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HOST-PLANT PROTEIN AND PHENOLIC RESIN EFFECTS ON LARVAL GROWTH AND SURVIVAL OF A BUTTERFLY

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Abstract—*Euphydryas chalcedona* prediapause larvae were reared on fertilized and control shrubs of the host plant, *Diplacus aurantiacus*. Larval growth was enhanced by high leaf nitrogen content and inhibited by high leaf phenolic resin content. Larvae fed less on leaves near the branch tip which contained a higher leaf resin content. The results agree with prior laboratory investigation that the dietary content of nitrogen and *D. aurantiacus* leaf resin are major determinants of *E. chalcedona* larval growth and suggest that the phenolic leaf resin of *Diplacus* may both deter and inhibit leaf herbivores.

Key Words—*Diplacus aurantiacus*, Scrophulariaceae, *Euphydryas chalcedona*, Lepidoptera, Nymphalidae, flavonoid, herbivory, nitrogen.

INTRODUCTION

Strong effects of leaf nitrogen content on insect herbivore feeding and nutrition have been demonstrated for a wide range of plants and insects (White, 1978; Mattson, 1980; Slansky and Feeny, 1977; Scriber and Slansky, 1981; Lincoln et al., 1982). Common responses to increased leaf nitrogen levels are increasing insect larval growth rates and decreased feeding rates with increasing available protein. Similarly, the effects of plant secondary chemicals on herbivores has also been widely demonstrated (Fraenkel, 1959; Ehrlich and Raven, 1964; Swain, 1977; Feeny, 1976). Many of the studies demonstrating the effects of secondary chemicals have taken place, however, under artificial diet or laboratory circumstances (Goldstein and Swain, 1965; Soo Hoo and Fraenkel, 1966; David and Gardner, 1966; Rice et al., 1978). Those studies which examined effects under more natural conditions have sometimes yielded results which con-

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tradicted the laboratory observations or suggested that leaf nitrogen content was a more important determinant of herbivore success than secondary chemical content (Fox and Macauley, 1977; Morrow and Fox, 1980; Bernays, 1981; Coley, 1983). Phenolic leaf chemicals, in particular, have been implicated in herbivore inhibition because of their hypothesized generalized mode of action of protein-complex formation (Goldstein and Swain, 1965; Feeny, 1970). Several studies have suggested that the proposed generalization mode of action may not occur and that phenols may not deter herbivores under field conditions (Fox and Macauley, 1977; Bernays, 1981; Zucker, 1983).

The objective of the present study was to test the effectiveness of *Diplacus aurantiacus* leaf resin in inhibition of herbivores. The growth and survival of *Euphydryas chalcedona* larvae were used to assess the leaf resin as well as the influence of leaf nitrogen under field conditions. *Diplacus aurantiacus* (Scrophulariaceae) produces large quantities (up to 30% of the leaf dry weight) of a low-molecular-weight phenolic resin on the external surfaces of the leaves (Lincoln, 1980). A previous laboratory study, using an artificial diet, has shown that the dietary content of *D. aurantiacus* resin chemicals reduces growth and survival of the larvae of the checkerspot butterfly, *Euphydryas chalcedona* (Lepidoptera: Nymphalidae), the principal herbivore of this plant (Lincoln et al., 1982). In addition, the influence of the resin interacted with the protein content of the food. High nitrogen content led to enhanced larval growth and survival, but the effect was dependent on the level of resin present in the diet. The effects of dietary nitrogen and resin were most striking at levels found in plants.

METHODS AND MATERIALS

Ten pairs of plants were chosen at Jasper Ridge Biological Preserve, Stanford University, Stanford, California. Pairs of plants with similar vigor and size (1–1.5 m in height with multiple stems) were chosen. An area 1 m in radius was cleared around each plant and one pair member was fertilized on January 6, 1983 and March 29, 1983 with 10–10–10 slow-release NPK fertilizer to elevate its leaf protein content, while the other shrub remained as an untreated control. Inspection in May revealed that fertilized plants had grown much more than the control plants: fertilized plants had longer stems from the current year's growth than did unfertilized plants.

On May 23, 1983, 16 already-mated adult females of *E. chalcedona* were collected in a large subpopulation on Jasper Ridge where *D. aurantiacus* is the host plant. The adults were kept for two weeks, and egg masses were collected after oviposition on leafy stems of an alternative host plant, *Scrophularia californica.* Some egg masses were cooled to 6°C to synchronize hatching. On June 9, 1983, freshly hatched larvae were separated into batches of 50, and each group was placed on two *D. auranticus* leaves in a large vial.

After the larvae had spun a feeding web on the two leaves in the vial (two days), they were placed on shrubs. Each group of 50 larvae was attached to a leaf in the middle of a stem and a mesh bag slipped over the stem and sealed at the bottom. Each plant had two bags with 50 larvae in each. Bags were about 1 m in length and circumference and enclosed a sufficient amount of leaves to ensure that larvae would not lack for food and were situated so that the larvae could bask in the sun. The bags usually covered no more than 50% of the leaves of the plant. The mesh size of the bags were not small enough to retain the first-instar larvae, and probably not the second instar. The amount of light reduction due to the bags was approximately 5% (determined with a Lamda Instruments Quantum sensor which measures irradiance in the 400 to 700-nm band).

By June 17, 1983, larvae had spun visible feeding webs near the site where the larvae were originally placed. Although the number of larvae inside the bags could not be accurately estimated, no larvae were ever observed to have escaped from the bags despite repeated observations. The high survival at the end of the experiment ($>50\%$) confirms that there was little or no escape. On June 27, 1983, the primary branch of one bag broke at the base. The bag contained 24 larvae and, by this time, the larvae were too large to pass through the mesh bags. The data for this bag were retained and the larval weight at diapause was computed using the average larval weight in the other bag on the plant.

Rates of larval growth and development could not be measured because neither the number of active larvae nor the time of entry into diapause could be accurately determined. The experiment was therefore terminated when all larvae had obviously entered diapause (fourth instar). On July 13, 1983 all branches and intact bags were collected. The larvae were separated from the plants, counted, and weighed as a group. The average weight per larva was calculated by dividing the total larval weight by the number of larvae. Larval feeding was determined on 40 branches (20 from control plants and 20 from fertilized plants) by visually estimating the amount of leaf area consumed for the leaf pairs at the first six nodes below the branch tip. Feeding was estimated after all larvae had entered diapause. Branches which had not been fed upon at any nodal position were excluded.

Leafy branches were collected from each plant on June 11, 1983 and June 30, 1983 for chemical analyses of leaves and dried intact at 60°C. These dates correspond to the beginning of the experiment and an ending time when most of the larvae would have entered diapause. The nitrogen content was determined for a group of four to five leaves pooled from several positions on the branch for each plant on each date. Nitrogen content was determined with a Technicon Autoanalyzer II using Technicon Industrial Method 146171A and a modified digestion procedure of Isaac and Johnson (1976). From stems of each plant and date, leaf resin content was determined at three nodal positions, one to two expanded nodes below the tip, as well as two to four nodes and four to six nodes below the tip. Phenolic leaf resin content was determined by weighing individual

leaves, rinsing them with 5 ml of methanol, diluting 50-fold, measuring the absorbance at 292 nm, and assuming an average molecular weight of 440 and an extinction coefficient of 1.375×10^4 (Lincoln, 1980).

RESULTS

The fertilizer treatment of the shrubs significantly increased the level of leaf nitrogen in the treated plants over the matched control plants by an average of 24% ($t = 5.20$, $P < 0.001$, t test for paired comparison; Table 1). Leaf resin content, on the other hand, did not differ significantly between the two groups of plants at any of the three measured positions on the stem ($P > 0.05$, t test for paired comparison), although the fertilized shrubs tended to have slightly higher levels than did the untreated controls.

Larval growth, measured by weight gain to diapause, differed significantly between treatment and control groups ($P < 0.046$, t test for paired comparison; Table 1). Simple linear regression of the effect of leaf nitrogen on larval growth, however, did not reveal a significant effect. A general linear model approach was used for an analysis of covariance to assess the simultaneous effects of continuous variables (leaf nitrogen and resin contents) while adjusting for a class variable (treatment vs. control shrubs). Using a partial sum of squares (columns 3–5, Table 2), a statistically significant effect on larval growth was observed for the resin content of leaves at nodes 1–2 and nodes 4–6 below the tip, but not for leaf nitrogen content. A stepwise procedure was used to determine if the effects of resin were independent of possible effects of nitrogen. The stepwise method showed that leaf nitrogen had a very significant influence on larval growth (column 8, Table 2). The variance attributable to leaf nitrogen is also attributable to

TABLE 1. LARVAL PERFORMANCE AND HOST-PLANT CHARACTERISTICS FOR CONTROL AND FERTILIZED SHRUBS

	Control		Fertilized	
	Mean	Standard deviation	Mean	Standard deviation
Leaf nitrogen content (mg/g) (June 11 + June 30)/2	12.3	1.6	15.2	2.3
Leaf resin content (mg/g)				
Nodes 1–2 (June 11 + June 30)/2	233	42	256	17
Nodes 2–4 (June 11 + June 30)/2	180	33	200	43
Nodes 4–6 (June 11 + June 30)/2	136	31	160	31
Survival (%)	54.3	10.6	58.2	18.5
Fresh weight per larva (mg)	11.9	2.1	14.4	2.3
Total larval biomass (mg)	321.2	69.8	433.5	177.3

TABLE 2. ANALYSIS OF COVARIANCE FOR EFFECTS OF PLANT PAIR, LEAF NITROGEN, AND LEAF RESIN ON LARVAL GROWTH (FRESH WEIGHT AT DIAPAUSE), $R^2 = 0.88$

		Partial			Stepwise		
Source	*df*	Sum of squares	*F* ratio	$P <$	Sum of squares	*F* ratio	$P <$
Pair	9	76.8784	3.60	0.067	34.3879	1.61	0.289
Leaf nitrogen	1	8.5186	3.59	0.107	32.4021	13.65	0.010
Leaf resin							
Nodes 1–2	1	30.9713	13.05	0.011	14.0083	5.90	0.051
Nodes 2–4	1	10.1673	4.28	0.084	2.0913	0.88	0.384
Nodes 4–6	1	24.5420	10.34	0.018	24.5420	10.34	0.018
Error	6	14.2392			14.2392		

leaf resin at nodes 1–2 and nodes 2–4 (compare columns 5 and 8, Table 2), depending on the order of their entry into the computation. Thus, there appears to be a high degree of colinearity and lack of independence between the effect of leaf nitrogen and the effect of leaf resin at nodes 1–2 on larval growth. However, the effect of leaf resin at nodes 4–6 on larval growth was independent of leaf nitrogen.

Assessment of the influence of leaf resin on larval growth is best considered in the perspective of larval feeding. Larval feeding was reduced on leaves near the tip, especially for leaves at nodes 1–2 below the tip of the branch (Figure 1). This discrimination among leaves at different nodal positions did not differ appreciably between control and fertilized plants. The elongation of *D. aurantiacus* stems at Jasper Ridge is controlled by water availability and ceases in late June–early July (Mooney et al., 1980). Because of the limited potential for plant growth and the greater larval feeding in the latter stages of the experiment, the observed pattern of herbivory reflects larval feeding choice. The partial and stepwise regression coefficients suggested that the effect of leaf resin at nodes 1–2 on larval growth was positive, while the effect at nodes 4–6 was negative, with leaf resin at nodes 2–4 having little influence on larval growth. Hence, the influence of leaf resin is inhibitory (nodes 4–6) or potentially stimulatory (nodes 1–2). Because prediapause larvae of *E. chalcedona* feed primarily on leaves at nodes 3–6 below the tip (Figure 1), the positive correlation between larval growth and resin variation at nodes 1–2 appears to be spurious.

Larval survival did not differ significantly between the control and treated shrubs (Table 1). Survival was high, but was slightly lower on the control plants, perhaps because the initial attempt to place larvae on a control plant resulted in the lowest survival of any group (4%). In addition to a lack of difference between treatment and control, there was no statistically significant effect of leaf

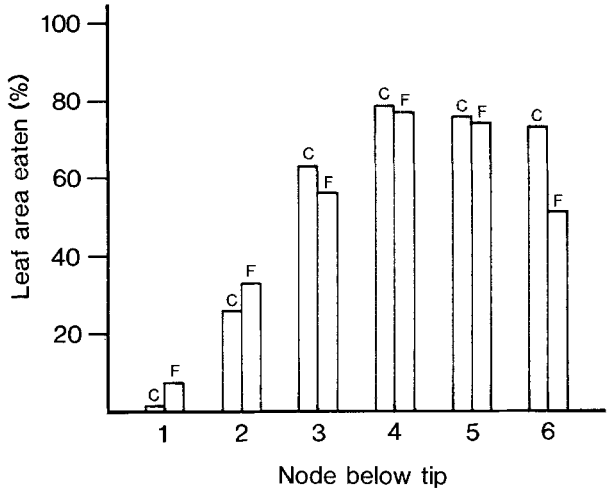

FIG. 1. Feeding of *Euphydryas chalcedona* prediapause larvae on control (C) and fertilized (F) shrubs of *Diplacus aurantiacus* according to leaf position below the tip of the branch (20 branches from each group).

nitrogen on survival and leaf resin content was significant only at nodes 1–2 (Table 3). As with the effect on larval growth, the regression coefficient of resin at nodes 1–2 on survival was positive, suggesting a stimulation of survival by leaf resin. There was, in addition, a significant difference in survival among plant pairs.

DISCUSSION

The stimulatory effect of leaf nitrogen content on larval growth under field conditions is concordant with previous laboratory results for *Euphydryas chal-*

TABLE 3. ANALYSIS OF COVARIANCE FOR EFFECTS OF PLANT PAIR, LEAF NITROGEN, AND LEAF RESIN ON LARVAL SURVIVAL (PROPORTION SURVIVING AT DIAPAUSE), $R^2 = 0.92$

		Partial			Stepwise		
Source	*df*	Sum of squares	*F* ratio	*P* <	Sum of squares	*F* ratio	*P* <
Pair	9	0.2971	5.24	0.028	0.3333	5.88	0.022
Leaf nitrogen	1	0.0073	1.16	0.322	0.0057	0.91	0.377
Leaf resin							
Nodes 1–2	1	0.0721	11.45	0.015	0.0661	10.50	0.018
Nodes 2–4	1	0.0042	0.67	0.445	0.0004	0.06	0.812
Nodes 4–6	1	0.0088	1.40	0.282	0.0088	1.40	0.282
Error	6	0.0378			0.0378		

cedona prediapause larvae (Lincoln et al., 1982). In addition, the results also suggest that the growth of *E. chalcedona* is limited by leaf nitrogen content under field conditions. Leaf nitrogen level has long been hypothesized to be a limiting factor to the growth of individual insects and to insect population growth (Smith and Northcott, 1951; White, 1978; Mattson, 1980). The covariance of nitrogen content and resin yield at nodes 1–2 suggests that nitrogen fertilization led to a greater resin production. Some studies have suggested that phenolic chemical production was enhanced under conditions of low nitrogen supply (Janzen, 1974; McKey et al., 1978), and Mihaliak and Lincoln (1985) have shown that carbon allocation to volatile terpenes in the composite *Heterotheca subaxillaris* increases as nitrogen supply declines.

Phenolic leaf chemicals have been implicated in inhibition of specialist feeding herbivores because of their hypothesized generalized mode of action of protein-complex formation (Goldstein and Swain, 1965; Feeny, 1970). Several studies have found no evidence that the proposed generalized mode of action occurs and suggest that the presence and variation of leaf phenols may not be related to herbivore pressure (Fox and Macauley, 1977; Bernays, 1981; Coley, 1983; Zucker, 1983). The present evidence shows that for *E. chalcedona*, the level of leaf resin in *D. aurantiacus* leaves has a substantial influence on growth. Thus, these results confirm the reduction in larval growth by *Diplacus* resin observed in the previous study using controlled conditions and artificial diets. The principal resin constituents (flavonoids) contain an orthodihydroxy group (Lincoln, 1980), and the presence of this chemical group appears to enhance the lepidopteran larval growth inhibition of flavonoids (Elliger et al., 1980).

The limited effect of nitrogen or leaf resin on larval survival could be due simply to the greater degree of variability in survival compared to growth of individuals. It had been noted in the previous artificial diet study that measures of survival tend to be more variable than growth (Lincoln et al., 1982). A relatively high degree of variability among plants and even among pairs of bags on a single plant was observed in the present study. Alternatively, the significant difference in larval survival among plant pairs implies that factors beyond nitrogen or resin influence larval survival under field conditions. Presumably, large predators were not influential because of the protective net bags used in the experiment.

Euphydryas larvae have the capacity to both choose among leaves and to adjust feeding rate. Current and previous observations (Mooney et al., 1980) have shown that prediapause larvae of *Euhydryas chalcedona* feed on *Diplacus* leaves with low resin contents and on leaves several nodes below the branch tip (Williams, 1983). Larvae also chose leaves with low resin contents in the laboratory (unpublished data). Thus, the observed results suggest that the leaf resin acts as both a deterrent and an inhibitor of larval growth. Because older leaves at lower nodal positions have lower nitrogen levels (Mooney et al., 1981), inhibition of feeding near the tip of the stem could result in an inhibition of growth.

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REFERENCES

BERNAYS, E.A. 1981. Plant tannins and insect herbivores: An appraisal. *Ecol. Entomol.* 6:353–360.

COLEY, P.D. 1983. Herbivory and defensive characteristics of tree species in a lowland tropical forest. *Ecol. Monogr.* 53:209–233.

DAVID, W.A.L., and GARDNER, B.O.C. 1966. Mustard oil glucosides as feeding stimulants for *Pieris brassicae* larvae in a semisynthetic diet. *Entomol. Exp. Appl.* 9:247–255.

EHRLICH, P.R., and RAVEN, P.H. 1964. Butterflies and plants: A study in coevolution. *Evolution* 18:586–608.

ELLIGER, C.A., CHAN, B.C., and WAISS, A.C., Jr. 1980. Flavonoids as larval growth inhibitors. *Naturwissenschaften* 67:358–360.

FEENY, P.P. 1970. Seasonal changes in oak leaf tannins and nutrients as a cause of spring feeding by winter moth caterpillars. *Ecology* 51:565–581.

FEENY, P.P. 1976. Plant apparency and chemical defense. *Recent Adv. Phytochem.* 10:1–40.

FOX, L.R., and MACAULEY, B.J. 1977. Insect grazing on *Eucalyptus* in response to variation in leaf tannins and nitrogen. *Oecologia* 29:145–162.

FRAENKEL, G. 1959. The raison d'etre of secondary plant substances. *Science* 129:146–170.

GOLDSTEIN, J.L., and SWAIN, T. 1965. The inhibition of enzymes by tannins. *Phytochemistry* 4:185–192.

ISSAC, R.A., and JOHNSON, W.C. 1976. Determination of total nitrogen in plant tissue, using a block digestor. *J. Assoc. Off. Anal. Chem.* 59:98–100.

JANZEN, D.H. 1974. Tropical blackwater rivers, animals, and mast fruiting by the Dipterocarpaceae. *Biotropica* 6:69–103.

LINCOLN, D.E. 1980. Leaf resin flavonoids of *Diplacus aurantiacus*. *Biochem. Syst. Ecol.* 8:397–400.

LINCOLN, D.E., NEWTON, T.S., EHRLICH, P.R., and WILLIAMS, K.S. 1982. Coevolution of the checkerspot butterfly *Euphydryas chalcedona* and its larval food plant *Diplacus aurantiacus:* Larval response to protein and leaf resin. *Oecologia* 52:216–223.

MATTSON, W.J. 1980. Herbivory in relation to plant nitrogen content. *Annu. Rev. Ecol. Syst.* 11:119–161.

MCKEY, D., WATERMAN, P.G., MBI, C.N., GARTLAN, J.S., and STRUHSAKER, T.T. 1978. Phenolic content of vegetation in two African rainforests: Ecological implications. *Science* 6:61–64.

MIHALIAK, C.A., and LINCOLN, D.E. 1985. Growth pattern and carbon allocation to violate leaf terpenes under nitrogen limiting conditions in *Heterotheca subaxillaris* (Asteraceae). *Oecologia.* 66:423–426.

MOONEY, H.A., EHRLICH, P.R., LINCOLN, D.E., and WILLIAMS, K.S. 1980. Environmental controls on the seasonality of a drought-deciduous shrub, *Diplacus aurantiacus* and its predator, the checkerspot butterfly, *Euphydryas chalcedona. Oecologia* 45:143–146.

MOONEY, H.A., WILLIAMS, K.S., LINCOLN, D.E., and EHRLICH, P.R. 1981. Temporal and spatial variability in the interaction between the checkerspot butterfly, *Euhydryas chalcedona* and its principal food source, the Californian shrub, *Diplacus aurantiacus. Oecologia* 50:195–198.

MORROW, P.A., and FOX, L.R. 1980. Effects of variation in *Eucalyptus* essential oil yield on insect growth and grazing damage. *Oecologia* 45:209–219.

RAUSHER, M.D. 1981. Host-plant selection by *Battus philenor* butterflies—the roles of predation, nutrition, and plant chemistry. *Ecol. Monogr.* 51:1–20.

RICE, R.L., LINCOLN, D.E., and LANGENHEIM, J.H. 1978. Palatability of monoterpenoid compositional types of *Satureja douglasii* to a generalist molluscan herbivore, *Ariolimax dolichophallus*. *Biochem. Syst. Ecol.* 6:45–53.

SCRIBER, J.M., and SLANSKY, F. 1981. The nutritional ecology of immature insects. *Annu. Rev. Entomol.* 265:183–211.

SLANSKY, F., and FEENY, P.P. 1977. Stabilization of the rate of nitrogen accumulation by larvae of the cabbage butterfly on wild and cultivated plants. *Ecol. Mongr.* 47:209–228.

SMITH, D.S., and NORTHCOTT, F.E. 1951. The effects on the grasshopper, *Melanoplus mexicanus mexicanus* (Sauss.) (Orthoptera: Acrididae), of varying the nitrogen content in its food plant. *Can. J. Zool.* 29:297–304.

SOO HOO, C.F., and FRAENKEL, G. 1966. The consumption, digestion and utilization of food plants by a polyphagous insect, *Prodenia eridania* (Cramer). *J. Insect Physiol.* 12:711–730.

SWAIN, T. 1977. Secondary compounds as protective agents. *Annu. Rev. Plant Physiol.* 28:479–501.

WHITE, T.C.R. 1978. The importance of a relative shortage of food in animal ecology. *Oecologia* 33:71–86.

Williams, K.S. 1983. The coevolution of *Euphydryas chalcedona* butterflies and their larval host plants III. Oviposition behavior and host plant quality. *Oecologia* 56:336–340.

ZUCKER, W.V. 1983. Tannins: Does structure determine function? An ecological perspective. *Am. Nat.* 121:335–363.