Trail marking and following by larvae of the small ermine moth *Yponomeuta cagnagellus*

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TRAIL MARKING AND FOLLOWING BY LARVAE OF THE SMALL ERMINE MOTH *YPONOMEUTA CAGNAGELLUS*

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STELLINGEN

1. De door Weyh & Maschwitz (1982) uitgevoerde experimenten met betrekking tot het bestaan van spoorstoffen bij de rupsen van *Iphiclides podalirius*, vormen onvoldoende rechtvaardiging voor hun conclusie dat er sprake is van een vluchtige signaalstof.

   Weyh, R. & Maschwitz, U. (1982) Individual trail marking by larvae of the scarce swallowtail *Iphiclides podalirius* L. (Lepidoptera; Papilionidae). *Oecologia* **52:** 415-416.

   Sabelis, M.W. & Dicke, M. (1985) Long range dispersal and searching behaviour. In: W. Helle & M.W. Sabelis (eds.) Spider mites, Their Biology, Natural Enemies and Control. *World Crop Pests* **1B**: 141-160. Elsevier, Amsterdam.

2. De door Borst (1984) voorgestelde methode om via electroantennogrammen aantallen geur-receptorcellen te bepalen, kan - in het licht van de resultaten van onder andere De Jong & Visser (1988) - niet langer als juist beschouwd worden.

   Borst, A. (1984) Identification of different chemoreceptors by electroantennogram-recording. *J. Insect Physiol.* **30**: 507-510.

   De Jong, R & Visser, J. H. (1988) Specificity-related suppression of responses to binary mixtures in olfactory receptors of the Colorado potato beetle. *Brain Research* **447**:18-24.

3. In plaats van de door Fitzgerald & Peterson (1988) gebruikte term "sociaal" voor sommige gregair levende rupsesoorten verdient de aanduiding "pre-sociaal" de voorkeur.

   Fitzgerald, T.D. & Peterson, S. C. (1988) Cooperative foraging and communication in caterpillars. *BioScience* **38**: 20-25.

4. De methodologie voor de analyse van extracellulaire zintuigfysiologische afleidingen is onvoldoende gedefinieerd.

5. In het kader van milieu maatregelen moet het als een verbijsterend voorbeeld van kortzichtigheid beschouwd worden dat men, kennelijk op grond van economische motieven, niet verder komt dan het geleidelijk afbouwen van de produktie van Chloorfluorkoolwaterstoffen.

   De Volkskrant, 15 oktober 1988.

6. Geïntegreerde plaag bestrijding heeft geen werkelijke toekomst zonder een drastische verandering in de houding van de consument ten opzichte van niet volledig gave produkten.

7. Risico's van moderne technologieën worden veelal gedomineerd door menselijke factoren. Het voorstel van Islam & Lindgren (1986) om risicoschattingen te baseren op een analyse van de werkelijke frequentie's van ernstige ongelukken, in plaats van op combinatie van de geschatte faalkansen van afzonderlijke onderdelen, verdient daarom nadrukkelijk aandacht.

Freudenberg, W.R. (1988) Perceived risk, real risk: Social science and the art of probabilistic risk assessment. *Science* **242**: 44-49.

Islam, S. & Lindgren, K. (1986) How many reactor accidents will there be? *Nature* **322**: 691-692.

8. Het invoeren van "road pricing" door middel van het aanbrengen van sensoren in het wegdek brengt ernstige risico's voor de privacy met zich mee.

9. Niet zozeer het feit dat er sprake is van het opzetten van spermabanken met sperma van "genieën" moet verbazing wekken, als wel het feit dat het mogelijk zou zijn deelnemers voor zo'n project te vinden.

10. Arts assistenten zijn de slaven van deze tijd, jonge wetenschappers de voortdurend rondtrekkende zigeuners.

11. De kunstmatig intelligente systemen van de toekomst zullen als hulpeloze baby's ter wereld komen en zullen moeten beschikken over een immuunsysteem tegen virussen en wormen.

Kuperstein, M. (1988) Neural model of adaptive hand-eye coordination for single postures. *Science* **239**: 1308-1310.

Marshall, E. (1988) Worm invades computer networks. *Science* **242**: 855-856.

Stellingen behorende bij het proefschrift van Peter Roessingh: Trail marking and following by larvae of the small ermine moth *Yponomeuta cagnagellus*.

Voorwoord

Nu het proefschrift afgerond is wil ik graag van de gelegenheid gebruik maken om de vele mensen die me geholpen hebben te bedanken.

In de eerste plaats mijn promotor Louis Schoonhoven. Zijn inspirerende colleges dierfysiologie en oecofysiologie vormden een belangrijke factor in mijn besluit te kiezen voor een zoölogische invulling van mijn biologie studie. Zijn steun en vertrouwen in latere jaren en tijdens het hier beschreven onderzoek betekenen veel voor mij. Wim Herrebout, mijn co-promotor wijdde me in, in het *Yponomeuta* onderzoek. Zijn enthousiasme voor het onderwerp, zijn vele ideeën en suggesties en zijn speciale humor vormden een voortdurende bron van inspiratie.

Met veel plezier denk ik ook terug aan mijn tijd bij de vakgroep dierfysiologie, waarbij ik speciaal Wim Bijlsma zou willen bedanken voor het bouwen van de electrofysiologische versterker. Ook de samenwerking met het TFDL was prettig, en vooral Felix Thiel wil ik bedanken voor zijn vakkundige advies en alles wat hij voor me gedaan heeft. De hulp van Teris van Beek en Maarten Posthumus (vakgroep Organische Chemie) waren onmisbaar bij mijn pogingen de geuren van de kardinaalsmuts te karakteriseren. Birgit Nübel zorgde voor de insectenkweek. Haar hulp bij het verzorgen van de rupsen, vormde een belangrijke steun tijdens het onderzoek, evenals de hulp van de gecombineerde diensten, waarbij met name de zorg voor de kardinaalsmuts door de kassengroep, het tekenwerk van Frederik von Planta en Piet Kostense en de dia's en foto's van de mensen van de Fotolocatie Binnenhaven vermeld moeten worden.

De prima tijd die ik gehad heb tijdens het onderzoek werd voor een groot deel bepaald door de goede sfeer in het lab. De dagelijkse praatjes met Lucas, Oscar, Louise en vele anderen; de gesprekken met Joop, vooral tijdens de tijd bij dierfysiologie, en de discusies met Ruurd na onze training-sessies droegen allen direct of indirect belangrijk bij aan het tot stand komen van dit proefschrift. In het bijzonder wil ik hier Wim Frentz noemen, bij wie ik altijd terecht kon als ik het eens niet meer zo zag zitten. Ik denk nog steeds met veel plezier terug aan onze vele lange gesprekken. Verder was zijn hulp tijdens de periode dat ik al in Zwitserland werkte van onschatbare waarde.

Mijn ouders verdienen een speciale plaats. Hun interesse, steun en bemoediging vormden steeds een stevige basis gedurende mijn studie en dit onderzoek.

Finally I wish to thank all visitors of the lab that I met during the years, who brought many new ideas and helped me to gain a wider perspective. Clythia, Julian, Steve, Tom, Dan and David, thank you for everything.

Zonder het organisatietalent van Ineke tenslotte, zou dit proefschrift nu nog niet klaar zijn.

Peter, December 1988

Voor mijn Oma en mijn Opa, in wiens studeerkamer ik de grondslag voor dit proefschrift heb gelegd.

CONTENTS

Figure 1 Rasterscanning electronenmicroscopische foto van de kop van *Yponomeuta cagnagellus*, van onderen gezien. In het midden van de foto is het spinselorgaan zichtbaar, met een nog steeds aanwezig stuk spinseldraad. Daarboven zijn de spitse palpen en de wat bredere galea zichtbaar, die gebruikt worden voor het waarnemen van reuk en smaakprikkels. Deze organen zijn zo geplaatst dat ze de ruimte voor de mondopening bestrijken. Hierboven zijn de twee kaken te zien afgedekt door de plaatvormige bovenlip. Aan weerszijde hiervan zijn de gedeeltelijk ingetrokken antennen zichtbaar die reukzintuigen bevatten. Nog verder naar de buitenzijde ligt een cirkelvormige groep van 6 enkelvoudige ogen. In de hals is de uitgang van het halsorgaan zichtbaar juist voor het voorste paar poten. De gehele kop is bedekt met lange tastharen.

Rasterscanning electron micrograph of the ventral side of the head of *Yponomeuta cagnagellus* . In the center of the picture the spinneret can be seen with a silken thread still attached to it. Anterior from this structure the maxillary palps and galea, used for olfaction and taste are visible, conveniently situated in front of the mouth. The two mandibles covered by the labrum are placed further anterior.The (partly retracted) antennae, bearing olfactory sensilla are located on each side of the mandibles, and further lateral, 6 simple eyes can be seen. In the neck region, just anterior of the first thoracal legs, the exit of the prothoracic organ is visible. The whole headcapsule is covered with tactile hairs.

1a. Inleiding en samenvatting

Chemische verbindingen spelen een belangrijke rol in het gedrag van insekten. Bekende voorbeelden zijn de sex-lokstoffen van motten en vlinders en de aggregatiesignalen van bastkevers. Sociale insekten, zoals bijen, wespen, mieren en termieten, produceren chemische stoffen die een rol spelen in de organisatie van de kolonie. Veel soorten gebruiken ook spoorstoffen (spoorferomonen) voor orientatie en voor het markeren van routes in hun omgeving. Het gebruik van spoorstoffen is echter niet beperkt tot sociale insekten, maar wordt ook gevonden bij verschillende rupsesoorten.

J.H. Fabre beschreef al in 1922 nauwkeurig het volggedrag van de processierups. Hij verklaarde zijn waarnemingen door te wijzen op de aanwezigheid van spinseldraden die deze (en vele andere rupsesoorten) produceren, en die door soortgenoten worden gevolgd. Hoewel dit argument tegenwoordig nog steeds geldt (Hoofdstuk 2), is het duidelijk geworden dat spoorferomonen ook een rol kunnen spelen. De best gedocumenteerde voorbeelden worden gevonden bij de Lasiocampidae. In het geslacht *Malacosoma*, de ringelrupsen, zijn spoorferomonen aangetoond die informatie overbrengen over de kwaliteit van het voedsel. Sporen die naar een hoogwaardige voedselbron leiden worden geprefereerd boven sporen naar andere locaties. Ondanks dit goed bestudeerde voorbeeld is de kennis over chemische communicatie bij rupsen nog zeer beperkt.

Om meer inzicht te verwerven in het markeren en volgen van sporen door rupsen wordt dit gedrag in dit proefschrift bestudeerd bij stippelmotten, een groep waaraan veel werk gedaan is in het kader van een multi-disciplinair onderzoeksprogramma naar soortvormingspocessen. De waardplantselectie van de vrouwlijke stippelmot wordt verondersteld een belangrijke rol te spelen bij de soortvorming. De voedselpreferentie van de rupsen hangt samen met voorkeur van het vrouwtje. Dit maakt het interessant te bekijken of het soortvormingsproces zich weerspiegelt in een differentiatie tussen de sporen van de verschillende rupsesoorten naar hun voedsel.

Er zijn echter nog andere redenen om het spoorvolgen van rupsen te onderzoeken. Verschillende eigenschappen van deze insekten maken dat zij zich goed lenen voor onderzoek over de regulatie van de voedselopname. Het gedrag van een rups is relatief eenvoudig, en de primaire functie van het dier (zoveel mogelijk eten) wordt niet gecompliceerd door andere activiteiten zoals het zoeken naar een partner, of de verzorging van nakomelingen.

Daarnaast heeft een rups een relatief beperkt zintuigsysteem. Slechts ongeveer 90 zintuigcellen zijn beschikbaar voor het doorgeven van chemische prikkels uit de omgeving naar het centrale zenuwstelsel. Ondanks deze beperkte mogelijkheden kunnen rupsen zeer sterke voedselvoorkeuren vertonen. Veel soorten zullen liever van honger omkomen dan te eten van een andere plant dan hun eigen voedselplant. Deze combinatie van factoren maakt rupsen zeer geschikt voor onderzoek naar het verband tussen zintuiglijke prikkels en gedrag. Daarnaast biedt ook de mogelijke integratie van verschillende prikkels een interessant perspectief. Receptoren voor sexferomonen zijn in het algemeen gescheiden van die voor het waarnemen van prikkels die samenhangen met voedsel. Spoorferomonen daarentegen, bezitten in dit geval een nauwe band met voedsel. Dientengevolge kan verwacht worden dat ook de zintuiglijke verwerking niet langs strikt gescheiden kanalen zal lopen.

De doelstellingen van dit onderzoek kunnen als volgt worden samengevat: Ten eerste, worden er spoorferomonen gebruikt door de stippelmotlarven, en zo ja, bestaan er dan verschillen tussen de verschillende soorten? Vervolgens moet worden vastgesteld welke receptoren gebruikt worden voor de waarneming van deze prikkel. Het is dan interessant te bepalen of deze informatie al dan niet geintegreerd verwerkt wordt met prikkels van het voedsel.

Als eerste stap in een analyse van dit systeem moet echter basis-kennis verzameld worden over het spoorvolggedrag zelf, de chemische stoffen die daar een rol bij spelen, de zintuigen die gebruikt worden en de oecologische betekenis ervan voor het dier. Deze vragen vormen de belangrijkste onderwerpen in dit proefschrift.

De meeste experimenten zijn uitgevoerd met *Y. cagnagellus* (Fig. 1). Deze soort komt algemeen voor in Nederland en is zeer geschikt voor gedragsstudies. Bovendien blijven de rupsen gedurende hun gehele ontwikkeling bij elkaar, waardoor ze voor de hand liggende kandidaten zijn voor onderzoek naar spoorferomonen. In sommige delen van het onderzoek werden ook *Malacosoma* rupsen gebruikt, waardoor een vergelijking mogelijk werd met een soort met goed beschreven spoorvolggedrag en een geïdentificeerd spoorferomoon.

Spoorvolggedrag van *Y. cagnagellus*

In hoofdstuk 2 werd onderzocht of *Y. cagnagellus* inderdaad spoorvolggedrag vertoont. Uit de resultaten van tweekeuzetoetsen bleek duidelijk dat dit het geval is. Bovendien werd duidelijk dat de aanwezigheid van spinseldraden een rol kan spelen.

Y. cagnagellus maakt geen onderscheidt tussen zijn eigen sporen en die van een vijftal andere stippelmotsoorten, maar wel ten opzichte van sporen van ringelrupsen. Dit gebrek aan soortspecificiteit komt ook voor bij andere insektensoorten m.b.t. spoorferomonen, en berust waarschijnlijk op de, in vergelijking met sexferomonen, indirekte koppeling tussen het signaal en het voortplantingssucces.

De spoorstof

Hoewel het bestaan van spoorvolggedrag werd aangetoond in hoodstuk 2, is daarmee nog niet bewezen dat hierbij een spoorferomoon betrokken is. Het bewijs dat een chemische verbinding in het volgedrag een rol speelt wordt in hoofdstuk 4 gegeven. Deze signaalstof is in water oplosbaar, en blijft (in het laboratorium) langdurig werkzaam. Gedragsexperimenten met extracten van klieren en delen van het lichaam laten zien dat de stof uitsluitend aanwezig is in de spinselklieren. Van daar uit wordt het waarschijnlijk met het spinsel mee uitgescheiden.

Het gebruikte zintuig

In hoofdstuk 3 wordt vastgesteld welk zintuig gebruikt wordt voor de waarneming van het spoor. In dit hoofdstuk wordt een vergelijking gemaakt met de ringelrups *Malacosoma americanum*, een bekende spoorvolger. Bij rupsen zijn zintuigen aanwezig op de antennen, de tasters (palpen) van de onderkaak, en aan de binnenzijde van de bovenlip. Door systematisch verwijderen van de verschillende belangrijke zintuigen werd aangetoond dat zowel bij *Malacosoma* als bij *Yponomeuta* de palpen noodzakelijk zijn voor de waarneming van het spoor. Aangezien zowel de plaats van herkomst als de aard van de spoorstoffen in hoge mate verschilt bij deze twee soorten, behorend tot verschillende families, is deze overeenkomst waarschijnlijk het gevolg van convergentie van de receptorfunctie.

De palpen bevatten receptoren voor zowel smaak als reuk, maar de spoorstoffen worden kennelijk waargenomen met de smaakzin.

Dit volgt uit de observatie dat met fijnmazig nylon gaas afgedekte sporen niet meer kunnen worden gevolgd. Deze conclusie is in overeenstemming met het feit dat de spoorstoffen een grote stabiliteit en lange levensduur bezitten, eigenschappen die op een geringe vluchtigheid wijzen.

Electrofysiologie van de palp

De palp bevat een aanzienlijk deel van de gehele zintuigelijke uitrusting van de rups (30-40 chemoreceptoren, meer dan 1/3 deel van het totaal). Desondanks is slechts heel weinig over deze zintuigen bekend. Daarom werd een electrofysiologische inventarisatie van de aanwezige receptoren uitgevoerd (Hoofdstuk 5). De zintuigharen zijn te klein voor het toepassen van de normale afleidtechniek via de tip van de sensillen. Smaakprikkels werden daarom aangeboden aan alle zintuigen op de palptop met een relatief wijde stimuluspipet, terwijl de activiteit van slechts enkele zintuigcellen selectief werd opgepikt met behulp van een capillaire micro-electrode en vastgelegd op een magnetische band voor latere analyse.

Om dit analyseproces te vereenvoudigen werd een computer programma geschreven (Hoofdstuk 7). De gekozen methode is gebaseerd op een voortdurende interactie tussen het programma en de gebruiker, waarbij het programma gebruikt wordt als instrument voor de manipulatie en weergave van de gegevens.

Als stimuli voor de reukzintuigen werden plantengeuren gebruikt, terpenen en C6 verbindingen uit de vetzuurstofwisseling, de zgn 'groene geuren'. Deze prikkelstoffen werden gedeeltelijk gekozen op grond van de resultaten van een chemische analyse van het geurcomplex rondom de waardplant van *Y. cagnagellus*, de kardinaalsmuts (*Euonymus europaeus*). De smaakzintuigen werden gestimuleerd met zoutoplossingen en met extracten van spinsels.

Er zijn aanwijzingen gevonden voor het bestaan van twee groepen van geur-receptoren. De ene groep is het meest gevoelig voor (E)-2-hexenal en hexanal (aldehydes) en de andere voor (Z)-3-hexen-1-ol en 1-hexanol (alcoholen). Ook werden receptoren gevonden die reageren op een extract van (spoor)spinsel, en dus waarschijnlijk op de spoorstof. Deze cellen hebben niet die hoge mate van specialisatie die vaak aangetroffen wordt bij receptoren voor sexferomonen van vlinders, maar lijken meer op de minder gespecialiseerde receptoren voor stimuli uit voedsel.

De resultaten uit de voorgenoemde hoofdstukken vormen tezamen een sterke indicatie voor het bestaan van een spoorstof bij *Y. cagnagellus*, die wordt uitgescheiden met het spinsel en waargenomen door contact-chemoreceptoren in de palpen.

Oecologische en evolutionaire aspecten

De vraag hoe de spoorstof gebruikt wordt, en wat de oecologische en evolutionaire betekenis van zo'n signaal kan zijn, wordt gesteld in hoofdstuk 6. Bij sociale insekten en ook bij de ringelrupsen worden spoorstoffen gebruikt om soortgenoten naar een goede voedselbron te leiden (de zgn. recruitering). Dit blijkt niet het geval te zijn bij de stippelmotrupsen. Uit veldobservaties is gebleken dat groepen rupsen hun gezamenlijk spinselnest tijdens de larvale levensduur enige malen verplaatsen, soms over vrij

aanzienlijke afstanden. De spoorstof kan een rol spelen in het bijeen houden van de groep gedurende deze verhuizingen. Dit roept de vraag op waarom het voordelig zou zijn voor de rupsen een groep te blijven vormen. Verscheidene auteurs hebben hiervoor goede redenen aangevoerd. Er moet echter opgemerkt worden dat er ook duidelijke nadelen verbonden zijn aan groepsvorming, bijvoorbeeld doordat er voedselconcurrentie kan ontstaan. In hoofdstuk 6 wordt een simpel evolutionair model gebruikt als hulp bij een beschouwing over de invloed van deze positieve en negatieve effecten op de evolutionaire stabiliteit van groepsgedrag. Een van de resultaten hiervan is dat het waarschijnlijk zinvol is om groepsgedrag te klassificeren aan de hand van het moment waarop een overgang plaatsvindt van een strategie die het bijeenblijven van de groep bevordert naar een strategie die resulteert in solitair voedselzoeken.

1b. Introduction and summary

The importance of chemical cues in insect behaviour is well established (Bell & Cardé, 1984). The best known examples include the sex pheromones of butterflies and moths, and the aggregation pheromones of bark beetles. In eusocial insects (bees, wasps, ants, and termites) pheromones are widely used to maintain the organization of the colony. Many of these species produce chemical markers (trail pheromones) and deposit them on terrestrial trails that lead to food sources or nesting sites. Trail pheromones may also serve as cues in home range orientation and can facilitate migration of colonies (Attygalle & Morgan, 1985). However, trail following is not confined to eusocial species and is, for instance, also found in the Lepidoptera. Fabre (1922) already described the striking following behaviour of the procession caterpillar *Thaumetopoea pityocampa* (Denis & Schiffermüller). To explain his observations, he stressed the importance of tactile stimuli from the silken treads that these (and other) caterpillars produce, and that can be followed. Although this argument still holds today (Chapter 2), it has become clear that, in addition to silk, chemical trail markers may also be important in the social behaviour of caterpillars (Fitzgerald & Peterson, 1988). The best documented examples are found in the Lasiocampidae. In *Eriogaster lanestris* trail marking was demonstrated by Weyh & Maschwitz (1978), and in the genus *Malacosoma* chemical trails convey information about the quality of a feeding site, and recruit other larvae to these places (Fitzgerald & Peterson, 1983; Peterson, 1988). In spite of these thoroughly studied examples, knowledge about chemical communication in caterpillars is limited, and mainly restricted to the Lasiocampidae. To gain more insight in trail following and trail marking in the Lepidoptera it is necessary to study this behaviour in other families.

This thesis focuses on caterpillars of small ermine moths, members of the genus *Yponomeuta*. This group has been studied in the context of a long term multi-disciplinary research program on speciation, and host plant selection is thought to be an important element in the speciation process (Wiebes, 1976). Food

preferences of larvae are related to host preferences of female moths. This makes it interesting to see whether speciation is accompanied by interspecific differences in larval trails to feeding sites.

There are additional reasons to investigate trail marking and following in the Lepidoptera. Caterpillars have been advocated as model systems in the study of feeding behaviour (Schultz, 1983; Schoonhoven 1987), in part because their behaviour is relatively simple. A caterpillars primary function, gathering as much food as possible, is not complicated by tasks such as mate finding or taking care of offspring. In addition the sensory system is limited. Only about 90 chemosensory cells function in translating chemical messages from the environment into signals for the central nervous system (Albert, 1980; Devitt & Smith, 1982; Schoonhoven,1987). In spite of this restricted number of input channels, caterpillars can display striking food preferences, and will often die from starvation, rather than accept a non-host plant. Such behaviour, together with the possibility of tracing sensory connections into the central nervous system (Kent & Hildebrand, 1987) make caterpillars a good choice for studying the relationship between neurophysiology and behaviour.

A further point of interest is the possible integration of sensory information. The receptors for sex pheromones are in general separated from those that perceive stimuli associated with food. Trail pheromones of caterpillars bear a close relation to food finding. Therefore these insects may have an integrated receptor system that responds to both food and trail pheromone stimuli.

The objectives of this study were (1) to determine whether trail pheromones are employed by *Yponomeuta* and, if so, whether they differ in different species, (2) To identify receptors responsible for pheromone detection, and (3) to determine whether these receptors operate in an integrated way with receptors for food perception. However, as a first step in the analysis of this system basic knowledge must be gained about trail following behaviour itself, the chemicals involved, the senses used and the oecological context in which it functions. These questions form the main topics of this thesis.

Most experiments were performed on larvae of *Y. cagnagellus* (Hübner) (Fig. 1). This species is common in the Netherlands and suitable for behavioural studies. In addition, the caterpillars are gregarious throughout their development, suggesting that they may use a trail marker. *Malacosoma* caterpillars were used in some experiments to allow comparison to a species with well defined trail following behaviour, and an identified trail pheromone.

Outline of the thesis

Trail following in *Y. cagnagellus*

The study begins by asking whether *Y. cagnagellus* in fact exhibits trail following behaviour (chapter 2). Two-choice tests on filter paper Y-mazes show clearly that this is the case. In addition it is demonstrated that a tactile component of the trail (the silk) can be used as a cue. *Y. cagnagellus* does not discriminate between its own trails and those of 5 other *Yponomeuta* species, but does prefer its own trails over those of *M. neustria*. This lack of species specificity within the genus is, in contrast to sex pheromones, not uncommon for trail pheromones, possibly because the relationship between mating success and the signal is indirect.

A chemical marker

The existence of trail following behaviour does not by itself prove that a chemical marker is involved. Evidence for the presence of a chemical signal is presented in chapter 4. The marker appears to be water soluble, and highly stable under laboratory conditions. Behavioural responses to extracts from several glands and body parts show that the marker is present in the labial glands (the silk gland) only. Therefore, the marker is probably secreted with the silk.

The receptors involved in trail following behaviour

Chapter 3 describes the sensory organ used for the perception of the trail. In this chapter a comparison is made with the American tent caterpillar *Malacosoma americanum*, a known trail follower (Fitzgerald, 1976). Chemoreceptors in caterpillars are located on the antennae, the maxillary palps, the galea and on the inner side of the labrum (Fig. 1). Systematic removal of various relevant structures shows that the maxillary palps are necessary for the detection of the trail in both *M. americanum* and *Y. cagnagellus*. Since the source of the trail marker, as well as its chemical composition, differs between the two species, this is most likely an example of convergence of chemoreceptor function.

Although the maxillary palps contain olfactory as well as gustatory receptors, the trail markers seem to be perceived only by contact chemoreception. This follows from the observation that trails covered with fine nylon mesh do not elicit any response. Moreover the long lifetime and stability of the markers, suggest that they have a low volatility.

Electrophysiology of the maxillary palp

Although the palps house a considerable fraction of the sensory equipment of a caterpillar (30-40 cells, more than 1/3 of the total), only very little is known about these organs. Therefore an electrophysiological survey of the chemoreceptors was conducted (Chapter 5). Because the sensilla are too small for tip recording, gustatory stimuli were applied to the whole tip of the palp. Electrical activity of only a few cells at a time was recorded with a glass microcapillary electrode. To aid analysis, a computer program was developed (Chapter 7). Following the ideas of van Drongelen *et al.* (1980), the program was designed to be highly interactive and to function as both as a display- and manipulation tool.

Plant volatiles were used as olfactory stimuli (terpenoids and C6 fatty acid derivatives or 'green odours', Visser & Avé, 1978). These were chosen in part on the basis of the results from a

dynamic headspace analysis (Cole, 1980) of *Euonymus europaeus*, the host of *Y. cagnagellus*. Silk extracts and salt solutions were employed as gustatory stimuli. Evidence was found for the existence of two groups of olfactory receptor cells, sensitive to (E)-2-hexenal and hexanal (aldehydes) or to (Z)-3-hexen-1-ol and 1-hexanol (alcohols). Receptors responsive to the silk extracts (and probably to the trail pheromone) were also identified. These cells do not show the degree of specificity typical of cells specialized for lepidopteran sex pheromones but, rather, resemble the generally more broadly tuned receptors for food components.

The results from this and the preceding chapters strongly suggest the existence of a chemical trail marker in *Y. cagnagellus*, secreted with the silk and detected by contact-chemosensory neurons housed in the maxillary palps.

Oecological and evolutionary aspects

Chapter 6 addresses the the oecological and evolutionary relevance of such a signal. In eusocial insects as well as in *Malacosoma*, trail pheromones are often used to recruit siblings to high quality feeding sites (Peterson, 1988). In *Y. cagnagellus* this does not happen, but field observations have shown that a groups of caterpillars moves its silken nest over considerable distance, on average four times during development. The trail marker could help to maintain gregariousness during these migrations. Thus, it is of interest to ask whether gregariousness is advantageous. While many authors have discussed the benefits of larval aggregation (e.g. Tsubaki, 1981; Fitzgerald & Peterson, 1988; Weaver, 1988), gregariousness may also be associated with distinct disadvantages, for instance those arising from competition for food (Charnov et al., 1976). In chapter 6 a simple evolutionary model is presented to analyze the influence of these conflicting parameters on the evolutionary stability of gregarious behaviour. One result from this study is that it would be informative to classify larval behaviour in terms of the time of which larvae switch from gregariousness to solitary food searching.

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2. The trail following behaviour of *Yponomeuta cagnagellus*.

Peter Roessingh

Abstract

In this study the trail following behaviour of the caterpillar *Yponomeuta cagnagellus* (Hübner) (Lepidoptera, Yponomeutidae) is investigated. It is demonstrated that these caterpillars follow trails made by conspecifics. Ablation experiments show that at least part of this behaviour is directed by the tactile senses, but additional chemical cues cannot be excluded. In choice experiments, using trails from different species, *Y. cagnagellus* strongly preferred conspecific trails over those from *Malacosoma neustria* , but did not prefer conspecific over other *Yponomeuta* trails. This lack of species-specificity within *Yponomeuta* is discussed and it is concluded that trail following is probably of little help in the elucidation of the evolutionary history of the genus.

Introduction

Trail following is well known from many eusocial insects (e.g. ants, termites and bees). It is often based on chemical markers conveying information about potential food sources, mates or resting sites (Butler, 1970). In ants this system is often highly refined, and facilitates finely-tuned interactions between colony members (Bradshaw & Howse, 1984). However, trail following is, not confined to eusocial species and has been documented for a number of other taxa (Fitzgerald, 1976; Cook, 1979, Maschwitz & Gutmann,1979; Tietjen & Rovner 1982; Chelazzi *et al.*, 1985). Lepidopterous larvae provide some of the best known examples, and Fitzgerald & Peterson (1983) even suggest that the diversity of trail systems in the Lepidoptera might parallel that found in the Formicidae. To evaluate such a claim, we must substantially

improve our presently poor understanding of trail following behaviour in this group (Silberglied, 1977; Fitzgerald & Peterson, 1983).

In this study, trail following in *Yponomeuta cagnagellus* (Hübner) (Lepidoptera, Yponomeutidae) is investigated. Caterpillars of this species live gregariously in silken webs on the spindle tree *Euonymus europaeus*, and remain aggregated through pupation (Hoebeke, 1987). Feeding takes place at the periphery of the web, which is gradually extended to encompass new leaves. If the local food supply is exhausted, the group moves to a new feeding site. During locomotion silken threads are produced. This results in trails composed of silk and possibly chemical markers. In this respect, it is important to separate the concept of "trail following", a behaviour which might be based on several cues, from the existence of a "trail pheromone", one of the possible cues that elicits trail following. Here the trail following behaviour of *Y. cagnagellus* is investigated with special attention to the importance of tactile cues, a trail component that has received little attention to date.

Y. cagnagellus is one of nine European *Yponomeuta* species which show various degrees of taxonomic relationship (Povel 1984), and which have been used to study the stages in a speciation process (Herrebout *et al.*, 1976; Wiebes 1976). Therefore, the response of *Y. cagnagellus* towards trails from other *Yponomeuta* species is also evaluated, to determine whether speciation in this group has been accompanied by differentiation of trail following behaviour. It should also provide more insight in the specificity of lepidopterous trail pheromones in general.

Materials and Methods

Insects

Yponomeuta evonymellus (L.), *Y. cagnagellus* (Hübner), *Y. malinellus* Zeller, *Y. padellus* (L.), *Y. rorellus* (Hübner) and *Y. vigintipunctatus* (Retz.), were collected in the field from their host plants. Species in the *padellus*-complex, (see Povel, 1984 for

discussion), were identified on the basis of the hosts from which they were collected. The larvae were reared in the laboratory in petri-dishes on host foliage at 25 °C under a 18:6 h L:D cycle. *Yponomeuta* caterpillars migrate during the day as well as during the night, but all experiments were conducted during the photophase. To obtain silk from another species, *Malacosoma neustria* (Hübner) egg batches were collected from oak and larvae were reared on leaves of apple seedlings. *Yponomeuta* caterpillars were used during the first 4 days of their fifth stadium. *Malacosoma* caterpillars were used during their third stadium. At these stages the larvae were comparable in size.

Bioassay

The trail following behaviour was measured using a Y-maze test modified from Fitzgerald and Edgerly (1979). Trails were produced by placing caterpillars on 80 cm long, 4 mm wide filter paper strips, and allowing them to crawl to the other end. Twenty-five caterpillars were permitted to move along the paper to produce the test strip. The arms of the Y-maze were formed by 4 cm. long sections cut from either the test strip or a clean control. The maze was laid out on a piece of perspex. Its smooth surface effectively prevented the larvae from moving off the strips.

Individual caterpillars were tested by placing them at the start of a marked strip that formed the stem of the Y. In a typical experiment the larva would follow the strip toward the branch point and, after palpating both arms, choose one. A larvae was considered to have made a choice when it moved completely on to one arm. Caterpillars that left the maze or refused to choose were re-tested up to 4 times. If they still failed to choose they were excluded from the experiment. Caterpillars that did not move within 3 min were also excluded (in a sample of 400 larvae, only 4 were not testable). To prevent contamination from deposited silk or pheromones during the experiment, the Y-maze arms were changed after each choice. The stem was re-used. The experimental arena received diffused light and was shielded from the environment with white paper to prevent orientation to visual

stimuli. The positions of the experimental and control arms were reversed after each test.

The specificity of trail following in *Y. cagnagellus* was determined by comparison of the response to conspecific trails with response to trails of other *Yponomeuta* species. The same caterpillars were also presented with a choice between *Y. cagnagellus* and *M. neustria* trails in order to determine whether the caterpillars were capable of discriminating between trails.

Ablations

Ablations were conducted using a 50 MHz. radio microcautery device (Murphy Developments, Hilversum, The Netherlands) modified from Unwin (1978). This device allowed removal of sensory organs, in most cases without bleeding. Insects that did not stop bleeding within 60 seconds were rejected. During the operation the larvae were strapped to a glass surface with small strips of masking tape.

The chemosensory organs of caterpillars are present on the antennae, the maxillary palpi, the galeae and on the inside of the labrum (Schoonhoven, 1987; see also Fig. 1). Except for the epipharyngeal organs which do not touch the surface, all structures were removed. In a series of experiments each structure was first removed individually. Combinations of the operations, (removing several sensory structures at the same time) were also performed. Control larvae were handled in the same way (i.e. strapped) as the experimental group, with the exception of the ablations.

Statistical analysis

The Y-maze experiments were analyzed using a G-test (Sokal & Rohlf 1981), to determine if the choice ratio between the two arms differed significantly from the 1:1 ratio expected under the null hypothesis.

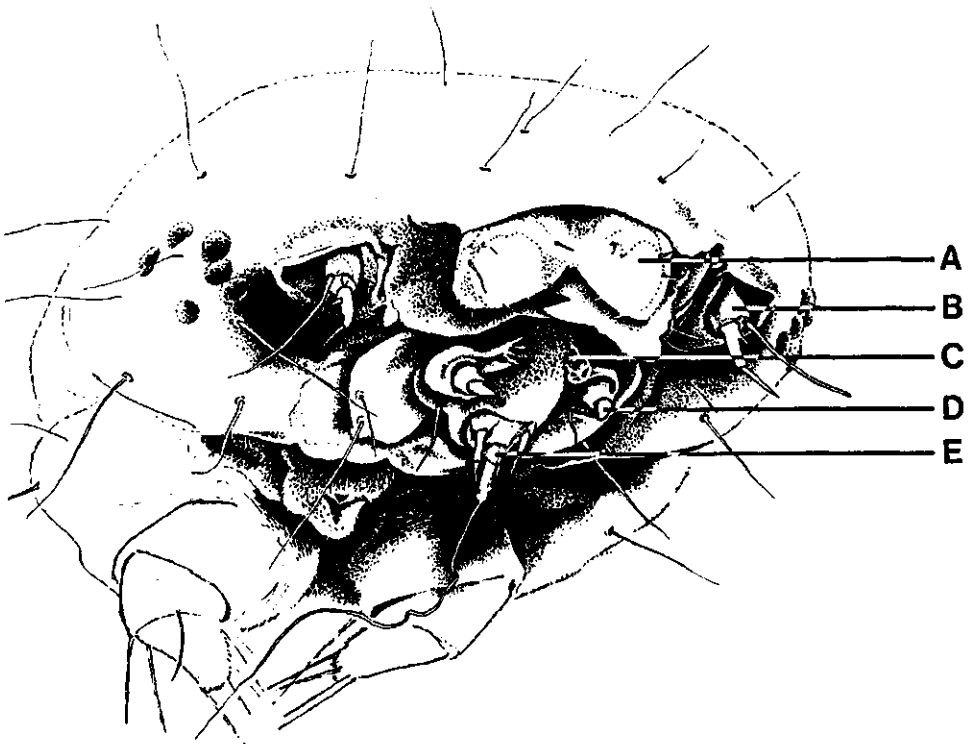

Figure 1 Ventral view of the head of the fifth stadium larvae of *Yponomeuta cagnagellus* **A:** Labrum, **B:** Antenna, **C:** Galea, **D:** Maxillary palp, **E:** Labium (Spinneret). Only the most prominent of the numerous tactile hairs on the head have been drawn.

Results

Trail following

Trail following behaviour in *Y. cagnagellus* and its relation to the strength of the trail was demonstrated in a series of experiments in which trails produced by different numbers of caterpillars were used. In Y-maze tests, these trails were compared to clean control arms. As shown in Fig. 2, a significant preference emerges for trails made by five or more larvae.

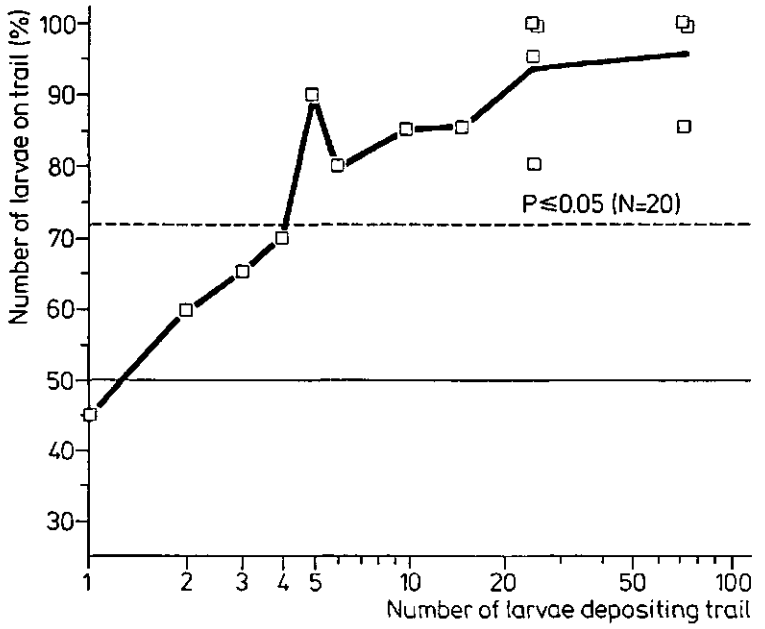

Figure 2. Choice behaviour of fifth stadium *Yponomeuta cagnagellus* larvae in Y-maze tests on trails made by different numbers of *Y. cagnagellus* larvae. Control arms free of trails, N=20 for all data points.

To investigate the influence of tactile cues from the silk, and to discriminate between these effects and chemical signals, selective ablation experiments would have to be performed,

removing either all chemosensory or all tactile inputs. In addition to chemoreceptors (see Schoonhoven, 1987 for review), caterpillars are richly equipped with tactile receptors. These are partly associated with the chemosensory organs (Schoonhoven & Dethier, 1966; Hanson, 1970), and partly located in setae distributed over the body (Tautz, 1977, 1978; Levine *et al.*, 1985). Since it is the head of the caterpillar that is most actively involved in scanning the trail, the hairs on this part of the body (Fig. 1) could be especially important. Fig. 3 gives an example of an input-response curve from one of these hairs showing that it is indeed innervated. The hairs contain a single cell that is sensitive to deflections as small as three degrees from the resting position.

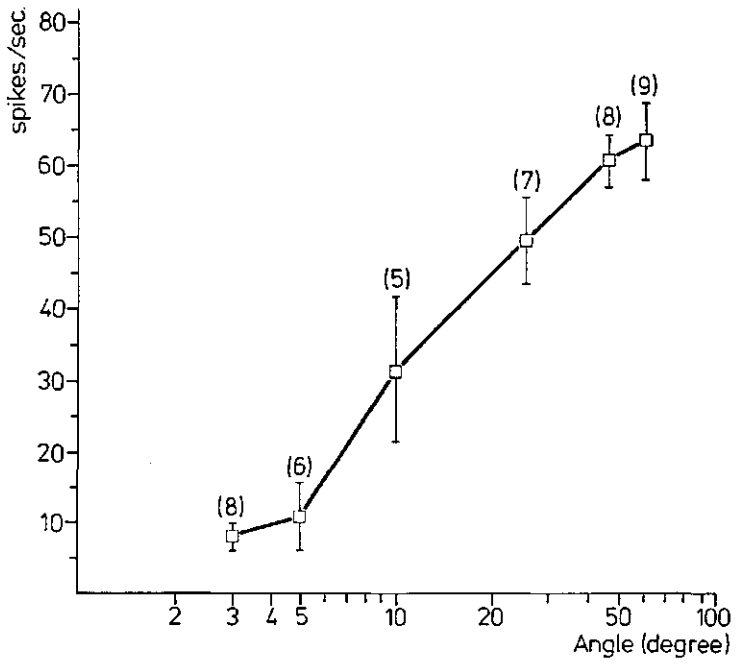

Figure 3 Stimulus-response curve for the large hair ventro-medial of the maxillary palp of *Y. cagnagellus.* in the fifth stadium. One spike was observed in response to displacement of the hair with a tungsten hook. The number of replications is given between brackets. Vertical bars indicate 95 % confidence interval.

Since the tactile receptors are so widely distributed over the body, ablating the chemosensory organs is more feasible. Table 1 summarizes the results of this experiment. Even when all sensory organs were ablated, effectively eliminating external chemoreceptors, caterpillars still significantly preferred trails over clean controls.

Table 1. Effect of sensory ablations on trail following behaviour in Y-maze tests of fifth stadium *Yponomeuta cagnagellus* larvae .

Treatment	N	*Y.cagnagellus* larvae on trail	control	G
Control	80	75	5	70.84 **
Antennae removed	20	17	3	9.18 **
Palpi removed	20	16	4	6.40 **
Galea removed	20	17	3	9.18 **
Antennae & palpi removed	40	29	11	7.46 **
Galeae & palpi removed	40	31	9	11.60 **
Antennae, galeae & palpi removed	45	35	10	13.49 **

Trails were made by 25 *Y.cagnagellus* larvae, and tested against clean controls. G-test for goodness of fit to 1:1 ratio.
ns = $p > 0.05$ ** = $p \leq 0.01$

In another series of experiments *Y. cagnagellus* larvae were tested on substrates with varying similarity to *Y. cagnagellus* trails. This is another way to exclude the possibility that trail recognition occurs solely via chemicals.

From Table 2 it can be seen that silk from *Y. evonymellus*, from a species of *Malacosoma*, from a cocoon of *Bombyx mori*, and fibers pulled from cottonwool were all preferred over clean controls.

Table 2. Trail following behaviour of fifth stadium larvae of *Yponomeuta cagnagellus* in Y-maze tests on trails from different sources.

		Y.cagnagellus larvae on		
source of trail	N	trail	control	G
Yponomeuta cagnagellus	20	19	1	17.07 **
Yponomeuta evonymellus	20	19	1	17.07 **
Malacosoma castrensis	40	33	7	16.85 **
Bombyx mori cocoon	20	17	3	9.18 **
Cotton wool fibers	20	15	5	4.20 *

Trails were made by 25 larvae, and tested against clean controls. G-test for goodness of fit to 1 :1 ratio.
ns = $p > 0.05$ * = $p \leq 0.05$ ** = $p \leq 0.01$

Trail specificity

To test whether *Y. cagnagellus* larvae prefer their own trails over that of other species, trail specificity experiments were conducted. *Y. cagnagellus* did not discriminate among trails made by conspecifics and trails made by other *Yponomeuta* species (Table 3). However, *Y. cagnagellus larvae* prefer a congeneric trail over a *Malacosoma* trail.

Table 3. Trail following behaviour of fifth stadium larvae of *Yponomeuta cagnagellus* in Y-maze tests providing a choice between conspecific and heterospecific trails.

a. test series

exp. number	species producing test trail	N	larvae on trail of		G
			Y.cagnagellus	other species	
1	*Y.evonymellus*	20	8	12	0.45 ns
2	*Y.padellus*	40	14	26	3.06 ns
3	*Y.malinellus*	40	20	20	0.02 ns
4	*Y.rorellus*	40	15	25	2.04 ns
5	*Y.vigintipunctatus*	40	20	20	0.02 ns

b. control series

exp. number	species producing test trail	N	larvae on trail of		G
			Y.cagnagellus	*M.neustria*	
1	*M.neustria*	20	19	1	17.07 **
2	*M.neustria*	40	35	5	23.42 **
3	*M.neustria*	40	34	6	19.95 **
4	*M.neustria*	40	33	7	16.85 **
5	*M.neustria*	40	36	4	27.32 **

The same groups of caterpillars were tested in control series on *Malacosoma neustria* trails to verify that they were capable of discriminating between trails. G-test for goodness of fit to 1:1 ratio.
ns = $p > 0.05$ * = $p \leq 0.05$ ** = $p \leq 0.01$

Discussion

Detailed information about trail following behaviour in Lepidoptera is limited. The most thoroughly studied examples are in the genus *Malacosoma*. Evidence has been reported for the presence of one or more chemical factors eliciting trail following (Fitzgerald, 1976; Fitzgerald & Gallagher, 1976; Fitzgerald & Edgerly 1979; Fitzgerald & Costa, 1986; Crump *et al.*, 1987), as

well as recruitment to feeding sites (Fitzgerald & Peterson, 1983; Peterson 1986, 1987, 1988). In addition to these chemical factors, an influence of tactile input from the silk was found (Fitzgerald & Edgerly, 1982). This possibly dual nature of caterpillar trails has not received much attention. Some authors do not differentiate between tactile cues from the silk and chemical signals (Long, 1955; McManus and Smith, 1972). Others fail to make an unambiguous distinction due to the uncontrolled effect of solvents on the silk structure (Gallagher & Lanier, 1977; Weyh & Maschwitz, 1982), or focus only on the chemical cues (Weyh & Maschwitz, 1978; Capinera, 1980). Kalkowski (1958) concluded that although tactile input played a role in trail following in *Y. evonymellus*, a volatile chemical factor was more important. This result was not easily reproducible for *Y. cagnagellus* (Roessingh *et al.*, 1988, Chapter 3).

In the present paper it is shown that trail following does occur in *Y. cagnagellus* and that at least part of this behaviour may be explained by responses to tactile cues provided by the silk strands (Fig. 1, Table 1 & 2). From this it can be concluded that when studying trail following behaviour, care must be taken to include controls for the tactile component of the trail.

The fact that tactile cues are important does not exclude the existence of chemical cues. Results from the specificity experiments in which *Yponomeuta* caterpillars differentiated between their own trails and trails made by *M. neustria* suggest that such chemical factors exist. This possibility is examined in more detail elsewhere (Chapter 4).

From Fig. 2 it can be deduced that trails from single caterpillars do not evoke trail following. This is in contrast to *Malacosoma*, where a single caterpillar is able to recruit its tentmates (Fitzgerald, 1976). The significance of this fact is difficult to assess. It might indicate that the trail system in *Yponomeuta*, in contrast to that of *Malacosoma*, does not function in recruitment behaviour but, rather, facilitates aggregation during exploration. This function for trail following has been mentioned by several authors (Long,1955; Capinera, 1980; Stamp, 1984), and might not require the stronger effects typical of mass recruitment systems. Interestingly, the gregarious larvae of the bug *Elasmucha*

grisea (L.) produce a trail that functions in this manner and elicits significant following only after the passage of about 5 insects (Maschwitz & Gutmann 1979).

In trail pheromone systems, lack of species specificity seems to be common. Attygalle & Morgan (1985) and Morgan (1984) conclude that in ants trail pheromones are frequently not species specific. In termites interspecific interactions also occur (Prestwich, 1983). In Lepidoptera it has been shown that different species in the genus *Malacosoma* readily follow each other's trail (Fitzgerald & Edgerly, 1979; Crump *et al.*, 1987; Peterson, 1988). A notable exception to this pattern is the larva of the scarce swallowtail, *Iphiclides podalirius* L., in which trail recognition at the level of the individual has been described (Weyh & Maschwitz, 1982).

The general lack of species specificity in trail pheromone systems provides a contrast to the usually striking specificity in sexual communication systems. Most of the mechanisms proposed to explain distinct chemical communication channels are relaying on a direct influence of the signal on reproductive success. (Hölldobler, 1984; Cardé, 1986; Hölldobler & Carlin, 1987).
Trail pheromones do not have this direct link to reproduction and consequently may change at a low rate, resulting in a limited degree of species specificity. West-Eberhard (1983; 1984), suggested that intra-specific communication may be either co-operative or competitive. Co-operative signals have the function of coordinating the activities of different individuals in the performance of a task that has survival value for the interactants. Competitive signals, on the other hand, lead to differential success in obtaining some resource. It is thought that the stronger selection resulting from social competition can lead to more rapid divergence of the communication signal, resulting in greater species specificity. According to this hypothesis, highly specific signals can also be expected in case of competition for food. This might explain the observed specificity of the trails made by *Iphiclides* larvae. These larvae are solitary and show marked aggressiveness towards each other. Trail marking in this species seems to function in demarcating a foraging territory in a habitat with limited resources.(Weyh & Maschwitz, 1982), These signals

have a competitive nature and would consequently be more specific.

In contrast, in cases where trail communication is non-competitive and serves only to coordinate the behaviour of colony members, a limited degree of species specificity is expected. This expectation is met for *Y. cagnagellus* (Table 3). To date, only interactions between *Y. cagnagellus* and other *Yponomeuta* spp. have been investigated. However, in the light of the discussion above, it is unlikely that trail following behaviour will be more specific in other species of this genus. Prestwich (1983) concludes that the lack of specificity of trail pheromones in termites argues against their use in systematics. Given the reported lack of specificity in the trail following behaviour within the genus *Malacosoma* (Fitzgerald & Edgerly, 1979; Crump *et al.*, 1987; Peterson, 1988) and the results for *Y. cagnagellus* presented here, this conclusion also seems justified for gregarious Lepidoptera. Therefore, the study of trail following in *Yponomeuta* is probably of little help in the elucidation of the evolutionary history of this genus.

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3. The sensory basis of trail following in some lepidopterous larvae: contact chemoreception.

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Physiological Entomology **13**, 219-224 (1988).

Abstract

Caterpillars of the genus *Malacosoma* follow trails of the chemical 5-beta-cholestane-3,24-dione, but nothing is known of how they perceive this compound, or more generally about the sensory basis of trail following in caterpillars. By selective ablations of chemosensory organs we show that, in *Malacosoma*, the trail chemical is perceived by the maxillary palpi. In another lepidopteran species, *Yponomeuta cagnagellus*, the palpi are needed to discriminate their own trails from a trail of *Malacosoma*.. *Malacosoma* larvae also lose their specificity for conspecific trails when their palpi are ablated. Volatile cues evidently do not play a role in trail following behaviour, since neither *Malacosoma* nor *Yponomeuta* can orient a trail covered with fine nylon mesh. These data indicate that for *Malacosoma*, and probably also for *Yponomeuta*, contact chemoreception mediated by the maxillary palpi is the primary mode of pheromone perception. The evolution or receptor sensitivity to trail chemicals in caterpillars is discussed.

Introduction

Trail following behaviour in lepidopterous larvae has been demonstrated in a number of cases (Fitzgerald, 1976; Capinera, 1980; Weyh & Maschwitz, 1978, 1982). In the best-documented example, *Malacosoma americanum*, this response depends on a chemical marker (trail pheromone) secreted from a site on the sternum of the last abdominal segment (Fitzgerald & Edgerly, 1982). Recently, the steroid 5-beta-cholestane-3,24-dione has

been identified as an active component of this marker (Crump *et al.*, 1987). This non-volatile chemical is most likely perceived by contact chemoreception, but nothing is known about the receptors involved. We have tested the hypothesis that contact chemoreception is important in trail following and used an ablation technique to locate the pheromone receptors.

For other caterpillars species in which trail following is thought to occur, no data concerning the chemical nature of the trail or the senses involved are available, although volatile markers have been implicated (Kalkowski, 1958; Wojtusiak, 1972; Weyh & Maschwitz, 1982). A problem in these cases is that no pure trail chemicals are available for testing. An alternative way to assess the importance of sensory structures in trail following is to examine the preference of larvae for trails. *Malacosoma* caterpillars of different species follow each other's trails, but are able to discriminate their own trails from those made by more distantly related species (Fitzgerald & Edgerly, 1979). Since this specificity must have a sensory basis, it is possible to determine the importance of the different chemoreceptors using larvae with selectively ablated sensory organs. Following this reasoning we investigated the preference of another trail following species, *Yponomeuta cagnagellus*, for its own trails in comparison with *Malacosoma neustria* trails and evaluated the sensory basis of this behaviour.

Materials and Methods

Insects

Egg batches of *Malacosoma americanum* (Fabritius), *Malacosoma neustria* (Hübner) and *Yponomeuta cagnagellus* (Hübner) were collected in the field and kept at 6°C and 80% relative humidity until needed (cf. Bucher, 1959). The larvae were reared at 25°C in petri-dishes on host foliage. In all experiments, third stadium larvae of *Malacosoma* and fifth stadium larvae of *Yponomeuta* were used. Caterpillars in these stadia are of comparable size. Because of the limited number of *M. americanum*

available, most work was done on *M. neustria*. This species readily follows the chemical isolated from *M. americanum* (Crump et al., 1987).

Ablation technique

The ablations of sensory structures were performed using a 50 Mhz HF radio microcautery device (Murphy Developments, Hilversum, The Netherlands) modified from Unwin (1978). Three types of operations were carried out; removal of the antennae, removal of the maxillary palpi and removal of the galeae. The three operations together remove all known chemosensory organs (Schoonhoven, 1986) except the epipharyngeal taste sensilla on the inner side of the labrum, but these do not contact the substrate in a walking caterpillar.

During the operations, *Malacosoma* larvae were immobilized by strapping them down on the top of an ice-filled petri-dish with small pieces of parafilm. The dish was placed in a larger container filled with ice and a layer of frozen CO_2 (dry ice) to keep the water ice cool. For *Yponomeuta*, no cooling was necessary and the strapping was done with small strips of masking tape. After the ablations, larvae were fed and given at least 20 h at 25°C to recover. Control larvae were handled in the same way but were not cauterized.

Bioassays

Two types of bioassays were used, one for testing responses to synthetic trails and one for testing specificity for natural trails.

Synthetic trails were produced using 5-beta-3,24-dione (henceforth referred to as dione) dissolved in methanol (Merck) to from a pheromone solution of 0.1 mg/ml. With this solution, trails of 10 cm long and 2 mm wide were laid out on white paper with the aid of a microcapillary pipette calibrated to deliver 4 µl per 10 cm of trail. This resulted in a pheromone dilution of 4×10^{-9} g/mm of trail.

The caterpillars were placed with their heads on one end of the trail and observed. A typical following response consisted of a larva crawling to the other end of the trail and stopping there. If the trail was followed all the way this was called following. If the caterpillar left the trail before reaching the end the response was called not following. A caterpillar that lost the trail was given a second opportunity. Caterpillars that did not move within 3 min were excluded from the experiment.

For testing the specificity of the trail following response on natural trails, a Y-maze test was used, modified from Fitzgerald & Edgerly (1979). The trails were produced by placing twenty-five caterpillars one by one on a long, 4 mm wide paper strip and allowing them to traverse to the other end. These paper strips were divided into sections of 4 cm. One section was used as the experimental branch of a Y-maze laid out on a plexiglass plate. The control branch was a similar section bearing the trail of another species. The smooth surface of the plexiglass prevented the caterpillars from moving off the strips. The branches were at an angle of 60° to each other and connected to a 5 cm long paper runway with a trail on it, forming the stem of the Y.

A caterpillar placed at the base of this runway could be observed to choose one of the two branches. After each choice the branches were replaced to prevent contamination of the trails by newly deposited silk and pheromone. The runway was re-used. To exclude bias due to a possible side preference, the experimental and control branch were interchanged. All tests were conducted in a shielded area with diffuse light to prevent larval orientation to visual stimuli. A choice for either side was counted if the caterpillar completely passed onto one of the branches. Caterpillars that refused to choose and moved back on the stem were given four additional tries before they were rejected. Caterpillars that did not move or choose within 3 min were also excluded from the test.

To investigate if volatile factors are important in the trail following behaviour, both bioassays were performed with a fine nylon mesh (90 μm mesh size) drawn taut over the trails. In this way the caterpillars were unable to contact the silk or the chemicals on the surface, but any volatile components would still be available.

Statistical analysis

The results were analysed using a G-test (Sokal & Rohlf, 1981), either testing for significant differences between treatments or for a difference from the expected 1:1 ratio in the Y-maze tests.

Results

The results for *Malacosoma* caterpillars on a dione trail are given in Table 1. It is clear that after removal of the palpi the trail following response disappeared. All other operations did not affect trail following behaviour. This was true for both *M. americanum* and *M. neustria.* These results indicate that the maxillary palpi are necessary for trail following. This correlates well with our observation that the caterpillars continuously palpate the surface during walking.

Table 1. Effect of sensory ablations on trail following behaviour of *Malacosoma* spp. on a linear trail of dione (0.1 mg/ml).

	M. americanum			*M. neustria*		
	Following	Not following	G	Following	Not following	G
Control	10	0	-	20	0	-
Antennae removed	8	1	1.04^{ns}	31	0	0.0^{ns}
Palpi removed	0	9	24.35^{*}	0	30	65.12^{*}
Galeae removed	-	-	-	19	2	2.22^{ns}

ns $p>0.05$, * $p\leq0.01$, G-test, two-way comparison with control.

Table 2 summarizes the results of the preference experiments to assess the specificity of the trail following response. Both *Malacosoma* and *Yponomeuta* preferred trails laid down by conspecifics over trails made by the other species. This preference was preserved after removal of galeae and antennae, but disappeared after ablation of the palpi.

Table 2 Effect of sensory ablations on preference for trails in *M. neustria* and *Y. cagnagellus* given a choice between their own and each other's trails in Y-maze tests.

	M. neustria Choosing:			*Y. cagnagellus* Choosing:		
	Conspecific trail	Hetrospecific trail	G	Conspecific trail	Hetrospecific trail	G
Control	76	4	78.65*	67	13	39.65*
Antennae removed	19	3	12.69*	32	8	15.23*
Palpi removed	22	19	0.22^{ns}	31	30	0.02^{ns}
Galeae removed	17	4	8.46*	18	2	14.36*

^{ns} $p>0.05$, * $p\leq0.01$, G-test, goodness of fit to 1:1 ratio.

In the investigation of the role of volatile components, none of the *M. neustria* caterpillars tested responded to a covered dione trial (Table 3). If the cover was removed, however, all of these insects followed the trail.

Table 3. Effect of covering trails with nylon mesh in *M. neustria* and *Y. cagnagellus* on linear trail following and Y-maze preference tests.

		M. neustria		
Assay	Cover	Larvae following	Larvae not following	G
Linear trail test	No	10	0	
	Yes	0	10	25.79*
		Y. cagnagellus		
Assay	Cover	Larvae on trail	Larvae on control	G
Y-maze test	No	53	7	39.63*
	Yes	34	26	1.06^{ns}

^{ns} $p>0.05$, * $p\leq0.01$, G-test, for *M. neustria* two-way comparison with control, for *Y. cagnagellus* goodness of fit to 1:1 ratio.

Y. cagnagellus also showed a strong preference for uncovered natural trails compared to clean paper strips when offered a choice on a Y-maze, but if both arms were covered with mesh, no significant preference could be detected. These experiments indicate that contact cues are important in the trail following response of both species.

Discussion

In summary, the following observations have been made: the maxillary palpi are needed for chemical trail following in *Malacosoma*, when the palpi are ablated preference for conspecific trails is lost in both *Malacosoma* and *Yponomeuta*, and contact with the trail is necessary for the following response in both *Malacosoma* and *Yponomeuta*.

The main conclusion from these results is that the maxillary palpi are the appendages involved in the perception of trail pheromones. In *Malacosoma* there is no doubt about this conclusion, because the pure chemical was used in testing.

For *Yponomeuta* it can only be said that the palpi are important for trail following behaviour. In this species there is no direct evidence that a chemical factor is involved. Subtle differences in the structure of the silk could, for instance, be responsible for the observed discrimination between their own trails and those of *Malacosoma*.. If this is the case, trail following would merely depend on tactile cues rather than on the presence of a pheromone. It is known that caterpillars have an acute tactile sense (Hanson, 1970, Tautz, 1977). The observation that the removal of only the tops of the maxillary palpi suffices to inhibit the preference response to their own trails can be interpreted in three ways: in *Yponomeuta* an extreme tactile sensibility is located at the distal end of the palps, chemoreceptors which are tuned to trail chemicals are involved, and chemoreceptors detecting deterrent chemicals in the control trails made by *Malacosoma* are involved. This last explanation is unlikely considering that *Yponomeuta* caterpillars prefer *Malacosoma* trails over clean controls, indicating that a possible deterrent has limited influence (Chapter

2). At present no discrimination between the other two explanations, extreme tactile sensibility or trail pheromone detection, is possible, but given the general importance of chemical signals in insect behaviour the latter explanation is more likely. Further work is in progress on the relative significance of silk and pheromone in the trail following behaviour of *Yponomeuta*.

The role of the palpi in lepidopterous larvae is not well understood. It appears that although they house a considerable fraction of the caterpillar's total sensory equipment, their role in the process of food testing and recognition is less prominent than that of the taste hairs on the galeae (Schoonhoven, 1986). From our research, it can be concluded that at least one other function in some species is the perception of trail pheromones.

The fact that these trail pheromones seem to be contact stimuli differs from the situation in other trail following species. In ants and termites, identified chemicals are mostly volatile (Howse, 1984; Morgan, 1984). Although several authors have expressed the idea that lepidopteran trail pheromones might also be volatile (Kalkowski, 1958; Merz, 1959; Wojtusiak, 1972; Weyh & Maschwitz, 1982), no conclusive evidence has been presented. Rather, it seems that trail following in caterpillars is more analogous to the situation in spiders. In this group some of the chemicals used in communication are thought to be contact cues bound to the silk (Tietjen & Rovner, 1982; Roland, 1984).

The trail pheromone of *Malacosoma* is very similar to beta-sitosterol, a common sterol present in the surface wax of many plants (Caldicott & Eglinton, 1973). Since caterpillars palpate the surface when testing their food and during feeding (Devitt & Smith, 1985), sensory receptors tuned to this type of compound can be expected to be present in the palpi. For example, in *Bombyx mori* the maxillary palpi are sensitive to beta-sitosterol (Ishikawa *et al.*, 1969). One might speculate that some modification of these receptors could lead to pheromone detectors, thus providing an evolutionary pathway from the detection of leaf compounds to sensing trail pheromones. A similar idea has been proposed for bark beetles (Shorey, 1976), where the pheromones show a striking resemblance to the terpene resins from the host tree. To set this process in motion, a suitable chemical must become

available as a pheromone. The accidental leakage of substances into the environment as a basis for the evolution of chemical communication has been proposed by several authors (Wynne-Edwards, 1962; Kittredge & Takahashi, 1972; Shorey, 1976; Tietjen & Rovner, 1982). In insects, steroids are known to occur in cuticular waxes (Blomquist & Dellwith, 1985) so wax glands may have acquired a pheromone-producing function. In this respect it is interesting to note that the trail marker in *Malacosoma* does not seem to be stored in a special gland, but is given off directly from the body surface (Fitzgerald & Edgerly, 1982). Thus a body surface chemical, at first inadvertently contaminating the environment, may have come into play as a trail pheromone, sensed by a receptor that was pre-adapted for this function.

For *Y. cagnagellus*, a similar tentative hypothesis is less easy to construct. Although the same sensory organ is involved, the chemical nature of the pheromone, if any, is unknown. There is no reason to assume that the pheromone of *Y. cagnagellus* is chemically related to the pheromone used in *Malacosoma*. The dragging of the abdomen over the substrate during deposition of trail pheromone by *Malacosoma* is clearly observable (Fitzgerald & Edgerly, 1982). Such marking behaviour has not been observed in *Yponomeuta*, suggesting that the pheromone is produced at another site. Moreover, the pheromone of *Malacosoma* does not stimulate trail following in *Yponomeuta*.. In ants it is thought that trail communication has arisen independently many times (Hölldobler, 1984). This could also be the case in Lepidoptera, resulting in chemically unrelated trail pheromones in different species, which are, however, perceived by means of receptors located in the same morphological structure.

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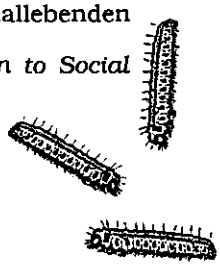

4. A chemical marker from the silk of *Yponomeuta cagnagellus.*

Peter Roessingh

Abstract

Trail following in lepidopterous larvae is often attributed to chemical markers but only a few clear cut examples are found in the literature. In this paper evidence is presented for a chemical basis of the trail following behaviour of *Yponomeuta cagnagellus.* (Lepidoptera: Yponomeutidae)

The marker is shown to be very persistent under laboratory conditions and is water soluble. Several possible secretory sites were investigated and it is concluded that the marker is probably secreted together with the silk from the labial gland. Problems associated with the demonstration of trail markers in caterpillars are discussed.

Introduction

Trail following has been described for several species of lepidopterous larvae (See Fitzgerald & Peterson, 1988 for review). This behaviour is thought to facilitate orientation and plays a role in larval aggregation (Long, 1955; Fitzgerald & Peterson, 1983, Fitzgerald & Costa, 1986). In addition to these functions it has been shown that in the genus *Malacosoma* "recruitment trails" are produced by successful foragers. Such trails can convey information about the quality of a distant food source and are preferred by colony members over exploratory trails (Fitzgerald, 1976; Fitzgerald & Peterson 1983; Peterson, 1986,1988).

Although trail following in Lepidoptera is often attributed to chemical markers associated with the trail, only a few clear cut demonstrations of a chemical basis for this behaviour have been reported in the literature. Except for the results in *Malacosoma*

(Fitzgerald & Gallagher, 1976; Fitzgerald & Edgerly, 1982; Fitzgerald & Costa, 1986; Peterson, 1988) only two other unequivocal cases have been found. The European birch tent moth *Eriogaster lanestris* has been shown to possess a trail marker (Weyh & Maschwitz, 1978). This species belongs to the same family as *Malacosoma*, i.e. the Lasiocampidae. The other example is the saturniid *Hemileuca oliviae* (Capinera, 1980).

It can be concluded that trail pheromones are predominantly known from the Lasiocampidae. To broaden the knowledge of trail pheromone systems and the types of substances used in the Lepidoptera, trail following in *Yponomeuta cagnagellus* (Yponomeutidae) was investigated. Caterpillars of this species live gregariously in groups of 20-50 on the Spindle tree *Euonymus europaeus* and produce conspicuous webs that may envelop the leaves of their food plant. During their development the larvae gradually extend the limits of this web to encompass new leaves. When the local food supply is exhausted the colony often moves to a new feeding site, sometimes at a great distance from the original web. The larvae remain gregarious during this process as well as during their migration to the vegetation on the ground at the time of pupation (Hoebeke, 1987). A trail pheromone could facilitate this gregarious behaviour.

It has been shown that *Yponomeuta* larvae are able to follow each other's trails (Chapter 2) Tactile cues from the silk play a role in this behaviour, while an additional chemical factor has been hypothesized (Roessingh *et al.*, 1988, Chapter 3; Chapter 2). In this paper experiments are described which establish the existence of such a trail marker. In addition the longevity of the trail under laboratory conditions was studied, as well as the effects of washing trails with water and organic solvents.

An important piece of evidence in the validation process of a trail pheromone is the localization of the secretory site (Howard *et al.*, 1976). Several sites have been identified or proposed for caterpillars. In the genus *Malacosoma* a trail marker is produced by cuticular glands on the ventral side of the abdomen (Fitzgerald & Edgerly, 1982; Fitzgerald & Costa, 1986; Peterson, 1988). Another possibility, suggested by Weyh & Maschwitz (1978), is that the marker originates in the labial glands (the silk glands), and is

excreted together with the silk. This could also be the case for *Hemileuca oliviae* (Capinera, 1980) An alternative idea was formulated by Povel & Beckers (1982) in a paper describing the prothoracic organ, a gland on the ventral side of the prothoracic segment, that traditionally has been designated a defensive role. These authors stated that this gland does not seem to function in this manner in *Yponomeuta*, and they suggested that it might be the source of a trail pheromone. In addition to the glands already mentioned, *Yponomeuta* caterpillars are also equipped with prominent mandibular- and pharyngeal glands as well as glands of filippi (Berlese, 1909).

In an attempt to answer the question whether a trail pheromone is produced in *Yponomeuta*, extracts from glands and body parts were assayed for their ability to induce trail following behaviour. Additional information about the possible role of some of these structures was collected in experiments in which the release of chemicals was blocked, and in tests with trails made out of chemicals wiped directly from the body surface of the caterpillars.

Materials and Methods

Insects and plants

Egg batches of *Yponomeuta cagnagellus* (Hübner) were collected in the field on *Euonymus europaeus* and kept at 6°C. and 80 % relative humidity until needed (cf. Bucher, 1959). Rearing was done in petri-dishes at 25°C and an 18:6 h. LD photoperiod on young *Euonymus* leaves taken from potted plants in the greenhouse. Plants were periodically cut back to induce development of new shoots with young leaves, essential for the survival of the first instar. The insects had continuous access to food and were used during the first four days of their fifth stadium.

Bioassay

All tests on natural trails were performed on filter paper Y-mazes modified from Fitzgerald & Edgerly (1979) and described in detail elsewhere (Roessingh *et. al.*, 1988, Chapter 3; Roessingh, Chapter 2). Filter paper proved to be unsuitable for testing extracts, probably because the extract is absorbed in the paper and only small quantities of active material are present on the surface (Crump *et al.*, 1987). To overcome this problem stainless steel segments of 50 x 2.5 mm wide were used. Forty of these strips were placed in line and 25 caterpillars were allowed to lay a trail by crawling over this pathway. After the production of the trail all sections were washed 3 times for 5 min. in aqua dest. to inactivate the trail. The first 2 cm of 20 of these silk covered metal segments were painted with 5 µl of extract, while the first 2 cm of the remaining twenty segments were painted with the plain solvent. One segment from each group was used to form an arm in the Y-maze (Fig. 1). Since *Y. cagnagellus* caterpillars are reluctant to enter silk free substrates (Kalkowski, 1958; pers. observations), the presence of the silk on both arms of the Y is important and facilitates the choice process.

Washing and extraction procedures

Washing of trails was done in petri-dishes in 100 ml. of solvent. The liquid was refreshed three times and gently shaken during the whole procedure.

Extracts were made from silk produced by caterpillars which had just moulted into their fifth stadium. When starved these larvae display extensive locomotion and produce large amounts of silk, only minimally contaminated with fecal pellets. The few pellets that are produced can easily be removed. Quantities of silk with known weights were soaked in 1 ml of aqua dest. and stirred on a vortex for 5 min. The silk strands were then taken out and the remaining solution was centrifuged for two minutes at 10.000 g to remove solids. The supernatant was subsequently concentrated at room temperature to yield 100 µl of silk extract.

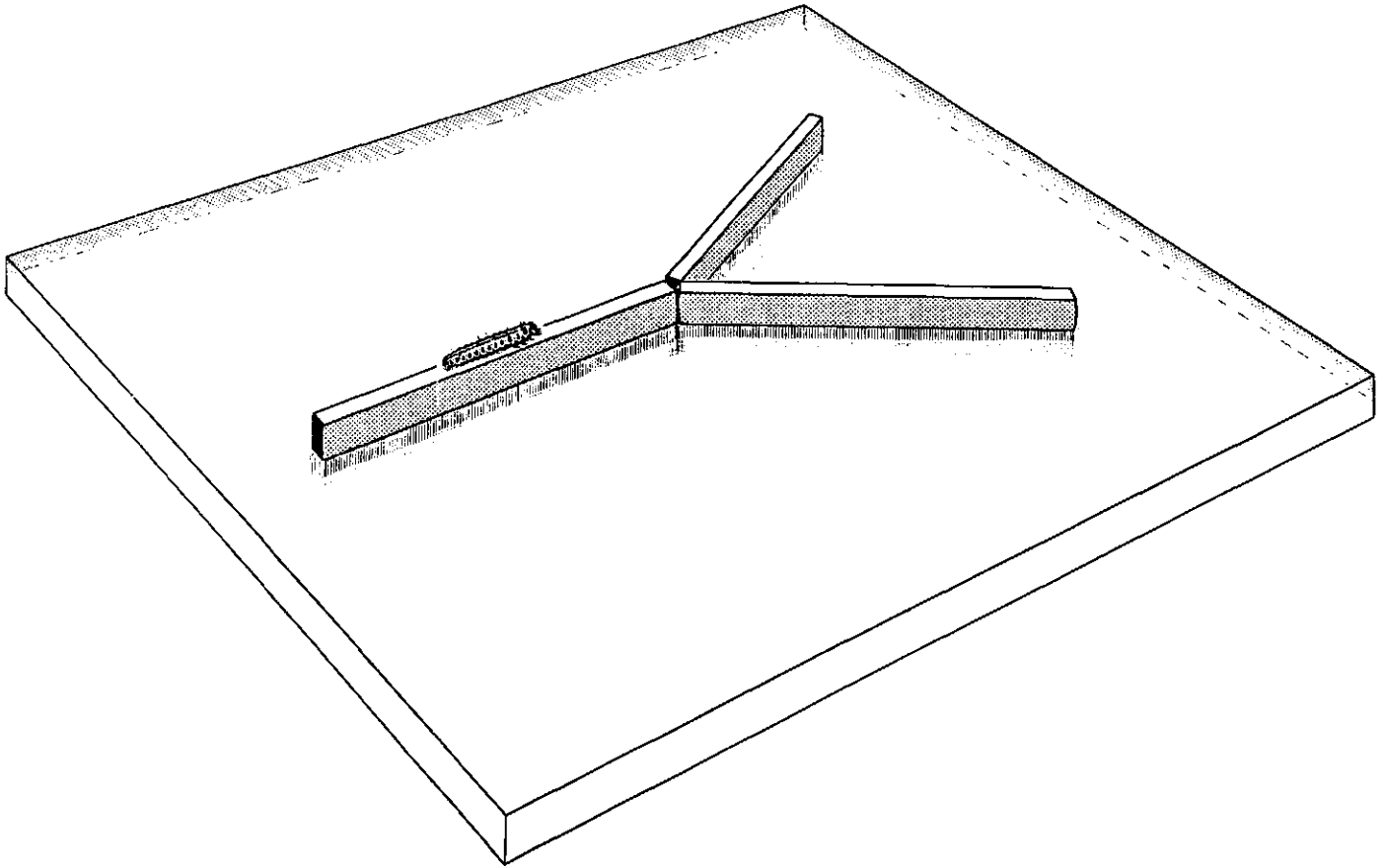

Figure 1 Y-maze used to determine the response of Yponomeuta cagnagellus caterpillars to extracts. The Y is made out of stainless steel segments, to prevent absorbtion of the extract.

For the gland and tissue extracts fifth stadium larvae were killed by immersion in dichloromethane for about 20 sec and dissected in distilled water. Labial glands, mandibular glands and the prothoracic organ were removed. Also the last two segments of the abdomen and the head (containing the pharyngeal glands, the glands of filippi and the extreme distal parts of the labial and mandibular glands) were dissected. Tissues from 40 or 50 larvae were immersed in one ml aqua dest. and disintegrated with a sonifier cell disrupter (Model B-12, Branson sonic power Co. Soest, The Netherlands, equipped with a 3 mm micro tip for use with small volumes). During this procedure the tissue solution was kept chilled at 0°C. After homogenization the solution was centrifuged for 2 min at 10.000 g and the supernatant was concentrated at room temperature to yield 100 µl of tissue extract.

Aging of trails

The trails for the aging experiment were made by 25 caterpillars on filter paper strips. Aging of these trails was done in the lab in the dark in petri-dishes. No special measures were taken to control humidity or temperature. Aged trails were compared to fresh ones also made by 25 caterpillars. The test started within 45 minutes after production of the fresh trail.

Ablations, wiping and sealing experiments

The ablations were conducted with the help of a HF microcautery device (Murphy Developments, Hilversum, The Netherlands) following the method described in detail elsewhere (Roessingh *et al.*, 1988, Chapter 3)

Sealing of the prothoracic organ was done using wax with a low melting point and with the help of a laboratory build temperature controlled needle. Caterpillars were given 24 hours to recover from the treatment before they were used to produce trails. Before and after the trail production the wax cover was

checked to prevent contamination of the trail. Control trails were made by sham treated larvae.

The test for an active surface residue from the prothoracic organ was done following the method used in the *Malacosoma* studies (Fitzgerald & Costa 1986; Peterson, 1988). Fifth stadium larvae were held firmly between two fingers and the creased edge of a piece of filter paper was wiped over the exit of the prothoracic organ. The filter paper was then unfolded and the linear trail that results was tested using a fresh caterpillar.

Statistical analysis

The results of the Y-maze tests were analyzed using a G-test for goodness of fit (Sokal & Rohlf, 1981). The numbers of larvae found on both arms of the Y were compared to the 1:1 ratio that is expected under the hypothesis that there is no difference in attractiveness between the two arms of the Y-maze. For the linear trails from the wiping experiment a comparison was made with the results of a control experiment.

Results

The existence of a trail pheromone can be demonstrated by determining behavioural responses to extracts from trails or trail silk. Washing trails with different solvents, and testing the washed trails to see if the behavioural activity is lost, allows the identification of solvents that removes the pheromone from the silk.

The effects of washing trails are summarized in Table 1. The effect of washing with hexane is small, no significant difference with the untreated controls could be detected. Washing with H_2O on the other hand, exhibits a large effect on the attractiveness of the trail and almost all insects prefer untreated controls over H_2O washed trails. Extended washing with Dichloromethane (DCM) also influences trail attractiveness. The last two rows in the table show

a direct comparison between washing with H_2O and hexane, again indicating the strong effect of H_2O on the trail following response.

Table 1 Effects of different solvents on the following response of *Yponomeuta Cagnagellus* caterpillars.

Solvent used on:		Larvae on branch:		Wash time	N	G
A	B	A	B	(min)		
-	Hexane	12	8	5	20	0.45 ^{ns}
-	Hexane	12	8	5	20	0.45 ^{ns}
-	H_2O	18	2	5	20	12.66 **
-	H_2O	18	2	5	20	12.66 **
-	H_2O	16	4	5	20	6.40 *
-	H_2O	18	2	60	20	12.66 **
DCM	H_2O	13	7	180	20	1.26 ^{ns}
DCM	H_2O	12	8	180	20	0.45 ^{ns}
Hexane	H_2O	17	3	5	20	9.18 **
Hexane	H_2O	18	2	5	20	12.66 **

^{ns} $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, G-test for goodness of fit to 1:1 ratio.

More direct evidence for the existence of a trail pheromone is provided by the data in Fig 2. In these experiments extracts of trail silk have been applied to a stainless steel Y-maze. Differential effects of the solvents on the structure of the silk (as might occur in the experiment described above) can now be excluded. From this figure it is clear that extracts from 25 mg silk/ml or more, are preferred over the plain solvent, indicating that a chemical marker is involved in the trail following response of *Y. cagnagellus*. To demonstrate that this factor is characteristic for *Y. cagnagellus* and is not a general constituent of silk, or brought about by contamination with fecal pellets, an additional series of assays was done. The results in Table 2 indicate that neither extracts from *M. neustria* or *B. mori* silk, nor fecal pellet extracts evoke a following response, but an extract of *Y. cagnagellus* silk is again significantly preferred over the control.

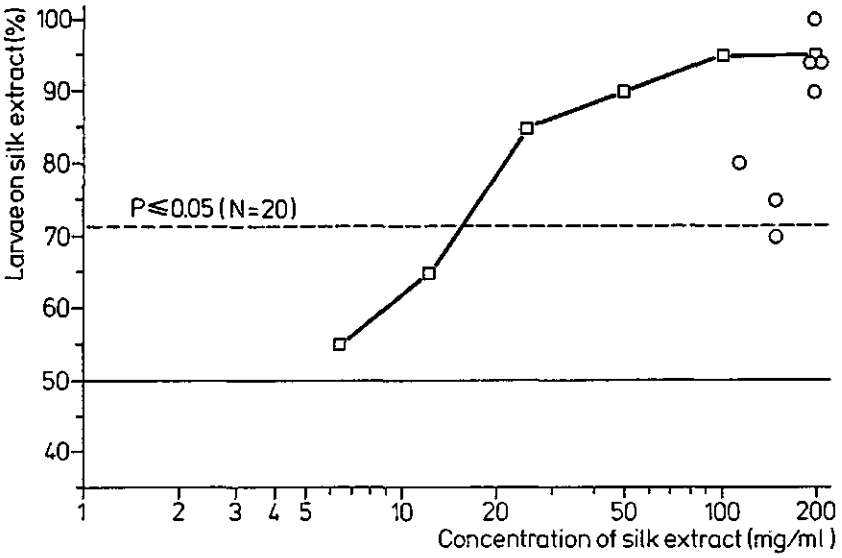

Figure 2 Results of Y-maze tests with extracts from trail silk of *Y. cagnagellus*. The circles are results from tests with different batches of silk.The points on the curve are based on a dilution sequence of a single silk extract.

Table 2. Response of *Yponomeuta cagnagellus* larvae to extracts from fecal pellets and to heterospecific silk extracts.

	Extract on branch:		Larvae on branch:		N	G
	A mg/ml)	B	A	B		
Silk *M.neustria*	200	H_2O	8	12	20	0.45 ns
Silk *B. mori*	200	H_2O	7	13	20	1.26 ns
Silk *Y.cagnagellus*	200	H_2O	18	2	20	12.66 **
Silk *Y.cagnagellus*	200	Fecal pellets 20 mg/ml	19	1	20	17.07 **
Fecal pellets	16	H_2O	9	11	20	0.05 ns
Fecal pellets	20	H_2O	16	4	20	6.40 *
Fecal pellets	20	H_2O	9	11	20	0.05 ns
Fecal pellets	67	H_2O	10	10	20	0.05 ns

ns p> 0.05, * p <= 0.05, ** p <= 0.01, G-test for goodness of fit to 1:1 ratio.

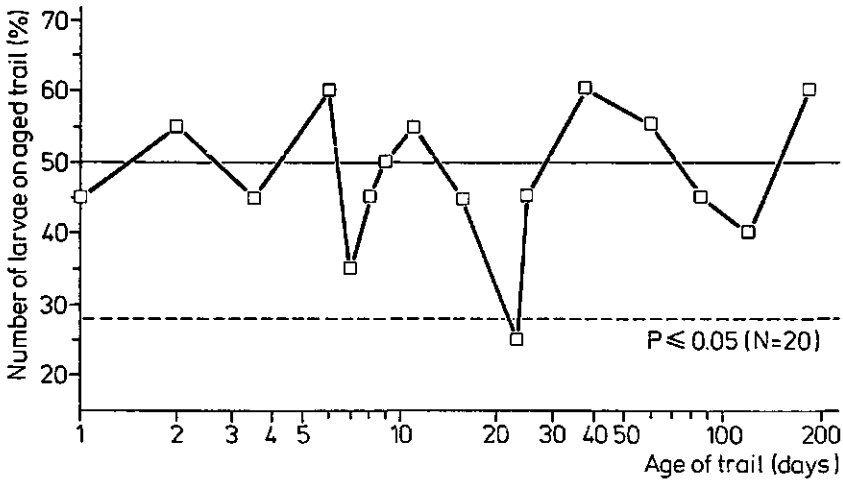

Figure 3 Effect of aging on the preference for these trails over fresh controls. N=20 for all data points.

Table 3. Responses of *Y. cagnagellus* larvae to extracts from glands and body tissue.

Extract of:	Larvae on:		N	G
	Extract	Control		
Head	35	25	60	1.36 ns
Abdomen	16	24	40	1.23 ns
Prothoracic organ	17	23	40	0.63 ns
Mandibular gland	17	23	40	0.63 ns
Labial gland	45	15	60	14.62 **

ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, G-test for goodness of fit to 1:1 ratio.

To investigate the longevity of the marker, experiments were performed in which freshly produced trails were compared to

trails aged in the laboratory for up to 200 days. Fig. 3 gives an overview of the results. It can be concluded that even extensive aging of trails does not decrease their attractiveness compared to fresh trails. This indicates that the trail marker is non-volatile and very persistent under laboratory conditions.

To determine the site of secretion of the marker five possible sources have been examined: i) The terminal part of the abdomen (cf. Fitzgerald & Edgerly, 1982), ii)The labial gland (cf. Weyh & Maschwitz, 1978; Capinera, 1980), iii) The prothoracic organ (cf. Povel & Beckers, 1982), iv) The mandibular glands (cf. Mudd & Corbet, 1984), and v) The head, with the glands of filippi and the pharyngeal glands. Tissue extracts from all of these sources were tested (Table 3). The only extract that is consistently active in the bioassay is that of the labial gland (the silk gland). Attempts to identify the exact region of the gland were the trail marker is produced were unsuccessful.

In other experiments to investigate whether or not the prothoracic organ produces the trail pheromone, this organ was sealed with wax. Trails made by these larvae were compared to those of sham treated insects. No difference was found between the trails of larvae with sealed and unsealed prothoracic organs (Table 4). Also trails produced by wiping with filter paper over the exit of the gland did not induce following behaviour.

Table 4. Results of Y-maze tests on trails made by *Y. cagnagellus* larvae with sealed prothoracic organs.

	Larvae on trail made by larvae with sealed organ	Larvae on trail made by larvae with unsealed organ	N	G
	13	7	20	1.26 ns
	12	8	20	0.45 ns
	7	13	20	1.26 ns
	11	9	20	0.05 ns
Total	43	37	80	0.31 ns

ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, G-test for goodness of fit to 1:1 ratio.

Discussion

The main aim of this paper is to demonstrate the existence of a trail pheromone associated with the silk trails of *Y. cagnagellus*. When the methods used to authenticate trail pheromones in lepidopterous larvae are reviewed it appears that a standard procedure does not exist.

Several authors have suggested the presence of trail pheromones on the basis of the observation that caterpillars prefer conspecific trails over heterospecific trails (Fitzgerald & Edgerly, 1979 for *Archips*; Roessingh *et al.*, 1988, (Chapter 3) for *Yponomeuta* and Weyh & Maschwitz, 1982 for *Iphiclides*). While such results might provide good indications for the existence of trail markers, they do not yield unequivocal evidence. It has, for instance, been demonstrated that *Y. cagnagellus* can use tactile information from the trail (Chapter 2) and therefore subtle cues from the silk structure can not be excluded as a basis for the observed discrimination.

Other authors relied on washing of trails with solvents to demonstrate trail markers (Gallagher & Lanier, 1977 for *Lymantria*; Masaki & Umeya, 1977 for *Hyphantria* and Weyh & Maschwitz, 1982 for *Iphiclides*). Conclusions from these experiments are based on the implicit assumptions that: (a) the marker can be removed by the solvent used, and (b) The solvent does not change any other aspect of the trail that can be sensed by the caterpillars. Neither of these two assumptions can be justified. The results of Table 1 show the risk of relying on extraction without prior knowledge of the type of chemical involved. In the case of *Yponomeuta* attempts to remove the trail marker with hexane are unsuccessful. Concerning the second point, it has been shown that silk can irreversibly change its shape and conformation in response to wetting (Hepburn *et al.*, 1979; Denny, 1980; Gosline *et al.*, 1986). For spider silk a 45% axial shrinkage has been reported together with a volume increase of about 15% (Work, 1977). Because of these effects a change in silk structure rather than the removal of a chemical marker could explain the responses of the larvae.

Unequivocal evidence for the existence of chemical markers can be obtained by demonstrating trail following activity in response to solvent extracts of trails. This method was applied by Fitzgerald & Gallagher, (1976) and Fitzgerald & Edgerly, (1979) for *Malacosoma*, by Weyh & Maschwitz, (1978) for *Eriogaster*, and by Capinera, (1980) for *Hemileuca*. Water extracts of trail silk from *Y. cagnagellus* showed activity in the trail following bioassay described in the present paper. This indicates that a chemical marker is indeed associated with the silk. The marker is not a general constituent of silk, since extracts of silk from other species do not evoke following behaviour. These findings contribute strong evidence that a pheromone is involved in the trail communication of *Y. cagnagellus*.

A plausible source of this marker is the prothoracic organ, as suggested by Povel and Beckers (1982). This gland terminates in a slit-like opening on a cone shaped protrusion that is movable to some degree. Silk threads from the spinneret pass over this protrusion, and therefore a marker from this gland could be readily added to the newly spun silk. In view of the results presented in this paper it must be concluded that this hypothesis is no longer tenable. All approaches used to substantiate a role for this organ (blocking of the gland, assays of tissue extracts and surface residues) yielded negative results. In contrast, extracts from the labial glands did induce trail following behaviour and this is taken as evidence that the trail marker in *Yponomeuta* is secreted together with the silk.

Experiments using labial gland extracts from 40 or 50 larvae showed that even this large amount of material yielded a trail that evoked only cautious reactions by the caterpillars and often induced extensive searching behaviour. This result differs, for instance, from findings in termites and ants, which strongly respond to extracts from a single gland (Traniello, 1983; Jaffe & Howse, 1979). A possible explanation is that the trail marker in *Yponomeuta* is not stored in significant quantities in the gland, a situation comparable to that found in *Malacosoma* (Fitzgerald & Edgerly, 1982).

In gypsy moth larvae, *Lymantria dispar*, a water soluble kairomone was found that elicits searching behaviour in the braconid parasite *Cotesia* (=*Apanteles*) *melanoscela* (Weseloh, 1976, 1987). The labial gland was identified as the principal source of this substance (Weseloh, 1976, 1977). It has been argued that a kairomone also must possess some biological advantage for the emitter, or otherwise production of the chemical would be selected against (Vinson, 1976). Since in *Lymantria* larvae trail following behaviour has been described (McManus & Smith, 1972; Gallagher & Lanier, 1977), it is conceivable that the primary function of this kairomone is that of a trail marker, which is secreted together with the silk, like in *Yponomeuta*.

From the work on ants and termites it has become clear that longevity of trail pheromones is correlated with their function. Markers for orientation in the home range largely retain their information content over time and can be persistent. In snails and slugs orientation cues have been described that persisted for days (Cook, 1979; Chelazzi *et al.*, 1985). For several termite species it has been shown that extremely stable markers are produced that (under laboratory conditions) stay active over periods of months or years. (Traniello, 1982; Runcie, 1987). Recruitment signals, on the other hand, carry information about food sources and when these become exhausted the signal should disappear. Consequently these signals are in general volatile substances that are often only effective over periods of minutes (Hölldobler, 1977; Traniello 1982; Bradshaw & Howse 1984; Runcie, 1987). More durable markers are used in situations with a stable food supply (Hölldobler, 1977). This might explain the relatively long lifetime of trail markers in phytophagous insects like leaf cutting ants and caterpillars (Lewis *et al.*, 1974; Fitzgerald, 1976; Peterson, 1988), as well as in *Yponomeuta*.

The extreme persistence of the *Yponomeuta* marker indicates that it must be largely non-volatile. This is consistent with results from tests on *Y. cagnagellus* in a bioassay where trails were shielded with nylon mesh. The mesh inhibited following behaviour, although possible volatiles from the trail were still accessible to the larvae (Roessingh *et al.*, 1988 , Chapter 3). It was

concluded that if a trail marker exists it will be a contact cue. The observed persistence reported in this paper corroborates this conclusion.

These facts are not consistent with data published on a near relative of *Y. cagnagellus*, i.e. *Y. evonymellus*. Kalkowski (1958) reported a volatile trail marker for this species. The cause of this discrepancy is not clear. Given the close taxonomic relationship of the two species it seems unlikely that these species use completely different trail pheromones. One possibility is that the marker is a multi-component system consisting of a stable contact cue, and a short lived rapidly evaporating volatile cue. A volatile marker disappearing within 30 minutes might have escaped attention in the present research on *Y. cagnagellus*. This explanation assumes that Kalkowski tested his trails within this time span, but the information on experimental procedures given does not allow conclusions on the likelihood of this interpretation.

From experiments in which *Y. cagnagellus* trails were treated with different types of chemicals it is clear that the trail following behaviour of the larvae is not influenced by the apolar solvent hexane, while a polar solvent like H_2O effectively removes the following response. This result indicates that the marker has a polar character and is water soluble.

A water soluble marker seems to be of limited use under natural conditions, and has led to some doubts about the adaptive value of some of these substances (Prokopy, 1975). On the other hand, water soluble semiochemicals are not at all uncommon. Prokopy (1981) gives an extensive list of epideictic pheromones and many of these have been found to be water soluble. The contact sex pheromone bound to the silk of the lycosid spider *Pardosa lapidicina* also is readily inactivated by water (Dondale & Hegdekar, 1973), as is the chemical present in the silk of the gypsy moth *Lymantria dispar* (Weseloh, 1976, 1977). Thus it may be concluded that many semiochemicals are easily washed away by rain or inactivated by dew, but this does not seem to prevent their successful use in nature (Averill & Prokopy, 1987). In some cases this may be related to the fact that only a limited activity period is needed for the substance to function. The oviposition deterring

pheromone of the leaf miner *Agromyza frontella*, for instance, is washed away in heavy rains, but a 24 hours activity period seems to suffice to give the larvae a competitive advantage over later deposited ones (Quiring & McNeil, 1984).

In the case of *Yponomeuta* the water solubility might cause periodic updating of the information in the trail system and offset the long lifetime of the marker.

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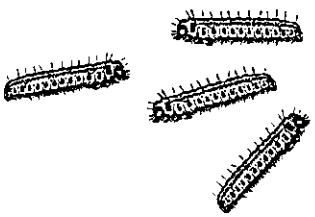

5. An electrophysiological survey of chemoreceptors on the maxillary palps of *Yponomeuta cagnagellus* larvae.

Peter Roessingh

Abstract

In lepidopterous larvae the maxillary palps contain a relatively large portion of the sensory equipment available to the insect. Yet, only very little is known about the sensitivity of these cells. In this paper an electrophysiological investigation of the maxillary palps of *Yponomeuta cagnagellus* (Lepidoptera: Yponomeutidae) is presented. Evidence is reported for the existence of two groups of receptor cells sensitive to plant volatiles (C6 fatty acid derivatives or 'green odours'). Cells mainly sensitive to (E)-2-hexenal and hexanal (aldehydes) or to (Z)-3-hexen-1-ol and 1-hexanol (alcohols) were observed. The maxillary palps also contain gustatory neurons and evidence for the existence of receptors responding to this species' chemical trail marker is presented. In addition, responses to CO_2 were recorded, as well as responses from receptors sensitive to temperature change (cold receptors). The external morphology of the palp was studied using a cryo-SEM technique and the electrophysiological results are discussed in relation to the morphology and ultrastructure of palpal sensilla in lepidopterous larvae in general.

Introduction

Lepidopterous larvae can display striking food preferences. Yet they have only a limited set of chemoreceptors to translate messages from the outside world into signals for the central nervous system (Schoonhoven 1987). In addition, the behaviour of a caterpillar is relatively simple, and its primary function, gathering as much food as possible, is not complicated by tasks

such as mate finding or taking care of offspring. For these reasons caterpillars have been advocated as model systems for the study of feeding behaviour (e.g. Schultz, 1983; Schoonhoven, 1987) and attempts have been made to analyse the relation between sensory input and behavioural output (Ma, 1972; Blom, 1978; Schoonhoven & Blom, 1988). Such an approach requires knowledge about the receptors involved.

External chemoreceptors in caterpillars are located on the antennae, on the maxillae, and on the epipharynx. The sensilla styloconica on the galea and the epipharyngeal organs on the labrum are the most important in defining food choice, and a large body of data is available about their sensitivity (see Schoonhoven, 1987 for review). The two other sets of receptors, those on the antennae and the maxillary palps, are more difficult to study and consequently less well known, but their combined influence on feeding behaviour is commensurate with that of the taste hairs on the galea (Hanson & Dethier 1973)

The olfactory receptors of the antennae were studied in *Manduca sexta* and *Hyalophora gloveri* by Schoonhoven & Dethier (1966) and Dethier & Schoonhoven (1969), in *Bombyx mori* by Morita & Yamashita (1961) in *Malacosoma americanum* by Dethier (1980), and recently in *Pieris brassicae* by Visser & de Jong (1988).

The maxillary palps, on the other hand, have received very little attention. Except for the pioneering studies of Schoonhoven & Dethier (1966) and the recordings from the maxillary nerve of the silkworm by Hirao (1976), no electrophysiological data are available. Behavioural experiments (Dethier, 1937; Ishikawa *et al.*, 1969; Hanson & Dethier, 1973) together with the above mentioned electrophysiological work indicate olfactory as well as gustatory functions for the palp, and this is confirmed by the structure of the sensilla (Hanson & Dethier, 1973; Albert, 1980; Devitt & Smith, 1982). In this paper morphological and electrophysiological aspects of the maxillary palps in larvae of *Yponomeuta cagnagellus* are investigated. The feeding behaviour of this species is relatively well known (Gerrits-Heybroek *et al.*, 1978; Van Drongelen, 1980; Kooi & v.d.Water, 1988). In addition, van Drongelen (1979) investigated the sensitivity of the sensilla

styloconica on the galea. To add to this framework the first inventory of palpal sensilla is presented here.

The maxillary palps in *Y. cagnagellus* are also involved in the detection of a non-volatile trail marker (Roessingh *et al.*, 1988; Chapter 4), and an attempt is made to identify receptors for this stimulus.

Materials and Methods

Insects

Egg batches of *Yponomeuta cagnagellus* (Hübner) were collected in the field from their host plant *Euonymus europaeus* and kept at 6°C and 80% relative humidity until needed. Larvae were reared in 10 cm-wide petri-dishes (25°C, ambient humidity, 18:6 hour LD photoperiod) on host foliage from potted plants grown in the greenhouse. During the rearing period larvae were fed ad lib, but they were starved for two to three hours prior to the experiments. All experiments were done with 1 to 4 days old 5th stadium larvae.

Morphology

The morphology of the palps was studied using scanning EM. The most critical steps in this procedure are the fixation and drying, necessary before the preparation can be introduced in the vacuum of the microscope. These procedures often induce shrinking and cause collapse of soft tissue parts. One way to avoid this is to skip fixation and drying. The preparation is stabilized instead by quick immersion in nitrogen slush (60 K) and viewed in the frozen state (Cryo-SEM). This approach was taken here. Larvae were used directly after moulting into the fifth stadium. After coating with a 200 Å thick layer of gold, they were examined in a Philips 535 SEM equipped with a Hexland CT1000/CP2000 and in a Jeol 35C SEM equipped with an EMSCOPE SP2000A cryo preparation assembly.

It has become clear that a classification of sensilla based on external morphology is no longer tenable and a new typology based on ultrastructural characteristics has been developed (Altner & Prillinger, 1980; Zacharuk, 1980). Since ultrastructural details are not yet available for *Yponomeuta* larvae, the old nomenclature will be used here.

Host plant odour composition

One of the problems in determining the function of relatively unknown sensory organs is the choice of the stimuli to be tested (Johnson *et al.*, 1988). To identify a set of relevant substances an attempt was made to characterize the chemical composition of the headspace of *Euonymus europaeus*, the host of *Y. cagnagellus*. Branches (30 cm long) of *E. europaeus* growing in the field in Wageningen were cut with sharp garden scissors and transferred to the lab. About two kg of branches was placed in a 20-l stainless steel vessel within 30 minutes after cutting. Care was taken not to damage the leaves. Air from a membrane pump was passed through KOH pellets, molecular sieves 5A and 3X, active charcoal and a tenax pre-trap. This cleaned air flowed for 7 to 15 hours at a rate of 70 ml/min through the stainless steel vessel and from there through the tenax-TA sample trap. The collected volatiles were released from the tenax by heating in a Thermo-desorption Cold Trap Unit (Chrompack, Middelburg, The Netherlands) at 250°C for 3 minutes. The desorbed compounds were collected in a Sil5CB coated, fused silica capillary at -100°C. Flash heating of the cold trap provided sharp injection of the compounds into the GC/MS unit (Chrompack Sil19CB column (25 m x 0.25 mm, df 0.25 µm). Electron impact ionisation was carried out at 70 eV on a VG MM 7070F mass spectrometer.

Electrophysiological recordings

A diagram of the recording set-up is presented in Fig.1. An isolated caterpillar head was mounted on a silver wire loop and connected via a 10-mm connector to the input terminal of a laboratory built amplifier ($R_i > 10^9\ \Omega$, $C_i < 1$ pF, $I_b < 1$ pA). The sensory cells of the sensilla on the palp are located below the base of the terminal segment (Schoonhoven & Dethier,1966; Albert,1980). To gain access to these cells, the connecting membrane between the two most distal segments was pierced with a sharpened tungsten wire. The actual recording was made using glass capillary electrodes (impedance about 40 MΩ) inserted through the hole. The glass electrode, filled with 3M KCl, served as the indifferent electrode and was connected to ground.

The preparation was placed in a continuous stream of clean, moistened air (80 ml/sec, 25 cm/sec). Odour stimuli were injected in the main air stream by switching a second stream (2ml/sec) for one second through a pasteur pipette that contained the stimulus. Stimulation with CO_2 required the following modifications: the stimulus and control flow were replaced with pure CO_2 and the control flow was not injected into the main air stream. The stimulus flow was adjusted to produce the desired concentration of CO_2 in the main stream during stimulation. Pure compounds were diluted in paraffin oil (10^{-3}, 10^{-2} and 10^{-1} v/v). 25 µl of these solutions was applied to filter paper strips (6.0 x 0.5 cm) and placed in pasteur pipettes. The following compounds (99% pure) were used: 1-hexanol, benzaldehyde (Fluka Switzerland); (Z)-3-hexenyl acetate, (E)-2-hexenal, (Z)-3-hexen-1-ol (Roth, West Germany); hexanal (98% pure) (Merck, USA); limonene, citral (97% pure), geraniol, (98% pure.) (Aldrich, Germany) and CO_2 (Hoekloos, the Netherlands). Taste stimuli (extracts from *Y. cagnagellus* silk, *Bombyx mori* silk and KCl solutions), were applied to the apex of the palp with an insulated capillary filled with the stimulus solution. Silk extracts were made by washing quantities of silk in distilled water and concentrating the extract at room temperature to obtain the desired concentration.

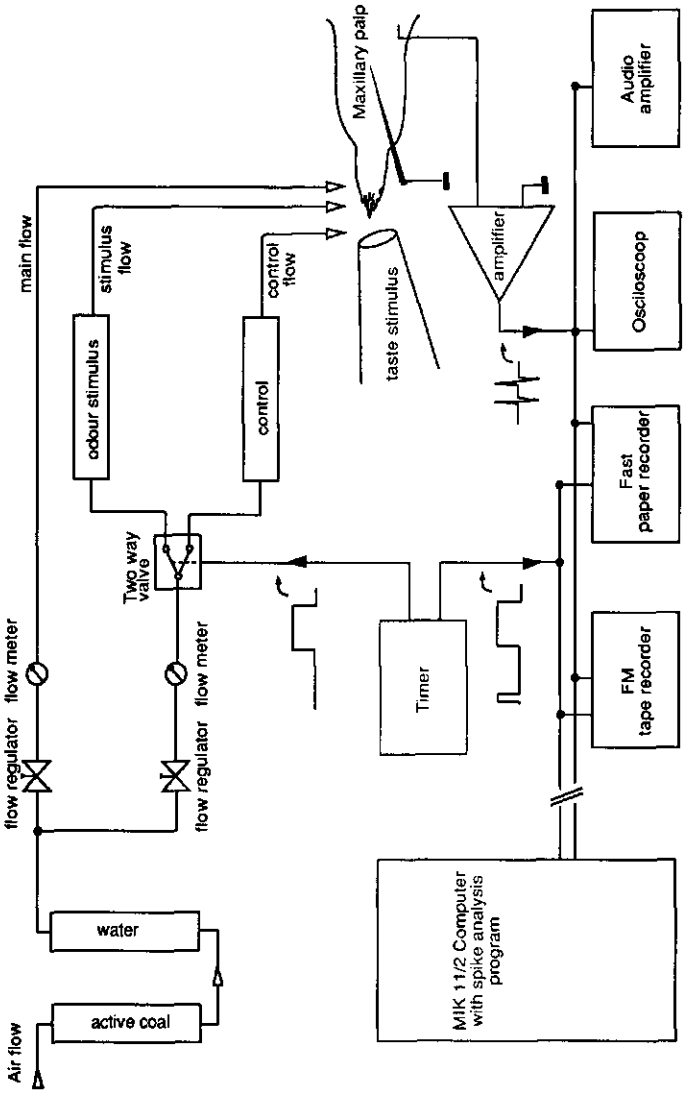

Figure 1 Schematic diagram of the electophysiological set-up used to monitor responses to both gustatory and olfactory stimuli. Open arrows indicate air flow. Black arrows electrical signals.

All stimuli were applied in random order with at least 30 seconds between them. Longer intervals were used after strong stimuli. Preparations were discarded after 60 minutes and changed after a successful recording.

The AC amplified action potentials from the sensory cells were recorded on tape (Racal FM taperecorder). To facilitate computer assisted analysis, the valve signal and a pre-pulse one second earlier were recorded on an additional channel.

Analysis of recordings

The recordings made with the system described above contained activity from 1 to 4 sensory cells but usually not more than 2. Recordings with more than 3 cells were discarded. Multi-cellular responses were analysed with the help of an interactive computer program based on the approach of van Drongelen *et al.* (1980) and described in more detail in chapter 7. Spikes were characterized primarily by amplitude. Amplitude boundaries for different cells were taken from an amplitude vs. time plot. The separation of the cells is a cyclic process in which spike amplitude and spike interval distribution are used as criteria to judge the separation. After the separation, responses for each cell were calculated as the difference in action potential frequency between the last second before the onset of stimulation and the first reaction second. A cell was assumed to respond to a stimulus if an increase of more than 10 spikes was found. Cells with weaker reactions were assumed to be sensitive to other stimuli and were not further analysed. Relative response spectra were obtained by setting a cell's 'best' stimulus to 100 % and scaling all other responses accordingly. To test the significance of observed differences in spike frequency, a two way ANOVA was used on log transformed response data.

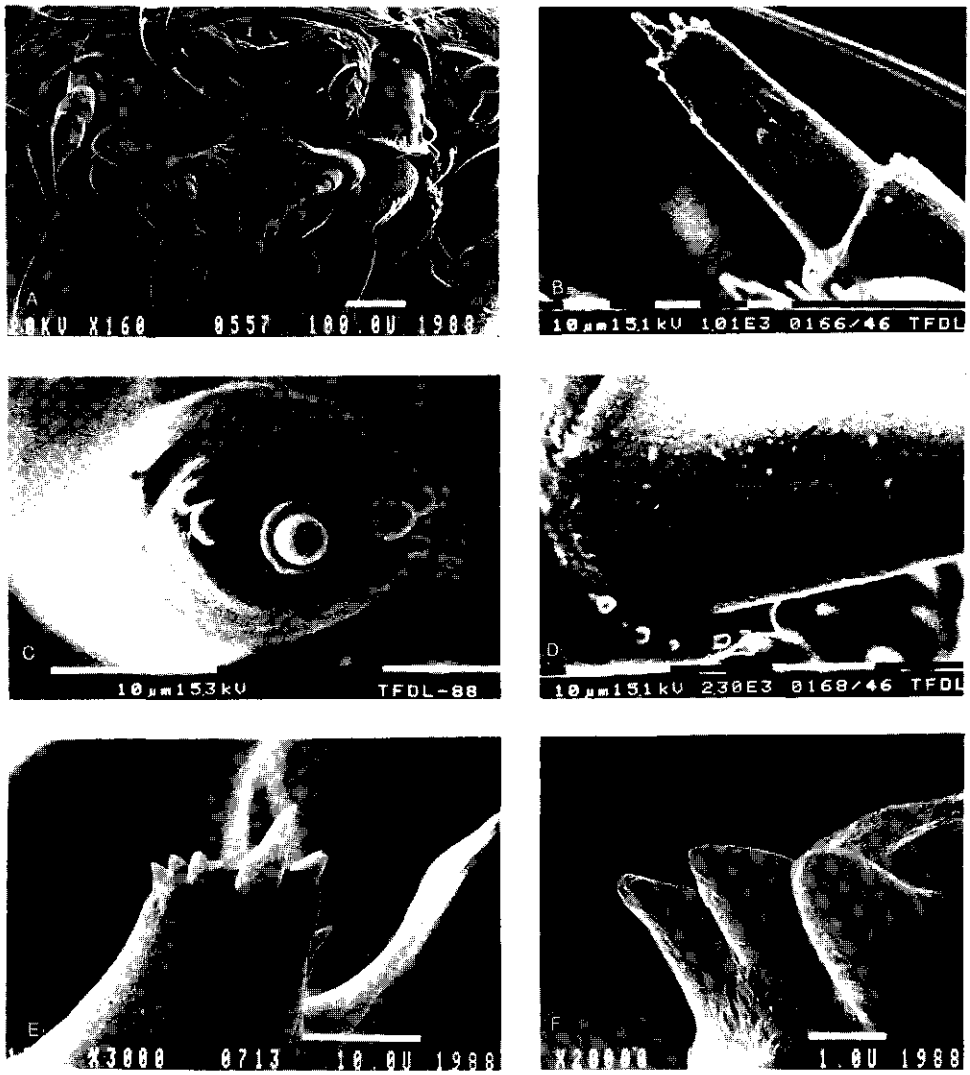

Figure 2 Cryo-SEM micrographs of *Y. cagnagellus*. **A.** Ventral view of the head. **B.** Maxillary palp showing the digitiform sensillum and a large campaniform sensillum. **C.** Overview of the tip of the most distal segment. Note the relatively linear arrangement of the sensilla. **D.** Detail of the digitiform sensillum. **E.** On the apex of the palp, seven sensilla basiconica and one sensillum styloconicum are present. From left to right, L1, L2, L3, A1, a styloconic A2, A3, a partly hidden M1, and M2 can be seen (Terminology after Grimes & Neunzig, 1986). **F.** High magnification of the the lateral side of the apex. The two leftmost sensilla belong to the lateral group. The slightly more blunt sensillum on the right is A1. Note the absence of surface structures.

Results

Morphology

A description of the external structure of the maxillary palps of lepidopterous larvae was recently published by Grimes & Neunzig (1986). From their results it is clear that location, size and shape of palpal sensilla are relatively constant in lepidopterous larvae. Eight sensilla are always found on the apex. In addition, a digitiform sensillum and up to four campaniform sensilla are present in the wall of the most distal segment.

The palps of *Y. cagnagellus* are more elongated than in many other species (see Grimes & Neunzig, 1986), but the sensory equipment does conform to the general pattern. At the apex seven sensilla basiconica and one sensillum styloconicum can be distinguished. On the side of the segment a digitiform sensillum and at least one campaniform sensillum are visible (Fig 2). With the exception of the styloconic sensillum, which has a prominent base, the sensilla on the apex are simple blunt cones. The lateral and medial sensilla seem to be slightly more tapered (Fig. 2E, 2F) but there is no clear distinction. It was not possible to observe details like grooved surfaces or apical pores.

Host plant odour composition

The compounds used for olfactory stimulation were chosen partly on the basis of the headspace analysis of the insect's hostplant *E. europaeus*. Fig. 3 shows an example of a gas-chromatogram. The need to use whole plants instead of macerates has recently been stressed by several authors (Buttery *et al.*, 1985; Tollsten & Bergström, 1988). However, whole plants release only limited amounts of volatiles (Buttery *et al.*, 1985; Dicke, 1988), and indeed only small quantities were found in the present study, thus complicating analysis. The only compounds that could be detected reliably were fatty acid derivatives ('green odours', Visser & Avé,

1978). Other compounds (limonene, beta-ocimene, alpha-copaene, beta-bourbonene, benzaldehyde), could be detected only occasionally.

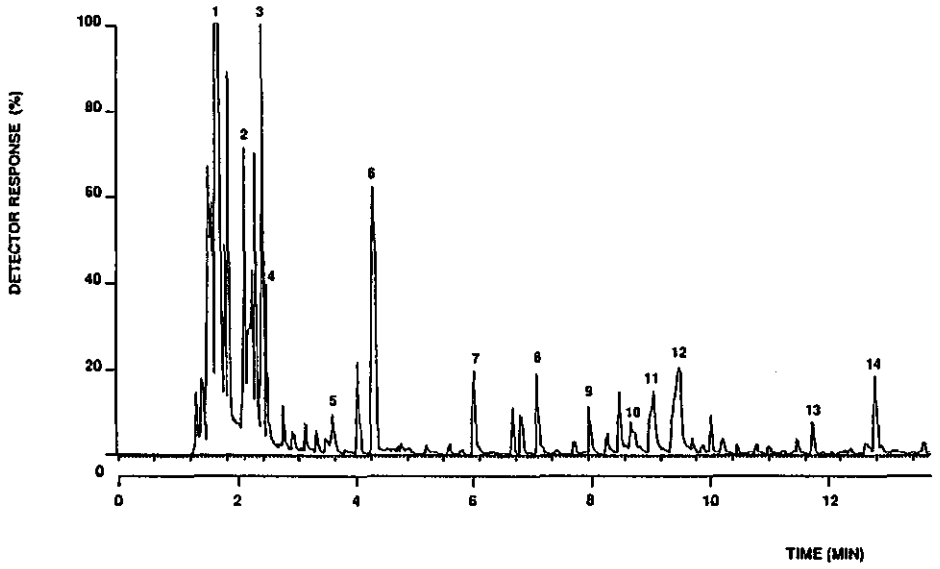

Figure 3 Gaschromatogram of volatiles from undamaged *E. europaeus* leaves collected on Tenax-TA. **1**. hexane, **2**. ethyl acetate, **3**. chloroform, **4**. benzene, **5**. 1-butanol, **6**. toluene, **7**. hexanal, **8**. m/p xylene, **9**. (E)-2-hexenal, **10**. 3-hexen-1-ol, **11**. (Z)-3-hexen-1-ol, **12**. 1-hexanol, **13**. limonene, **14**. (Z)-3-hexen-1-yl acetate. Note the relatively high contribution of compounds from the atmosphere.

Electrophysiology

In experiments on 69 preparations, a total of 85 single cells were analysed. Twentyfour cells did not respond to any of the applied stimuli. Of the remaining receptors, 22 responded strongly to 'green' odours (Fig. 4), whereas 22 other cells showed no response to odours, but responded to taste stimuli, i.e. silk extracts or salt solutions (Fig. 5).

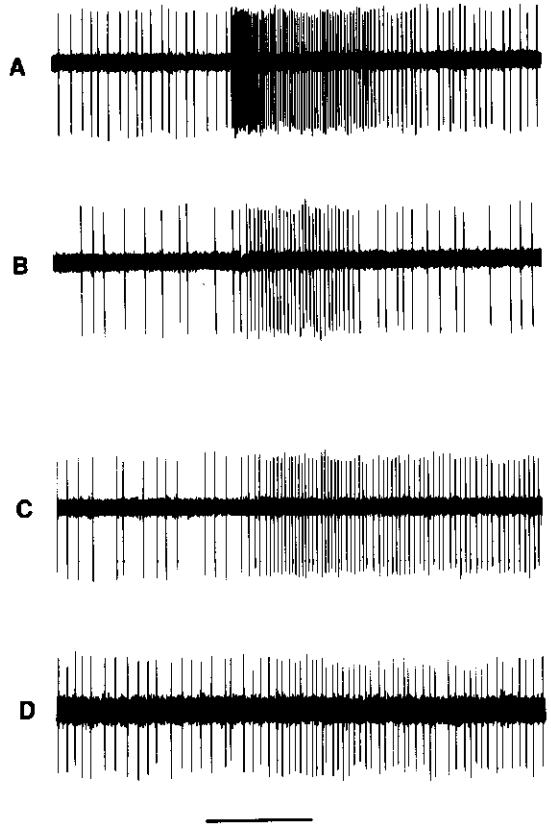

Figure 4A Responses of a (E)-2 hexenal 'best' cell in the maxillary palp of *Y. cagnagellus* to plant odour compounds. **A.** Hexanal 10^{-1}, **B.** Hexanal 10^{-2}, **C.** (Z)-3-hexen-1-ol 10^{-1}, **D.** (Z)-3-hexen-1-ol 10^{-2}. The stimulus period (1 second) is indicated by the horizontal bar.

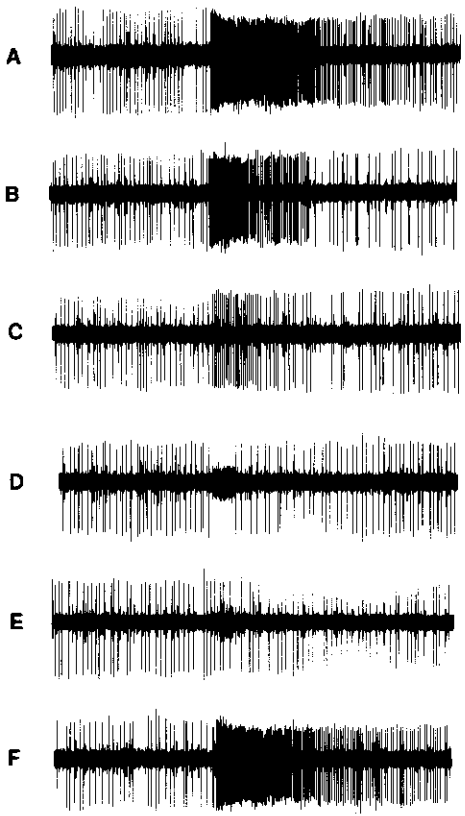

Figure 4B Responses of a (Z)-3-hexen-1-ol 'best' cell in the maxillary palp of *Y. cagnagellus* to plant odour compounds. **A.** (Z)-3-hexen-1-ol 10^{-1}, **B.** (Z)-3-hexen-1-ol 10^{-2}, **C** (Z)-3-hexen-1-ol 10^{-3}, **D** (E)-2-hexenal 10^{-2}, **E.** hexanal 10^{-2} **F** benzaldehyde 10^{-2}. Note in **D** and **E** the presence of an aldehyde-sensitive cell in the noise. The stimulus period (1 second) is indicated by the horizontal bar.

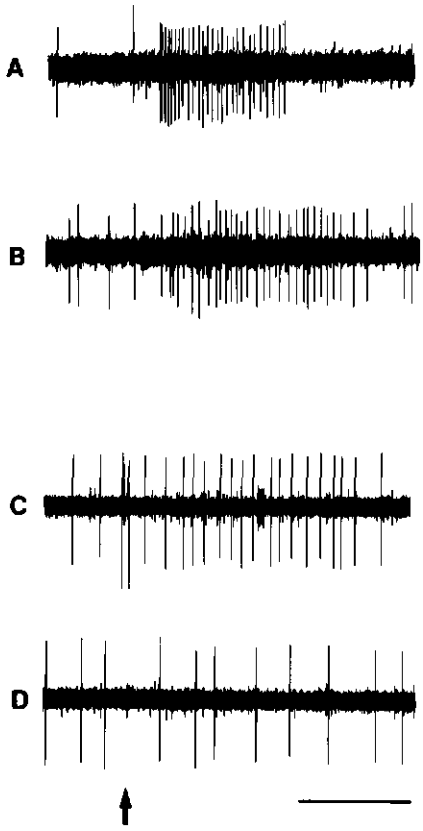

Figure 5 Responses of cells in the maxillary palps of *Y. cagnagellus* to gustatory stimuli. **A** & **B** Extract of *Y. cagnagellus* silk (200 mg/ml) **C** 100 mM KCl, **D** 10 mM KCl. The arrow indicates the onset of stimulation. The time marker indicates 1 second.

Response spectra of the 22 cells sensitive to the green odour complex are given in Fig. 6. Two groups can be distinguished. Cells 1 to 6 respond to the aldehydes (E)-2-hexenal and hexanal. The other cells respond to the leaf alcohol (Z)-3-hexen-1-ol and to 1-hexanol. In this latter group a continuity exists from cells responding mostly to 1-hexanol (cells 7-10) to cells responding strongly to (Z)-3-hexen-1-ol (cells 11-18). No cell responded to (Z)-3-hexen-1-yl acetate.

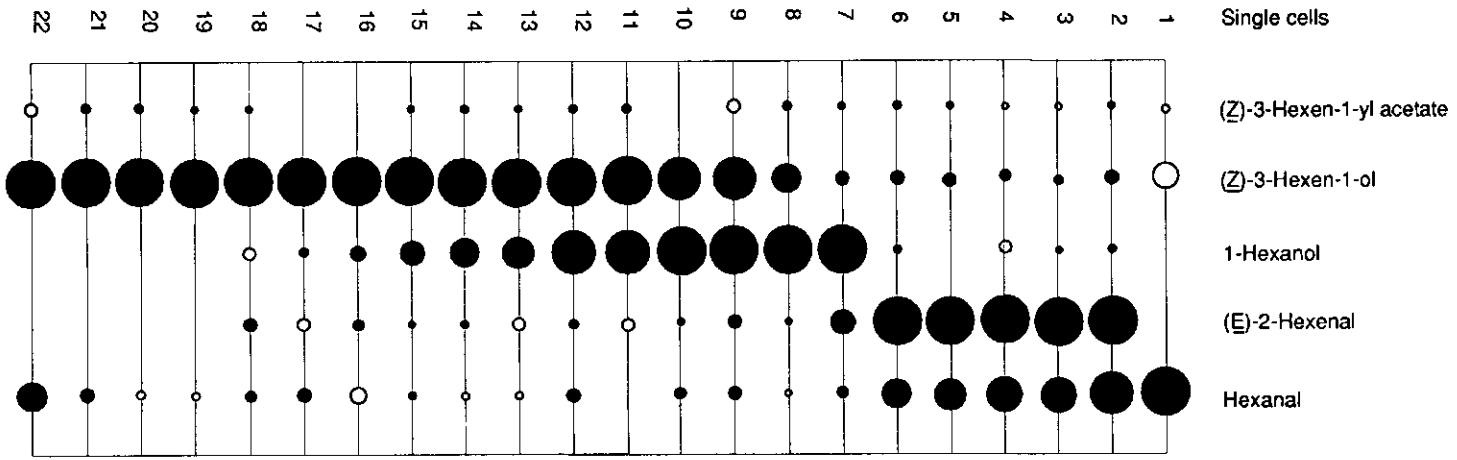

Figure 6 Relative response spectra for 22 cells sensitive to plant odour compounds in the maxillary palps of *Y. cagnagellus*. The spectra were obtained by setting a cells best response to 100 % and scaling all other responses accordingly. Relative response intensity is indicated by the diameter of the circles. Black circles indicate stimulation while open circles indicate inhibition. Note that empty spots are missing observations.

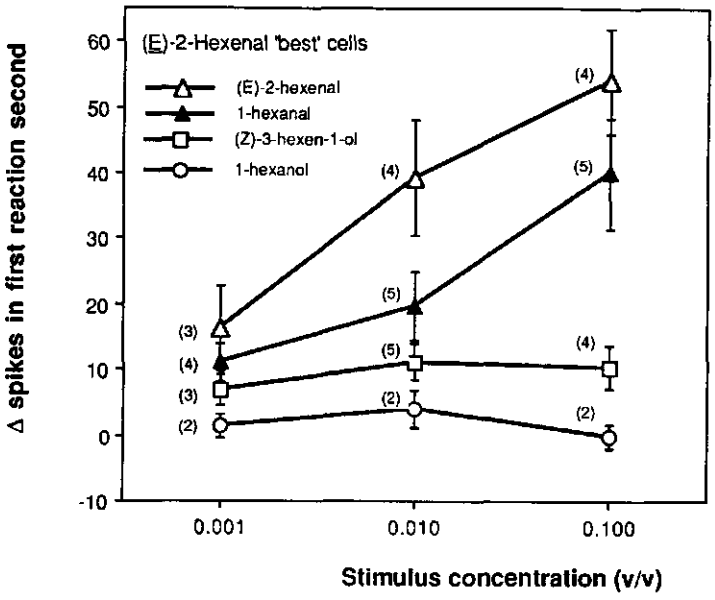

Figure 7A Dose-response relations of (E)-2-hexenal 'best' cells in the palp of *Y. cagnagellus*

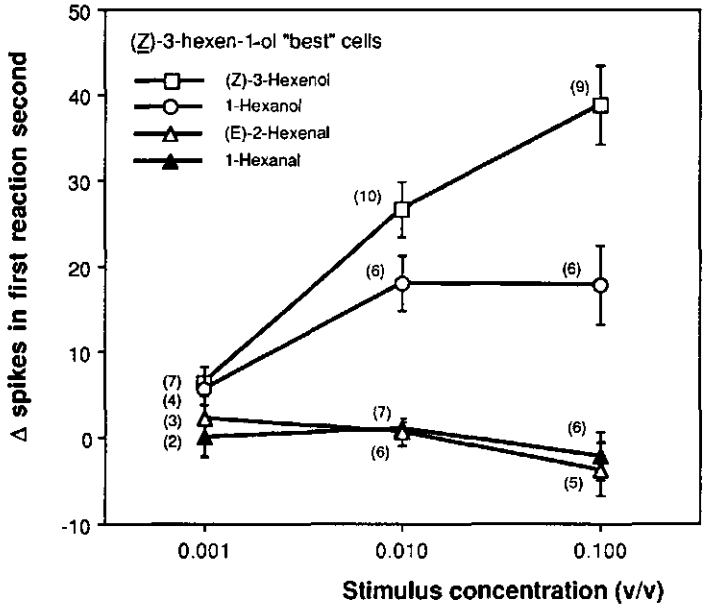

Figure 7B Dose-response relations of (Z)-3-hexen-1-ol 'best' cells in the palp of *Y. cagnagellus*

Dose respons relations for the two main cell groups are given in Fig. 7. In Fig. 7A the responses of the (E)-2-hexenal 'best' cells to the different odours are plotted. The same is done in Fig. 7B for the (Z)-3-hexen-1-ol 'best' cells.

The (Z)-3-hexen-1-ol and 1-hexanol 'best' cells were consistently sensitive to benzaldehyde (Fig. 4F). Evidence indicating that the sensitivity could be attributed to another cell (e.g. superpositions, irregular spike interval histograms) was not observed. The aldehyde 'best' cells did not show this sensitivity. Only two cells responded weakly to benzaldehyde (19% and 23 % of the response to (E)-2-hexenal).

No responses to the terpenoids limonene, (6 cells tested), citral (3 cells tested) and geraniol (5 cells tested) have been found.

Fig. 8 displays the results obtained with taste stimuli. A classification was made in 'silk extract' cells and 'salt' cells, according to each cells 'best' stimulus. In Fig. 8A the dose-response curves for KCl are plotted for the two cell groups. The salt 'best' cells show a log-linear dose dependency. The silk extract 'best' cells are significantly less sensitive to KCl ($p \leq 0.001$), but stimulation with 100 mM KCl clearly evokes a response.

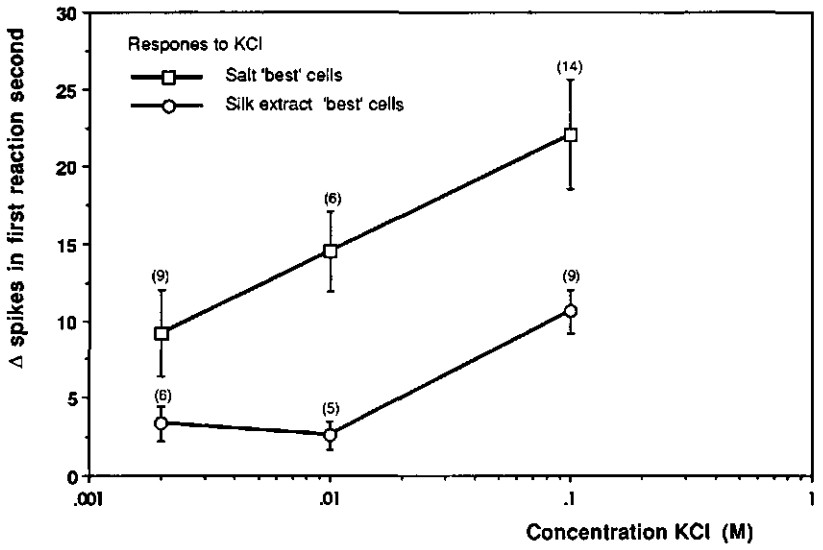

Figure 8A Dose-response relations of gustatory cell groups in the palp of *Y. cagnagellus* . Stimulation with KCl.

In Fig. 8B the sensitivity of the two cell groups to silk extracts is plotted. In this case the two curves overlap. At the two highest concentrations the salt 'best' cells respond also to the silk extract although the variation is large. Stimulation with an extract from *B. mori* silk (Fig 8B, black symbols) shows that the silk extract 'best' cells (more correctly:*Y. cagnagellus* silk extract 'best' cells) are in comparison to the salt 'best' cells significant less responsive to the *B. mori* extract (Anova on log transformed spike counts from individual stimulations, $p < 0.03$).

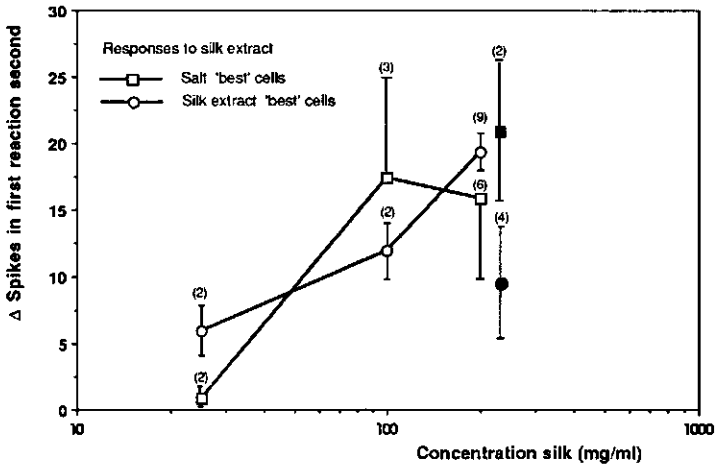

Figure 8B Dose-response relations of gustatory cell groups in the palp of *Y. cagnagellus*. Stimulation with silk extract from *Y. cagnagellus*. The black symbols represent *B. mori* silk extract (200 mg/ml). Vertical bars indicate SEM. The number of single cells on which the point was based is indicated in brackets

Fourteen cells responded selectively to CO_2 (Fig. 9). The dose-response curve (Fig. 10) is almost linear from 0.2 to 2.5 % CO_2. No significant response to other odour stimuli was found for these cells. All green odours sometimes inhibited spike activity, although this did not happen consistently, and only at high concentrations (10^{-1} v/v). The terpenoids limonene, citral and geraniol did not inhibit the CO_2 cells (N=14 cells).

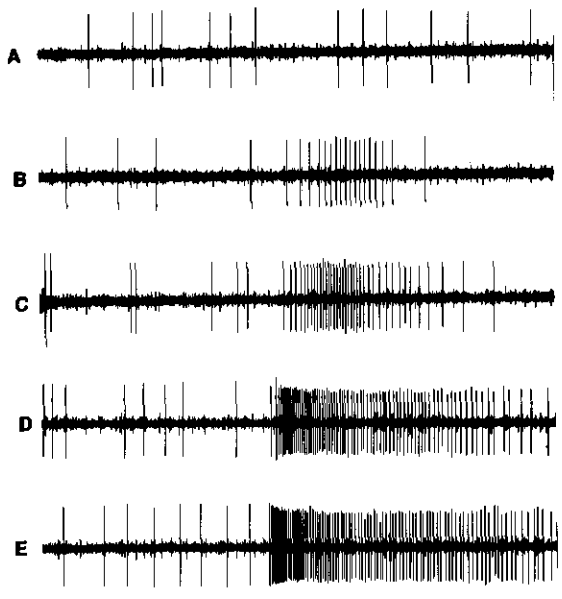

Figure 9 Response of a CO_2 sensitive cell in the maxillary palp of *Y. cagnagellus*. A to E are responses to respectively 0.26, 1.0, 1.5, 2.5 and 10 % CO_2. The time marker indicates 1 second.

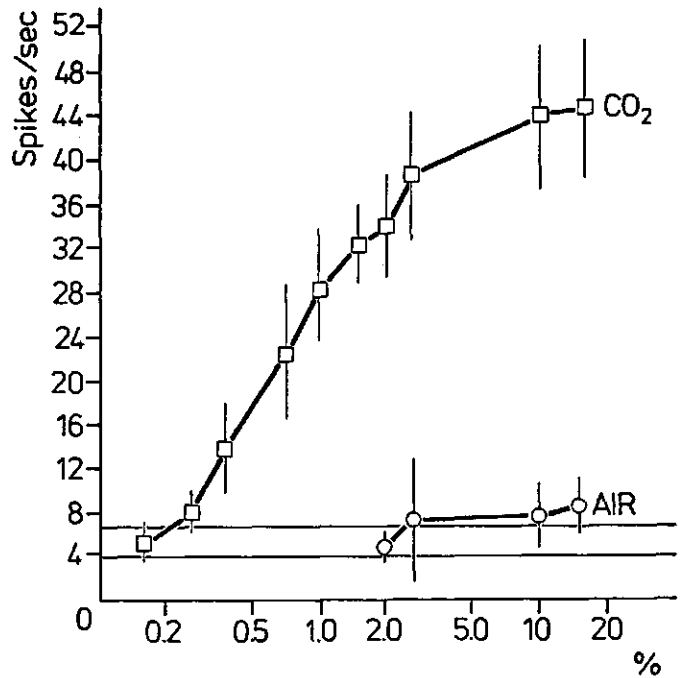

Figure 10 Dose-response relationship of 12 CO_2 sensitive cells in the palp of *Y. cagnagellus* stimulated with CO_2 or clean air. Vertical bars indicate 95 % confidence interval.

Three cells did not react to chemical stimuli but responded strongly to a drop in temperature (Fig 11).

The modalities were always clearly separated. Taste cells did not respond to odours, and olfactory cells were never observed to respond to taste stimuli. Moreover, cells with different modalities were also spatially separated. In multi-unit recordings olfactory and taste cells were never observed simultaneously. Also, since recordings containing either activity from a cold- or a CO_2 cell never contained any other active receptors with a comparable amplitude, it is likely that these cells are located at some distance from other receptor types.

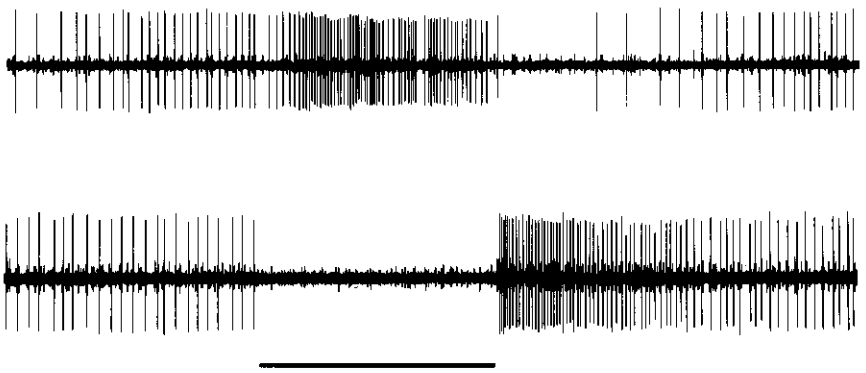

Figure 11 Response of a cold receptor in the maxillary palp of *Y. cagnagellus*. **A.** Stimulation with a cold metal rod at an upwind distance of about 10 mm. **B.** Stimulation with a warm metal rod at an upwind distance of about 10 mm. Stimulus bar 2.5 seconds.

Discussion

The external morphology of the sensilla on the maxillary palps conforms to the general pattern of ditrysian species as outlined by Grimes & Neunzig (1986). These authors divide the 8 sensilla on the apex in a medial group (M1, M2), an apical group (A1, A2, A3) and a lateral group (L1, L2). These groups can be recognised in *Y. cagnagellus*, although the layout of the sensilla is more linear than the generalised pattern (Fig. 2).

Grimes & Neunzig (1986) make a distinction between the more primitive, endophagous feeders, and larger, exophagous feeders. In endophagous species the walls of the A sensilla are smooth, and A2 is often two-tiered (styloconic). In the more advanced, exophagous groups A2 is basiconic and the A sensilla are wrinkled or pock-marked. The *Yponomeutidae* are regarded as relatively primitive within the ditrysia (Common, 1975), and indeed a styloconic A2 and smooth A sensilla are found (Fig. 2; see also van Drongelen 1979). It is possible that the slightly more tapered form of the L and M sensilla indicates a taste function for these structures, as found in many other species (Grimes & Neunzig, 1986). However, since neither apical nor wall pores were observed in *Y. cagnagellus*, definite conclusions about the modalities of these sensilla await ultrastructural investigations.

The wall of the most distal segment of the palp bears at least one campaniform sensillum. This sensillum is probably equivalent to the large plate sensillum described in *Euxoa messoria* (Devitt & Smith, 1982). The plate sensilla in this species are multiporous, multiple innervated, and possess branching dendrites. If this is also true for *Y. cagnagellus* these sensilla must be considered as olfactory organs, where the neural responses reported in this paper could originate.

In the wall of the most distal segment, a digitiform sensillum is present which is normally innervated by one neuron (Schoonhoven & Dethier, 1966; Albert, 1980; Devitt & Smith, 1982). In *E. messoria*, this sensillum appears to possess a laminated outer dendritic segment and no wall pores (Devitt & Smith, 1982). Laminated dendrites have been associated with cold receptors (see Steinbrecht, 1984; Altner & Loftus, 1985 for

reviews), as well as with CO_2 receptors (Chu-wang *et al.*, 1975; McIver & Siemicki, 1984; Lee *et al.*, 1985; Bogner *et al.*, 1986). In the latter case the neurons are housed in wall pore sensilla. In *Y. cagnagellus* both cold receptors and CO_2 receptors were found. Since digitiform sensilla have no pores (Devitt & Smith, 1982) the digitiform sensillum of *Y. cagnagellus* may function as a cold receptor.

Temperature reception could be used for monitoring the microclimate (Schoonhoven, 1967) or could play a role in determining leaf quality (Dethier & Schoonhoven, 1968). The function of the CO_2 receptor is less clear. CO_2 reception in phytophagous and saprophagous species is restricted mainly to soil dwelling organisms that must locate plant roots (Jones & Coaker, 1978). For larvae living above ground there seems to be no need for such a receptor, at least not in the context of host finding. During the day, CO_2 levels near the surface of green leaves will be 0.03% or lower, while this level may rise during nocturnal respiration. The observed sensitivity range of the CO_2 receptor makes it unlikely that this receptor is used to assess the respiratory status of the leaf. An alternative hypothesis is that the receptor monitors CO_2 levels during periods of tight clustering of larvae, or facilitates aggregation behaviour.

The interpretation of the responses to plant odour components is complicated by the fact that mechanisms of odour discrimination and identification in insects are still largely obscure (Visser, 1986; Boeckh & Ernst, 1987). Traditionally olfactory receptors have been divided into 'specialists' and 'generalists' (Boeckh *et al.*, 1965). Although still useful, it has become clear that these terms represent the two extremes of a continuum (see, for instance, de Jong & Visser, 1988), and numerous problems exist concerning their definition. Kafka (1987) has argued that true generalist receptors do not exist. He found that, although single cells in *Antheraea polyphemus* show broad and overlapping spectra, each is also highly selective. As a consequence he argued that odour generalists should be regarded as a subset of specialists.

In the experiments with *Y. cagnagellus* presented here not enough stimuli were tested to allow firm conclusions, but presently

two spectral types can be recognised: aldehyde sensitive cells and alcohol sensitive cells. The two groups are clearly separated from each other. It should be kept in mind, however, that this might be the result of the limited set of stimuli used. The reaction spectra for the Colorado potato beetle (Ma & Visser, 1978) also show only minor overlap between aldehyde and alcohol functional groups (see Visser, 1983). However, when the responses to (E)-2-hexen-1-ol are also taken into account, the separation disappears. Overlapping sensitivities are also found for the two aldehydes and for the two alcohols tested on *Y. cagnagellus*. In the last group all combinations (from cells mainly sensitive to 1-hexanol towards cells mainly sensitive to (Z)-3-hexen-1-ol) are present.

The fact that a single receptor cell reacts to different types of compounds is commonly interpreted as a reflection of the presence of several receptor 'sites' on the membrane (Kaissling, 1976; Selzer, 1984). In gustatory neurons this is even more clear (Hansen, 1978). The observed sensitivity from the (Z)-3-hexen-1-ol and 1-hexanol 'best' cells in *Y. cagnagellus* to benzaldehyde may arise from the presence of multiple receptor sites.

In the present study no response to (Z)-3-hexenyl acetate was found in the palps of *Y. cagnagellus*. However, receptors sensitive to this compound have been found in the antennae of this species (Roessingh, unpublished results) as well as in the antennae of *Pieris brassicae* larvae (Visser & de Jong, 1988). In this respect it is interesting to note that in *Manduca sexta* fibers from the maxillary nerve travel into a core of neuropile in the suboesophageal ganglion, that also receives antennal axons (Kent & Hildebrand, 1987).

The results from stimulation with taste substances indicate the presence of two cell types with limited specificity and respectively most sensitive to KCl and *Yponomeuta* silk extract. Each type shows a clear dose-response relationships for its best stimulus. The sensitivity of the salt 'best' cells to silk extract could be explained by the presence of other compounds in this rather crude stimulus mixture. This explanation is supported by the finding that these cells respond also to *Bombyx* silk, though it should be mentioned that the number of observations is rather

small. Just as with *Yponomeuta* silk, the *Bombyx* silk extract evokes activity in the salt 'best' cell. Apparently stimulatory compounds for this cell type are present in both silk extracts. The limited effectiveness of *Bombyx* silk on the silk extract 'best' cells is taken as evidence supporting the hypothesis that specific compounds in the *Yponomeuta* extract (e.g. the trail marker, Chapter 4) cause the response of this cell to *Yponomeuta* silk.

The maxillary palps of caterpillars contain 15-30 chemoreceptors (Schoonhoven & Dethier, 1966; Albert, 1980; Devitt & Smith, 1982). If the 85 cells in the present study had been chosen at random from the population in the palps, then the proportion of neurons sensitive to each of the applied stimuli could have been estimated. However, it must be stressed that the position of the electrode and details in the recording procedure strongly influence which cell type is found. A successful procedure for a certain cell type typically was continued until enough data were obtained to allow statistical analysis. As a consequence, the recordings by no means represent a random sample. Due to the limited lifetime of the preparation, only one recording per insect was made, further complicating the estimation of cell-type proportions. In spite of these limitations, however, it can be concluded that receptors for the tested terpenoids and for (Z)-3-hexen-1-yl acetate are relatively scarce (if present at all in this species), since no cells sensitive to these stimuli were found.

Acknowledgements

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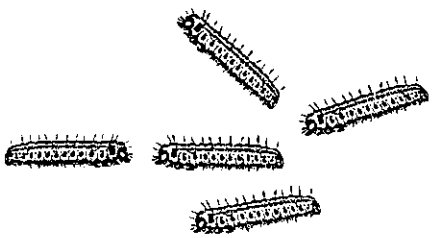

6 Foraging behaviour and the adaptive significance of trail following in larvae of *Yponomeuta cagnagellus*.

Peter Roessingh & Mart C. M. De Jong

Abstract

Larval foraging behaviour and migration patterns of *Yponomeuta cagnagellus* Hübner (Lepidoptera: Yponomeutidae) were studied. The larvae live in a communal nest on their host *Euonymus europaeus* and move to new locations up to 85 cm away about four times during development. Most movements take place after local depletion of food. In contrast to the central place forager *Malacosoma neustria* (Hübner), studied for comparative purposes, *Y. cagnagellus* larvae do not show recruitment behaviour. The possible advantages of trail following and gregarious behaviour are discussed with the help of a simple evolutionary model. It is suggested to classify larval foraging strategies on the basis of the moment at which larvae become solitary.

Introduction

The use of chemical signals to facilitate group formation and cooperative foraging, a principle well known from social insects, has recently also been studied in gregarious lepidopterous larvae (for review, see Fitzgerald & Peterson, 1988). In their analysis of the links between foraging behaviour and trail pheromones these authors broadly distinguish 3 types of foraging behaviour: patch restricted foraging, nomadic foraging, and central place foraging. The patch restricted strategy is defined as feeding on a contiguous patch of leaves by systematically moving to adjacent intact leaves. Nomadic foragers make frequent moves to new, often distant feeding sites and may abandon a feeding patch before it is completely depleted. The last, and least common, strategy is

central place foraging. The best known examples of this behaviour occur in the genus *Malacosoma* (Fitzgerald, 1976; Peterson 1987, 1988). In *Malacosoma americanum*, feeding takes place during excursions from the central nest site, and routes to food sources are marked by brushing a sternal secretory site against the substrate (Fitzgerald & Edgerly 1982; Peterson 1988). The signal appears to be used for elective recruitment and is contingent upon each larva's assessment of the food quality at the feeding site (Fitzgerald & Peterson, 1983; Peterson 1987; Peterson, 1988).

Fitzgerald and Peterson (1988) predict that the type of trail communication is dependent on the type of foraging behaviour. Patch restricted caterpillars are predicted to use relatively simple communication systems (e.g. demarcation of the foraging arena), while recruitment pheromones are expected in central place foragers. This theory is indeed supported for central place foragers (Fitzgerald & Peterson 1983; Fitzgerald & Peterson, 1988; Peterson, 1988) but more information is needed for the other behavioural types.

In this paper we investigate the foraging behaviour of *Yponomeuta cagnagellus* (Hübner). Trail following is known to occur in this species (Chapter 2; Roessingh *et al.*, 1988), and the use of a chemical trail marker has been established (Chapter 4). To determine how these observations relate to the biology of the caterpillars, details on the foraging behaviour were collected. Migration in the field was assessed for 17 groups during development from the first stadium to pupation. In addition, group movements of *Y. cagnagellus* in the laboratory, and for comparative purposes those of the central place forager *Malacosoma neustria* (Hübner), were studied.

Studies of lepidopterous larvae frequently invoke the possible advantages of group living as an explanation for the existence of trail following systems (Butler, 1970; Capinera, 1980; Fitzgerald & Costa, 1986; Fitzgerald & Peterson, 1988; Chapter 4) However, it should be noted that there are also distinct disadvantages associated with gregariousness if food is limited (Charnov *et al.*,

1976; Schultz, 1983). We developed a simple qualitative model to gain insight into the balance between advantages and disadvantages of gregarious behaviour, and thus into the evolutionary consequences of trail following.

Materials and methods

Field observations

Y. cagnagellus moths lay their eggs in batches. The larvae hatch at the end of the summer but remain as first instars under the egg cover until next spring. Around the time the leaves flush, the larvae migrate to the bursting buds and mine into them.

Field observations were conducted on groups of caterpillars on a naturally infested host tree (*Euonymus europaeus*) in Wageningen, The Netherlands. After larval establishment (in the first week of May), 20 infested buds were located and labelled. Nearby groups were removed to prevent interference at later stages of development. The position and size of each nest was recorded daily. If groups fragmented, the position of each subgroup was assessed. Distances were measured along the connecting branches. Nest size was measured as the largest diameter of the nest. The amount of food remaining in deserted locations was scored using 3 categories: no food available, food available at the periphery of the nest, and food available in the nest itself. For each of the groups the observation was continued until the larvae left the tree for pupation (in the first week of June). No counts of the exact number of larvae were made since this would have required the partial destruction of the nest, causing interference with normal behaviour.

Although care was taken to remove nests in the neighbourhood of each group under observation at the beginning of the experiment, 4 groups fused during their development. These were treated as one single group.

Lab observations

One day old, fifth instars of *Y. cagnagellus* and third instars of *M. neustria* were placed on branches of host trees (respectively *E. europaeus* and *Quercus robur*) in groups of 20. After the formation of a nest, the branch was connected by a paper strip (5 mm wide, 20 cm long) to a new branch.

When the food on the first branch was almost depleted (after about 24 hours) the larvae began to explore their environment and eventually found the new food on the other side of the strip. The onset of exploration was defined as the moment the first larva reached the paper strip. The foraging pattern was observed for 6 groups of *Yponomeuta* and 3 groups of *Malacosoma* larvae. All observations were conducted under continuous light at 21°C and ambient humidity.

Table 1 Summary of field observations on nest building and foraging behaviour of 17 groups of *Y. cagnagellus* larvae

<table>
<tr><th>Number of movements</th><th>Food situation at moment of movement</th><th>Observations on distance moved</th></tr>
<tr><td rowspan="4">57 movements to new locations</td><td>40 times no food left</td><td rowspan="5">74 distances measured</td></tr>
<tr><td>8 times food in periphery</td></tr>
<tr><td>5 times food in nest</td></tr>
<tr><td>4 missing values</td></tr>
<tr><td colspan="2">20 movements resulting in nest fusions</td></tr>
<tr><td colspan="2">22 movements away from the plant for pupation</td><td>3 missing values</td></tr>
</table>

Results

Field observations

A summary of the field observations on nest building and foraging behaviour of *Y. cagnagellus* is given in Table 1. From 17 groups a total of 99 nests (and the accompanying location shifts) were observed. The frequency distribution of the movements (Fig.1) has a modus and a median of 4, and is skewed towards higher numbers of shifts.

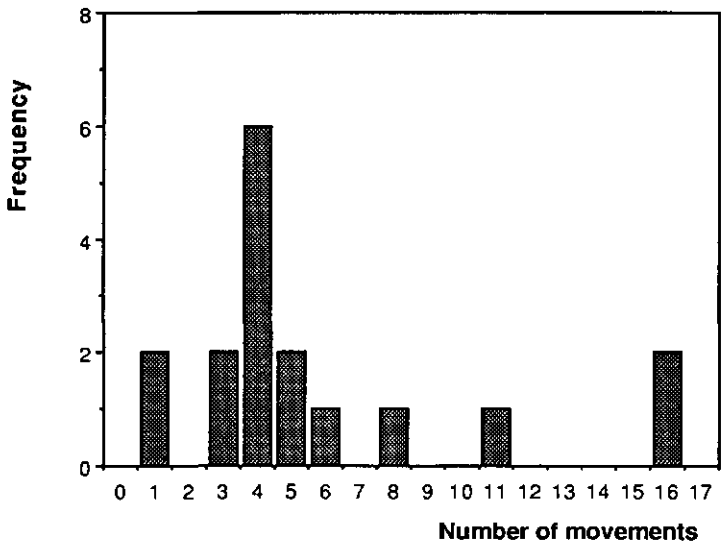

Figure 1 Frequency distribution of 99 movements of 17 groups of *Yponomeuta cagnagellus* on the host tree *Euonymus europaeus* during development from first instar to pupation.

When the total number of movements for each group is plotted against the final nest size (as an indicator for the number of larvae), a significant positive correlation is found (Fig. 2, r = 0.74; p<0.001). A significant correlation remains even when the large fused group is excluded from the analysis (r=0.6; p< 0.013). This indicates that bigger groups move more often.

The distribution of the distances travelled is presented in Fig. 3. The final movements to the ground for pupation (22 cases), as well as 3 missing observations, are excluded from this figure. The resultant distribution has a long tail; although the median movement is 10 to 15 cm, movements up to 85 cm were observed.

In abandoned nests (53 cases) the availability of food was determined. (Fig. 4) (22 pupation movements, 20 nest fusions and 4 missing observations were excluded). 75,5 % of all movements (40 cases) took place when all available food was eaten. For 15% (8 cases) there was food available in the periphery of the nest, and for the remaining 9.5% (5 cases) there also was food left in the nest itself.

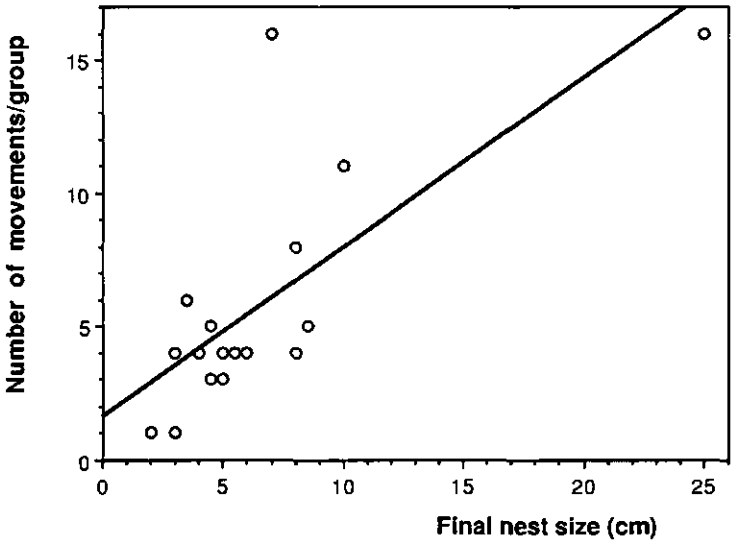

Figure 2 Relation between the total number of movements and the final size of the last nest for 17 groups of *Yponomeuta cagnagellus* on the host tree *Euonymus europaeus*.

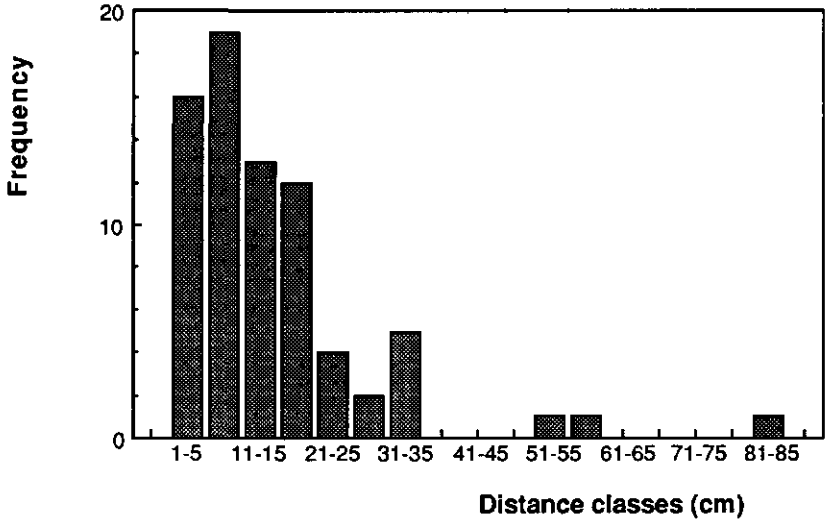

Figure 3 Frequency distribution of travelled distances to new locations for 57 movements of groups of *Yponomeuta cagnagellus* on the host tree *Euonymus europaeus*.

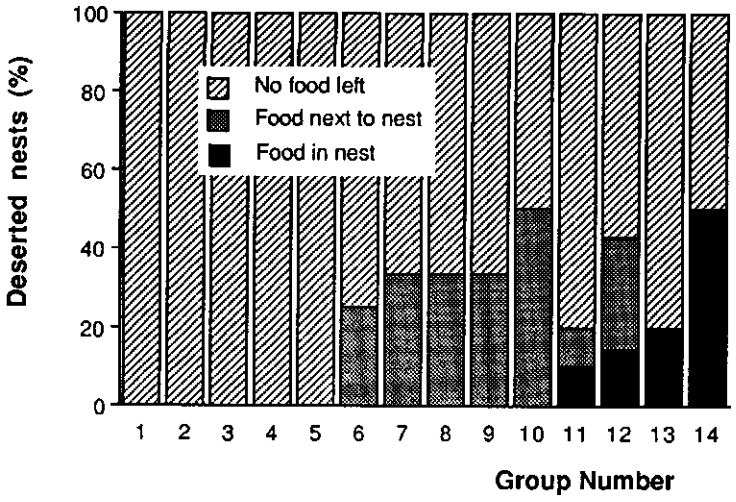

Figure 4 Availability of food in 53 deserted nest of 14 groups of *Yponomeuta cagnagellus* on the host tree *Euonymus europaeus*.

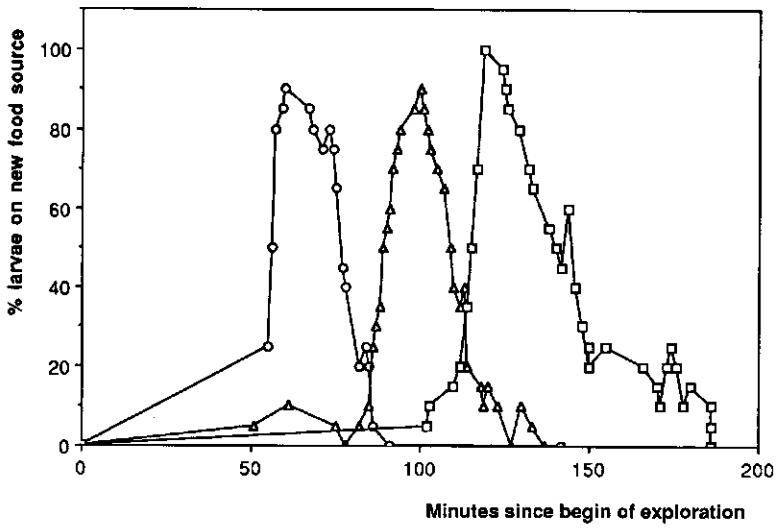

Figure 5 Foraging behaviour of 3 groups of third stadium *Malacosoma neustria* larvae in a laboratory assay with two disjunct food patches. Symbols indicate different experiments.

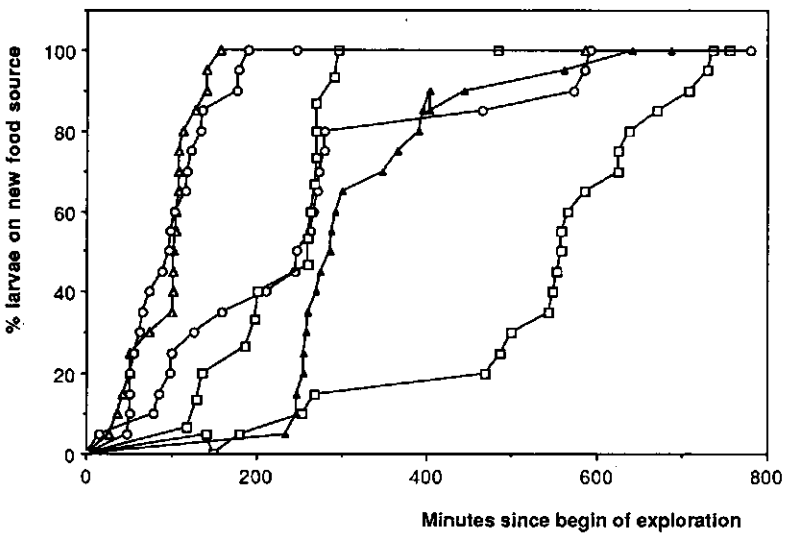

Figure 6 Foraging behaviour of 6 groups of fifth stadium *Yponomeuta cagnagellus* larvae in a laboratory assay with two disjunct food patches. Symbols indicate different experiments.

Lab observations

The results from observations of the foraging behaviour of *Y. cagnagellus* and *M. neustria* in experiments with one old and one new feeding station are given in Fig. 5 and 6. The percentage of larvae present on the new food source is plotted against time. *Malacosoma* larvae display a typical central place foraging strategy. After feeding at the new location, all caterpillars return to the old nest site. Foraging excursions are synchronized. While foraging, larvae often move back and forth between the two locations, as indicated by the waves superimposed on the main peaks in Fig. 5.

Yponomeuta larvae on the other hand, display a different foraging pattern. Although migration is still synchronized, the process takes longer than in *Malacosoma* (Mann-Whitney U test, $p \leq 0.05$) After finding the new food source the larvae do not return to the old nest. From the 116 observed larvae in 6 groups, only 2 individuals went briefly back (1.7%). Since returning to the old nest is a prerequisite for recruitment behaviour, this strategy is not used by *Y. cagnagellus* (Fig. 6).

Discussion

Experimental data

From our results it follows that, in contrast to the situation in the genus *Malacosoma*, recruitment cannot be considered as an important factor underlying the trail following behaviour of *Y. cagnagellus*. The caterpillars show a combination of patch restricted and nomadic foraging. The nomadic aspect is shown in the repeated movements to new feeding sites (Fig. 1), and the occasionally long distances travelled (Fig. 3). Patch restricted behaviour is reflected in the fact that when sufficient food is available the nest may be expanded continuously to form a single patch. Also, in general all available food is consumed before the group moves to a new feeding site (Fig. 4). Together with the observation that larger groups (with high overall food consumption)

move relative often, this indicates that migration in *Y. cagnagellus* is induced by food shortage.

Given this type of behaviour, the use of a trail pheromone could be advantageous if maintaining group cohesion is beneficial. Many authors have discussed the possible advantages of living in a group. An important aspect is the ability to produce a silk nest. A group can make a bigger and stronger structure than a single caterpillar. The nest may facilitate control of the microclimate (Strong *et al.*, 1984; Tsubaki, 1981), improve thermoregulation (Porter, 1982; Knapp & Casey 1986), and function as holdfast and anti-predator structure (Capinera, 1980; Tsubaki & Kitching, 1986). In addition, gregariousness can increase survival rate, at least for early instars (Ghent, 1960 (for sawfly larvae); Capinera 1980; Tsubaki, 1981; Weaver, 1988), and defense against predators may be more efficient (Meyers & Smith, 1978; Stamp, 1981). However, it should be noted that distinct disadvantages of gregariousness also exist. The caterpillars are faced with a set of conflicting demands (Schultz, 1983). Members of a group depress the availability of food to themselves (Charnov *et al.*, 1976), and frequent movement to new feeding sites may be associated with an increased risk of mortality (Morimoto 1979, cited in Tsubaki & Shiotsu 1982; Capinera 1980; Bergelson & Lawton 1988; Steward *et al.*, 1988). This cost has not always been recognized. For instance, the conclusion of Tsubaki & Shiotsu (1982) that group feeding can be beneficial because it promotes more efficient resource utilization relies on the assumption that the costs of frequent movements to new feeding sites are negligible.

In summary, the question emerges why larvae of *Y. cagnagellus*, and in a more general context all gregarious caterpillars, stay together in spite of the resulting competition for food.

Evolutionary interpretation

To gain insight into this problem and the possible role of trail pheromones, it is useful to consider larval behaviour from an evolutionary point of view. *Y. cagnagellus* larvae initially start as a

group because females deposit eggs in batches. Since there is a disadvantage associated with gregarious behaviour (local food depletion) solitary foraging will be favored by natural selection unless group foraging offers commensurate advantages. When such advantages exist, any Evolutionary Stable Strategy (ESS) will require that larvae remain together until the local food supply is depleted. Following depletion of food, larvae can either adopt a strategy that leads to solitary food searching, or one that leads to re-grouping at a new feeding site.

As long as gregarious behaviour confers a net advantage upon the individual, the ESS for a larva will be to follow the others when they leave the depleted site. To do this, the larva might use cues, left by departing larvae. Furthermore, if being a member of a group is advantageous, it will also be to the advantage of the departing larvae to assure that others will follow. One way to achieve this is to produce trail pheromones. Thus, to gain insight into the evolutionary consequences of the use of trail communication, the advantages and disadvantages of gregarious behaviour must be evaluated.

The adaptive significance of larval gregariousness is at present not well understood (Sillén-Tullberg, 1988). We therefore developed a simple model, in which larvae follow different strategies at the moment of food depletion. The aim of the model is to determine the circumstances under which gregarious behaviour is an ESS, i.e. circumstances under which the population can not be invaded by larvae with solitary behaviour.

Two larval types are modelled: 1] A type with a group strategy, which responds to, and/or produces trail pheromones, i.e. a larva whose behaviour after food depletion contributes to the maintenance of the group, and 2] a solitary foraging type that neither produces nor responds to trail pheromones. In this analysis we only evaluate the larval strategies and ignore possible evolutionary changes in oviposition behaviour. As a consequence, the initial group size is only determined by the size of the egg batch produced by the female moth. For simplicity it is also assumed that each larva follows only one of the two strategies and that generations are discrete.

The results in this paper show that food shortage effects the migration behaviour of *Y. cagnagellus* We therefore distinguish in the model between two factors that influence larval mortality: mortality due to local food depletion (i.e. risks involved in searching for a new feeding patch after local food depletion), and all non-food related mortality factors, e.g. predation, temperature effects, etc. The following symbols are used:

g_t	=	Number of larvae with a group strategy in generation t
s_t	=	Number of larvae with a solitary strategy in generation t
R	=	Number of offspring per surviving larva
$P_{food,1}$	=	Probability of finding sufficient food to survive for a larva with a solitary strategy
$P_{food,n}$	=	Probability of finding sufficient food to survive for a larva with a group strategy in a group of size n
$P_{other,1}$	=	Probability of surviving non-food-related mortality factors for a larvae with a solitary strategy
$P_{other,n}$	=	Probability of surviving non-food related mortality factors for a larva with a group strategy in a group of size n

The number of larvae of each type in the next generation can be calculated from the number in the current generation multiplied by the number of offspring and the survival probabilities:

$$s_{t+1} = s_t \cdot R \cdot P_{food,1} \cdot P_{other,1} \quad (1a)$$

$$g_{t+1} = g_t \cdot R \cdot P_{food,n} \cdot P_{other,n} \quad (1b)$$

If one is interested only in the frequency of the group strategy in the population (cf. De Jong & Sabelis 1988; Sabelis & De Jong 1988) it suffices to determine whether the growth rate for the group strategy ($R \cdot P_{food,n} \cdot P_{other,n}$) is larger than that of the solitary strategy ($R \cdot P_{food,1} \cdot P_{other,1}$) or not. Thus for R being the same for both strategies the outcome of the evolution is determined by:

$$P_{food,n} \cdot P_{other,n} - P_{food,1} \cdot P_{other,1} \quad (2)$$

When (2) is positive the group strategy will be the ESS and when (2) is negative the solitary strategy will be the ESS. After division by $P_{food,1} \cdot P_{other,n}$,to allow convenient graphical analysis we obtain:

$$(P_{food,n} / P_{food,1}) - (P_{other,1} / P_{other,n}) \qquad (3)$$

When (3) is positive the group strategy is evolutionary stable, and when (3) is negative, the solitary strategy is stable.

The value of (3) depends on the balance between the advantages and the disadvantages of being a member of a group of size n. In Fig. 7A $P_{food,n}/ P_{food,1}$, (the relative 'advantage' due to food competition of an individual in a group of size n over a solitary individual), is plotted against n. For a group size of one, $P_{food,n}$ is the same as $P_{food,1}$, and thus the ratio of these probabilities will equal one. For a group size larger than one, the probability that a larva dies due to local food shortage increases strictly with n. The slope depends on the amount of locally available food and the probability to find more food in the immediate vicinity. If this is sufficient to sustain all larvae, there will be no competition for food in the group and the slope will be zero (Fig. 7A, curve c). As food becomes increasingly limited, competition becomes more intense, i.e. the slope becomes steeper (Fig. 7A, curve a & b). As Fig. 4 indicates, competition for food is likely in *Y. cagnagellus*.

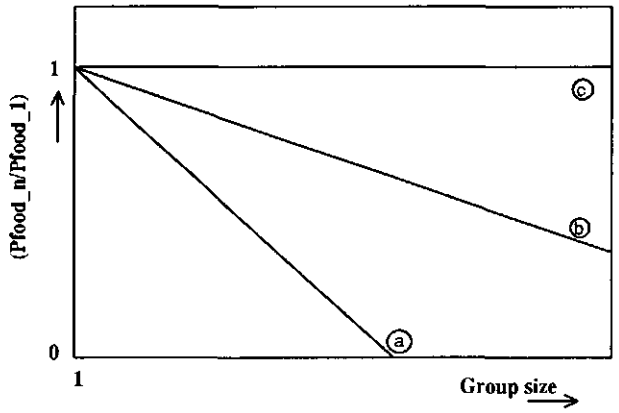

Figure 7A The relative advantage due to food competition of an individual in a group size n over a solitary individual. The curves a) to c) apply to situations with an increasing amount of available food.

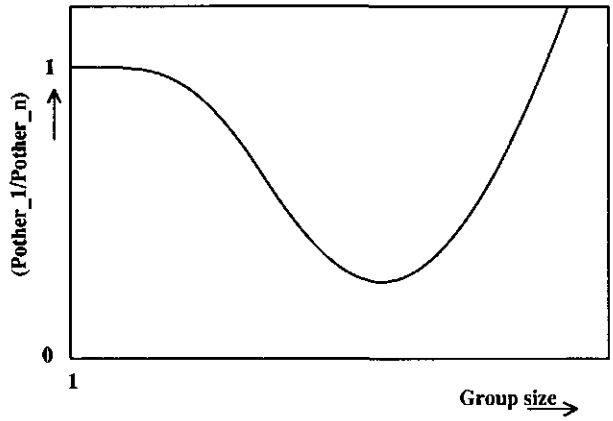

Figure 7B The relative advantage due to non-food causes of a solitary individual over an individual in a group size n. See text for explanation of the curve shape.

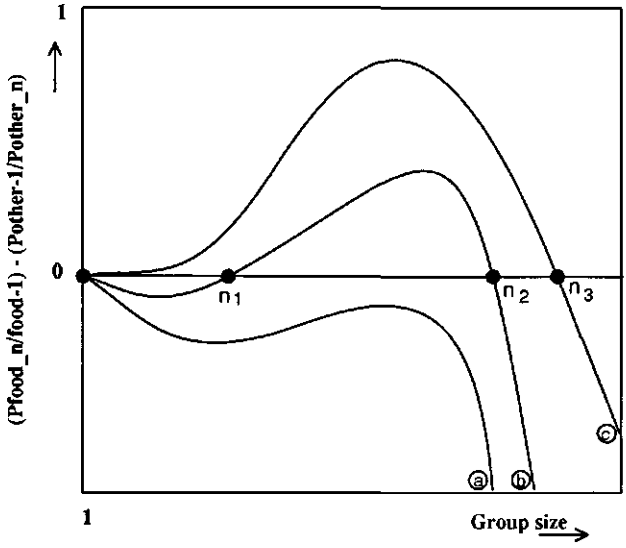

Figure 7C The difference between the relative advantages for gregarious and solitary individuals as given in Fig. 7A and 7B. For positive values of this expression gregarious behaviour is an ESS.

Fig. 7B depicts $P_{other,1}$ / $P_{other,n}$ (the relative 'advantage' due to non-food related factors of a solitary individual over an individual in a group of size n). We assume that the chance of survival in a group increases

with the number of resident larvae (for instance due to positive effects of communal nest building); thus, $P_{other,1}$ / $P_{other,n}$ initially decreases as group size increases. However, this advantage may disappear at larger group sizes, for instance because a larger production of faeces may attract predators or may increase the chance of mortality from disease. Thus, the ratio increases above a certain group size (Fig. 7B).

Subtraction of $P_{other,1}$ / $P_{other,n}$ from $P_{food,n}$/ $P_{food,1}$ yields expression 3, which is presented in Fig. 7C, and from which inferences about evolution of larval behaviour can be made. Three situations can be distinguished:

a) Food is such a limited resource that its effect on survival always outweighs the risks of leaving the group (curve a). Under such circumstances, the group strategy is never an ESS

b) Competition for food only outweighs non-food related factors if the group size is small or large relative to the food supply. The group strategy is an ESS if the initial group size is located between n_1 and n_2 (curve b)

c) When sufficient food is available it is always an advantage to be a member of a group, unless the group is larger than n_3 (curve c).

In summary, the favored larval strategy depends on the initial group size (egg batch size), and on the relative advantages and disadvantages to larvae of being in a group of that size. This in turn depends partly on the distribution of food. Curves a to c in Fig. 7C reflect situations with increasingly more food and/or increasingly less advantage from being in a group. For positive values of these curves the group strategy is evolutionary stable.

From this analysis a number of predictions can be made, some of which can be easily tested. Gregarious behaviour and trail following are not expected in habitats with small isolated patches

of food, because then the disadvantages of local food depletion are the largest (curve a). From the model, it is also clear that when food competition is less intense (curve c), e.g. in trees or when plants are clumped, it is more likely that gregarious behaviour will evolve. These predictions seem to be supported by the work of Stamp (1980) and Courtney (1984), and can be tested by a review of the literature on host plants of solitary and gregarious species.

The disadvantage due to food competition that a larva in a group experiences will increase with any increase in group size. On the other hand, the positive effects of a group are expected to arise mainly at larger group sizes. Because of this asymmetry, the group strategy is not likely to be an ESS for small groups, as indicated by the position of n1. (curve b) Therefore, gregarious behaviour and trail following are not likely to evolve when the initial group size (i.e. egg batch size) is small. This result agrees with the view of Courtney (1984), who argues that batch laying is a necessary first step in the evolution of gregarious behaviour.

Although the model applies to lifetime strategies for larvae, the results can be extrapolated to age dependent strategies. The benefits of being in a group are especially important in early instars (Ghent, 1960; Capinera, 1980; Tsubaki & Kitching 1986). In Fig. 7B this may be depicted by a curve that extends less far towards zero. On the other hand, the competition for food will be more intense during later stadia, when the caterpillars consume food at a high rate. This results in steeper slope in Fig. 7A. The combined effect is that n1 and n2 in Fig. 7C will be closer together. As a consequence the range of group sizes for which gregarious behaviour is an ESS becomes smaller during development, and the probability that gregarious behaviour disappears in later instars will increase. This indeed is a common observation in many caterpillar species. (e.g. Tsubaki, 1981; Larsen, 1987; Fitzgerald & Peterson, 1988).

The behaviour of *Y. cagnagellus* does not fit the behavioural types distinguished by Fitzgerald & Peterson (1988), but can be easily explained from the evolutionary interpretation of individual behaviour given here. We suggest that larval behaviour should be classified by the age at which larvae switch from a gregarious to a solitary strategy.

Contrary to Fitzgerald & Peterson (1988) we do not consider it likely that trail following evolves from solitary behaviour but, instead suggest that selection favors trail following in batch laying species because it allows the initial group to remain together for a longer time.

Acknowledgements

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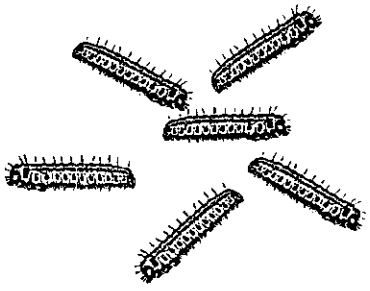


7. Computer-assisted analysis of electrophysiological data from insect sensilla.

Peter Roessingh, Hans Visser, Herman Ament & Harm Wezel

Abstract

Electrophysiological investigations of insect chemoreceptors are hampered by the massive amounts of data that must be analyzed. A variety of computer programs have been written to deal with this problem, but a fully satisfactory solution is not yet available. In this paper the development and use of such a program is reported. It is concluded that spike analysis software should be viewed as a set of tools to display and manipulate the data in an interactive way. These facilities should guide the user in his decisions concerning the course of the analysis. The possibility to re-analyze data with different parameter settings and flexible cross-referencing between different display facilities are identified as the most important aspects of a successful program.

Introduction

Single unit recordings from insect sensilla provide the basis for theories about perception of stimuli, sensory coding and neural integration (Boeckh & Ernst, 1987). In addition these data are essential for the correct interpretation of ultrastructural details of sensilla (Altner & Prillinger, 1980; Zacharuk, 1980). Although the electrophysiological technique is more then 30 years old (Hodgson *et al.*, 1955; Schneider & Hecker, 1956), progress in the field has been relatively slow. This is partly due to the extensive labor involved in the analysis and interpretation of raw spike data (Hanson *et al.*, 1986). It is generally accepted that the full potential of electrophysiology will not be realized until this analysis is made less burdensome.

Attempts to write computer programs that accomplish the task of data acquisition and analysis are numerous (e.g. Gerstein & Clark, 1964; Kent, 1971; O'Connell *et al.*, 1973; Piesch & Wieczorek, 1982; van der Molen *et al.*, 1978; van Drongelen *et al.*, 1980; Teyler, 1981; Hanson *et al.*, 1986; Mitchell & McIntyre, 1986; Mankin *et al.*,. 1987). Yet the facts that new programs continue to be proposed and that no standard method has arisen, suggest that none of these programs is fully satisfactory.

On the other hand, several techniques and algorithms for separation and classification of spikes are available (e.g. Gerstein & Clark, 1964; Smith *et al.*, 1985; see Frazier and Hanson, 1986 for review). In the present paper we describe the development of a spike analysis program and identify critical aspects in the design of this type of program.

The signal

Due to the problems associated with data analysis, electrophysiological research of insect sensilla focuses at present on systems with relative few active units. Recordings typically consist of activity from one to four sensory cells, often with a poor signal to noise ratio. Several aspects of the signal complicate automated data analysis.

In taste receptors, changes in the conformation of the apical pore may influence the responsiveness of the sensilla (Bernays *et al.*,1972; van der Wolk, 1984). These changes can be very rapid (Kramer & van der Molen, 1980) and can cause variations in latency time and contact artefacts. The unpredictable variability that results from these effects generally preclude a totally automatic analysis and requires great flexibility in the procedure and systematic monitoring of each step. In addition the spike-shape is dependent on characteristics of the preparation, the amplifier, and on details of the recording procedure. Amplitudes and shapes of spikes may also change with spike frequency (Fujishiro *et al.*, 1984; Bowdan, 1984). This limits the use of more rigid programs to only a few types of preparations. The constant need for operator control and supervision was recently stressed by

Hanson *et al.* (1986) and Frazier & Hanson (1986). It is therefore concluded that a truly interactive approach, as advocated by O'Connel (1973) and van Drongelen *et al.* (1980), is the most promising method for a successful analysis program.

The Process

The process of data analysis can be viewed as a chain of discrete steps:

1) Data acquisition. This step involves the translation of analog spike data, either directly from the preparation or from tape, into a machine readable form, i.e. analog to digital conversion.
2) Spike/noise separation. This step accomplishes the separation of spikes from the background noise.
3) Spike classification. Once the relevant portions of the signal are known, each spike must be assigned to one of several active neurons, i.e. the activities from different sensory cells must be sorted.
4) Cell characterization. After the assignment of spikes to specific sensory cells, the response characteristics of these cells can be described. This step yields the final quantification of the response, used for further analysis and interpretation.

As stated above, a high degree of operator supervision is necessary in order to prevent the generation of artefacts. In the program described here, a loop is created for each of the steps (Fig. 1).

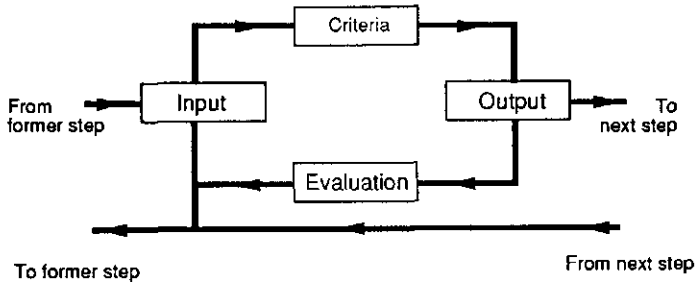


Figure 1 Each step in the analysis process should consist of a tight feedback loop in which the user repeatedly can specify criteria and evaluate the output.

In this loop, input data is taken and processed using user-defined criteria. The resultant output is displayed and evaluated by the operator. This interactive process of analysis and evaluation is repeated, with different criteria, until the output is acceptable. The next step uses this result as input (Fig. 2).

In the following section the implementation of the program will be discussed and attention will be given to some of the encountered problems.

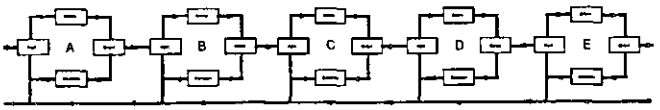


Figure 2 The complete analysis consists of series of steps. **A.** trigger level adjustment. **B.** AD-conversion, **C.** spike/noise separation, **D.** spike classification, **E.** cell characterisation.

The program

Data acquisition

There are two approaches to data acquisition. The first method involves taking a continuous stream of samples over the period of interest (2 to 3 seconds). Although this method is easy, it uses extensive amounts of memory. Given a minimum sample rate of 10 kHz (Frazier & Hanson, 1986), 3 seconds of data take up at least 60 kBytes of memory. Although this nowadays is not a real problem anymore, handling of the large datafiles can be complicated and slow. Therefore we use an alternative approach, and sample only those parts of the signal that contain relevant information. The timing diagram is given in Fig. 3. Two separate trigger units, controlled by the program, are used. The first two traces in Fig. 3 show the analog spike signal and a separate channel with a pulse indicating the stimulus period. This channel also contains a pre-pulse occurring one second before the stimulation.

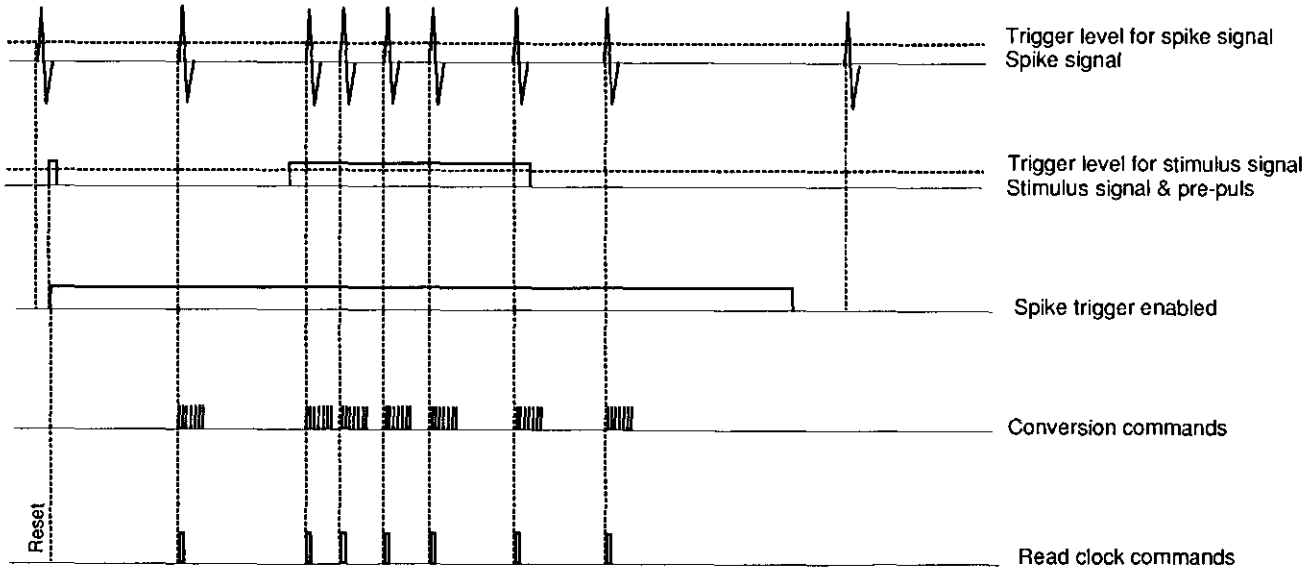


Figure 3 Timing diagram of the AD-conversion step. The spike trigger is enabled for 3 seconds by a pre-stimulus pulse. During this period trigger events in the spike channel cause a series of conversion commands (3 msec. max. 100 kHz.). Samples are stored in a transient recorder and afterwards transferred to disk.

This allows for a comparison between the neural activity before and after the stimulation. When tip recording is used, the stimulus signal can be replaced by the spike signal. The stimulus artefact starts the process in this case. When the pre-pulse activates the first trigger unit, the trigger in the spike channel is enabled for a user-defined period, 3 seconds in our case. During this interval each trigger event causes a burst of conversion commands, generated by an external clock. All the parameters in the conversion process (e.g. total sample time, sample frequency, duration of the sample burst) are interactively controlled by the user. The samples of the spike signal are saved in a special buffer. In addition, the interval since the last trigger event is recorded. The advantage of this method is that a series of recordings can be analysed with a high sample rate, without exhausting the available memory. The high sample rate (max. 100 kHz.) allows in principle a Fourier analysis of the spike shape. In addition time is saved because the separation of spikes from noise is done by the hardware. The only disadvantage of this simplification is that the conversion step must be repeated if the trigger levels were not set correctly. To facilitate a correct setting, an analog to digital conversion for the complete spike signal, including the noise, can be performed. The result is displayed, and the user can interactively choose the magnification factors of the X and Y axis. With a hairline cursor, the trigger level for the spike signal can be specified (Fig. 4).

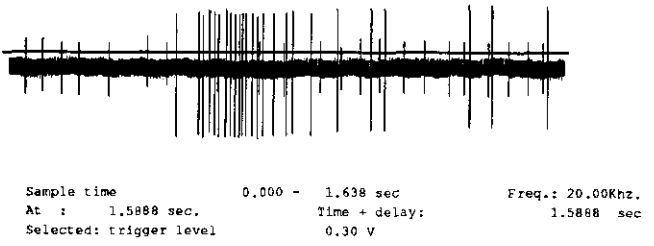


Figure 4 Display of the AD conversion process for the complete signal, including the noise. The magnification factors of the X and Y axis are interactively defined. With a hairline cursor, the trigger level for the spike signal can be specified.

Spike / noise separation

Although the spike/noise separation is accomplished largely during the preceding step, some noise will normally also be sampled. In the present step, all sample groups are compared to a set of criteria to separate valid spikes from noise peaks. We found that the distances between maximum and minimum in the spikes as well as the time until the first maximum or minimum provided a good separation from the noise peaks. To evaluate the fidelity of this process, a display function for individual spikes is available, together with a list of parameter values calculated for each spike. The following parameters were used: the top-top amplitude, a shape index following van Drongelen *et al.* (1980) and the slope of the connection between the maximum and minimum of the spike. Special care was taken to use a modular approach during the writing of this section. As a consequence, subroutines that extract other parameters (e.g. template matching techniques or results from Fourier analysis) can easily be added.

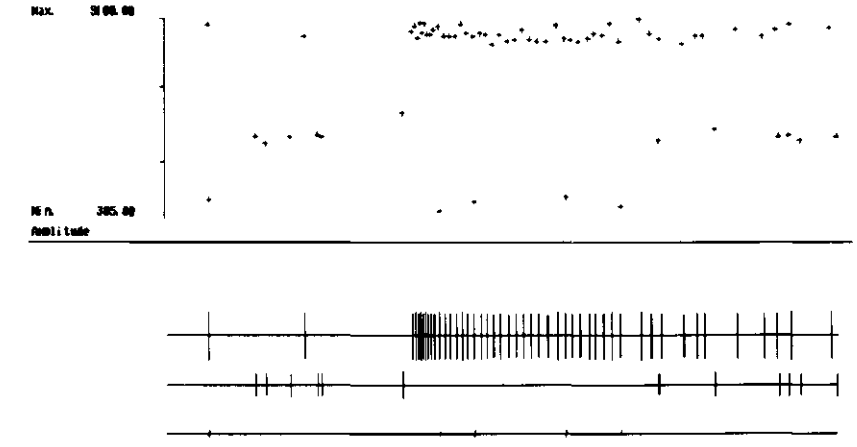
All parameters are kept in a data file, together with the sequence number and the time of occurrence of the spikes. In the classification step this file is extended with information indicating the cell to which each spike is assigned. The detected noise peaks are labelled as invalid, but not deleted, and can still be displayed in later steps. The valid spikes are counted.

Classification step

This step forms the heart of the analysis. Following the ideas of O'Connel (1973) and van Drongelen *et al.* (1980), the program produces a display of one of the parameters from the last step. Traditionally frequency histograms are used for this purpose, but here we plotted diagrams of each parameter against time (cf. Frazier & Hanson, 1986). Such a diagram displays all the information contained in a frequency histogram and, in addition, shows gradual changes of the parameter over time. The user specifies threshold values for the different spike classes, by moving a hairline cursor in the plot and indicating the boundaries. After

this specification the program immediately executes the separation and draws the spikes (as vertical lines with the appropriate top-top amplitude) for each cell on a separate axis (Fig. 5). The user can judge the result, change the position of one or more of the boundaries, repeat the analysis and again observe the result. In this interactive process, an acceptable separation can normally be reached after 2 to 3 cycles. In addition it is possible to produce scatter plots of one parameter against another.

This allows the user to gain insight in the way the spikes cluster, and which parameter yields the best separation.



Class	1	2	3	Invalid
Count	5	12	48	28
Boundary	2837.960	6144.880	9100.000	

Selected: Amplitude Value:305.00
N = New class, E = Enter boundary, D = Delete boundary <SPACE> = read value, Q = Quit

Figure 5 Display during the spike separation step. Cell type boundaries (small horizontal stripes on Y-axis) are specified with a hairline cursor in an amplitude vs. time plot. It is possible to add and remove boundaries. The program immediately displays the separated spikes with their proper amplitudes on their own time axis under the plot

Characterization step

This part of the process is relatively straightforward. The user specifies, again with the help of a hairline cursor, the moment of stimulus onset in the separation plot from the preceding step, and indicates the time interval to be used in the counting (Fig. 6). The program produces a table with the number of spikes per cell per time interval.This result can also be displayed in histogram format (Fig. 7).

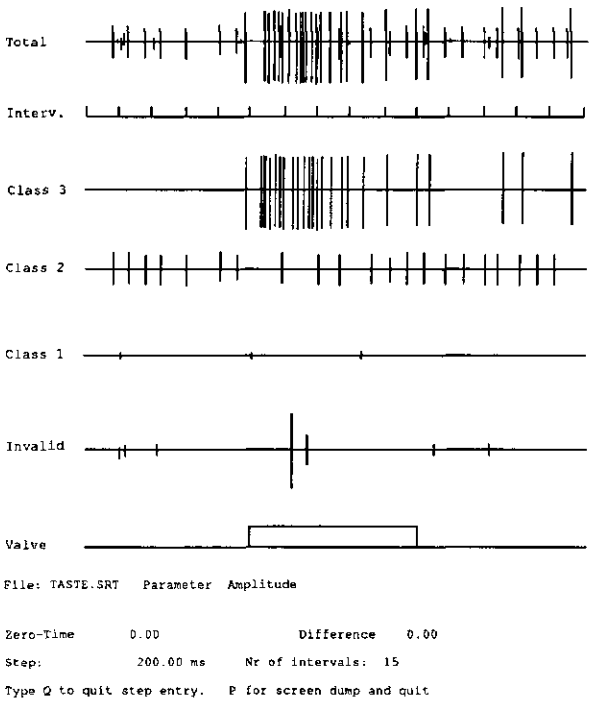


Figure 6 Video display during the Characterization step. The user specifies, again with the help of a hairline cursor, the moment of stimulus onset in a plot with separated spikes, and indicates the time interval to be used in the counting. The program produces a table with the number of spikes per cell per time interval.

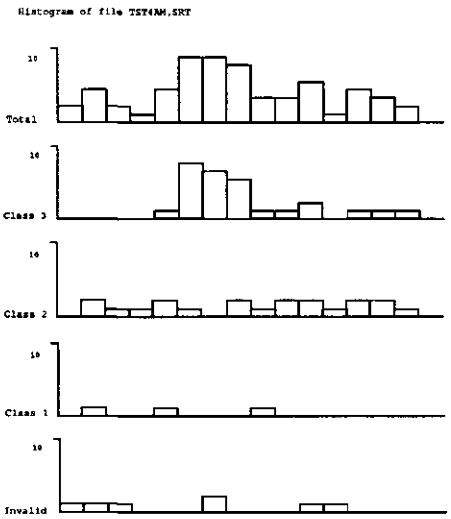


Figure 7 The results from the counting process can also be plotted as a histogram.

Discussion

The program package described here was tested on several types of electrophysiological data, i.e. on gustatory responses from tarsal receptors in *Pieris* butterflies, on olfactory receptor responses from the antennae of the Colorado Potato Beetle, *Leptinotarsa decemlineata*, and on recordings of gustatory and olfactory receptors from antennae and palpi of *Yponomeuta cagnagellus* larvae. From this experience critical points in the design of the program showed up clearly.

We have stressed several times the need for constant user supervision. To accomplish this, graphical display of the results is obligatory. In each of the analysis steps, some parameters (e.g. trigger levels, classification boundaries, display para-meters) must be changed. A graphical interface allows the user to inspect the data and change parameters in a single step. The feedback loop should be as tight as possible, to allow convenient repetition of the cycles.

As stated by Frazier & Hanson (1986), multiple representations of the data facilitate correct interpretation. The program should allow exploration of the structure in the data. Important in this respect is the integration of the whole package. It is possible that during the analysis the need arises to repeat a previous step; therefore, a quick way of entering an earlier program section should be available. To allow fast tracking of the origin of anomalies it must also be possible throughout the program to get information about the exact location of certain events. If, for instance, several large spikes appear in a plot of spike amplitudes vs. time, it should be possible to cross reference to the display of individual spikes to check if these amplitudes are caused by superpositions. This facility is implemented in the current package using cross hairline cursors, with which information about time or parameter values can be retrieved from the different plots.

To summarize, electrophysiological data often contain a high level of variability. As a consequence, it is unlikely that a completely automatic analysis program will be applicable. Instead, we view a spike analysis program as a set of tools, to manipulate and display the data interactively in a variety of ways, while the actual analysis is done by the user. From this perspective the main obstacles for writing useful spike analysis software are associated with the flexibility and interaction of data display and manipulation, rather than with the lack of algorithms or separation procedures.

Instrumentation

Camac system with MIK 11/2 microprocessor including EIS/FIS chip, (Standard Engineering Corporation). RT11 operating system (v5.0A). RT11 Fortran IV (v2.6) programming language. Dual trigger PDS 7901. Twelve bit AD convertor and data logger LRS 8212A/4 (Le Croy) with 32 kBytes memory. LRS 8800/12 interval timer PDS 8104. Pulse generator Hijtec 95. Clockgenerator LRS 8501 (Le Croy) Flexible disk Memory system (dual floppy disk) DSD-440 (Data systems). Cifer T5 terminal emulating DEC VT100 and Tektronix 4010. Adcomp X80SP printer-plotter.

Acknowledgements

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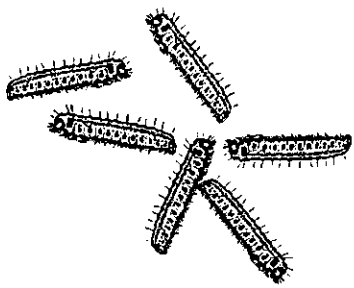

Curriculum vitae

Peter Roessingh werd op 19 september 1954 geboren te Wageningen. Na het doorlopen van de IVO-MAVO en de HAVO, behaalde hij in 1976 zijn eindexamen VWO aan de Rijksscholengemeenschap Wageningen. In hetzelfde jaar begon hij zijn studie biologie aan de Landbouwuniversiteit te Wageningen. De praktijkperiode bracht hij door in London, bij het Centre for Overseas Pest Research, waar hij onder begeleiding van E. A. Bernays and R F. Chapman werkte aan de regulatie van de voedselopname bij de woestijnsprinkhaan. In 1984 studeerde hij af met als hoofdvakken dierfysiologie en ethologie. Tevens behaalde hij een onderwijs bevoegdheid. Van 1984 tot 1987 werd bij de vakgroepen Dierfysiologie en Entomologie van de Landbouwuniversiteit het in dit proefschrift beschreven onderzoek verricht. Het onderzoek is gedurende 3 jaar gefinancieerd door BION (NWO). Sinds mei 1988 is hij werkzaam bij de Eidgenössische Forschungsanstalt für Obst-, Wein- und Gartenbau in Wädenswil, Zwitserland.