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CUTICULAR WAX OF *EPILACHNA VARIVESTIS*

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Abstract—Cuticular lipids of larvae, pupae and adults of the Mexican bean beetle (*Epilachna varivestis*) have been examined using gravimetric and thin layer densitometric techniques. The effects of rearing on different hostplants and of rearing temperature on lipid composition were studied.

Total lipid varied with developmental stage as well as hostplant. The amount of lipid extracted ranged from 3.0 mg/g wet weight, in the case of larvae reared on snapbeans in the field, to 5.2 mg/g wet weight for pupae reared in limas under field conditions. Total lipid increased with increasing temperature for larvae reared under controlled climatic conditions.

Thin layer densitometry was used to quantify lipid classes. Epicuticular lipids included hydrocarbons, wax esters, triacylglycerols, fatty alcohols, free fatty acids, sterols, three unidentified materials and alkaloid(s). Lipids of larval and pupal stages were composed primarily of wax esters, hydrocarbons and fatty alcohols in roughly equal proportions; free fatty acids, triacylglycerols, sterols, alkaloid(s) and two unknown materials made up of the remainder. Adult cuticular lipids consisted mainly of hydrocarbons (49–60% of total lipid).

Key Word Index: *Epilachna varivestis*, Coccinellidae, Mexican bean beetle, cuticular lipids, thin layer densitometry, temperature, diet

INTRODUCTION

Although composition of insect cuticular lipids is well documented, the function of these components has received less attention (Ramsey, 1935; Beament, 1945, 1976; Ebeling, 1973; Edney, 1977, 1981; Gilby, 1980; Machin, 1981). Numerous reports of epicuticular wax analyses have appeared in the literature (for reviews see Jackson and Baker, 1970; Jackson and Blomquist, 1976; Blomquist and Jackson, 1979; Lockey, 1980). Unfortunately, few papers have examined these materials from a structure–function point of view. Several reports have related specific components of the wax with pheromonal or allomonal properties (Uebel *et al.*, 1978; Huyton *et al.*, 1980; Howard *et al.*, 1980). Some papers (Davis, 1974; Hadley, 1977, 1980; Toolson and Hadley, 1977) have shown that composition of the waxes appears to be linked with climatic change, implying that cuticular lipid composition may change to regulate climate-induced water stresses.

The above studies have, for the most part, been limited to desert dwelling arthropods. However, virtually nothing is known of the alterations in cuticular lipids which occur as a function of climate in insects reared in less extreme environments. For this reason a study of epicuticular wax of the Mexican bean beetle, as a function of developmental stage, hostplant and rearing temperature has been carried out in our laboratories. Choice of this insect arose from its increasing significance as a pest of soybeans, and observations of its sensitivity to temperature and humidity stress. Early in this century, field entomologists noted the susceptibility of *E. varivestis* to high temperatures in combination with low or high humidities as it fed on *Phaseolus* species (Graf, 1925; Marcovitch and Stanley, 1930; Miller, 1930; Sweetman, 1932). These observations have since been extended to *E. varivestis* feeding on soybeans (*Glycine max*), another member of the legume family (Kitayama *et al.*, 1979; Lockwood *et al.*, 1979; Sprenkel and Rabb, 1981; Wilson, 1979, 1982). In the course of studying environmental factors which affect bean beetle population dynamics, Wilson (1982) demonstrated that cuticular transpiration rates were higher for soy reared larvae compared to those fed lima beans. Transpirational water loss in this paper is defined as being water loss via the cuticle as opposed to spiracular or respirational water loss. Respiration is, of course, a major factor in insect water loss.

MATERIALS AND METHODS

Larvae, pupae and adult of *E. varivestis* were obtained from fields in Wake, Sampson and Camden counties in North Carolina during the summer of 1981 (July–August). Temperatures ranged from 12.8 to 36.7°C during this time. The lima reared field insects were collected from the Henderson bush variety, the snapbean reared insects from Blue Lake variety plants, and the soybean reared insects from Bragg soybeans. Insufficient numbers of soybean reared pupae were obtained to be included in the analyses. Insects and the leaves on which they were feeding were placed in paper bags over dry ice for transport to the laboratory. Insects used for the controlled temperature experiments were obtained as eggs from microsporidia-free cultures. Newly hatched larvae were placed on bouquets of plants grown at the N.C.S.U. Experiment Farm, Clayton, North Carolina. These plants were held in Erlenmeyer flasks containing water in environment controlled chambers (Wilson, 1979) at relative humidities of 50–65% and at tem-

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peratures of 23 and 27 C. Insects were removed from the plant on reaching the third instar, placed in vials under N_2, and stored at −10°C prior to extraction.

Cuticular lipids were extracted by washing weighed groups of 100 insects with 25 ml of a 1:1 (v/v) solution of pentane–methylene chloride over two 2 min periods (50 ml total). A minimum of four replications were carried out. Prior TLC work had shown that essentially quantitative removal of wax was effected after two washes and that glyco- and phospho-lipids, indicative of internal lipid contaminants, appeared only after five washes.

Thin layer chromatography was carried out using 250 μ Analtech silica gel G plates. These plates were predeveloped in methanol, dried at 110 C, and stored in a desiccator. A line was scribed at 18.5 cm to isolate the resulting band of contaminants at the top of the plate. Samples and standards were spotted as 10 mg/ml solutions. A triple development procedure was adopted using hexane to 18.5 cm, benzene to 18.5 cm and hexane–diethyl ether–acetic acid (80:20:1 by volume) to 10 cm. Plates were thoroughly dried in a fume hood between developments. Spots were visualized for densitometry by use of a chromic acid–sulphuric acid mixture (Privett *et al.*, 1973). The acid treated plates were heated slowly to a maximum temperature of 230°C on a stainless steel block on top of a hotplate (Downing, 1980). A Kratos Model SD3000 Thin Layer Spectrodensitometer equipped with Schoeffel SDC300 Density Computer, a Digital Specialties Lilliputer was used for quantitation of lipid classes. Scanning was first carried out on standards that were applied in amounts of 0.1, 0.5, 1.0, 5.0 and 10.0 μl of 10 mg/ml solutions in order to verify that response was linear over a range of sample sizes. These standards were pure compounds which corresponded to the lipid classes found in insect cuticles. The split width was set to equal that of the broadest charred spot in the lane scanned. A minimum of four analyses were run on each sample.

Lipid classes were identified by comparison with authentic standards and literature R_f values, and by the use of several specific and nonspecific spray reagents. Table 2 lists the reagents employed.

RESULTS

Table 1 presents the results of the gravimetric analyses. Among stages of field reared insects, the greatest amount of lipid per wet weight was extracted from pupae. Larval and adult cuticular lipids occurred in similar amounts in the *Phaseolus* reared insects, whereas soybean reared larvae contained much higher amounts of wax than adults. In addition, the amount of cuticular lipid per insect wet weight is higher in soy fed larvae and adults than in corresponding *Phaseolus* reared insects. The increase in wax/insect wet weight demonstrated in soy reared adults and larvae may be explained if the average weight of each insect for *Glycine* versus *Phaseolus* fed animals is considered. Baker *et al.* (1979a) showed that younger (and therefore smaller) larvae of the black carpet beetle had higher percentages of cuticular lipid than did the larger larvae. A similar relationship may explain the observations in this study.

Larvae reared under the controlled environmental regimens displayed an increased ratio of weight of lipid to insect wet weight at the higher rearing temperature. Differences in extracted weight of lipid were significantly different (95% confidence interval) as a function of temperature, regardless of host/plant.

Table 1. The effect of developmental stage, hostplant and rearing temperature on the amount of extractable cuticular lipid of *Epilachna varivestis*

Developmental stage	Host	Environmental conditions	Average wt of insects (g)	Average wt of extracted lipid (mg/g)
Larval (3rd and 4th instars)	*P. lunatum*	Field	0.0214	3.76
Pupal	*P. lunatum*	Field	0.0241	5.15
Adult	*P. lunatum*	Field	0.0249	4.00
Larval	*P. vulgaris*	Field	0.0221	3.03
Pupal	*P. vulgaris*	Field	0.0263	3.89
Adult	*P. vulgaris*	Field	0.0271	3.25
Larvel	*G. max*	Field	0.0190	4.72
Adult	*G. max*	Field	0.0218	3.67
Larval	*P. lunatum*	23°C 50–65% r.h.	0.0183	3.89
Larval	*P. lunatum*	27°C 50–65% r.h.	0.0176	4.43
Larval	*G. max*	23°C 50–65% r.h.	0.0167	3.65
Larval	*G. max*	27°C 50–65% r.h.	0.0148	4.39

Table 2. Identification of *Epilachna varivestis* epicuticular lipid classes

Identity	Method										Present in		
	A	B	C	D	E	F	G	H	I	J	Larvae	Pupae	Adult
Alkaloid (struck. unk.)	×	×					×				×	×	×
Sterols	×	×	×		×					×	×	×	×
Fatty alcohols	×	×			×				×	×	×	×	×
Fatty acids	×	×				×				×	×	×	×
Unknown 1	×	×											×
Triacylglycerols	×	×						×	×	×	×	×	×
Unknown 2	×	×		×					×	×	×	×	×
Unknown 3	×	×						×			×	×	×
Wax esters	×	×						×	×	×	×	×	×
Hydrocarbons	×	×							×	×	×	×	×

A $K_2Cr_2O_7/H_2SO_4$; B—phosphomolybdate; C—H_2SO_4 (sterols turn pink); D—DNPH (carbonyls); E—vanillin/H_2SO_4 (carbonyls, alcohols); F—Bromocresol green/NaOH (acidic groups); G—Dragendorff's Reagent (amines); H—disappearance following basic hydrolysis (ester); I—i.r. examination; J—comparison with standards.

This data would seem to support previous experimental evidence of increased amounts of lipid with increasingly more rigorous environmental conditions. Hadley (1977) demonstrated this phenomenon when he compared the amounts of extractable hydrocarbon in winter and summer active desert tenebrionids (*Eleodes armata*). He showed that summer active beetles had approximately three times the extractable hydrocarbon of winter active beetles. A puzzling aspect of correlations between bulk wax and resistance to desiccation which Hadley (1977) pointed out is the apparent failure to link increases in amount of wax with reduced transpiration in plants. Similar observations were also made by Beament (1945) using waxes coated on artificial and insect wing membranes. In addition, Grncarevic and Radler (1976) showed in model experiments that the type of lipid is of greater importance than is the total amount of lipid in determining resistance to water loss. Thus, greater amounts of extractable wax associated with pupae or with insects reared under more extreme enviroments may not be a direct correlate of desiccation resistance.

Results of thin layer examinations are shown in Tables 2, 3 and 4. Table 2 shows various classes of lipid identified in these experiments and the methodologies employed. Those classes identified are typical of components observed in previous epicuticular wax analyses with the exception of the alkaloidal fraction. Alkaloids have been identified as defensive secretions in a number of Coccinellids (Happ and Eisner, 1961; Tursch *et al.*, 1975). These alkaloids have not been isolated and their structure(s) elucidated in *E. varivestis* although a number of structurally related alkaloids have been identified in other Coccinellids. Table 3, which presents the quantitative analysis of larval, pupal and adult lipid classes from insects collected in the field, shows that this alkaloidal fraction occurs in significantly higher amounts in pupal stages. (Significance in this and following cases was determined using Student's t-test of small sample differences in mean at the 95% confidence level.) Increased alkaloid in pupae is perhaps a reflection of this stage's immobile and otherwise defenceless nature.

Other materials found in the bean beetle cuticular wax include sterols (St), fatty alcohols (FAlc), fatty acids (FA), an unknown (Uk1), triacylglycerols (TG), another unknown (Uk2), an unidentified ester material (Uk3, possibly acetyl ester), wax esters (WE) and hydrocarbons (HC). Fatty alcohols, wax esters and hydrocarbons occur in approximately equal proportions in larval and pupal stages on all hosts and are the major lipid classes for these two stages, comprising from 73.3 to 82.8% of total extracted wax. In adults, hydrocarbons are the predominant component, ranging from 49.6% of the total extractable wax in soy reared insects to 60.7% in lima fed insects. High percentages of hydrocarbons have been shown to be characteristic of adult insect cuticular waxes (see for example Baker *et al.*, 1979a, b). Other components occur in significantly higher amounts in the adults; unknown 1 is found only at that stage. Triacylglycerols levels are approximately twice as high in snapbean reared adults compared to larvae and pupae. In lima reared animals, the increase is

Table 3. Thin layer densitometry of lipid classes; analysis of cuticular wax of field reared insects*

Developmental stage / hostplant	Larvae *P. vulgaris*	Pupae *P. vulgaris*	Adults *P. vulgaris*	Larvae *P. lunatum*	Pupae *P. lunatum*	Adults *P. lunatum*	Larvae *G. max*	Adults *G. max*
Lipid classes								
Alkaloids	3.3 ± 0.8	6.4 ± 1.2	1.5 ± 0.1	2.5 ± 0.3	8.6 ± 0.6	2.3 ± 0.4	2.6 ± 0.4	1.2 ± 0.2
Sterols	5.8 ± 0.2	5.0 ± 0.4	0.4 ± 0	3.3 ± 0.4	7.4 ± 0.8	0.7 ± 0.2	6.8 ± 0.4	tr
Fatty alcohols	21.2 ± 1.5	25.4 ± 1.1	5.7 ± 0.3	22.4 ± 2.0	30.6 ± 1.9	7.1 ± 0.9	19.1 ± 1.6	2.8 ± 0.1
Fatty acids	5.5 ± 0.6	3.7 ± 0.3	1.4 ± 0.2	2.6 ± 0.2	4.0 ± 0.5	1.4 ± 0.3	5.3 ± 0.3	1.7 ± 0.1
Unknown 1	—	—	2.6 ± 0.6	-	—	tr	—	3.8 ± 0.6
Triacylglycerols	4.7 ± 0.7	4.5 ± 0.3	9.4 ± 0.5	1.4 ± 0.3	tr	7.3 ± 0.1	6.2 ± 0.9	10.0 ± 0.3
Unknown 2	1.9 ± 0.3	tr†	11.3 ± 0.7	tr	tr	8.3 ± 0.6	2.1 ± 0.3	11.0 ± 0.8
Unknown 3	5.7 ± 0.4	3.1 ± 0.3	6.3 ± 0.5	7.3 ± 0.3	tr	5.7 ± 1.0	3.3 ± 1.1	9.1 ± 0.5
Wax ester	24.4 ± 1.9	29.2 ± 0.9	3.9 ± 0.7	29.1 ± 0.2	26.8 ± 0.8	6.5 ± 0.4	25.4 ± 2.0	10.8 ± 0.8
Hydrocarbon	27.9 ± 1.1	23.7 ± 0.9	58.0 ± 3.1	31.3 ± 2.1	23.0 ± 0.6	60.7 ± 2.6	29.9 ± 2.8	49.6 ± 1.3

*Composition equals % ± SD from a minimum of four replicate. †tr = Trace.

Table 4. Thin layer densitometry of lipid classes; analysis of cuticular wax of 3rd instar larvae reared at different temperatures*

	Temperature (°C)			
	23	27	23	27
	P. lunatum		Host plants	*G. max*
Lipid classes				
Alkaloids	2.1 ± 0.1	0.9 ± 0	1.6 ± 0.2	1.1 ± 0.1
Sterols	5.1 ± 0.5	5.7 ± 0.3	8.1 ± 0.5	5.3 ± 0.4
Fatty alcohols	27.4 ± 1.0	23.2 ± 0.7	28.4 ± 1.3	22.1 ± 1.3
Fatty acids	3.1 ± 0.2	2.2 ± 0.1	5.7 ± 0.1	4.3 ± 0.2
Unknown 1			—	
Triacylglycerols	0.7 ± 0	1.0 ± 0.2	3.5 ± 0.1	1.9 ± 0.3
Unknown 2	tr†	tr	tr	1.2 ± 0.2
Unknown 3	7.1 ± 0.5	6.9 ± 0.3	6.9 ± 0.3	10.4 ± 0.4
Wax ester	20.2 ± 0.3	24.8 ± 3.3	19.1 ± 2.0	28.0 ± 2.0
Hydrocarbon	30.8 ± 2.7	33.6 ± 1.7	26.6 ± 1.3	25.8 ± 0.2

*Composition equals % ± SD from a minimum of four replicate groups of insects.
†tr = Trace.

even greater. Unknown 2, which may be a long chain ketone or aldehyde, occurs in small percentages or at trace levels in all immature insects, but is found in amounts from 8.3 to 11.3% in the adult insects. Unknown 3, which is hydrolyzed upon treatment with aqueous base, occurs in slightly higher amounts in adults. The R_f of this component was similar to that of fatty acid methyl ester standards and may be an acetyl fatty alcohol ester (see Jackson and Armold, 1981).

Table 5 shows the composition of larval cuticular waxes at two temperature regimens using *Phaseolus lunatum* and *Glycine max* as hosts. Classes found are the same and generally occur in amounts similar to that of the field reared larvae. Significant differences as a function of hostplant are not apparent. Soy reared larvae do appear to have somewhat less hydrocarbon and more sterol, triacylglycerols and fatty acids than their lima fed counterparts although these differences are not significant at the 95% confidence interval.

Several interesting patterns emerge from examination of the cuticular lipids as a function of temperature. First, the amount of wax ester increases significantly (95% confidence interval) at the higher temperature for larvae reared on both host plants. In addition, the amount of fatty alcohol decreases significantly with increasing temperature in larvae reared on both hosts. A similar trend was noted for fatty acids although these decreases are not significant at the 95% confidence level. Alkaloids also decrease with temperature. The other lipid classes present do not exhibit consistent trends.

DISCUSSION

An overview of lipid class composition in these studies can be considered from three vantage points: as a function of development, diet or rearing temperature. In some cases, significant variations in lipid class ratios are evident, while in others clear patterns do not emerge.

Changes in composition which occur as a function of development are substantial between the immature and adult insects. This parallels observations by other workers (Baker *et al.*, 1979a) that composition shifts from a mixture of components in roughly equal proportions in the larvae, to a composition in the adult in which hydrocarbons predominate. Baker *et al.* (1979a) pointed out that this shift may possibly be related to the need for a high melting, abrasion resistant wax in larval animals. Such a wax would be advantageous to immature stages where the underlying cuticulin layer is considerably less sclerotized and is therefore less resistant to water loss. Unsclerotized, extensible cuticle is a necessity in larval insects, which must accommodate considerable growth between moults, unlike adult insects. Corresponding changes in cuticular lipid might therefore reflect changes in the chemical and physical nature of the cuticle.

Examination of changes in lipid class composition as a function of diet (hostplant) revealed no readily discernible correlations. At this level of investigation examination of factors such as the degree of unsaturation or the chain length of individual components within each class is not possible. Therefore, it cannot be determined with certainty what effect dietary lipid changes exert on cuticular lipid composition.

Changes in cuticular lipid class patterns in larvae as a function of rearing temperature reveal correlations of potential significance. A comparison of the ratios of nonpolar lipids (hydrocarbons, wax esters and triacylglycerols) to "amphiphilic" lipids (alcohols, sterols and free fatty acids) indicates a significant (95% confidence interval) shift in nonpolar compared with amphiphile lipid. This ratio increases at higher temperatures for insects reared on either soy or lima beans. Unknown materials were not taken into account.

Locke (1965) observed that a prominent feature of insect cuticles are the so-called pore or wax canals which contain filaments of wax. He postulated that these wax filaments were actually lipid–water liquid crystals in the "middle" or hexagonal phase. Lipid–water liquid crystals are composed of three basic components: nonpolar lipid, amphiphile and water. The crystalline structure of such materials is dependent on the relative ratios of all three components as well as the temperature of the system. [See Friberg (1976) and Brown and Wolken (1979) for introductory discussions of these materials and their properties.] The preceding analyses show that the ratio of nonpolar lipid to amphiphilic lipid increases with increasing temperature. Since water loss rates

rise with increasing temperature, it would be advantageous for an insect to alter its cuticular chemistry to compensate for such increases. Over the long term (i.e. days and weeks compared with hours) it appears that an increase in nonpolar wax and/or a decrease in amphiphilic lipids are among the compensatory changes that result in the Mexican bean beetle when it is subjected to a higher temperature regimen.

If pore and wax canals of insects are indeed a major site of transpirational water loss (along with water lost through respiration or excretion), alterations in equality (and quantity?) of lipid materials in these canals should be a primary means of regulating this loss. The changes which occur as a function of temperature in *Epilachna* larval wax correspond to alterations one might expect based on lipid–water liquid crystal properties.

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