Zintuiglijke en voedingsfysiologische effecten van aminozuren en fenolische plantestoffen op de rupsen van twee *Pieris* soorten

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Sensory and nutritional effects of amino acids and phenolic plant compounds on the caterpillars of two *Pieris* species

Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
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in het openbaar te verdedigen
op vrijdag 8 januari 1988
des namiddags te vier uur in de aula
van de Landbouwuniversiteit
te Wageningen.

To eat or not to eat is one question;

How much to eat is another question.

J.L.E.M. Frantzen

STELLINGEN

1. De toepassing van genetische manipulatie-technieken voor de overdracht van resistentie tegen insekticiden naar predatoire of parasitaire insekten die als biologische vijanden in de bestrijding van plaaginsekten kunnen worden ingezet, is een stap terug in het noodzakelijke proces van sterke terugdringing van het gebruik van de milieu-belastende insekticiden.

   Carson, R., 1963. Dode lente. H.J.W. Becht's Uitg. Mij., Amsterdam.

2. De landbouwkundige toelating van plantmateriaal of microörganismen welke via genetische manipulatie zijn verkregen dient aan een gefundeerde wetgeving onderworpen te worden in plaats van aan de regelgeving die door ad-hoc commissies tot nog toe inderhaast werd geopperd, om mogelijke nadelige ecologische consequenties van toelating achteraf te kunnen rechtvaardigen.

3. In het door privatiseringsdrift begeleide enthousiasme over de mogelijkheid die de moleculaire genetica momenteel biedt om resistentiefactoren die strict op één gen berusten, over te brengen naar planten, gaat voorbij aan de naar verwachting korte duur waarin de doelinsekten een dergelijke monofactoriële resistentie doorbreken.

   McGaughey, W.H., 1985. Insect resistance to the biological insecticide Bacillus thuringiensis. Science 229: 193-195.

4. Peulvruchten zijn bij uitstek geschikt als 'vierde gewas' ter verruiming van de vruchtwisseling in de Nederlandse landbouw en verdienen meer aandacht in het door de overheid gefinancierde landbouwkundig onderzoek.

5. Om tot een diepergaand inzicht te kunnen komen in de werkingsmechanismen en duurzaamheid van resistenties in planten tegen pathogene schimmels of fytofage insekten is samenwerking tussen fysiologen en genetici een conditio sine qua non.

6. Directe calorimetrie is methodologisch te verkiezen boven alle andere bestaande methoden in balans-studies die ten doel hebben de stofwisselingsefficiëntie van organismen te bepalen.

   Dit proefschrift

7. De weerstand tegen aantasting door insekten die vele plantesoorten ontlenen aan het secundair metabolisme van aromatische aminozuren, wordt veelal onderschat als gevolg van een psychologische kloof tussen het kwantitatieve karakter van deze bescherming en de voorkeur die onder onderzoekers bestaat om spectaculaire kwalitatieve effecten te bestuderen.

   Isman, M.B & S.S. Duffey, 1982. Entomol. exp. appl. 31: 370-376.
   Dit proefschrift

8. De inhoudelijke kwaliteit van hetgeen tegenwoordig gepubliceerd wordt lijkt in toenemende mate ondergeschikt te zijn aan de kwantiteit van de publikaties die onderzoekers geacht worden af te leveren per tijdseenheid.

9. Het wetenschapsbeleid in Nederland wordt in zorgwekkende mate bepaald door korte-termijn financieel-economische overwegingen en nauwelijks door een lange-termijn visie op de toekomstige ontwikkeling van de wetenschap en haar culturele en educatieve functie in de maatschappij.

   Ministerie van Onderwijs en Wetenschappen, 1987. Hoger Onderwijs en Onderzoek Plan.

10. Een hoofdargument dat universitair geschoolde medici aanvoeren tegen de erkenning van een aantal als 'alternatief' aangeduide geneeswijzen, nl. dat er geen natuurwetenschappelijke basis is welke de effectiviteit van deze geneeswijzen waarschijnlijk maakt, is evenzeer van toepassing op vele erkende geneeswijzen en verhult bovendien dat in feite meer prozaïsche argumenten van financiële aard een hoofdrol spelen om de gewraakte erkenning op de lange baan te schuiven.

11. Het zou een goed academisch gebruik zijn wanneer elke opponent tenminste één stelling met een gericht tegenargument ter discussie zou stellen om zodoende te voorkomen dat stellingen slechts als een soort academische folklore zijn te beschouwen.

12. Het proefschrift en de bijbehorende stellingen van J.J.A. van Loon munten niet uit in beknoptheid.

13. Leven komt pas dan tot volle bloei, wanneer het niet langer beknopt is.

Stellingen behorende bij het proefschrift: "Sensory and nutritional effects of amino acids and phenolic plant compounds on the caterpillars of two *Pieris* species" door J.J.A. van Loon.

Wageningen, 8 januari 1988

INHOUD / CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

Insect - plant relationships

The relationships between insects and plants offer a vast array of phenomena that have stimulated scientists and amateurs to detailed investigations. One of the most intriguing problems has been, and often still is, why many plant-feeding insects accept only a limited number of often related plants (oligophagy), while other species exploit a much wider range of host plants (polyphagy) (Dethier, 1954). These widely different degrees of association between insects and host plants have been the subject of numerous evolutionary considerations (Jermy, 1984). A primary question for the physiologist in this respect is how the insect is able to select particular plant species from the usually complex vegetation in nature. It seems a characteristic of insect - plant relationships that there is no general answer to this central question and several quite different mechanisms have been described (Dethier, 1982; Schoonhoven, 1982).

Host plant preference behaviour and sensory physiology

By now it is evident that such often categorical behaviours as preference and rejection are guided by a chain of physiological processes. This chain invariably starts in the pre-ingestive phase with sensing the potential food source. Insects possess elaborate sensory organs that serve to gather olfactory and gustatory information on the chemical composition of the food. This information is transmitted in an encoded form, the action potential, to the central nervous system. At this central level decisions on acceptance or rejection of the plant material as food are made. Apart from the chemosensory input, the central nervous system receives messages from other sensors that measure post-ingestive parameters like the level of satiety and the nutrient status (Bernays & Simpson, 1982). On a short term (i.e. seconds to minutes) chemosensory activity has often been found to

play a major role in food selection behaviour (Dethier & Crnjar, 1982; Schoonhoven, 1982). For this reason, the study of insect chemoreceptors with electrophysiological techniques has revealed several clues to a better understanding of food preference behaviour. The chemoreceptors of phytophagous insects display sensitivity to chemical stimuli that are ubiquitous in plants, like sugars, amino acids and salts (Schoonhoven & Dethier, 1966; Dethier & Kuch, 1971; Dethier, 1973). The discovery of gustatory cells specifically reacting to so-called secondary plant compounds gave a physiological entrance to the study of the apparent 'botanical instinct' guiding food preference behaviour (Schoonhoven, 1967; 1982; Dethier, 1980).

Secondary plant substances and food selection behaviour

Since their earliest isolation and identification by chemical methods at the end of the nineteenth century, secondary plant substances have been puzzling compounds for plant physiologists for several decades, as their metabolic role in the plant itself was a mystery (Schoonhoven, 1972). The appreciation of the ecological rather than physiological function of secondary plant chemicals has founded a central paradigma in the field of insect - plant relationships. Again typical for this field, evidence was found for both stimulatory token functions as well as for the opposite viz. strongly deterrent effects of secondary compounds. Thus, many insect species seem to perform negative food selection by avoiding plants that contain sensory deterrents ('antifeedants', 'unpalatable' compounds) while other species positively select plants that produce specific secondary substances which the insects utilize as token stimuli (Fraenkel, 1959; Jermy, 1966; Schoonhoven, 1968; Whittaker & Feeny, 1971; Dethier, 1982; Hsiao, 1985). The term 'allelochemical' (i.e. a compound produced by one organism (the plant) that is not a nutrient and influences behaviour, growth, or reproduction of another organism (the insect)) was proposed for secondary plant substances to express their ecological role (Whittaker & Feeny, 1971). The role of secondary plant chemicals as feeding deterrents for phytophagous insects is illustrated by the fact that several species can be successfully reared on artificial diets that are devoid of these compounds. At the same time, this indicates the role of the common nutrient chemicals that exert apparently sufficient stimulation for food acceptance and continued intake.

From the foregoing historical sketch it appears that food selection behaviour has received much attention. The role of secondary plant substances as factors guiding decisions on acceptance or rejection via chemosensory perception is well documented. Knowledge of the sensory mechanisms by which feeding deterrents act is considered desirable by several authors as it may provide a basis to design novel strategies for crop protection. Influencing insect behaviour in a pre-ingestive phase certainly is an attractive idea but up till now no extensive application of the knowledge on this subject has been developed (Bernays, 1983).

The classical case of *Pieris*

One of the earliest examples of the ecological role of secondary plant substances was reported by Verschaffelt (1910). He studied the food preference behaviour of the caterpillars of *Pieris brassicae* L. and *P. rapae* L. (Lepidoptera: Pieridae; the large and small white butterflies respectively) that normally feed on plants belonging to the family of the Cruciferae. He demonstrated that the glucosinolate sinigrin, a common secondary constituent of cruciferous plants, may cause food acceptance of otherwise rejected, non-cruciferous plant species. The sensory basis of this behavioural recognition was revealed when sensory cells were discovered on the mouthparts of *P. brassicae* caterpillars that reacted specifically to sinigrin and other glucosinolates (Schoonhoven, 1967; Ma, 1972). The acceptance of an artificial diet was much improved by the addition of these compounds (David & Gardiner, 1966). *P. brassicae* and *P. rapae* are examples of specialist feeders that have overcome the glucosinolate defence barrier that is effective against non-adapted species (Blau et al., 1978).

Physiology of nutrition

When the primary sensory information leads to acceptance of a food source and food intake has started, actual nutrition just begins. The physiology of nutrition of phytophagous insects is a field of study which encompasses subjects such as basic requirements (essential nutrients), proportions and concentrations of nutrients, the effects of allelochemicals and quantitative food consumption, utilization and their regulation (review

by Slansky & Scriber, 1985). It is apparent that an understanding of these post-ingestive processes is only beginning to emerge. Many interactions may play a role. Apart from chemosensory input, mechanoreceptors in the gut wall and putative internal chemoreceptors monitoring nutrient status have been implicated to supply the central nervous system with information that is integrated to guide the timing and amount of food intake (Bernays & Simpson, 1982; Simpson & Abisgold, 1985).

Secondary plant substances and nutrition

Apart from their sensory and behavioural effects, secondary plant substances have been found to influence insect nutrition and growth (Reese, 1979; Bernays, 1982; Slansky & Scriber, 1985). When the nutrient content of the leaf tissues of green plants is considered and compared to the known basic requirements of phytophagous insects (Dadd, 1985), one would conclude that these leaf tissues should provide adequate nutrition. This conclusion is again substantiated by the adequacy of artificial diets in which the levels and proportions of nutrients as they occur in plant tissue can be mimicked to yield an apparently adequate supply of nutrients. In several cases, it has been demonstrated that allelochemical plant substances play a major role in preventing successful utilization of plant tissue as food by insects (Schoonhoven, 1972; Scriber, 1984). Considering the latter statement from the plant's point of view, another way of saying the same, is that plant allelochemics in many cases constitute defensive chemicals that confer plant resistance against insect attack (Maxwell, 1972).

The post-ingestive effects of secondary plant substances are probably as varied as their chemical nature and again no generalizations can be made. In many cases, however, it has proved difficult to separate such seemingly simple effects as reduction of food intake from reduced post-ingestive utilization of food. Growth inhibition may be the result of either of both or due to their interaction and to reveal the mechanism of action of secondary compounds, knowledge of the process or processes that are primarily affected must be considered basic (Reese, 1979). A serious obstacle experienced in the present study turned out to be that the measurement of food intake using gravimetric techniques (Waldbauer, 1968) is subject to rather serious errors (Schmidt & Reese, 1986).

The relationship between Pieris caterpillars and Brassica oleracea plants

In this thesis, the relationship between two species of Pieris caterpillars and Brassica oleracea L. is investigated against the background of the main themes in the foregoing, viz. chemosensory perception and nutrition.

As indicated in previous sections, the sensory and behavioural effects of glucosinolates, secondary metabolites typical for cruciferous plants, have been comparatively well studied in P. brassicae and other cruciferous specialists (Nielsen, 1978; Rodman & Chew, 1980). These compounds do not seem to have any antibiotic effects on these specialized feeders. However, glucosinolates are not the only group of secondary metabolites in cruciferous plants (Hegnauer, 1973). In the genus Brassica, phenolic acids and flavonoids are commonly occurring secondary constituents (Durkee & Harborne, 1973; Herrmann, 1976). The biological effects of these products on Pieris have not been investigated up to date.

In the present study, attention was focussed on how caterpillars reacted to acceptable food sources and on possible post-ingestive differences in a no-choice, ad libitum situation. The main themes are the analysis of the perception and post-ingestive utilization of amino acids and the perception and nutritional effects of phenolic secondary plant compounds that are biosynthetically derived from the two aromatic amino acids and that naturally occur in B. oleracea. The simplest phenolic derivatives are synthesized by only a few enzymatic conversions from the amino acids phenylalanine and tyrosine and thus are originating close to a branching point of primary and secondary plant metabolism (Hanson & Havir, 1979). Furthermore, it has recently been demonstrated that phenylalanine is a limiting nutrient in the hemimetabolous Schistocerca gregaria due to large investment of aromatic amino acids in the cuticle (Bernays & Woodhead, 1984). It was of interest to see if the same is true for the holometabolous Pieris.

Using a variety of approaches, it has been investigated whether phenylpropanoid secondary metabolites can be assigned an allelochemical role in the defence against the caterpillars of both Pieris species. It was explicitly tried to separate pre- and post-ingestive effects. Both natural and artificial diets have been used comparatively during the study as far as possible.

Parallel to the themes nutrients - secondary substances, sensory (pre-

ingestive) - post-ingestive and natural - artificial diets, the entire study was performed on two taxonomically related species, which will be introduced briefly in the next section.

The cabbage white butterflies, Pieris brassicae and Pieris rapae

While P. brassicae may be considered as one of the best investigated phytophagous insect species (Feltwell, 1982), it is often unknown if the features found for one insect species are specific or will apply also to a related species, such as P. rapae. Clearly, both species strongly resemble each other in adult appearance, but differ considerably in the larval stage and in several ecological respects. The adult female butterfly of P. brassicae oviposits batches of eggs, while P. rapae females lay single eggs. The young larval stages of P. brassicae are gregarious, while the larvae of P. rapae show strong competition and cannibalistic behaviour. The larvae of P. brassicae are coloured aposematically and expose themselves during feeding at leaf edges, while P. rapae caterpillars have a cryptic appearance, display countershading when resting and often feed from central portions of leaves. P. rapae migrates to the younger parts of the host plant, which often becomes a compact head of leaves growing tightly together in the 'capitata'-group of cultivated cabbages. There is also a remarkable difference in geographical distribution between the species. P. brassicae is restricted to the Palaearctic region (Feltwell, 1982), while P. rapae is notorious for its cosmopolitic occurrence. Both species can cause serious economic damage to cabbage crops (Feltwell, 1982; Dickson & Eckenrode, 1975). Probably because of its smaller size and difficulties in rearing, P. rapae, although economically more important, has little been studied compared to P. brassicae on which c. 3000 references were available in 1982, about half of which are cited by Feltwell (1982). This study will show that such a large number of studies on an insect species does not necessarily imply that fundamentals of its larval biology are well understood.

Outline of the thesis

A comparative analysis of amino acid chemoreception in maxillary gustatory organs was undertaken to reveal the specificity and sensitivity with which this group of nutrients, among which ten essential ones, are perceived (Chapter 2). Subsequently, sensory effects of phenolic acids and flavonoid compounds are investigated in connection with some behavioural experiments (Chapter 3). The quantitative study of longer-term food intake in final instars posed several methodological problems. Prior to routine experiments in which the effects of phenolic acids and flavonoids on food consumption, utilization and growth were assessed, it was judged necessary to measure respiratory expenditure and its variation in order to reliably estimate food intake. To this end, a flow-through gas analysis system was developed that allowed the continuous measurement of respiration in final instars. The effects of some phenolic acids and flavonoids on energy utilization have been investigated (Chapter 4). In studies on the effects of defined phytochemical compounds on phytophagous insects, artificial diets have frequently been used as convenient substrates of which the composition can be manipulated and in which single experimental factors can be varied. These are apparent advantages as compared to the variable and practically intraceable chemistry of living host plant tissue. The nutritional adequacy of artificial diets is mostly measured by parameters such as survival, rate of development, pupal weights and reproductive success. Data on the effect of artificial substrates on the absorption and metabolic efficiency with which nutrients are used as compared to the natural feeding situation, the host plant, are conspicuously lacking for many insect species. From a methodical point of view, it was considered fundamental to study the utilization of amino acids, especially the aromatic phenylalanine and tyrosine, on both natural and artificial food to find out to what extent differences occurred (Chapter 5). The results of this comparison raised no serious objections against the use of an artificial diet to test the effects of continuous exposure to phenolic and flavonoid compounds on the performance of both caterpillar species (Chapter 6). The term performance as used in the present study comprises the extent to which the insects are successfully surviving, developing and growing on a particular food source. A preliminary test of the hypothesis that also on the host plant phenolic acids and flavonoids exert inhibitory effects on both species of Pieris caterpillars, was carried out by studying the performance of the

caterpillars on seven different cultivars and a concomitant phytochemical analysis of the concentrations of the most common phenolic acids and flavonoids in the leaves of these cultivars (Chapter 7).

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CHAPTER 2

CHEMORECEPTION OF AMINO ACIDS IN LARVAE OF TWO *PIERIS* SPECIES

ABSTRACT

Specificity and sensitivity of gustatory neurones in response to 22 amino acids was studied in larvae of *Pieris brassicae* L. and *Pieris rapae* L. (Lepidoptera: Pieridae) using electrophysiological methods. In both species 14 amino acids were stimulating a receptor cell in one of the styloconic sensilla on the maxilla. Four amino acids stimulated cells in a different sensillum. Significant differences were found in stimulatory effectiveness between the amino acids. Nutritionally essential amino acids were generally more effective.

A correlation analysis suggested the presence of 4 sites. One of these is postulated to recognize several amino acids carrying non-polar R-groups (leucine, isoleucine, methionine, alanine, asparagine, valine) while the other three may recognize proline, phenylalanine and tryptophane respectively. Similarities with postulated sites in some Diptera were found. Data on five other lepidopteran species could be interpreted on the basis of the site model proposed.

Phytochemical data on concentrations of free amino acids in a common host plant, *Brassica oleracea* L., were compared to concentration - response parameters of the gustatory neurones. It appears that amino acids are effective stimuli at their natural concentrations and that gustatory neurones can transmit information about concentration differences in the plant tissue.

INTRODUCTION

Amino acids stimulate feeding behaviour in several phytophagous insects. The behavioural reaction to a particular amino acid is different for different species (Bernays and Simpson, 1982). At the same time, nutritionally essential amino acids and especially the aromatic phenylalanine are considered to be limiting nutrients for phytophagous insects (Bernays, 1982).

Analysis of specificity, structure-activity relations and sensitivity in the chemoreception of amino acids occurring in plant tissues has been performed in only few phytophagous insects. The first report on the existence of a gustatory cell specific to amino acids discovered electrophysiologically has been published by Schoonhoven (1969b) for the larva of Pieris brassicae L. (Lepidoptera: Pieridae). A partial correlation with behavioural results was found by Ma (1972). Mitchell and Schoonhoven (1974) found amino acid sensitive cells in larval Leptinotarsa decemlineata. Their sensitivity pattern correlated well with behavioural responses (Hsiao and Fraenkel, 1968). Amino acid receptor cells in adult Leptinotarsa are highly specific to L-alanine and γ-amino butyric acid (GABA) as demonstrated in a recent structure-activity study (Mitchell, 1985).

In the present study a comparative analysis of the specificity of amino acid gustation in two caterpillar species is performed. The two species, Pieris brassicae L. and Pieris rapae L. are considered as taxonomically closely related (Geiger and Scholl, 1985). To date such a comparison is lacking (Mitchell, 1985). A correlation analysis is introduced to assist the interpretation of the specificity pattern found. The interpretation offered is related to structure-activity considerations. A comparison with data on five lepidopterous species (Dethier, 1973) will be presented to investigate if generalizations are possible. Finally, concentration-response (C/R) characteristics of the amino acid receptor cells will be related to phytochemical data on amino acid concentration ranges in a common host plant, Brassica oleracea L.

MATERIALS AND METHODS

Insects

Pieris brassicae and P. rapae cultures were maintained in the laboratory under conditions described by David & Gardiner (1962). Both cultures were initiated using field-collected material; inbreeding was limited by introductions of field-collected individuals into the cultures every summer. Both species of caterpillars were reared on Brassica oleracea L. var. gemmifera DC. cv. Titurel (Sluis and Groot, Enkhuizen, The Netherlands) under ad libitum conditions. Rearing temperature was 25 ^{o}C, relative humidity varied between 50 and 70% . Photo/scotophase was 15/9. For electrophysiology only those caterpillars were used that had completed their development ab ovo up to the final larval moult at the normal rate of 15 days (P. brassicae) or 13 days (P. rapae) at 25 ^{o}C. Final instars of P. brassicae used for experiments had weights ranging from 250-400 mg, P. rapae larval weights ranged from of 80-120 mg. Caterpillars of both species were 24-48 h in the final instar. In this way, it was attempted to work with individuals of defined physiological age and nutritive status, as these parameters are known to affect chemosensory response strength (Blaney et al., 1986). Prior to the experiments, larvae were deprived of food during 2-3 hours. This starvation-period was found to improve the signal-to-noise ratio recorded, as is the case in locusts (Bernays et al., 1972). The head and the first thoracic segment were amputated for recording. A U-shaped silver wire recording electrode was inserted through the foramen magnum of the isolated head and was protruded into the maxillary-labial complex to fix the maxillae in a more prognathous position.

Morphology

Location, morphology and ultrastructure of the lateral (abbreviated as L) and medial (M) styloconical sensilla of the maxillary galea were described by Ma (1972) for P. brassicae. In this species, a gustatory neurone specifically sensitive to amino acids is located in the L-sensillum in addition to three other taste cells (Schoonhoven, 1969a,

b). All evidence available suggests that the same structural situation is present in P. rapae.

Stimuli and stimulation-procedures

Stimuli studied are given in table 1. They were of 99% purity. The compounds listed were obtained from the following commercial sources: Merck, the compounds 1, 2, 6, 9, 11, 14, 18-22; Baker: 3, 4, 5, 7, 8, 10, 12, 13, 16; Fluka: 17; British Drug Houses: 15.

Table 1 : List of amino acids (all in L-form) applied as gustatory stimuli

Essential amino acids	abbreviation
arginine	Arg
histidine	His
isoleucine	Ile
leucine	Leu
lysine	Lys
methionine	Met
phenylalanine	Phe
threonine	Thr
tryptophane	Trp
valine	Val
Non-essential amino acids	
alanine	Ala
asparagine	Asn
aspartic acid	Asp
cystein	Cys
glutamic acid	Glu
glycine	Gly
γ-aminobutyric acid	GABA
ornithine	Orn
proline	Pro
4-hydroxyproline	Hyp
serine	Ser
tyrosine	Tyr

Amino acids are referred to throughout the paper by their abbreviations

All stimuli were dissolved into 2 mM (P. rapae) or 10 mM (P. brassicae) potassium chloride solutions in distilled water, which served as the respective control stimulations. The pH of 10 mM solutions ranged from 5.0-5.5, except for the acidic Asp and Glu (pH 4.5) and the basic His (pH 6.0), Arg (pH 8.0) and Lys (pH 7.5). To reduce evaporation of solvent at the stimulus capillary tip, fluid from the body of the capillary was sucked through the tip less than 10 s preceding stimulus onset. To the same end, local humidity around the preparation and stimulus-capillary was kept high (± 80%), both being encased in an aluminum box guarded to the amplifier input. Stimuli were delivered in a glass capillary (tip diameter 30 - 50 µm) used as stimulating electrode. The stimulus delivery sequences were random. In an experimental series carried out on an individual preparation, the control stimuli and an effective stimulus that evoked only single cell reactions, were replicated 2-3 times to check the reproducibility of cell reactions in time. Preparations were used no longer than 90 minutes although reproducible responses could be obtained over several hours. Stimulus duration was 2-4 seconds; interstimulus periods were at least 30 seconds. Adaptation experiments had shown that, following such stimulus durations, complete disadaptation occurs within 10 seconds in the sensilla studied.

Recording of electrophysiological responses

A modification of the tip recording technique of Hodgson et al. (1955) was used to record the responses of gustatory chemosensory cells. The stimulating electrode served as indifferent electrode, while the recording electrode was in contact with the insect tissue. The recording electrode was connected to a preamplifier by a short (10 mm) connector. The amplifier was adapted from van Drongelen (1979). In brief, it used an AD 515-K (Analog Devices) integrated circuit in the first stage, yielding < 1 pA input bias current, 10^{15} Ohm and 0.8 pF input impedance. The amplifier included a band-pass filter, set at 100 Hz - 3 kHz (-3 dB). The amplifier case was connected to a guard voltage. Spike amplitudes recorded ranged from 0.5 - 3 mV; spike-to-noise ratios varied from 2 to 10. The blocking artefact at stimulus onset lasted 5 - 40 ms. Amplified responses were recorded on tape (Akai GX-255) at 19 cm/s, for subsequent reproduction on paper, using a

Siemens Oscillomink-E inkjet recorder (0 - 1000 Hz) at 25 or 50 cm/s. For detailed analysis of spike shape, recordings were re-recorded at 60 ''/s (Racal Store-7 instrumentation recorder) and reproduced on paper at 3.75''/s. Environmental temperatures during recording were 22 $\pm$ 2°C.

Analysis of the recordings

Chemoreceptor responses are quantified as the number of action potentials generated from 50 ms until 1050 ms after stimulus onset, further on designated as F_{ap}, frequency of action potentials, expressed in spikes/s. Action potentials were counted based on visual identification. Spike amplitude and interspike-interval duration served as criteria to assign spikes to the different neurones present in multineural responses (Bowdan, 1984).

Statistical analysis

The following statistical procedures have been employed: Shapiro-Wilk test to investigate if data belonged to a normal distribution-type (the conservative null-hypothesis (i.e. the distribution is normal) could not be rejected since $p > 0.1$ or 0.5 in all cases, $n > 10$); Student's t-test either for paired or independent samples to test significance of differences between treatment means; testing was limited to comparing ranked adjacent means and, if no significance was calculated, extended till the first significant difference emerged along the ranking order to avoid the accumulation of the experimentwise error rate (Jones, 1984). As part of Student's test, the F-statistic was calculated to decide on equality of variances observed. Correlation analysis was performed using Spearman's rank correlation coefficient r_s, for sample sizes $n > 11$ an approach assuming a normal distribution was applied; both were corrected for ties (Siegel, 1956). This correlation analysis was applied on a series of paired response values in which each pair consisted out of two values registered from an individual cell.

RESULTS

Specificity pattern of the amino acid receptor cell in P. rapae.

Fig. 1 represents the response profile of the L-sensillum of P. rapae to twelve amino acids. Eight other amino acids (to know: Tyr, Orn, Glu, Asp, Gly, Cys, Arg and Lys) did not stimulate when tested at 10 mM. Five of the twelve incorporated into the profile of fig. 1 stimulated essentially one cell, whereas the remaining seven compounds evoked responses also from a second neurone (see column at the right hand side in fig. 1).

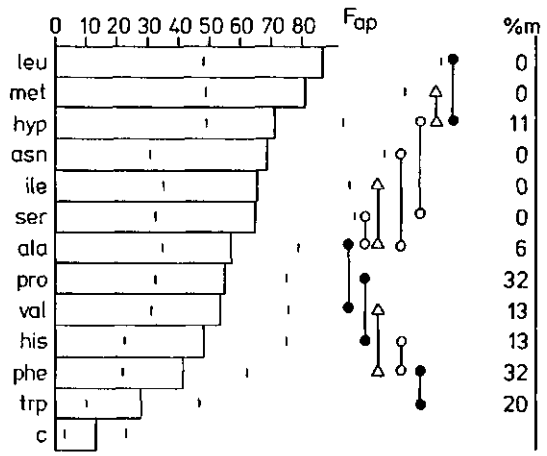

Figure 1:
Response profile of amino acid receptor cell of P. rapae (all compounds tested at 10 mM; data from 10 individuals, 806 recordings). Top axis is ordinate, representing response intensity, F_{ap} in spikes/s. Column at the right indicates the percentage of responses with a multineural character (%m). Significance of differences between mean responses along the vertically ranked sequence is indicated by the vertical lines delimited by the following symbols: 0 means $p < 0.05$; △ $p < 0.01$; • $p < 0.005$.

Especially Pro and Phe and, though to a lesser degree, Trp, Val, His, Hyp and Ala seem to stimulate more than one cell. Still, even when stimulated with these amino acids, in 70-95% of the sensilla only one neurone was active. The second neurone, from which a smaller spike-amplitude was occasionally recorded, was moderately active (F_{ap} 20-30 spikes/s). Recordings displaying multineural responses were excluded from statistical analysis unless a discrete separation on the basis of spike-amplitude and additional criteria could be made. Thr and GABA

were excluded from further analysis as they produced activity in more than two cells in 60% and 66% of the recordings respectively. These responses showed a high degree of variation as compared to the rather consistent reactions of the twelve other compounds presented in fig. 1. It is concluded that in the L-sensillum of P. rapae one cell is especially sensitive to 12 out of 22 amino acids tested.

It can be noticed from fig. 1 that significantly different grades of effectiveness exist in the whole range established. More in particular, a comparison of stimulatory effectiveness within five pairs of amino acids that differ structurally only one functional group within the pair is illustrative. With Leu-Ile and Pro-Hyp, the first one is active while the second one is significantly less stimulatory (fig. 1). Within the pairs Asp-Asn, Phe-Tyr and GABA-Orn, the first one is active while the second one is ineffective.

Spearman's r_s was calculated for all possible pair-wise combinations of the 12 adequate stimuli. The combinations that proved statistically significant at three values of the type I error probability value, p, are listed in table 2. Starting at the best stimulus and going down the effectiveness ranking order (fig. 1), an amino acid is assigned to the same group as the previous and more effective one if $p < 0.001$ for the correlation between the two or if $p < 0.01$ more than once with amino acids already assigned to a group. Five groups emerge using this grouping procedure (table 2): group I: Leu, Met, Asn, Ile, Ala, Val; group II: Hyp and His; group III: Pro; group IV: Phe and group V: Trp. All amino acids were tested also on the M-sensillum. Four of the 22 compounds tested evoked responses: Asp, Glu, Arg and Trp. Upon stimulation with the acidic Asp and Glu, the M-sensillum responded with large spikes in a tonic mode, which is characteristic for the so-called deterrent-neurone, also present in this sensillum (Schoonhoven, 1969a). In some cases a multineural response was recorded (Asp 26%, Glu 6%). Asp was more effective than Glu (Asp: mean $F_{ap} \pm SD$ 38 ± 16 spikes/s; Glu 24 ± 8). Trp, the only one among these four that is also effective in the L-sensillum, elicited also large spikes (26 ± 10 spikes/s; 23% multineural). Quite different from the preceding three, the basic Arg was the best stimulant (43 ± 9 spikes/s), producing a smaller spike, probably originating in the cation receptor cell present in this sensillum (Ma, 1972).

Table 2 : Grouping of amino acids that stimulate the amino acid receptor cell of P. rapae

amino acid	code	significance of r_s at p < 0.001	0.005	0.01	group	R-group
Leu	1	2,5,7		9	I	NP
Met	2	1,5,6,7	9	3,10	I	NP
Hyp	3	10		2	II	PU
Asn	4	5	10	6	I	PU
Ile	5	1,2,4,7	6,9	10	I	NP
Ser	6	1	5,9,10	4,7	I	PU
Ala	7	1,2,5	9	10,12	I	NP
Pro	8				III	NP
Val	9		5,6	1,7	I	NP
His	10	3	4,6	1,5	II	PB
Phe	11				IV	NP
Trp	12			1,7	V	NP

Amino acid code numbers reflect their effectiveness in descending order (cf. fig. 1). R-group refers to classification given by Lehninger (1977); NP means non-polar R-group, PU - polar, uncharged; PB - polar and basic

Specificity pattern of the amino acid receptor cell in P. brassicae

Response intensities to various amino acids in P. brassicae have been ranked into the profile of fig. 2. When comparing this profile to the one for P.rapae, several differences appear. Firstly, the order of effectiveness seems almost reversed, e.g. His, Phe and Trp are the strongest stimulants in P. brassicae, while in P. rapae they were found to rank among the weakest. Secondly, Cys and Thr appear in the profile. GABA was again yielding inconsistent multineural responses in a fashion similar to that found in P. rapae and is therefore likewise excluded (26 ± 11 spikes/s). Altogether, 14 amino acids (out of 22 tested) were found to be adequate stimuli for this cell, among which 12 are identical for both species. A third contrast to be mentioned is the single unit character of most responses; multineural responses were found only rarely. Reference can be made to the description of the five pairs that differ one functional group in P. rapae, except that in P. brassicae Leu and Ile are equally effective.

Table 3 presents a similar grouping of amino acids as described for P. rapae. The Roman numbers used to designate groups are similar to

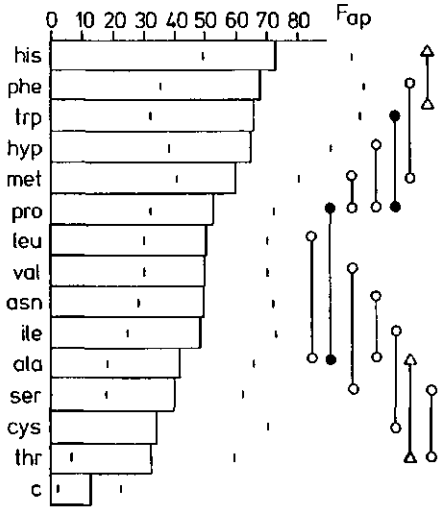

Figure 2:
Response profile of amino acid receptor cell of P. brassicae. Data from 9 individuals, 687 recordings. Legends see fig. 1.

Table 3 : Grouping of amino acids that stimulate the amino acid receptor cell of P. brassicae

amino acid	code	significance of r_s at p < 0.001	0.005	0.01	group	R-group
His	1			4	VI	PB
Phe	2			3	IV	NP
Trp	3			5	V	NP
Hyp	4	5			II	PU
Met	5	4		3,7	II	NP
Pro	6		11	14	III	NP
Leu	7			5,9	II	NP
Val	8				(-)	NP
Asn	9			7	II	PU
Ile	10	13			(-)	NP
Ala	11	14	6		III	NP
Ser	12				(-)	PU
Cys	13	10			(-)	PU
Thr	14	11		6	III	PU

Legends cf. table 2. (-) means grouping unclear

those used in P. rapae if the group has a comparable composition. The following subdivision emerges: group II: Hyp, Met, Leu and Asn; group III: Pro, which however displays links to Ala and Thr also; group IV: Phe; group V: Trp; group VI: His. The assignment of Val and the relatively weak stimulants Ile, Cys and Ser to the groups given is not well possible on the basis of the criteria applied. For the M-sensillum, similar observations were made as in P. rapae. The same four amino acids (Asp, Glu, Arg, Trp) were active, though the order of effectiveness differs (mean $F_{ap} \pm SD$): Asp (50 ± 10), Glu (45 ± 19), Arg (30 ± 7), Trp (20 ± 8). Control solutions elicited a typical response of 15 ± 5 spikes/s. The exact cellular origin of the spikes in the M-sensillum has not been established.

Sensitivity of the amino acid receptor cell in P. rapae

Fig. 3 shows the semi-logarithmic C/R-curves for the amino acids Met, Asn, Pro, His and Phe, representatives of four of the groups I - V mentioned above. Data were obtained using individuals taken from a batch of insects reared 6 months later than the larvae yielding the data summarized in fig. 1. The threshold for responses to Met must be lower than 0.2 mM, while for the other four compounds it lies around 0.2 mM. Responses to Phe and His appear to be saturated in the same range as for the other three amino acids (25-50 mM) but do not reach the same maximal response frequencies. For Phe the response seems saturated at a maximum response frequency of 34 spikes/s, while the response to Met reaches a saturated level of 90 spikes/s. The values of K_b, i.e. the concentration at which 50% of the maximal response frequency occurs (Mitchell and Gregory, 1979), was determined graphically. They are listed in table 4.

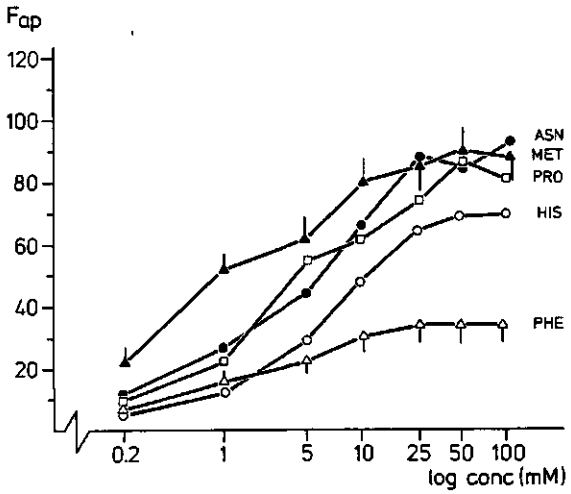

Figure 3:
Concentration - response curves for the amino acid sensitive cell of P. rapae. Data from 5 individuals, 500 recordings. Abscissa: concentration (log mM); ordinate response intensity (spikes/s). To avoid confusion due to overlapping S.E.M.-bars, only for methionine and phenylalanine S.E.M.-bars are indicated (one-sided). S.E.M.-values for the other three compounds were largely comparable.

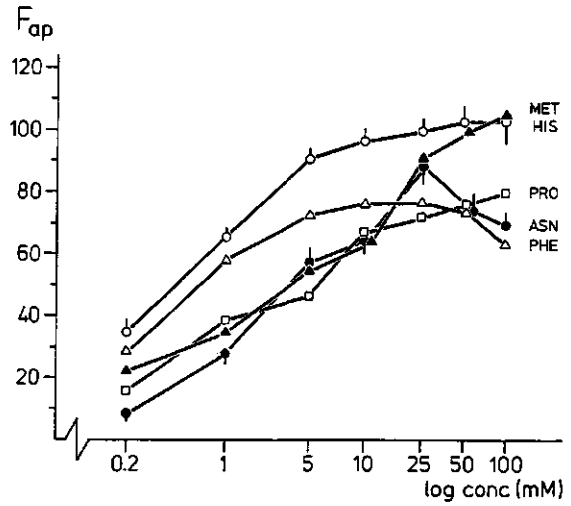

Figure 4:
Concentration - response curves for amino acid sensitive cell of P. brassicae. Data from 5 larvae, 512 recordings. Legends see fig. 3, S.E.M.-values indicated only for histidine and asparagine.

Sensitivity of the amino acid receptor in P. brassicae

In fig. 4 C/R-curves for the same five amino acids as tested on P. rapae are depicted. With P. brassicae also, the sample group was reared 6 months later than the one yielding the data of fig. 2. The threshold concentration for responses to Asn appears to occur around 0.2 mM, the lowest concentration tested. For the other four amino acids, it must be assumed to occur at still lower concentrations. Response intensities to His and Asn reach their maximum at 25 mM, while for Asn and Phe it declines beyond this concentration. The maximum response frequency evoked by Phe (77 spikes/s) is significantly lower than for His (103 spikes/s). The curves for Pro and Met still show a slight increase going from 50 to 100 mM, while presumably their respective maximum responses differ considerably. K_b values range from 0.3 mM (Phe) to 5.8 mM (Met) (see table 4).

DISCUSSION

Interpretation of specificity using data from individual neurones

The consistency of the specificity data on both species reported here is corroborated by a comparison with two existing studies. The quantitative ranking order in the sensitivity to twelve amino acids for P. brassicae as given by Schoonhoven (1969b) and the semi-quantitative pattern presented by Dethier and Kuch (1971) for P. rapae are both in satisfactory accordance with our findings, although they used 100 mM as test concentration. In general, few if any data are available on the reproducibility of sensory patterns in insect populations separated in time and space.

A comparison of the specificity profile of both species reveals a remarkable shift of three amino acids, His, Phe and Trp, from the lowest positions in P. rapae (fig. 1) to the highest three positions in P. brassicae (fig. 2). This observation raised the idea to investigate if the sensitivity to these three was quantitatively independent from the other stimulatory compounds in both species. To analyze this, a correlation analysis was employed. Significance of correlation at $p < 0.01$ between responses to different amino acids over a series of cells is used to discern certain groups. The hypothesis about the underlying physiological processes that may bring about this correlation is, that two or more amino acids interact with the same receptor site. An alternative explanation, however, would be that two amino acids thus correlated each interact with their own specific receptor sites which are correlated in their density in the sensory cell membrane by some coordinated mechanism. Both explanations are based on the rationale that the density of multiple receptor sites that possibly exist, should be detectable in quantitative electrophysiological studies on individual neurones, at least in a relative manner, when more than one site is present. The absolute number of physiologically active receptor sites mediating chemosensory transduction in insects is an unknown and poorly understood factor, although it is assumed to be of physiological relevance (Hansen and Wieczorek, 1981; Menco and van der Wolk, 1982; Morita and Shiraishi, 1985). In the fly Boettcherisca peregrina the concept of receptor sites having a protein structure has been adopted as responsible for amino acid recognition (Shimada and Tanimura, 1981).

At this stage, we have no data allowing a discrimination of the two alternative explanations given above. However, with this limitation in mind, we will briefly examine the data under the assumption that the first alternative reflects a physiological reality.

Considerations on specificity and number of multiple sites

The fact that four or five pairs of amino acids that differ chemically by one functional group exert significantly different response intensities supports the presence of specific receptor sites. Structural modifications of cystein present in Cruciferae also resulted in clear effects on response strengths in P. brassicae (Schoonhoven, 1969b). In both species, altogether 6 groups of amino acids result from the correlation analysis (tables 2 and 3, groups I-VI). In P. rapae, Pro and Phe appear unlinked to any other amino acid. The position of Hyp in both species can be interpreted as a capacity to interact with both the group I site and a His-site that seemed independent in P. brassicae (VI). Free concentrations of Hyp in hostplants of Pieris are unknown. Most probably they are low as it generally has phytotoxic properties and is rapidly incorporated into specialized inducible defence proteins (Esquerre-Tugaye et al., 1985). Disregarding Hyp, group II in P. brassicae then shows similarities to group I discerned in P. rapae.

In P. brassicae, the links His-Hyp, Phe-Trp and Trp-Met at $p < 0.01$ makes the separation into different entities disputable. As they belong to the most effective stimulants, these links may be caused by saturation phenomena, as appearing from the C/R curves of fig. 4. At the lower concentrations the correlation coefficients were lower also and were no longer significant. Within the less effective group I-amino acids correlations were significant at $p < 0.001$. When the three problematic links mentioned above would be due to interaction with common sites, together with saturation phenomena significance of correlation would be expected to be at least comparable to the latter value, while it is clearly lower. In the lower range of the profile of P. brassicae (fig. 2), the pattern is less clear. However, it is realized that we deal here with relatively ineffective stimulants.

In summary, correlation analysis together with the additional considerations given supply indirect evidence that in P. rapae four sites are

present when Hyp is assigned to group I. If this is done for P. brassicae also, the same four sites are present, together with a fifth, specific His-site.

Structure-activity relations

Amino acid structure-activity relations in insect chemoreceptors have been studied in detail only in the saprophagous fleshfly Boettcherisca peregrina (Shimada, 1975; Shimada and Isono, 1978; Shimada and Tanimura, 1981). Two amino acid receptor sites have been identified pharmacologically to date, both of which are present on a gustatory cell also sensitive to a range of sugars. The so-called T-site or 'aliphatic carboxylic anion'-site is sensitive to Leu, Ile, Met and Val. In contrast, Phe and Trp, previously thought to interact with the F-site responsible for fructose recognition (Shimada, 1975), are hypothesized to interact with a fourth site on the sugar cell (Shimada and Tanimura, 1981). Indeed, in view of threshold and K_b values, Phe is a stronger stimulus to this cell than the best stimulating sugar, sucrose (Shiraishi and Kuwabara, 1970). A detailed model of the two amino acid receptor sites has been proposed, suggesting three and four subsites for the T- and Phe-Trp-sites respectively (Shimada and Tanimura, 1981). Several similarities occur between the Boettcherisca situation and Pieris. Group I shows a considerable overlap with the T-site amino acids. The clearly separate Phe-Trp site in B. peregrina corresponds to two separate sites postulated in Pieris.

Amino acids can be classified into four groups according to polarity and charge carried by the R-group (Lehninger, 1977). It appears that the amino acids with a non-polar R-group constitute the most effective ones. Within this group, Pro, possessing an aliphatic ring, Phe, having an aromatic ring and Trp, characterized by an indole moiety, occupy special positions. They accordingly emerge as separate groups from the correlation analysis. The fifth group is made up only by His, having an imidazole ring that is positively charged at neutral pH values.

Comparison with other Lepidoptera

Dethier and Kuch (1971) and Dethier (1973) presented qualitative data on amino acid gustation in 15 species of Lepidoptera. Comparison with the two Pieris species is limited here to five species that were tested with buffered 100 mM solutions (Dethier, 1973) to exclude the possibility of non-specific pH effects. It is concluded, surprisingly enough, that the pattern found for Pieris can be recognized in the other five species also, especially for the L-sensillum. Predominant in the spectra of all seven species are the group I amino acids, His and Pro. Four species display sensitivity to Trp, while only the two Pieris species react to Phe. Within the essential amino acids, only Arg is ineffective in all six species tested, while Glu, Asp, and Tyr are ineffective in all species tested. Generally, essential amino acids are scored as frequent as the non-essential ones. When the pattern in the five species referred to (Dethier, 1973) is rearranged according to the groups I-V postulated for Pieris, a total of 50 combinations of groups is possible. Because not all compounds were tested on all five species, thirty of these could be screened. It turned out that in 17 cases (57%) sensitivity to one group of a pairwise combination was present and to the other one absent. In 7 cases (23%), both were absent and in 6 cases (20%) both were present. This reinforces the idea that the sensitivity to the groups discerned behaves independently also in other species and may correspond to separate sites.

Comparison with Coleoptera

A still different situation has been found in the beetle species Entomoscelis americanum (Mitchell and Gregory, 1979) and Leptinotarsa decemlineata (Mitchell, 1985). In the latter, an amino acid cell has been studied that seems to possess one site that recognizes specifically Ala and GABA. It has been suggested that amino acid receptors may have evolved several times within the Insecta (Mitchell, 1985).

Coding of food quality

In both Pieris species, nutritionally essential amino acids predominate the sensitivity spectrum. This observation holds also for

other Lepidoptera (Dethier, 1973). These findings fit into a teleological picture, i.e. the functional significance of the specificities of the amino acid receptor is to signal nutritionally relevant compounds in the host plant. Moreover, indirect evidence is available that a separate site is present for Phe. This aromatic amino acid is the precursor of both cross-linking phenolic elements in insect cuticle proteins (Bernays, 1982) and of plant phenolic metabolism (Camm and Towers, 1973). This supplied the basis for a study of the sensory effects of plant phenolics to test this discriminatory ability further (van Loon, in preparation).

Dose-response characteristics in relation to amino acid concentrations in plant tissue

Knowledge about the amino acid concentrations that actually reach the dendritic membranes during feeding on plant tissue is virtually lacking. Indirectly, however, the C/R-characteristics of the neurones studied and especially the K_b parameter, are considered to render information regarding these concentrations (Mitchell and Gregory, 1979). Transformation of the hyperbolic or sigmoid C/R-curves into straight lines was not applied in this study, as several methodological problems are connected with this practice. Linearizing is commonly justified by assuming an analogy with enzyme-substrate or reversible binding kinetics. However, the quantitative measure of phasic-tonic gustatory cell responses used by us and most other authors is not in accordance with the steady state situation required for this analogy. Secondly, as mentioned above, little if anything is known about receptor protein density in the cell membrane, which should be identical for all stimuli tested to permit the application of the Michaelis-Menten equation. In fact, differences in these densities may explain the hitherto problematic phenomenon of different saturation levels (Morita and Shiraishi, 1985). Thirdly, response variability has been shown to exert a profoundly obscuring effect on relevant C/R-curve characteristics as estimated by linear transformation (Maes, 1985). For these reasons the curve parameters as assessed in the present paper are graphically estimated from the semi-logarithmic C/R-curves.

In table 4, the values of amino acid concentrations and their reported ranges of variation, as known from the phytochemical literature on Brassica oleracea, are compared with the concentration-range in which the sensory neurones show their strongest change in response intensity, i.e. usually around the K_b value. Evidently, the amino acid receptor cell encounters all amino acids at the same time; the differential effects this may have (addition, synergism or even inhibition) are unknown. Also, histochemical localization of free amino acids, e.g. in vascular tissues where they are present in higher concentrations than in the surrounding laminar tissue, is not reflected by calculations based on total leaf samples. Apart from the compounds featuring in table 4, Asp and Glu are the dominant free amino acids in Brassica oleracea, reaching values of 1-4 mM and 0.6-2.6 mM respectively. It is remarkable that they stimulate a different cell, in the M-sensillum, of both Pieris species. On the basis of the present information it can be concluded that amino acids represent gustatory stimuli at concentrations normally occurring in their host plants.

Table 4 : Comparison of C/R curve parameters of the amino acid sensitive cells of P. rapae and P. brassicae with concentration (mM) of free amino acids in leaves of Brassica oleracea

amino acid	threshold conc.		minimum leaf conc.	K_b		maximum leaf conc.
	P.b.	P.r.		P.b.	P.r.	
proline	<0.2	0.2	0.4	1.7	2.7	15.0
asparagine*	0.2	0.2	1.0	2.2	5.0	9.0
phenylalanine	<0.2	0.2	0.05	0.3	1.4	0.6
histidine	<0.2	0.2	0.01	0.5	6.0	0.5
methionine	<0.2	<0.2	(0)**	5.8	0.9	0.2

Data on Brassica leaf total free amino acid concentrations taken from Benepal and Hall (1967), van Emden and Bashford (1971) and Dodd and van Emden (1979). * includes glutamine, **trace amounts. Recalculated from dry weight basis to fresh weight assuming a water content of 85%

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CHAPTER 3

CHEMORECEPTION OF PHENOLIC ACIDS AND FLAVONOIDS IN LARVAE OF TWO PIERIS SPECIES

ABSTRACT

Chemosensory responses in two maxillary sensilla styloconica to stimulation with phenolic acids and flavonoids were studied using electrophysiological methods in caterpillars of Pieris brassicae L. and Pieris rapae L. Stimulus compounds and the concentrations tested were selected on the basis of reported occurrence in a common host plant, Brassica oleracea L. Of the five phenolic acids tested, chlorogenic and protocatechuic acids were the most effective stimulants. Out of seven examined, catechin was the most effective flavonoid stimulant in the lateral sensillum styloconicum of both species. P. brassicae showed a higher sensitivity to the three anthocyanins tested than P. rapae. The flavonols kaempferol, quercetin and quercetin-3-rutinoside were not evoking responses at any of the concentrations tested. The medial sensillum was less sensitive to flavonoid stimulation than the lateral one. Chemosensory responses were generally increasing with increasing stimulus concentrations in the range tested (0.2 - 5.0 mM). P. rapae generally was less sensitive as judged by higher thresholds. Mixture experiments suggested that in the lateral sensillum one and in the medial sensillum two cells were especially sensitive. Cyanin caused inhibition of spiking activity in several other neurones. Caterpillars reared on an artificial diet showed reduced sensitivity as compared to rearing on a host plant. Chemosensory responsiveness was reflected in preference behaviour in dual choice situations. Set against the background of phytochemical data, dose-response relations permit the conclusion that naturally occurring levels of phenolic acids and flavonoids are stimulating to some chemosensory neurones and can cause inhibition of activity in others.

INTRODUCTION

Phenolic acids and flavonoids are ubiquitous secondary metabolites in plants (Harborne, 1979). They have the first part of their biosynthesis in common and serve regulatory physiological functions within the plant. There is ample evidence for their ecological function in the biochemical defense of plants against fungal pathogens and herbivores (Levin, 1971; McClure, 1975). The effects on phytophagous insects differ to a large extent, depending on the species (Schoonhoven, 1972a; Harborne, 1979). Flavonoids may either stimulate or inhibit feeding behaviour in oligophagous as well as polyphagous insects (Matsuda, 1978; Dreyer & Jones, 1981; Larsen et al., 1982; Woodhead & Cooper-Driver, 1979). In contrast to other classes of secondary compounds however, the sensory physiology of phenolic compounds is scarcely documented (Schoonhoven, 1982). A few electrophysiological studies report qualitatively on one arbitrary concentration of a few flavonoids tested (Ishikawa, 1966; Wieczorek, 1976; Dethier, 1973; 1980; van Drongelen, 1979).

The caterpillars of Pieris brassicae and P. rapae are oligophagous on Cruciferae. Their host selection behaviour can to a large extent be explained by their gustatory recognition of glucosinolates from cruciferous plants (David & Gardiner, 1966; Schoonhoven, 1967). Host plants in the genus Brassica contain varying amounts of several flavonoid compounds (Hegnauer, 1973; Durkee & Harborne, 1973), visual evidence of which is the anthocyanin accumulating red cabbage B. oleracea var. rubra. Schoonhoven (1969) reported gustatory sensitivity to anthocyanins from B. oleracea var. rubra in maxillary receptors of P. brassicae. Crucifer feeding flea beetles employ both glucosinolates and flavonoids as gustatory stimuli guiding host selection behaviour (Nielsen, 1978; Larsen et al., 1982).

In the present study, the contact chemosensory effects of host plant borne phenolics, flavonoids and some closely related compounds are investigated by electrophysiological methods. The sensitivity to both groups of compounds is put into the perspective of quantitive data on concentrations in host plants and is compared for both closely related species. In P. brassicae, the degree of specialization of receptor cells responding to these compounds is studied. Finally, behavioural data are presented to investigate if electrophysiological characteristics are reflected in feeding behaviour.

MATERIALS AND METHODS

Insects

Pieris brassicae and P. rapae cultures were maintained in the laboratory under conditions described by David & Gardiner (1962). Both cultures were started with field-collected material; every year new field-collected individuals were introduced into the cultures. Caterpillars of both species studied in electrophysiological experiments were reared on Brassica oleracea L. var. gemmifera DC. cv. Titurel (Sluis and Groot, Enkhuizen, The Netherlands) under ad libitum conditions. Rearing temperature was 25 °C, relative humidity varied between 50 and 70%. Photo/scotophase was 15/9.

For electrophysiology only those caterpillars were used that had completed their development ab ovo upto the final larval moult at the normal rate of 15 days (P. brassicae) or 13 days (P. rapae) at 25 °C. They were 24-48 h in the instar since ecdysis. P. brassicae-weights ranged from 250-400 mg, P. rapae individuals used had weights of 80-120 mg. In this way, it was attempted to work with individuals of defined physiological age and nutritive status, as these parameters are known to affect chemosensory response strength (Blaney et al., 1986). Prior to the experiments, larvae were deprived of food during 2-3 hours. The head and the first thoracic segment were amputated for recording. A U-shaped silver wire recording electrode was inserted through the foramen magnum of the isolated head and was protruded into the maxillary-labial complex to fix the maxillae in a more prognathous position. Location, morphology and ultrastructure of the styloconical sensilla of the maxillary galea were described by Ma (1972) for P. brassicae. All evidence available indicates that the same structural situation is present in P. rapae. In the following, the lateral styloconical sensillum is abbreviated as the L-sensillum, the medial one as M-sensillum.

For behavioural experiments, P. brassicae larvae were reared ab ovo on a semi-defined artificial diet (Ma, 1972). This diet routinely contains the phenolic methyl-p-hydroxybenzoate at a rate of 184 mg per 100 g fresh weight as an inhibitor of microbial contaminants. Phenolic compounds added to the standard diet were dissolved in ethanol 96% and admixed just prior

to solidification at a temperature of 45 °C.

Stimuli

Chemicals studied were the following: 1. sucrose, 2. proline, 3. sinalbin, 4. helveticoside. Organic acids: 5. ascorbic acid, 6. cinnamic acid, 7. citric acid. Phenolic acids: 8. protocatechuic acid, 9. caffeic acid, 10. ferulic acid, 11. sinapic acid, 12. chlorogenic acid. Flavonoids: 13. cyanin, 14. malvin, 15. oenin, 16. quercetin, 17. rutin, 18. kaempferol, 19. DL-catechin. They were of 99% purity and obtained from the following commercial sources: Merck, the compounds 1, 2, 5. Sigma: 4, 6, 8-12, 16-18. Baker: 7, 15, 19. Serva: 13, 14. Roth: 3.

All stimuli were dissolved into 2 mM (P. rapae) or 10 mM (P. brassicae) potassium chloride solutions in distilled water. Phenolics and flavonoids were presolubilized in 100 µl of methanol, subsequently diluted to a final maximal concentration of 1% of methanol. A solution of methanol and potassiumchloride served as the control stimulus. In C/R-experiments, concentrations were chosen to include the concentration levels found to occur in the tissues of host plants, on the basis of known data (see discussion). The pH of phenolic acid solutions at four concentrations were determined using an Orion 701A electronic pH-meter. Details on stimulus application and procedures are given in chapter 2 .

Recording of electrophysiological responses

A modification of the hair-tip recording technique of Hodgson et al. (1955) was used to record the responses of gustatory chemosensory cells (van Drongelen, 1979). Details on recording methods and equipment are to be found in chapter 2 . Room temperatures during recording were 22 ± 2 °C.

Analysis of the recordings

Chemoreceptor sensitivity is quantified as the total number of action potentials generated from 50 ms until 1050 ms after stimulus onset, further

on designated as F_{ap}, frequency of action potentials, expressed in spikes/s. Action potentials were counted after visual identification on the basis of typical biphasic wave-form (fig. 6 - 8). Spike amplitude, duration of interspike interval and adaptation rate served as criteria to assign spikes to the different neurones active in multi-neural responses. Mixtures of two compounds that elicit single unit activity when applied separately assisted the analysis of cellular origin of spikes (Bowdan, 1984).

RESULTS

Concentration-response relations

Phenolic acids

Dose-response curves of the electrophysiological reactions of L- and M-sensilla of P. brassicae and P. rapae are shown in fig. 1 and 2 respectively. In the L-sensillum of both species, chlorogenic acid and protocatechuic acid were the best stimulants . Thresholds were lower than 0.5 mM. The responses to protocatechuic acid in both species and to caffeic acid in P. rapae were lower at 5.0 mM than at the next lower concentration tested. Sinapic acid and ferulic acid elicited comparatively weak dose dependent responses and no further increase was induced at a dose of 5.0 mM.

In the M-sensillum of both species, chlorogenic and protocatechuic acid also were the best stimulants. Thresholds in this sensillum were around the lowest concentration tested. Responses to protocatechuic acid in P. brassicae and caffeic acid in P. rapae did not decline at 5.0 mM. Ferulic acid in P. rapae at 5.0 mM equalled the effectiveness of chlorogenic and caffeic acids.

Flavonoids

In the L-sensillum of both species, catechin was the most effective stimulant with a threshold lower than 0.5 mM. In P. brassicae, cyanin chloride was the next best stimulant. Sensitivity to this compound was absent in P. rapae. Oenin and malvin showed dose-dependent effects in P. brassicae that were less distinct in P. rapae. Kaempferol, quercetin and rutin did not evoke responses at any of the concentrations tested in neither the L-sensillum nor the M-sensillum.

In the M-sensillum of both species, catechin was only weakly effective causing a small increase in response at higher concentrations. Cyanin evoked a response at concentrations beyond 0.2 mM in P. brassicae, while in both species oenin and malvin evoked distinct reactions only at 5.0 mM.

Generally P. rapae was less sensitive to phenolic acid or flavonoid

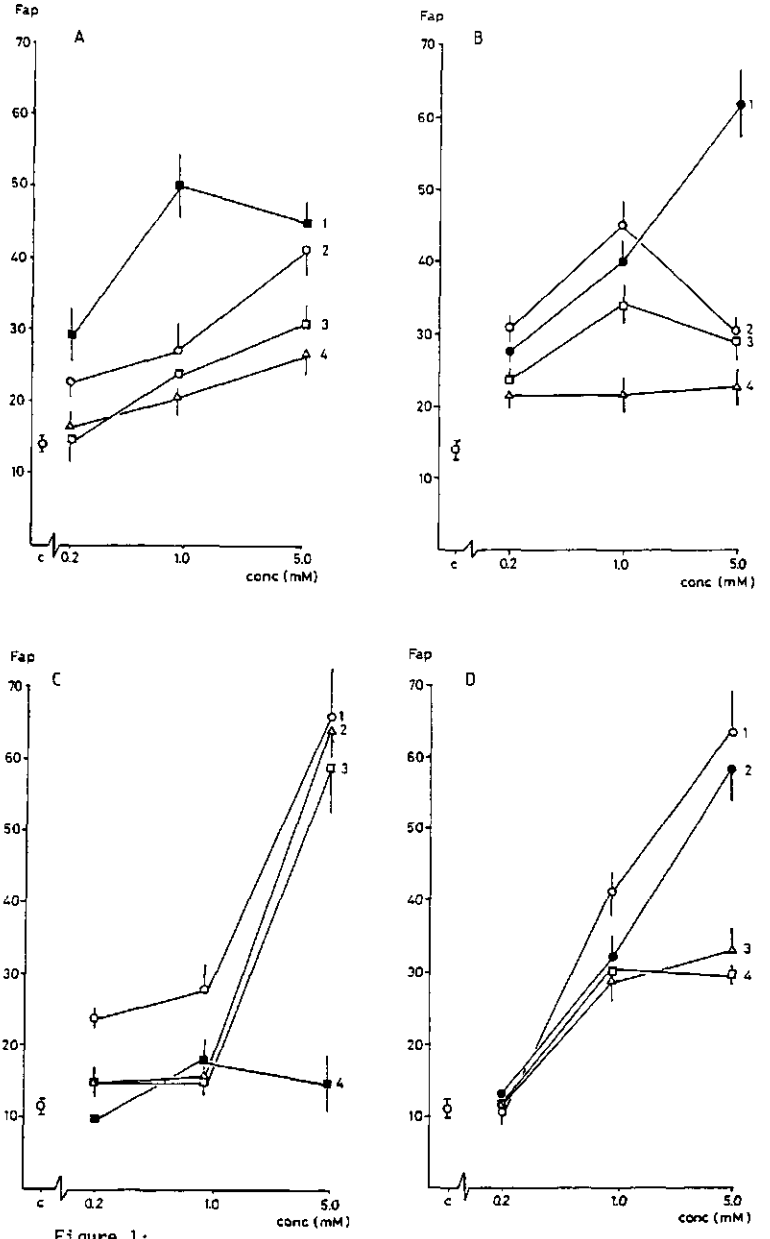

Figure 1:
Concentration-response curves for flavonoids (A,C) and phenolic acids (B,D) in P. brassicae. Graph A,B: L-sensillum; graph C,D: M-sensillum. Abscissa: concentration (mM), logarithmic scale. Ordinate: response frequency (Fap, in action potentials/s). Vertical bars represent S.E.M. (one- or two-sided). Graph A: catechin (1), cyanin chloride (2), oenin (3), malvin (4). Graph B: chlorogenic acid (1), protocatechuic acid (2), ferulic acid (3) and sinapic acid (4). Graph C: cyanin chloride (1), malvin (2), oenin (3), catechin (4). Graph D: protocatechuic acid (1), chlorogenic acid (2), sinapic acid (3) and ferulic acid (4). Data from 7-14 individuals.

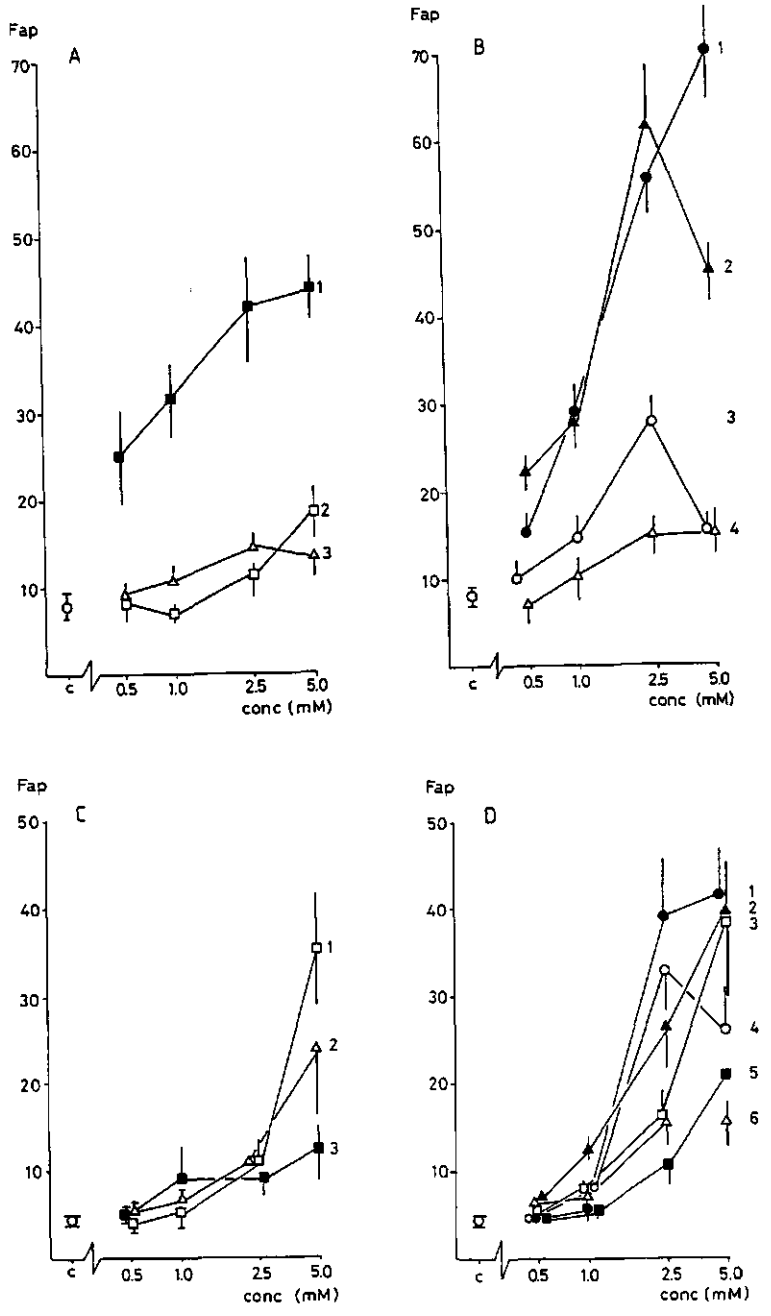

Figure 2:
Concentration-response curves for flavonoids and phenolic acids (B,D) in P. rapae. Graph A,B: L-sensillum, graph C,D: M-sensillum. Details as for fig. 1. Graph A: catechin (1), oenin (2), malvin (3). Graph B: chlorogenic acid (1), caffeic acid (2), protocatechuic acid (3), sinapic acid (4). Graph C: oenin (1), malvin (2), catechin (3). Graph D: chlorogenic acid (1), caffeic acid (2), ferulic acid (3), protocatechuic acid (4), cinnamic acid (5), sinapic acid (6). Data from 9-16 individuals.

stimulation as response values at 1.0 mM relative to those at 5.0 mM are lower in the latter species (fig. 2).

Response profiles

Response profiles combining both groups of compounds at a single intermediate concentration in the range tested have been constructed as a means of comparison. Only those compounds are incorporated which evoked significantly higher spiking activity from the sensilla than the control level. It is seen that in both species the phenolic acids as a group and especially those with hydroxyl-groups at the 3- and 4-positions of the aromatic ring were more effective stimuli to both L- and M-sensilla than the flavonoids. An exception is catechin in the L-sensillum. Although response variability was high, significant differences were calculated between mean response values (fig. 3 and 4).

The effect of pH

Response frequencies in the L-sensillum to three non-phenolic organic acids and three phenolic acids at 5.0 mM showed no apparent relationship with the pH of the stimulant solution (fig. 5). In the M-sensillum the responses to caffeic acid in P. rapae and to protocatechuic acid in P. brassicae are too high to infer a positive correlation between the free hydrogen-ion concentration as such and the sensory response.

Cellular origin of spikes

The responses to phenolics and flavonoids in the L-sensillum were originating predominantly in a single cell in both species, judged on the basis of a single spike-type with a large amplitude. The time characteristic of the spiking activity was of a tonic nature as compared to the phasic-tonic responses to sucrose, proline and sinalbin (fig. 8). A smaller spike was occasionally present at lower frequencies (fig. 6). With the anthocyanins, responses occasionally showed an extended initial latency period (fig. 6).

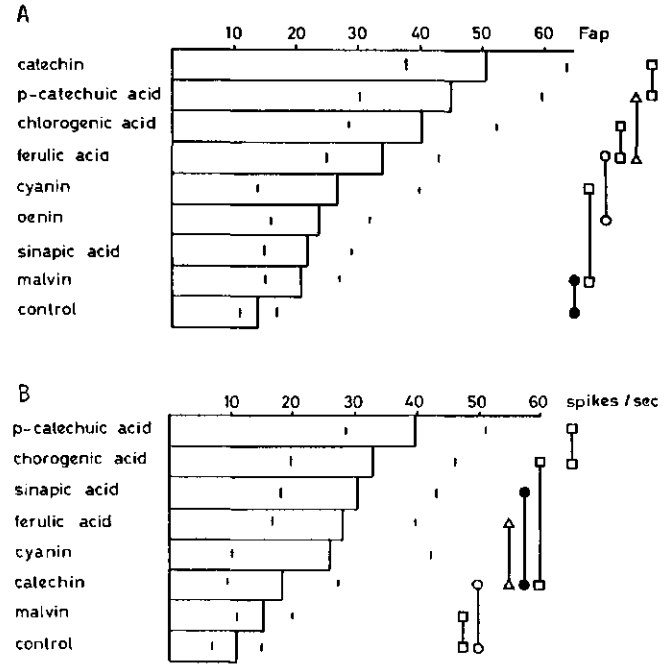

Figure 3:
Response profile of P. brassicae to phenolic acids and flavonoids at 1 mM. Top axis is ordinate (response frequency, F_{ap}, spikes/s). *Significance of differences between mean* response values (Student's t-test) is indicated by the vertical lines at the right hand side delimited by the following symbols: 0 : $p < 0.05$; $p < 0.025$; $p < 0.01$; $p < 0.005$. Graph A: L-sensillum; Graph B: M-sensillum. Data from 7-12 individuals.

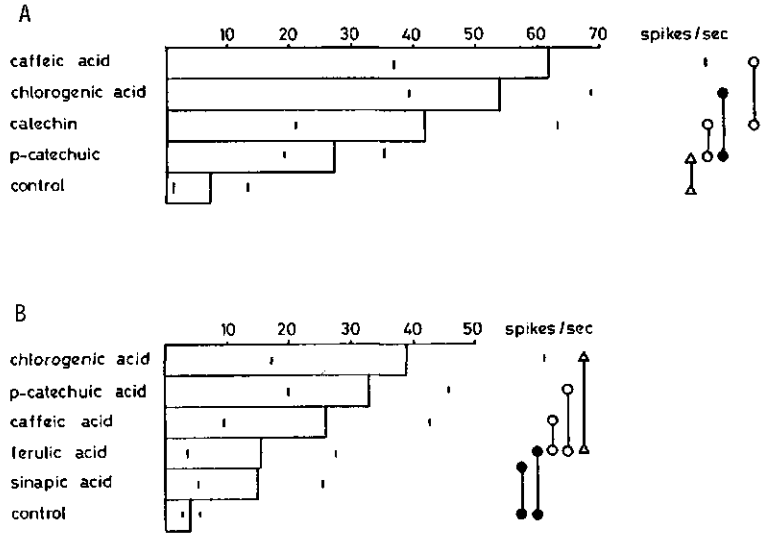

Figure 4:
Response profile of P. rapae to phenolic acids and flavonoids at 2.5 mM. Details as for fig. 3. Data from 9-14 individuals.

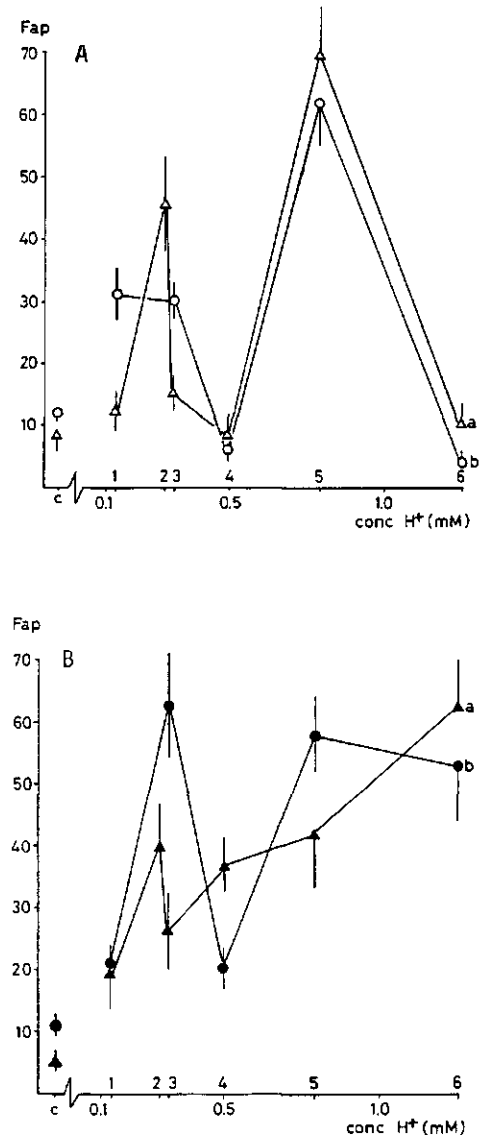

Figure 5:
Dose-response curves for P. brassicae (a) and P. rapae (b) in response to hydrogen-ion concentration (mM, abscissa) in 5 mM organic acid solutions. Ordinate: response frequency, Fap (spikes/s). Graph A: L-sensillum, graph B: M-sensillum. pH-values of solutions marked along abscissa: cinnamic acid, pH = 3.89 (1), caffeic acid, pH = 3.54 (2), protocatechuic acid, pH = 3.51 (3), ascorbic acid, pH = 3.35 (4), chlorogenic acid, pH = 3.03 (5), citric acid, pH = 2.85 (6).

In the M-sensillum, single cell responses were observed at concentrations below 2.5 mM. In contrast, in part of the recordings taken at 2.5 mM and most of the recordings at 5.0 mM, two or three neurones were activated (fig. 7).

Responses to mixtures

In the L-sensillum, mixing cyanin-chloride at 2.5 mM with proline or sinalbin induced the firing of two neurones with different amplitudes (fig. 8). Results with sucrose-cyanin mixtures were less conclusive, partly caused by the high response frequency to 15 mM sucrose alone. In some cases very large spikes occurred, probably due to electrical addition, but their number was much smaller than expected (table 1). Responses to helveticoside-cyanin mixtures were very similar to the responses to helveticoside alone, showing only one large spike type.

Results for the M-sensillum were generally more difficult to explain because responses to cyanin alone frequently were multineural at 2.5 mM. When it is assumed that the smaller spikes in the latter recordings originated in the cation-sensitive cell, the findings for the M-sensillum were comparable with those described for the L-sensillum. In all cases, response frequencies to mixtures were significantly lower than expected on the basis of simple additivity of reaction frequencies upon stimulation with the single constituents. With proline and helveticoside in the L-sensillum and sucrose in both sensilla, response frequencies of the corresponding single cells were significantly inhibited during mixture stimulation ($p < 0.05$ or better).

Effect of rearing diet on chemosensory responses

Caterpillars raised on the artificial diet showed a significantly lower or decreased chemosensory sensitivity to chlorogenic acid in both sensilla and to cyanin chloride in the L-sensillum compared with caterpillars reared on B. oleracea leaf material (table 2).

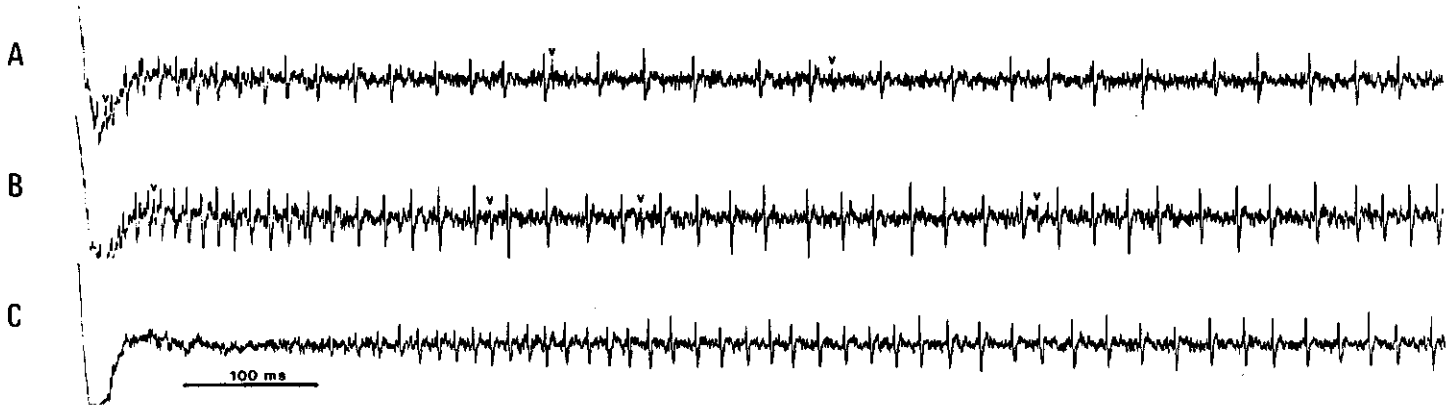

Figure 6:
Electrophysiological responses of lateral sensilla of P. brassicae to (a) 1 mM chlorogenic acid, (b) 2.5 mM chlorogenic acid, (c) 2.5 mM cyanin-chloride. (a) and (b) from the same sensillum, (c) from a different sensillum showing a considerable latency time prior to a tonic response. Distortion at beginning of traces due to stimulus onset artifact. Arrows indicate occasional small action potentials from another cell. The solvent alone (10 mM KCl) evoked only few small spikes (not shown)

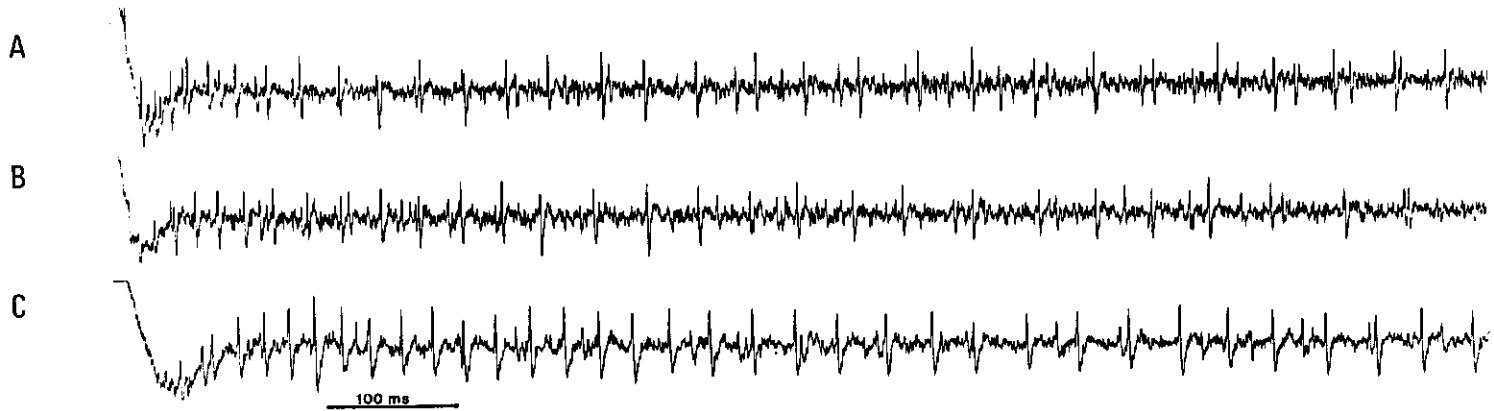

Figure 7:
Responses of medial sensilla of P. brassicae to (a) 2.5 mM chlorogenic acid, (b) 2.5 mM cyanin-chloride and (c) 5 mM cyanin-chloride. Three different sensilla, displaying tonic spiking activity of at least two different chemosensory neurones in varying ratios.

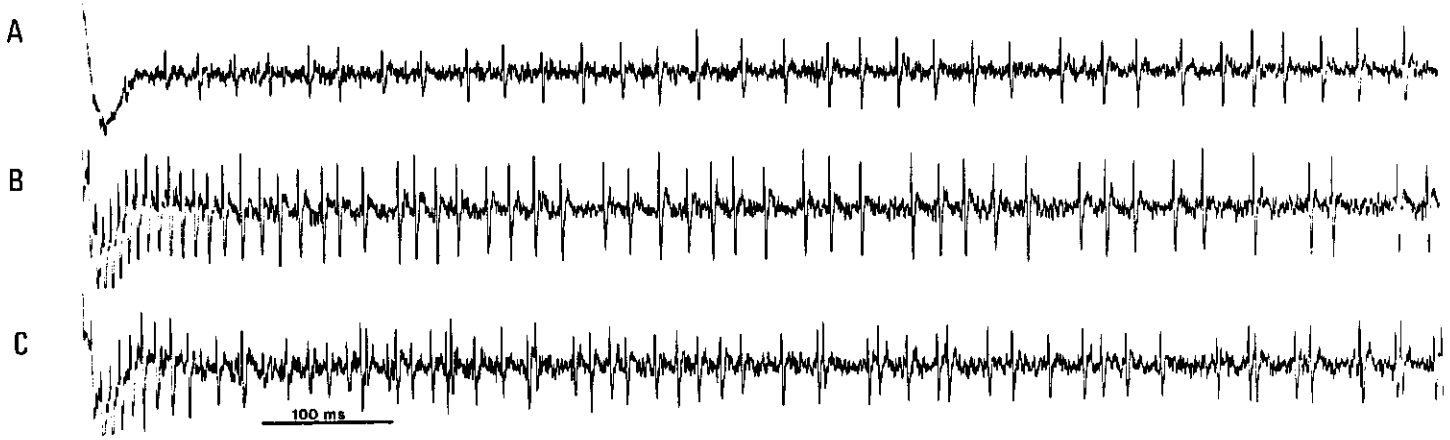

Figure 8:
Responses of a single lateral sensillum of P. brassicae to (a) 2.5. mM cyanin-chloride, (b) 0.2 mM sinalbin and (c) a mixture of 2.5 mM cyanin-chloride and 0.2 mM sinalbin. Recording (a) illustrates a tonic reaction-type in contrast with the phasic reaction to sinalbin (b). Recording (c) demonstrates the simultaneous activity of the neurones from (a) and (b) as judged from both the different amplitudes and irregular interspike-intervals. The activity of the neurone producing the larger spike (b) is inhibited.

Table 1. Responses to mixtures of cyanin and four other compounds known to evoke single cell responses

sensillum	stimulus	conc [a] mM	response [b] (A)	response to cyanin 2.5 mM (B)	observed response to mixture (C)	expected response under additivity (D)		spike height classes (mixtures)
L	sucrose	15	150 ± 11	28 ± 9	133 ± 18	178 ± 16	* [c]	1-2
	proline	5	70 ± 4	26 ± 10	50 ± 18	96 ± 6	*	2
	sinalbin	0.2	59 ± 4	27 ± 9	61 ± 12	86 ± 17	*	2
	helveticoside	0.01	61 ± 9	26 ± 9	53 ± 9	87 ± 11	*	1
M	sucrose	15	60 ± 15	43 ± 10	27 ± 20	103 ± 24	*	2
	sinalbin	0.2	71 ± 20	40 ± 16	89 ± 28	111 ± 27	*	2-3
	helveticoside	0.01	27 ± 7	57 ± 3	47 ± 8	80 ± 3	*	1-2

a: in solutions of both single compound and mixtures.
b: response values in spikes/s ± SD. c: * indicates D significantly higher than C according to Wilcoxon's matched pair signed rank test ($p < 0.05$, $n = 5-7$) using values obtained from individual sensilla.

Table 2. Effect of diet experienced during development upto the fifth instar on response frequencies in chemosensory sensilla of P. brassicae

compound	concentration	sensillum					
	mM	diet			diet		
		plant[a]	artificial	p[b]	plant	artificial	p
sucrose	30	138 ± 25[c]	142 ± 23		68 ± 13	79 ± 18	
proline	5	86 ± 24	72 ± 10	0.05	14 ± 12	13 ± 6	
chlorogenic	1	40 ± 12	37 ± 11		32 ± 14	14 ± 7	0.005
acid	5	62 ± 18	42 ± 11	0.025	58 ± 16	37 ± 9	0.01
cyanin	1	25 ± 14	11 ± 6	0.025	28 ± 14	19 ± 14	
	5	42 ± 11	19 ± 10	0.001	66 ± 30	65 ± 12	

a: B. oleracea.

b: level of significance according to Student's t-test (n = 6-10).
no value means P > 0.05.

c: response frequencies in
spikes/s ± SD.

Behavioural experiments

In dual choice experiments lasting the initial 24 hours of the fifth instar, diet-reared caterpillars showed significant preferences for the control diet over oenin and chlorogenic acid containing diets (table 3). A higher concentration of chlorogenic acid caused a stronger preference for the control diet. On the other hand, with cyanin chloride and sinapic acid, a significant preference for the diets containing these compounds was observed. Incorporation of rutin at 5.0 mM failed to induce a preference for either of both diets offered.

Correlation between behaviour and electrophysiology

Following the dual choice test, individuals in test group 6 (table 3) were deprived of food. They were subjected to electrophysiological experiments to establish their responsiveness to chlorogenic acid solutions within 6 hours after the onset of food deprivation. Individual correlations between preference score and sensitivity in either the L- or the M-sensillum revealed no significant correlations. A significantly negative rank correlation was calculated when the difference between the frequency of impulses in L- and M-sensilla were used as the chemosensory parameter. Spearman's rho is -0.75, $p < 0.05$ according to the Hotelling-Pabst test (table 4).

Table 3. Feeding preference behaviour in a dual choice situation of newly ecdysed fifth instars of P. brassicae during the first 24h in the instar. All tests were run simultaneously.

Test	Test choice	Concentration mM	Amount consumed mg ± SD	P[a]
1	control vs.		4 ± 4	
	cyanin	2.5	62 ± 7	0.001
2	control vs.		13 ± 5	
	cyanin	5.0	64 ± 14	0.001
3	control vs.		74 ± 6	
	oenin	2.5	4 ± 2	0.001
4	control vs.		66 ± 8	0.001
	oenin	5.0	4 ± 3	
5	control vs.		29 ± 13	
	rutin	5.0	37 ± 11	0.05
6	control vs.		48 ± 14	
	chlorogenic acid	2.5	23 ± 10	0.005
7	control vs.		65 ± 7	
	chlorogenic acid	5.0	11 ± 11	0.001
8	control vs.		15 ± 8	
	sinapic acid	5.0	53 ± 13	0.001

a: Student's t-test for paired observations (n=8). mg are dry weight.

Table 4. Chemosensory sensitivity and feeding preference behaviour of 7 individuals in response to 2.5 mM chlorogenic acid solutions and a dual choice between control and 2.5 mM chlorogenic acid-containing diet respectively.

individual	amount consumed mg[c]		preference index[a]	response[b] spikes/s sensillum		difference
	control	treated		L	M	L - M
1	40	23	0.63	52	40	+ 12
2	56	13	0.81	45	53	- 8
3	45	21	0.68	61	43	+ 18
4	39	41	0.49	54	51	+ 3
5	56	15	0.79	31	36	- 5
6	55	22	0.71	13	16	- 3
7	38	21	0.64	38	36	+ 2

a: preference index is amount consumed of control diet divided by total amount consumed.

b: mean of 3 tests on the same sensillum.

c: mg refer to dry weight

DISCUSSION

Dose-response relations and phytochemistry

The dose-response curves reveal that in the concentration range investigated, most compounds tested evoked higher responses at higher concentrations. Exceptions to this generalization are protocatechuic acid and caffeic acid, to which responses were distinctly lower at 5.0 mM in some cases. Quantitatively the dose-dependency differs widely, however. Chlorogenic, caffeic and protocatechuic acids are characterized by very steep dose-response curves, in contrast to the small increase in response frequencies with sinapic acid or malvin for example. Structurally the former have ortho-substituted hydroxyl-groups at the 3- and 4-positions of the aromatic ring in common. Threshold-concentrations cannot be determined with great accuracy as only few concentrations have been tested. Yet it is of interest to compare quantitative phytochemical data on the occurrence of phenolic acids and flavonoids in B. oleracea-cultivars with the dose-response relations established. Over different B. oleracea varieties (i.e. gemmifera, sabauda, rubra, sabellica) as well as over four to five different cultivars within these varieties, a high degree of variation in levels of these compounds has been reported (Wildanger & Herrmann, 1973; Schmidtlein & Herrmann, 1975; Hanefeld & Herrmann, 1976; Herrmann, 1976; 1977; Brandl & Herrmann, 1983). Maximum amounts of caffeic acid in leaf material ranged from 0.23 to 1.7 mM (values recalculated from mg/kg fresh weight divided by the molecular weight). Maximum levels of sinapic acid varied between 0.4 and 3.2 mM and of ferulic acid between 0.15 and 1.4 mM. A fourth predominant phenolic acid that has not been screened in the present study is p-coumaric acid, found to vary between 0.1 and 0.9 mM. Protocatechuic acid was found only in B. oleracea var. rubra at a maximum level of 0.6 mM. When the total concentration of 4-hydroxylated aromatic acids, carrying either an additional free or methoxylated hydroxyl group at the 3- or 3- and 5-positions are summed, var. sabellica has the highest level with 6.5 mM, followed by var. rubra with 3.5 mM, the gemmifera cultivars screened contained maximally 1.8 mM and sabauda cultivars 1.3 mM. The cultivars belonging to the alba-group contain minimal amounts of these constituents. These analyses pertain to aglycones. On the basis of

these data it can be concluded that several phenolic acids tested may represent actual stimuli to caterpillars feeding on B. oleracea leaf material. As mixtures of these acids have not been tested, it is unknown if additive, synergistic or inhibitory interactions occur at the chemosensory level.

The predominant flavonoid aglycones in B. oleracea are quercetin, kaempferol and isorhamnetin (Hegnauer, 1973; Durkee & Harborne, 1973; Wildanger & Herrmann, 1973) and cyanidin as the prevalent anthocyanidin (Hrazdina et al., 1977). In the sabellica-group the highest amounts of flavonol aglycones have been reported, being around 1.0 mM (Wildanger & Herrmann, 1973; Herrmann, 1976). The presence of rutin as a flavonol glycoside in the genus Brassica has been disputed (Durkee & Harborne, 1973). Catechin has not been reported as a common flavonoid in Brassica. No quantitative data are available on cyanidin-levels in mature leaves of the rubra group. Malvin and oenin have not been found in Brassica. Threshold for the response to cyanin in P. brassicae was below 0.2 mM and this anthocyanin may be assumed to constitute a natural stimulant.

Several factors complicate the references to phytochemical data made above. Phenolic acids and flavonoids accumulate predominantly in the vacuole (McClure, 1975; Matile, 1984). In this compartiment they occur for 95% or more as several species of glycosides (Harborne, 1979). Cellular disruption during feeding releases enzymatically the respective aglycones. The phytochemical analyses express amounts as aglycones, based on total leaf homogenates. In view of the dimensions of the sensillar pores (0.2 μm (Ma, 1972)) it is conceivable that subcellular localization mentioned may result in contacting higher levels than calculated on the basis of total leaf analyses. The exact concentrations of aglycons and glycosides actually encountered by the sensillar tips contacting the damaged leaf edge are unknown and the references given above are approximations.

The two species differ considerably in their sensitivity to the compounds tested. P. brassicae clearly is the more sensitive of the two. This may bear connections to differences in larval host selection behaviour. P. rapae readily feeds on B. oleracea cultivars in the rubra-group, while these are accepted only after a period of rejection by larvae of P. brassicae. Furthermore, P. rapae tolerates higher levels of phenolics and flavonoids during development than P. brassicae (van Loon, unpublished results/Chapter 6). For both species, the capacity of sensory

discrimination of these secondary compounds can be thought to influence foraging behaviour within a single host plant. The selection of younger plant parts that contain lower amounts of these compounds (Herrmann, 1976) is thus made possible. Higher levels of both phenolic acids, flavonols and anthocyanins can be induced by several kinds of stress agents (Del Moral, 1973; Hrazdina, 1982; McClure, 1982). Plant tissues where such induction has occurred can be avoided.

Structure-activity considerations

Some deductions on the importance of structural characteristics of the stimulus compounds can be made, although no systematic series of structural modifications were tested, due to the emphasis placed on the study of host-plant borne compounds. Phenolic acids that possess free hydroxyl-groups at both the 3- and 4-positions of the aromatic ring are more effective than when one of these groups is methoxylated. The same is true for the B-ring of the flavonoids in P. brassicae; methoxylation of the hydroxyl-groups here result into decreased responsiveness. In the L-sensillum of P. rapae, the quinic ester part of chlorogenic acid does not contribute to the stimulating effectiveness. The high effectiveness of catechin in the L-sensilla of both species as compared to the less effective anthocyanins as well as the ineffective flavonols may be ascribed to an increased oxidized state of the C-ring. The high responsiveness to catechin demonstrates also that glycosylation is not a requirement for effectiveness. This can be considered a functional characteristic when it is realized that aglycons are released during feeding, as already mentioned. In the M-sensillum of P. brassicae sensitivity to catechin is absent in the presence of anthocyanin sensitivity, which suggests different receptor-types responsible. As a comparable contrast, catechin sensitivity is present in the L-sensillum of P. rapae in the absence of cyanin-sensitivity.

The possibillity that responses depended merely on free hydrogen ion concentrations accompanying phenolic acids was ruled out for the L-sensillum, though it may play a role in the M-sensillum for chlorogenic acid. Structure-activity studies on flavonoids have been performed on the behavioural level (Norris, 1977; Elliger et al, 1980; Lane et al., 1985). Ortho-hydroxylation of the B-ring was found a key structural characteristic

of flavonoids in their toxicity to Heliothis zea (Elliger et al., 1980), which corresponds to the present results at the chemosensory level in two Pieris species. In contrast, feeding by Scolytus multistriatus was inhibited especially by flavonoids possessing an oxidized C-ring like kaempherol and quercetin, which were ineffective in the present study (Norris, 1977). In chrysomelid beetles, differences in sugar residues explained feeding inhibition of flavonoids better than the phenolic substitution pattern of the aglycons (Matsuda, 1978). In conclusion, several structural characteristics of phenolic and flavonoid compounds seem important for the degree of their effectiveness as chemosensory stimulants.

Cellular specificity

The specificities of the gustatory neurones in both sensilla of P. brassicae have been investigated in detail (Schoonhoven, 1967; 1969; 1972; Ma, 1972). This made it possible to determine which neurones were activated by stimulation with phenolic acids or flavonoids. Spike-amplitude served as the main criterion to decide on the number of neurones active in multicellular recordings. The amplitudes measured from the four neurones present at the basis of the sensilla invariably show a ranking order in that for example the deterrent-neurone in the M-sensillum always is represented by a greater amplitude than the glucosinolate sensitive cell, which can be recognized by a greater amplitude than the sucrose-sensitive cell, while the cation-receptor shows the smallest amplitudes. This ranking order is very constant from individual to individual and from recording to recording, though the absolute amplitudes are not. When stimuli that evoke single-unit activity are mixed together with a compound for which the sensitive neurone is unknown, the appearance of two spike amplitude classes in the recording is considered a proof that another neurone is also excited. This reasoning recently posed problems in two dipterous species, where amplitudes from different neurones were similar (Bowdan, 1984) or where amplitude rankings varied (Fujishiro & Morita, 1984). On the basis of the known specificities and this mixture-technique it can be decided that in the L-sensillum of P. brassicae a neurone separate from the sucrose-, amino acid- and glucosinolate-'best' cells was activated by the effective phenolic acids and flavonoids. For anthocyanins this has already

been suggested (Schoonhoven, 1969). This fourth lateral cell is at the same time very sensitive (threshold about 2 μM) to the steroidal compound helveticoside, which occurs in some Cruciferae (van Loon, unpublished results; cf. Nielsen, 1978). It is, however, insensitive to a range of steroidal compounds to which the medial deterrent neurone reacts (Ma, 1972). Additional similarities between this 'fourth' neurone and the medial deterrent neurone are the slow adaptation rates (Schoonhoven, 1977) and the frequent occurrence of an extended latency time prior to the first spike (van Drongelen, 1979). Cellular specificity in the M-sensillum is more difficult to interpret. At higher concentrations, phenolic acids and anthocyanins evoke multineural responses when they are applied as pure compounds. This phenomenon has been described for sinalbin also (Ma, 1972). It seems probable that in the M-sensillum, both the deterrent-neurone and the sinalbin-sensitive cell are excited. This would mean that the presence of an aromatic ring is the structural requirement for activity of the so-called aromatic glucosinolate-sensitive neurone (Schoonhoven, 1969). A resemblance with the glycoside receptor of Mamestra brassicae emerges in this respect (Wieczorek, 1976). The third neurone that is activated at 5.0 mM most probably is the cation-receptor, which is labile to pH-values lower than 3.0 .

The mixture-experiments revealed the operation of peripheral interactions (table 1). Significant decreases in responses to sucrose and proline were established, while in no case simple additivity of responses occurred. It appears that cyanin both stimulates deterrent receptors in both sensilla and at the same time inhibits other receptor cells. Moreover, the response of the lateral cyanin-sensitive cell seemed suppressed by the simultaneous application of sucrose. Several mechanisms have been proposed for such interactions (Bowdan, 1984). The data available do not provide a better insight into which of these were operative in the experiments described, however. At this point it must be stressed that also those plant compounds that by themselves do not evoke responses (e.g. rutin) theoretically have the potential of inhibiting the sucrose receptor for example (Schoonhoven, 1982). This possibility has not been checked here.

The dual choice experiments demonstrate that in P. brassicae electrophysiological properties are reflected in behavioural discrimination. As sensitivity to the compounds mentioned resides in both the L- and the M-sensillum, the resulting behaviour is most likely based upon a decision process at a central level. The outcome can apparently be both negative (avoidance) or positive (preference), depending on the compound and its concentration. In several respects, L- and M-sensilla mirror each other; they both contain cells sensitive to sucrose and to glucosinolates, organic acids and several other secondary plant substances. The specificity and sensitivity spectra overlap but are not identical (Ma, 1972; van Loon, unpublished results). Coding of food quality, as for example the presence of phenolic compounds, can in part come about by the weighing of chemosensory input from the anatomically separated maxillary sensilla. This simple model has been suggested also for Manduca sexta, although exceptions were found (Schoonhoven & Dethier, 1966; Dethier & Crnjar, 1982).

Artificial diets offer the possibility of manipulation and strict control of dietary composition which are impossible in experiments with plant material. The decreased sensitivity to chlorogenic acid and cyanin in diet-reared caterpillars is an undesired and surprising phenomenon. The continuous exposure to the commonly applied phenolic preservative methylparahydroxybenzoate during larval development may be a factor in this respect. Sensory effects of this synthetic compound have been documented (Vinson et al., 1976). The duration of the dual choice test (24 h) theoretically allowed post-ingestional effects on feeding behaviour to come into play. It also allows effects on gustatory cells to come about by prolonged exposure to the chemical under test. The latter considerations pertain also to experiments with P. brassicae using application of phenolic acids and flavonoids on leaf discs in a no-choice situation during 24 h (Jones & Firn, 1979). The compounds are not distributed homogeneously but rather presented in a concentrated layer at the leaf surface. Despite these differences and with some exceptions, essentially similar results were reached in this behavioural setup (Jones & Firn, l.c.). It is concluded that although the available evidence for direct involvement of chemosensory sensitivity on food preference behaviour is suggestive, the experimental set-up does not permit definite conclusions (cf. also Bernays, 1987).

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CHAPTER 4

RESPIROMETRY VS. GRAVIMETRY AS METHODS TO DETERMINE THE ENERGETIC EFFICIENCY OF FOOD UTILIZATION IN LARVAE OF TWO *PIERIS* SPECIES

ABSTRACT

The energetic efficiency of growth was determined for *Pieris brassicae* L. and *P. rapae* L. (Pieridae, Lepidoptera) caterpillars in their final instars on different artificial diets and host plants. Two methodologically independent methods were used on the same insects, the conventional gravimetric method and a respirometric method which allowed continuous monitoring of caterpillar energy exchange.

In 7 out of 8 experiments, discrepancies in the calculated energetic efficiency between both methods were found for both species, differences ranging from -13% to +51%. Differences due to dietary treatments showed for the gravimetric method relatively large variations in both absolute metabolic rate (-17% to +65%) and energetic efficiency (-36% to +11%). The respirometric method on the other hand showed smaller variations of -9% to +18% in energetic efficiency. Host plant borne polyphenolics incorporated into an artificial diet affected growth rate but did not negatively influence energetic efficiency according to respirometric results although this was suggested by gravimetric calculations.

It is argued that gravimetric budget calculations are subject to a number of random and systematic errors, only partly compensated by certain recently proposed corrections. For example, a systematic error of only +5% in the dry matter content of the food could explain 75% of the discrepancies. Large variations in insect food utilization (200-300%) are common in the literature. An estimation of total heat production by means of chronic respirometry during the final instar of an insect is presented for the first time, offering a methodological check that was not previously available. The results are compared to respiratory rates measured in closed vessel respirometers.

Several sources of error in gravimetric budget studies, especially those

with phytophages, are discussed. It is suggested that protein catabolism should be accounted for in investigations on metabolic efficiency in insects. Estimations of energy channeled to maintenance, biosynthesis and work are presented. It is concluded that the majority of published values on variations in energetic efficiency as a result of differences in food quality, determined using the gravimetric method, should be critically reconsidered.

INTRODUCTION

The measurement of food utilization by insects is usually performed using the nutritional index-technique, which is based on the construction of gravimetric budgets (Waldbauer, 1968; Scriber & Slansky, 1981). Efficiency of food utilization is one of the few available physiological parameters to assess the effect of differences in food quality on the performance of insects (Slansky & Scriber, 1985). It has been used to quantify suitability and resistance of plants to phytophagous insects (Scriber, 1984) and the effects of quality and quantity of specific phytochemicals as applied in artificial diets (Reese, 1979).

The reliability and accuracy of gravimetric dry matter budgets in studies employing artificial diets have recently been disputed by Schmidt & Reese (1986). Their results indicate that especially the efficiency of conversion of digested food into body mass (ECD) is subject to accumulation of errors. The ECD, also termed metabolic efficiency (Woodring et al., 1979) generally shows a large degree of variation within a species as a function of food source (see reviews by Mattson, 1980; Scriber & Slansky, 1981; Slansky & Scriber, 1985).

Also in our investigations on the potential effect of host-plant borne phenolics on food utilization in Pieris brassicae L. and P. rapae L. larvae inconsistent results were obtained. Despite several precautions, the reproducibility of ECD-values was too low to be acceptable from a methodological point of view.

For these reasons, it was felt necessary to develop an independent way of measuring the metabolic efficiency of growth in both caterpillar species. A possible way to achieve this independency is the use of respirometry to determine the metabolic rate. Relating this to the growth attained yields a measure of energetic efficiency independent of food intake. For such metabolic data to be representative, respirometry should ideally be continuous during the budget study.

The present paper describes a flow-through respirometer that allows such continuous measurement of metabolic expenditure during the complete final instars. It specifically aims at a methodological comparison of the gravimetric versus the respirometric determination of energetic efficiency of body growth and at the measurement of the physiological variation that may occur. The effects of three host plant borne phenolics as well as the

effect of different food plants are assessed employing groupwise comparisons. Some attention is paid to interindividual variation in respiration rate. The findings are discussed in relation to literature data on gravimetric budgets, respiratory rate, protein catabolism and fat synthesis of *P. brassicae*.

General implications for the methodology of studies on insect food utilization are indicated.

MATERIALS AND METHODS

Insects

Caterpillars of P. brassicae and P. rapae were reared ab ovo in the laboratory. Eggs were obtained from continuous laboratory stock cultures that were four years old at the onset of this study. Inbreeding was limited by introduction of wild caught adults into the cultures once a year. Batches of one hundred eggs collected within 24 hours made up the starting material of one experiment. Rearing upto the final larval ecdysis took place in a climatic room at 25 ± 1 °C, 60% RH and 16 hours photoperiod. Light sources were four 60 W overhead fluorescent strip-lights. Newly ecdysed fifth instars used for experiments had moulted within less than five hours time difference and ranged from 100-120 mg (14 days ab ovo, P. brassicae) or 40-45 mg (13 days ab ovo, P. rapae) fresh body weights. The caterpillars of both species were fed either an agar-based semi-defined diet (Ma, 1972) or reared on intact plants of Brassica oleracea L. var. gemmifera cultivar Titurel (exp. 5) and cultivar Stiekema (exp. 6).

Groups of equal size, usually 6 larvae of P. brassicae and 8 of P. rapae were introduced into each of both respiration chambers. Handling of caterpillars e.g. for weighings, introducing fresh food, removing food remains and faeces was kept to a minimum and usually took place five times during 15-30 minutes during the experimental period. Experiments lasted 90 hours (except exp. 6, 72 hours) and this period in all cases allowed the larvae to complete their development by reaching the pupal stage. In experiments with individual larvae (exp. 7 and 10), these were kept in their rearing situation in glass petri dishes apart from handling during respirometry. As a check for the possible effect of residence in the submersed respiration chambers, comparable groups were kept continuously in their normal rearing circumstances on the same diets as the experimental groups.

Food sources

Artificial diet

The composition of the artificial control diet was described by Ma (1972). The experimental treatments were for P. brassicae the lowering of protein content to 60% of the control diet (which contained 22.5% casein on dry weight basis) (exp. 1) or addition of phenolic compounds: sinalbin at 0.4 mM (exp. 2), caffeic acid at 1.0 mM (exp. 3), quercetin at 1.0 mM (exp. 4). For P. rapae the additions were caffeic acid at 2.5 mM (exp. 8) and quercetin at 2.5 mM (exp. 9). All three compounds were of 99% purity and obtained from commercial sources (sinalbin from Roth, caffeic acid and quercetin from Sigma).

Plant material

In two experiments (exp. 6 and 7, P. brassicae) the effect of a switch to an acceptable host plant, differing from the host on which the caterpillars had been reared (B. oleracea var. gemmifera), was studied. They were B. oleracea var. sabauba cultivar Savoy Chieftain (exp. 5) and B. oleracea var. rubra cultivar Extase (exp. 6). All three cultivars were reared in a greenhouse at 17 ^{o}C. Mature leaves from the middle part of the stem of 90-110 days old plants were used. Both alternative hosts have been reported to possess a higher field resistance to P. rapae caterpillars (Benepal and Hall, 1967; Chalfant and Brett, 1967). In addition, these B. oleracea varieties have been shown to contain relatively high amounts of phenolic compounds as compared to other cabbage varieties (Schmidtlein and Herrmann, 1975).

Gravimetric budget construction

Gravimetric determination of budget parameters and concomitant calculations were performed as originally proposed by Waldbauer (1968). In brief, the dry matter budget is of the form:

$$C = G + F + R,$$

in which C is the amount of food consumed, G is the amount of growth of insect body, F is the amount of faeces egested (including urinary waste products) and R is the amount of substance lost from the body in the gaseous phase due to oxidative energy metabolism. All amounts are expressed in mg dry matter.

The following efficiency parameters have been calculated: approximate digestibility (AD) which equals (C - F)/C; the efficiency of conversion of digested food to body substance (ECD), calculated as G/(G + R) or G/(C-F). A third type of efficiency parameter was calculated as G/R (mg dry matter of growth per mg of dry matter lost by respiration) and is termed growth-efficiency. The latter gravimetric measure of the cost of growth allows a comparison with the respiratory parameter defined hereafter.

Dry weights were taken after drying to constant weight at 70°C. Dry matter content of artificial diets ranged from 15.97% to 17.77% (mean $\pm$ SD 17.17 $\pm$ 0.50) over the experiments. Within a batch of diet, dry matter content had a coefficient of variation ranging from 0.5 - 3.0% (mean $\pm$ SD: 1.50 $\pm$ 0.80). With plant material (exp. 6 and 7), dry matter content ranged from 12.4 - 21.8%. Aliquots used for dry matter determinations were the longitudinally matched halves of the same leaves offered to the larvae (Waldbauer, 1968). Coefficient of variation between leaves within a host species ranged from 1.4 - 6.0%. Corrections for leaf respiration were performed according to Axelsson and Agren (1979). The value of α in the correction formula was set at 0.65. The respiration rate, r, of leaf tissue was determined using a volumetric respirometer (Scholander, 1950). A value of 0.032 was calculated for r, based upon respiration rates found to be 1.0 $\pm$ µl of oxygen per mg DW per hour at 25 ^{o}C for all three host species. This value corresponded in a satisfactory way with data on leaf respiration obtained via the flow-through gas analysis system.

A sensitivity analysis was carried out to estimate the extent to which random errors could affect the calculated budget parameters (Schmidt and

Reese, 1986). Initial fresh weight of food offered was related to the standard deviation in dry matter determinations of aliquots to calculate the error in the R-parameter. Results are given as the absolute amount (mg DW) that is either an overestimation or an underestimation of R when the relative error in dry matter content of the diet is 2.5% ($R_{2.5\%}$, table 2).

For example, when the diet contains 18% dry matter, $R_{2.5\%}$ is calculated at 18 x 0.025, multiplied by the absolute amount of dry matter offered at the start of the experiment.

Respirometry and gas analysis

A gas analysis system was developed that allowed the automatic and continuous monitoring of gas exchange by undisturbed, actively feeding caterpillars (fig. 1). The set-up basically consisted out of two glass respiration chambers that were continuously purged with purified air of known composition (medical quality); they were kept at constant temperature by submersion in a thermostated water bath at 25 ^{o}C. The air-flow through the chambers was regulated at a known and strictly constant level by an electronic air-flow regulator (Brooks 5850 TR mass flow controller). Solenoid valves switched either from one respiration chamber (control groups) to the other (containing the treated group) or to a reference gas stream to check for possible thermal drift in the gas analyzers which operated at high sensitivity. Switching between experimental groups was programmed to occur once every 60 minutes using electrical timers. Oxygen concentrations were determined by a differential paramagnetic oxygen analyzer (Taylor Servomex OA 184). Carbon dioxide production was detected by a differentially operating diaferometer or catharometer (Pieters, 1971). Analyzer signals were converted to voltages and fed to millivolt recorders. Intermittent calibration was performed by sampling the outcoming gas stream and subsequent volumetric gas analysis using a Sonden-apparatus with an accuracy of 20 ppm (van Es, 1958).

Flow rate was 2000 or 3000 ml/h, partial flow-rate through the diaferometer was 1500 ml/h. Respiration chamber volume was 200 ml. Resolution capacity for oxygen was better than 50 ppm (100-150 µl/h), for carbon dioxide better than 10 ppm (20-30 µl/h). Response times were 2 min

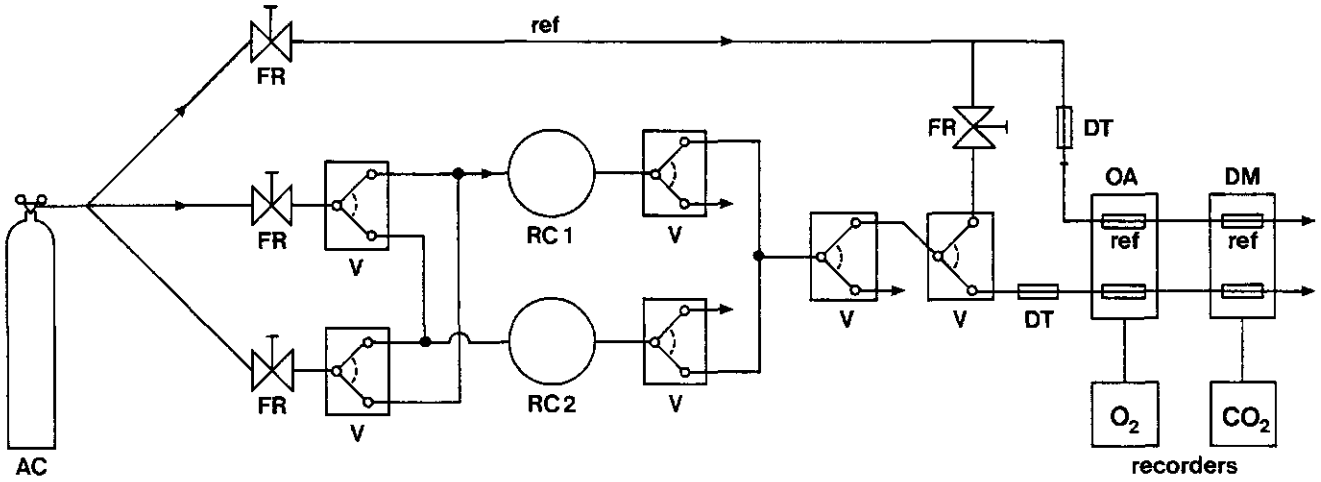

Figure 1:
Schematic drawing of the flow-through gas analysis system used to monitor continuously insect respiratory metabolism.
Legends: AC - air cylinder; FR - flow regulator; V - solenoid valve (two-way); RC - respiration chamber; OA - paramagnetic oxygen analyzer; DM - carbon dioxide analyzer (diaferometer); DT - air drying tube.

(O_2) and 1 min (CO_2). Technical details of this system can be obtained from the author upon request.

The continuous records of the voltage output of both gas analyzers were converted to oxygen and carbon dioxide concentrations in the respired gas stream using calibration values from the Sonden-analysis. During the three months of experimentation, no significant changes in calibration values were found. For the purpose of data reduction, continuous records were processed using the averaged values over one hour. As the diaferometer is not specific to carbon dioxide, corrections were made for oxygen content of the respired air. The sensitivity of the diaferometer to oxygen was 10.0% of that to carbon dioxide. Only absolutely dry air was fed to both analyzers by passing it through 50 ml glass tubes filled with pumice stones dusted with phosphorous pentoxide. The performance of these drying tubes was tested intermittently using the catharometer as a highly sensitive water vapour detector (Pieters, 1971). Oxygen consumption and carbon dioxide production were calculated using the concentration differences between incoming and outcoming air flowing through the respiration chambers multiplied by the flow-rate. The Haldane-correction for the cases in which the RQ differed from 1.00 had a neglegible influence.

Contributions of leaf respiration (exp. 6 and 7) were corrected for using respiration rates of leaf tissue and leaf biomass offered as food. Corrections were made using the formula of Axelsson and Agren (1979). Excised leaf portions served as food material no longer than 16 h. Except for the first 6-8 hours of both experiments, leaf contribution was maximally 10% of total gas-exchange in the respiration chambers just after introduction of fresh leaf portions and declined because of caterpillar feeding to yield a contribution of 5% on the average.

The values obtained were corrected to standard temperature and pressure (STP, 273 K and 760 mm Hg). Heat production (H) was calculated using the formula of Brouwer (van Es, 1961). The nitrogen term in this formula was neglected (see discussion). The respiratory quotient, RQ, was calculated as STP-volume of carbon dioxide produced divided by STP-volume of oxygen consumed.

Calculations and statistics

Body growth (G, table 1) was measured for individual caterpillars. Significance of differences, due to dietary treatment, between means of control and treated groups was tested using Student's t-test (table 1). Consumption, egestion, respired dry matter and heat production (table 1) were measured as group totals. Individual values were calculated by dividing the group total by the number of caterpillars in a group. Differences between control and treatment were expressed as the percentage of difference (% T/C, table 2), which is defined as treated/control x 100% minus 100%. As a comparative measure of variation, the coefficient of variation (CV) was used. Between individuals within an experimental group CV was calculated as the standard error of the mean divided by the mean over the individuals. CV between group means from different experiments was calculated as the standard deviation over these mean values divided by the grand mean.

RESULTS

Food consumption

The amount of food consumed by an individual caterpillar, C (mg dry matter), is given in table 1 for all 18 experimental groups of both species. Coefficients of variation in food consumption in P. brassicae were less than 7% over the control groups of exp. 1-5 and less than 1.8% between the individuals of exp. 5. The mean food consumption over the full duration of the final instar was 351 mg DW per caterpillar. Food consumption on leaf material of B. oleracea var. gemmifera was 27% higher (445 mg DW, difference 10%, exp. 6 and 7).

It is seen from table 1 that in exp. 3 and 4 considerable reduction in the rate of ingestion occurred due to treatment (27% and 29% respectively). In exp. 6, food intake on B. oleracea var. sabauda was 20% lower than the control value of 467 mg DW. On red cabbage (exp. 7), food intake was 11% higher.

Over the control groups of exp. 8-10 with P. rapae, food consumption varied 8% CV, while between the individuals of exp. 10 CV was 2.1%. Reductions in ingestion rate as a result of caffeic acid or quercetin addition were less than 10%.

The percentage of food eaten relative to the amount of food offered (Schmidt & Reese, 1986) was greater than 50% in all experiments. In exp. 1, 2, 5, 6 and 7 this percentage was greater than 70% in both control and experimental groups.

Growth

Several dietary treatments resulted into significant growth reductions as tested with Student's t-test (table 1). A casein content of 60% of the amount in the control diet supported a significantly lower growth rate. The phenolic glucosinolate sinalbin did not affect growth, while the phenolic caffeic acid at 1.0 mM significantly reduced growth in P. brassicae. Caffeic acid at 2.5 mM was ineffective on the growth of P. rapae . Quercetin inhibited growth in both P. brassicae at 1.0 mM and P. rapae at 2.5 mM.

Table 1 - Gravimetric budget parameters (C - food consumption, G - growth, F - faeces production and R - respired dry matter, all in mg DW), respirometric data (O_2 and CO_2, ml STP), heat production (H, Joules) and respiratory quotient (RQ) of P. brassicae (exp. 1-7) and P. rapae (exp. 8-10) on different diets. Mean values for an individual caterpillar, based on measurements on 6-8 larvae. Duration of experiments 90 h, except exp. 6, 72 h

Exp.	diet	C	G	F	R	O_2	CO_2	H	RQ
1	control	326	87	173	66	38.2	40.2	821	1.05
	casein 60%	347	78 c	208	61	36.7	38.3	787	1.04
2	control	394	108	211	75	43.0	47.0	933	1.09
	sinalbin 0.4 mM	383	109	206	68	43.7	48.7	953	1.11
3	control	342	96	186	60	47.9	60.9	1032	1.06
	caffeic acid 1.0 mM	250	77 c	123	50	36.5	37.2	778	1.02
4	control	358	96	187	75	40.4	41.7	864	1.03
	quercetin 1.0 mM	255	67 c	110	78	24.4	23.8	515	0.98
5	control	336	95	188	53		42.9[a]	874[a]	
	(individuals)	(1.8)[b]	(2.5)	(3.5)	(2.5)		(2.8)	(2.83)	
6	control	467	79	321	67	37.4	38.0	797	1.02
	sabauda	375	84	221	70	43.9	43.9	932	1.00
7	control	422	100	265	57	48.8	51.2	1032	1.05
	rubra	467	105	268	94	52.3	60.9	1154	1.16
8	control	125	42	55	28	15.2	15.6	325	1.02
	caffeic acid 2.5 mM	114	42	43	29	14.4	14.9	308	1.04
9	control	112	34	56	22	13.9	13.1	291	0.94
	quercetin 2.5 mM	108	31 c	49	28	11.0	10.6	232	0.96
10	control	104	28	58	18		14.0[a]	297[a]	
	(individuals)	(5.7)[b]	(4.2)	(4.9)	(11.3)		(5.3)	(5.3)	

a - value interpolated based on shorter measuring periods (cf. table 4 and 5)
b - values between brackets are CV (%)
c - value significantly lower than control ($p < 0.01$ or better)

Growth of P. brassicae on leaf material of alternative host plants was higher in both cases but below significance.

The mean value of CV of growth in P. brassicae was 2.3% both between the individuals of the control groups (exp. 1-5) and 6.6% over the means of these control groups.

The mean CV of growth over all treated groups was of similar magnitude (7.6%). In exp. 6 and 7 with leaf material, the difference between both control groups was 21%.

Variation in growth within control groups was 3.2% in P. rapae (exp. 8-10), in treated groups it was 3.9%. Between control group means CV was 16%, due to a relatively low growth rate in exp. 10.

Groups of caterpillars maintained to check for a possible positive or negative effect of residence in the respiration chambers showed that growth in the submersed respiration chambers was in all cases slightly but not significantly better than under normal rearing circumstances.

Respiration in dry matter equivalents

In P. brassicae, the amount of dry matter respired, R, showed a CV of 2.5% between the individuals of exp. 5. Over control group means (exp. 1-5), CV was 13%. Reductions in R due to treatment were calculated in exp. 1-3 (-8%, -9% and -17% respectively), while in exp. 4 and 6 small (4% in both cases) and in exp. 7 a very large increase in R (65%) were calculated as a result of the respective dietary differences.

In P. rapae, individual variation (exp. 10) amounted up to a CV of 11.3%, over the control groups of exp. 8-10 this was 18% CV. While in exp. 8, R was only 4% larger in the treated group, in exp. 9 it was calculated to be 27% higher.

Heat production

Variation in heat production in P. brassicae (table 1) showed a CV of 8% between the control group means of exp. 1-5 and 2.8% as individual variation (exp. 5, table 3). The lower protein concentration (exp. 1) nor sinalbin (exp. 2) exerted appreciable effects on heat production (-4% and 2%

Table 2 - Efficiency parameters and variables interrelating gravimetric and respirometric data. For explanation of symbols, see table 1 and text. 2

		AD %	ECD %	G/R mg/mg	G/H µg/J	G/R_H mg/mg	R_{G-H} mg	Δ%DM %	$R_{2.5\%}$ mg	H/R J/mg
1	control	46.9	56.9	1.32	106	1.84	19	4.0	12	12.4
	casein 60%	40.1	56.1	1.28	99	1.72	16	3.3	12	12.9
	%T/C			-3	-7					
2	control	46.4	58.7	1.42	116	2.01	22	4.6	12	12.3
	sinalbin 0.4 mM	46.2	61.2	1.58	114	1.98	14	2.7	13	13.8
	%T/C			+11	-2					
3	control	45.6	61.5	1.60	93	1.61	0	0	11	17.2
	caffeic acid 1.0 mM	50.8	60.6	1.54	99	1.72	5	1.0	12	15.6
	%T/C			-4	+6					
4	control	47.8	56.1	1.28	110	1.91	15	3.1	12	11.7
	quercetin 1.0 mM	56.9	46.2	0.86	130	2.25	48	9.2	13	6.6
	%T/C			-33	+18					
5	control	44.0	64.2	1.81	109	1.89	3	0.6	13	17.5
6	gemmifera	31.3	54.1	1.18	99	1.72	21	3.8	14	11.9
	sabauda	41.1	54.5	1.20	90	1.56	26	5.4	12	13.3
	%T/C			+2	-9					
7	gemmifera	37.2	63.4	1.75	95	1.65	-4	-0.5	19	18.4
	rubra	42.6	52.8	1.12	91	1.58	28	4.1	17	12.3
	%T/C			-36	-5					
8	control	56.0	60.0	1.50	129	2.24	13	5.4	6	11.6
	caffeic acid 2.5 mM	62.3	59.2	1.45	137	2.38	16	6.7	6	10.6
	%T/C			-3	+6					
9	control	50.0	61.1	1.55	117	2.03	5	2.5	5	13.2
	quercetin 2.5 mM	54.6	51.7	1.07	134	2.32	16	6.7	6	8.0
	%T/C			-31	+15					
10	control	44.2	60.9	1.56	95	1.65	1	0.5	5	16.5

Table 3 - Results of a sensitivity analysis assuming a 2.5% error in the estimation of percentage dry matter of the food, reflected in G/R, or in the estimation of heat production (reflected in G/H) regarding its effect on the percentual difference between treated and control groups (% T/C, cf. table 2).

experiment	2.5% error range in % T/C			
	G/R		G/H	
	min	max	min	max
1	-34	43	-11	-2
2	-20	59	-7	4
3	-37	50	1	12
4	-51	-6	12	24
6	-32	52	-13	-4
7	-64	3	-9	0
8	-37	48	1	12
9	-56	10	9	20

respectively). Heat production was reduced by both caffeic acid incorporation (-25%) and addition of quercetin (-40%) at the levels applied. In contrast, heat production was enhanced by both alternative host plants in exp. 6 (17%) and 7 (10%).

In P. rapae, heat production varied 5.3% between the individuals of exp. 10 (table 4) and 5% between the group means of exp. 8-10. Both caffeic acid and quercetin resulted into a lower heat production (-5% and -20%) than in the control groups.

Table 4 - Variation in carbon dioxide production (µl/mg FW/h) of individual P. brassicae larvae (n=8) during four periods of 2 h in the final instar (exp. 7). Values at 25 °C, corrected for gut contents (estimated at 22% of fresh body weight)

		period			
		15-18 h	41-52 h	64-72 h	81-91 h
minimum	mean	1.85	1.83	1.84	1.74
	CV	6	2.5	1.8	6.0
maximum	mean	2.38	2.22	2.18	2.08
	CV	5	3.5	1.8	6.4
ratio max/min	mean	1.29	1.20	1.18	1.20
	CV	1.1	1.8	1.8	2.8

Respiratory quotient

Diet exerted only minor effects on RQ. Differences in respiratory quotient were greater than or equal to 5% only in exp. 4 (-5%) and exp. 7 (+10%). Over the values from exp. 1-4 (P. brassicae on artificial diet), RQ had a mean value of 1.06 (CV 2%). For P. rapae on artificial diet (exp. 8 and 9), this was 0.98, with a difference of 9%.

Digestive efficiency

Variation in approximate digestibility, AD (table 2), between individuals of P. brassicae (exp. 5) was 1.1%, between control group means of exp. 1-5 CV was 3%. The lower casein content (exp. 1) yielded a 14% decrease in AD as compared with the control group. Given the value of CV, the number of individuals in each group and the mean values of AD (46.9 and 40.1; exp. 1) a difference of 10% is significant at $p < 0.005$ (Student's t-test), under the assumption that individual variation in the treated group is equal to that in the control group (cf. section on growth). Even when variation in

the treated groups would be twice as high, significance at $p < 0.025$ is calculated.

Sinalbin addition did not affect AD. In exp. 3, 4, 6 and 7, values of AD were higher by more than 10%. These increases may likewise be considered significant.

In P. rapae, variations in AD were higher than in P. brassicae (CV 2.5% within the group of exp. 10, CV of 10% between control group means (exp. 8 - 10). The differences due to dietary treatments are significant at $p < 0.05$ under the assumption given above.

Losses of matter and energy during growth

To meet convention, the values of ECD are given in table 2. The discussion will here be limited to the expressions G/R and G/H.

Regarding the data presented in table 2, two points are of interest: the effect of diet on growth efficiencies, both gravimetric (G/R) and calorimetric (G/H) and a comparison between the results of both methods. Only differences (% T/C, table 2) greater than 10% will be considered.

It is seen that the growth efficiency that is calculated gravimetrically, results into a positive effect of diet on metabolic efficiency (expressed in material equivalents) in exp. 2 and in negative effects in exp. 4, 7 and 9. In contrast, using the calorimetric measure (G/H), in exp. 4 and 9 the treated groups showed a better energy utilization. In 7 out of 8 experiments (the exception being exp. 1), the results of both methods differ 10% or more (range -13% to +51%). To convert heat production (H) to dry weight (R), an energy content of 17.3 J/mg has been used, based upon a 1:1 ratio of carbohydrate to protein oxidation (see discussion). The extent of these differences in absolute terms is given by the variable R_{G-H} (table 2), that gives the difference in R (mg DW) between both methods. This variable shows that the differences can be considerable as 10 mg DW represents about 15% of the total amount of R in P. brassicae. A clear trend towards a positive difference is present.

The variable Δ %DM (table 2) gives the relative error in estimation of percentage dry matter of the food that explains R_{G-H}. Errors of -0.5% to 5% explain more than 75% of the cases in which differences between both methods emerge.

The expected ranges of variation in the difference percentages (% T/C, table 2) when a 2.5% error is present in either the estimation of the dry matter content of the food ($R_{2.5\%}$, table 2) (reflected in G/R) or in the estimations of respiratory gaseous exchange (reflected in G/H), are given in table 3. These introduced errors obviously affect the calculated %T/C for the gravimetric method far more serious (range -64% to +59%) than for the calorimetric method (range -13% to +24%). Moreover, there seems to be no correlation between the results of both methods.

Finally, a most sensitive test variable that interrelates both methods is the quotient H/R, which should reflect a realistic energy content in Joules per mg DW. While in exp. 3, 5 and 10 probable values are calculated (16.5 - 17.2 J/mg), the value of H/R is improbably low in all other cases.

Individual variation in respiration rates

Coefficient of variation in respiration rates between individuals of *P. brassicae* (table 4) was relatively high in the initial and final phases of the instar (CV 5 - 6.5%). During mid-instar, in which the highest growth rate is achieved, CV was 1.8 - 3.5%. The ratio maximum to minimum respiration rate of a single individual varied from 3-8%. In both maximum and minimum respiratory rates, a slight decline is noticed during the instar. Interindividual variation in *P. rapae*, was 1.8 - 3.9%, (table 5).

Table 5 - Variation in carbon dioxide production (µl/mg FW/h) of individual *P. rapae* larvae (n=8) during three periods in the final instar (exp. 10). Values at 25 °C, averaged over 20' periods, not corrected for gut contents.

	period		
	23-27 h	50-53 h	75-77 h
mean	1.86	1.77	1.81
CV	1.8	2.8	3.9

Time pattern in respiratory rate

Examples of the observed time patterns in respiratory rate are presented in fig. 2 and 3.

The higher protein level (exp. 1, fig. 2) was associated with a continuously higher respiratory rate until 70 h of feeding, after which this situation was reversed for the remaining 20 hours in the instar. Initial and final respiration rates were almost equal for both groups, maximum metabolic intensity was reached at about the same moment but differs 23%. Respiratory quotient was both initially (6-12 h) and finally (75-90 h) just below 1.00, while in both groups it remains almost continuously beyond 1.05 in the intermediary phase.

While the time course in respiration intensity for the control group of exp. 4 (fig. 3) is very similar to the corresponding curve in fig. 2, a distinctly different pattern was caused by addition of quercetin at 1.0 mM. The effect became apparent only after 17 h, when the treatment resulted in a lower respiration rate. After 23 h, a steady decline was observed, changing to a slight increase after 50 h. Afterwards, respiration fluctuated around 275 µl/h for an individual and showed a slow decline from 80 to 90 h. In the control group a maximum value of 775 µl/h was reached around 57 h, in the quercetin treated group this was 360 µl/h. RQ was fluctuating in an irregular way in the treated group, while in the control group a trend in RQ similar to that in fig. 2 may be noticed, be it somewhat less pronounced.

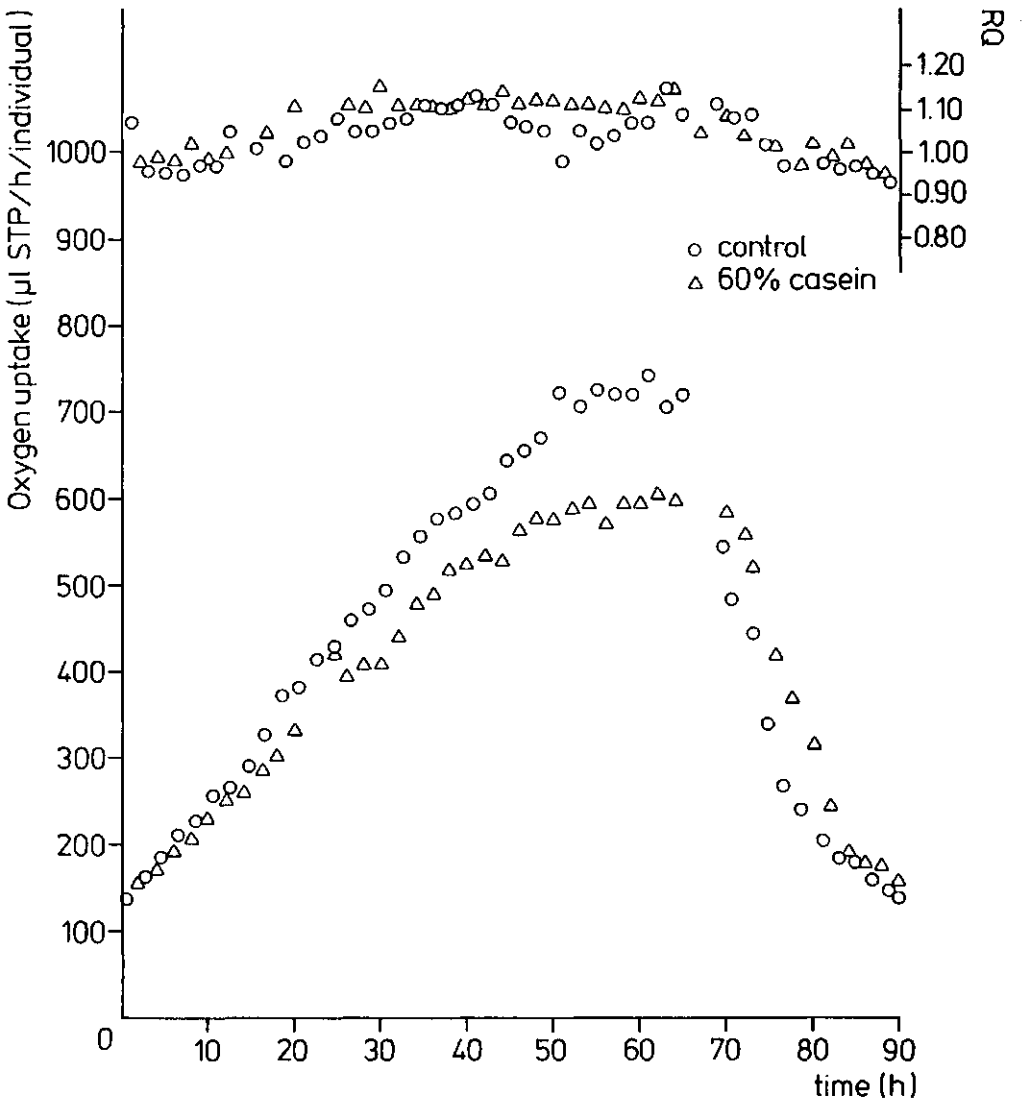

Figure 2:
Time pattern in respiratory rate and respiratory quotient as observed in exp. 1. Abscissa: time in hours after the start of the experiment. Left ordinate: oxygen uptake in microliters STP per hour per individual caterpillar. Right ordinate: respiratory quotient.

respirometers in P. brassicae reveals that the former are 1.3-1.8 times higher than those found by Moreau and Gourdoux (1971) and 2.6-3.6 times higher than those presented by Breugnon (1972). These differences can be interpreted as in concordance with the suggestions on 'flask effects' of Wightman (1977, 1981). On the other hand, respiratory rates in young pupae in this study were equal to the values reported by Fourche et al. (1977) using a closed vessel method.

The existence of a calorigenic effect of feeding in phytophagous insects has been offered as a third type of explanation, as insects are usually starved prior to and during respirometry (Aidley, 1976; McEvoy, 1984). When offered food, however, respiratory rates of the caterpillar species involved increased by factor 2.05 (Aidley, 1976) and 1.6 (McEvoy, 1984) in experiments conducted in Gilson respirometers and lasting a few hours. In the present study, short-term differences between maximum and minimum respiratory rate in P. brassicae were factor 1.18-1.29 (table 4) and likewise associated with feeding. Experiments on the effect of starvation performed separately showed a fast decline (within one hour time) in respiratory rate to about 75% of the initial value that kept slowly decreasing during the following 4-5 hours to 60-65% of the metabolic rate during ad libitum conditions, although locomotion was intensified. During this period about 80% of the gut contents had been egested (van Loon, unpublished observations). A similar course of decline in metabolic rate was observed in Manduca sexta (Johanssen) larvae, which showed a further decrease to a stable level of 30% when starvation was extended to a period of 20 hours (Ziegler, 1984). These results confirm that ad libitum availability of food can be expected to neutralize 'flask effects' to a considerable extent (Aidly, 1976; Wightman, 1981; McEvoy, 1984).

The effects of protein catabolism

In the calculation of both the gravimetric budget and heat production, protein catabolism has been neglected, as nitrogenous excretory products in faeces were not determined separately. This may produce an underestimation of the assimilated fraction of the food (Bhattacharya & Waldbauer, 1972) and an overestimation of the calculated heat production (van Es, 1961). These estimation-errors will be examined using published data on P. brassicae. The summed total amounts of uric and allantoic acids egested by the

final instar feeding on cabbage were approximately 10 mg, while the uric acid accumulation in the body was about 2 mg (Mauchamp & Lafont, 1975). Amino acid balance sheets for P. brassicae feeding on the standard artificial diet led to conclude that 22 mg of amino acids had been utilized for fuel (van Loon, unpublished observations). This would equal a total of 11 mg of nitrogenous waste substances. Both types of data indicate that the fraction of food assimilated is underestimated by about 10 mg. For the control groups of experiments 1-5, corrected AD values are calculated to be 2.6-3.1% higher than the ones tabulated (table 2). Conversely, as R must have been accordingly higher, ECD and G/R are computed 2.7 4.1% and 10.5-16.7% lower respectively. On the other hand, when the nitrogen term in Brouwer's formula is taken into account (van Es, 1961), heat production would be lower by about 20 J. This represents a reduction of 2 - 2.6% relative to the original calculations (table 1, exp. 1-5).

This suggests that protein catabolism cannot be neglected in view of its influence on both gravimetric and respirometric energetic efficiency parameters especially because the influence on both is exerted in opposite directions, which enlarges the discrepancies between both methods even more. Indeed, the amount of protein catabolism is considerable and in fact is estimated to be almost equal to carbohydrate oxidation. It is for this reason that in the calculation of G/R_H a carbohydrate:protein ratio of 1:1 was adopted, yielding the average energy content of 17.3 J/mg that was used to convert heat equivalents to material equivalents (table 2). Alternative assumptions regarding this ratio result into relatively small changes in the tabulated values of G/R_H. Gravimetric budget studies express metabolic efficiency in material equivalents (mg DW) which disregards the widely different calorific content of several possible fuel substrates (16.8 J/mg for carbohydrates vs. 39 J/mg for lipids).

Respiratory quotient and fat synthesis

The 1:1 ratio of protein to carbohydrate oxidation would be expected to render a RQ of 0.93. The fact that the measured values are higher is consistently explained by the considerable amount of fat synthesis that has been shown to occur in P. brassicae (Chippendale & Kilby, 1969; Kastari & Turunen, 1977), as in many other larval insects (Slansky & Scriber, 1985). Fat synthesis from carbohydrates is accompanied by excess of carbon dioxide

release (Kleiber, 1961). Though not reproduced here, it could be calculated that the amount of fat synthesis reported, in connection with a 1:1 ratio of carbohydrate to protein oxidation, can explain quantitatively the measured RQ values in the control groups of exp. 1-5. Fat synthesis at the same time explains for a major part the systematic differences between dry matter and energy budgets when the latter are calculated using calorific contents of dry matter determined with bomb-calorimetry (Schroeder, 1981; Slansky, 1985).

Comparison with literature data on gravimetric budgets in Pieris

Gravimetric budgets have been published for both P. brassicae (Chlodny, 1967; Shrihari, 1977; Schoonhoven & Meerman, 1978) and P. rapae (Slansky & Feeny, 1974). It must be concluded that for both species the amount of growth realized during the final instar varies considerably between studies. Growth achieved under the conditions of continuous respirometry is the highest reported for both species. Variations in energetic efficiency, expressed in dry matter equivalents (G/R) is still greater. When different varieties of B. oleracea were offered as food, values for energetic efficiency for P. brassicae differ by factor 2 (Schoonhoven & Meerman, 1978) and by as much as factor 7.7 between studies. For P. rapae differences range from factor 2 - 4.4 (Slansky & Feeny, 1977). The comparative approach presented here makes such large effects of food quality on energetic efficiency improbable. They are most likely to be explained by both measuring errors and invalid assumptions. Moreover, the differences are predominantly caused by variations in R. When differential experimental durations are accounted for, this would imply differences in absolute metabolic rates. That these are unlikely comes from the realization that the rates of basic metabolic processes have both lower and upper physiological limits that have been tentatively established in the present study. The maximum range of variation of energetic efficiency (G/H) under ad libitum conditions as it emerges from respirometric data in this study is within 25% over 7 experiments with P. brassicae (table 2). The expression for energetic efficiency G/H combines a gravimetric with an energetic measure (μg/J). The energetic content of the accumulated biomass has not been determined. It is however conceivable that the latter parameter also varies under different

dietary regimes, although the pupal calorific content seems fairly defined with values close to 23.5 J/mg for different species (Slansky & Feeny, 1985).

Comparison with data on other insect species

The foregoing discussion on the physiological probability of reported variations in gravimetrically determined energetic efficiency and their possible causes in both Pieris species pertains to many literature reports on other species (see for bibliography and review Slansky & Scriber, 1982; 1985). Recalculations of data from investigations on both smaller and larger phytophagous species feeding on both plants and artificial substrates demonstrated that the resulting ranges in both absolute metabolic rates (in dry matter equivalents) and metabolic efficiencies are improbably wide and most likely caused by measuring errors rather than by physiological causes. One reason for the fact that this has not been recognised is the insensitivity of the ECD to variations in R. Schmidt & Reese (1986) reached similar conclusions along a different line of reasoning.

It is, however, beyond the scope of this paper to present these recalculations here. A few studies combined gravimetric techniques with Gilson respirometry and thus allow a comparison of the outcomes of both methods (Woodring et al., 1979; Wightman, 1981; Bailey & Singh, 1977). The study of Woodring et al. (1977) implies that flask-effects are not operative to the same extent in all species or circumstances.

Sources of error in gravimetric budget studies with plant-feeding insects

Several sources of error that may become especially prominent with respiring leaf material in excised condition have been described (Waldbauer, 1968; Axelsson & Agren, 1979). However, appropriate corrections for leaf respiration are rare in the literature concerning food utilization in phytophagous insects (Slansky & Scriber, 1985). Additionally, errors caused by differences in excess of food and those inherent in dry matter determinations on aliquots (Slansky, 1985; Schmidt & Reese, 1986) influence experimental results with plant food in the same way as with artificial

diets.

An additional source of error is indicated by the systematic deviation between G/R and G/R_H, the latter being consistently higher. An explanation for this phenomenon may be that the determination of dry matter content of food material yielded values that are too high. The method used for drying that has been most commonly applied is drying to constant weight. This however does not guarantee that the resulting material is absolutely free from water and a check is usually not performed. Temperatures at which drying is performed have not been standardized. Evaporation of water during sampling and storage of wet material also may influence the accuracy of dry matter determinations. It seems clear that drying method is another potential source of systematic errors in constructing gravimetric budgets.

Condition of plant food in gravimetric budget studies

Excision of leaf material from the intact plant is a prerequisite for the application of the gravimetric approach. As Wightman (1981) already stressed, in the vast majority of food utilization studies on phytophagous insects, as in this investigation, the leaf material is not in its natural condition. Following excision it looses turgescence and thereby the capacity for photosynthesis. Even when turgescence is maintained by water supply through the petiole (Scriber, 1979), light intensities have been too low for photosynthesis to occur. Light intensity is in no case specified. The amount of dry matter fixed by photosynthesis in an attached leaf can readily become so large that feeding of an individual insect is in part compensated and cannot be measured reliably using the gravimetric method. A further complication is the possibility of wound respiration in the leaf tissue, which can be five times higher than normal (Uritani & Asahi, 1980), as a reaction to the mechanical damage of feeding. These considerations lead to the conclusion that there seems to be no single case documented in which the calculated efficiency of a phytophagous insect represents a true reflection of the feeding situation on its natural food. It is evident that especially respirometric techniques have the potential to overcome this lack in our knowledge.

Effect of dietary phenolics on energetic efficiency

One of the objectives of this study was to investigate the metabolic effect of food quality as modified by the addition of host plant-borne phenolics. While the gravimetric results suggested a decrease of metabolic efficiency in the presence of polyphenolics, the energetic efficiency of growth was increased judged on the basis of the respirometric results. Addition of caffeic acid or quercetin at the levels applied reduced food consumption and growth in the final instar of P. brassicae and quercetin at 2.5 mM reduced the growth rate in P. rapae. The 1 mM concentration is near the upper limit of the levels reported for both single compounds in B. oleracea (Wildanger & Herrmann, 1973; Hanefeld & Herrmann, 1976). These compounds reduced survival and growth in earlier instars (van Loon, unpublished results). The mechanism of action of these inhibitory effects is as yet unclear. Reese & Beck (1976 a, b, c) studied the effects of several phenolics on Agrotis ipsilon (Hufnagel). They calculated significant reductions in metabolic efficiency. Their values exhibit a variation range that is not very likely to be realistic despite certain precautions (cf. Schmidt & Reese, 1986). In Heliothis zea (Boddie), the phenolics chlorogenic acid and rutin did not affect dietary utilization (Isman & Duffey, 1982). During chronic feeding, 90% of the ingested dose of these compounds was excreted unchanged (Isman & Duffey, 1983).

It has been suggested by several authors that 'neutralization' of allelochemical compounds by means of detoxification, excretion or accumulation may require considerable amounts of energy (Feeny, 1976; Schoonhoven & Meerman, 1978; Brattsten, 1979; Reese, 1979; Scriber, 1984). This hypothesis has only recently been adequately tested, wich yielded no support for the latter suggestion (Neal, 1987). No technical means are available to measure the amount of energy involved in detoxification processes separately from the main energy requiring processes such as maintenance, growth and work. From the present results, some indications about this channelling can be derived. The dry matter body composition of the young pupa of P. brassicae is documented (Chippendale & Kilby, 1969; Lafont et al., 1975; Kastari & Turunen, 1977). The energetic requirement of growth leading to this body composition can be theoretically estimated using the generally observed energetic efficiencies of heterotrophic biosynthesis (Schroeder, 1981). These calculations showed that 32% - 37% of the heat produced can be

attributed to biosynthesis processes, varying with the assumption regarding protein turnover rate (1 - 2 respectively). Adding costs for digestion, absorption and membrane transport, an estimation of 45% - 50% seems realistic. Minimum maintenance requirements are estimated as 30% (Ziegler, 1984). Work dedicated to muscular movements during locomotion (Casey, 1983) and feeding (McEvoy, 1984) seems to be of minor importance in caterpillars from a budgetary point of view. From these estimations, it follows that maintenance and growth together explain the major part (a minimum of 80%) of heat production in P. brassicae. The energy directly dedicated to neutralization processes will constitute a minor fraction in view of the constraints to increase in metabolic rate. It also follows that for example inhibition of feeding, that increases the duration of development, can result into a reduction in energetic efficiency per se caused by the greater relative contribution of maintenance respiration, which in itself is considerable. This in turn implies that duration of experiments should be identical for all experimental groups to be compared, a requirement that is met in only a part of insect food utilization studies.

Conclusions and recommendations

The continuous respirometry-approach renders an independent method to determine the energetic efficiency of growth. Important discrepancies with gravimetric results were found despite precautions and corrections. Several random as well as systematic errors are inherent in gravimetric budget construction. These notions justify the conclusion that the reliability of results reached with gravimetric methods in the majority of published reports must be critically reconsidered. The respirometric method, although technically tedious, is to be preferred. The influence of protein catabolism on heat production cannot be neglected and the variable amount of nitrogenous excretory products under different dietary regimes should be taken into account.

There is no methodologically sound alternative to continuous calorimetry to determine energetic efficiency. A simplifying modification in gravimetric budget calculations can thus be proposed. Food consumption should be estimated by an assumption about metabolic efficiency, the latter based on respirometric data obtained from feeding insects. A sensitivity analysis

can subsequently reveal the effect of a set of alternative values that lie within the probability range of physiological variation. Determinations on the amount of protein catabolism are indispensable to achieve accuracy. This alternative prohibits the calculation of the ECD, but increases the reliability of the estimation of food consumption, the main source of error, and will allow a physiologically realistic calculation of the assimilation efficiency in phytophagous insects. At the moment, growth rate under optimized environmental conditions for an insect species and its food seems to constitute the most representative parameter available to assess food quality.

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CHAPTER 5

AMINO ACID AND NITROGEN UTILIZATION BY CATERPILLARS OF TWO *PIERIS* SPECIES FEEDING ON AN ARTIFICIAL DIET AND A HOST PLANT, *BRASSICA OLERACEA*

ABSTRACT

Amino acid and nitrogen utilization by *Pieris brassicae* and *P. rapae* final instars were studied on a host plant, *Brassica oleracea* and on an artificial diet to assess diet suitability. Food consumption differed significantly between both diets and was affected by leaf amino acid content. Relationships were present between total dry weight of food ingested over the final instar on the one hand and absorption efficiencies of dry matter, total amino acids, several individual essential amino acids, tyrosine and proline on the other, when data on both types of diet were pooled. Amino acid absorption efficiency was higher on the artificial diet than on *B. oleracea*, differences between both diets were small for most amino acids except for glycine, cystein and serine that were absorbed more readily on the plant. Amino acid metabolic efficiency was inversely related to amino acid content of the diet. Utilization patterns were very similar for both caterpillar species. An extensive conversion of phenylalanine to tyrosine was calculated to occur on the basis of the balance sheet procedure applied. Low degrees of phenylalanine oxidation correlated with lower degrees of tyrosine accumulation in the prepupal body mass. Indications were obtained that tyrosine and cystein may easily become limiting nutrients for growth on both the artificial diet and the host plant when dietary protein levels are low. This also applied to both species.

The data obtained are discussed with reference to literature data on amino acid absorption and metabolic utilization. The significance of relationships between overall food consumption and amino acid utilization patterns is inferred.

INTRODUCTION

The effects of specific plant compounds on the consumption and utilization of food by phytophagous insects is usually tested by adding such compounds in known amounts to an artificial diet (Reese & Beck, 1976; Reese, 1979). This approach has the advantage that the nutritional background against which to test defined chemical factors can be experimentally manipulated and reproduced in time to a large extent. The utilization of diets differing in one or more controlled factors is usually quantified by gravimetric determination of nutritional indices which reflect absorption efficiency and metabolic efficiency. Higher values of the latter two parameters are commonly interpreted as indicating a better food quality (Waldbauer, 1968; Scriber & Slansky 1981; Scriber, 1984).

The extent to which artificial diets as such are comparable to the natural food of the insect is often judged by their ability to support development and growth at normal rates and to sustain at least several generations when reared continuously on the artificial diet. Few comparative studies on the quality of artificial diets and host plants as food sources as reflected in nutritional indices are known to date (Cohen & Patana, 1984). Recently it has been demonstrated that these indices can be subject to rather serious errors if no appropriate precautions are made (Schmidt & Reese, 1986). The accurate measurement of food consumption is a prerequisite for reliable determination of food utilization and conclusions on food quality.

A study that combined gravimetric methods with continuous respirometry (van Loon, submitted/chapter 4) allowed accurate measurement of food consumption by caterpillars of Pieris brassicae L. and Pieris rapae L. (Lepidoptera: Pieridae). It appeared that food consumption on the common host Brassica oleracea L. was considerably higher than that on an artificial diet, while growth rates were similar on both diets. Prior to a study on the effects of phenolic allelochemical compounds occurring in B. oleracea by incorporating them into a nutritionally adequate artificial diet (David & Gardiner, 1966), it was considered essential to examine in more detail absorption and metabolic efficiencies of essential nutrients on both diets. To this end attention was focussed on amino acids. The aromatic

amino acids phenylalanine and tyrosine are known to be the precursors of phenolic compounds synthesized by higher plants in the phenylpropanoid pathway (Hahlbrock & Grisebach, 1975). In view of this biosynthetic origin, it is conceivable that allelochemic effects of phenolic compounds could come about by interactions at the nutritional level (Reese, 1979). In this budget study the overall economy of utilization of amino acids is investigated comparatively for an artificial and a natural diet and for the two related caterpillar species. The diets are compared in terms of quantity and composition of amino acids. Results are discussed with reference to physiological data on absorption and metabolism of amino acids in lepidopterous insects and their possible significance in the regulation of quantitative feeding behaviour is inferred.

MATERIAL AND METHODS

Insects

Cultures of *P. rapae* and *P. brassicae* were maintained in the laboratory under circumstances described by David and Gardiner (1962) on *Brassica oleracea* variety gemmifera DC. cultivar Titurel. Eggs laid within 24 h time difference were collected from these cultures as starting material for experimental groups. Prior to experiments, caterpillars were reared ab ovo upto the final larval ecdysis in a climatic room at 24 ± 2 °C, 60-70 % RH and 16 hours photoperiod. They were fed either on cabbage leaves or on a semi-synthetic diet (Ma, 1972). Newly ecdysed fifth instars used in experiments had moulted within less than four hours time difference and ranged from 100-120 mg (14 - 15 days ab ovo, *P. brassicae*) or 40-45 mg (13 - 14 days ab ovo, *P. rapae*) fresh body weights. When fed on artificial diet as the experimental food source, they were placed individually in glass petri dishes (diameter 12 cm) and given ad libitum access to the diet. On plant food, they were confined individually in cylindric clear plastic containers (diameter 20 cm, height 25 cm) with a ventilation opening covered with nylon gauze. At the start of an experiment a larva was placed in this container on an individual cabbage leaf the petiole of which was inserted into a flask with tap water. Leaves were replaced with fresh ones as necessary to ensure ad libitum availability of food. Temperature during experiments was 25 ± 1 °C, photoperiod was 16 h. Illumination was provided by overhead fluorescent strip lights at an intensity of c. 5 W/m^2. Duration of all experiments was 90 h, which under the circumstances given was known to be long enough to reach the prepupal (pharate) stage. Prepupae were immobile and had spun a girdle of silk. In this phase the gut had been emptied to contain only minute quantities of mainly reddish excretory material. No shedding of the larval cuticle had occurred (see results).

Experimental diets

Basically two types of diet were studied, the *B. oleracea* variety on which the cultures of both species were maintained and the semi-synthetic

diet described by Ma (1972).

The basic diet contained 22.5 % casein (Nutritional Biochemical Co., vitamin-free) relative to the dry matter part of the diet, which corresponded to 3.6 g of casein relative to fresh weight of diet immediately after preparation. It is abbreviated as 'HC' (high casein level) in the following. In addition to the basic diet itself, two experimental variants of this diet were studied. These were a diet in which the casein content was reduced to 15% of dry matter (hereafter indicated as 'LC', low casein) and the latter diet supplemented with 0.41% of dry weight phenylalanine ('LC+P'). This quantity of phenylalanine supplemented the 15% casein diet to the phenylalanine content of the basic diet. To the three diets an amount of casein-hydrolysate was added equivalent to 5% of the amount of casein. An additional protein source in the basic diet was present in the form of a wheat germ fraction, which contributed c. 5% protein relative to the dry matter of diet.

Individual leaves of B. oleracea offered as food were taken from 90 day old plants grown in a greenhouse at 16 ^{o}C under natural lighting. They were raised from seed in a potting soil of known composition in 3 litre containers and received 100 ml of a nutrient solution every two weeks. Experimental leaves were provisionally divided into two age groups. One group was made up by 'mature' (abbreviated as 'M') leaves that showed no signs of senescence; these leaves were taken from nodes 10 - 20. The second group consisted out of 'young' ('Y') growing leaves from nodes 21 - 25. Six plants were used in this way for each of both species of caterpillars. Samples of leaf material for chemical analyses were taken at the start and at end of the experiment and analysed separately. In addition to the five diets described above a sixth dietary regime involved a change in feeding history. In this case, caterpillars reared on the synthetic diet ab ovo upto the onset of the fifth instar were subsequently offered leaves of the 'young' category as the experimental food source. This situation is referred to as 'Y(D)' in this text.

Gravimetric budget construction

Dry matter budget parameters were determined gravimetrically (Waldbauer, 1968). In calculations the equation: amount of food consumption (C) =

amount of faeces egested (F) + amount of growth of body substance (G) + amount of fuel substance used in respiration (R). All values refer to dry weight . Dry matter budgets were determined for individual caterpillars. Food, insects and faeces were lyophilized at -35 °C and 0.02 bar during 48 h (WKf L 05 freeze dryer) and subsequently dried at 70 °C for 24 h, after which the constancy of dry weights was checked at 2 h intervals. Growth was calculated as the difference between the body weight at the time of sacrifice (90 h after the onset of the experiment) and the individual dry weight of a newly ecdysed fifth instar, determined using an aliqout of 20 insects sacrificed within 1 h of ecdysis. Faeces were collected once, at the end of the experimental period. Under the circumstances given, faeces dried rapidly to become firm discrete pellets. Different from the method used by Waldbauer (1968), in which it is assumed that food consumption can be measured directly, the amount of food ingested was estimated by summing G and F and an estimate of R, the amount of dry matter respired (cf. Simpson, 1982). This estimation was based upon continuous measurements of caterpillar respiration during circumstances comparable to those in the present study that yielded a measure of the energetic efficiency of growth (van Loon, submitted/ cf. chapter 4). For P. brassicae the energetic efficiency of growth was set at 0.56*G, for P. rapae a value of 0.5*G was used. The consequences of alternative assumptions about the actual values of the amount of dry matter respired, based on a physiological variation of 15% (coefficient of variation) found under different dietary regimes (van Loon, submitted/ cf. chapter 4) were calculated to be small and are not further considered in the following.

Budgets for total nitrogen, total amino acids and 17 individual amino acids were obtained by multiplying the values of the C-, F- and G dry matter budget parameters by the content of the latter components (expressed as percentage of dry matter) that resulted from the chemical analyses. In these calculations, group means for the budget parameters were used as insects belonging to the same experimental group had been pooled prior to analyses.

As measures of efficiency, absorption efficiency (AE) and metabolic efficiency (ME) according to the terminology of Woodring et al. (1979), were calculated. The latter two parameters are equivalent to the coefficient of approximate digestibility and the efficiency of conversion of digested food to body substance as defined by Waldbauer (1968).

Nitrogen and amino acid analyses

Total nitrogen was determined using a micro-Kjeldahl system (Büchi 322/342, coupled to a Mettler DL 40 electronic titrator). Analyses were run in duplicate or triplicate when the initial duplicates differed by more than 5%.

Prior to analysis, the dried samples of insects, faeces and food were ground in a Culatti apparatus and forced through a 0.5 mm metal sieve. A portion of the pulverized material was weighed and hydrolysed in 100 ml of 6N hydrochloric acid under reflux at 110 ^{o}C for 22 h. After evaporation of the hydrolysate fluid, the remaining concentrated fraction was redissolved into citrate buffer (pH 2.0). Amino acids were separated by ion-exchange chromatography and quantified using the ninhydrin-reaction (Moore & Stein, 1951; Spackman et al., 1958). This was accomplished using a Biotronik LC 2000 automatic amino acid analyzer. The main column in this system had a length of 26 cm and a diameter of 6 mm. It was packed with Durrum DC 6A resin. The pre-column contained Durrum DC 3. Fifteen amino acids (table 1) could be quantified after acid hydrolysis. Spectrophotometric detection was performed at 570 nm, except for proline, which was measured at 440 nm. Glutamine and asparagine were measured as glutamic and apartic acids respectively, they are referred to as Glx and Asx respectively. Instead of the usual three-buffer system, a four-buffer system was used to produce a complete separation between peaks of phenylalanine and tyrosine on one hand and glucosamine and galactosamine on the other hand. The sulphur-containing amino acids methionine and cystein were determined as methionine sulphone and cysteic acid respectively, after performic acid oxidation. Samples were analyzed in duplicate or triplicate when the initial duplicates differed by more than 10%.

Leaf material was sampled at the start, halfway and at the end of the experimental period and values on amino acid content averaged. Amino acids are referred to by their three-letter code (cf. Chapter 2, table 1).

RESULTS

Food consumption, assimilation and growth

In both species, significant differences in food consumption over the complete final instar occurred depending on the diet offered (tables 1 and 2). On Brassica leaves more food was consumed than on the three artificial diets. An exception is the Y(D)-group of P. rapae that showed a very poor performance. This was caused especially by four out of ten caterpillars that achieved an unusually poor growth rate. None of the caterpillars in this group reached the prepupal stage within the 90 h period. The exceptional position of the latter group was reflected in several other parameters discussed below and will not be explicitly mentioned in the following . Unless stated otherwise, the descriptions refer to both species.

The lower casein level did not affect the amount of food intake in both species. On the Phe-supplemented diet, P. brassicae showed an increased consumption, while P. rapae did not. Both species consumed more food on mature leaves than on young leaves.

Variations in growth on different diets were smaller than in food consumption. Although some significant differences between diets occurred these were not consistently between plant material and artificial diet, as with food consumption (tables 1 and 2).

Higher values of AE correlated with lower values of food consumption over the diets (Spearman's rho was -1 ($p < 0.01$) for P. rapae, except for the outlying value for the Y(D)-group, for P. brassicae rho was - 0.77 ($p < 0.1$) (fig. 1)). These inverse relations were reflected in a smaller degree of variation in the amount of assimilation (equals C - F) compared to the amount of food consumed on the different diets. In P. rapae the coefficient of variation in the means for diets was 18% for the C parameter, while for the assimilated fraction this was 8%, for P. brassicae these values were 9% and 4% respectively.

Table 1. Dry matter budget parameters and absorption efficiency for *P. brassicae* fifth instars in six dietary situations. C, F and G in mg dry matter, AE in %

diet	C	F	G	AE
artificial				
LC	340 ± 23 a	201 ± 16 a	89 ± 7 a	41.8 ± 1.8 c
LC + P	372 ± 21 b	230 ± 13 b	91 ± 6 ab	39.0 ± 1.1 b
HC	339 ± 25 a	190 ± 14 a	95 ± 9 b	44.8 ± 2.1 d
Brassica				
M	434 ± 20 d	291 ± 24 d	92 ± 9 ab	34.0 ± 3.4 a
Y	398 ± 38 c	246 ± 27 bc	97 ± 9 b	39.1 ± 1.7 b
Y (D)	399 ± 15 c	245 ± 11 c	99 ± 13 b	39.5 ± 3.4 b

Means + SD (n = 10). Means in a column are significantly different when different letters are present at the right and there is no letter in common ($p < 0.05$, multiple application of Student's t-test, starting with the two lowest values). For abbreviations see Methods

Table 2. Dry matter budget parameters and absorption efficiency for *P. rapae* fifth instars in six dietary situations. C, F and G in mg dry matter, AE in %

diet	C	F	G	AE
artificial				
LC	105 ± 20 b	59 ± 13 a	30 ± 5 b	45.1 ± 1.9 c
LC + P	102 ± 24 b	58 ± 15 a	30 ± 6 b	45.4 ± 2.4 c
HC	101 ± 12 b	51 ± 10 a	33 ± 5 bc	51.3 ± 6.0 d
Brassica				
M	157 ± 17 d	102 ± 13 c	36 ± 4 c	36.0 ± 2.8 b
Y	132 ± 4 c	82 ± 5 b	33 ± 1 b	39.2 ± 2.0 b
Y (D)	73 ± 25 a	54 ± 19 a	13 ± 7 a	27.1 ± 11.1 a

Means ± SD (n = 10-12). Details see table 1

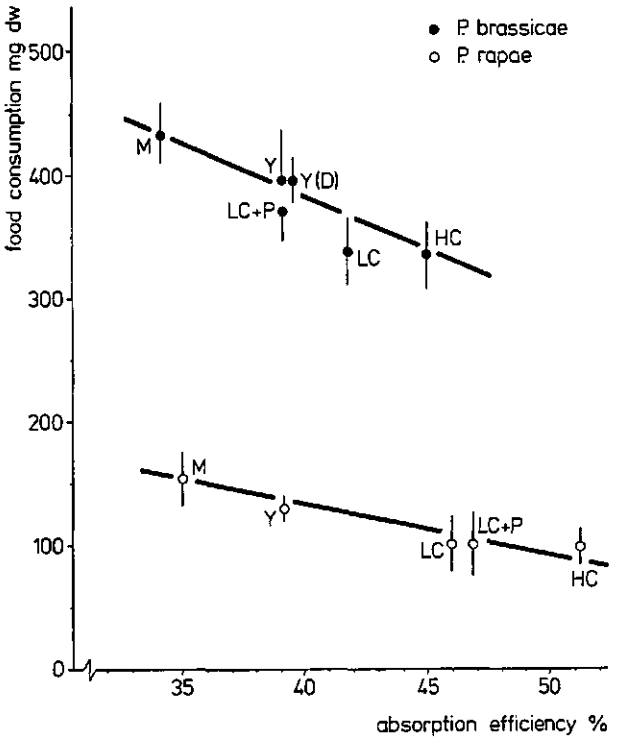

Figure 1:
Relationships between absorption efficiency and total food consumption over the final instar of P. brassicae and P. rapae feeding on different diets (letter codes see Methods). Regression equations of fitted lines: y = -10x + 760 (r = 0.92, P. brassicae); y = -3.8x + 284 (r = 0.91, P. rapae).

Total nitrogen and amino acid budgets

Nitrogen ingestion on either artificial diets or Brassica was in both species generally higher on diets with a higher nitrogen content (tables 3 and 4). Increased nitrogen intake was accompanied by a higher nitrogen egestion but not proportionally as is seen from the decrease in AE. Nitrogen absorption efficiency was higher on artificial diets in both species. The ME-values for nitrogen varied from 85 - 99% in P. brassicae and from 84 - 115% in P. rapae. The concomitant balance inequality was smaller than 10%, either positive or negative. The inequality tended to be higher with higher nitrogen-levels in the diet.

Amino acid ingestion, accumulation and absorption efficiency were higher on diets containing higher amino acid concentrations. Absorption efficiency of amino acids was substantially higher than comparable values for either total dry matter (table 1 and 2) or nitrogen (tables 3 and 4).

Table 3. Nitrogen budget and efficiency parameters and nitrogen content of diet for P. brassicae final instars in six dietary situations. Analyses of hydrolysates in which amino acids have been determined. C, F and G mg, AE and ME %. N-content in % of dry weight of diet

diet	C	F	G	AE	ME	(C-F-G)/C[a]	N-content
artificial							
LC	12.12	4.44	7.50	63.4	98	+ 1.5	3.53
LC + P	13.05	5.11	7.02	60.8	88	+ 7.0	3.48
HC	15.11	6.29	7.46	58.4	85	+ 9.0	4.43
Brassica							
M	14.19	6.43	7.86	54.7	99	- 0.7	3.27
Y	18.19	8.81	8.86	51.6	94	+ 2.9	4.57
Y (D)	18.75	9.24	8.34	50.7	88	+ 6.2	4.70

Group means (n = 10). a: balance inequality, expressed as percentage of C. Abbreviations see Methods

Table 4. Nitrogen budget and efficiency parameters and nitrogen-content of diet for P. rapae final instars in six dietary situations. Analyses of hydrolysates in which amino acids have been determined. C, F and G mg, AE and ME %. N-content in % dry weight of diet

diet	C	F	G	AE	ME	(C-F-G)/C[a]	N-content
artificial							
LC	3.69	1.17	2.45	68.2	98	+ 1.7	3.53
LC + P	3.56	1.20	2.32	66.3	98	- 0.8	3.56
HC	4.52	1.46	2.67	67.7	87	+ 8.7	4.43
Brassica							
M	4.98	2.15	3.00	56.9	106	- 3.3	3.17
Y (D)	6.01	2.63	2.83	56.2	84	+ 9.0	4.58
Y (O)	4.09	2.93	1.34	28.3	115	- 4.4	5.17

Group means (n = 10-12). Details see table 3

Accumulation of amino acids in the body was higher on Brassica than on artificial diet. The total amount of amino acids oxidized was increased at higher dietary levels of amino acids, reflected also in lower values of ME (fig. 2). In the Y(D)-group of P. rapae AE and especially ME showed much lower values than the other five groups.

A weak negative correlation was present when total food consumption was plotted as a function of the overall absorption efficiency of amino acids (fig. 3). Values for the latter parameter were considerably higher than for total nitrogen. The ratio of nitrogen to amino acids in faeces was 0.32 - 0.36 on a weight basis during feeding on artificial diets, while it was 0.30 - 0.42 on leaf material in P.brassicae (table 3 and 5). Nitrogen to amino acid ratios in P. rapae ranged from 0.30 - 0.34 on artificial diets and from 0.27 - 0.47 on Brassica (table 4 and 6).

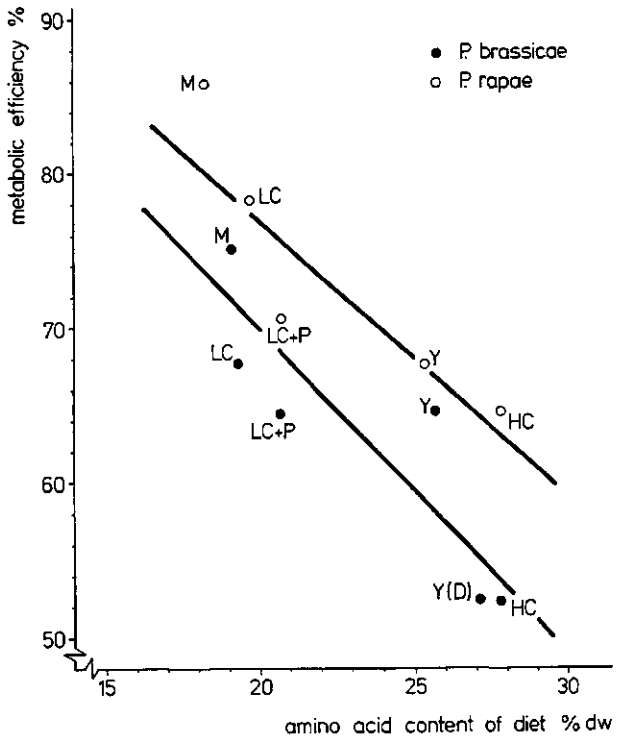

Figure 2:
Relationships between amino acid content of different diets (letter codes, see Methods) and metabolic efficiency of amino acid utilization. Regression equations: y = -2x + 109 (r = 0.88, P. brassicae); y = -1.9x + 116 (r = 0.89, P. rapae).

Table 5. Amino acid budget and efficiency parameters and amino acid content of diets for P. brassicae final instars. Summed totals of seventeen amino acids. Amino acid content in % of dry weight of diet

diet	C	F	G	R[a]	AE	ME	amino acid content
artifical							
LC	66.3	13.7	36.2	16.4	79.3	68.8	19.3
LC + P	77.4	16.1	39.6	21.8	79.2	64.5	20.6
HC	94.7	17.6	40.4	36.7	81.4	52.4	27.8
Brassica							
M	82.7	21.6	46.1	15.1	73.9	75.3	19.1
Y	102.7	22.9	51.8	28.0	77.7	64.9	25.7
Y (D)	108.6	22.1	45.2	41.2	79.7	52.3	27.2

Group means (n = 10) a: R refers to apparent overall net oxidations, irrespective of interconversions. Abbreviations see Methods

Table 6. Amino acid budget and efficiency parameters and amino acid content of diets for P. rapae final instars. Summed totals of seventeen amino acids. Amino acid content in % of dry weight of diet

diet	C	F	G	R	AE	ME	amino acid content
artificial							
LC	20.6	3.9	13.1	3.6	81.1	78.4	19.7
LC + P	20.7	3.5	12.1	5.0	83.1	70.3	20.7
HC	28.3	4.7	15.3	8.3	83.4	64.8	27.8
Brassica							
M	28.6	7.9	17.8	2.9	72.4	86.0	18.2
Y	33.2	8.9	16.5	7.8	73.2	67.9	25.4
Y (D)	19.7	6.3	6.4	7.1	68.0	47.8	24.9

Group means (n = 10-12). Details see table 5

Absorption efficiency

Considering individual amino acids, it is seen that AE values differ little between groups of P. brassicae caterpillars feeding on artificial diets (table 7). Within the essential amino acids, only His shows a maximum difference exceeding 3% (7%). Of the non-essential amino acids, AE for Gly and Cys differ by more than 5% between the three diets. Differences in AE

Table 7. Absorption efficiency (%) of individual amino acids in six dietary situations in P. brassicae

	artificial			Brassica		
amino acid	LC	LC + P	HC	M	Y	Y(D)
Essential						
Phe	89	89	90	73	76	78
Met	90	88	87	78	79	79
Leu	88	88	90	74	76	78
Ile	79	77	80	74	76	77
Val	85	84	86	76	79	79
Thr	78	79	79	74	75	76
Lys	91	91	92	81	83	83
Arg	85	85	87	80	87	87
His	69	67	62	71	67	66
Non-essential						
Ala	81	79	81	70	79	81
Pro	83	83	86	73	81	82
Tyr	90	89	91	76	77	80
Cys	21	28	52	37	48	70
Gly	46	42	49	65	66	71
Ser	59	59	64	76	77	74
Asx	76	75	79	74	79	79
Glx	77	77	80	75	79	80

Values rounded to integer percentage points

Table 8. Absorption efficiency (%) of individual amino acids in six dietary situations in *P. rapae*

	artificial			Brassica		
	LC	LC+P	HC	M	Y	Y(D)
Essential						
Phe	89	90	92	72	77	68
Met	82	92	93	73	75	76
Leu	90	90	91	70	74	70
Ile	81	82	83	71	74	69
Val	87	87	88	75	76	76
Thr	80	82	83	74	72	67
Lys	89	89	89	78	73	66
Arg	87	87	88	77	82	83
His	74	78	72	69	71	53
Non-essential						
Ala	82	81	82	72	73	75
Pro	85	88	89	69	73	70
Tyr	89	90	92	79	77	68
Cys	69	63	45	73	63	49
Gly	34	41	40	60	62	58
Ser	61	66	67	74	74	59
Asx	78	79	81	74	76	66
Glx	82	83	83	75	74	68

Values rounded to integer percentage points

between groups feeding on Brassica were greater than 5% with Leu (11%) and Arg (7%) and with Gly, Cys and Pro. Generally, AE was higher on artificial diet than on Brassica, maximal differences ranging from 5 - 16% for essential amino acids. For Arg, differences were minimal while for His, Gly, Ser and Cys the reversed situation occurred. With the remaining non-essential amino acids, AE values for Tyr and Pro differed maximally 15 and 13% respectively while differences for Ala, Asx and Glx were small.

The pattern found in AE values for P. rapae is essentially similar, with only few exceptions (table 8). Variability in AE is slightly higher for groups feeding on Brassica when the Y(D) group is taken into account. Differences in absorption efficiency of individual amino acids between groups feeding on artificial diets and those offered plant food were greater than in P. brassicae (9 - 22%, disregarding the Y(D) group).

In a comparable way as in fig. 3, food consumption has been plotted as a

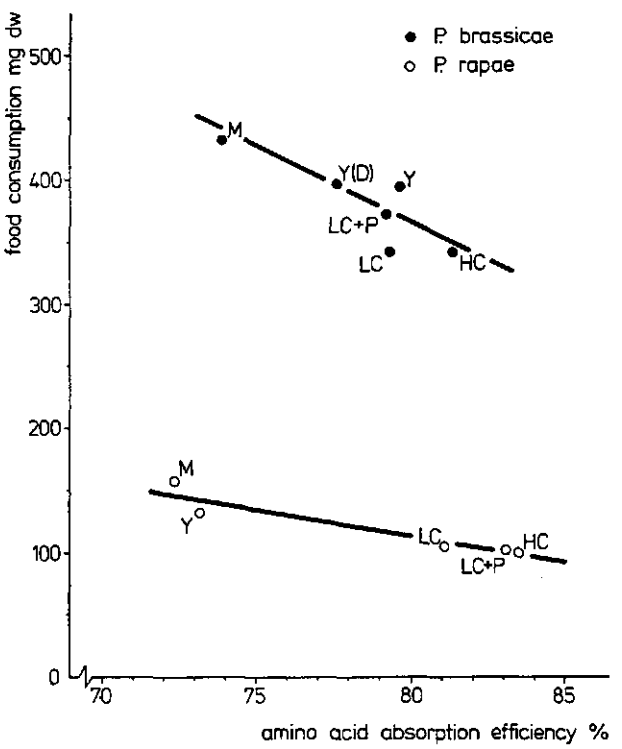

Figure 3:
Relationships between absorption efficiency of amino acids and food consumption over the final instars of P. brassicae and P. rapae on different diets (letter codes see Methods). Regression equations: $y = -12x + 1330$ ($r = 0.83$; P. brassicae); $y = -4.3x + 458$ ($r = 0.95$, P. rapae).

function of AE for some representative amino acids (fig. 4 and 5). In both species food consumption is lower at higher values of AE, although these relations are not strict. Spearman's rank correlation test showed significant correlations for Val and Lys ($p < 0.01$), for Phe, Tyr, Leu, Ile and Pro ($p < 0.025$) and for Thr ($p < 0.05$) in *P. brassicae* ($n = 6$). For *P. rapae* significant rank correlations were calculated for all essential amino acids except for Lys and for the non-essential Tyr ($p < 0.05$, $n = 5$, disregarding the values for the Y(D)-group).

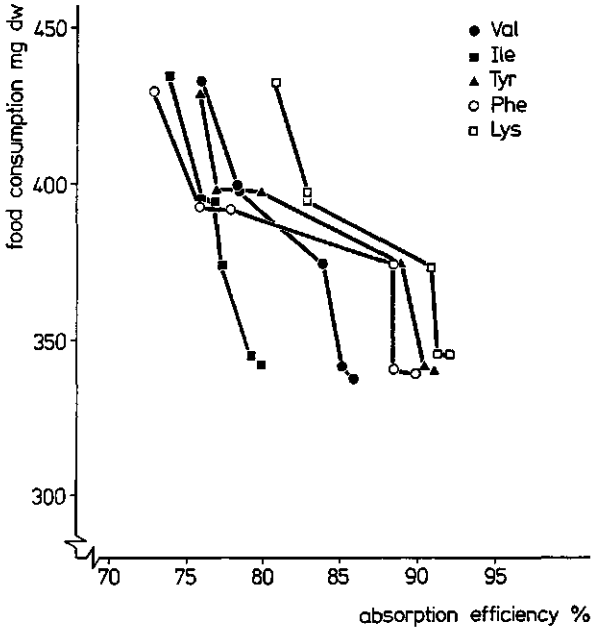

Figure 4:
Relationships between the absorption efficiency of a particular amino acid and the amount of food consumed over the final instar of *P. brassicae*.

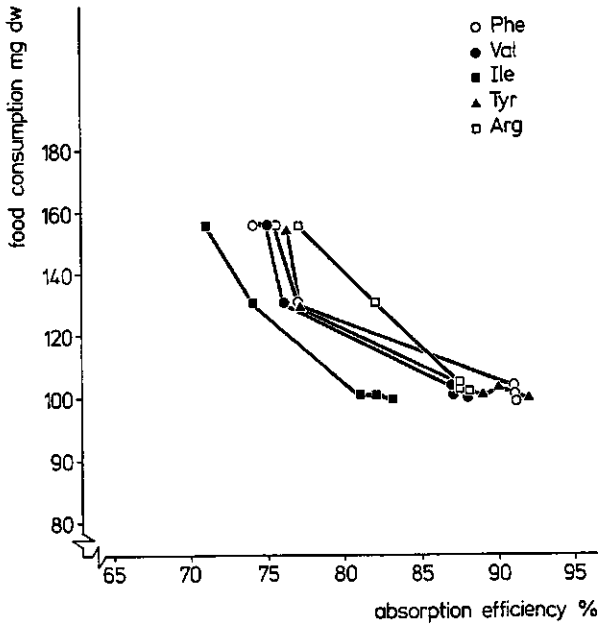

Figure 5:
Relationships between the absorption efficiency of a particular amino acid and the amount of food consumed over the final instar of P. rapae.

Metabolic utilization

Metabolic utilization efficiencies of individual amino acids showed a higher degree of variability over different diets than AE values (table 9 and 10). In P. brassicae ME of most amino acids ranged from 40 - 80%, for P. rapae this was 50 - 90%. In both species, especially Pro and Glx were utilized less efficiently, together with Leu and Ala on Brassica. In the Y(D)-group of P. rapae, another six amino acids were utilized with comparatively low efficiencies. In both species, especially His, Lys and Arg showed values of ME higher than 80 or 90% on all diets. Values of ME greater than 100%, indicating net synthesis, were found for Tyr on all diets and in both species. On artificial diets, Cys and Gly and Asp also were characterized by ME values exceeding 100% in both species, except for

Table 9. Metabolic efficiency (%) of individual amino acids in six dietary situations in P. brassicae

	artificial			Brassica		
amino acid	LC	LC+P	HC	M	Y	Y(D)
Essential						
Phe	72	51	57	61	59	50
Met	70	68	58	88	91	75
Leu	54	51	42	59	56	45
Ile	80	83	60	64	62	60
Val	66	62	50	73	67	52
Thr	75	73	57	66	62	52
Lys	70	55	45	92	80	64
Arg	81	71	60	76	46	44
His	98	96	82	97	96	93
Non-essential						
Ala	73	69	58	55	46	32
Pro	30	30	20	65	37	29
Tyr	125	133	103	144	149	149
Cys	234	222	87	166	120	40
Gly	168	187	136	63	60	49
Ser	69	63	44	55	51	52
Asx	102	101	77	75	65	61
Glx	44	42	29	78	66	58

Values rounded to integer percentage points

Table 10. Metabolic efficiency (%) of individual amino acids in six dietary situations in *P. rapae*

	artificial			Brassica		
	LC	LC+P	HC	M	Y	Y(D)
Essential						
Phe	70	52	68	69	59	42
Met	74	60	64	89	90	33
Leu	48	56	50	68	60	34
Ile	79	75	69	81	71	44
Val	72	70	62	86	50	35
Thr	81	73	70	77	67	46
Lys	78	64	66	98	91	56
Arg	93	80	86	92	65	31
His	97	93	95	98	95	80
Non-essential						
Ala	98	92	83	61	57	22
Pro	50	28	22	87	55	28
Tyr	129	146	121	133	136	142
Cys	117	198	140	54	65	61
Gly	295	239	232	82	82	54
Ser	94	80	66	63	59	64
Asx	115	103	101	84	68	67
Glx	50	41	42	90	78	58

Values rounded to integer percentage points

Cys and Asp on the HC-diet. Both over the artificial diets and over the Brassica groups, ME generally decreased at higher amino acid levels in the food, a trend also presented in fig 2 for total amino acids. This trend encompasses the main part of the variation in ME and was present also for several individual amino acids. Negative rank correlations calculated for essential amino acids were significant for Thr ($p < 0.01$), Arg ($p < 0.025$), Phe ($p < 0.05$) and Ile and Lys ($p < 0.1$) in P. brassicae ($n = 6$). In P. rapae such correlations were present for Val ($p < 0.01$), Thr ($p < 0.025$), Arg ($p < 0.05$) and Phe and Ile ($p < 0.1$).

Utilization of phenylalanine

The observation of considerable degrees of Tyr synthesis on all diets and in both species made it relevant to consider the utilization of assimilated Phe, the only precursor of Tyr, in more detail (table 11 and 12). It appeared that in both species the fraction of Phe oxidized relative to the total amount assimilated was strongly dependent on the diet. On the LC- and M-diets small quantities of Phe were oxidized as opposed to the other diets. The amounts of Phe and Tyr accumulated in the prepupal body also showed a considerable variation, being higher in the groups feeding on Brassica. At lower levels of Phe oxidation, Tyr content of the pupal body is also lower, except for the Y(D) groups of both species. Supplementing the LC diet with Phe resulted into a considerable decrease of the relative amount of Phe devoted to growth.

Amino acid composition of diets.

Amino acid composition of the artificial diet (i.e. the content of individual amino acids divided by the total amino acid content) was clearly different from that of Brassica (table 13). Differences were smaller than 10% only with Ser, Val, Ile and Tyr. Thr, Phe, Arg, Cys, Asp, Ala and Gly were abundant in Brassica, while the artificial diet contained relatively more Met, Lys and His. Minor differences (smaller than 5%) occurred in amino acid patterns of mature and young leaves. Pro and Arg were more prominent in young leaves while the opposite was the case with Ser and Tyr.

Table 11. Utilization of assimilated phenylalanine for growth, tyrosine synthesis and oxidation and phenylalanine and tyrosine content of pre-pupae (% of dry weight) in P. brassicae

diet	Phenylalanine utilization			Phenylalanine content	Tyrosine content
	growth	Tyr-synthesis	oxidation		
artificial					
LC	72	27	2	2.27	3.95
LC + P	51	24	25	2.41	4.28
HC	57	3	40	2.55	4.43
Brassica					
M	61	35	4	2.62	4.80
Y	59	33	8	2.78	5.12
Y (D)	50	30	20	2.46	4.29

Table 12. Utilization of assimilated phenylalanine for growth, tyrosine synthesis and oxidation and phenylalanine and tyrosine content of pre-pupae (% of dry weight) in P. rapae

diet	Phenylalanine utilization			Phenylalanine content	Tyrosine content
	growth	Tyr-synthesis	oxidation		
artificial					
LC	70	27	3	2.23	3.66
LC + P	52	33	15	2.27	4.18
HC	68	21	11	2.42	4.15
Brassica					
M	69	29	2	2.61	4.30
Y	59	27	14	2.73	4.65
Y (D)	42	19	39	2.50	3.52

Table 13. Average amino acid composition of artificial diet, nature and young B. oleracea - leaves as percentages of total amino acid content of these diets

	diet		
	artificial	Brassica leaves	
amino acid		mature	young
Essential			
Phe	4.50	6.16	5.86
Met	2.45	2.10	2.01
Leu	8.07	9.13	8.71
Ile	4.94	5.35	5.12
Val	6.33	6.65	6.53
Thr	4.45	5.42	5.19
Lys	7.26	6.14	6.26
Arg	4.48	5.55	7.03
His	2.84	2.52	2.59
Non-essential			
Ala	3.66	6.51	6.29
Pro	9.52	5.19	6.27
Tyr	4.53	4.20	3.90
Cys	0.99	1.37	1.32
Gly	2.80	5.61	5.31
Ser	5.76	5.72	5.32
Asx	7.32	10.49	10.51
Glx	20.10	11.75	11.78

Amino acid composition of prepupal body

The contribution of particular amino acids to the total amount of amino acids accumulated in the prepupal body (table 14) displayed a coefficient of variation smaller than 5% over the six experimental groups for the majority of amino acids in both species. In P. brassicae Cys and His contents were more variable (13% and 10% respectively). The coefficient of variation of five amino acids was greater than 5% in P. rapae. These were Cys (20%), Pro (16%), Val (12%), Leu (9%) and Ser (8%). The differences in amino acid pattern between both species were smaller than 0.3% for thirteen amino acids. For Met this difference was somewhat greater. P. brassicae had a comparatively low average Cys level and high Met level, while the opposite was true for P. rapae. Differences were greatest for Ala (0.35%) and Tyr (0.57%).

Table 14. Amino acid composition of prepupae of P. brassicae and P. rapae expressed as percentages of total amino acid content of prepupal bodies. Average values from groups reared on six diets, except for Y(D) group of P. rapae

amino acid	P. brassicae	P. rapae
Essential		
Phe	5.20	5.08
Met	2.66	2.35
Leu	7.06	6.81
Ile	5.30	5.07
Val	6.36	6.11
Thr	4.73	4.67
Lys	7.66	7.82
Arg	5.83	5.84
His	3.39	3.45
Non-essential		
Ala	4.65	4.99
Pro	4.39	4.59
Tyr	9.25	8.68
Cys	1.17	1.38
Gly	4.95	4.67
Ser	4.70	5.01
Asx	10.36	10.11
Glx	12.43	13.38

DISCUSSION

Dietary levels of amino acids and nitrogen and quantitative food consumption

A significant increase in the amount of food ingested occurred when caterpillars of both species were fed on mature leaves, which contained c. 18.5% amino acids, as compared to younger leaves, having 25.5%. Food intake was distinctly lower on artificial diets than on plant food and, in contrast, not influenced by a reduction of the protein content from 28% to 19% of dry matter. These observations point to the importance of the amino acid composition, which was clearly different (table 13) (McGinnis & Kastings, 1972; Rock, 1972).

Behavioural compensation in response to reduced nitrogen or protein levels has been documented for several phytophagous insects. Slansky & Feeny (1977) found that final instar P. rapae caterpillars consumed different amounts of leaf material (ranging from 124 to 180 mg dry matter) on different cultivars of B. oleracea. They explained this by differences in foliar nitrogen levels, which varied between 1.5 and 3.1% for different cultivars reared under similar conditions (cf. tables 4 and 6). Employing five levels of five unusual protein or amino acid sources, Horie & Watanabe (1983) found different levels of food consumption by Bombyx mori, both between protein sources and between levels within a particular source. On a 20% casein diet, 1.29 times as much food was consumed as on a 25% casein diet. On a diet containing 20% of an amino acid mixture, 1.14 times as much food was ingested than on the 25% level. Behavioural compensation was also found in Locusta migratoria that ate 1.48 times as much on an artificial substrate containing 13.7% protein than on the same diet having 27.5% protein (Simpson & Abisgold, 1985).

The absence of an effect of a reduced protein content on the amount ingested by P. brassicae was unexpected. In preliminary experiments employing four casein levels, significant effects on food consumption occurred. On a 17.5% casein diet, 363±19 mg dry matter was consumed, while on a 22.5% diet (comparable to the HC diet) 305±23 mg were consumed, a difference of a factor 1.20 ($p < 0.001$, Student's t-test ($n = 7$)). Although there was a considerable time lag between the latter and the present ex-

periments, preventing a direct comparison, it is unknown why the expected degree of compensation on the LC diet in the present experiments did not occur. As different lots of casein were used, differences in casein quality may have caused the discrepancy (Davis, 1972).

Nitrogen and amino acid budgets

Contrary to total dry matter budgets, nitrogen budgets offer the possibility of checking the balance sheet. In theory, assuming that nitrogen cannot escape in a gaseous form from the different budget items (food, insect, egesta), the difference C - F - G should equal zero and the ME for nitrogen should be 100%. From tables 3 and 4 it appears that deviations from these theoretical expectations occurred. In only few published reports the nitrogen balance is given in full. In ecologically oriented studies nitrogen utilization efficiency is often calculated as the amount of nitrogen accumulated in the insect body divided by the estimated amount of nitrogen consumed (Slansky & Feeny, 1977; Mattson, 1980; Grabstein & Scriber, 1982). Nitrogen balances for Bombyx mori (Horie & Watanabe, 1983) showed inequalities, reflected in ME values ranging from 88 - 110%. In Heliothis zea deviations were even more serious (Cohen & Patana, 1984). Several sources of error in constructing nitrogen balance sheets are identical to those inherent in dry matter budget studies (Schmidt & Reese, 1986), while an additional source of error is introduced in the chemical determination of nitrogen. An explanation for an apparent overshoot of nitrogen may be that nitrogen could escape in a gaseous form by microbial activity in food or egesta.

The physiological significance of absorption efficiency of total nitrogen or nitrogen derived protein amounts is limited as it disregards the fact that part of the apparent undigested nitrogen actually may have been absorbed and excreted afterwards. In part this can be overcome by measuring excretory end products of nitrogen catabolism like uric acid (Bhattacharya & Waldbauer, 1972). The high nitrogen to protein ratios in faeces calculated in the present study indicate indirectly that a considerable part of faecal nitrogen is not contained in amino acids. Uric acid excretion with faeces can display large variation depending on the dietary source and concentration of protein (Horie & Watanabe, 1983). In P.

brassicae total uric acid excretion in faeces was c. 5 mg and faecal allantoic acid excretion amounted upto 2 mg during the final instar (Mauchamp & Lafont, 1975). The latter authors also found uric acid accumulation in the body amounting to 2 mg and they further mentioned that these quantities seemed to depend strongly on the host plant.

Balance sheets for amino acids yield more insight into the actual efficiency of absorption and utilization of this quantitatively most important group of nitrogen containing nutrients. Absorption efficiency for total amino acids was found to be higher than for nitrogen, as expected on the basis of the foregoing considerations. It is also seen that considerable quantities of amino acids apparently are oxidized and that this strongly depends on the amino acid content of the diet (tables 5 and 6; fig 2). It can be estimated that in the HC-groups of both species amino acid oxidation represented c. 70% (P. brassicae) and 50% (P. rapae) of the total dry matter of fuel oxidized. Comparable data on directly determined amino acid or protein absorption efficiencies for other insects are scarce. In larval Acheta domesticus overall AE for protein was 81% and ME was 80% (Woodring et al., 1979). The highest value of AE for protein reached in nymphs of Locusta migratoria was 70% and was correlated with the peak in net dry weight growth efficiency (Simpson, 1982). The latter studies employed nitrogen derived protein values that were corrected for faecal uric acid.

At this point it must be noted that the balance sheet method applied in the present investigation also has limitations. It is realized that the AE parameter only reflects an apparent, overall or net efficiency as in theory it is possible that amino acids have been absorbed and excreted again, while non-essential amino acids may have been subjected to interconversions in the meantime. The ME parameter likewise reflects only overall values for the non-essential amino acids while it reflects true values for essential ones. The extent to which post-absorptive excretion of amino acids occurs e.g. by Malphigian tubules or midgut is largely unknown. It would seem an inefficient but not impossible strategy that those amino acids showing a low AE value would have been preferentially excreted as they are the same that are synthesized. A simpler explanation is that indeed their absorption itself is for some reason poor.

Absorption of amino acids

In lepidopterous larvae, amino acids are predominantly absorbed in the anterior region of the midgut, while no evidence exists for a role of the hindgut (reviews by Turunen, 1985; Dow, 1987). Transport of several amino acids from the gut lumen into the haemolymph is active and most probably carrier mediated. The absorption of phenylalanine has been especially well studied in the model Bombyx mori (Sacchi et al., 1981; Giordana et al., 1982). An energy requiring cotransport with potassium occurred from gut lumen to haemolymph without accumulation in the gut epithelium. The energy required for the powerfull electrogenic potassium pump in lepidopterous midgut has been shown to be derived predominantly from amino acids, especially alanine, glutamate and glutamine (Parenti et al., 1985). These data come from in vitro studies with a few amino acids. Data comparable to those presented in this study on the apparent in vivo efficiency of absorption of individual amino acids from complex diets are lacking in the literature.

Recently evidence has been obtained that absorption of amino acids may occur also in the form of oligopeptides (Turunen, 1985). Digestive breakdown of larger protein molecules is accomplished especially by the protease trypsin. Levels of tryptic activity in the gut in response to a range of dietary casein levels differed between the polyphagous lepidopterans Spodoptera exigua and Heliothis zea (Broadway & Duffey, 1986). Endopeptidases, leucineaminopeptidases and other proteases have been obtained from P. brassicae (references in Feltwell, 1982). The observation that absorption efficiency of most amino acids was lower on a natural than on an artificial food may be explained by the absence of cellular structures in the artificial diet, which conceivably protect at least part of the plant protein from digestion or absorption. Enzymatic digestion of food protein is essential as only a relatively small quantity of amino acids is present in free form in the diets. The amount of free amino acids in B. oleracea is variable with plant age and may depend on the cultivar (Dodd & van Emden, 1979) but seems not to exceed 10% of the protein content. A different explanation for the lower AE on plant material may be that it contains chemical factors that reduce digestion or absorption.

Metabolic utilization of amino acids

Amino acid metabolism has been studied in many insects with qualitative methods to trace metabolic pathways (review by Chen, 1985). Especially the haemolymph pool of free amino acids has been studied quantitatively and the haemolymph of lepidopterous larvae has been found to contain exceptionally high levels (review by Mullins, 1985). From the data of Junnikkala (1969) on P. brassicae it can be calculated that the haemolymph free amino acid pool is c. 1.6 mg on B. oleracea and 1.35 mg on the artificial diet. The five most abundant amino acids were asparagine and glutamine (together 16 mM, while aspartate and glutamate levels were neglegible), proline (14 mM), histidine (11mM) and γ-amino-n-butyric acid (9 mM), which together make up 56% of the total amount. These data refer to the wandering phase, assuming a haemolymph volume of 150 µl and are averaged for both sexes, which were found to be quite different in this respect (Junnikala, 1969; 1976). The amount of amino acids contained in haemolymph proteins in P. brassicae seems much more important and can be calculated to be around 11 mg at its peak near the end of the fifth instar without significant differences between plant and artificial diets (van der Geest, 1968; Chippendale & Kilby, 1969). Apart from the haemolymph pool, little is known about the overall utilization of amino acids (Chen, 1985). Indeed, this requires a reliable estimate of food intake (McGinnis & Kastings, 1972), which is rather difficult to obtain especially with living food (van Loon, submitted/chapter 4).

The inverse relationship between amino acid content of the diet and their overall metabolic utilization suggests a mechanism to operate that regulates oxidation of amino acids according to needs. While the overall relation is not strict in P. brassicae caused by an outlying value for the Y-group, rank correlations were significant for several essential amino acids. It is noted also that with several amino acids low values for AE correlate with high values for ME. This is evident for histidine and for cysteine and glycine on the artificial diet. The latter amino acids apparently are suboptimally absorbed from the artificial diet and seem to require net synthesis. It is remarkable that in both species the relative utilization of glutamate and aspartate differs considerably between the artificial diet and the natural one. While absorption efficiency was not or only slightly affected, utilization in the Y(D) groups, especially that of

P. rapae was importantly reduced. In P. rapae this poor utilization may have been the cause of poor growth, although the ultimate cause, probably related to an irreversible adaptation to the pattern of absorbed amino acids on the artificial diet ,is unknown. The difference in capability to utilize the plant protein after previous development on an unusual protein source was one of the few striking differences between both caterpillar species.

Metabolism of phenylalanine and tyrosine

Tyrosine is generally classified as non-essential to insects, but its only metabolic precursor, phenylalanine is essential. Tyrosine is known to accumulate in considerable amounts in both the haemolymph and fat body of final instars (Chen, 1985; Keeley, 1985). Only part of the storage is made up by free tyrosine. Important storage forms in several insect species are β-glycosyl-O-tyrosine and tyrosine-rich proteins such as calliphorin and manducin (Chen, 1985). In P. rapae β-glycosyl-O-tyrosine has been demonstrated and it has been suggested that the tyrosine-rich peptide in P. brassicae postulated by Junnikkala (1976) is in fact β-glucosyl-O-tyrosine (Chen, 1985). Tyrosine levels in P. brassicae have been studied in somewhat more detail and were found to display a cyclical pattern with peak levels just prior to ecdysis (Junnikkala, 1969; 1976; Post & de Jong, 1973). From the data of Junnikkala (1969; 1976) and those presented here it appears that in the wandering phase a relative important amount of tyrosine (c. 25%) is circulating in the haemolymph in 3 - 4 mM concentrations as free tyrosine and 40 - 50 mM as β-glucosyl-O-tyrosine.

The balance sheet approach followed in the present study yielded the significant finding that an extensive net conversion of phenylalanine to tyrosine must have occurred. Also, where the apparent surplus amount of phenylalanine oxidized was calculated to be small, tyrosine content of the prepupal body was reduced. These results suggest that tyrosine may readily become a limiting compound for growth (Gordon, 1972), certainly at still lower dietary levels of both aromatic amino acids than applied in the present investigation. This is illustrated by plotting the amino acid pattern of the absorbed fraction against the pattern of the accumulated

biomass (Gordon, 1972). Judged by the degree of deviation from the line y = x, on Brassica cystein and tyrosine are equally limiting. The essential histidine and lysine may also become limiting (fig. 6). For the artificial diet, again cystein and tyrosine are potentially limiting followed by glycine and aspartic acid/asparagine (fig. 7).

The general role of tyrosine as an important precursor of cross-linking compounds like N-acetyl-dopamine in the cuticle of insects is well established by now (reviews by Brunet, 1980; Andersen, 1985). This implies that the timing of sampling material for tyrosine analysis is critical. When conversion of tyrosine to diphenolic derivatives has started, actual tyrosine accumulation is underestimated. The degree to which phenylalanine was converted to tyrosine in this study warrants that such an underestimation was unlikely. Indeed, caterpillars were sacrificed prior to shedding of the larval cuticle and sclerotization of the pupal exoskeleton.

Few studies on the quantitative requirement for aromatic amino acids are known and none on the effects that suboptimal supply and accumulation may have on the quality and quantity of body protein or on performance parameters such as reproduction. Bernays & Woodhead (1984) presented evidence that phenylalanine is a limiting nutrient for the locust Schistocerca gregaria. Phenylalanine supplementation improved food to body conversion efficiency and there was an indication that higher food intake occurred to compensate apparently insufficient phenylalanine intake. Tyrosine, however, was not adequate as supplementary nutrient for reasons unknown. Acquisition of aromatic compounds during the larval stage may be considered more critical in Lepidoptera than in Orthoptera, as adults of the former group feed predominantly on nectar which probably contains relatively low levels of aromatic amino acids.

The fact that phenylalanine supplementation did not reduce food intake in both Pieris species may have several explanations. The content of amino acids to which phenylalanine was added was still rather high (20%) and thus may not have been limiting. Also the way of supplementing was different. In the case of Schistocerca phenylalanine was given discontinuously and separate from the main food sources, while in this study it was mixed with the main food source. This may have given rise to a saturation of the phenylalanine uptake mechanism (Giordana et al., 1982).

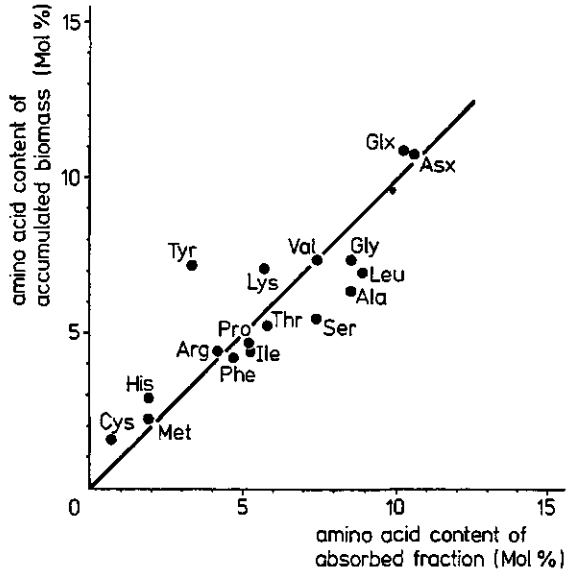

Figure 6:
Relationship between the amino acid content (Mol %) of the absorbed fraction of the food and the amino acid content (Mol %) of the accumulated biomass of P. brassicae final instars feeding on mature leaves of B. oleracea.

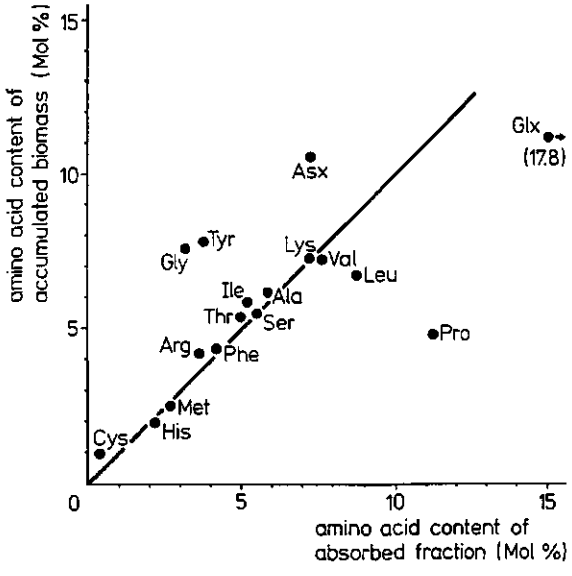

Figure 7:
Same as fig. 6, P. brassicae feeding on the LC-diet.

Relations between amino acid absorption efficiency and food consumption - their possible significance in regulation of food intake.

Inverse relations between overall dry matter food utilization parameters and food intake have been found in several insect - plant combinations (Slansky & Feeny, 1977; Scriber, 1984). Such static relations a posteriori do not necessarily indicate a causal relationship, as clearly the question of cause and consequence is involved. Slansky & Feeny (1977) addressed this question in a study on P. rapae. They proposed that caterpillars showed an increased food intake in response to a lower nitrogen content of the plant food. The higher rate of food intake tended to decrease the overall utilization of food. They conclude that the caterpillars were compensating for a low nitrogen content of their host plant so as to stabilize the rate of nitrogen accumulation. It remains unexplained, however, why food consumption rate varied over a much greater range than the relatively narrow range in which a maximum level was already reached. This suggests that nitrogen as such is not a representative indicator of nutritive limiting factors. Nonetheless, dietary nitrogen levels have been shown to influence food intake and growth of insects in many cases (reviews by McNeill & Southwood, 1978; Mattson, 1980; Scriber and Slansky, 1981). It is general practice to assume a direct relation between nitrogen level on the one hand and protein level on the other, using a conversion factor. Evidently, elementary nitrogen in itself is not a nutrient. Bernays & Woodhead (1984) concluded that total protein concentration in plants derived from N-content is not in all cases a reliable indicator of protein quality. The question if some nitrogen containing nutrients and especially amino acids are more important than others in mediating the apparent effects on quantitative food intake via a postulated nutrient feedback mechanism has yet to be answered for phytophagous insects (Simpson & Abisgold, 1985). The inverse relationships between the absorption efficiency of especially essential amino acids and tyrosine in both Pieris species, consistent over diets that differ in many more factors than amino acid levels and composition on one hand, and total dry matter food consumption on the other may be considered as indirect evidence for the existence of such a mechanism and the role of amino acids therein. At this level of actual nutrients, the cause and consequence problem is in part resolved by the observation that relationships were absent or even positive (with serine and glycine in both

species) for all non-essential amino acids except tyrosine and proline, indicating that a higher rate of food intake does not automatically reduce absorption efficiency.

A related question at the ecological level is what advantage a reduced food consumption rate has for it to make a nutritional regulation mechanism of value. From a teleological viewpoint, lower food consumption rates are considered advantageous (Slansky & Feeny, 1977). Also, it would be of interest to study the nutritional and possibly endocrine mechanisms that control the decision to stop food intake at relatively low levels of accumulation of essential nutrients, as occurred on an artificial diet in the present study.

An experimental approach in which individual amino acids or mixtures in known amounts are fed directly into the haemolymph via canulation and the simultaneous study of concomitant effects on food intake behaviour will be needed to prove causal relationships. This study provides a basis to guide such experiments.

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CHAPTER 6

EFFECTS OF PHENOLIC ACIDS AND FLAVONOIDS ON LARVAL PERFORMANCE OF TWO PIERIS SPECIES

ABSTRACT

The phenolic acids caffeic and chlorogenic acid and the flavonoids quercetin-3-rutinoside and oenin (an anthocyanin) inhibited larval survival, growth and development when Pieris brassicae and P. rapae larvae were continuously exposed to these compounds presented in an artificial diet at levels from 0.4 to 6.25 mM. Inhibition generally was stronger at higher concentrations. P. brassicae was distinctly more sensitive at lower levels of these compounds than P. rapae. This was also reflected in a much smaller degree of reduction of pupal weights and retardation of pupation in the latter species. Final instars of both species that had not previously been exposed to the compounds were not influenced by the lower concentrations tested. In the final instar P. rapae tolerated higher levels without showing effects on food consumption, absorption efficiency and growth than P. brassicae. It is suggested that the inhibition of growth found in final instars is caused primarily by a reduction of food intake and that this reduction in turn is caused by sensory mechanisms. Such mechanisms may operate also in earlier instars. The potential role of phenolic acids and flavonoids that occur in a common host plant, Brassica oleracea, as defensive compounds against caterpillars of both Pieris species is discussed.

INTRODUCTION

Plants belonging to the Cruciferae family are known to contain glucosinolates (Van Etten & Tookey, 1979). Representatives of this group of secondary plant compounds have been shown to serve a defensive function against phytophagous insects (Blau et al., 1978). The deterrent and toxic actions of glucosinolates have received much less attention than their attractive and stimulating effects on those insects that are specialized feeders on Cruciferae like caterpillars of the genus Pieris (Pieridae, Lepidoptera) (David & Gardiner, 1966; Schoonhoven, 1967; Rodman & Chew, 1980). Such oligophagous insects have apparently overcome the glucosinolate biochemical barrier that prevents acceptation and survival by most other insect herbivores, although the mechanism of neutralization of glucosinolates has not been studied in detail (Dowd et al., 1983). All nine glucosinolates investigated by David & Gardiner (1966) stimulated feeding behaviour of P. brassicae over the concentration range tested. There seemed to be no effect of variation in quality and quantity of glucosinolate patterns in wild and cultivated Cruciferae on the larval growth rate of P. rapae (Slansky & Feeny, 1977). These data decrease the likelihood that glucosinolates could play a significant role in the defense against Pieris caterpillars. Moreover, none of the atypical steroidal secondary compounds that occur in some non-cultivated Cruciferae were toxic to P. rapae larvae (Usher & Feeny, 1983).

Apart from glucosinolates, the occurrence of phenolic acids and flavonoids in Cruciferae and more specific in the genus Brassica is well documented (Hegnauer, 1973; Durkee & Harborne, 1973; Das & Rao, 1975; Classen & Nozzolillo, 1980). It has been demonstrated that levels of both groups of compounds vary depending on cultivar and environmental conditions (Wildanger & Herrmann, 1973; Herrmann, 1975). The allelochemic effects of phenolic compounds in plants on phytophagous insects are less overt and often of a more quantitative nature than the evident qualitative effects like acute deterrency or toxicity of glucosinolates. The nature of the effects of phenolic compounds seem to escape generalization (Schoonhoven, 1972; Harborne, 1979; McClure, 1982). These circumstances may explain why few studies have been devoted to this subject.

In a search for potential resistance mechanisms in cultivated Brassica against Pieris, it was considered of interest therefore to examine the

effects on P. brassicae and P. rapae of some phenolic and flavonoid compounds occurring in Brassica or close analogs of them. The effects of continuous exposure were studied by incorporating such compounds into a semi-synthetic diet and assessed using long term survival, growth, pupal weights and final instar food consumption and utilization as overall performance criteria.

MATERIAL AND METHODS

Insects

Cultures of P. rapae and P. brassicae were maintained in the laboratory under circumstances described by David and Gardiner (1962) on Brassica oleracea variety gemmifera DC. cultivar Titurel. Eggs laid within 24 h time difference on plant material were collected from these cultures as starting material for experimental groups. Experiments were performed in a climatic room at 25 $\pm$ 1 ^{o}C, 60-70 % RH and 16 hours photoperiod. Illumination was provided by overhead fluorescent striplights, yielding an intensity of c. 5 W/m^2 at the level on which the larvae were reared.

Experimental diets

The compounds tested were added to a semi-synthetic diet described by Ma (1972), which differed only in a few quantitative respects from the diet developed for P. brassicae by David & Gardiner (1966). Both species were reared on this diet. The standard diet incorporates the phenolic methyl-p-hydroxybenzoate as a microbial inhibitor at a level of 184 mg per 100 g fresh weight of diet. Apart from this compound, the diet was found to contain negligible amounts of phenolic acids or flavonoids, as these could not be detected by thin layer chromatography (van Loon & van Beek, chapter 7).

Three phenolic acids and three flavonoids were examined. They were obtained from a commercial source (Sigma Co.) and were of high purity (more than 99%): sinapic acid (4-hydroxy-3,5-methoxycinnamic acid), caffeic acid (3,4-dihydroxycinnamic acid), chlorogenic acid (3-(3,4-dihydroxy-cinnamoyl)quinic acid), quercetin (3,3',4',5,7-pentahydroxyflavone), rutin (quercetin-3-rutinoside) and oenin (3-(glucosyloxy)-4',5,7-trihydroxy-3',5'-dimethoxyflavylium-chloride). These compounds were admixed at different concentrations by solubilizing the crystalline compounds in pure methanol (200 ul) and dissolving this concentrated solution in the vitamin mixture which was added immediately afterwards as the last component during diet preparation, below 50 ^{o} C. The level of vitamin C, added as a pure

substance, i.a. serving an anti-oxidative function to protect the phenolic compounds, is calculated to be 23 mM in the freshly prepared diet.

Dose effect bioassays

The effects of the six compounds on larval survival, growth, development and pupation in both species was investigated by placing groups of 25 neonate larvae within 6 h since hatching randomly on the control and treated diets. The diet was offered in glass petri dishes (diameter 9 cm) in slices (P. brassicae) or as a layer of 3 or more mm thick in the bottom half of the dish that was placed upside down (P. rapae). Four concentrations were assayed for each compound (see results). For each combination of compound and concentration two or three replicate groups were studied and this setup was repeated at least once in time. Since results of the repeated series were similar, data from only one series are given as average values over replicates. Survival and development were scored every 48-60 h during the first 12 days of the experiment. From day 4 onwards, also the weight gain of the group was recorded. For the sake of data reduction, results on survival, development and growth are presented for one discrete moment only, viz. when about 50% of the control group had reached either the penultimate instar (survival and growth) or the last larval ecdysis (development). Pupal weights were determined within 12 h after the occurrence of larval to pupal ecdysis. Pupation occurring later than 40 days after egg hatch was not further recorded.

Food consumption, absorption efficiency and growth trials

Newly ecdysed fifth instars subjected to experiments that had not previously been into contact with the compounds, had moulted within less than four hours time difference and ranged from 100-120 mg (14 - 15 days ab ovo, P. brassicae) or 40-45 mg (13 - 14 days ab ovo, P. rapae) fresh body weights. They were placed individually in plastic boxes (200 ml) and given ad libitum access to the diet. Gravimetric estimation of food consumption and calculation of absorption efficiency employed a measure of respiratory

expenditure. The rationale of this procedure and the effects of phenolics are described in detail elsewhere (van Loon, submitted/Chapter 4; Simpson, 1982). The fifth instar trials lasted 96 h in both species. Caterpillars in control groups reached the prepupal stage in all cases within this period. When caterpillars were still feeding, they were deprived of food during a 4 h period in order to let the major part of gut-emptying proceed prior to sacrifice and determination of body dry weight.

RESULTS

Dose-effect bioassays

Survival

Continuous exposure to sinapic and caffeic acids resulted into a minor reduction in survival rate with increasing dietary concentrations in P. brassicae, a trend which was absent in P. rapae (fig. 1 and 2). Chlorogenic acid severely reduced survival in P. brassicae, while the reduction was smaller in P. rapae. Similarly, survival of P. rapae stabilized at 85% of the control value in response to oenin in the range 1 - 6.25 mM while survival of P. brassicae falls to c. 20% at the two highest concentrations tested. At the two lower concentrations, rutin caused the most pronounced decrease in survival in both species and quercetin in P. brassicae was similar in this potency. Mortality induced by rutin occurred predominantly during the first larval ecdysis.

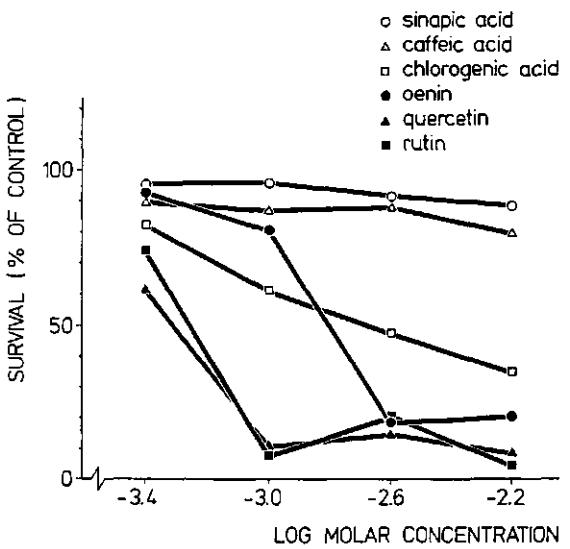

Figure 1:
Semi-logarithmic plot of the survival of P. brassicae larvae 8-9 days after egg hatch relative to survival in control groups (no phenolics added) as a function of the dietary level of several phenolics or flavonoids.

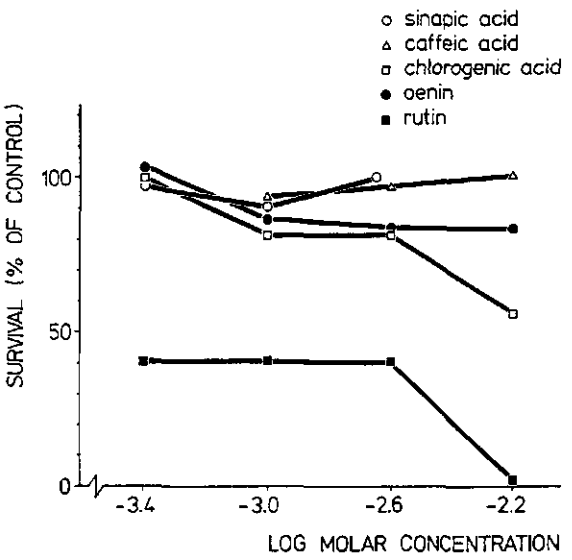

Figure 2:
Same as fig. 1, P. rapae.

Growth during the earlier instars

The presence of sinapic acid in the diet of P. brassicae resulted into a small increase in weight gain after 9 days of larval development, except for the 6.25 mM level. All other compounds, except for oenin in P. rapae, seriously inhibited growth and the ranking order of their efficacy in this respect differed little from that found for the deleterious effects on survival (fig. 3 and 4). As with survival, both species clearly differ in their sensitivity to oenin. In both species, rutin exerts the strongest inhibitory effects on growth of the first two instars. Inhibition of growth seems a more sensitive parameter to assess effects of the compounds than survival, i.e. caterpillars may survive but still suffer considerable growth inhibition.

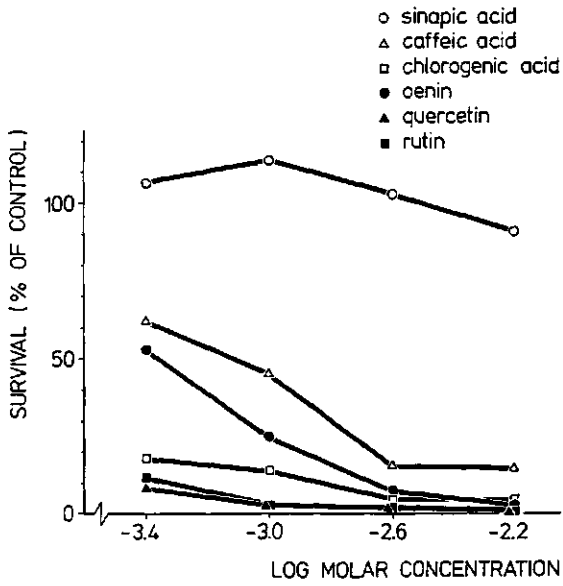

Figure 3:
Semi-logarithmic plot of the weight gain of individual P. brassicae larvae 8-9 days after egg hatch relative to the weight gain of larvae in control groups as a function of the dietary level of different phenolics or flavonoids.

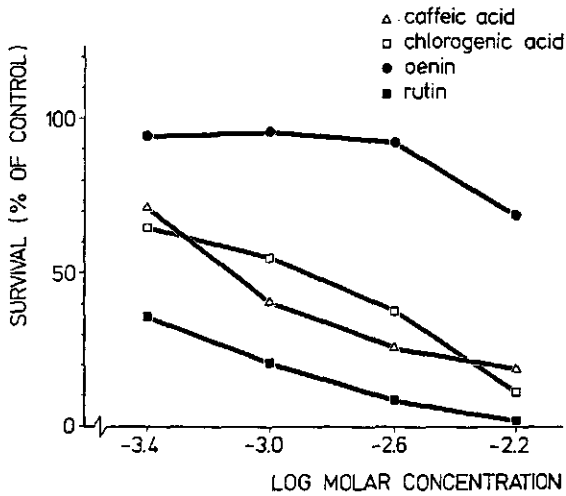

Figure 4:
Same as fig. 3, P. rapae.

Development to late fourth and young fifth instar

Caffeic and chlorogenic acid as well as rutin caused a retardation in developmental speed at the 0.4 and 1.0 mM levels. Oenin exerted such an effect only in P. brassicae. This appears from fig. 5 and 6, in which the contribution of penultimate and final instars to the larval population surviving to a particular advanced stage has been depicted. A reduced proportion of final instars in several cases coincides with an increased proportion of penultimate instars.

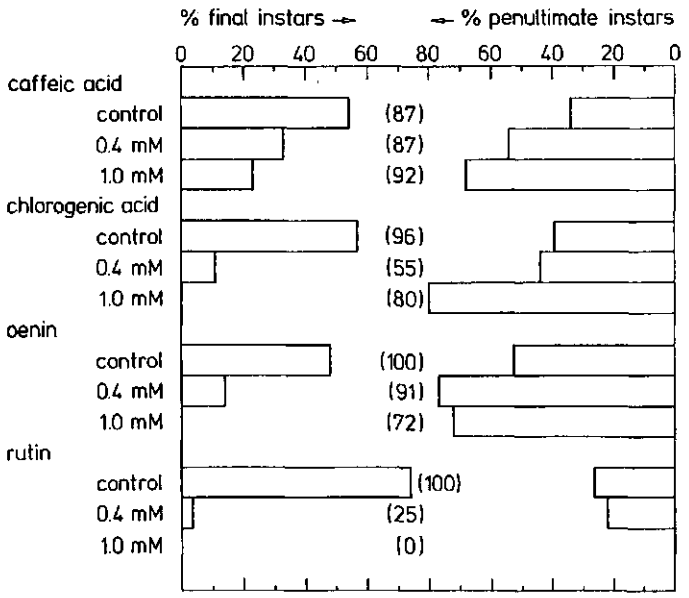

Figure 5:
Frequency of occurrence of penultimate and final instars relative to the total number of surviving caterpillars in control groups and groups exposed to two dietary levels of phenolics or flavonoids in P. brassicae 11-13 days after egg hatch. Figures between brackets are the summed percentages of final and penultimate instars.

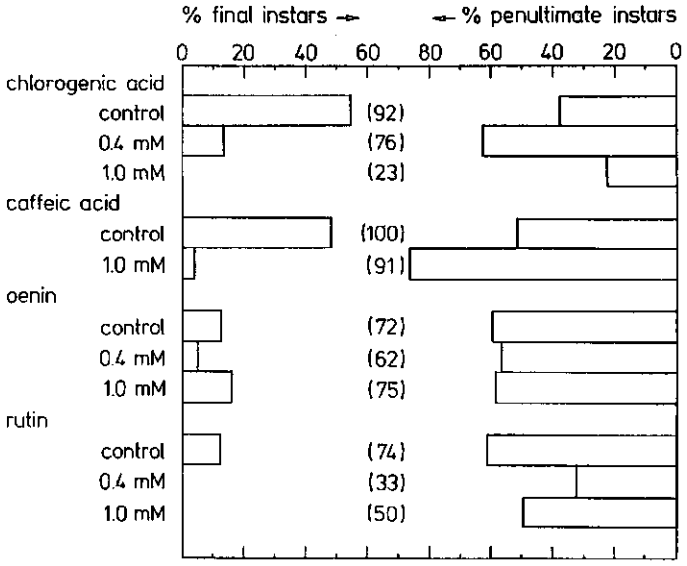

Figure 6:
Same as fig. 5, P. rapae 10-11 days after egg hatch.

Pupal weights

Chlorogenic acid affected pupal weight negatively, and desynchronized and postponed the moment of pupation at the 0.4 mM level in P. brassicae (table 1). Oenin had a comparable effect at the 1 mM level. Sinapic acid did not influence the latter parameters in P. brassicae. In P. rapae sinapic acid led to an increase in pupal weight at 1.0 mM (table 2). Pupal weights in the latter species were not affected by chlorogenic acid at any of the four levels tested, while effects with oenin and rutin occurred only at levels higher than those sufficient to cause reduction in P. brassicae. Retardation of pupation also occurred in P. rapae as a result of exposure to the respective compounds but was less pronounced. Pupal weights in the treated groups (table 2) were obtained from caterpillars which showed a developmental speed considerably greater than the average of the treated group and pupated in most cases within two days after pupation in the control group had occurred.

Table 1. Pupal weights of P. brassicae (mg fresh weight) and duration of larval development from egg hatch to pupation (days) as a result of development ab ovo on diets containing phenolic acids or flavonoids at the level (mM) indicated

compound	conc	pupal weight	n	duration
sinapic acid	control	343 ± 28 a	9	19
	0.4	348 ± 22 a	9	19
	1.0	347 ± 36 a	11	19
	2.5	349 ± 19 a	9	19
	6.25	359 ± 23 a	9	19
chlorogenic acid	c	372 ± 21 a	6	16
	0.4	336 ± 23 b	8	19
	1.0	310 ± 18 b	11	21-23
	2.5	237 ± 29 c	6	27-33
	6.25	213 ± 9 c	4	33-40
oenin	c	356 ± 21 a	10	17
	0.4	349 ± 32 a	20	17-21
	1.0	306 ± 17 b	9	21-22
	2.5	272 ± 53 b	4	25-35

Means ± SD. Weights refer to fresh weight recorded within 12 h after larval-pupal ecdysis. Means in a column referring to a particular compound differ significantly when followed by a different letter ($p < 0.01$, Student's t-test)

Table 2. P. rapae pupal weights (mg fresh weight) as a result of development on diets containing phenolic acids and flavonoids at the levels (mM) indicated

compound	conc	pupal weight	n
sinapic acid	control	148 ± 12 a	6
	0.4	163 ± 22 ab	5
	1.0	176 ± 21 b	5
	2.5	163 ± 33 ab	4
	6.25	163 ± 20 ab	8
chlorogenic acid	control	157 ± 29 a	6
	0.4	164 ± 40 a	5
	1.0	167 ± 6 a	3
	2.5	153 ± 13 a	5
	6.25	165 ± 25 a	5
oenin	control	152 ± 18 a	5
	0.4	160 ± 26 a*	5
	1.0	169 ± 5 a	5
	2.5	156 ± 17 a	5
	6.25	92 ± 25 c	5
rutin	control	173 ± 18 a	14
	0.4	190 ± 10 a	3
	1.0	133 ± 50 b	7

Means ± SD. Legends see table 1, except $p < 0.025$.

Food consumption, utilization and growth in the final instar

Caterpillars of P. brassicae in their final instar exposed without prior experience to chlorogenic acid or rutin showed reduced food consumption and growth at the 1.0 mM concentration (tables 3, 4 and 5). This reduction was more severe at both higher levels examined. With oenin such effects became apparent from the 2.5 mM concentration onward. The extent of feeding and growth inhibition was substantial. At the 2.5 mM level, ingestion was 45%

Table 3. Food consumption (C), body growth (G) and absorption efficiency (AE) for P. brassicae final instars feeding on diets containing increasing levels (mM) of chlorogenic acid. C and G in mg dry matter, AE in %

diet	conc	C	G	AE
control		305 ± 18 a	87 ± 5 a	45.6 ± 1.1 a
chlorogenic acid	1.0	253 ± 29 b	74 ± 9 b	46.7 ± 3.4 a
	2.5	138 ± 21 c	39 ± 9 c	44.5 ± 6.7 a
	6.25	138 ± 16 c	39 ± 5 c	44.8 ± 1.8 a

Means ± SD (n = 10). Means in a column followed by a different letter and with no letter in common are significantly different (Student's t-test, $p < 0.01$). Duration of experiment 96 h

Table 4. Food consumption (C), body growth (G) and absorption efficiency (AE) for P. brassicae final instars feeding on diets containing indicated levels (mM) of oenin. C and G in mg dry matter, AE %. Results of two experiments performed with a 9 months interval

diet	conc	C	G	AE
exp. 1				
control		336 ± 32 a	83 ± 8 a	39.5 ± 1.1 a
oenin	0.17	347 ± 17 a	84 ± 6 a	38.7 ± 1.8 a
	0.50	336 ± 14 a	86 ± 4 a	40.8 ± 1.7 a
	1.5	300 ± 20 b	77 ± 5 b	45.9 ± 4.1 b
exp. 2				
control		350 ± 34 a	80 ± 8 a	39.0 ± 1.1 a
oenin	1.0	298 ± 19 b	82 ± 6 a	45.6 ± 5.2 b
	2.5	252 ± 37 c	64 ± 18 b	44.6 ± 1.6 b
	6.25	158 ± 41 d	41 ± 12 c	45.4 ± 4.3 b

Means ± SD (n = 8, exp. 1; n = 9, exp. 2). Statistical details see table 3

Table 5. Food consumption (C), body growth (G) and absorption efficiency (AE) for P. brassicae final instars feeding on diets containing indicated levels (mM) of oenin. C and G mg dry weight, AE %

diet	conc	C	G	AE
control		399 ± 47 a	95 ± 13 a	39.8 ± 5.9 a
rutin	0.4	350 ± 59 a	95 ± 14 a	46.3 ± 1.8 b
	1.0	216 ± 74 b	59 ± 22 b	46.6 ± 2.3 b
	2.5	115 ± 29 c	27 ± 9 c	38.9 ± 5.3 a
	6.25	108 ± 17 c	28 ± 6 c	43.8 ± 2.6 ab

Means ± SD (n = 9). Statistical details see table 3

(chlorogenic acid), 72% (oenin) and 29% (rutin) of the value found for the control groups, while growth was reduced to 45% (chlorogenic acid), 80% (oenin) and 28% (rutin) of control values. In contrast, absorption efficiencies were equal to or higher than those in the control groups. Thus, reduced ingestion was in some instances in part compensated for by increased absorption efficiency. The most important cause of growth reduction, however, turned out to be reduced food consumption.

The sensitivity of P. rapae to chlorogenic acid was markedly different from that observed in P. brassicae. Food consumption nor growth were reduced in treated groups. At 6.25 mM absorption efficiency was increased, coinciding with a somewhat lower ingestion rate (table 6). With rutin on the other hand, ingestion and growth were reduced, but to a lower extent than in P. brassicae. At 2.5 mM ingestion was 71% and growth 74% of control values. Absorption efficiency was not significantly affected (table 7). With regard to oenin, it was found that up to a concentration of 6.25 mM no significant effect was found on any of the parameters measured.

When caterpillars were confronted with chlorogenic acid or rutin at 1 mM for the first time just after the final larval ecdysis, this produced a minor growth inhibition after 96 h of feeding as compared to first instars exposed immediately after hatching and of which the average individual weight gain was recorded after 8-9 days (fig. 7). Caterpillars that succeeded to pupate, be it with retardation, after continuous exposure to both compounds showed a degree of growth inhibition that resembled more that of the group treated only during the final instar.

Table 6. Food consumption (C), body growth (G) and absorption efficiency (AE) for P. rapae final instars feeding on diets containing increasing levels (mM) of chlorogenic acid. C and G mg dry matter, AE in %

diet	conc	C	G	AE
control		119 ± 21 ab	39 ± 7 ab	50.6 ± 2.0 a
chlorogenic acid	1	130 ± 18 b	44 ± 5 b	51.9 ± 2.6 ab
	2.5	120 ± 9 ab	40 ± 3 ab	51.4 ± 2.2 a
	6.25	109 ± 19 a	38 ± 6 a	54.5 ± 3.0 b

Means ± SD (n = 10). Statistical details see table 3

Table 7. Food consumption (C), body growth (G) and absorption efficiency (AE) for P. rapae final instars feeding on diets containing increasing levels of rutin. C and G mg dry matter, AE %

diet	conc	C	G	AE
control		126 ± 16 a	39 ± 5 a	48.0 ± 2.5 a
rutin	1	109 ± 15 ab	34 ± 5 ab	48.8 ± 2.4 a
	2.5	90 ± 21 bc	29 ± 8 bc	49.5 ± 2.9 a
	6.25	76 ± 20 c	25 ± 6 c	50.4 ± 2.0 a

Means ± SD (n = 10). Statistical details cf. table 3

FIG. 7

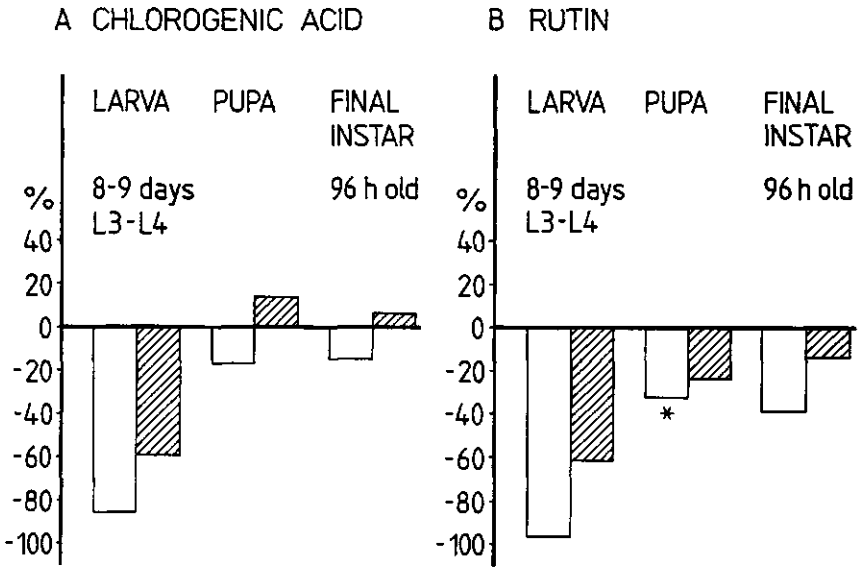

Figure 7:
Percentual differences in growth relative to control groups as a result of treatment with chlorogenic acid (A) or rutin (B) at 1.0 mM. Effects have been plotted comparatively for three developmental stages exposed either continuously during development (L3-L4 larva and pupa) or for the first time at the start of the final instar (final instar, 96 h old). Open bars P. brassicae, hatched bars P. rapae. * : at 0.4 mM.

DISCUSSION

The present results indicate that phenolic acids and flavonoids can exert inhibitory effects on several performance parameters of both Pieris caterpillars feeding on an artificial diet. Though dose - effect relationships were not uniform in nature, effects were more severe at higher concentrations. A clear-cut interspecific difference was found, P. rapae being much less sensitive to these compounds as judged by several parameters. Also, chronic exposure of first and subsequent instars results into considerably stronger inhibition of growth and development than exposure of final instars. Phenolic acids as well as flavonoids have been shown to influence behaviour, development, growth and reproduction in several phytophagous insect species, belonging to different taxonomic orders (reviews by Schoonhoven, 1972; Harborne, 1979; Hsiao, 1985; Kogan, 1986). Inhibitory as well as stimulatory effects have been found. At the behavioural level specific flavonoids may strongly stimulate food intake, as occurs in the silkworm, Bombyx mori (Hamamura et al., 1970). In chrysomelid beetles the response to different flavonoids can differ strikingly between related species (Matsuda, 1978; Nielsen, 1978; Larsen et al., 1982). Many cases are known in which flavonoids or phenolic acids constitute feeding deterrents or exert post-ingestional effects which remained mostly undefined. On these grounds they have consequently been implicated to play a role in plant resistance (Todd et al., 1971; Reese & Beck, 1976a, b, c; Adams & Bernays, 1978; Jones & Firn, 1979; Dreyer & Jones, 1981; Kogan, 1986). The bioassays employed to demonstrate inhibitory effects varied greatly. Artificial and natural substrates, often parallelling the respective absence and presence of adequate nutrition, and choice vs. no-choice situations have been used. Another crucial parameter clearly influencing results is the tested concentration of the compounds. Also, the degree of feeding specialization differs widely between the species investigated. In some insect species a certain amount of phenols in the diet is indispensable (Kato, 1978) or can be utilized as nutrients especially for cuticle synthesis (Bernays & Woodhead, 1982). These circumstances prohibit comparative and generalizing conclusions on the role of phenolic compounds as defensive compounds against phytophagous insects. Indeed, by now such generalizations may be considered irrelevant in view of the wide-spread

occurrence of phenolic acids and flavonoids in taxonomically unrelated plant families and the variable effects on polyphagous and oligophagous insects (Isman & Duffey, 1982).

The few examples of structure-activity studies with phenolic acids and flavonoids stress this point. They reveal that the structural characteristics of the molecules important for biological activity differ for different insect species, although ortho-dihydroxylation of the aromatic ring (the B-ring of the flavonoids) ranked among the most effective molecular characteristics in the three species investigated (Todd et al., 1971; Elliger et al., 1980; Dreyer & Jones, 1981). The present results show that methylation of the hydroxyl group at the 3-position, together with the presence of a methoxygroup at the 5-position (sinapic acid) leads to a lack of inhibitory activity. The quinic acid ester of caffeic acid (chlorogenic acid) is more effective than the free acid. Comparing the effects of quercetin and rutin in P. brassicae, it appears that the rutinose part is not essential for activity.

The types of biological parameters measured to quantify effects of phenolic compounds, e.g. growth reduction, in most cases fail to reveal which biological processes in the insect are affected. The mechanism or mechanisms of action of phenolic and flavonoid compounds have hardly been investigated and still remain obscure (Elliger et al., 1980; Isman & Duffey, 1982; Isman & Duffey, 1983). The polyphagous Heliothis zea reacts quite differently to the exposure to chlorogenic acid and rutin from both Pieris species. At levels substantially higher than investigated in the present study (14 - 28 mM) both compounds did not affect growth, food consumption or absorption efficiency when fed to fifth instars (Isman & Duffey, 1982). In the final instar, growth inhibition in Pieris can be explained by inhibition of food intake. Although it has been suggested that flavonoids or phenolic acids may decrease absorption efficiency by non-specific inactivation of digestive enzymes or by reducing the availability of dietary protein, such a mechanism is unlikely to play a role in Pieris as absorption efficiencies were in some cases higher following dietary exposure to the compounds. This increase in absorption efficiency may in turn simply be a consequence of a reduced rate of food intake which leads to an increased retention time of food in the gut lumen. An overall measure of food absorption efficiency as employed here is, however, unlikely to reflect the possible occurrence of decreased or blocked availability of

specific essential nutrients such as thiamine or lysine as discussed by Isman & Duffey (1982). An interaction with phenylalanine or tyrosine utilization is a conceivable mechanism that has not been investigated.

Inhibition of feeding is assumed to be caused by two main physiological mechanisms i.e. a direct influence of chemosensory neural activity on food intake behaviour or post-ingestional feed-back effects either nutritional, induced by toxicosis or as a result of food aversion learning. Post-ingestional feed-back may operate via neural, endocrine or combined pathways (Dethier, 1980 a, b; Schoonhoven, 1982; Bernays & Simpson, 1982). In an electrophysiological study (van Loon, submitted/chapter 3) it has been established that maxillary gustatory neurones in both Pieris species are sensitive to several phenolic acids and anthocyanins and this chemosensory activity was inferred to have behavioural consequences in artificial and natural contexts. The involvement of chemosensory activity as a mechanism contributing to the feeding inhibition in the final instars of both Pieris species is further supported by the present results. The chemosensory sensitivity pattern found for phenolic acids and anthocyanins matches the pattern of effectiveness on performance parameters in this study. The relative insensitivity of P. rapae on the chemosensory level is likewise reflected in these parameters. The effects of rutin and quercetin, however, cannot be explained in this way. These compounds did not evoke chemosensory activity per se. This observation, however, still leaves open the involvement of sensory mechanisms, as several possibilities for effects on the sensory level that are less overt than spiking activity are known (Dethier, 1980; 1982; Schoonhoven, 1982). Indeed, such a hidden sensory mode of action has been found for the anthocyanin cyanin, occurring in Brassica oleracea (van Loon, submitted/chapter 3).

Sensory effects have been examined in final instars. It is unknown if similar mechanisms operate in earlier instars, which suffer stronger inhibitory effects. It is conceivable that the mode of action of the phenolic compounds changes during development (Isman & Duffey, 1982). A possible approach to investigate the importance of sensory mechanisms in earlier instars could be the electrophysiological study of individuals that suffer a relatively mild growth inhibition during development. The foregoing reasoning predicts that such individuals display a relatively low chemosensory sensitivity to the compounds to which they have been exposed.

A more detailed knowledge of the modes of action of phenolic compounds

is desirable in view of the need for well-founded selection of both relevant and practical parameters of insect development to assess quantitative effects resulting from continuous exposure to these defensive phytochemicals. The action of these compounds on chemoreceptor cells as found in Pieris and the resulting effects on feeding is one of the few examples available from the literature to date and may thus well be specific for these species.

The present results were reached using an artificial diet. Such an approach allows manipulation and control of single chemical factors and their combinations to analyse their possible role as allelochemicals. In this connection it would be of interest to study mixtures of several compounds at lower levels than tested here as this reflects a more natural situation (Adams & Bernays, 1978). To what extent such studies yield results that are representative for the natural diet must be investigated separately. Relating to this aspect, it has been established that the utilization of aromatic amino acids, the main precursors in plant phenolic biosynthesis, were quantitatively comparable for both diets (van Loon, submitted/chapter 5). An important link between studies employing artificial and natural diets, i.e. host plants, is knowledge of the range of concentrations that insects encounter when feeding on the plant. Phytochemical data on levels of phenolics in Brassica oleracea leaves of different varieties indicate that the 0.4 mM concentration is easily reached for individual compounds (Wildanger & Herrmann, 1973; Herrmann, 1975). In this study this was the lowest level tested and yet was found to affect several larval performance parameters, especially so in P. brassicae. In conclusion, the present results point to a potential role of phenolic acids and flavonoids as substances that confer resistance of B. oleracea against Pieris caterpillars.

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CHAPTER 7

LARVAL PERFORMANCE OF TWO *PIERIS* SPECIES ON *BRASSICA OLERACEA* L. CULTIVARS AS RELATED TO THE FOLIAR LEVELS OF PHENOLIC ACIDS AND FLAVONOID COMPOUNDS

with T.A. van Beek

ABSTRACT

Survival, development and growth from egg to pupa and pupal weights were used as parameters to assess the performance of *Pieris brassicae* L. and *P. rapae* L. caterpillars on seven cultivars of a common host plant, *Brassica oleracea* L. Final instar performance was determined using growth, food consumption and absorption efficiency as indices. Four of the seven cultivars have been reported to possess resistance to *P. rapae*. The concentrations of the main aglycones of phenolic acids and flavonoids were determined in the leaves of five of the cultivars examined by high-performance liquid chromatography (HPLC).

In both caterpillar species, significant differences in performance parameters were found between the cultivars tested. Some differences in the ability to deal with alternative hosts were found between both species. On a particular cultivar, some performance parameters were negatively affected while others, determined in advanced developmental stages, were influenced positively. This was interpreted as a result of selection in the experimental group. These observations made the construction of an overall ranking order of host plant suitability arbitrary. Reported resistance could not in all cases be confirmed.

HPLC-determinations demonstrated that each of the cultivars had its own quantitative pattern of phenolic acids and flavonoids. The foliar levels found for some of the individual compounds have been found to reduce larval performance in an artificial context. Several unidentified components were apparent in the chromatograms. Based on UV-spectra and retention times,

these were most probably flavonoid aglycones with a more polar character than quercetin. The concentrations of these components differed considerably between cultivars.

It is suggested that phenolics and flavonoids in B. oleracea may be involved in defence against Pieris. Several factors that complicate interpretations and that are inherent to studies on suspected allelochemics in plants, are discussed.

INTRODUCTION

Caterpillars of Pieris brassicae L. and P. rapae L. (Lepidoptera, Pieridae) are notorious for their capacity to inflict damage upon crops of Brassica oleracea L. (Radcliffe & Chapman, 1966; Feltwell, 1982). Few studies, however, are known on the possible differences in host plant suitability between cultivated forms or varieties of B. oleracea to support larval survival and development. At present there seem to be no defined genetic sources of resistance available against both insect species. Resistance against P. rapae has been reported to exist in Savoy Chieftain, a savoy cabbage (Benepal & Hall, 1967; Chalfant & Brett, 1967) and in 'glossy', dark leaved derivatives of 'Blightproof' (PI 234599), a cauliflower from Australia characterized by a mutant structure of cuticular wax crystals (Macey & Barber, 1970; Dickson & Eckenrode, 1975; 1980). However, resistance was tested in the field by assessing damage which was caused by more species than by P. rapae only.

In previous studies it was found that phenolic acids and flavonoids incorporated into artificial diets inhibit larval growth and development of both species and evoke sensory reactions in sensilla crucial in regulating feeding behaviour (van Loon, submitted/chapters 3 & 6). The dietary concentrations at which these effects occur are in the range of reported levels of identical or related compounds naturally occurring in B. oleracea (Herrmann, 1976; 1977). Thus it seems interesting to investigate larval performance of both Pieris species on different cultivars of B. oleracea, including the 'glossy' lines, and to analyse leaf tissues of these host species to test the hypothesis that phenolic metabolism of B. oleracea has a function in the defence against Pieris caterpillars.

MATERIAL AND METHODS

Insects

Continuous stock cultures of P. brassicae and P. rapae were maintained in the laboratory under circumstances specified by David & Gardiner (1962) on Brassica oleracea L. variety gemmifera DC. cultivar Titurel. The cultures were three years old at the onset of the first experiments reported here (exp. 1, both species). Inbreeding in both species was in part alleviated by introduction of field collected individuals once a year. Eggs laid on cabbage leaves within 16 h time difference were obtained from these cultures and made up the starting material for experiments. Tests of larval performance were conducted in a climatic room at 25 ^{o}C, 60-80% RH and 16 h photoperiod. Illumination was provided by overhead fluorescent striplights, yielding an intensity of c. 5 W/m^2 at the level on which the larvae were feeding.

Plant material

Seven different cultivars or breeding lines of Brassica oleracea L. were investigated in two experiments, performed in two successive years. In both experiments, B. oleracea L. var. gemmifera DC. (Brussels sprouts) cultivar Titurel was used as a reference host on which many generations had been completed with both species without any signs of diseases or deficiencies. The six alternative hosts all belonged to the 'capitata' (L.) Alef. -taxon discerned within B. oleracea. In exp. 1, three alternative hosts were tested that belong to the var. alba DC. (white cabbage) ('cv' indicates cultivar, all commercial): cv Langendijker Vroege and the breeding lines G-8329 and G-10131 (G is an abbreviation of Geneva, see below). In exp. 2, the three alternative hosts investigated were: var. alba DC. cv Predena, var. sabauda L. cv Savoy Chieftain (a savoy cabbage) (abbreviated in the following as 'Chieftain') and var. rubra DC. cv Extase (a red cabbage). Seed material of the five commercial cultivars were kindly provided by Dutch seed growing companies and seeds of the G-breeding lines were a gift of Prof. M.H. Dickson, New York State Agricultural Experiment Station of

the Cornell University, Geneva, U.S.A.

In exp. 1, mature leaf material of Titurel was taken from 80-100 days old plants grown in a greenhouse at 20 °C (day) - 15 °C (night) under daylight and additional artificial light in a defined potting soil. Leaf material of the three host plants tested in exp. 1 was taken from 80-100 days old plants grown in an experimental field at the Institute for Horticultural Plant Breeding (IVT), Wageningen. It was used immediately after harvesting. Plant material of all four hosts in exp. 2 was grown in a greenhouse of the IVT under conditions similar as described above for Titurel. Leaf material of five of the above mentioned host plants analysed for their contents of phenolic acids and flavonoids (see table 6) were grown in the field at the IVT until 85-90 days of age. Mature leaves of intermediate age were harvested and extracted on the same day.

Testing of larval performance

Four parameters describing larval performance from egg to pupa were assessed. Survival and development were monitored every 48-60 h during the first 12 days after egg hatch in both experiments. In exp. 2 also the weight of the group was registered and divided by the number of caterpillars in the group to yield the mean individual weight at a particular moment. In order to achieve data reduction, results on survival, growth and development are given for one discrete moment only, chosen to be the time at which c. 50% had reached the middle or end of the penultimate instar. Pupal weight, taken within 12 h after pupation, constituted the fourth parameter.

In exp. 1, groups of 25 neonate larvae were confined in glass petri dishes (diameter 12 cm) on excised portions of leaf material of the respective cultivars. Freshly excised material was offered every 48 h, when leaf remains and frass were removed. There were four replicate groups. In exp. 2, groups of 10 larvae were placed on intact excised leaves, the petiole of which was inserted into a flask of tap water. Such an individual leaf with a group of larvae on it was confined in a cylindric clear plastic container (diameter 20 cm, height 25 cm) that was provided with a ventilation opening covered with nylon gauze. Freshly excised leaves were offered every 48-60 h. There were five replicate groups for each cultivar.

Growth and faeces production of final instars were measured gravimetrically. Food consumption was estimated as the sum of growth, faeces production and an assumption on respiratory expenditure proportional to the amount of growth. These procedures and the calculation of absorption efficiency are described in detail elsewhere (van Loon, chapter 4; cf. also Simpson, 1982). Newly ecdysed fifth instars were used that had moulted within less than 8 h time difference and ranged from 100-120 mg fresh body weights in P. brassicae (14-15 days after egg hatch) or 40-45 mg in P. rapae (13-14 days after egg hatch). Larvae of both species were confined individually on the leaf material under test in the same circumstances as already described for the performance trials of both experiments. Duration of the different final instar trials is indicated in the results section. When caterpillars had not yet entered the prepupal stage, they were deprived of food during a 4 h period allowing a substantial amount of gut-emptying to occur prior to sacrifice by rapid freezing and subsequent determination of body dry weight.

Phytochemistry

Solvents.

All solvents used were either distilled prior to use or were of analytical (pro analysi) quality. For HPLC HPLC-quality solvents were used.

Extraction.

All plantmaterials were extracted using three different methods:

Method 1 (acid hydrolysis only, for determination of flavonoid and furan aglycones):
140.0 g leaves were cut into small pieces and thrown piecemeal in 300 ml of boiling distilled water. After approx. 10-15 min the solution was cooled and chopped for 5 min in a Waring blender. The solution was boiled for another 5 min and filtrated over a Büchner funnel while still hot. The

plantmaterial was extracted two times more with 2x 200 ml of distilled water in the same manner. The filtrates were combined and brought to a strength of 2N HCl with concentrated HCl. The solution was placed in a boiling waterbath for 30 min with occasional shaking and with a reflux condenser placed on top of the flask. After cooling the solution was extracted three times with respectively 300, 200 and 100 ml of EtOAc. The EtOAc fractions were pooled, dried over anhydrous Na_2SO_4 and evaporated in vacuo.

Method 2 (combined acid/basic hydrolysis for determination of hydroxycinnamic acid aglycones):
See method 1 until the aqueous extracts have been combined. The pH of the combined extracts was brought to 7-8 and 2 g $NaBH_4$ was added in small portions under stirring. Then approx. 8 g of solid $Ba(OH)_2$ was added and the solution was boiled for 15 min under stirring. After cooling the solution was neutralised with 10% H_2SO_4. Next ca 8 ml of concentrated H_2SO_4 was added and the solution was boiled once again for 15 min. The hot solution was filtrated over a Büchner funnel. The precipitate was collected and extracted again with 100 ml of distilled H_2O, filtrated and added to the first filtrate. After cooling it was extracted 3x with 3x 250 ml EtOAc. The EtOAc fractions were collected, dried and evaporated in vacuo.

Method 3 (glycosides):
140.0 g of plantmaterial was cut into small pieces and extracted with 600 ml MeOH for 5 min in a Waring blender. The suspension was filtrated over a Büchner funnel. The plantmaterial was extracted another time with 100 ml MeOH of 50 ^{o}C in the blender, filtrated and combined with the first extract. The solution was evaporated. When not used all extracts were stored at -20 ^{o}C in the dark.

Fractionation.

75.0% of each extract (method 1, 2 or 3) was suspended in 50 ml H_2O -MeOH-36% HCl = 979-20-1 by means of ultrasonic sound waves and heavy mechanical agitation. This suspension was led over a small column of ca 10 g C18 material for flashchromatography (Baker 7025-0). Subsequently it was washed with the same volume of this mixture as needed to prepare the column

(discard). Next the column was sucked dry by means of vacuum and then washed with 50 ml of hexane-EtOAc = 9-1 (discard), 50 ml EtOAc-hexane = 7-3 (fraction 1), 50 ml H_2O-MeOH-36% HCl = 500-500-1 (fraction 2) and finally 50 ml MeOH-36% HCl = 999-1 (fraction 3). Fraction 2 and 3 were later combined. Fraction 1, 2 and 3 of extraction method 1 have been analysed for the presence of flavonoids by means of TLC and HPLC. Fraction 1 of extraction method 2 has been analysed for the presence of hydroxycinnamic acids by means of TLC and HPLC.

Thin layer chromatography.

For the separation of the compounds two different types of plates were used:
T1: silica gel, 0.2 or 0.25 mm thickness (Merck Kieselgel 60F254)
T2: RP18, 0.25 mm thickness (Merck RP18 F254s, cat.nr. 15685). In combination with T1 plates the following solvents were used:
S1: EtOAc-HCO_2H-HOAc-H_2O=100-11-11-26
S2: toluene-tBuMeO-HOAc=30-30-1
In combination with T2 plates the following solvent was used:
S3: MeOH-H_2O-HOAc=35-65-5
After development in a saturated chamber, drying and viewing under UV 254 and 366 nm the plates were sprayed with one of the following reagents:
D1: 1% g/v diphenylboric acid β-ethylaminoester in MeOH followed by viewing under UV 366 nm
D2: 0,5% g/v anisaldehyde in HOAc-MeOH-H_2SO_4=10-85-5 followed by heating with hot air
D3: 1.73 g $CuSO_4.5H_2O$, 17.3 g Na-citrate and 10 g Na_2CO_3 in 100 ml H_2O followed by viewing under UV 366 nm.

Column chromatography.

This was carried out with a Merck Lobar column filled with Lichroprep 40-63 µm. The solvent, toluene-tBuMeO-HOAc = 50-5-1 was pumped through the column with a low pressure pump. The purity of the collected fractions was checked

with TLC. Pure fractions were pooled and evaporated in vacuo. Apart from several hydroxycinnamic acids two other compounds were isolated and identified as 2-furoic acid and 5-hydroxymethyl-furfural.

High-performance liquid chromatography (HPLC)

The HPLC analysis was carried out with a Gilson gradient analytical system equipped with a Rheodyne 7125 injection valve (5 or 20 µl sample loop), a Gilson 116 variable wavelength UV detector and a Shimadzu CR 3A integrator. The column used for the analysis of the hydroxycinnamic acids and 2-furoic acid was a 20 cm x 4.6 mm stainless steel column packed with Spherisorb S10 ODS 2 material. The column used for the analysis of the flavonoids was a Rainin Dynamax column (10 cm x 4.6 mm) filled with Microsorb C18 3 µm material. Before the main column a 1.5 cm x 4.6 mm guard column filled with the same material was installed.
The following gradient was used for the analysis of the hydroxycinnamic acids and 2-furoic acid:
A: MeOH-H_2O-HOAc = 15-84-1
B: MeOH-HOAc = 99-1
0-5 min: 0% B, 5-15 min: 0-17.9% B, 15-25 min: 17.9% B, 25-30 min 17.9-22.18% B, 30-32 min: 22.18-0% B. Flow 1.0 ml/min. Detection wavelength: 254 nm for 2-furoic acid, 325 nm for hydroxycinnamic acids.
The following gradient was used for the analysis of quercetin and kaempferol:
A: H_2O-HCO_2H = 95-5
B: THF = 100
0-10 min: 25-37.5% B, 10-17.5 min: 37.5% B, 17.5-18.5 min: 37.5-25% B. Flow 0.8 ml/min. Detection wavelength: 370 nm.

Spectroscopic apparatus.

UV spectra were recorded in MeOH. ^{1}H-NMR spectra were recorded either at 90 MHz on a Varian EM 390 or at 300 MHz on a Bruker CXP 300. EI-MS (70 eV) and FD-MS were recorded on a MS902 equipped with a VG ZAB console.

Spectral data 2-furoic acid

UV: max 244 nm.
^{1}H-NMR ($CDCl_3$): 6.55 (dd, J =1.5 and 3.5 Hz), 1H), 7.30 (d, J=3.5 Hz, 1H), 7.63 (d, J=1.5 Hz, 1H), 9.33 (bs, 1H).
MS m/z (rel. int.): 112 (M^+, 100), 101 (9), 95 (55), 57 (7), 55 (5), 39 (16), 38 (5).
TLC: Rf = 0.25 in S2, identical with a reference (Merck). Colour with D2: light blue.

Spectral data 5-hydroxymethyl-furfural.

UV: max 280 nm, shoulder 215 nm
^{1}H-NMR ($CDCl_3$-CD_3OD): 4.67 (s, 2H), 6.51 (d, J=3.5 Hz, 1H), 7.20 (d, J=3.5 Hz, 1H), 9.56 (s, 1H).
MS m/z (rel. int.): 126 (M^+, 84), 99 (6), 97 (100), 95 (14), 69 (29), 60 (16), 45 (17), 43 (21), 41 (36).
TCL = Rf = 0.20 in S2, identical with a reference (Aldrich). Colour with D2: green-blue.

The other compounds were identified by their UV spectra, their HPLC retention times, their Rf-values and their specific colours with spray reagents D1, D2 and D3.

Modifications in the quantitative analysis of the content of phenolic acids and flavonoids in B. oleracea.

After method 2 extraction, designed to determine hydroxycinnamic acids (Schmidtlein & Herrmann, 1975; see methods for details), a purification step was necessary prior to the final HPLC-analysis. This was carried out by means of a RP18 column. Three steps were employed, viz. adsorbing and subsequent washing with water-methanol-36% hydrochloric acid (979:20:1), a washing with hexane-ethyl acetate (9:1) followed by selective elution

with an ethyl acetate-hexane mixture (7:3). The result was a highly purified mixture of phenolic acids which could readily be analysed by HPLC (fig. 1). The flavonols quercetin and kaempferol were determined by means of HPLC after acidic hydrolysis (method 1). The same purification procedure as applied for the hydroxycinnamic acid fraction was used. Determination of flavonoids without any sample pretreatment was not possible due to the presence of many interfering compounds. As flavonoids are somewhat more polar than hydroxycinnamic acids, part of the flavonoids remained on the column after the elution with ethyl acetate-hexane (7:3) and were then eluted using 50% methanol in water. Both fractions were investigated by HPLC and the total content of the flavonoids was found by summation of the two values. The tailing (asymmetry factor 5 to 6) which occurred when gradients of methanol or acetonitril and water with 1-5% acetic or formic acid were used (Wulf & Nagel, 1976; Vanhaelen & Vanhaelen-Fastre, 1980; Vande Casteele et al., 1982) was found to disappear when tetrahydrofuran was used as elution solvent. This significantly improved both the separation of kaempferol and isorhamnetin, which eluted in between quercetin and kaempferol, and the separation of other, non-identified compounds (fig. 2).

RESULTS

Performance on seven B. oleracea cultivars from egg to pupa

Survival of P. brassicae as examined 8 days since egg hatch and transfer to the cultivars tested was only slightly lower on the G-breeding lines while both survival and growth during earlier instars were significantly lower on Chieftain and Extase (table 1). The G-breeding lines caused a minor retardation of development but pupal weights after completion of larval development were considerably higher. In contrast, both developmental rate and pupal weights were significantly reduced after feeding on the three commercial varieties tested in exp. 2.

Table 1. Performance parameters of P. brassicae fed on seven different B. oleracea cultivars, measured at indicated moments since egg hatch

cultivar	survival % day 8	growth mg day 7	development % L4 day 8	pupal weight mg	n
Exp. 1					
Titurel	85[&]	-	98[&]	357±38 a	10
Langendijker	86	-	95	420±20 b	10
G-8329	74	-	89	418±19 b	10
G-10131	74	-	88	428±29 b	4
Exp. 2					
Titurel	86±11 a	26±3 a	93±9[†] a	374±17 a	11
Predena	78±25 a	25±7 a	45±21 b	313±22 b	11
Chieftain	40±12 b	9±4 b	5±10 c	281±26 c	8
Extase	25±17 b	16±9 b	53±36 b	-	10

Means ± SD. Means in a column followed by a different letter differ significantly (Student's t-test, $p < 0.05$) Weights refer to fresh weight. n: number of pupae. & : observations on replicates in exp. 1 pooled (n = 100). - : not recorded. † : % final instars at day 10 since egg hatch

P. rapae showed a higher mortality on G-10131 (table 2). Developmental speed was lower on both G-10131 and Langendijker. In contrast, G-10131 supported higher pupal weights, while pupal weights on Titurel, G-8329 and Langendijker were equal. The commercial varieties on the other hand all led to reduced growth after 9 days, retarded development and gave rise to lower pupal weights.

Table 2. Performance parameters of P. rapae fed on seven different B. oleracea cultivars, measured at indicated moments since egg hatch.

cultivar	survival % day 8	growth mg day 9	development % L4 day 7	pupal weight mg	n
Exp.1					
Titurel	84[&]	-	77[&]	144±15 a	10
Langendijker	80	-	31	141±22 a	7
G-8329	73	-	84	149±14 a	10
G-10131	65	-	48	166±16 b	10
Exp. 2					
Titurel	-[†]	73±25 a	90±9 a	177±13 a	11
Predena	-	31±6 b	17±21 c	163±19 b	10
Chieftain	-	33±2 b	63±16 b	152±11 b	9
Extase	-	36±9 b	25±10 c	165±12 b	10

Means ± SD. Details see table 1. † : survival in exp. 2 was not reliably recorded due to unintended effects of leaf replacement on the adherence of larvae to the leaf

Performance on seven B. oleracea cultivars of final instars

Food consumption was reduced and absorption efficiency increased by switching to alternative hosts in exp. 1 A (table 3). Growth was not affected significantly. When reared continuously on three alternative hosts, growth and absorption efficiency were enhanced relative to caterpillars reared on Titurel. For both G-varieties ingestion and growth were higher relative to the groups offered these hosts for the first time during the final instar, while absorption efficiency was not affected. The highest values for all three parameters were reached on G-8329.

Growth was similar when feeding on the three alternative hosts in exp. 2 (table 3). In contrast to exp. 1, food consumption was equal to or higher than on Titurel. Food intake was highest on Predena, which at the same time produced the lowest absorption efficiency.

Offering final instars of P. rapae Titurel instead of the host previously experienced, Langendijker, gave rise to an increased food ingestion and

Table 3. Food consumption (C), body growth (G) and food absorption efficiency (AE) of P. brassicae final instars tested on seven cultivars of B. oleracea after rearing from egg hatch to final larval ecdysis on the cultivar indicated

cultivar	C	G	AE	n
	mg	mg	%	
Exp. 1 A, reared on Titurel				
Titurel	386±26 a	55±5 a	24.0±2.0 a	6
Langendijker	244±37 b	60±11 a	41.7±2.2 b	5
G-8329	238±16 b	59±4 a	42.2±0.3 b	5
G-10131	220±63 b	49±18 a	36.4±6.5 b	5
Exp. 1 B, reared on the same cultivar as tested on				
Langendijker	265±36 b[&] ns[†]	65±9 b ns	41.8±1.6 c ns	9
G-8329	295±16 b s	76±4 c s	43.6±1.3 c ns	6
G-10131	282±16 b s	65±5 b s	39.2±1.8 b ns	7
Exp. 2, reared on Titurel				
Titurel	356±29 a	78±5 a	35.1±0.7 a	10
Predena	397±19 c	80±5 a	32.3±1.6 b	10
Chieftain	378±28 b	78±5 a	33.1±1.0 a	10
Extase	356±15 a	79±6 a	35.4±1.9 a	10

Means ± SD. Means in a column followed by a different letter are significantly different according to Student's t-test ($p < 0.05$). & : values in the row tested against the value for Titurel in exp. 1 A. † : tested for a significant difference with the value for the identical cultivar in exp. 1A (ns: $p > 0.05$; s: $p < 0.05$). Duration of experiments: 1 A and 1 B: 48 h, of exp. 2: 72 h

reduced absorption efficiency, while growth was not influenced. In contrast, food intake, growth and absorption efficiency all were reduced on G-10131 (exp. 1 A, table 4). Rearing P. rapae larvae continuously on G-8329 likewise resulted into reduced food consumption and growth as compared to continuous rearing on Langendijker, while absorption efficiency was equal.

Table 4. Food consumption (C), body growth (G) and food absorption efficiency (AE) of P. rapae final instars tested on seven cultivars of B. oleracea subsequent to rearing from egg hatch to final larval ecdysis on the cultivar indicated

cultivar	C	G	AE	n1	n2
	mg	mg	%		
Exp. 1 A, reared on Langendijker					
Langendijker	119±27 a	30±8 a	43.2±2.2 a	7	3
Titurel	181±46 b	28±7 a	26.1±2.1 b	4	-
G-10131	71±30 c	17±8 b	40.9±1.5 c	4	1
Exp 1 B, reared on the same cultivar as tested on					
G-8329	80±30 b[&]	21±8 b	44.3±1.6 a	7	3
G-10131	110±26 a s[†]	29±6 a s	44.5±2.2 a s	9	1
Exp. 2, reared on Titurel					
Titurel	108±27 a	25±7 a	36.4±5.3 a	10	-
Predena	104±46 a	20±10 a	27.6±9.7 b	9	1
Chieftain	103±34 a	21±6 a	33.3±6.7 ab	10	-
Extase	97±47 a	19±10 a	27.3±9.8 b	8	2

Means ± SD. Means in a column followed by a different letter and having no letter in common are significantly different (Student's t-test, $p < 0.05$). &: values in the row tested against the value for Langendijker in exp. 1A. †: tested for a significant difference against the value for the identical cultivar in exp. 1A (s: $p < 0.05$). n1: number of caterpillars on which tabulated values are based; n2: number of caterpillars in the experimental group that showed poor growth (< 10 mg DW), not considered in calculations. Duration of experiments: 1 A and 1 B: 72 h; exp. 2: 90 h

Rearing from egg to final larval ecdysis on G-10131 and testing final instar performance on this host led to values equal to those obtained after continuous rearing and testing on Langendijker while considerably higher values were reached than after a switch from Langendijker as the previous host.

Growth and food consumption were not significantly different between Titurel and the alternative hosts in exp. 2 (table 4), although growth tended to be reduced. Food absorption efficiencies all were lower and significantly so when feeding on Predena and Extase.

Comparison of performance of both species

A qualitative overview of the performance of both species on the seven B. oleracea cultivars examined as measured using seven parameters is presented in table 5. It is seen that, with few exceptions, performance on the host plants in exp. 1 is similar for both species, except for the relatively poor performance of P. rapae feeding on G-10131. Also for the hosts tested in exp. 2, the patterns for both species were similar, minor differences being that P. brassicae seems to utilize Savoy Chieftain less well than P. rapae, while the reverse occurs for Predena.

Contents of phenolic acids and flavonoids in five B. oleracea cultivars

Results of the HPLC-analyses of the concentrations of phenolic acids and flavonoids are presented in table 6. It is seen that each of the cultivars has its own quantitative pattern, both for phenolic acids (fig. 1) and flavonoids (fig. 2). Titurel contained high or intermediate levels of all compounds identified relative to the other four cultivars. Extase displayed by far the lowest levels of all four phenolic acids and at the same time had the highest levels of both flavonoids. Chieftain showed the highest concentration of *p*-coumaric acid. Predena had the highest levels of ferulic and sinapic acids. G-8329 was relatively low in kaempferol and quercetin. Isorhamnetin turned out to be present in minor quantities only. When either the amounts of the four phenolic acids or those of both flavonoids are summed and the sums compared between the five cultivars, a wide range emerges, covering an order of magnitude in some cases.

Table 5. Qualitative comparison of performance of P. brassicae (P.b.) and P. rapae (P.r.) on seven B. oleracea cultivars as measured by seven parameters

cultivar	egg to pupa on same host								final instars after host switch					
	survival		growth		development		pupal weight		consumption		growth		AE	
	P.b.	P.r.	P.b.	P.r.	P.b.	P.r.	P.b.	P.r.	P.b.	P.r.	P.b.	P.r.	P.b.	P.r.
Exp. 1														
Titurel	e	e	-	-	e	e	↓	e	h	h[&]	e	e	↓	↓↓
Langendijker	e	e	-	-	e	↓↓	e	e	↓	↓	e	e	e	h
G-8329	↓	↓	-	-	e	e	e	e	↓	-	e	-	e	h
G-10131	↓	↓↓	-	-	e	↓↓	e	↑	↓	↓↓	e	↓↓	e	-
Exp. 2														
Titurel	e	-	e	h	h	h	h	h	e	e	e	e	e	e
Predena	e	-	e	↓	↓	↓↓	↓	↓	↑	e	e	e	↓	↓
Chieftain	↓	-	↓	↓	↓↓	↓	↓↓	↓	↑	e	e	e	↓	e
Extase	↓	-	↓	↓	↓	↓↓	-	↓	e	e	e	e	e	↓

Legends: e: equal values (column-wise); h: highest value; ↓: decrease, ↓↓: strong decrease and ↑: increase relative to e or h, based on statistical tests where possible. - : not done. & : in exp. 1, prior to switch P. rapae was reared on Langendijker instead of Titurel. AE : absorption efficiency. For details on performance measures see tables 1-4.

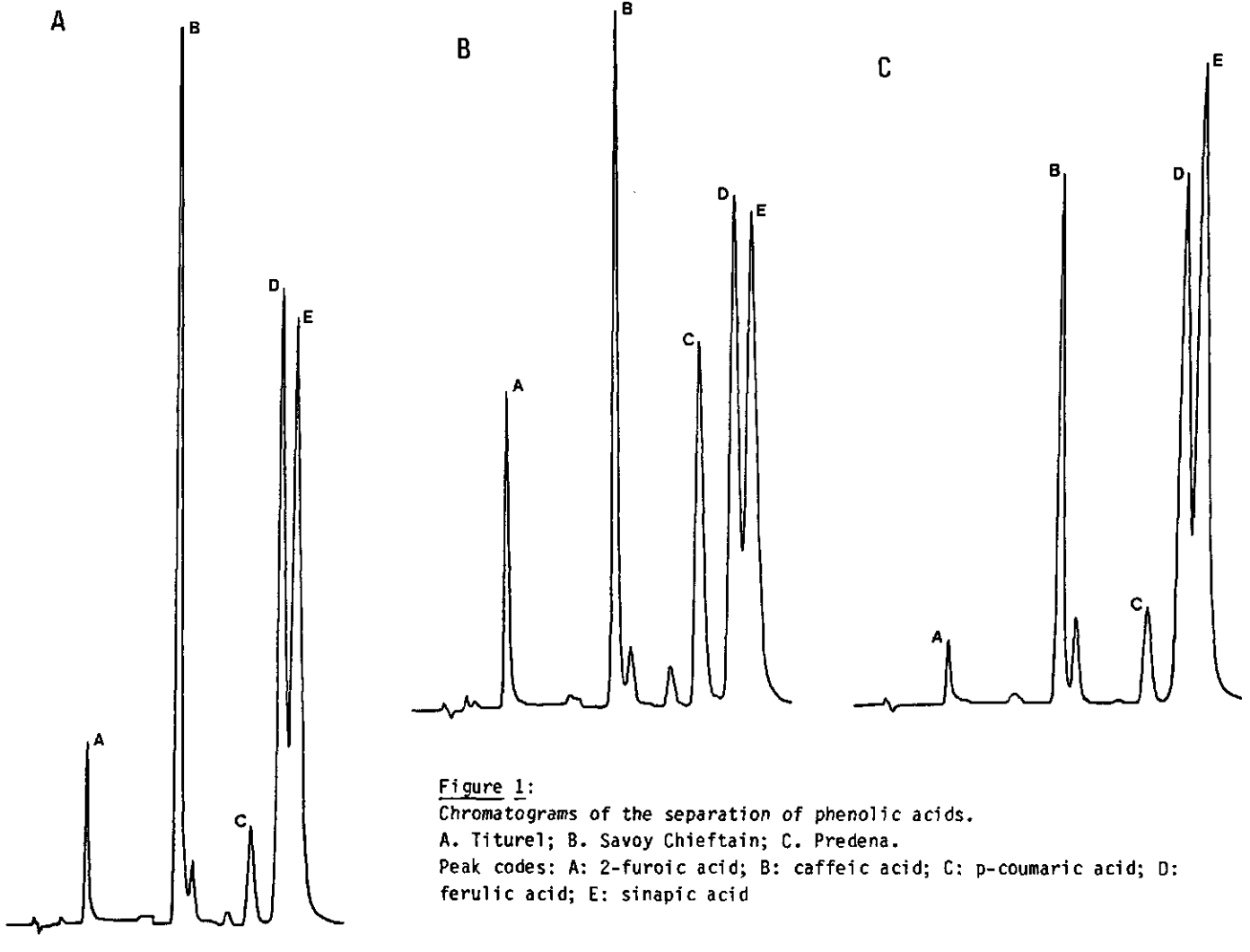


Figure 1:
Chromatograms of the separation of phenolic acids.
A. Titurel; B. Savoy Chieftain; C. Predena.
Peak codes: A: 2-furoic acid; B: caffeic acid; C: p-coumaric acid; D: ferulic acid; E: sinapic acid

Table 6. Contents of phenolic acids and flavonoids as determined by HPLC analyses of leaf extracts of five B. oleracea cultivars. Concentrations expressed as mM

cultivar	compound					
	p-coumaric acid	caffeic acid	ferulic acid	sinapic acid	quercetin	kaempferol
Titurel	0.08	0.57	0.55	0.55	0.19	0.13
G-8329	0.11	0.42	0.66	0.55	0.01	0.03
Predena	0.15	0.48	0.84	1.04	0.05	0.08
Chieftain	0.34	0.41	0.52	0.52	0.06	0.09
Extase	0.01	0.01	0.03	0.03	0.33	0.20

Results of HPLC-analyses were originally expressed as mg/kg (ppm) fresh weight and recalculated to mM by dividing this value by the molecular weight of the respective substances, assuming one kg fresh weight of leaf material has an approximate volume of one liter.

Apart from caffeic-, *p*-coumaric-, ferulic- and sinapic acids, only one other peak of importance could be distinguished in the HPLC chromatograms (method 2, fraction 1; UV detection either at 254, 300 or 325 nm). This compound was also present after application of acid hydrolysis (method 1) and could be isolated by means of column chromatography. It was identified by means of UV, MS, ^{1}H-NMR and TLC with a reference as 2-furoic acid. In another fraction of this separation 5-hydroxymethyl-furfural could be identified. This compound could not be observed in the chromatogram of the purified hydroxycinnamic acid fraction (method 2-hydrolysis) because its reduction product is probably not retained on RP18 material and causes no UV-absorption at 254 nm.

In the original plant material both hydroxycinnamic acids and flavonoids must be present as more polar esters or acetals as in methanolic extracts (method 3) of the various leaf samples no free hydroxycinnamic acids, furans or flavonoid aglycones could be detected.

Fig. 2 A

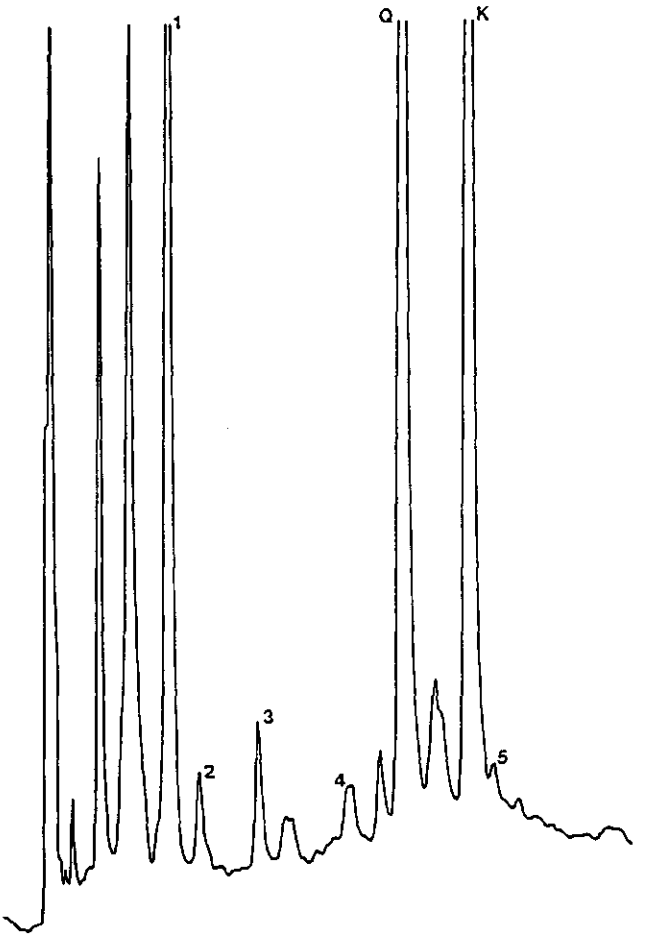


Figure 2:
Chromatograms of the separation of flavonoid aglycons.
A. Titurel; B. Savoy Chieftain; C. Predena
Peak codes: Q: quercetin; K: kaempferol; 1 - 5: unidentified flavonoid substances.

Fig. 2 B

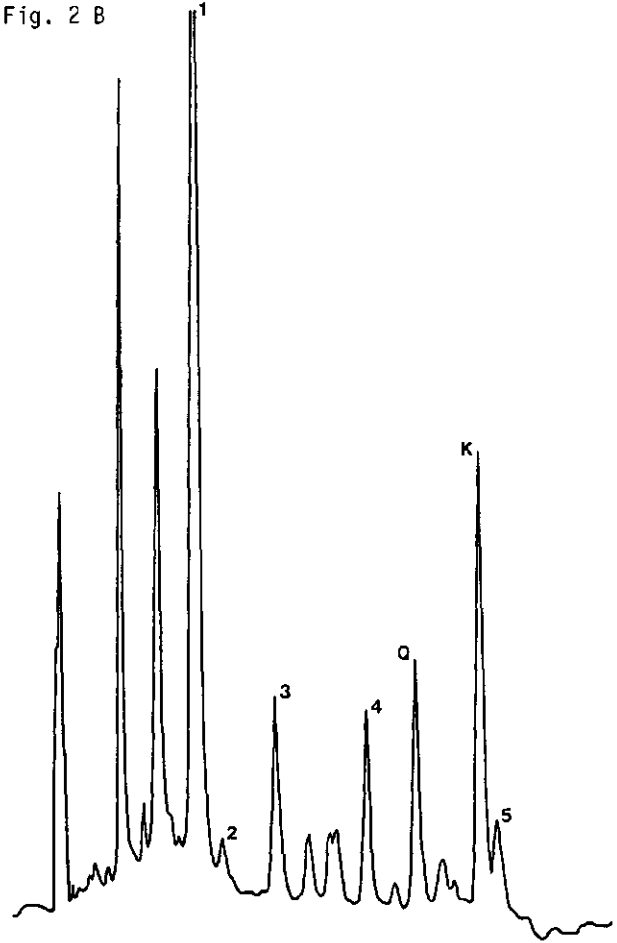
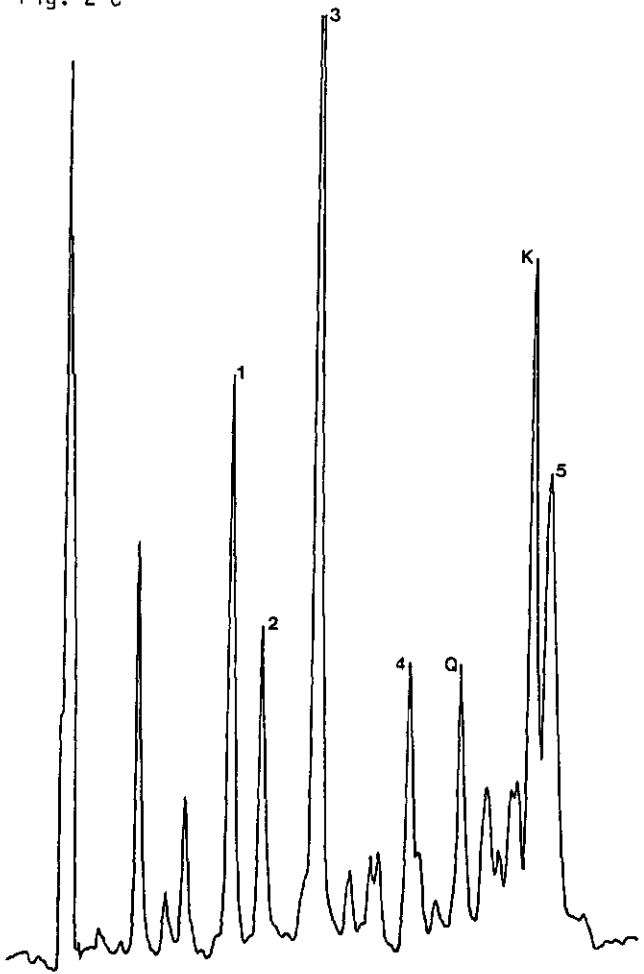


Fig. 2 C



A gradient system employing tetrahydrofuran was used (see methods) to improve the separation of several unknown compounds eluting before quercetin (fig. 2). From their characteristic UV spectra, which were recorded on line and highly resembled those of quercetin, they are most likely other more polar flavonoid aglycones. Their identity was not further pursued. It appeared, however, that the quantities of these compounds differed considerably between cultivars (fig. 2). HPLC-analysis of the crude extracts after hydrolysis (not shown) demonstrated that for several of these unidentified flavonoid substances contents could differ factor 10 between cultivars, when the integrated areas under peaks occurring at identical retention times were compared. G-8329 had only minor quantities while Extase had the highest levels, followed by Chieftain, Predena and Titurel.

DISCUSSION

The present results demonstrate that larval performance of both Pieris species differs on different B. oleracea cultivars and indicate that the two species differ in their capacity to deal with alternative hosts. Comparable studies on host plant suitability of cultivated B. oleracea to both Pieris species as assessed by larval performance are not known from the literature. Several field studies have been devoted to localizing sources of resistance to a complex of cabbage caterpillars, among which P. rapae (Pimentel, 1961; Radcliffe & Chapman, 1966; Chalfant & Brett, 1967), but results differed considerably probably due to variable environmental factors. Dickson & Eckenrode (1975) suggested that an antibiotic factor was responsible for low feeding damage by P. rapae to G-8329 in field tests. However, this suggestion has not been experimentally substantiated since then. To date, the only documented resistance in B. oleracea that appeared to be of an antibiotic nature was published by Lin et al. (1983) for Plutella xylostella L. Resistance of glossy lines, like G-8329 or G-10131, derived from the B. oleracea var. botrytis accession PI 234599 (Dickson & Eckenrode, 1975; 1980) have been tested only in field situations. Such results do not allow conclusions on the mechanism of resistance. An environmental factor seems important for the expression of resistance as in laboratory and greenhouse studies, the field results could not be repro-duced (Dickson & Eckenrode, 1975). In the present study, survival was somewhat reduced on both G-breeding lines using field grown leaf material. Pupal weights and final instar performance was not affected. More detailed studies on survival of first and second instars of both Pieris species and Mamestra brassicae L. on intact field grown plants showed no signs of antibiotic resistance in both G-lines in contrast to the results of Lin et al. (1983) with Plutella xylostella (van Loon, unpublished results). In field experiments, resistance as assessed by larval survival after artificial infestation, appeared present in one season but was absent the next season (de Ponti & Steenhuis, unpublished results).

It was surprising to find that larval performance was negatively influenced by all three alternative hosts in exp. 2. Poor performance on the anthocyanin accumulating red cabbages and Savoy Chieftain has been documented for P. rapae (Benepal & Hall, 1967; Dickson & Eckenrode, 1975). In Savoy

Chieftain, resistance was accompanied by extremely low amounts of free phenylalanine and tyrosine and a relatively high level of pipecolic acid (Benepal & Hall, 1967). This was interpreted by us as indications for the involvement of phenolic metabolism, in which phenylalanine and tyrosine are primary precursors. In this study, Savoy Chieftain was found to contain relatively high amounts of several phenolic acids and of both identified and unidentified flavonoids.

In a separate study (van Loon, submitted/chapter 6), the incorporation into a synthetic diet of caffeic acid and its quinic acid ester and of quercetin and quercetin-3-rutinoside at 0.4 mM and 1.0 mM levels resulted into negative effects on larval performance of both species, thus showing the potential of these compounds in the defence against Pieris species. From the present study it appears that the 0.4 mM and 1.0 mM concentrations applied in the artificial diet experiments correspond to natural levels in cultivated forms of B. oleracea (table 6).

Several complications hamper a comparison of the present results, obtained from insects fed plant material, with the results of experiments using artificial diets. In the latter type of experiments, only one compound was tested at increasing levels. The effects of the simultaneous presence of all four phenolic acids and both main flavonoid aglycones are unknown. There may be additive, synergistic or even mutually inhibitory interactions when they occur together. Another important consideration is that the phytochemical analyses as presented here reflect only total amounts of aglycones, while in the plant tissue phenolic acids and flavonoids predominantly occur as glycosides, esters or other bound forms (Harborne, 1979; Brandl & Herrmann, 1983; Winter & Herrmann, 1986). Although both aglycones and glycosides were effective in the diet studies, it is conceivable that the naturally occurring glycosidic form is relevant for its inhibitory biological activity. This problem of structure - activity relationships is difficult to investigate as many different glycosidic forms of flavonoids are known to occur simultaneously in one plant species (Harborne, 1979). Furthermore, a quantitatively important group of flavonoids within B. oleracea, viz. anthocyanins was not examined in the present study. At least eight different glycosides of the main anthocyanidin in B. oleracae, cyanidin, have been detected in a cultivar of red cabbage (Hrazdina et al., 1977).

The possible involvement of still other variables requires due carefullness in drawing conclusions on the significance of phenolic substances in

influencing larval performance on host plants. One such variable is the unknown degree of inbreeding and selection that has occurred in the laboratory colony from which the experimental insects were obtained. In between introductions of individuals caught from the wild, a certain degree of selection on vigorous performance on the host used to maintain the colony (cv. Titurel) will have occurred. This cultivar contains comparatively high levels of the phenolics analysed. However, trials with insects from a laboratory colony imported from Geneva, N.Y. (a gift from Dr. A.M. Shelton, Geneva Experimental Station) produced very similar results on the hosts tested in exp. 1 (van Loon, unpublished data). Other variables that may be of influence are rearing circumstances and physiological age of the host plants. B. oleracea plants grown in a greenhouse are known to contain lower amounts of phenolic acids and flavonoids than field grown plants and mature leaves contain higher amounts than younger ones (Herrmann, 1976). Levels of phenolic compounds have also been found to increase in some cases as a reaction to insect feeding damage (Ryan, 1983). It is unknown if this occurs in B. oleracea too. Interesting in this respect is the observation that an important amount of the phenolic acid pool in the cultivars is present as sinapic acid. This compound causes no inhibition. A conceivable mechanism, that can be induced, would be enzymatic change of methoxy-groups to hydroxy-functions which will result into higher biological activity as predicted on the basis of the present results. Generally, concentrations found in the present study are within the range of published values for varieties and cultivars of B. oleracea, which range in itself is quite wide (Wildanger & Herrmann, 1973; Schmidtlein & Herrmann, 1975; Hanefeld & Herrmann, 1976; Herrmann, 1976; 1977; Brandl & Herrmann, 1983).

It is tempting to speculate on the possible role of phenolic acids or flavonoids in determining host plant suitability of B. oleracea to Pieris caterpillars. On the basis of the present results, however, no definite conclusions can be drawn. In the foregoing, considerations have been discussed that complicate the matching of the pattern of differential larval performance on the one hand and the quantitative pattern of phenolic acids and flavonoids on the other. Bioassays of purified fractions, enriched in the latter compounds, and incorporation into artificial diets would be useful to further substantiate their suggested defensive significance that may contribute to resistance of B. oleracea cultivars to Pieris caterpillars.

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CHAPTER 8

GENERAL DISCUSSION

In the present study, attention was focussed on sensory and nutritional aspects of the relationships between two species of Pieris caterpillars and a host plant, Brassica oleracea. The specific role that glucosinolates play in this relationship has been well investigated and it can be concluded that they do not constitute a defensive barrier against exploitation by Pieris, while they are clearly toxic to other insect species. In contrast, the possible allelochemic significance of phenolic acids and flavonoids occurring in Brassica and other cruciferous plants has hardly been examined, while the red cabbages clearly indicate the quantitative potential of flavonoid synthesis in Brassica. One reason why glucosinolates have received much attention is the fact that their toxicity to non-adapted insect species is evident and seems an almost qualitative barrier against exploitation. The study of quantitative effects of phytochemicals, relevant in the case of phenolic acids and flavonoids, became possible only after identified and purified compounds became readily available. Phenylpropanoid-based defence is considered interesting for several reasons. Firstly, the synthesis of phenolics and flavonoids in plants is closely linked to the metabolism of phenylalanine (Camm & Towers, 1973; Hanson & Havir, 1979). Only recently evidence was found that this aromatic amino acid is a limiting nutrient for insects due to its essential function in cross-linking of the cuticle (Bernays & Woodhead, 1984; Andersen, 1985). Secondly, the toxicity of phenolic compounds to humans and cattle seems much lower than that of glucosinolates (Singleton & Kratzer, 1973; VanEtten & Tookey, 1979), which is a requirement when they are to be considered relevant resistance factors that can be selected for in plant breeding programmes.

Sensory data described in the present study suggested that most of the essential amino acids stimulate an amino acid receptor cell in both species. Nutritional analyses revealed a relationship between the absorption efficiency of essential amino acids and longer-term food intake. Of the non-essential amino acids only tyrosine showed such a relationship in both species. At the sensory level, differences in amino acid sensitivity between both species were apparent, while nutritional utilization of amino

acids was remarkably similar.

After it had been established that the basic nutritional requirements of insects resembled those of mammals to a high extent (Dadd, 1985), physiological research into insect nutrition has not been extensive (Slansky, 1982). Waldbauer (1968) made an important contribution by an attempt to standardize measuring methods and parameters of insect quantitative nutrition. Since then, over 500 papers have appeared that studied food utilization against the background of nutritional ecology (Scriber & Slansky, 1981). However, in the present study the use of gravimetric techniques to measure food intake, based on a decrease in food weight, was found to be methodologically inadequate (Chapter 4). The high degree of variation in metabolic efficiency as a result of dietary conditions that is found in many papers in this field are most probably be due to measuring errors rather than reflecting a physiological reality. The continuous respirometry that yielded this insight may be considered a fundamental improvement in the methodology of constructing insect matter and energy budgets, and consequently to the field of quantitative insect nutrition. The balance sheet approach employed in the subsequent chapters rely on the values for the metabolic efficiency that were obtained in the respirometric experiments. This practice is subject to criticism, as diets and other experimental conditions were not identical. However, the maximum degree of variation in energetic efficiency of growth found by using respirometry (coefficient of variation c. 20%) was calculated to affect the estimation of food intake to a minor extent. To my opinion, subtle differences in food consumption and utilization efficiencies can only then be reliably measured when respirometric or calorimetric techniques are used on insects that still can perform their normal behaviour in the experimental situation. Due attention should also be paid to the physiological condition of the food source, especially in the case of living leaf tissues that have hardly if ever been studied in a condition representative for the natural state, e.g. capable of photosynthesis.

A balance sheet based on the foregoing argument that was applied to quantitative amino acid nutrition revealed that, during feeding on diets of low protein content, in both species tyrosine is liable to become a limiting nutrient (Chapter 5). This resembles the situation in another insect, Schistocerca gregaria (Bernays & Woodhead, 1984). The suggestion that the requirement for aromatics would be higher for hemi-

metabolous than for holometabolous insects (Bernays, 1987) disregards the fact that, at least in the case of butterflies, a large cuticular investment is directed to the pupal exoskeleton.

As with amino acids, a correspondence between sensory data and post-ingestive parameters was also present with phenolic acids and anthocyanins (Chapter 6). Food utilization experiments demonstrated that growth reduction in final instars was primarily due to reduction in food intake. The chemosensory effects that were recorded are most probably responsible for at least part of this reduction. Such a relation between sensory and post-ingestive phenomena was not found for the flavonols. As these compounds caused the strongest degree of inhibition in both species, an extended analysis of their mechanism of action would be interesting. Quercetin has been found to interfere with trans-membrane ion transport by inhibiting a calcium-dependent ATP-ase pump (Racker et al., 1980). Thus, non-specific effects of this compound on the sensory level, that were not registered in the present study, may have played a role. Inhibitory effects on several chemoreceptor cells that can be classified as non-specific, were found for the main anthocyanin in B. oleracea, cyanin.

The sensitivity of P. brassicae to phenolic acids and flavonoids was markedly higher than that of P. rapae (Chapter 6). This was true both at the sensory and at the nutritional level. The reasons for this difference are unknown. Generally, the mechanisms of action of phenolic and flavonoid compounds in insects have rarely been studied and, consequently, are poorly understood (Isman & Duffey, 1982; 1983). It would require combining of biochemical techniques with methods employed in the present study to follow the fate of phenolic compounds after ingestion to investigate if post-ingestive degradation, modification or excretion occur. In the present study, no experimental link was made between the utilization of phenylalanine and tyrosine and the effects of phenolic acids on this utilization, which seems an obvious candidate process to be negatively affected. Although indications exist that inhibition of food intake could be due to chemosensory effects on behaviour, the involvement of post-ingestive processes such as toxicosis and food-aversion learning cannot be ruled out as possible factors responsible for decreased ingestion of food.

Both literature data and results of the present study indicate that cultivars of B. oleracea display considerable variation in the concentrations of phenolic acids and flavonoids (Chapter 7). Furthermore, cultivars

were found to differ in their suitability as host plants for both Pieris species. However, on the basis of the preliminary results obtained no firm conclusions can be drawn. It is not well possible to compare the patterns of performance with the pattern of phenolic and flavonoid contents in the cultivars investigated in a straightforward manner. Such a comparison is obstructed by several factors. An important factor is that both performance and phytochemistry are multivariate. Furthermore, only aglycones have been determined while it is conceivable that glycosides or other conjugated forms are more relevant in terms of biological activity. Still then, the cultivars were most certainly different in more respects than their contents of the phytochemicals studied. This is in most insect - plant studies the main methodological obstacle, preventing decisive conclusions on the significance of the few specific compounds investigated as defensive phytochemicals against phytophagous insects in a natural context.

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SUMMARY

The relationships between caterpillars of Pieris brassicae L. and Pieris rapae L. (Lepidoptera: Pieridae) and a common host plant Brassica oleracea L. were studied using chemosensory and nutritional techniques. Attention was focussed on amino acids, which are in part essential nutrients, and on phenolic and flavonoid derivatives of two aromatic amino acids, that are products of the secondary metabolism in the host plant.

An electrophysiological study of amino acid gustation showed that in both species 14 out of 22 amino acids were stimulants to a receptor cell in a maxillary sensillum. The nutritionally essential amino acids were generally stronger stimuli than dispensable ones. A correlation analysis provided indirect evidence that the amino acid receptor possessed four sites, one less specific and three or possibly four specific ones. A comparison of data on free amino acid concentrations in B. oleracea with dose-response relations of the amino acid cell showed that this cell can quantitatively sense foliar amino acids.

Phenolic acids and an anthocyanin that naturally occur in B. oleracea elicited neural responses from two to three maxillary gustatory cells. Chlorogenic and protocatechuic acids, both carrying ortho-substituted hydroxyl groups on the aromatic ring, were the most effective stimulants. A steep increase in responsiveness was occurring with increasing concentrations in the range 0.2 - 5.0 mM. P. rapae was the less sensitive of both species. Flavonols were ineffective. The predominant anthocyanin in B. oleracea, cyanin, evoked neural activity in some cells but inhibited the activity in gustatory cells sensitive to sugars, amino acids and glucosinolates in P. brassicae. Chemosensory responsiveness was reflected in preference behaviour. Naturally occurring levels of phenolic acids in B. oleracea as found in phytochemical studies are able to affect sensory processes in the caterpillars (Chapter 3).

Assessment of possible metabolic effects of dietary phenolics and some other dietary variations was performed using a flow-through respirometer. This was designed to monitor continuously the gas exchange of feeding caterpillars during the complete final instar. The results of these measurements were compared to the results obtained using standard gravimetric techniques that make use of the measurement of food intake to calculate metabolic efficiency. Respirometric results yielded small effects on

the energetic efficiency of growth, which was in contrast to gravimetric results. The causes of the discrepancies between both methods and the consequences of these findings for studies on insect food utilization in general are discussed (Chapter 4).

The nutritional utilization of amino acids and nitrogen was studied comparatively for caterpillars of both species on an artificial diet and on B. oleracea. Food consumption in the final instar was lower on the artificial diet. More food was consumed when leaf amino acid content was lower. Relationships were found between food consumption and the absorption efficiencies of most of the essential amino acids. Absorption efficiencies for glycine, cystein and serine were lower on the artificial diet, differences for other amino acids were small between the diets. Amino acid utilization patterns were similar for both species. Balance sheet calculations showed that an extensive conversion from phenylalanine to tyrosine occurred. For both species indications were obtained that tyrosine and cystein may become limiting for growth when dietary protein levels are low (Chapter 5).

Phenolic acids (caffeic and chlorogenic acids) and flavonoids (oenin and quercetin-3-rutinoside) inhibited survival, development and growth when larvae of both species were continuously exposed to these compounds present in an artificial diet. P. brassicae was distinctly more sensitive at lower levels of the compounds (0.4 and 1.0 mM). Final instars of both species were much less sensitive than earlier instars. Growth inhibition in final instars was primarily due to reduced food consumption. The results suggest a potential role of phenolic acids and flavonoids, normal constituents of leaves of B. oleracea, in defence against Pieris caterpillars (Chapter 6).

Seven cultivars of B. oleracea were offered as food to study their suitability as a host plant for larvae of both caterpillar species. Parameters of larval performance showed differences between cultivars. High-performance liquid chromatography was used to analyse leaf tissues of five cultivars with respect to concentrations of phenolic acids and flavonoids. Each of the cultivars was found to have its own quantitative pattern of these compounds. Unidentified polar flavonoid components were detected in highly variable amounts. These preliminary results further support a potential role of phenolic and flavonoid compounds in resistance of B. oleracea against Pieris (Chapter 7).

SAMENVATTING

Plantenetende insekten vertonen veelal een gedragsmatige voorkeur voor bepaalde waardplanten. Een voorbeeld van een insekt - plant relatie waarvoor de fysiologische basis van dit voorkeursgedrag uitgebreid is onderzocht, is de relatie koolwitjes - koolplanten (Pieris brassicae L. (het grote koolwitje) en Pieris rapae L. (het kleine koolwitje) - Brassica oleracea L. (de koolplant)). Een belangrijke rol in de herkenning van de koolplanten door zowel de vlinders als de rupsen spelen de zgn. mosterdolie-glucosiden. Dit zijn stoffen waarvan wordt aangenomen dat de plant ze niet zozeer gebruikt in zijn eigen stofwisseling, maar ze produceert als verdedigingsstoffen die de beschadiging van het plantenweefsel door insekten en andere belagers tegengaan. Dit verdedigingsmechanisme werkt tegen insekten die normaal niet op kool worden aangetroffen, maar kennelijk niet tegen koolwitjes. Deze maken juist gebruik van mosterdolieglucosiden als signaalstoffen waaraan geschikt voedsel wordt herkend met behulp van daarop afgestemde zintuigcellen.

In koolplanten worden echter ook zgn. fenolische stoffen aangetroffen die mogelijkerwijze eveneens een verdedigende functie dienen. Deze stoffen, fenolische zuren en de polyfenolische flavonoïden, worden door de plant gemaakt uit de twee aromatische aminozuren (fenylalanine en tyrosine), welke als voedingsstoffen door insekten vooral worden gebruikt in de aanmaak van hun exoskelet. De behoefte aan beide aminozuren is groot gedurende de snelle groei die larvale insekten, zoals rupsen, doormaken. De aandacht werd in dit proefschrift geconcentreerd op zintuigfysiologische en voedingsfysiologische aspecten van aminozuren en fenolische plantestoffen. De vraagstellingen welke centraal stonden waren: hoe vindt de zintuiglijke waarneming van aromatische en andere aminozuren plaats (hoofdstuk 2); hebben de fenolische verbindingen zintuiglijke effecten, hoe zijn deze afhankelijk van de chemische structuur (hoofdstuk 3); zijn er effecten op de stofwisselingsintensiteit (hoofdstuk 4); zijn er effecten op overleving en groei gedurende de larvale ontwikkeling en op de voedselopname of de verteringsefficiëntie in het laatste larvale stadium, waarin verreweg het grootste deel van de voedselopname gedurende de larvale ontwikkeling plaatsvindt (hoofdstuk 6). Daarnaast werd onderzocht hoe de absorptie en benutting van aminozuren verliep op twee verschillende voedselbronnen nl. een kunstmatig dieet en een natuurlijke voedselbron, koolblad. Dit onderzoek had tot doel vast te stellen in hoeverre de aminozuurhuishouding op beide substraten verschilde, met name wat betreft de benutting van de beide

aromatische aminozuren, alvorens routinematig het kunstmatige dieet te gebruiken voor de toetsing van fenolische plantestoffen (hoofdstuk 5). Tenslotte werd onderzocht of er een verband bestond tussen de gehalten van fenolische verbindingen in verschillende kool-cultivars en de geschiktheid van deze cultivars als voedselplant (hoofdstuk 7). In het volgende worden de belangrijkste bevindingen weergegeven.

Van de 22 aminozuren bleken er 14 zintuigfysiologische activiteit op te wekken in bepaalde sensilla aanwezig op de monddelen. Binnen die 14 waren het vooral de essentiële aminozuren die activiteit in de smaakcellen opriepen. Zo veroorzaakte fenylalanine zintuigreacties, terwijl tyrosine geen reacties teweegbracht. Er was een duidelijk verschil tussen beide soorten rupsen. Een correlatie-analyse leverde indirecte aanwijzingen op dat de aminozuurspecifieke zintuigcel aminozuren niet als groep herkent maar mogelijk een aantal aparte structuren voor herkenning bezit. Een vergelijking van fytochemische gegevens over de concentraties van vrije aminozuren in koolblad met het concentratie-traject waarin de aminozuurgevoelige cel actief is, leverde als conclusie op dat deze cel informatie kan doorgeven over verschillen in concentraties zoals deze in koolblad voorkomen (hoofdstuk 2).

Fenolische zuren en een anthocyaan, het flavonoïde pigment dat de rode kool zijn kleur geeft, wekten zintuigfysiologische activiteit op in dezelfde smaakharen waarin ook de aminozuurgevoelige cel ligt. De activiteit opgewekt door deze verbindingen was echter afkomstig uit andere cellen. Sommige verbindingen wekten geen activiteit op. Het anthocyaan onderdrukte de activiteit van cellen die reageren op de aanwezigheid van suikers, mosterdolieglucosiden en van de aminozuurgevoelige cel. Uit gedragsproeven in een keuzesituatie bleek dat de gemeten zintuigfysiologische activiteit in keuzegedrag werd vertaald. Ook hier gold dat het concentratietraject waarin zintuigreacties optraden een overlap vertoonde met concentraties van de fenolische stoffen zoals deze in koolblad zijn aangetroffen (hoofdstuk 3).

De vraagstelling aangaande mogelijke effecten van fenolische stoffen op de stofwisseling van de rupsen mondde uit in een studie van methodologische aard. Daar de voor insekten gebruikelijke gravimetrische methode geen reproduceerbare resultaten opleverde en onwaarschijnlijk hoge variaties in de efficiëntie van de stofwisseling suggereerde, werd een gasanalyse-opstelling opgebouwd waarin het mogelijk was om de gaswisseling van de rupsen continu te meten en zo hun warmteproduktie te berekenen. Deze methode bleek wel reproduceerbaar en leverde aanzienlijk geringere varia-

ties op dan de gravimetrische. De fenolische verbindingen bleken geen negatieve invloed uit te oefenen op de energetische efficiëntie van de groei. De fysiologische waarde van gravimetrisch bepaalde stofwisselingsefficiënties, onderwerp van veel publikaties, wordt bediscussieerd (hoofdstuk 4).

De utilisatie van aminozuren van rupsen op een kunstmatig dieet en op koolblad vertoonden veel gelijkenis. Enkele niet-essentiële aminozuren werden wat slechter geresorbeerd op het dieet. Er bleken omgekeerd evenredige relaties te bestaan tussen de voedselopname gedurende het laatste larvale stadium en de absorptie-efficiëntie van de meeste essentiële aminozuren en van proline en tyrosine. Balans-berekeningen aan aminozuren lieten zien dat een belangrijk deel van het benodigde tyrosine gemaakt werd uit fenylalanine, op zowel koolblad als op dieet met een laag totaal aminozuurgehalte. In deze situatie kan tyrosine beperkend worden voor de groei of voor de kwaliteit van het opgebouwde eiwit (hoofdstuk 5).

Een tweetal fenolische zuren (chlorogeenzuur en koffiezuur) en twee flavonoïden (rutine en oenine) bleken de overleving nadelig te beïnvloeden, ontwikkeling en groei te remmen en popgewichten te verlagen, wanneer deze stoffen werden toegepast in een kunstmatig dieet. P. brassicae rupsen waren gevoeliger voor deze verbindingen dan de rupsen van P. rapae. De jongere larvestadia waren duidelijk gevoeliger dan het laatste larvale stadium. Groeiremming in het laatste stadium werd vooral veroorzaakt door een verminderde voedselopname. Deze resultaten tonen aan dat de fenolische stoffen in koolblad een rol als beschermingsstof kunnen spelen. Het gehalte waarin ze in het voedsel aanwezig zijn is daarbij een belangrijke factor (hoofdstuk 6).

De waardplantkwaliteit van zeven verschillende kool-cultivars vertoonde duidelijke onderlinge verschillen. Met behulp van hoge-druk vloeistofchromatografie werden eveneens verschillen aangetroffen in de gehalten van fenolische stoffen in bladmateriaal van vijf van deze cultivars. Ook werden een aantal niet nader geïdentificeerde flavonoïden gevonden. De patronen van waardplantkwaliteit en concentraties zijn echter niet zonder meer op elkaar te passen. Voortgezet onderzoek is nodig om de beschermende functie van fenolische stoffen in kool tegen rupsen van koolwitjes overtuigender aan te tonen dan in dit proefschrift is gebeurd (hoofdstuk 7).

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CURRICULUM VITAE

De schrijver van dit proefschrift werd op 8 juli 1956 geboren te Amersfoort. In juni 1974 behaalde hij het diploma Gymnasium-β aan het Eemland College te Amersfoort. In september 1974 begon hij aan zijn studie biologie aan de Landbouwhogeschool te Wageningen. In mei 1978 haalde hij zijn kandidaatsexamen (cum laude), studiedifferentiatie 'organisme'. In maart 1981 rondde hij zijn biologiestudie af (cum laude) met als verzwaard hoofdvak Dierfysiologie en hoofdvak Celbiologie.

Van augustus 1981 tot november 1985 was hij in dienst bij de Vakgroep Dierfysiologie van de Landbouwhogeschool. Hier verrichte hij aanvankelijk onderzoek naar de zintuig- en gedragsfysiologie van vraatremmende plantestoffen bij enkele insektensoorten en kreeg gaandeweg het onderzoek vorm dat in dit proefschrift wordt beschreven. Daarnaast begeleidde hij doctoraalstudenten en gastmedewerkers en was tevens betrokken bij practica en colleges.

Sinds mei 1986 is hij werkzaam bij de Stichting voor Plantenveredeling als onderzoeker op het gebied van resistentie tegen schimmels en insekten.