areas (grey) proximally on the epipharynx (labeled c)). In total 20-21 sensilla are located on the epipharynx. The broken line indicates the border of the exterior surface of the labrum.

was tested only once with one concentration and the experiment was repeated 6-12 times for each concentration. Concentrations between 0.1 mM and 10 mM were tested. The drimane was not tested at 10 mM, because of its limited solubility at this concentration.

Electrophysiology. For recording responses from the epipharyngeal sensillum we used a modified 'tip recording technique' after Hodgson *et al.* (1955) and van Loon (1990). Before the experiments, the larvae were starved for 3-6 h and subsequently kept at -20°C for *c.* 2 min. The starvation period improved the signal to noise ratio in the recordings. The 2 min chilling period reduced the chance of regurgitating of the insect on decapitation of its head, and thus improved the chance of successful recording. Severed insect heads were mounted on a silver wire electrode that was connected to the input probe of an amplifier (Syntech UN-03b, Hilversum, The Netherlands). The heads were positioned with the rostral-ventral side upwards. A micro glass hook mounted on a micromanipulator was used to lift the labrum backwards, to expose the inner surface. Stimulus solutions were provided in glass capillaries (tip diameter *c.* 10 µm) connected to ground to serve as reference electrode. Amplified signals were digitized (DAS 16 Metrabyte Co. AD conversion board) and sampled into computer memory (Intel 486 DX) at 10 kHz sampling frequency. We stimulated the epipharyngeal sensillum (Figure 2) by carefully positioning the tip of the glass capillary perpendicular towards the surface of the

epipharynx. When the solution in the capillary tip touched the sensillum, a 'solution bridge' between the capillary and the sensillum was formed. When applied in this way, leakage of solution out of the capillary was mostly prevented. To increase the local humidity, a water bath was positioned *c.* 10 cm below the preparation. *c.* 20 Seconds before each stimulation, the solution in the capillary tip was drawn out with filter paper, to prevent possible increase of concentration by evaporation of water from the tip. We recorded the first 3 seconds of each stimulation. Time between two stimulations was at least 2 min, to allow for disadaptation. Impulses were counted with the aid of SAPID Tools computer software (Smith *et al.*, 1990) and sorted visually on the basis of action potential amplitude, temporal pattern of firing and shape. From each preparation, responses from either the left or right epipharyngeal sensillum (Figure 2) were recorded; the sensillum that showed the highest signal / noise ratio was chosen. Preparations that did not respond to the control solution were omitted. The proportion of preparations that could be recorded from successfully was about 30%.

The drimane was tested at concentrations between 0.01 mM and 2.5 mM. Because of its limited solubility concentrations above 2.5 mM were not tested. Sinigrin was tested at concentrations between 0.01 mM and 10 mM. The original intention was to stimulate each larva with the control solution and with the full range of concentrations of one of the deterrent compounds. This was not possible for all preparations because of early deterioration of the signal. The different concentrations were offered in random order. The sequence did not noticeably influence the responses. Each concentration was tested on 6-11 larvae. The response during the 1st 1.5 s of stimulation with sucrose, dissolved in 5 mM KCl, was measured as well (doses between 0.05 mM and 10 mM; each concentration was tested on 5-6 larvae). Furthermore, a dose-response experiment with potassium chloride was performed (doses between 0.1-100 mM; each concentration was tested on 5-10 larvae.

To examine whether the drimane, sinigrin and sucrose stimulate the same, or different receptor cells in the epipharyngeal sensillum, nerve impulse firing patterns on stimulation with mixtures (1 mM drimane + 1 mM sinigrin, 1 mM drimane + 1 mM sucrose and 1 mM sinigrin + 1 mM sucrose) were examined. Further, we examined whether peripheral interactions occurred by comparing the response of the receptor cells on stimulation with pure 1 mM solutions and these mixtures. The responses that were compared were obtained from the same epipharyngeal sensillum in the same insect. Each solution was tested on 4-6 larvae.

Results

Electron microscopy. The epipharyngeal sensillum examined by us was identified at the ultrastructural level by its position (middle of the row of three sensilla; Figure 2). Cross sections of this sensillum reveal five separate dendrites (Figure 3). The sensillum must therefore be innervated by five receptor cells (Figure 3).

Figure 3. Electron micrograph of a cross section through the epipharyngeal sensillum at the level of the dendrites (bar = 500 nm).

Behavioural microsyringe test. The microsyringe test was employed to examine whether *L. decemlineata* larvae can perceive the feeding deterrents with their mouthpart sensilla. In the majority of experiments (*c.* 75%), after a larva had contacted a droplet of feeding deterrent solution interruption of feeding activity occurred immediately. The remaining larvae reacted with a slight delay, varying between 2-12 seconds. At the highest concentrations, the larvae typically reacted by withdrawing the head and making quick chewing movements, upon which they turned away from the feeding spot. After this, while palpating the leaf surface, they searched for another feeding spot on the leaf disc. At lower concentrations, larvae resumed feeding within shorter time, or did not seem to be disturbed. Figure 4 shows that the mean disturbance time is dose dependent for both sinigrin and the drimane. The threshold concentration for reaction to drimane solutions (between 0.1 and 0.5 mM) is lower than the threshold for reaction to sinigrin solutions (between 0.5 and 1.0 mM). For concentrations $\geq$ 1 mM, the disturbance time caused by the two feeding deterrents does not differ significantly.

Electrophysiology. The behavioural microsyringe test indicates that *L. decemlineata* larvae detect the presence of feeding deterrents with their mouthpart sensilla. Since it had been shown before that the maxillary galeal sensilla did not respond to stimulation

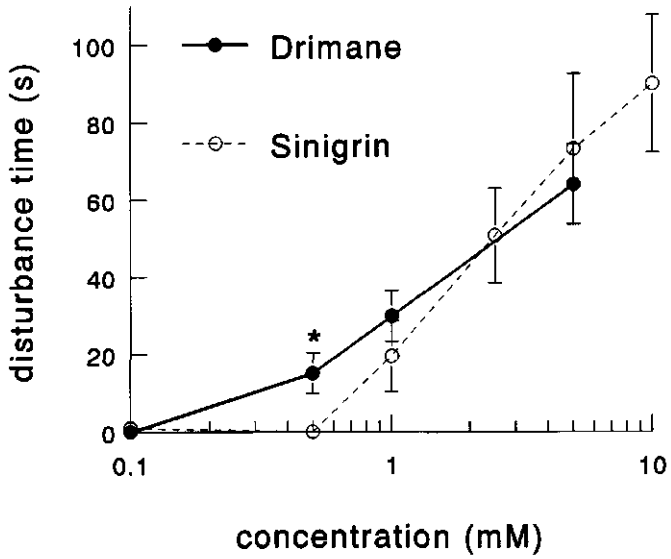

Figure 4. Disturbance time at various concentrations of the feeding deterrents in the behavioural microsyringe test. (means±s.e., for ascending concentrations of the drimane n = 11, 15, 16 and 11 respectively, for sinigrin n = 6, 6, 11, 11, 10 and 12 respectively). Statistics: Mann-Whitney U; * $P < 0.05$.

with the drimane (Messchendorp, unpubl.) we continued with examining one of the epipharyngeal sensilla on the inner surface of the labrum, which function was unknown until now (Figure 2). The two epipharyngeal sensilla situated medially and laterally of the sensillum that we examined had a chemosensory function, but were more difficult to stimulate (the success ratio was low and the evoked responses were irregular). We did not further examine these epipharyngeal sensilla nor the ones positioned more proximally on the epipharynx.

Figure 5 shows electrophysiological recordings from the epipharyngeal sensillum examined by us during the first 0.5 (of the totally 1.5-3) seconds of stimulation. Figures 5A and 5B show recordings of responses on stimulation with distilled water and 5 mM KCl respectively. In response to both stimuli, generally one action potential type with a phasic firing pattern occurs. In a small percentage of the recordings (< 10%) a second, irregular and infrequent occurring action potential type with smaller amplitude was found. In general, the responses to distilled water showed a low signal / noise ratio. The potassium chloride dose-response curve (Figure 6) shows that, except for the small increase in response at concentrations between 10-50 mM, the responding receptor cell is not highly sensitive to potassium chloride. Remarkably, the action potential amplitudes recorded changed with potassium chloride concentration: peak amplitudes were found at

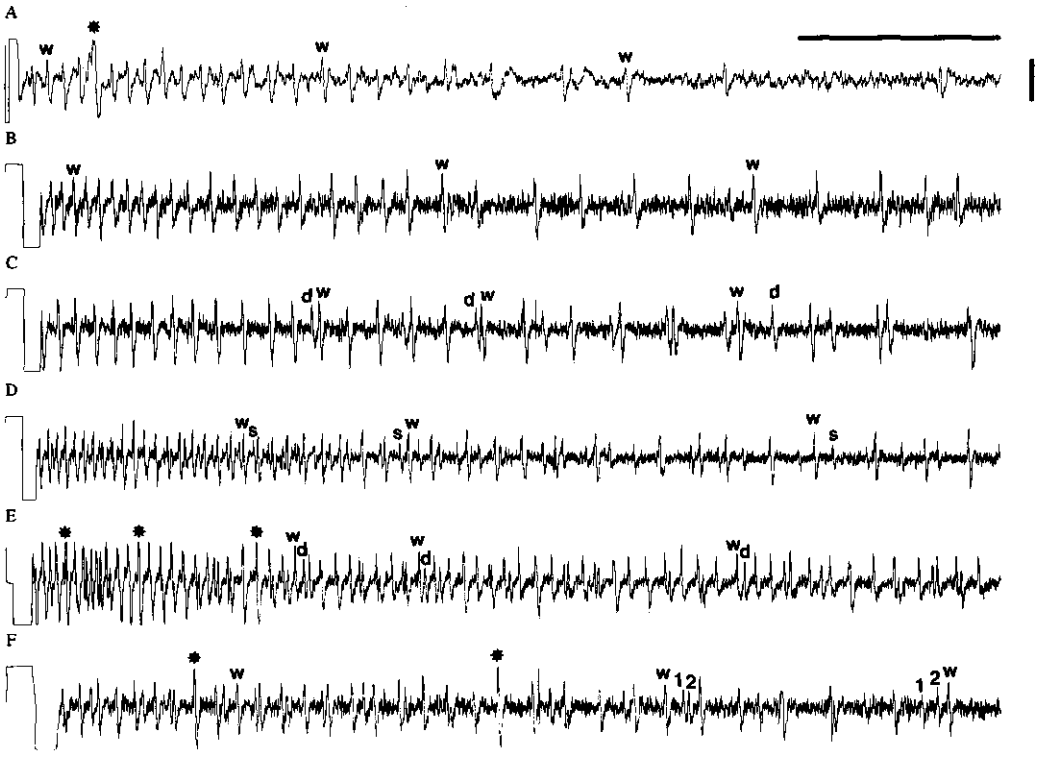

Figure 5. Electrophysiological recordings from responses of the epipharyngeal sensillum (1st 500 ms; horizontal bar = 100 ms, vertical bar = 3 mV) to distilled water (A), 5 mM KCl and 5% ethanol in distilled water (control solution) (B), 1 mM drimane (C), 1 mM sucrose (D), 1 mM drimane + 1 mM sinigrin (E) and 1 mM drimane + 1 mM sucrose (F). w, d and s indicate action potentials generated by the water cell, the deterrent cell and the cell sensitive to sucrose, respectively: three examples have been marked in each recording; 1 and 2 indicate action potentials from either the deterrent cell or the cell sensitive to sucrose; * indicates a probable addition of two spikes.

concentrations between *c.* 0.1-5 mM KCl and at lower and higher doses recorded amplitudes were considerably smaller. The similarity in temporal pattern of firing and the absence of a dose-response relationship in the range of 0.1-5 mM KCl led us to assume that the action potentials in the responses to distilled water and KCl solutions originate from the same cell, a putative 'water cell'. We chose 5 mM KCl as control solution because it gave a good signal/noise ratio in the recordings and because this concentration is in the range of free potassium ion concentrations found in plants.

The recordings of responses to the drimane (Figure 5C) and sinigrin show, besides the action potentials from the water cell, a second action potential type with a

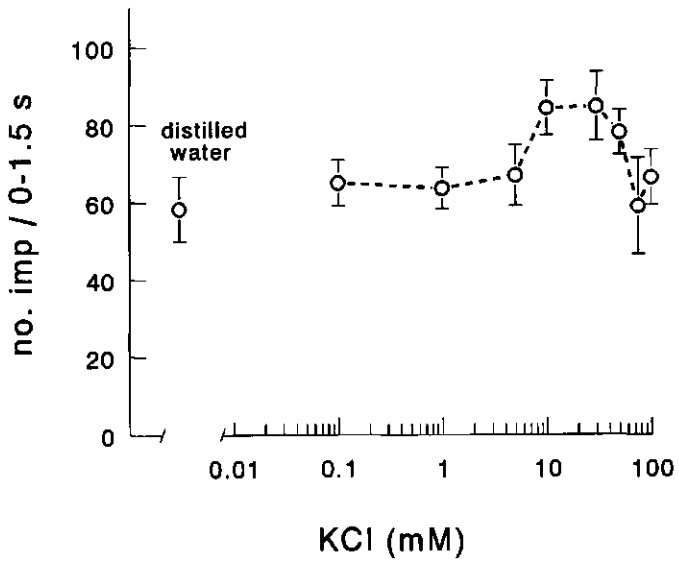

Figure 6. Numbers of action potentials from the water cell on electrophysiological stimulation with distilled water and various concentrations of potassium chloride during the 1st 1.5 seconds of stimulation (means±s.e., for distilled water and ascending concentrations of potassium chloride n = 5, 9, 10, 8, 8, 6, 10, 5 and 8 respectively).

smaller amplitude. The response to sucrose shows, besides the water cell action potentials, a second type with small amplitudes as well (Figure 5D). Some of the action potentials in Figures 5D-F are clipped because additions of spikes occurred. It was technically not possible to record the full amplitude of the latter without reducing the gain, which would make the action potentials from the sucrose sensitive and the deterrent cells less discernable. Figure 5E shows recordings of the response of the epipharyngeal sensillum on stimulation with a mixture of 1 mM drimane + 1 mM sinigrin. Similar to the response pattern on stimulation with a single feeding deterrent solution, two action potential types occur in the recordings. We conclude that the drimane and sinigrin probably stimulate the same 'deterrent cell' in the epipharyngeal sensillum. In Figure 5F the response to a mixture of 1 mM drimane + 1 mM sucrose is shown. In these responses, and also in the responses to mixtures of 1 mM sinigrin + 1 mM sucrose three types of action potentials can be discerned. Therefore, in addition to a water cell and a deterrent cell, the epipharyngeal sensillum must contain a third cell that responds to stimulation with sucrose.

Figures 7A-D shows the dose-response curves of the epipharyngeal sensillum to drimane and sinigrin solutions. The response of the 'water cell' was stable at the different concentrations of drimane and sinigrin. Its impulse frequency strongly decreased during

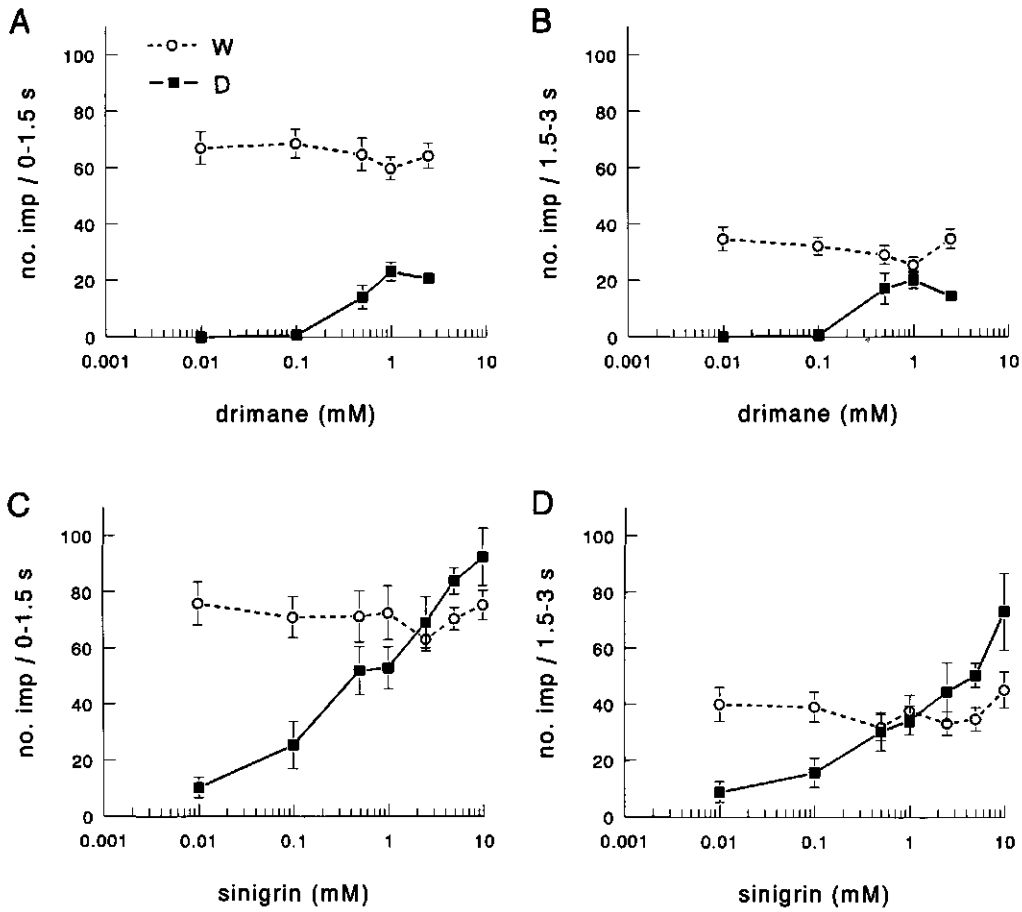

Figure 7. Numbers of action potentials from the deterrent cell (D) and the water cell (W) on electrophysiological stimulation with various concentrations of the feeding deterrents during the 1st (A,C) and 2nd (B,D) 1.5 seconds of stimulation (means±s.e., for ascending concentrations of drimane during the 1st and 2nd 1.5 seconds n = 9, 11, 10, 9 and 10 respectively, for ascending concentrations of sinigrin during the 1st 1.5 seconds n = 7, 11, 7, 8, 9, 8 and 7, and during the 2nd 1.5 seconds n = 7, 11, 6, 8, 9, 8 and 7 respectively). A,B drimane. C,D sinigrin.

the second 1.5 s of stimulation. The response of the 'deterrent cell' increased with increasing doses of drimane and sinigrin, and shows little adaptation in the second 1.5 s of stimulation. The threshold concentration for response to the drimane (*c.* 0.1 mM) is higher than for sinigrin (below 0.01 mM). Sinigrin evokes more nerve impulses from the deterrent cell than the drimane at all concentrations tested. The drimane evokes a

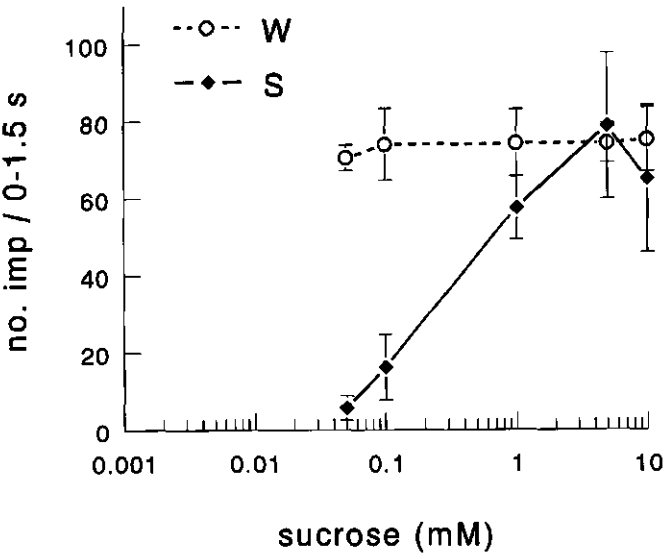

Figure 8. Numbers of action potentials from the sucrose sensitive cell (S) on electrophysiological stimulation with various concentrations of sucrose during the 1st 1.5 seconds of stimulation (means±s.e., for ascending concentrations n = 6, 5, 6, 6 and 5 respectively).

maximum response at 1 mM. This might be caused by the fact that the solution is saturated at this concentration. Figure 8 shows that the threshold for response to sucrose is 0.05 mM and that 5 mM causes a maximal response.

In Figures 9A,B the number of nerve impulses recorded from the epipharyngeal sensillum on stimulation with a mixture of 1 mM drimane + 1 mM sinigrin is compared with the number of nerve impulses on stimulation with 1 mM pure solutions. The number of action potentials from the water cell on stimulation with the three different solutions did not significantly differ. The response of the deterrent cell to the mixture is higher compared with the response to 1 mM drimane, at least in the second 1.5 second of stimulation (Figure 9B), but not different when compared with the response to sinigrin. This is in agreement with the dose-response curves for the two deterrents (Figures 7A-D). Sinigrin is more effective than the drimane in stimulating the deterrent cell, but a concentration of 2 mM sinigrin does not give a highly increased response compared to 1 mM. Therefore, under the assumption that the two deterrents stimulate the same deterrent cell, a mixture of 1 mM sinigrin + 1 mM drimane is also not expected to give a large increase in response compared to sinigrin alone. Figure 9 clearly shows that the drimane and sinigrin do not additively evoke action potentials from receptor cells in the epipharyngeal sensillum, which would be expected if the compounds were stimulating

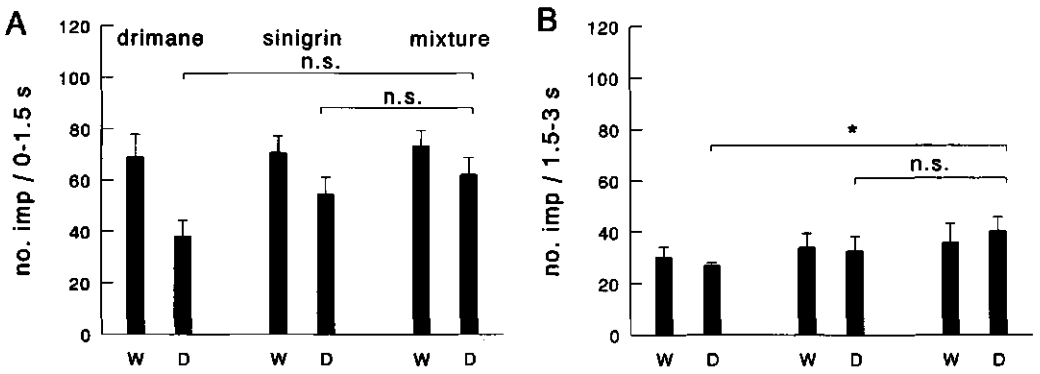

Figure 9. Numbers of action potentials from the deterrent cell (D) and the water cell (W) on electrophysiological stimulation with a mixture of 1 mM drimane + 1 mM sinigrin and 1 mM pure solutions during the 1st (A) and 2nd (B) 1.5 seconds of stimulation (means±s.e., n = 7(A) and 5(B)). Statistics: Mann-Whitney U; * $P < 0.05$.

two different cells. These considerations strengthen the hypothesis that the drimane and sinigrin stimulate the same receptor cell in the epipharyngeal sensillum.

Except for the action potentials from the water cell, it proved to be very difficult to objectively separate the other two types of action potentials in the recordings of responses to mixtures with deterrents and sucrose. Therefore, in Figures 10A,B the total number of nerve impulses from the deterrent and the sucrose sensitive cell on stimulation with a mixture of 1 mM drimane + 1 mM sucrose is compared with the sum of nerve impulses from the cell responsive to sucrose (evoked by a 1 mM solution of sucrose) and from the deterrent cell (evoked by a 1 mM solution of drimane). The number of nerve impulses on stimulation with the mixture is significantly lower than the sum of the nerve impulses recorded on stimulation with pure solutions (mean difference = 64% of the sum in the 1st 1.5 s of recording and 62% in the 2nd 1.5 s of recording). When sinigrin instead of drimane was presented in the mixture (Figures 10C,D), the difference with the sum of nerve impulses evoked by pure solutions was less (mean difference = 18% in the 1st 1.5 s of recording and no significant difference in the 2nd 1.5 s of recording). The number of action potentials from the water cell on stimulation with the different solutions did not significantly differ. Therefore, peripheral interactions must occur at the level of the cell responsive to sucrose and/or the deterrent cell.

Discussion

In this work we report the first deterrent receptor identified for *L. decemlineata* larvae. In electrophysiological experiments, we showed that at least one of the epipharyngeal

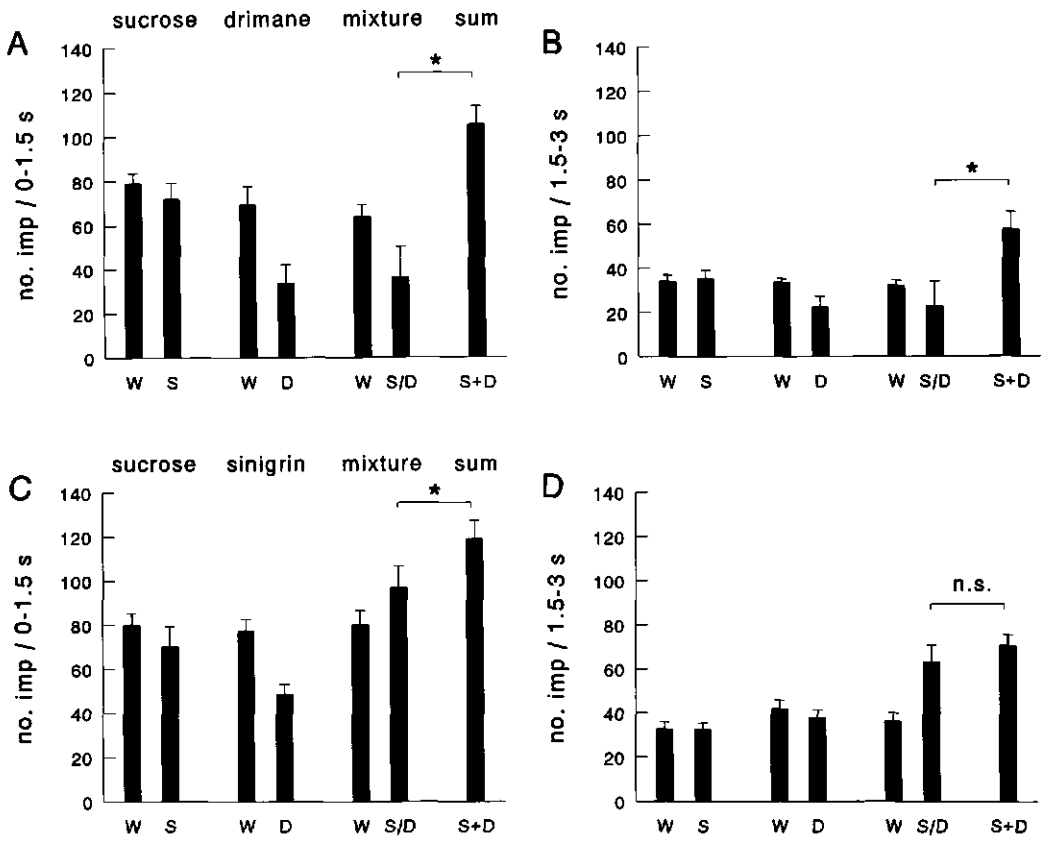

Figure 10. Numbers of action potentials from the water cell (W), the sucrose sensitive cell (S) and the deterrent cell (D) on stimulation with 1 mM sucrose, 1 mM drimane and (1 mM sucrose + 1 mM drimane) (S/D indicates numbers of action potentials from the sucrose sensitive cell + deterrent cell) (A,B), or on stimulation with 1 mM sucrose, 1 mM sinigrin and (1 mM sucrose + 1 mM sinigrin) (C,D), compared to the sum of action potentials from the sucrose sensitive cell and the deterrent cell when stimulated with 1 mM pure solutions (S+D) (means±s.e., n = 6(A), 5(B,C) and 4(D)). Responses during the 1st (A,C) and 2nd (B,D) 1.5 s of stimulation are shown. Statistics: Mann-Whitney U; * $P < 0.05$.

sensilla has a contact chemosensory function. Responses from three receptor cells in this sensillum could be recorded: one cell responded to water, a second to sucrose and a third to feeding deterrents. With the aid of electron microscopy we showed that this epipharyngeal sensillum contains five sensory cells. The function of the remaining two cells is still unknown. In pilot tests GABA and chlorogenic acid, two compounds that were earlier found to effectively stimulate cells in galeal sensilla (Mitchell & Schoonhoven,

1974), did not evoke responses. We did not find indications for the presence of a mechanoreceptor cell.

Stimulation of the epipharyngeal sensillum with a range of known feeding deterrents from different chemical classes is needed to determine if the deterrent cell in the epipharyngeal sensillum is a general deterrent cell such as are described for many lepidopteran species (Schoonhoven *et al.*, 1992). Cells responsive to water have rarely been found in phytophagous insects. To our knowledge, no water cells have been found on mouthparts of *L. decemlineata* or other phytophagous insects. In the cabbage fly *Delia radicum* L. indications for a water receptor were found in tarsal 'B'-sensilla (Städler, 1978; P. Roessingh, unpubl.). Recently, cells responsive to water were found in tarsal and ovipositor taste sensilla of *Plutella xylostella* L. female moths (Qiu *et al.*, 1998).

Although in this work only one of the 20-21 epipharyngeal sensilla was examined we tried to derive a neural code for the tested feeding deterrents in *L. decemlineata* larvae by relating the behavioural responses to the electrophysiological responses from this epipharyngeal sensillum. For the drimane, the sensory threshold and behavioural threshold are approximately identical (between 0.1 mM and 0.5 mM). This suggests a direct influence of the deterrent cell on feeding behaviour. For sinigrin however, the sensory threshold (below 0.01 mM) and behavioural threshold (between 0.5 mM and 1 mM) do not correspond. The sensory threshold for sinigrin is lower than the drimane threshold. Sinigrin also evokes more nerve impulses from the deterrent cell than the drimane at all concentrations tested. In contrast, the behavioural threshold for sinigrin is higher than the drimane threshold. Thus it is clear that the behavioural reaction can not fully be explained by responses of this deterrent cell. In the behavioural tests a mixture of feeding deterrents and chewed leaf substance is experienced by the larvae. The leaf substance contains a blend of chemicals (among others sucrose) that stimulate the larvae to feed (Hsiao & Fraenkel, 1968 a,b). Differential peripheral interactions (Chapman, 1995) at the receptor membrane could take place on stimulation with a mixture of leaf substance and different feeding deterrents. Therefore, the electrophysiological experiments with mixtures of sucrose and feeding deterrents could elucidate the relationship between behaviour and the sensory response. On stimulation with mixtures of sucrose and drimane we measured a dramatically lower number of nerve impulses than would be expected on the basis of the sum of nerve impulses in responses to single compounds (Figures 10A,B). When sinigrin is used there is hardly any difference in nerve impulse number (Figures 10C,D). From the latter result we derive that sucrose does not strongly influence the response of the deterrent cell and deduce that the decrease in numbers of action potentials on stimulation with mixtures of drimane and sucrose must be due to inhibition of the response of the sucrose cell by the drimane. In a model on brain functioning in insects proposed by Blom (1978) and Schoonhoven & Blom (1988), it is assumed that information from receptor cells sensitive to feeding stimulants and receptor cells sensitive to feeding deterrents is subtracted algebraically in the central nervous system. Thus, applying this model on *L. decemlineata* larvae, it can be reasoned that sinigrin has a lower feeding inhibiting effect than expected on the basis of the nerve impulse frequency from the deterrent cell because, in contrast to the drimane, it does not (or hardly) inhibit the response of the receptor cell sensitive to sucrose.

We conclude that the deterrent cell in the epipharyngeal sensillum contributes to the perception of feeding deterrents by *L. decemlineata* larvae. Further, peripheral interactions on the sucrose sensitive cell probably determine the potency of feeding deterrents to *L. decemlineata* larvae. To directly determine the sensory response of the epipharyngeal sensillum when the larvae are exposed to the behavioural microsyringe test electrophysiological experiments with mixtures of leaf juice and feeding deterrents are needed. Possibly more epipharyngeal sensilla, or sensilla located at other mouthparts are important in the perception of feeding deterrents by *L. decemlineata* larvae. Experiments with labrally ablated larvae could answer the latter question, although these would practically be difficult to conduct. Extended research on the electrophysiological characteristics of this and the other epipharyngeal sensilla is needed to further uncover their function in feeding behaviour in *L. decemlineata* larvae.

The finding that an epipharyngeal sensillum is involved in mediating the perception of feeding deterrents in *L. decemlineata* larvae gives rise to an evaluation of the function of epipharyngeal sensilla in taste perception in *L. decemlineata* and other insects. Little is known about the sensory perception of feeding deterrents in coleopterans. In two other chrysomelid species, *Diabrotica virgifera virgifera* (Chyb *et al.*, 1995) and *Entomoscelis americana* (Mitchell & Sutcliffe, 1984) galeal sensilla were examined but no specific 'deterrent cells' were found. Epipharyngeal sensilla were not electrophysiologically examined.

Research on sensory taste mechanisms in phytophagous insects has been mainly focussed on the larval stages of lepidopteran insects e.g. *Manduca sexta*, *Pieris* and *Spodoptera* species. In several of these species, the frequency of nerve impulses from deterrent cells in galeal sensilla can be related to the behavioural effects of feeding deterrents (Blaney *et al.*, 1987; Luo *et al.*, 1995; Simmonds *et al.*, 1995; Messchendorp *et al.*, 1996). This suggests that signals from these deterrent cells to the central nervous system directly influence feeding behaviour. Feeding deterrents also have been shown to interfere with the response of cells sensitive to feeding stimulants (Simmonds & Blaney, 1983; Simmonds *et al.*, 1990, Schoonhoven & Luo, 1994; Luo *et al.*, 1995; Messchendorp *et al.*, 1996). In few lepidopteran species, e.g. *P. brassicae* (Ma, 1972; Blom, 1978) and *M. sexta* (de Boer *et al.*, 1977), also epipharyngeal sensilla have been examined. In most species examined in the literature an epipharyngeal sensillum was shown to contain three contact chemoreceptor cells, among which one deterrent cell. No mechanosensory cells were identified in the epipharyngeal sensilla.

In *L. decemlineata* larvae, we found a varying number of epipharyngeal sensilla, mostly 20 or 21 (Figure 2). Compared to lepidopterans, in which the examined species possess a number of epipharyngeal sensilla varying between zero and six, *L. decemlineata* larvae thus have many more of these sensilla. This might reflect a greater importance of epipharyngeal taste sensilla in food selection by *L. decemlineata* larvae than by lepidopteran caterpillars, in which the galeal sensilla are generally considered as the decisive sensory organs affecting food selection. According to Mitchell (1988), "video observations of adult *L. decemlineata* (Mitchell, unpubl.) suggest that ... epipharyngeal sensilla ... are the first gustatory organs to taste leaf sap" when feeding on a leaf is started, which might also indicate that epipharyngeal sensilla are important in taste perception by *L. decemlineata*.

Despite many studies, reviewed by Mitchell (1988) and Jermy (1994), the perception of feeding deterrent compounds, known to play an important role in food plant recognition in *L. decemlineata*, remained for several decades an enigma. The present study, by attributing a major role to the epipharyngeal organs, puts a new perspective on the problem of how L. decemlineata larvae unerringly recognize their food plants.

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mechanism(s) of action of deterring compounds against aphids, and the sensilla involved to detect them could assist in utilizing such compounds as crop protection agents.

In this work, eleven synthetic drimane compounds, applied to the surface of artificial diet sachets, were tested for their deterring activities on nymphs of two aphid species, *Myzus persicae* and *Aphis gossypii*. Both species are important pests on many crops throughout the world (Eastop, 1983; van Steenis, 1995), especially *A. gossypii* which rapidly exhibits resistance against many insecticides (Kerns & Gaylor, 1992). Further, in both *M. persicae* and *A. gossypii* nymphs, with use of 24-48 h behavioural interval observations and ablation studies, it was examined how drimanes influence the distribution pattern of the nymphs over artificial diet test-rings in time, and which sensilla the aphids use to detect the drimanes.

Materials and methods

Aphids. Parthenogenetically reproduced, apterous *M. persicae* (clone WMp3: Reinink *et al.*, 1989) were reared on 3-4 weeks old oil seed rape plants (*Brassica napus*, cultivar 'Olymp') in the greenhouse at T= 16±2°C (night) - 20±5°C (day) and L16:D8. Field collected *A. gossypii* aphids were obtained from the 'Centro de Ciencias Medioambientales, CSIC', Madrid, Spain. Parthenogenetically reproduced, apterous nymphs were reared on 5-8 weeks old cucumber plants (*Cucumis sativus* L., cultivar 'Chinese slangen') in the greenhouse, under the same conditions as *M. persicae*. 5-6 Day old *M. persicae* and 1-3 day old *A. gossypii* nymphs reared on plants were used in the dual-choice tests. For the other experiments nymphs reared on artificial diet (Harrewijn, 1983) were used.

Chemicals. Eleven synthetic drimanes (Figure 1) were obtained from the Department of Organic Chemistry, Wageningen Agricultural University (Jansen, 1993; C.T. Bouwman, unpubl.). All compounds are racemic (only one of the two enantiomeres is drawn in Figure 1). Although compounds **1**, **2**, **3**, **5** and **8** can be isolated from plants (Jansen, 1993), in all experiments 0.1% (1 g/l) synthetic drimane solutions (*c.* 4-5 mM) in ethanol (> 99%) were used.

Dual-choice test. Artificial diet test-rings were prepared by stretching a layer of parafilm (*c.* 5*5 cm) over a plastic ring (2.7 cm diameter and 1.7 cm high) and painting half of the lower surface of the parafilm in the ring with 10 µl drimane solution (*c.* 3.5 µg drimane/-cm^2) and the other half with 10 µl ethanol. Two drops (*c.* 30-50 µl) of artificial diet (Harrewijn, 1983) were kept between two layers of parafilm that were sealed together in two halve circles (*c.* 1-2 cm diameter), thereby forming two sachets containing artificial diet (for control and treated half). After the first layer was dried, the sachets containing artificial diet (for control and treated half) were stretched over the first layer on the plastic ring. We used artificial diet to prevent possible phytotoxic effects of the drimanes (Asakawa *et al.*, 1988; Perczel, 1994), that could influence the test results. We applied the solutions to a separate layer of parafilm because it was previously found in our laboratory that solvents necessary to dissolve the drimanes, such as ethanol, diglyme or

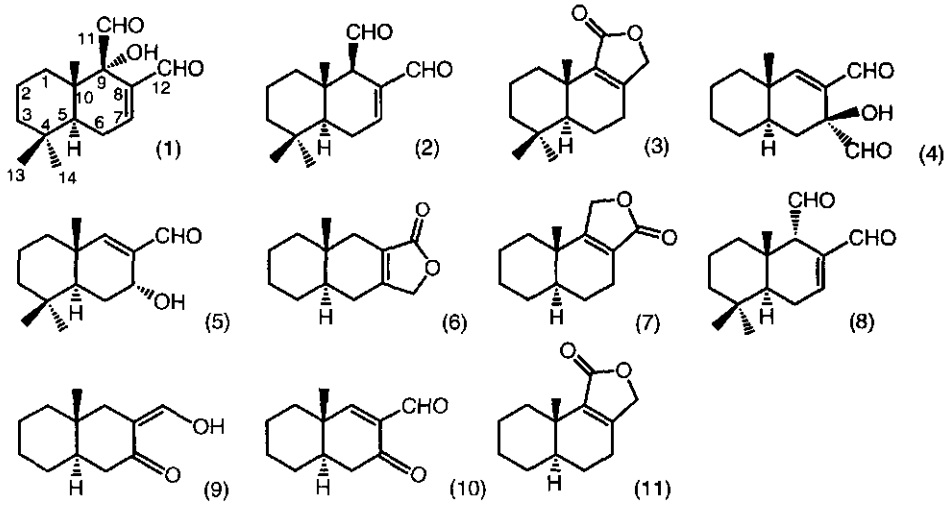


Figure 1. Chemical structures of the drimanes. Trivial names: warburganal (**1**), polygodial (**2**), isodrimenin (**3**), polygonal (**5**) isotadeonal (**8**).

Tween-80, penetrate into the diet when directly applied to the sachet surface and subsequently have deterring and/or toxic effects themselves (Perczel, 1994), which could influence the test results as well. 20-25 Nymphs reared on plants were put on the inside surface of the ring and the ring was placed on a plastic petri-dish with the diet on top. Nymphs, instead of adults, were used because previous work had shown that the latter are less sensitive to drimanes (Perczel, 1994) and because adults keep producing progeny, which could result in early depletion of the diet. Each drimane was tested with 20 replicates in a climate chamber at T = 22°C with continuous illumination (2 high frequency Philips PL-L 24 W, colour 84 fluorescent lamps) from above. Thin filter paper was attached just below the lamps to make the light more diffuse. After 24 h most *M. persicae* nymphs had moved to the diet surface to penetrate into the diet and the number of nymphs on control and treated half was counted. *A. gossypii* nymphs were still relatively restless at 24 h and were therefore counted at 48 h. Data on test-rings with less than 10 nymphs on the diet surface were omitted. A deterrency-index (D.I.) was calculated for each ring: D.I. = C-T/C+T (C = number of nymphs on control half; T = number of nymphs on treated half). Wilcoxon's matched pairs signed rank test was used to assess significance.

24 h interval observations of dual-choice tests. The same diet test-rings and methods as described for the dual-choice test were used. Aphids were reared on diet to avoid

transition effects by the change from plants to diet at the start of the test. The aphids on the lower surfaces of the diet sachets were video recorded during the 24 h of the test (Panasonic CCD camera (WV-CD 20) equipped with Ernitec 8-16 mm zoom lens, connected to a Panasonic time lapse video cassette recorder (AG 6010)). *A. gossypii* nymphs that were reared on diet were less restless than nymphs reared on plants and settled within 24 h in these tests. Every hour the number of aphids that walked, and that remained stationary on the control- and treated sides and on the middle line (the seal between control and treated diet sachet), were counted. *M. persicae* was observed on test-rings treated with polygodial (**2**) and *A. gossypii* on test-rings with polygodial (**2**) and isotadeonal (**8**). The numbers of replicates were 6-10.

24 h interval observations of dual-choice tests after ablation of the distal parts of the antennae. Aphid nymphs reared on artificial diet were anaesthetized for *c.* 1-2 min with CO_2 and from both antennae the distal parts (1/3 - 2/3 of the total length) were cut with a razor blade to remove the contact chemosensilla at the tip of the flagellum (Anderson & Bromley, 1987). The olfactory sensilla on the distal parts of the antennae that were removed by the operation as well are not likely to play a role in the detection of the drimanes, since these compounds exhibit very low volatilities. Control nymphs were anaesthetized with CO_2 for 1-2 min. After ablation the nymphs were left on artificial diet rings for 24 h to recover. The same methods as described for the 24 h interval observations of the dual-choice tests were used. Control and treated groups of *M. persicae and A. gossypii* nymphs were video recorded on dual-choice test-rings treated with polygodial (**2**). The numbers of replicates were 7-8.

48 h interval observations of tests with A. gossypii nymphs reared on plants. The same methods as described for the 24 h interval observations of the dual-choice tests were used. *A. gossypii* nymphs reared on plants were video recorded during the 48 h dual-choice test with polygodial (**2**). Two more sets of nymphs were video recorded during 48 h on control diet rings and on rings closed with a parafilm layer but without diet sachets. The number of replicates was 6.

Results

Dual-choice test. In general, *A gossypii* was less sensitive to the drimanes than *M. persicae* (Figure 2). The highest deterring indices were found in response to the dialdehydes warburganal (**1**) and polygodial (**2**) in *M. persicae*. Three compounds with a lactone substituent (**3**, **6** and **7**), the dialdehyde (**4**) and the mono-aldehyde (**5**) are also effective deterrents. Surprisingly, most *A. gossypii* nymphs reared on plants were found dead within the first 24 h of the tests with warburganal (**1**) and polygodial (**2**) while nymphs reared on artificial diet stayed alive and were significantly deterred by polygodial (hatched bar) in a 24 h test (see results of the 24 h interval observations). Also when these compounds were tested at 0.05%, the majority of the nymphs was dead at 24 h. When tested at 0.01% no deterring effects occurred and most nymphs survived. (data no

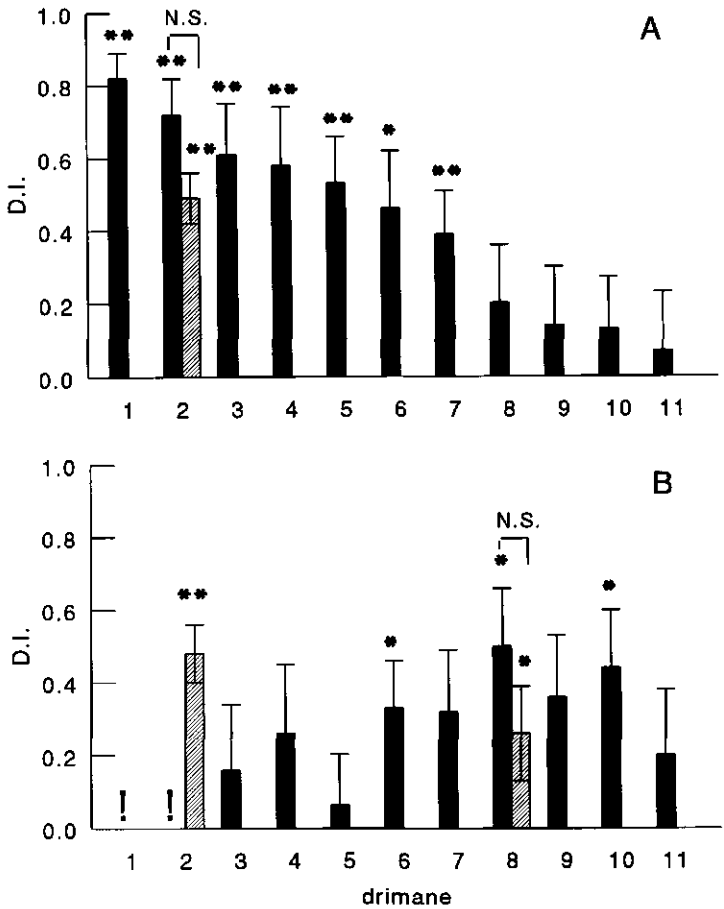
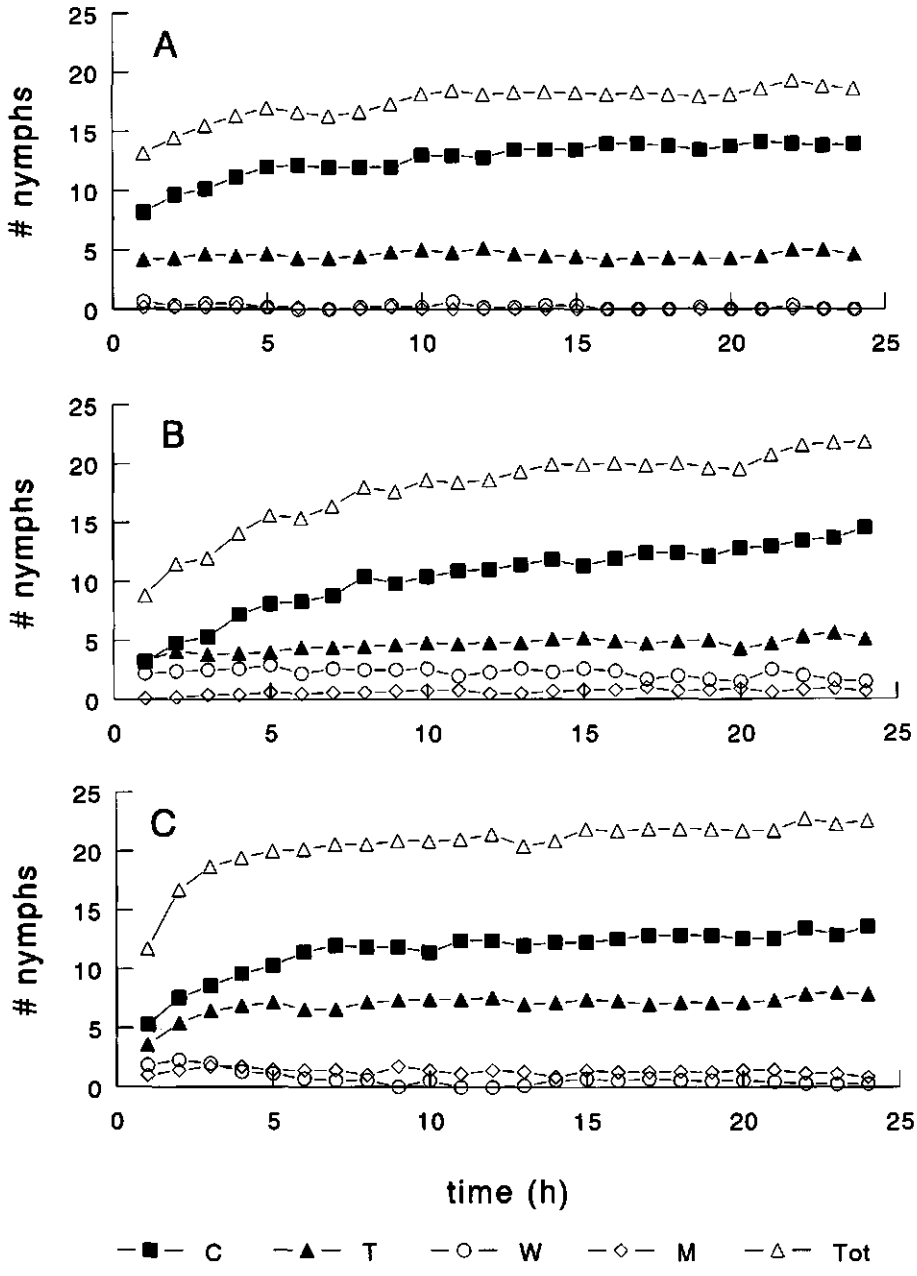


Figure 2. Deterrency indices (D.I.) (means±s.e.) of the dual-choice tests with nymphs reared on plants: (A) *M. persicae*, n = 14-20, calculated at 24 h; (B) *A. gossypii*, n = 19-20, calculated at 48 h. Also D.I.'s of nymphs reared on artificial diet (hatched bars) are given (see caption Figure 3). '!': The majority of the nymphs in these tests were dead at 48 h. Statistics: Wilcoxon's matched pairs signed rank test (for deterrency indices) and Mann-Whitney U (to compare the results of nymphs reared on plants and nymphs reared on diet); * $P < 0.05$; ** $P < 0.01$.

shown in Figure). Among the other compounds, **6**, **8** and **10** had significant activity against *A. gossypii*. Nymphs reared on artificial diet (hatched bars, see also results of the 24 h interval observations) seemed to be less sensitive to polygodial (**2**) (*M. persicae*) and isotadeonal (**8**) (*A. gossypii*) than nymphs reared on plants (black bars), but no significant differences occurred.


A
B
C
nymphs
nymphs
nymphs
0
5
10
15
20
25
time (h)
C
T
W
M
Tot

24 h interval observations of dual-choice tests. In both species *c.* 1/3 - 1/2 of the total number of nymphs entered the parafilm membrane within the first hour (Figure 3). During the next 10-15 h of the tests, the total number of nymphs on the diet surface gradually increased. After the first hour, the mean number of nymphs of both species on the polygodial treated sides hardly increased while the mean number of nymphs on the control sides did, at a similar rate as the total number of nymphs on the diet surface. The mean number of *A. gossypii* nymphs on the isotadeonal treated sides increased until *c.* 3 h to remain constant afterwards. The mean number of *M. persicae* nymphs that walked during the tests was very low (0-1), while the mean number of walking *A. gossypii* nymphs seemed slightly higher (0-3). The deterrent indices of the tests at 24 h (mean±s.e.) are given in Figure 2 (hatched bars).

24 h interval observation of dual-choice tests after ablation of the distal parts of the antennae. The day after the ablation of the distal parts of the antennae > 90% of the nymphs had survived. During the dual-choice tests with polygodial, the non-ablated, anaesthetized nymphs of both species showed a distribution pattern (Figure 4) similar to the pattern of non-anaesthetized nymphs (Figure 3). At 24 h the deterrent indices of these tests were (mean±s.e.) 0.55±0.09 ($P < 0.01$) and 0.50±0.1 ($P < 0.01$) for *A. gossypii* and *M. persicae* respectively. The ablated nymphs of both species spread evenly over the control and treated sides of the diets throughout the tests (Figure 4). After 13 h some *A. gossypii* nymphs moved from the treated to the control sides, but no significant difference between numbers on treated and control sides developed within 24 h (Wilcoxon's matched pairs signed rank test). The mean deterrent indices at 24 h were 0.32±0.2 (N.S.) and 0.31±0.2 (N.S.) for *A. gossypii* and *M. persicae* respectively.

48 h interval observations of tests with A. gossypii nymphs reared on plants. To investigate the cause of the mortality of *A. gossypii* nymphs reared on plants after transfer to polygodial dual-choice test rings, we examined the distribution pattern of the nymphs during 48 h and also examined the distribution pattern of nymphs that were transferred to control diet and of nymphs without diet. Because the distribution patterns of the nymphs on the diets remained relatively stable after the first 24 h we show only the first day of the observation (Figure 5).
After being transferred from plants to control diet the mean number of walking nymphs decreased during the first 15 h from *c.* 10 to *c.* 0-3 nymphs, while the mean total number of nymphs on both halves of the diet conversely increased. At 48 h most nymphs survived. When transferred to polygodial dual-choice rings, the distribution pattern of the nymphs was similar but the number of walking nymphs remained low throughout the test.

Figure 3. (page 82) 24 h Distribution pattern during the dual-choice test of nymphs of (A) *M. persicae* on polygodial (n = 6); (B) *A. gossypii* on polygodial (n = 10) and (C) *A. gossypii* on isotadeonal (n = 7). Indicated are the mean numbers of nymphs out of 20-25 introduced in total (Tot), walking (W), and stationary on the control (C) and treated (T) surface and on the middle line (M) (see legends). S.e.'s ranged between 0-1.8. The deterrency indices of the tests at 24 h are given in Figure 2.

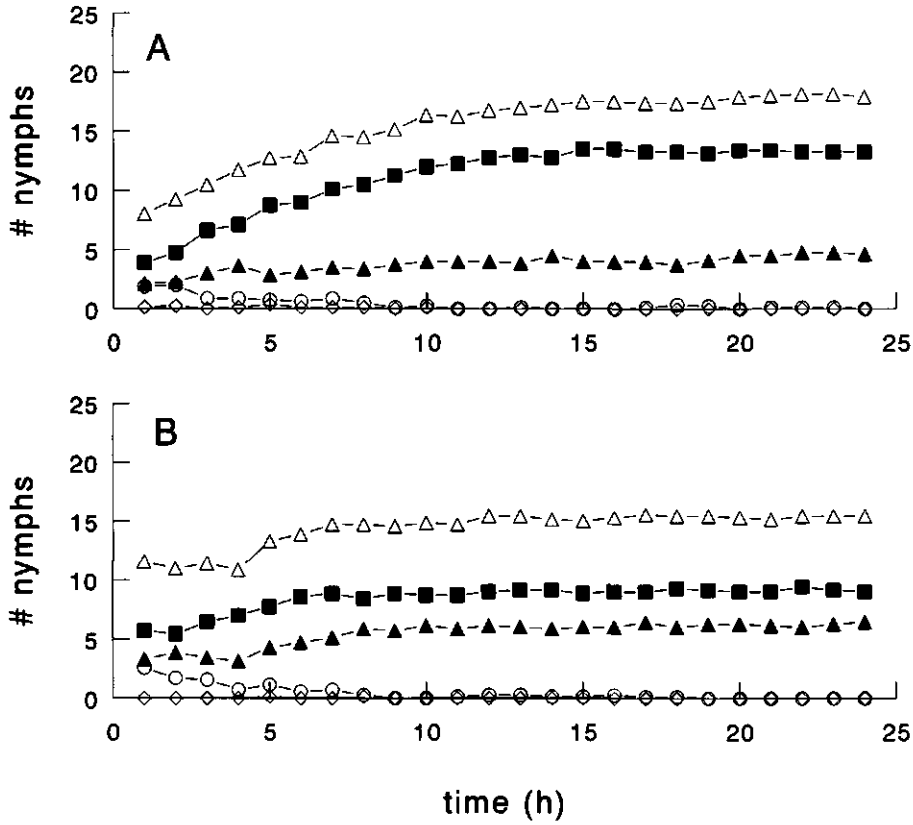


Figure 4. (A,B) 24 h Distribution pattern during the dual-choice test after ablation of the distal parts of the antennae or anaesthetization only of nymphs of *M. persicae* ((A) control, n = 8; (B) ablation, n = 8) and *A. gossypii* ((C) control, n = 8; (D) ablation, n = 7). S.e.'s ranged between 0-1.8. See caption Figure 3 for explanation of the legends.

The nymphs did not significantly choose for either control or treated side. At 48 h *c.* 75% of the nymphs was dead. From the video recordings we could not deduce at what time they had died because most dead aphids remained on the parafilm and could not be distinguished from penetrating aphids. Nymphs that were transferred to rings without diet walked more during the first 10 h than nymphs on the control or polygodial dual-choice diet. After *c.* 10 h the mean number of stationary aphids on both halves conversely increased with the decrease in mean number of walking aphids. After *c.* 40 h no walking occurred anymore. At 48 h *c.* 85% of the nymphs was dead.

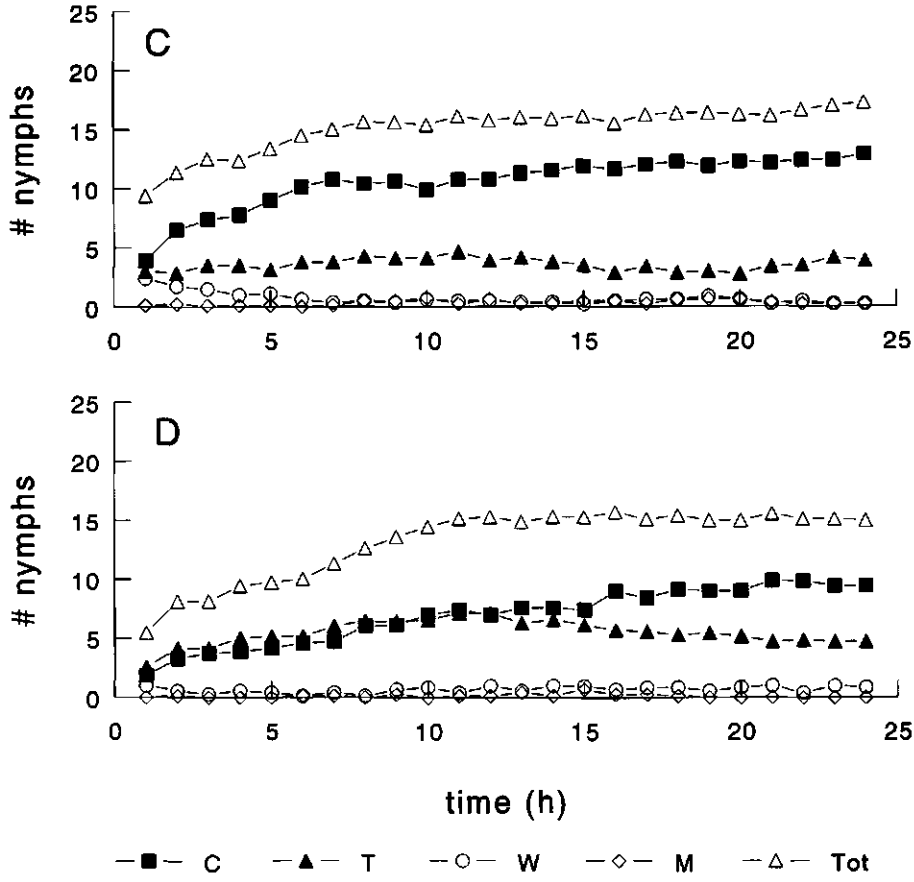
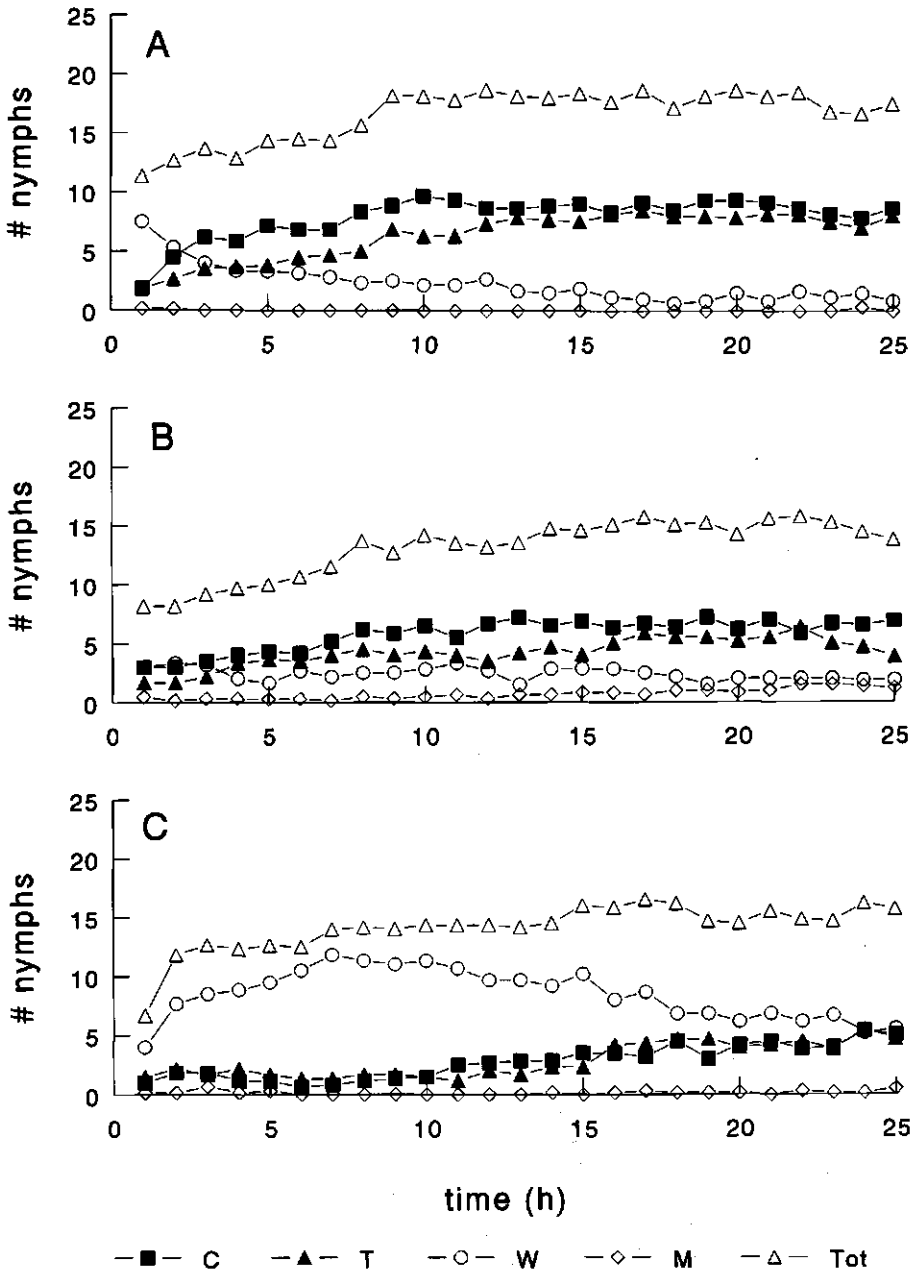


Figure 4. (C,D)

Discussion

Figure 2 shows that the SAR of the drimanes for the two aphid species differs. Warburganal (**1**) and polygodial (**2**), with a ß-dialdehyde configuration and double bond at C7-C8, are highly effective as deterrents and/or feeding inhibitors against both species. It is clear that not only the presence of substituents on the double ring drimane structure, but also their configuration and combination with other substituents (compare *e.g.* compounds **1**, **2**, **4** and **8** or compounds **3**, **6**, **7** and **11**) determine the activity of the drimanes. Several authors (*e.g.* Blaney *et al.*, 1987; Asakawa *et al.*, 1988 and Gols *et*


A
B
C
nymphs
nymphs
nymphs
0
5
10
15
20
25
time (h)
C
T
W
M
Tot

al., 1996) have shown earlier that a C9 ß-configured dialdehyde, combined with a C7-C8 double bond gives highly effective drimane antifeedants, which is confirmed by our results. Remarkably, the relatively simple synthetic compound **10** shows fairly high deterring activity against *A. gossypii,* while it was not effective at all against *M. persicae* and the earlier tested species *L. decemlineata* (Gols *et al.*, 1996) and *P. brassicae* (Messchendorp *et al.*, 1996). This result indicates that synthetic analogues of natural deterrents could be developed into highly selective feeding deterrents.

The 24 h interval observations of the dual-choice tests (Figure 3) show that the number of walking nymphs remained low throughout the tests while the total number of nymphs on the diet surface increased after the onset of the tests. We deduce that the majority of the nymphs of both species settled within an hour after having entered the diet surface and subsequently remained immobile for the largest part of the time. From the 24 h ablation studies (Figure 4) it is clear that nymphs with ablated antennae do not differentiate between control and polygodial treated surfaces throughout the test. After *c.* 12 h some *A. gossypii* nymphs switched from the treated to the control surface but no significant difference developed within 24 h. We conclude that nymphs of both *M. persicae* and *A. gossypii* detect polygodial and possibly also the other drimanes with the contact chemosensilla located at the tips of their antennae. This is in agreement with the results of Powell *et al.* (1995), who showed that adult *M. persicae*, in short-term behavioural tests, detect polygodial with the contact chemosensilla at the tips of their antennae. The ablation studies also show that in both species no epipharyngeal, tarsal or labial sensilla are involved in detecting polygodial (when applied to the parafilm surface) within 24 h (Figure 5), which is in agreement with the hypothesis that among the latter two types of sensilla no chemoreceptors are present in aphids (Tjallingii, 1978). Since aphids 'wave' their antennae and contact the surface while walking (Powell *et al.*, 1995) but fold their antennae backwards when penetrating or remaining immobile, the choice for the control side must be made before penetrating.

The fact that *A. gossypii* nymphs reared on plants, in contrast to nymphs reared on diet, do not survive in the dual-choice tests with warburganal (**1**) and polygodial (**2**) is rather surprising. In the 48 h test on polygodial the nymphs remained relatively immobile, in contrast to nymphs that were kept in test-rings without diet, and spread evenly over control and treated side (Figure 5). It is possible that, because nymphs reared on plants experience a more extreme difference in taste compared with their previous diet than nymphs reared on artificial diet, the deterrent action of warburganal and polygodial is so strong that the nymphs are inhibited from ingesting any diet at all, resulting in death. This phenomenon could be related to a 'central nervous system inhibitory state' caused by extreme deterrency, in which the insect does not respond to feeding stimuli that are

Figure 5. (page 86) 48 h Distribution pattern of *A. gossypii* nymphs reared on plants during three tests: (A) control diet on both sides, (B) polygodial (dual-choice) and (C) no diet test. n = 6 for all tests. S.e.'s ranged between 0-2.9. Because the distribution patterns of the nymphs on the diets remained stable after the first 24 h only the first day of the observations is shown. See caption Figure 3 for explanation of the legends; for the tests without diet and with control on both sides C and T indicate the two (indifferent) halves of the diet.

present, as described by Jermy (1971). Earlier, with *Aphis fabae* (Hardie *et al.*, 1992) and *M. persicae* adults (Powell *et al.*, 1993), it was found that a 24 h pre-exposure to leaves or green paper painted with polygodial caused a reduction in the subsequent number of penetrations and an increase in the mean duration of these penetrations. These effects of polygodial on adult aphids and the effects on nymphs in our experiments could have a similar origin.

The ecological role of deterrents on the leaf surface in the process of food plant selection by aphids is not yet fully elucidated (Klingauf, 1987; Niemeyer, 1990; Pickett *et al.*, 1992; Tjallingii, 1995). The feeding habit of aphids by penetrating the plant and feeding from phloem sap means that they contact leaf surface compounds as well as compounds inside the plant. Instances of influences of leaf surface compounds (*e.g.* Klingauf *et al.*, 1978) as well as phloem components (Tjallingii, 1995) on aphid feeding behaviour are known. The relative importance of feeding behaviour modifying compounds on both locations is not known and might differ between aphid species. Our study shows that both *M. persicae* and *A. gossypii* nymphs detect drimane deterrent compounds, applied to the surface of artificial diets, with the contact chemosensilla at the tips of their antennae. It is possible that these sensilla also play a role in the selection of host plants by these and other aphid species. The difference in the sensitivity of the two species for the drimanes possibly reflects differences in the perception of deterrents at the molecular level in the antennal sensilla. This research shows that drimane compounds, applied to surfaces of artificial diets, can have strong, species specific, deterring and feeding inhibiting effects on *M. persicae* and *A. gossypii* aphids. This indicates that spraying plants with deterring compounds, through interference early in the host plant selection sequence by aphids, could be a promising way to protect plants against aphid infestation.

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7

Feeding inhibiting effects of drimanes and related analogues on *Spodoptera exempta*, *S. exigua*, *Mamestra brassicae*, *Pieris brassicae* and *Leptinotarsa decemlineata* larvae and *Locusta migratoria* nymphs

Abstract

Some drimanes and related analogues were screened for their effects on feeding of *Spodoptera exempta* (Walker), *S. exigua* (Hübner), *Mamestra brassicae* (L.) (Lepidoptera: Noctuidae), *Pieris brassicae* (L.) (Lepidoptera: Pieridae) and *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae) larvae and *Locusta migratoria* (L.) (Orthoptera: Acrididae) nymphs in dual-choice tests. However, their efficacy was low. Therefore no further experiments have been performed.

Introduction

Drimane compounds were originally isolated from plants that were known to deter feeding in many insect species. Knowledge on the feeding inhibiting effects of pure compounds against different insect species is needed to estimate their potential for application as crop protection agents. This chapter presents the results of standard dual-choice tests with drimanes and simplified related analogues. Test insect species were *Spodoptera exempta, S. exigua, Mamestra brassicae, Pieris brassicae, Leptinotarsa decemlineata* and *Locusta migratoria.*

Materials & Methods

Chemicals. The drimanes and related analogues were synthesized at the Department of Organic Chemistry, Wageningen Agricultural University (Jansen, 1993; C.T. Bouwman, unpubl.) (Figure 1). All compounds are racemic mixtures. The compounds were presolubilized in ethanol (>99%) and a detergent (Tween-80), which was used to promote an even distribution of the solution on the leaf discs. Distilled water was then added to obtain a final solution of 2% ethanol and 2% Tween-80. Distilled water with 2% ethanol and 2% Tween-80 served as control solution.

Insects. Larvae of *S. exempta* and *S. exigua* were reared on wheat seedlings (*Triticum aestivum*, cv. Okapi). Sixth and 5th instar larvae were used for the experiments, respectively. Larvae of *M. brassicae* and *P. brassicae* were reared on cabbage (*Brassica oleracea*, var. gemmifera cv. Titurel) and 6th and 5th instar larvae were used for the experiments, respectively. Larvae of *L. decemlineata* were reared on excised foliage of potato plants (*Solanum tuberosum*, cultivar Surprise) and 4th instar larvae were used for the experiments. Gregarious nymphs of *Locusta migratoria* were reared in a mass-

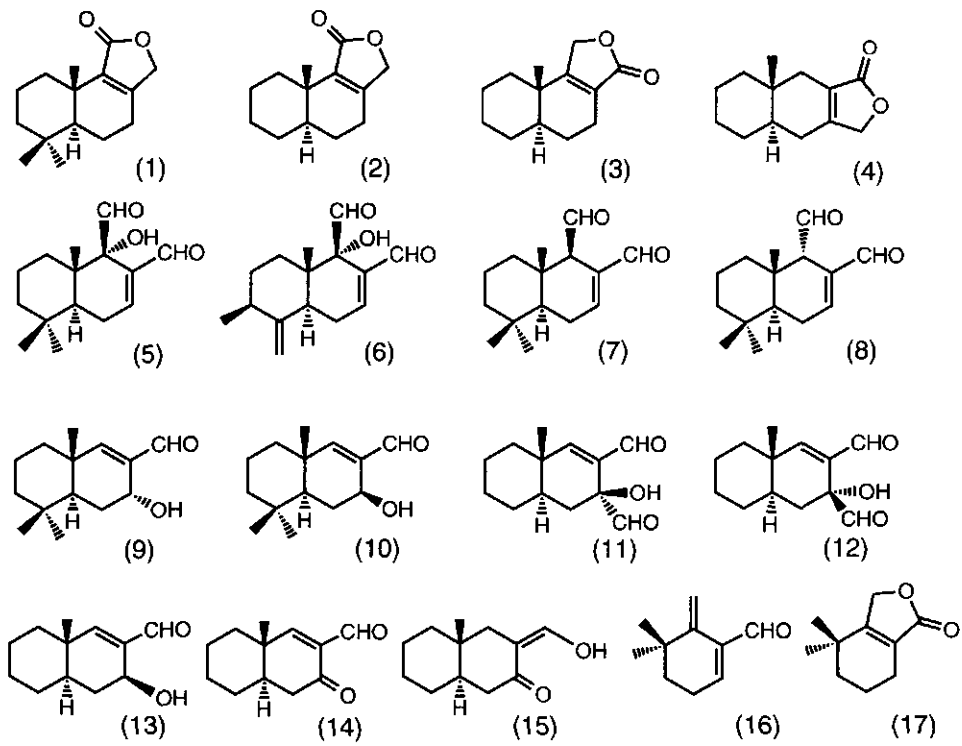


Figure 1. Molecular structures of the drimanes. Trivial names: (1) isodrimenin, (5) warburganal, (6) muzigadial, (7) polygodial, (8) isotadeonal, (9) polygonal, (10) isopolygonal.

rearing culture on diverse grasses and 3 days old 5th instar nymphs were used for the experiments.

Dual-choice feeding tests. For the tests with *S. exempta*, *S. exigua*, *M. brassicae*, *P. brassicae* and *L. decemlineata* the same test procedure as earlier described for *P. brassicae* (chapter 2) was used. On *S. exempta*, drimanes were tested at 1 and 5 mM, applied to maize (*Zea mays*, cv. Anjo) or sorghum (*Sorghum bicolor*) leaf discs (10 µl per disc of 3.8 cm^2), in 3 h dual-choice tests. On *S. exigua*, drimanes were tested at 1 and 5 mM in dual-choice tests that lasted for 3, 4½, 7 or 21 h. The drimanes were applied either to maize (10 µl per disc of 3.8 cm^2) or cabbage (5 µl per disc of 2.3 cm^2). On *M. brassicae*, drimanes were tested at 5 mM, applied to cabbage leaf discs (10 µl per disc of 3.8 cm^2). The 3 h dual-choice tests were performed in the dark. On *P. brassicae*, drimanes were tested at 1 and 5 mM, applied to cabbage leaf discs (10 µl per disc of 3.8 cm^2). On *L. decemlineata* drimanes were tested at 5 mM, applied to potato leaf discs (10

Table 1. Antifeedant indices (A.I.; means±s.e.) of the dual-choice tests with 6th instar *Spodoptera exempta* larvae

Compound		A.I.	s.e.	sign.	n	day
1 mM:						
5		0.50	0.15	*	11	14
5		0.08	0.09	NS	20	16
6		0.11	0.10	NS	12	2
10		0.09	0.13	NS	10	4
11		0.04	0.10	NS	11	4
7		0.01	0.11	NS	20	15
1		0.00	0.11	NS	11	14
5 mM:						
14		0.36	0.11	**	12	3
14		0.33	0.17	NS	12	2
11		0.35	0.13	NS	11	6
11		0.34	0.08	**	11	7
11		0.32	0.13	*	12	5
11		0.24	0.13	NS	20	9
11		0.13	0.11	NS	12	3
11	(s)	0.03	0.10	NS	20	9
12		0.34	0.05	**	12	8
10		0.27	0.09	*	12	5
5		0.23	0.12	NS	20	16
1		0.11	0.12	NS	12	13
9		0.08	0.11	NS	11	13
16	(s)	0.08	0.11	NS	12	10
7		0.08	0.12	NS	20	15
4		0.07	0.08	NS	12	1
2		0.03	0.09	NS	20	11
8		0.02	0.12	NS	12	1
15		0.01	0.11	NS	12	1
3		-0.08	0.09	NS	20	12
17	(s)	-0.26	0.13	NS	12	10

Statistics: Wilcoxon's signed-rank matched pairs test; * $P < 0.05$; ** $P < 0.01$

(s): tests performed with sorghum leaf discs

day: compounds with the same number were tested on the same day

Table 2. Antifeedant indices (A.I.; means±s.e.) of the dual-choice tests with 5th instar *Spodoptera exigua* larvae

Compound		A.I.	s.e.	sign.	n	day
1 mM (3 h):						
5		0.34	0.65	NS	10	1
1 mM (7 h):						
5		-0.05	0.53	NS	12	3
5 mM (3 h):						
14		0.39	0.70	NS	11	1
11		0.23	0.61	NS	11	1
3	(c)	0.18	0.66	NS	17	7
3	(c)	0.14	0.67	NS	15	6
2	(c)	0.14	0.70	NS	15	6
5 mM (4.5 h):						
10	(c)	0.29	0.36	*	12	5
4		0.22	0.58	NS	11	4
11	(c)	0.10	0.23	NS	12	5
5 mM (7 h):						
10		0.35	0.45	NS	12	3
14		0.14	0.39	NS	12	3
5 mM (21 h):						
9		0.01	0.08	NS	10	2
8		-0.01	0.13	NS	10	2

Statistics: Wilcoxon's signed-rank matched pairs test; * $P < 0.05$; ** $P < 0.01$

(c): tests performed with cabbage leaf discs

day: compounds with the same number were tested on the same day

Table 3. Antifeedant indices (A.I.; means±s.e.) of the dual-choice tests with 6th instar *Mamestra brassicae* larvae

Compound	A.I.	s.e.	sign.	n	day
3	0.28	0.14	NS	20	1
7	0.27	0.10	*	20	3
2	0.14	0.12	NS	19	1
6	0.14	0.13	NS	20	2

Statistics: Wilcoxon's signed-rank matched pairs test; * $P < 0.05$; ** $P < 0.01$
day: compounds with the same number were tested on the same day

Table 4. Antifeedant indices (A.I.; means±s.e.) of the dual-choice tests with 5th instar *Pieris brassicae* larvae

Compound	A.I.	s.e.	sign.	n	day
1 mM:					
16	-0.02	0.13	NS	12	1
17	-0.05	0.18	NS	12	1
5 mM:					
13	0.30	0.13	NS	12	2
16	-0.12	0.15	NS	12	1
17	-0.26	0.14	NS	12	1

Statistics: Wilcoxon's signed-rank matched pairs test; * $P < 0.05$; ** $P < 0.01$
day: compounds with the same number were tested on the same day

Table 5. Antifeedant indices (A.I.; means±s.e.) of the dual-choice tests with 4th instar *Leptinotarsa decemlineata* larvae

Compound	A.I.	s.e.	sign.	n	day
16	0.42	0.13	**	12	1
17	0.15	0.13	NS	12	2

Statistics: Wilcoxon's signed-rank matched pairs test; * $P < 0.05$; ** $P < 0.01$
day: compounds with the same number were tested on the same day

Table 6. Antifeedant indices (A.I.; means±s.e.) of the dual-choice tests with 5th instar *Locusta migratoria* nymphs

Compound	A.I.	s.e.	sign.	n	day
on paper:					
sinigrin (0.5%)	0.50	0.05	**	12	1
11	0.77	0.06	**	12	2
14	0.76	0.06	**	12	2
14	0.49	0.08	**	12	2
10	0.55	0.09	**	12	1
on bamboo:					
15	0.34	0.22	NS	12	3
11	0.06	0.26	NS	11	3
on mais:					
15	-0.03	0.16	NS	17	4
11	0.14	0.15	NS	19	4

Statistics: Wilcoxon's signed-rank matched pairs test; * $P < 0.05$; ** $P < 0.01$,
day: compounds with the same number were tested on the same day

µl per disc of 2.3 cm^2).

For the tests with *L. migratoria* the nymphs were individually placed in white, light transmitting, cardboard mugs of 12 cm height and 13 cm diameter. On the bottom of each mug a 1½ cm thick triplex disc (13 cm diameter) was placed, on which the feeding substrates (either filter paper discs or cut parts of maize or bamboo (*Fargesia spathacea*) leafs: one control and one treated) were attached with needles. The mugs were closed with a plastic Petri dish lid, and shaded from direct light by placing a black, plastic tray on top of the mugs. Leaf materials were painted with 5 mM drimane (or control) solution (in distilled water with 2% Tween-80 and 2% ethanol) (2.6 µl / cm^2) and left to dry before they were placed in the mugs. Filter paper discs (of 4 cm diameter and 84 mg weight) were made palatable by treating them with 200 µl 58 mM sucrose solution in ethanol (% sucrose = 5% of dry weight of the paper discs). After drying, 200 µl of 4000 ppm (*c.* 16-20 mM) drimane solution (% drimane = 1% of dry weight of the paper discs) or ethanol (control discs) was added and the discs were again left to dry for *c.* 30 min before they were placed in the mugs. Sinigrin treated paper discs (% sinigrin = 0.5% of dry weight of the discs) were tested as a reference.

For all tests an antifeedant index (A.I.) was calculated: A.I. = (C-T)/(C+T)* 100% (C = area consumed from control leaf discs and T = area consumed from treated leaf discs).

Results and Discussion

The results of the dual-choice tests with *S. exempta, S. exigua, M. brassicae, P. brassicae, L. decemlineata* and *L. migratoria* are presented in Tables 1, 2, 3, 4, 5 and 6, respectively. Some drimanes significantly inhibited feeding in *S. exempta*, but no strong effects were seen. *S. exigua* consumed relatively little food during the 3 h tests and therefore longer lasting tests were also performed. However, the drimanes did not significantly inhibit feeding (except for compound 10 in one test). The drimanes tested on *M. brassicae* and *P. brassicae* did not give promising results. Compound 16 inhibited feeding in *L. decemlineata* only slightly. The drimanes tested on *L. migratoria* only inhibited feeding when tested on filter paper discs. However, the concentrations used in the paper substrate were extremely high so that these results probably have little relevance with respect to plant substrates.

Reference

Jansen B.J.M., 1993. Total synthesis of insect antifeedant drimane sesquiterpenes. Ph.D. thesis, Wageningen Agricultural University.

8

Feeding inhibiting effects of synthetic analogues of natural *neo*-clerodane diterpenes on larvae of *Pieris brassicae* and *Leptinotarsa decemlineata* and nymphs of *Myzus persicae*

Abstract

Several synthetic analogues, derived from the C9 side chains of clerodin and ajugarin I, were tested for their effects on feeding of *Pieris brassicae* (L.) (Lepidoptera: Pieridae) and *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae) larvae and *Myzus persicae* (Sulzer) (Homoptera: Aphididae) nymphs. Several compounds showed moderate activity against *P. brassicae* larvae. Interestingly, the compound that was most effective in the dual-choice tests (16) is the analogue that most resembles the structure of the furopyran-fragment of azadirachtin, which compound had previously been shown to be a highly effective feeding deterrent against *Spodoptera littoralis*. The compounds tested against *L. decemlineata* and *M. persicae* were not (or only slightly) effective.

Introduction

Many plants belonging to different plant families produce *neo*-clerodane diterpenes (Luteijn, 1982; Warthen, 1990) of which some are known to inhibit feeding by insects at very low concentrations (*e.g.* 1-100 ppm). Two examples are shown in Figure 1: clerodin (A) and ajugarin I (B) have been tested for their feeding inhibiting effects on many insect species (*e.g.* Kubo *et al.*, 1976; Blaney *et al.*, 1988; Griffiths *et al.*, 1988). An extended overview of the feeding inhibiting effects of natural and (semi)synthetic *neo*-clerodane diterpenes reported in the literature is given by klein-Gebbinck (1998).

From earlier work (Kojima & Kato, 1981; Geuskens *et al.*, 1983; Belles *et al.*, 1985; Blaney *et al.*, 1988, 1990) it is known that simplified synthetic compounds, derived from (fragments of) *neo*-clerodane diterpenes, can still inhibit feeding in insects, although these compounds generally have a much lower efficacy than the original. Also (fragments of) the highly effective antifeedant azadirachtin (Figure 1; (C)) have served as model for synthetic derivatives. Two furopyran-fragments of azadirachtin, synthesized by Ley *et al.* (1987) (Figure 1; (D) and (E)), were highly effective at 10 ppm against *Spodoptera littoralis* larvae in a bioassay with glass fibre discs (antifeedant indices of 0.98 and 0.69 respectively).

In the work presented in this chapter, 33 synthetic analogues (Figure 2), derived from the C9 side chains of clerodin and ajugarin I (Figure 1) were tested for their feeding inhibiting effects. The C9 furofuran-fragment of clerodin (Figure 1 (A)) shows some structural resemblance to the furopyran-fragments of azadirachtin (Figure 1 (D) and (E)). Therefore, further examination of structures related to these fragments might lead to new, effective feeding deterrents. Larvae of *Pieris brassicae* and *Leptinotarsa decemlineata* and nymphs of *Myzus persicae* were used in the tests.

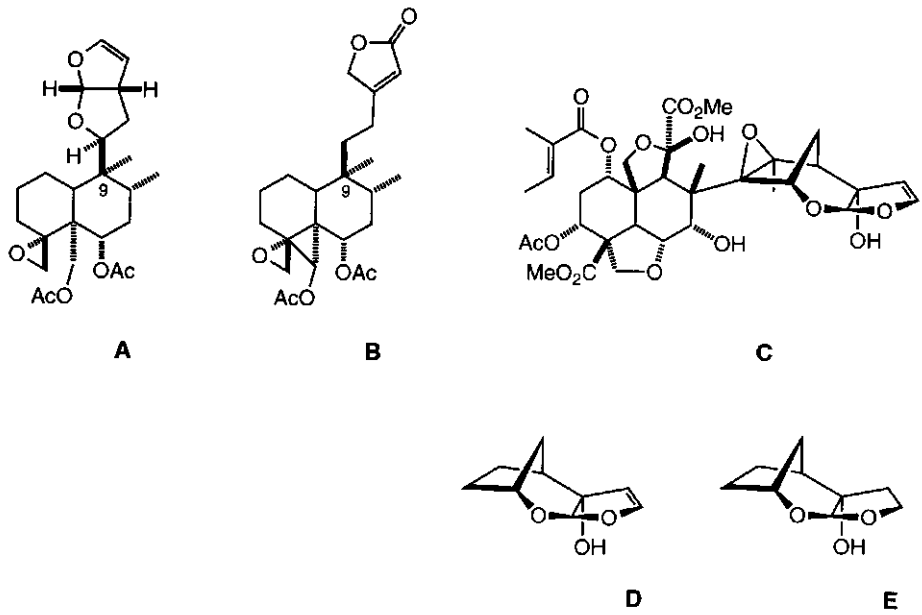


Figure 1. Molecular structures of clerodin (A), ajugarin I (B), azadirachtin (C) and two furopyran derivatives (D) and (E).

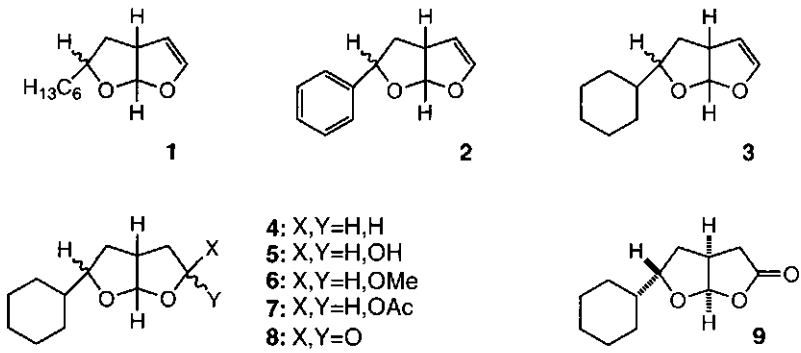
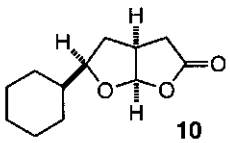


Figure 2. (page 100 and 101) Molecular structures of the synthetic analogues of clerodin (1-21) and ajugarin I (22-33).



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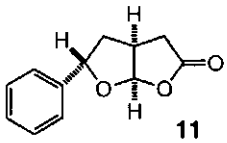

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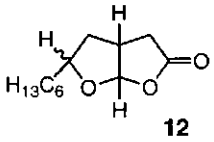

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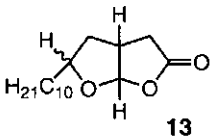

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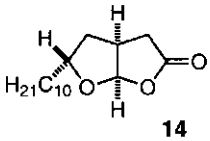

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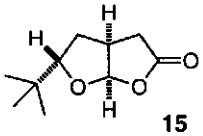

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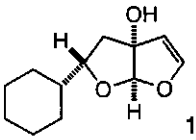

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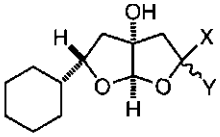
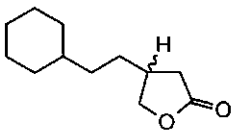


17: X,Y=H,H
18: X,Y=H,OH
19: X,Y=H,OMe
20: X,Y=H,OAc
21: X,Y=O



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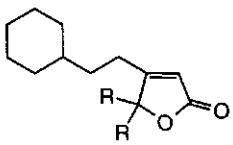

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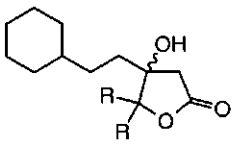

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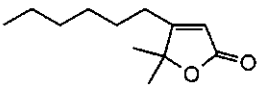
26: R=Me



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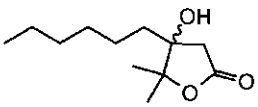

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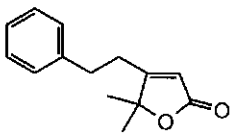

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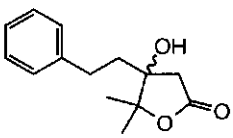

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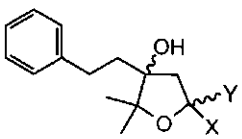

31: X,Y=H,H
32: X,Y=H,OMe
33: X,Y=H,OH

Materials & Methods

Chemicals. The 33 *neo*-clerodane diterpene analogues were synthesized at the Department of Organic Chemistry, Wageningen Agricultural University (E.A. klein-Gebbinck; C.T. Bouwman; G.A. Stork; M.B. Bourgois, unpubl.) (Figure 2). All compounds are racemic mixtures. In the tests with *P. brassicae* and *L. decemlineata* 5 mM solutions were used. Because the molecular weights of the compounds ranged between 188-270, this is equivalent to concentrations between 940-1350 ppm (mg/l). The compounds were presolubilized in ethanol (>99%) and a detergent (Tween-80), which was used to promote an even distribution of the solution on the leaf discs. Distilled water was then added to obtain a final solution of 2% ethanol and 2% Tween-80. Distilled water with 2% ethanol and 2% Tween-80 served as control solution. For the tests with *M. persicae* 0.1% (1000 ppm; *ca.* 4-5 mM) solutions in ethanol (> 99%) were used and ethanol (> 99%) served as control solution.

Insects, dual-choice and no-choice tests. Rearing conditions of the insects and procedures of the dual-choice and no-choice tests were described in earlier chapters. For *P. brassicae* in chapter 2 and 3; in this research we used cabbage plants (*Brassica oleracea* var. gemmifera) of cv. Titurel (for the tests with compounds 1-21) and cv. Cyrus (for the tests with compound 22-33), for *L. decemlineata* in chapter 4 and for *M. persicae* in chapter 6, respectively.
For the dual-choice tests with *P. brassicae* and *L. decemlineata* an antifeedant index (A.I.) was calculated: A.I. = (C-T)/(C+T)*100% (C = area consumed from control leaf discs and T = area consumed from treated leaf discs) and for the dual-choice tests with *M. persicae* a deterrency index: D.I. = $(C_n-T_n)/(C_n+T_n)*100\%$ (C_n = number of nymphs on control side and T_n = number of nymphs on treated side). For the no-choice tests with *P. brassicae* and *L. decemlineata* inhibition percentages were calculated: I.P. = $(C_{(\text{mean of controls})}-T)/C_{(\text{mean of controls})} * 100\%$ (C = area eaten/weight of control larva and T = area eaten/weight of treated larva).

Results & Discussion

Dual-choice and no-choice tests with P.brassicae larvae. Compounds 1-33 were tested in dual-choice tests (Table 1), and compounds 1-8 were tested in no-choice tests as well (Table 2). On each test day (except for day 3 and 4), also a reference drimane compound was tested (for its molecular structure see chapter 2, Figure 1; compound 4). The efficacy of the reference drimane varies between the test days, indicating that other individual or environmental factors, such as condition of food on which the insect was reared or weather conditions (Lewis & van Emden, 1986; Bernays & Wege, 1987) might have influenced the test results. Also the results of repeated tests (with compounds 2, 8, 9 and 21) vary, but the antifeedant indices (A.I.) did not differ more than 11% indicating that the dual-choice tests are probably precise enough to distinguish fairly active from inactive compounds. Compounds with an average antifeedant index > 0.2 are all significantly effective (Table 1). Interestingly, the most effective compound (16; Figure 2)

Table 1. Antifeedant indices (A.I.; means±s.e.) of the dual-choice tests with 5th instar *Pieris brassicae* larvae.

Compound	A.I.	s.e.	sign.	n	day
16	0.54	0.12	**	19	6
13	0.41	0.11	**	20	3
30	0.36	0.07	**	20	8
23	0.35	0.08	**	20	7
29	0.33	0.10	**	20	8
31	0.28	0.10	**	20	8
15	0.28	0.11	*	20	3
25	0.26	0.06	**	20	7
4	0.26	0.07	**	18	1
10	0.24	0.08	*	20	4
17	0.24	0.09	*	20	5
32	0.23	0.08	**	20	8
6	0.20	0.09	NS	20	2
9	0.19	0.07	*	20	5
9	0.08	0.07	NS	20	4
11	0.18	0.09	*	18	4
19	0.18	0.10	NS	19	6
18	0.16	0.06	**	19	5
7	0.16	0.09	NS	20	2
26	0.15	0.08	NS	20	7
2	0.15	0.08	NS	20	1
2	0.07	0.12	NS	17	4
21	0.14	0.08	NS	20	5
21	0.13	0.08	NS	22	3
8	0.12	0.10	NS	20	3
8	0.11	0.10	NS	20	2
8	0.07	0.12	NS	17	4
28	0.11	0.09	NS	19	7
1	0.11	0.09	NS	20	1
14	0.10	0.06	NS	20	4
5	0.10	0.09	NS	19	1
20	0.09	0.07	NS	21	6
27	0.09	0.09	NS	20	7
33	0.05	0.08	NS	20	9
3	-0.025	0.07	NS	20	1
24	-0.04	0.10	NS	20	7
22	-0.092	0.12	NS	19	9
12	-0.11	0.08	NS	20	4
REF	0.39	0.08	**	20	1
REF	0.23	0.10	NS	19	2
REF	0.31	0.05	**	20	5
REF	0.39	0.09	**	18	6
REF	0.48	0.07	**	20	7
REF	0.42	0.07	**	20	8
REF	0.62	0.10	**	20	9
REF	0.47	0.08	**	19	9

Statistics: Wilcoxon's signed-rank matched pairs test: * $P < 0.05$; ** $P < 0.01$

day: compounds with the same number were tested on the same day

Table 2. Inhibition percentages (I.P.; means±s.e.) of the no-choice tests with 5th instar *Pieris brassicae* larvae.

Compound	I.P.(0-1.5)	s.e.	sign.	I.P.(1.5-3)	s.e.	sign.	n
8	22.4	5.0	**	13.9	4.3	**	20
1	20.3	5.3	**	10.6	4.6	*	20
5	18.6	7.6	*	15.9	7.0	*	20
6	18.3	8.5	NS	9.5	5.8	NS	20
7	16.7	5.4	*	3.0	3.8	NS	20
4	6.7	6.8	NS	5.4	6.9	NS	20
2	-1.7	6.2	NS	-2.6	3.0	NS	20
3	-9.3	5.2	NS	-12.3	4.3	NS	20
REF	47.0	3.4	***	66.1	2.3	***	19

Statistics: Mann-Whitney U test; * $P < 0.05$; ** $P < 0.01$

Table 3. Inhibition percentages (I.P.; means±s.e.) of the no-choice tests with 4th instar *Leptinotarsa decemlineata* larvae

Compound	I.P.(0-1.5)	s.e.	sign.	I.P.(1.5-3)	s.e.	sign.	n
2	8.7	8.5	NS	15.9	5.5	**	20
1	-1.7	8.0	NS	1.0	5.9	NS	20
6	-4.8	9.2	NS	3.0	9.1	NS	19
8	-6.3	9.3	NS	-4.8	5.7	NS	20
3	-7.1	8.1	NS	-17.4	6.0	NS	20
5	-13.5	6.1	NS	3.8	4.7	NS	20
4	-20.2	11.7	NS	0.8	6.3	NS	20
7	-21.3	5.2	NS	6.3	4.9	NS	20
REF	28.2	6.1	**	29.7	5.0	**	20

Statistics: Mann-Whitney U test; * $P < 0.05$; ** $P < 0.01$

Table 4. Deterrency indices (D.I.; means±s.e.) of the dual-choice tests with *Myzus persicae* nymphs.

Compound	D.I.	s.e.	sign.	n	day
5	0.31	0.20	NS	19	2
9	0.29	0.14	NS	20	5
3	0.29	0.19	NS	18	2
8	0.20	0.19	NS	16	4
12	0.16	0.20	NS	15	3
12	0.09	0.14	NS	20	5
4	0.11	0.18	NS	14	1
4	-0.09	0.16	NS	19	5
7	0.10	0.19	NS	15	4
1	0.05	0.17	NS	18	4
1	0.02	0.17	NS	14	1
15	0.05	0.21	NS	15	3
2	-0.01	0.18	NS	16	5
2	-0.09	0.17	NS	17	1
6	-0.10	0.17	NS	19	2
21	-0.18	0.18	NS	18	3
14	-0.19	0.18	NS	17	3
12	-0.26	0.14	NS	19	1
11	-0.41	0.16	*	17	4
REF2	0.26	0.13	*	13	1
REF2	0.80	0.07	**	18	2
REF2	0.65	0.14	**	18	3
REF2	0.35	0.19	NS	18	4
REF2	0.85	0.04	**	20	5

Statistics: Wilcoxon's signed-rank matched pairs test; * $P < 0.05$; ** $P < 0.01$

day: compounds with the same number were tested on the same day

is the analogue that most resembles the structure of the furopyran-fragment of azadirachtin: it consist of a furofuran ringsystem with a combination of a double bond and a tertiary hydroxy group, in a similar spatial arrangement as the furofuran-fragment of azadirachtin (Figure 1; (D) and (E)). The other active compounds structurally differ in several ways (but only slightly) from inactive compounds, which makes it difficult to compose a structure-activity relationship. In the no-choice tests (Table 2), compounds 1, 5, 7 and 8 significantly inhibit feeding, but the inhibition percentages (I.P.) are rather low (up to 22%), indicating that probably no strong postingestive, feeding inhibiting effects occur within the three hours of the test (see also chapter 3).

No-choice tests with L. decemlineata larvae. Compounds 1-8 were tested in no-choice tests (Table 3). Only compound 2 significantly (but only slightly) inhibits feeding during the second 1.5 h period, indicating that probably no strong postingestive, feeding inhibiting effects occur within the three hours of the test (see also chapter 3).

Dual-choice tests with M. persicae nymphs. Compounds 1-15 and 21 were tested in dual-choice tests, applied to the outer surface of artificial diet sachets (Table 4). None of the compounds significantly deterred *M. persicae* from settling on the treated side of the diet, although deterrency indices (D.I.) > 0.2 occurred. On each test day also a reference drimane compound was tested (polygodial: for its molecular structure see chapter 2, Figure 1; compound 7). The efficacy of the reference drimane varies considerably between the test days, indicating that other factors (*e.g.* Lewis & van Emden, 1986) might have influenced the test results. Also the results of repeated tests (with compounds 1, 2, 4 and 12) vary considerably. This might be explained by the lack of deterring effectiveness of these compounds, resulting in a large variability of the (not significant) deterrency indices of repeated tests. Compound 11 significantly stimulates the nymphs to settle on the treated side.

When examining the results, it should be noted that the mechanisms of action by which the tested compounds (that were effective) inhibit feeding are not known. To determine a structure-activity relationship, it will be needed to distinguish the exact mechanism of action of each compound (postingestive and/or sensory). Direct behavioural observations (such as described in chapter 3) could assist in this.

In conclusion, considering the relatively high concentrations used in the tests, none of the tested *neo*-clerodane diterpene analogues is a highly effective feeding inhibitor or deterrent against *P. brassicae* and *L. decemlineata* larvae or *M. persicae* nymphs. Therefore, lower concentrations of these compounds were not tested. However, it should be reminded that in earlier work on *neo*-clerodane diterpene analogues in most cases tests were performed on glass fibre discs (instead of leaf discs) and with different insect species. Because the feeding inhibiting efficacy of test compounds generally is much higher when artificial diets rather than leaf material are used, the *neo*-clerodane diterpene analogues tested in this work probably are more effective against *P. brassicae* when tested on artificial diets. However, when examining their possible use in protecting crops against insect feeding, tests on leaf material are more appropriate.

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9

General discussion

This thesis describes a study on the behavioural and sensory effects of terpenoid antifeedants on several insect species.

The aim of the study was to provide more insight in the effectiveness of terpenoid antifeedants and their mechanisms of action, as a basis for estimating their potential use in crop protection against insects, and enlightening the role of antifeedants in host plant selection.

The effect on feeding behaviour of a number of terpenoid compounds was quantified and the relationship between molecular structure and effect was studied comparatively. The mechanism(s) of action of sesquiterpene drimanes were studied in different insect species, by measuring sensory responses and observing the effects on behaviour. Furthermore, long-term and toxic effects were studied.

Taste perception of drimane antifeedants

Pieris brassicae

Previously, the sensitivities of the contact chemosensory receptor cells of *Pieris brassicae* larvae have been studied rather thoroughly (*e.g.* Schoonhoven, 1967; Ma, 1972; Blom, 1978; van Loon, 1988; van Loon & van Eeuwijk, 1989). The presence of a 'deterrent cell' in the medial sensillum styloconicum, sensitive to a broad range of antifeedants, was shown by Ma (1969). Schoonhoven & Yan (1989) showed that the drimanes polygodial (compound 7; Figure 1), warburganal (compound 5) and muzigadial (compound 6) exert several sensory effects on fifth instar *P. brassicae* larvae, *i.e.* stimulating the medial deterrent cell, evoking 'bursts' in the medial and lateral sensilla styloconica and inhibiting the response to cells sensitive to feeding stimulants during and after long-term sensory stimulation with the drimane solutions.

Chapter 2 presents research with the aim to provide more insight in the sensory perception of drimane antifeedants by *P. brassicae* larvae, by comparing behavioural and sensory responses to 15 drimane antifeedants. We showed that the impulse frequency of the medial deterrent cell, measured during the first 1.5 s of stimulation, significantly correlates to the behavioural response in a 3 h dual-choice test, although a fairly large variation occurred in the data. The results indicate that the response of the medial deterrent cell significantly contributes to inhibition of feeding behaviour in *P. brassicae* larvae, which suggests that in this insect a 'labelled line' coding principle for the perception of antifeedants is used. Furthermore, we showed for one of the drimanes that, during the first 1.5 s of stimulation, also inhibition of sensory responses to feeding stimulants occurs. This mechanism could contribute to inhibition of feeding behaviour as well. When

different drimanes affect feeding stimulant receptors differently, this could (partly) explain the variation in the correlation between the response of the deterrent cell and behaviour.

Leptinotarsa decemlineata

The hypothesis that feeding deterrents in potential food plants are decisive in food plant selection by insects (chapter 1) was originally derived from experiments with the Colorado potato beetle (Jermy, 1961; Jermy, 1994). No food plant specific 'sign stimuli' to *L. decemlineata* have been identified, but in contrast many feeding deterrents to these beetles are known. Despite the importance of feeding deterrents in food plant selection by *L. decemlineata*, little was known about the sensory perception of such compounds by this beetle. Galeal sensilla have not been found to respond to stimulation with feeding deterrents (Mitchell & Schoonhoven, 1974). Although inhibition of galeal receptor cells sensitive to feeding stimulants was found, there was no correlation between these effects and inhibition of feeding (Mitchell, 1987).

The finding that larvae are immediately deterred from feeding when their mouthparts contact droplets of drimane solutions in a 'microsyringe test' (Chapter 5) led us to the hypothesis that a 'deterrent cell' must be present on Colorado potato beetle mouthparts. Because Chin (1950), Mitchell (1988) and van Haeften (1993) had mentioned the presence of epipharyngeal sensilla in *L. decemlineata* larvae and adults we decided to investigate the function of one of the *c.* 20 epipharyngeal sensilla in fourth instar larvae.

Electron microscopy revealed that the epipharyngeal sensillum is innervated by five neurons. Electrophysiological experiments showed that one of these cells responds to water, a second to sucrose and a third to two feeding deterrents (compound 4 and sinigrin) that were effective in a behavioural test as well. Furthermore, the response of the sucrose sensitive cell was strongly inhibited by the drimane and only slightly by sinigrin. From a comparison of the results of behavioural microsyringe tests and electrophysiological responses we concluded that probably both the response of the deterrent cell and peripheral interactions exerted by feeding deterrents on the sucrose sensitive cell determine the potency of feeding deterrents in *L. decemlineata* larvae.

These results suggest that the neural code for antifeedancy in *L. decemlineata* larvae parallels that of *P. brassicae* larvae, *i.e.* a 'labelled line' (the deterrent cell in the epipharyngeal sensillum) coding principle combined with inhibition of receptor cells sensitive to feeding stimulants. They confirm the model on brain functioning in insects proposed by Schoonhoven & Blom (1988).

Because previously no convincing coding principles for the perception of antifeedants in Colorado potato beetle had been shown, these results for the first time provide a physiological basis for the hypothesis that the presence of feeding deterrents in potential food plants is a decisive cue in food plant selection by Colorado potato beetle larvae.

The finding that an epipharyngeal sensillum is involved in mediating the perception of feeding deterrents in *L. decemlineata* larvae gives rise to an evaluation of the function of epipharyngeal sensilla in taste perception in *L. decemlineata* and other beetles. Little is known about the sensory mechanisms of taste in coleopterans. In two other species, galeal sensilla were examined but epipharyngeal sensilla were not. In adults of the chrysomelid beetle *Diabrotica virgifera virgifera*, Chyb *et al.* (1995) identified galeal sensilla that were involved in the perception of feeding deterrents. These sensilla

responded to feeding deterrents and feeding stimulants at doses that were also behaviourally effective. It was also shown that beetles with ablated galea were still able to detect the feeding deterrent strychnine. This suggests that in this species other, e.g. epipharyngeal sensilla are also involved in the perception of feeding deterrents. In larvae and adults of the chrysomelid beetle *Entomoscelis americana*, Mitchell and Sutcliffe (1984) showed that the deterrent strychnine stimulates a galeal chemoreceptor cell that also responds to feeding stimulating glucosinolates. These authors suggest that feeding inhibition by strychnine is mainly due to another effect, namely inhibition of the response of the sugar sensitive cell.

In *L. decemlineata* larvae, we found a varying number of epipharyngeal sensilla, mostly 20 or 21. Compared to lepidopterans, in which the examined species possess between zero and six epipharyngeal sensilla, *L. decemlineata* larvae thus have many more of these sensilla. This might reflect a greater importance of epipharyngeal taste sensilla in food selection by *L. decemlineata* larvae than by lepidopteran caterpillars. According to Mitchell (1988), "video observations of adult *L. decemlineata* (Mitchell, unpubl.) suggest that ... epipharyngeal sensilla ... are the first gustatory organs to taste leaf sap" when feeding on a leaf is started, which might also indicate that epipharyngeal sensilla are important in taste perception by *L. decemlineata*. In coleopteran species, the epipharyngeal sensilla might be more important for the perception of antifeedants than galeal sensilla. However, more research on *L. decemlineata* and other beetle species is necessary to test this hypothesis.

Myzus persicae and *Aphis gossypii*

Although it was known that drimanes, such as polygodial (7), deter *M. persicae* from settling on (leaf) surfaces painted with these compounds (*e.g.* Gibson *et al.*, 1982; Asakawa *et al.*, 1988), the chemoreception of drimanes by this aphid remained unclear for long. However, Powell *et al.* (1995) showed in short-term behavioural tests that adult *M. persicae* can detect the drimane polygodial (7), applied to Chinese cabbage leaf discs or to plastic and glass surfaces, with contact chemosensilla located at their antennal tips.

In the research presented in chapter 6, we examined the behavioural effects of 11 drimane deterrents, painted on the surface of artificial diet sachets, on *Myzus persicae* and *Aphis gossypii* nymphs during 24-48 h tests. Furthermore, it was investigated which sensilla these aphid species use in the perception of the drimanes. In 24-48 h standard dual-choice tests *A. gossypii* nymphs in general were less sensitive to the drimanes than *M. persicae* nymphs. In dual-choice tests, in which the upper parts of the nymphal antennae were ablated, it was found that both species do not detect polygodial (7) anymore. It was concluded that nymphs of *M. persicae* and *A. gossypii* detect polygodial (7) and probably the other drimanes tested with contact chemosensilla at the tips of their antennae. The ablation studies also showed that in both species no tarsal, labial or epipharyngeal sensilla are involved in detecting polygodial (7) within 24 h.

The fact that antennal contact chemosensilla immediately detect polygodial (7) when applied to dry surfaces (Powell *et al.*, 1995; chapter 6) raises the question how the receptor cells in these sensilla come into contact with the polygodial (7) molecules while there is no plant sap, or insect saliva, to act as a transfer medium between substrate and

sensory dendrite. Experiments performed by Gödde *et al.* (1991) might give an explanation; in chemosensilla of intact flies he found that upon contacting oil covered crystals, the latter were dissolved into the extracellular fluid in which the receptor cells are bathed. This phenomenon could give a good explanation on how insects can perceive chemicals present on (plant) surfaces, although more research is needed with other (herbivorous) insect species. Possibly it also occurs in the antennal contact chemosensilla of aphids, so that, after the antennae touched the surface, polygodial (7) molecules could be dissolved in the extracellular receptor fluid.

About the neural code for deterrency by the antennal contact chemosensilla in aphids barely anything is known. Preliminary experiments by Wadhams (pers. comm.) (Pickett *et al.*, 1992) suggest that polygodial (7) possibly distorts the normal response of the receptor cells, rather than stimulating a potential 'deterrent cell'.

The ecological role of deterrents on the leaf surface in the process of food plant selection by aphids is not yet fully elucidated (Klingauf, 1987; Niemeyer, 1990; Pickett *et al.*, 1992; Tjallingii, 1995). Because the work of Powell *et al.* (1995) as well as our experiments show that the contact chemosensilla at the tips of the antennae detect deterrents on (leaf) surfaces, they might play a role in the selection of host plants by these and other aphid species.

Long-term effects of drimane antifeedants

Pieris brassicae

In chapter 3 the behaviour of *P. brassicae* larvae, when exposed to five drimane antifeedants in a no-choice test, was studied with detailed 1 min interval observations. We had found that some of the drimanes that were effective in a 3 h dual-choice test did not inhibit feeding in a 3 h no-choice test. The behavioural interval observations showed that two of the five drimanes (polygodial (7) and warburganal (5)) inhibited feeding only during the beginning (0-30 min) of the tests and that later on 'habituation' to these compounds occurred. Two other drimanes (confertifolin (see chapter 3) and compound 4) did not inhibit feeding during the first 30-90 min, but were effective during the remaining time in the test. Furthermore, these compounds inhibited locomotion behaviour as well. The fifth compound (isodrimenin (1)) inhibited feeding during the whole 3 h period. The results indicate that in a no-choice situation some drimanes (*e.g.* confertifolin and compound 4) do not at all or only slightly inhibit feeding through sensory effects, but do have postingestive, toxic effects resulting in inhibition of feeding. This means that analogous drimane antifeedants can inhibit feeding in *P. brassicae* through multiple mechanisms of action. This is probably a second factor contributing to the fairly large variation found in the correlation between the sensory response of the medial deterrent cell and behaviour (chapter 2). The results suggest that in no-choice situations *P. brassicae* larvae soon habituate to drimane antifeedants, unless the drimanes are toxic.

The fact that habituation occurs to polygodial (7) and warburganal (5) within 30 min indicates that 'bursting effects', such as measured by Schoonhoven & Yan (1989) during long-term sensory stimulation, probably do not play a role in the perception of these antifeedants.

From this study it can be concluded that, when developing a structure activity relationship (SAR) for a series of antifeedants, it is essential to distinguish the mode of action which underlies inhibition of feeding.

Leptinotarsa decemlineata
The behaviour of *L. decemlineata* larvae was studied when exposed to 3 drimanes in 3 h no-choice tests as well (with 1 min interval observations; chapter 4). In contrast to the results with *P. brassicae* larvae, polygodial (7) and warburganal (5) inhibited feeding throughout the 3 h no-choice tests, while exploring behaviour (walking + palpating) was increased compared to the controls. Also compound 4 had contrasting effects on *L. decemlineata* compared to *P. brassicae*; habituation occurred after the first 15 min, but exploring behaviour remained increased throughout the test. The results indicate that polygodial (7) and warburganal (5) are either strong antifeedants or possess toxic properties as well, so that no habituation occurs. Topical application of polygodial (7) and warburganal (5) to the larval cuticle also inhibited feeding, which could indeed indicate that toxic properties of these molecules might contribute to feeding inhibition as well, although the effects of topical application are not necessarily the same as the effects after ingestion of the compounds (*e.g.* not in the case of *P. brassicae*; chapter 3).

Aphis gossypii and *Myzus persicae*
The aphid species *A. gossypii* and *M. persicae* were not tested in no-choice situations. However, the behaviour of nymphs that settled on the treated diet surfaces was not noticeably influenced by the compounds during the 24-48 h of the tests.

In the dual-choice tests with warburganal (5) and polygodial (7) (chapter 6) we found a remarkable effect on *A. gossypii*; nymphs reared on plants, in contrast to nymphs reared on diet, did not survive in these tests. It is possible that, because nymphs reared on plants experience a more extreme difference in taste compared with their previous diet than nymphs reared on artificial diet, the deterrent action of warburganal (5) and polygodial (7) is so strong that the nymphs are inhibited from ingesting any diet at all, resulting in death.

This phenomenon could be related to a 'central nervous system inhibitory state' caused by extreme deterrency, as described by Jermy (1971). Earlier, with *Aphis fabae* (Hardie *et al.*, 1992) and *M. persicae* adults (Powell *et al.*, 1993), it was found that a 24 h pre-exposure to leaves or green paper painted with polygodial (7) caused a reduction in the subsequent number of penetrations and an increase in the mean duration of these penetrations. These effects of polygodial (7) on adult aphids and the effects on nymphs in our experiments could have a similar origin.

Effectiveness of terpenoid antifeedants on different insect species and Structure Activity Relationships (SAR)

Figure 1 summarizes the effectiveness of the drimanes tested in this thesis on *P. brassicae*, *L. decemlineata*, *M. persicae* and *A. gossypii* (results of the standard dual-choice tests with 5 mM drimane treatment, presented in chapter 2, 4 and 6 respectively).

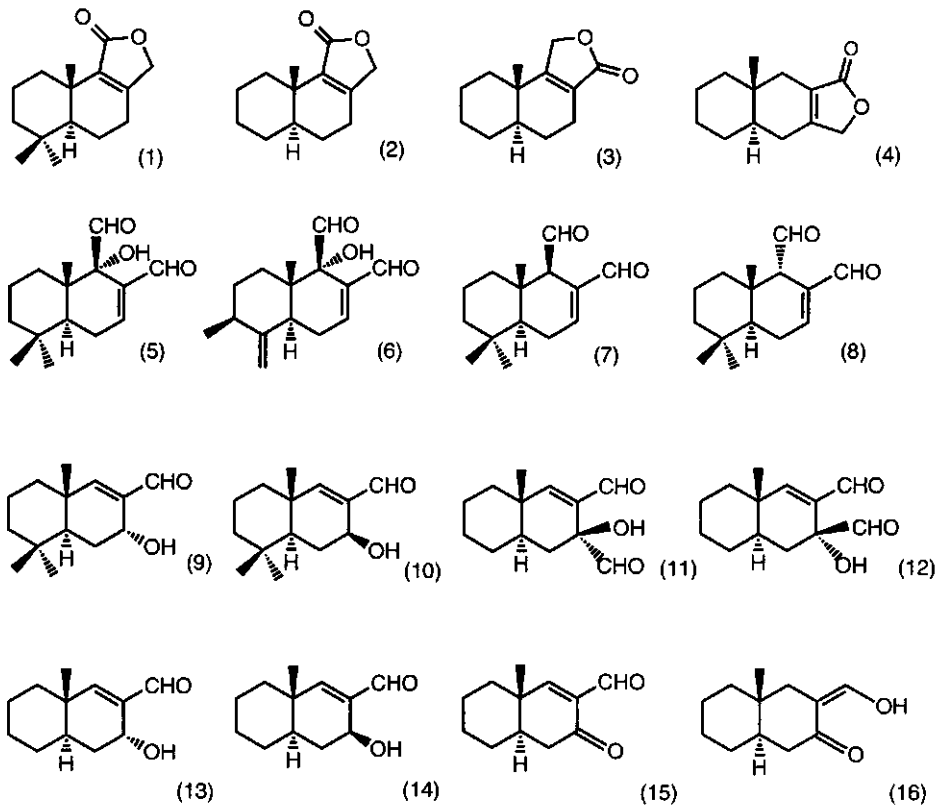
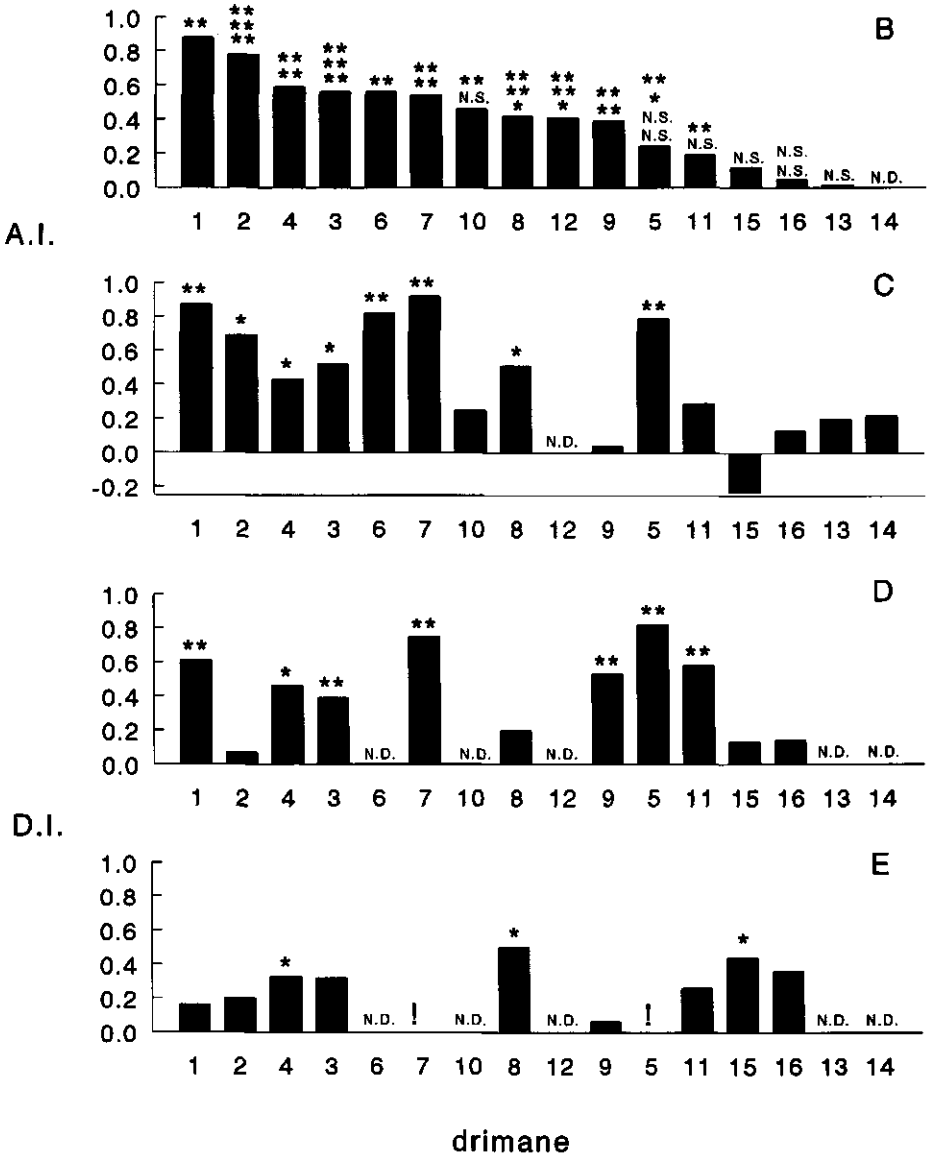


Figure 1. (page 114 and 115) Influence of 16 drimanes (Molecular structures: (A)) on the behaviour of *P. brassicae* (B) and *L. decemlineata* (C) (given as antifeedant index; A.I.) and *M. persicae* (D) and *A. gossypii* (E) (given as deterrency index; D.I.) with use of 5 mM drimane solutions (for calculation of A.I. and D.I. see chapters 2, 4 and 6). Wilcoxon's matched pairs signed rank test was used to assess significance; * $P < 0.05$; ** $P < 0.01$. N.D.: no data, !: The majority of the nymphs did not survive in these tests. For *P. brassicae* the weighted means of several tests are given, plus the significance levels of each test (N.S.: not significant). For the other insect species bars without marks indicate non-significant effects.

The Figure clearly shows that the four insect species all are affected by some of the compounds, but that the compounds that are most effective differ among species. This demonstrates that no generalizing SAR can be developed for the feeding inhibiting effectiveness of drimanes in insects. In *P. brassicae*, the drimanes with a lactone group on the B-ring appear to be the most potent antifeedants. In *L. decemlineata* and *M. persicae*, the highest deterrent indices were found in response to the dialdehydes polygodial (7) and warburganal (5) while in *L. decemlineata*, the lacton isodrimenin (1)


A.I.
B
1.0
0.8
0.6
0.4
0.2
0.0
1 2 4 3 6 7 10 8 12 9 5 11 15 16 13 14
N.S.
N.D.
C
-0.2
D
D.I.
E
!
drimane

was highly effective as well. Remarkably, a relatively simple synthetic compound (15) shows fairly high deterring activity against *A. gossypii*, while it was not effective at all against *M. persicae*, *L. decemlineata* and *P. brassicae*. When considering the sensory effects of drimanes in *e.g. P. brassicae* or *L. decemlineata* (chapter 2 and 5, respectively), *i.e.* stimulating a deterrent cell and inhibiting cells sensitive to feeding stimulants, it is conceivable that one or more of the membrane receptors involved may differ among species, or that the change in neural code caused by these compounds may have a different meaning in different insect species. The latter differences may contribute to the differences in SAR between insect species.

When developing a structure activity relationship, possible postingestive, toxic effects of antifeedants should be examined as well. For *P. brassicae* we showed that in no-choice tests some drimanes inhibit feeding through postingestive, toxic effects (chapter 3). In dual-choice tests, these toxic effects could contribute to the final feeding inhibiting effect as well. In fact, standard dual-choice tests are unsuitable for developing a SAR at the chemosensory level, because for some compounds the combined result of sensory and toxic effects will be measured. Also long-term effects, such as habituation, sensitization or food aversion learning can occur during the few hours that these tests usually last. However, standard dual-choice tests provide a quick method to screen if compounds have any feeding inhibiting effect at all. Detailed behavioural observations (*e.g.* chapter 3 or 4) could assist in determining which mode of action causes feeding inhibition.

Another phenomenon observed in the outcome of the dual-choice tests is the variability of the results of repeated tests with the same compound on the same insect species (chapter 2, 7 and 8). Although the tests were standardized as much as possible (*e.g.* for developmental state, degree of satiety, foodplant on which the insect was reared, temperature and light) there are probably many (individual and environmental) factors influencing insect feeding behaviour that we cannot measure or standardize. Variability in feeding tests and sensitivity of chemoreceptors has been described earlier, *e.g.* Bernays & Wege (1987) and Blaney *et al.* (1986).

Taste perception of plants

The research presented in this thesis contributes to our knowledge on the perception of antifeedants by comparing the outcome of standard behavioural tests with detailed behavioural studies and electrophysiological studies. In the electrophysiological studies with *P. brassicae* and *L. decemlineata*, we measured responses to single chemicals that were known to influence feeding, *e.g.* the feeding stimulant sucrose or the feeding inhibiting drimanes (chapter 2 and 5). When an insect is feeding on plant material treated with antifeedants, the sensilla do not contact single chemicals, but a blend of chemicals originating from plant saps and macerated cells, mixed with insect saliva and the antifeedants. Electrophysiological experiments in which mixtures of a feeding stimulant and a feeding deterrent are tested approach this situation more closely (chapter 2 and 5). As had been shown before (reviewed by *e.g.* Chapman, 1995), in these experiments 'peripheral interactions' occurred, *i.e.* the responses of cells to single compounds were

altered by the presence of the other compounds. Although responses to single compounds and mixtures have served as suitable information for developing theoretical models on insect taste perception (*e.g.* Schoonhoven & Blom, 1988), it is conceivable that the sensory responses to a mixture of leaf substance and deterrents are probably quite different from the responses to the binary mixtures that we tested. Therefore, to examine the neural code of antifeedancy, that the insect experiences when feeding on plants treated with antifeedants, electrophysiological experiments with (mixtures of) plant juices, insect saliva and feeding deterrents are needed. Although this might give some technical problems, *e.g.* in applying the stimulus and in interpreting from which sensory cells the recorded action potentials originate, some work on sensory responses to plant juices has been done, indicating that further investigation of the sensory responses to plant juices should be possible.

Dethier (1980), employing electrophysiological methods, tested leaf juices of up to 36 plant species on the medial and lateral maxillary sensilla of caterpillars of several species, *e.g. Danaus plexippus* and *Papilio* species. The saps were tested undiluted, directly after being pressed out of fresh foliage. Of the 36 plant species tested on *D. plexippus* (that were all behaviourally rejected), 83% evoked responses in three receptor cells in the sensilla. This indicates that the caterpillar brain generally receives information from multiple taste receptor cells when feeding on non-host plants.

Mitchell *et al.* (1990) and Haley Sperling & Mitchell (1991) tested leaf juices from several host and non-host plants on galeal sensilla of *L. decemlineata* adults. They mixed plant juices with 100 mM potassium chloride and found that the sensory responses to a five-fold dilution of this mixture is barely distinguishable from the original mixture. However, although Haley Sperling & Mitchell (1991) found that *L. decemlineata* sensilla rarely respond to 100 mM potassium chloride, it remains unclear if freshly expressed juices would give similar responses. In their experiments mostly two or three cells responded to the diluted plant juices. One of these cells responded vigorously and with little variation to all host plants (the 'primary response'). In contrast, responses to non-host plants were highly variable, suggesting that the 'primary response' might be important in the recognition of host plants by *L. decemlineata*.

Prospects for practical use of (terpenoid) antifeedants in crop protection

In the studies described in this thesis we tested the antifeedant efficacy of 18 drimanes (including related derivatives) and 33 derivatives of partial structures of *neo*-clerodanes in standard dual-choice and/or no-choice tests on several insect species. We did not study their effectiveness in field and/or greenhouse situations, which would be needed to assess their possible use as crop protection agents. However, from the laboratory studies some indications for the potential of their practical use can be derived.

In this paragraph, a hypothetical example on the use of one of the drimanes (*i.e.* isodrimenin; compound 1, Figure 1) as control agent against the Colorado potato beetle in a potato field will be described. Our laboratory bioassays and a field study on the use of an azadirachtin based product against the Colorado potato beetle (Wood *et al.*, 1995) were used as basis for the estimations. The example illustrates the dosis of isodrimenin

(1) that would be needed to control the beetle. Warthen Jr. (1990) mentions in his overview of laboratory studies on the efficacy of antifeedant compounds that warburganal (5) and polygodial (7) belong to the most potent antifeedants found as yet. However, these compounds have been shown to possess phytotoxic properties (*e.g.* chapter 4 or Asakawa *et al.*, 1988), and therefore were not chosen as an example here. Isodrimenin (1) is as effective against the Colorado potato beetle as warburganal (5) and polygodial (7) (chapter 4). At 5 mM it causes an antifeedant index of 0.87, but it is not effective at 1 mM. Therefore we estimate that a concentration of 5 mM will be needed for crop protection (assuming that isodrimenin in a field application is as effective as in laboratory bioassays, and that no habituation to the compound occurs). According to Wood *et al.* (1995) the product 'ALIGN^{TM}' (AgriDyne Technologies Inc.), that contains 3% azadirachtin, gave adequate crop protection in a potato field infested with Colorado potato beetle (in the treated plots 5% defoliation occurred, compared to 32% in the control plots) at a dose of 25 g azadirachtin/ha, with 470 litres solution/ha applied weekly. Combining these data, it can be estimated that weekly 470 litres of 5 mM isodrimenin/ha is needed to control the Colorado potato beetle, which equals weekly 550 g isodrimenin/ha (*c.* 1.2 g/l.).

The estimated dosis of isodrimenin (1) is *c.* 20 times higher compared to the azadirachtin dose needed. Because the costs of synthesizing isodrimenin (1) will probably be high, availability of compounds that are active at *c.* 50-100 times lower concentrations would be desirable when the use of synthetic compounds is aimed for crop protection in field situations (for comparison: synthetic deltamethrin is used at a dose of 7.5-12.5 g/ha against the Colorado potato beetle (van Geel, 1991). However, I figure that the chance that such compounds will be found is rather low: as yet only compounds with complex molecular structures, such as the triterpenoids azadirachtin (Blaney *et al.*, 1990) and toosendanin (Luo *et al.*, 1995) or *neo*-clerodane diterpenoids (Blaney *et al.*, 1988) have been shown to be effective at such low concentrations. Because their molecular structures are extremely difficult and costly to synthesize, it is doubtful if synthetic specimens of these compounds could be used in crop protection. Our results with the simple analogues of *neo*-clerodanes in this thesis (chapter 8) show that only few compounds had moderate effectiveness at a dosis of 5 mM, indicating that these simple analogues are probably not suitable for use in crop protection.

In the literature, also more promising examples of the use of drimane antifeedants in crop protection are reported. Pickett *et al.* (1987) found in field experiments with polygodial (7) against the aphid *Rhopalosiphum padi*, a vector of barley yellow dwarf virus, that the yield of barley could be raised by three treatments of polygodial (7) at only 50 g/ha (the yield in treated plots was 5,22 tonnes/ha, compared to 3,83 tonnes/ha in control plots).

The polygodial (7) used in the previous experiment was obtained from large scale production of the polygodial (7) producing plant 'water-pepper' (*Polygonum hydropiper*). This example leads us to alternative ways to obtain antifeedants for crop protection (besides the synthetic method). At first glance, it seems illogical to grow plants for producing antifeedants to assist in growing crops. However, it should be kept in mind that in Europe more and more agricultural area comes available for alternative uses because of overproduction of food crops (Wetenschappelijke Raad voor het Regerings

Table 1. Feeding inhibiting effect (antifeedant index (A.I.) ± s.e.) of single drimane compounds (1 mM) and binary mixtures (0.5 + 0.5 mM) on 5th instar *Pieris brassicae* larvae. The mixture and single compounds were tested at the same time.

Compound 1	A.I.	n	Compound 2	A.I.	n	A.I. (mixture)	n
(1)	0.23±0.5 (NS)	11	(6)	-0.02±0.3 (NS)	12	0.68±0.3 **	12
(1) (not tested)			(6)	0.20±0.5 (NS)	12	0.63±0.3 **	12
(1) (not tested)			(8)	0.45±0.3 **	12	0.47±0.4 **	12
(1) (not tested)			(9)	0.09±0.4 (NS)	12	0.12±0.4 (NS)	12
(8)	0.41±0.4 **	19	(9)	0.11±0.3 *	20	0.44±0.3 **	20
(5)	0.16±0.4 (NS)	12	(6)	0.20±0.5 (NS)	12	0.19±0.5 (NS)	10

Statistics: Wilcoxon's matched pairs signed rank test, * $P < 0.05$; ** $P < 0.01$

beleid, 1992). These areas may be used to produce natural crop protection agents, to replace conventional insecticides. Another way of obtaining antifeedants could be *in vitro* callus growth of antifeedant producing plant material. Kearney *et al.* (1994) showed that callus and shoot cultures of the neem tree *Azadirachta indica* suppressed feeding in no-choice bioassays using the desert locust *Schistocerca gregaria*. Jermy (1990) mentions some other possible cheap sources of insect antifeedants, such as by-products and waste materials of pharmaceutical and other organochemical and food industries, or products extracted from algae (Saleh *et al.*, 1984) or fungi (Rowan and Tapper, 1989).

A possible way of using antifeedants in crop protection could be a combination of several antifeedants with different mechanisms of action, *e.g.* one that acts through sensory effects and one that has toxic properties, or one that stimulates a 'deterrent cell' and another that inhibits cells sensitive to feeding stimulants. Such combinations may prevent the occurrence of habituation, or may synergistically increase the feeding inhibiting effect, *i.e.* the effect of the combination would be higher than the added effects of the single compounds. In this research, the effects of a few combinations of drimane antifeedants have been tested in dual-choice tests on *P. brassicae* (Table 1; concentration antifeedant = 1 mM). Only the combination of compound 1 and 6 synergistically increased feeding inhibition. This result is interesting, because compound 1 probably has postingestive, toxic properties (chapter 3) while compound 6 is not toxic, but does inhibit feeding in dual-choice tests at 5 mM (chapter 2). However, in combination with compounds 8 and 9 compound 1 does not cause increased feeding inhibition. More research is needed before final conclusions can be drawn. Considering the research on the

sensory perception of antifeedants by the Colorado potato beetle (chapter 5), it would be interesting to behaviourally test a combination of compound 4, that slightly stimulates the deterrent cell but strongly inhibits the sucrose cell, and sinigrin, that strongly stimulates the deterrent cell, but slightly inhibits the sucrose cell.

Another possible way of using antifeedants in crop protection could be a combined action with other (biological) control agents. In greenhouses antifeedants could be employed to protect only the most vulnerable, young parts or flowers of plants, while insect control is established by a slow acting, biological control agent. Applied in this way, only small quantities of antifeedants would be required. Recent studies have shown that antifeedants may effectively be used in conjunction with an endotoxin (see *e.g.* Murray *et al.* (1993) who combined citrus limonoid antifeedants with a *Bacillus thuringiensis* endotoxin) or with growth regulator insecticides (*e.g.* Griffiths *et al.*, 1991). Probably the use of combinations of innovative crop protection agents is the most promising method for future crop protection. Because many environmentally friendly crop protection agents are not effective enough when applied alone, the combined use of several control agents might give better results. Another advantage of combined uses is that resistance development is absent or delayed, because adaptation to several mechanisms of action is needed. However, for the grower combined uses of crop protection agents would be rather 'knowledge intensive', which could make the introduction of such methods problematic (Vereijken, 1989; Jansma *et al.*, 1993).

In conclusion, it can be said that for practical implementation of (terpenoid) antifeedants in crop protection still much work remains to be done. In order to get a marketable product, research should be focussed on selected insect species, that are important pests and for which no or little (environmentally friendly) control methods are available. It should be examined how large quantities of antifeedants can be obtained with low costs. The ecological effects of applying large quantities of antifeedant compounds should be known as well before their use in crop protection can be allowed. Further, more research on the combined use of antifeedants with different mechanisms of action or with other (biological) control agents should be done. Some of the terpenoid antifeedants studied in this thesis may perform appropriately in such combined applications.

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Summary

This thesis describes a study on the behavioural and sensory effects of terpenoid antifeedants on several insect species.

The main aim was to elucidate the mechanisms of action of terpenoid antifeedants. From a fundamental point of view, this will yield insight in the role of these compounds in host plant selection by insects. From an applied perspective, knowledge of the level of effectiveness and of putative structure-activity relationships, provides a basis to assess the potential for using terpenoids as crop protection agents.

The effects of specific molecular structures were studied comparatively in different unrelated insect species. The mechanism(s) of action of sesquiterpene drimanes were investigated by measuring sensory responses and observing their effects on behaviour. Furthermore, long-term and toxic effects were studied.

Larvae of the large white butterfly *Pieris brassicae* L. (Lepidoptera: Pieridae) were used as model insect for studying the sensory perception of sesquiterpene drimane antifeedants by insects. By comparing sensory and behavioural responses to 15 drimane antifeedants, it was shown that the frequency of impulses from the deterrent cell in the medial sensillum styloconicum significantly correlates to the behavioural response in dual-choice tests. In addition, the drimanes caused inhibition of sensory responses to feeding stimulants. The results suggest that in this insect a 'labeled line' coding principle is used for the perception of antifeedants, combined with inhibition of receptor cells sensitive to feeding stimulants (chapter 2).

The temporal aspects of the behavioural effects of five drimanes on *P. brassicae* larvae were studied with aid of detailed, 1 min interval behavioural observations in a no-choice test (chapter 3). It was found that two of the five drimanes (polygodial and warburganal) inhibited feeding only during the beginning (0-30 min) of the tests and that two other drimanes (confertifolin and 'compound 4') became effective 30-90 min after the onset of the test. The fifth compound (isodrimenin) inhibited feeding during the whole 3 h period. It is concluded that some drimanes (*e.g.* confertifolin and compound 4) have postingestive, toxic effects resulting in inhibition of feeding. These and the results of chapter 2 indicate that analogous drimane antifeedants can inhibit feeding in *P. brassicae* through multiple mechanisms of action. Probably *P. brassicae* larvae in no-choice situations soon habituate to drimane antifeedants, unless the drimanes are toxic. The results indicate that, when developing a structure-activity relationship for a series of antifeedants, it is essential to distinguish the mode of action which underlies inhibition of feeding.

The behaviour of larvae of the Colorado potato beetle, *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae) was studied when exposed to three drimanes in 3 h no-choice tests (chapter 4). In contrast to *P. brassicae* larvae *L. decemlineata* larvae were inhibited from feeding throughout the 3 h no-choice tests when treated with polygodial or warburganal, while to compound 4 habituation occurred after the first 15 min of the test. The results indicate that polygodial and warburganal are either strong

antifeedants or possess toxic properties as well, preventing that habituation occurs. Topical application of polygodial and warburganal to the larval cuticle also inhibited feeding. This could indicate that toxic properties of these molecules might contribute to feeding inhibition, although the effects of topical application are not necessarily the same as the effects after ingestion of the compounds (*e.g.* not in the case of *P. brassicae*; chapter 3).

The hypothesis that feeding deterrents in potential food plants are decisive in food plant selection by insects (chapter 1) was originally derived from experiments with the Colorado potato beetle. However, little was known about the sensory perception of such compounds by this beetle. In this study the role of an epipharyngeal sensillum in the perception of antifeedants was investigated (chapter 5). Electron microscopy revealed that the epipharyngeal sensillum is innervated by five neurons. Electrophysiological experiments showed that one of these cells responds to water, a second to sucrose and a third to two feeding deterrents (*i.e.* compound 4 and sinigrin) that had been found effective in a behavioural test. Furthermore, the response of the sucrose sensitive cell was strongly inhibited by the drimane and only slightly by sinigrin. From a comparison of behavioural and sensory responses it is concluded that probably both the response of the epipharyngeal deterrent cell and peripheral interactions exerted by feeding deterrents on the sucrose sensitive cell in this sensillum determine the potency of feeding deterrents in *L. decemlineata* larvae. The results provide a physiological basis for the hypothesis that the presence of feeding deterrents in potential food plants is a decisive cue in food plant selection by *L. decemlineata* larvae.

The sensitivities of nymphs of two aphid species, *Myzus persicae* Sulzer and *Aphis gossypii* Glover (Homoptera: Aphididae) to 11 drimane compounds, applied to the lower surface of artificial diet sachets, were compared in dual-choice tests. In general, *A. gossypii* nymphs were less sensitive to the drimanes than *M. persicae* nymphs. Warburganal and polygodial were highly active as deterrents and/or feeding inhibitors against both species. In dual-choice tests in which the upper parts of the nymphal antennae were ablated, it was found that both species do not detect the drimane polygodial anymore. It was concluded that nymphs of *M. persicae* and *A. gossypii* detect polygodial and probably the other drimanes tested with contact chemosensilla at the tips of their antennae. The ablation studies also showed that in both species no tarsal, labial or epipharyngeal sensilla are involved in detecting polygodial within 24 h. The results indicate that deterrents on the leaf surface might play a role in the selection of host plants by these and other aphid species.

Chapter 7 reports on additional bioassays with drimanes on several insect species. In chapter 8, several synthetic analogues, derived from the C-9 side chains of the diterpenoids clerodin and ajugarin I, were tested for their effects on feeding of *P. brassicae* and *L. decemlineata* larvae and *M. persicae* nymphs. Several compounds showed moderate activity against *P. brassicae* larvae. Interestingly, the most effective compound most closely resembled the structure of the furopyran-fragment of the triterpenoid azadirachtin, which fragment had previously been shown to be a highly effective feeding deterrent against *Spodoptera littoralis*.

In conclusion, the mode of perception as well as the structure-activity relationship of terpenoid antifeedants considerably differs between the insect species examined in

this study (chapter 9). For practical implementation of terpenoid antifeedants in crop protection, this means that research should be focussed on selected pest insect species. Furthermore, the possibilities for combined use with other (biological) control agents should be investigated.

Samenvatting

Dit proefschrift beschrijft onderzoek aan 'terpenoid antifeedants', ofwel 'vraatremmende terpenoïden'. De effecten van deze stoffen werden bij verschillende insectensoorten op gedrags- en zintuigniveau onderzocht.

Het doel van het onderzoek was het ophelderen van de werkingsmechanismen van vraatremmende terpenoïden. Fundamenteel gezien geeft dit inzicht in het effect van deze stoffen bij de selectie van waardplanten door insecten. Vanuit een toegepast perspectief is kennis van de mate van effectiviteit van vraatremmende terpenoïden en van mogelijke structuur-activiteitsrelaties belangrijk om het potentiële gebruik van deze stoffen als gewasbeschermingsmiddel te kunnen inschatten.

De effecten van specifieke moleculaire structuren werden vergeleken bij verschillende, niet verwante insectensoorten. De werkingsmechanismen van sesquiterpenoïde drimanen werden onderzocht door het meten van zintuigreacties en het observeren van de gedragseffecten. Daarnaast werden lange termijn- en toxische effecten bestudeerd.

Rupsen van het Grote koolwitje *Pieris brassicae* L. (Lepidoptera: Pieridae) werden gebruikt als modelinsect voor het bestuderen van de zintuiglijke waarneming van sesquiterpenoïde drimanen door insecten. Door de zintuig- en gedragsreactie op 15 drimanen te vergelijken werd aangetoond dat de impulsfrequentie van de 'deterrent cel' in de mediale smaakhaar significant correleert met de gedragsreactie in twee-keuze toetsen. Daarnaast veroorzaakten de drimanen een remming van de zintuiglijke reactie op vraatstimulerende stoffen. De resultaten suggereren dat bij dit insect sprake is van een 'labeled line' principe voor de neurale codering van vraatremming, in combinatie met remming van receptorcellen die gevoelig zijn voor vraatstimulerende stoffen (hoofdstuk 2).

De temporele effecten van vijf drimanen op het gedrag van *P. brassicae* rupsen werden bestudeerd met behulp van gedetailleerde gedragsobservaties (met een interval van 1 min.) tijdens een geen-keuze toets (hoofdstuk 3). Er werd aangetoond dat twee van de vijf drimanen (polygodial en warburganal) de vraat slechts gedurende het begin (0-30 min.) van de toets remden en dat twee andere drimanen (confertifolin en 'stof 4') pas 30-90 min. na het begin van de toets de voedselopname remden. De vijfde stof (isodrimenin) remde de vraat gedurende de hele periode van 3 uur. Geconcludeerd werd dat sommige drimanen (bijv. confertifolin en stof 4) na opname toxische effecten hebben die resulteren in vraatremming. Deze resultaten en die van hoofdstuk 2 geven aan dat analoge drimanen de vraat van *P. brassicae* via meerdere werkingsmechanismen kunnen remmen. Waarschijnlijk habitueren *P. brassicae* rupsen in geen-keuze situaties snel aan vraatremmende drimanen, tenzij de drimanen toxisch zijn. De resultaten geven aan dat het essentieel is om het werkingsmechanisme van de vraatremming te onderscheiden wanneer structuur-activiteitsrelaties binnen series van vraatremmende stoffen worden onderzocht.

Het gedrag van larven van de Coloradokever, *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae) werd bestudeerd bij blootstelling aan drie drimanen in een

geen-keuze toets van 3 uur (hoofdstuk 4). In tegenstelling tot *P. brassicae* rupsen werden *L. decemlineata* larven gedurende de hele periode in de vraat geremd bij behandeling met polygodial of warburganal, terwijl bij behandeling met stof 4 habituatie optrad na de eerste 15 min. van de toets. De resultaten geven aan dat polygodial en warburganal òf sterk vraatremmende stoffen zijn òf ook toxische eigenschappen bezitten die het optreden van habituatie voorkomen. Wanneer polygodial en warburganal plaatselijk op de cuticula van de larven was aangebracht werd de vraat ook geremd. Dit zou kunnen betekenen dat toxische eigenschappen van deze moleculen bijdragen tot de vraatremming, hoewel de effecten van externe toediening niet altijd hetzelfde zijn als de effecten na orale opname van de stoffen (bijv. niet in het geval van *P. brassicae*; hoofdstuk 3).

De hypothese dat vraatremmende stoffen in potentiële voedselplanten voor insecten beslissend zijn bij de selectie van voedselplanten was oorspronkelijk afgeleid van experimenten met de Coloradokever. Toch was er weinig bekend over de zintuiglijke waarneming van dergelijke stoffen door deze kever. In dit onderzoek werd de rol van een epifaryngeaal zintuigorgaan bij de waarneming van vraatremmende stoffen bestudeerd (hoofdstuk 5). Electronenmicroscopie liet zien dat het epifaryngeale zintuigorgaan dendrieten van vijf zintuigcellen bevat. Electrofysiologische experimenten toonden aan dat één van deze zintuigcellen op water reageerde, een tweede op sucrose en een derde op twee vraatremmende stoffen (nl. stof 4 en sinigrine), welke in een gedragstoets effectief waren bevonden. Daarnaast werd de reactie van de sucrose-gevoelige zintuigcel sterk door het drimaan geremd maar slechts in geringe mate door sinigrine. Uit een vergelijking van gedrags- en zintuigreacties werd geconcludeerd dat de reactie van de epifaryngeale 'deterrent cel' èn perifere interacties van vraatremmende stoffen met de sucrose-gevoelige zintuigcel in dit zintuigorgaan de activiteit van vraatremmende stoffen voor *L. decemlineata* larven bepalen. De resultaten verschaffen een fysiologische basis voor de hypothese dat de aanwezigheid van vraatremmende stoffen in potentiële voedselplanten beslissend is bij de selectie van voedselplanten door *L. decemlineata* larven.

Nymfen van twee bladluissoorten, *Myzus persicae* Sulzer en *Aphis gossypii* Glover (Homoptera: Aphididae), werden in een twee-keuze toets vergeleken op hun gevoeligheid voor 11 drimanen die aangebracht waren op de onderkant van twee lagen parafilm, waartussen zich kunstmatig dieet bevond. In het algemeen waren *A. gossypii* nymfen minder gevoelig voor de drimanen dan *M. persicae* nymfen. Warburganal en polygodial hadden een sterk afwerende en/of vraatremmende werking op beide soorten. Wanneer de bovenste gedeelten van de antennen werden verwijderd waren beide soorten niet meer in staat om polygodial waar te nemen. Hieruit werd geconcludeerd dat nymfen van *M. persicae* en *A. gossypii* polygodial en waarschijnlijk ook de andere geteste drimanen waarnemen met 'smaakzintuigen' die zich op de toppen van hun antennen bevinden. Deze experimenten tonen ook aan dat bij beide soorten binnen 24 uur geen tarsale, labiale of epifaryngeale zintuigen zijn betrokken bij de waarneming van polygodial. Uit de resultaten blijkt dat vraatremmende stoffen op het bladoppervlak mogelijk een rol spelen bij de selectie van waardplanten door deze en andere bladluissoorten.

In hoofdstuk 7 wordt de uitkomst van een aantal gedragstoetsen met drimanen bij verschillende insectensoorten beschreven. In hoofdstuk 8 worden een aantal synthetis-

che analoga die afgeleid zijn van de C-9 zijketen van de diterpenoïden clerodin en ajugarin I getest op hun vraatremmende effectiviteit tegen larven van *P. brassicae* en *L. decemlineata* en nymfen van *M. persicae*. Enkele stoffen hadden een gering effect op *P. brassicae*. Het is interessant dat de structuur van de meest effectieve stof de structuur van het furopyran-fragment van het triterpenoïde azadirachtine het meest benadert, daar dit fragment eerder hoogst effectief tegen *Spodoptera littoralis* was bevonden.

Concluderend verschillen zowel de werkingsmechanismen als de structuur-activiteitsrelaties van vraatremmende terpenoïden aanzienlijk voor de in dit onderzoek geteste insectensoorten (hoofdstuk 9). Voor toepassing van vraatremmende terpenoïden in de gewasbescherming betekent dit dat het onderzoek zich zal moeten richten op geselecteerde plaaginsecten. Daarnaast moeten de mogelijkheden van een gecombineerd gebruik met andere (biologische) gewasbeschermingsmiddelen worden onderzocht.

Publications

Chapters of this dissertation have been or will be published in journal articles (in slightly different versions):

Gols G.J.Z., J.J.A. van Loon & L. Messchendorp, 1996. Antifeedant and toxic effects of drimanes on Colorado potato beetle larvae. *Entomol. Exp. Appl.* 79: 69-76.

Messchendorp L., G.J.Z. Gols & J.J.A. van Loon, 1996. Behavioural and sensory responses to drimane antifeedants in *Pieris brassicae* larvae. *Entomol. Exp. Appl.* 79: 195-202.

Messchendorp L., H.M. Smid & J.J.A. van Loon, 1998. The role of an epipharyngeal sensillum in the perception of feeding deterrents by *Leptinotarsa decemlineata* larvae. *J. Comp. Physiol. A* 183: 255-264.

Messchendorp L., G.J.Z. Gols & J.J.A. van Loon, 1998. Behavioural effects and sensory detection of drimane deterrents in *Myzus persicae* and *Aphis gossypii* nymphs. *J. Chem. Ecol.* (in press).

Messchendorp L., J.J.A. van Loon & G.J.Z. Gols. Behavioural observations of *Pieris brassicae* larvae indicate multiple mechanisms of action of analogous drimane antifeedants. *Entomol. Exp. Appl.* (accepted).

Curriculum Vitae

Op 17 december 1967 werd ik, Lindy Messchendorp, geboren te Groningen. Van 1980 - 1986 volgde ik het atheneum-B aan De Aletta Jacobs Scholengemeenschap te Hoogezand. De opleiding werd in de periode 1986 - 1991 vervolgd met een studie Biologie aan de Rijksuniversiteit Groningen. Tijdens de afstudeerfase deed ik onderzoek aan het visuele systeem van vliegen bij de vakgroep Biofysica en aan elektrische eigenschappen van protoplastmembranen bij de vakgroep Plantenfysiologie. In 1991 werd ik aangesteld als onderzoeker in opleiding aan de Landbouwuniversiteit Wageningen, bij de vakgroep Entomologie. Daar verrichtte ik tot 1997 onderzoek aan gedrags- en zintuigeffecten van vraatremmende stoffen op diverse (plaag)insecten. In de zomer van 1998 werkte ik aan de Justus-Liebig-Universität Gießen in Duitsland, bij de afdeling Biologischer und Biotechnischer Pflanzenschutz, aan de waarneming van plantengeuren door Coloradokevers in het veld. Het in dit proefschrift beschreven onderzoek werd uitgevoerd in de periode 1991 - 1996, aan de vakgroep Entomologie in Wageningen.