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EFFECT OF IRIDOID GLYCOSIDE CONTENT ON OVIPOSITION HOST PLANT CHOICE AND PARASITISM IN A SPECIALIST HERBIVORE

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Abstract—The Glanville fritillary butterfly *Melitaea cinxia* feeds upon two host plant species in Åland, Finland, *Plantago lanceolata* and *Veronica spicata*, both of which produce iridoid glycosides. Iridoids are known to deter feeding or decrease the growth rate of many generalist insect herbivores, but they often act as oviposition cues to specialist butterflies and are feeding stimulants to their larvae. In this study, two iridoid glycosides (aucubin and catalpol) were analyzed by micellar electrokinetic capillary chromatography. We measured the spatial and temporal variation of iridoid glycosides in natural populations of the host plants of *M. cinxia*. We also analyzed the aucubin and catalpol content in plants in relation to their use by ovipositing females, and in relation to the incidence of parasitism of *M. cinxia* larvae in natural populations. The mean concentrations of aucubin and catalpol were higher in *P. lanceolata* than in *V. spicata*, and catalpol concentrations were higher than aucubin concentrations in both host species. *Plantago lanceolata* individuals that were used for oviposition by *M. cinxia* had higher aucubin concentrations than random plants and neighboring plants. Additionally, oviposition and random plants had higher catalpol concentrations than neighboring plants, indicating that ovipositing females select for high iridoid glycoside plants or that oviposition induces iridoid glycoside production in *P. lanceolata*. Parasitism by the specialist parasitoid wasp *Cotesia melitaearum* occurred most frequently in larval groups that were feeding on plants with low concentrations of catalpol, irrespective of year, population, and host plant species. Therefore,

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parasitoids appear to avoid or perform poorly in host larvae with high catalpol content.

Key Words—Åland, aucubin, catalpol, *Cotesia melitaearum*, iridoid glycosides, oviposition, *Melitaea cinxia*, parasitism, *Plantago lanceolata*, *Veronica spicata*.

INTRODUCTION

Secondary compounds in plants often vary considerably both qualitatively and quantitatively among plant individuals and populations, as well as temporally and spatially (e.g., Puttick and Bowers, 1988; Jones and Firn, 1991; Bowers and Stamp, 1992; Bowers et al., 1992; Herms and Mattson, 1992; van Tienderen, 1992; Adler et al., 1995; Darrow and Bowers, 1997). Chemical defense by secondary compounds necessarily affects herbivores, but generalist herbivores and pathogens are generally more severely affected than specialist herbivores (e.g., Bernays and De Luca, 1981; Bowers and Puttick, 1988; Puttick and Bowers, 1988; Zangerl and Berenbaum, 1993; Koricheva et al., 1998). In this paper, we focus on iridoid glycosides, which are one important group of secondary defensive compounds produced by plants.

Iridoids are optically active cyclopentanoid monoterpenes, which can be divided into four distinct groups according to differences in chemical structure: iridoid glycosides, nonglycosidic (aglycone) iridoids, secoiridoids, and bisiridoids. Several hundred different structures of iridoid glycosides have been identified (El-Naggar and Beal, 1980; Boros and Stermitz, 1990, 1991), making them the most numerous iridoids. In this study, we are concerned with two iridoid glycosides, aucubin and catalpol, which are found in the plant families Apocynaceae, Bignoniaceae, Buddleiaceae, Callitrichaceae, Cornaceae, Eucommiaceae, Globulariaceae, Hippuridaceae, Lentibulariaceae, Loganiaceae, Orobanchaceae, Plantaginaceae, Scrophulariaceae, and Verbenaceae (El-Naggar and Beal, 1980). Catalpol is biosynthetically derived from aucubin (Damtoft et al., 1983).

Iridoids are deterrent to or decrease the growth rate of many generalist insect herbivores (Bernays and De Luca, 1981; Bowers and Puttick, 1988; Puttick and Bowers, 1988), and iridoid glycosides in nectar have been shown to deter nectar thieves (Stephenson, 1981, 1982). There is some indication that herbivory may induce the production of iridoid glycosides in *Plantago lanceolata* leaves, but variation in iridoid glycoside concentration with leaf age appears to be much greater (Darrow and Bowers, 1999; Stamp and Bowers, 2000). The iridoid glycosides aucubin and catalpol have been shown to be oviposition cues for the specialist butterfly *Junonia coenia* (Pereyra and Bowers, 1988). Moreover, iridoid glycosides are feeding stimulants to specialist butterfly larvae (Bowers, 1983, 1984; Adler et al., 1995), which grow and survive less (Bowers, 1984; Bowers and Puttick, 1988) or even refuse to feed on a diet without the stimulant iridoid glycosides

(L'Empereur and Stermitz, 1990b). However, Camara (1997a) has shown that there is a cost of chemical defense in the specialist *J. coenia* feeding on food with high iridoid glycoside concentration. Of the two focal iridoid glycosides in this study, catalpol is more toxic to generalist herbivores than aucubin (Bowers and Puttick, 1986, 1988; Bowers, 1991, 1992).

Specialist herbivores sequester iridoids, presumably to use as defense against natural enemies. Melitaeini butterflies sequester iridoids from their host plants as larvae and, at least in some cases, retain the toxic or noxious compounds as adults (Bowers, 1980, 1991; Bowers and Puttick, 1986; Stermitz et al., 1986; Gardner and Stermitz, 1988; Belofsky et al., 1989; Bowers and Farley, 1990; L'Empereur and Stermitz, 1990a,b; Bowers and Collinge, 1992; Dyer, 1995). The sequestration of iridoids by caterpillars has been shown to deter generalist predators like birds (Bowers, 1980), and *Junonia coenia* caterpillars fed with diets containing a high concentration of iridoid glycosides are rejected by several ant species (Dyer and Bowers, 1996; Camara, 1997c), stink bugs and predatory wasps (Stamp, 1992), and spiders (Theodoratus and Bowers, 1999).

The development of parasitoids can be influenced by plant secondary compounds eaten by their herbivorous hosts (Awmack and Leather, 2002). For instance, the alkaloid tomatine in host diet reduces rate of eclosion, size, and longevity of the generalist parasitoid *Hyposoter exiguae* (Campbell and Duffey, 1979, 1981), and nicotine, another alkaloid, in the diet of the host *Manduca sexta* reduces the survival and increases the development time of the parasitoids *Cotesia congregata* and *Hyposoter annulipes* (Barbosa et al., 1986; Thorpe and Barbosa, 1986). Specialist parasitoid wasps cannot avoid the plant secondary compounds sequestered by their hosts. In fact, specialist natural enemies may avoid generalist competitors by using chemically defended hosts. However, there are few studies of the effects of plant-derived chemicals in the host on the performance of specialist parasitoid wasps. Barbosa et al. (1986) demonstrated that the specialist parasitoid *C. congregata* performs better with nicotine than does the generalist parasitoid *H. annulipes*, but there are insufficient data on other species to generalize. It is likely that there is some physiological cost to metabolizing or otherwise enduring the compounds sequestered by the host; hence, the performance of immature parasitoids, even those specialized to use only chemically defended hosts, may depend at least to some extent on the concentration of the sequestered compounds. If this is so, then variation in secondary compounds among plant individuals may cause variation in successful parasitism of herbivores by specialist parasitoids.

The focal herbivore species in this study is the melitaeine butterfly *Melitaea cinxia*, which feeds on two host plant species, *Plantago lanceolata* and *Veronica spicata*, in our study area on the Åland Islands, in Southwest Finland (Hanski, 1999). Both host plant species contain aucubin and catalpol. The possible role of iridoid glycosides as oviposition cues or larval feeding stimulants has not been studied. However, larvae refuse to feed on artificial diet without host plant material

added, which suggests a role for iridoid glycosides in larval feeding (M. Camara, personal communication).

Iridoid glycoside concentrations are known to be genetically controlled in *P. lanceolata* (Marak et al., 2000) and to vary among populations, among individuals, and among genotypes within populations. Iridoid glycoside concentration also varies with the developmental state of the plant, including leaf and plant age, and attributes of the environment such as time of day, weather, soil nutrient conditions, and presence of arbuscular mycorrhizal fungi (Teramura, 1983; Bos et al., 1986; Bowers, 1991; Bowers and Stamp, 1992, 1993; Bowers et al., 1992; Fajer et al., 1992; van Tienderen, 1992; Stamp and Bowers, 1994; Gange and West, 1994; Adler et al., 1995; Darrow and Bowers, 1997). Here, we report on a study in which we measured the spatial and temporal variation of aucubin and catalpol in the host plants of *M. cinxia* in natural populations. We analyzed the aucubin and catalpol content in plants with respect to oviposition by female *M. cinxia* and in relation to the incidence of parasitism of larvae in natural populations.

METHODS AND MATERIALS

Study Area and Focal Species. The main Åland Islands in Southwest Finland have ca. 1000 km^2 of land area. Open meadows and pastures, which contain the host plants of *Melitaea cinxia,* form a fragmented habitat. The mean and median patch sizes are 1502 m^2 and 300 m^2, respectively (for a thorough description of the study area see Nieminen et al., 2003).

Melitaea cinxia (L.) (Lepidoptera: Nymphalidae) uses two plant species as hosts in Åland, *Plantago lanceolata* L. and *Veronica spicata* L. Both plant species are currently considered to be in the family Plantaginaceae (see Judd et al., 1999; Olmstead et al., 2001). *Plantago lanceolata* is widespread in Åland, occupying ca. 4000 meadows. *Veronica spicata* has a more restricted distribution, occurring in ca. 630 meadows. It has many populations in the northern, northwestern, and western parts of Åland but is completely absent from eastern Åland. Between these areas, there are sporadic occurrences of *V. spicata*. *Melitaea cinxia* lays eggs on the two host plants in June. The larvae live gregariously in silken webs until the last instar the following May [for a detailed description of the life cycle see Hanski (1999)]. On average, ovipositing females prefer *V. spicata* to *P. lanceolata* in *V. spicata*'s main distribution area, have no oviposition preference in the middle part of Åland, and prefer *P. lanceolata* in the east where only *P. lanceolata* is present (Kuussaari et al., 2000).

Cotesia melitaearum (Wilkinson) (Hymenoptera: Braconidae) is a specialist parasitoid of checkerspot butterfly larvae. In the Åland Islands, it uses *M. cinxia* as a host, living as small populations in several tightly clustered habitat patch networks (Lei et al., 1997; van Nouhuys and Hanski, 2002). The parasitoid is an important

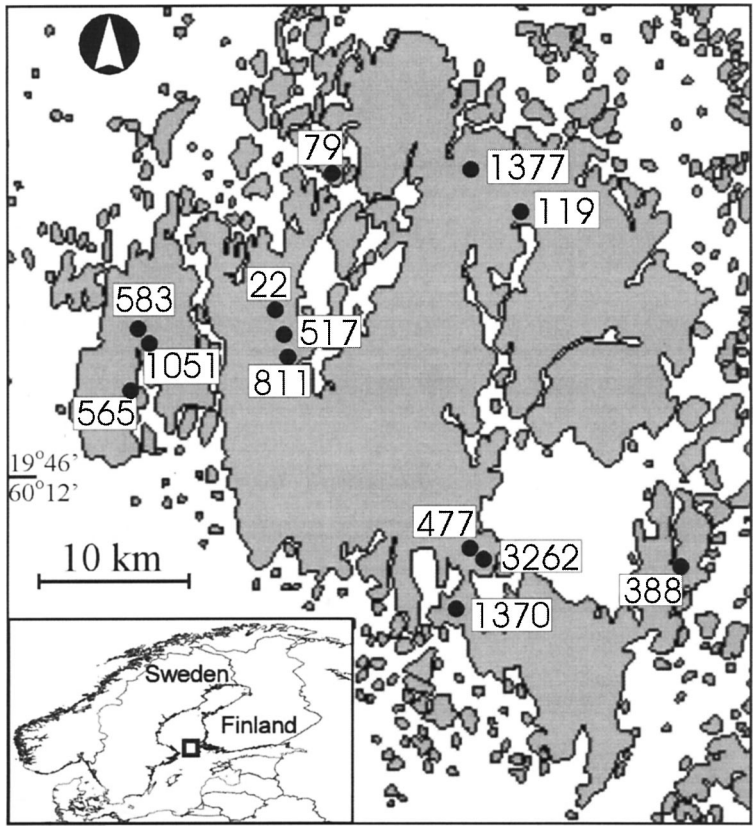

FIG. 1. Locations of sample sites in the Åland Islands. Inset map shows the position of Åland Islands in North Europe.

mortality agent for the butterfly, because the host larvae develop gregariously and there are two to three parasitoid generations per host generation (year). The parasitoid has been observed to cause local extinctions of host populations (Lei and Hanski, 1997).

Plant Samples. We took leaf samples from 13 habitat patches in different parts of Åland (Figure 1) at three times during the growing season in 1999 (Table 1). We selected habitat patches that were geographically representative and occupied by the butterfly. Randomly selected plant individuals were sampled from all parts of the habitat patches. Each *P. lanceolata* sample was an entire plant individual. *Veronica spicata* are larger, hence we collected two randomly selected stems from each plant, separating the leaves from the stems in the field.

TABLE 1. HOST PLANT INDIVIDUALS FOR WHICH IRIDOID GLYCOSIDE CONCENTRATIONS WERE ANALYZED[a]*

	Early summer, random		Mid-summer						Late summer, random	
			Oviposition		Neighboring		Random			
Patch	*Pl**	*Vs*	*Pl*	*Vs*	*Pl*	*Vs*	*Pl*	*Vs*	*Pl*	*Vs*
22		17		3	4	14	17	16	17	18
79			2	1			6	4		
119			2	1	15	5	4	6		
388	26	—					18	—	18	—
477				3	4		3	1		
517				2		12	3	5		
565			2	2	5	8	4	6		
583			24	4	19	10	4	4		
811							8	2		
1051							18	20	18	18
1370			1	—	9	—	10	—		
1377			3	2		14	4	6		
3262			2		5	2	6	4		

[a] Sample sizes from different habitat patches at different times of the season; for locations of the patches see Figure 1. **Pl* = *Plantago lanceolata*, *Vs* = *Veronica spicata*,—= corresponding host species missing from the habitat patch, empty cell = no material sampled.

We collected leaves into paper bags in the field and then left them in open plastic boxes between sheets of tissue paper to dry. In the laboratory, we stored plant samples in a freezer until analysis. After pretreatment, the extracts were analyzed as quickly as possible. If storage was necessary, they were stored at 4°C until analysis.

Host Plants Used for Oviposition. We collected data on host plant individuals used by egg-laying females from 10 habitat patches (Table 1, Figure 1) in order to study the factors that affect the oviposition plant use by female *M. cinxia*. We selected a random starting point within each patch, and around that point all host individuals were systematically searched for egg batches until at least two were found, or all plants had been investigated. If a patch had more than one separate subarea with host plants, the same procedure was repeated in each. If there was only one area with host plants, we searched the area from the random starting point until at least six egg batches were found, or all plants had been investigated. Plants that contained egg batch(es) or freshly hatched first instars are called oviposition plants. Fewer leaves were sampled from individual oviposition plants than from the other plants in order to avoid *M. cinxia* larval mortality due to starvation. These samples consisted of several leaves of different ages to avoid any potential bias due to leaf age.

In order to compare the characteristics of plants receiving eggs with those that did not, we sampled the 10 nearest host plants to each oviposition plant. These plants are called neighboring plants. Additionally, we sampled ten randomly selected plant individuals of each species from different parts of the patch. These plants are called random plants. Random plants were selected by throwing a stick (with one end sharpened) into the air, then throwing it again from the place where it dropped, and then sampling the host plant individual nearest to the sharp end of the stick. None of the random plants had eggs on them. These plants were also used for the measurement of seasonal and geographical variation in iridoid glycoside concentrations (the percentage of iridoid glycosides in dry weight) explained above.

We sampled oviposition, neighboring, and random plants for the determination of iridoid glycoside concentration as described above, except oviposition *P. lanceolata* plants of which maximally half of the leaves were sampled. Before taking leaf samples, we measured the following plant parameters: size (maximum diameter and height), number of stems for *V. spicata*, and number of leaves for both species (for *V. spicata* in two randomly selected stems), freshness (scale: 1 = completely fresh, 2 = withering, 3 = severely withered), and hairiness of the plant (scale: 1 = no hairs, 2 = moderately hairy, 3 = very hairy), growth form (mainly horizontal or erect leaves), flowering status, surrounding vegetation height, and percentage coverage of bare ground/rocks, lichen/moss, host plants, low herbs, low grasses, tall herbs, tall grasses, and scrub within a 30-cm radius. We also measured the location of the egg batch on the oviposition plant and the size of the leaf on which the egg batch was laid. We analyzed iridoid glycosides from all oviposition plants, but only a random subset of neighboring and random plants. The sample sizes from different habitat patches are given in Table 1.

Larval Samples. To detect whether *M. cinxia* larvae sequester iridoid glycosides from their food, we sampled prediapause *M. cinxia* larvae in July 1999 and postdiapause larvae in May 1999. We also analyzed postdiapause larvae reared on *P. lanceolata* in the laboratory in 2000. Larvae were starved for at least a day before freezing, dried at 50°C, and pretreated with the hot water extraction method optimized by Suomi et al. (2001).

Parasitism Rate. We measured the association of aucubin and catalpol concentration in individual plants with parasitism of *M. cinxia* larvae feeding on them in natural populations. In autumn 1999, we took leaf samples from all 34 plants with larval groups on them in patch 576, and all 22 plants with larval groups on them in patch 583. The samples were stored as described above, and the percent aucubin and catalpol in each sample was measured using the same procedure as described below. In spring 2000, we searched each of these larval groups for parasitoid cocoons. In autumn 2000, we took leaf samples from all plants with nests in four more populations occupied by the parasitoid (a total of 133 samples from patches 576, 583, 875, and 1071), and again searched for cocoons in the

following spring. Each population was known to be occupied by the parasitoid *C. melitaearum*. *Veronica spicata* occupied by larvae were sampled by collecting one stem (containing young and old leaves). *Plantago lanceolata* were sampled by collecting one young, one medium, and one old leaf. A few larval groups in each population were on plants that were too small to be sampled.

Reagents and Apparatus. We determined aucubin and catalpol concentrations using standards donated by Dr. S. R. Jensen (Department of Organic Chemistry, Danish Technical University, Lyngby, Denmark) with either an Hewlett-Packard ^{3F}CE capillary electrophoresis system (Agilent Technologies, Waldbronn, Germany) or a Beckman P/ACE 2000 capillary electrophoresis system (Beckman Instruments Inc., Palo Alto, California, USA) with a Compaq Prolinea 5100e computer system. The compounds had been extracted from plant material with ethanol (Damtoft et al., 1997), purified by RP-HPLC, and identified by UV detection at wavelengths 206 and 254 nm. We pretreated the plant and larval samples by hot water extraction in a heating block for test tubes.

Disodium tetraborate (borax, $Na_2B_4O_7 \cdot 10H_2O$) and standard solutions of 0.10 M and 1.0 M NaOH were obtained from Merck (Darmstadt, Germany). Sodium dodecyl sulfate (SDS, 99% pure) was supplied by BDH (Poole, UK). CHES [2-(*N*-cyclohexylamino)ethanesulfonic acid, $C_8H_{17}NO_3S$] was purchased from Sigma (St. Louis, Missouri, USA). The water used in the experiments was first distilled and then purified further with a Water-I instrument (Gelman Sciences, Ann Arbor, Michigan, USA) until its resistance was 18 MΩ.

Iridoid Glycoside Concentrations. In addition to iridoid glycosides, the plant samples contain hundreds of other substances, some of which can interfere with the analyses. We used a sample pretreatment to extract the iridoid glycosides before analysis. Most researchers have used methanol or ethanol as extraction solvents for iridoid glycosides (Gardner and Stermitz, 1988; Camara, 1997c; Damtoft et al., 1997). However, hot water extraction (HWE) is an excellent pretreatment method for the isolation of iridoid glycosides because no organic solvents are needed, the extraction is simple, and the extraction of aucubin and catalpol is more quantitative than when extracted using alcohols (Suomi et al., 2000). In the optimization of the pretreatment methods for heat-dried plant (Suomi et al., 2000) and larval samples (Suomi et al., 2001), we compared the results with those obtained by employing the more commonly used alcohol extraction technique. For larval samples, the hot water extraction was slightly more quantitative than extraction with methanol, while for plant samples the HWE was significantly more efficient than the reference method.

Iridoid glycosides generally tend to be hydrolyzed and subsequently undergo rearrangement even under mildly acidic conditions (Bianco, 1990). Thus, they must be treated and analyzed under strictly basic conditions. Still, some structures are more unstable and may hydrolyze even under basic conditions or if heated (Inouye, 1991). Usually, iridoid glycosides are analyzed with chromatographic techniques

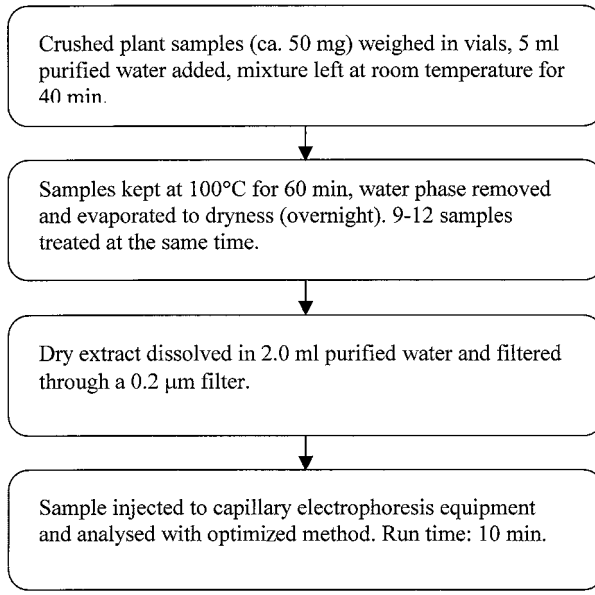

FIG. 2. Scheme of the pretreatment of plant samples. The weight of the sample was measured with an accuracy of 0.1 mg.

(gas chromatography, liquid chromatography, or thin-layer chromatography), but capillary electrophoresis (CE) with high speed and resolution was employed in this study. There are several different capillary electromigration techniques, of which micellar electrokinetic capillary chromatography (MECC) is suitable for neutral compounds like catalpol and aucubin. The separation in CE is based on different mobilities of the analytes in a high electric field across a narrow, fused silica capillary that is filled with an electrolyte solution. The mobilities depend on the size and charge of the compound. In MECC, the separation of neutral analytes is mainly based on partition between a micellar pseudostationary phase and the electrolyte solution.

A diagram of the hot water extraction procedure (Suomi et al., 2000) is shown in Figure 2, and the CE analysis conditions are listed in Table 2. We measured linearity using solutions of pure aucubin and catalpol prepared in purified water. The solutions were stored in a refrigerator when not in use. We observed no degradation of the molecules during a 4-month period. The same samples were analyzed with both CE instruments, verifying that the results were comparable.

In the Hewlett-Packard electrophoresis experiments, the concentration of micellar forming agent SDS was optimized. In the Beckman experiments, the concentrations of both components of the electrolyte solution were optimized.

TABLE 2. MICELLAR ELECTROKINETIC CAPILLARY CHROMATOGRAPHY (MECC) ANALYSIS CONDITIONS FOR QUANTITATIVE DETERMINATION OF AUCUBIN AND CATALPOL

Parameter	Hewlett-Packard 3F CE	Beckman P/ACE 2000
Capillary dimensions	50 μm ID, 375 μm OD, 41.5 cm to detection window, total length 50.0 cm	50 μm ID, 375 μm OD, 40.0 cm to detection window, total length 47.0 cm
Electrolyte solution	50 mM CHES, 120 mM SDS, pH 9.4	30 mM borax, 140 mM SDS, pH 9.4
Sample injection	Pressure: 50 mbars $\times$ 5 sec	Pressure: 35 mbars $\times$ 10 sec
Voltage and current	+15 kV, 35 μA	+15 kV, 90 μA
Analysis time	<5 min	<7 min
Limit of detection	Catalpol: 30 μg/ml Aucubin: 20 μg/ml	Catalpol: 20 μg/ml Aucubin: 20 μg/ml
Detection	200 nm, reference 250 nm	200 nm
Linearity	50–300 μg/ml for both compounds	50–400 μg/ml for both compounds
Analysis temperature	25°C	25°C
Cooling of the capillary	By air flow	By fluoro-organic fluid flowing outside the capillary
Detector	Diode array detector (spectra could be taken)	Single wavelength detector (six possible wavelengths)

The electrolyte was changed to borax to facilitate manual integration of analyte peaks.

Between analyses, the capillary was rinsed with the electrolyte solution for 8 min, and after three successive analyses it was rinsed with 0.1 M NaOH (2 min), water (5 min), and electrolyte solution (2 min). This was done to avoid blockage. The capillary was regenerated each morning by rinsing it with 0.1 M NaOH (10 min), water (15 min), and electrolyte solution (15 min). This was also done when the capillary was changed.

Statistical Methods. Iridoid glycoside concentrations were square-root transformed to normalize their distributions. For normally distributed data, we used one- or two-way analysis of variance, analysis of covariance, linear regression, *t* test, or Pearson correlation. To study the effects of plant category and freshness with their interaction, we used parametric ANOVA on ranks, because we were not able to normalize the iridoid glycoside distributions in this case. We analyzed the effects of the two iridoid glycosides on different variables using multivariate analysis of variance (MANOVA) because the iridoid concentrations are correlated. We also report the results of separate univariate ANOVA F tests for catalpol and aucubin with Bonferroni correction for significance (adjusted critical P value is 0.025). For testing goodness of fit in 2×2 tables, we used Fisher's exact test. We measured

the association between parasitism (presence/absence) and plant iridoid glycoside concentration using logistic regression. Year, population, host plant species, and the iridoid glycoside concentration were included in the logistic model as explanatory variables. We used SAS 6.0, Statistix 7, and Systat 8.0 for the statistical analyses.

RESULTS

Spatial and Temporal Variation in Iridoid Glycoside Concentrations in Host Plants. Figure 3 shows the mean aucubin and catalpol concentrations of both host plants for various classifications of the data. There was a significant positive correlation between aucubin and catalpol concentrations in both hosts (all samples included), but the correlation was stronger in *P. lanceolata* ($r = 0.761$, $P < 0.001$, $N = 347$) than in *V. spicata* ($r = 0.496$, $P < 0.001$, $N = 277$). The mean concentrations of aucubin and catalpol were higher in *P. lanceolata* than in *V. spicata* (all samples: Table 3; Figure 3A), and catalpol concentrations were higher than aucubin concentrations in both host species (all samples: Table 3; Figure 3A).

In early summer, the concentrations of aucubin and catalpol in randomly selected individuals of the two plant species did not differ between species (Table 3, univariate test; Figure 3B). However, because aucubin was slightly lower in *V. spicata* and catalpol slightly higher than in *P. lanceolata*, the overall iridoid makeup was different (Table 3, multivariate test; Figure 3B). Both aucubin and catalpol concentrations were higher in *P. lanceolata* than in *V. spicata* in mid- and late summer (Table 3; Figure 3B).

In randomly selected *P. lanceolata* individuals, there was no difference during the season in aucubin concentrations, but catalpol concentrations were higher in late and mid-summer than in late spring (Table 3; Figure 3B). In random *V. spicata* individuals, there was no difference during the season, either in aucubin or in catalpol concentrations (Table 3; Figure 3B).

Aucubin concentrations in random *P. lanceolata* differed among four habitat patches in mid-summer (Table 3; Figure 3C). There was no evident spatial pattern in this variation, because only patches 1370 (highest mean concentration) and 1051 (lowest mean concentration) differed significantly from each other (patches 22 and 388 did not differ from any other patch; Figure 1). There was also a significant difference in catalpol concentrations between the highest (patch 1370) and the lowest (388) mean concentration, and between patches 1370 and 1051 (Table 3; Figure 3C). In late summer, there were no differences among three habitat patches sampled (22, 388, and 1051) in aucubin or catalpol concentrations (Table 3; Figure 3D).

In random *V. spicata*, aucubin concentrations differed among five patches in mid-summer (Table 3; Figure 3C). Again, there was no apparent spatial pattern in

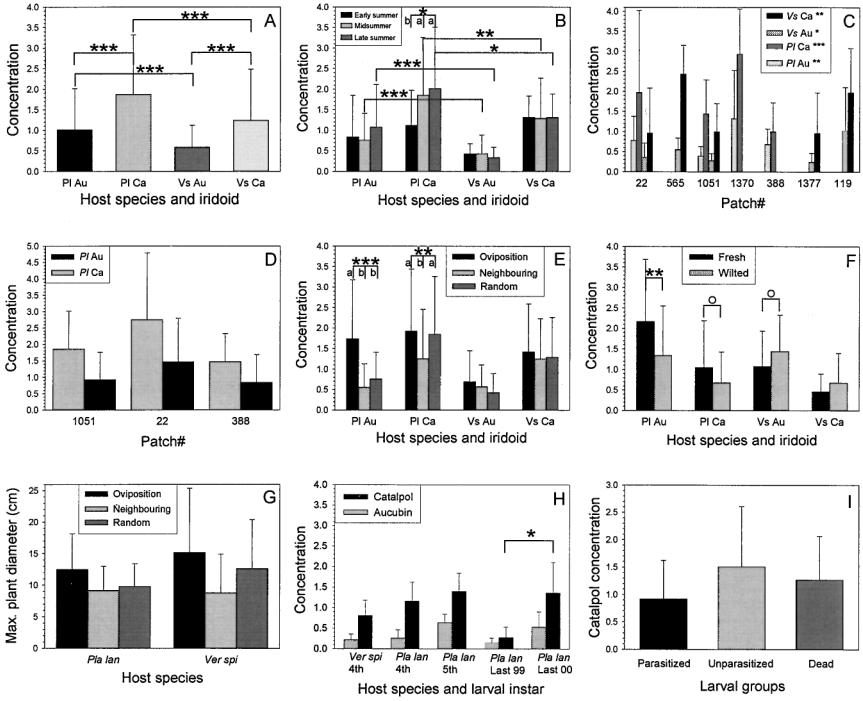

FIG. 3. (A) Average aucubin (Au) and catalpol (Ca) concentrations with standard deviations in all samples of *Plantago lanceolata* (Pl) and *Veronica spicata* (Vs). (B) Concentrations at three times during the growing season in random plants. (C) Concentrations in several habitat patches in mid-summer (random plants). See Figure 1 for the locations of the patches. (D) Concentrations in some habitat patches in late summer (only *P. lanceolata* samples; random plants). (E) Concentrations in oviposition, neighboring, and random plants in mid-summer. (F) Concentrations in completely fresh and somewhat wilted plant individuals in mid-summer (oviposition, neighboring, and random plants). (G) Average sizes with standard deviations of oviposition, neighboring, and random plants of both host plant species. The size measure used was the maximum diameter of the plant individual. *Plantago lanceolata* individuals used for oviposition were larger than neighboring plants, but random plants did not differ from oviposition or neighboring plants ($F = 3.24$, $df = 119$, $P = 0.043$). (H) Average aucubin and catalpol concentrations in *M. cinxia* larvae of different instars and fed with different hosts (99 = year 1999, 00 = year 2000). (I) Average catalpol concentrations with standard deviations in host plants with *M. cinxia* larval groups either parasitized by *C. melitaearum*, unparasitized or dead during winter. (See Table 3 for exact results of statistical tests. $^{\circ}P = 0.05$-0.025; $^{*}P < 0.025$; $^{**}P < 0.01$; $^{***}P < 0.001$; a and b indicate statistically significantly different comparisons (Tukey's test).)

TABLE 3. SUMMARY OF STATISTICAL TEST RESULTS[a]

Comparison	Panel	Test statistic	*df*	*P*
P. lanceolata versus *V. spicata*[b]				
Multivariate	A	20.4	2, 622	<0.001
Aucubin	A	37.3	1, 623	<0.001
Catalpol	A	30.7	1, 623	<0.001
Aucubin versus catalpol[b]				
P. lanceolata	A	$T = 19.2$	346	<0.001
V. spicata	A	$T = 15.9$	277	<0.001
P. lanceolata versus *V. spicata* in early summer[c]				
Multivariate	B	6.15	2, 40	0.005
Aucubin	B	2.43	1, 41	0.13
Catalpol	B	1.74	1, 41	0.19
P. lanceolata versus *V. spicata* in mid-summer[c]				
Multivariate	B	9.46	2, 175	<0.001
Aucubin	B	19.0	1, 176	<0.001
Catalpol	B	8.59	1, 176	0.004
P. lanceolata versus *V. spicata* in late summer[c]				
Multivariate	B	10.4	2, 86	<0.001
Aucubin	B	20.7	1, 87	<0.001
Catalpol	B	5.80	1, 87	0.018
Seasonal change in *P. lanceolata*[c]				
Multivariate	B	4.00	4, 360	0.003
Aucubin	B	1.64	2, 180	0.20
Catalpol	B	4.49	2, 180	0.013
Seasonal change in *V. spicata*[c]				
Multivariate	B	1.34	4, 248	0.26
Aucubin	B	1.09	2, 124	0.34
Catalpol	B	0.59	2, 124	0.56
Between patches in *P. lanceolata* in mid-summer[c]				
Multivariate	C	4.99	6, 118	<0.001
Aucubin	C	4.28	3, 59	0.008
Catalpol	C	6.13	3, 59	0.001
Between patches in *P. lanceolata* in late summer				
Multivariate	D	1.50	4, 100	0.21
Aucubin	D	1.66	2, 50	0.20
Catalpol	D	2.92	2, 50	0.063
Between patches in *V. spicata* in mid-summer[c]				
Multivariate	C	3.15	8, 98	0.003
Aucubin	C	3.35	4, 49	0.017
Catalpol	C	4.21	4, 49	0.005
P. lanceolata between oviposition, neighbor, and random				
Multivariate	E	16.7	4, 396	<0.001
Aucubin	E	19.3	2, 198	<0.001
Catalpol	E	6.26	2, 198	0.002
V. spicata between oviposotion, neighbor, and random				
Multivariate	E	1.50	4, 304	0.20
Aucubin	E	1.56	2, 152	0.21
Catalpol	E	0.07	2, 152	0.94

TABLE 3. CONTINUED

Comparison	Panel	Test statistic	*df*	*P*
Between fresh and wilted *P. lanceolata*[b]				
Multivariate	F	4.50	2, 117	0.013
Aucubin	F	8.94	1, 118	0.003
Catalpol	F	4.39	1, 118	0.038
Between fresh and wilted *V. spicata*[b]				
Multivariate	F	2.28	2, 102	0.11
Aucubin	F	4.09	1, 103	0.046
Catalpol	F	2.62	1, 103	0.11
Between fourth instar larvae on *P. lanceolata* and *V. spicata*				
Multivariate	H	1.66	2, 18	0.22
Aucubin	H	0.33	1, 19	0.58
Catalpol	H	3.36	1, 19	0.08
Between last instar larvae in 1999 and 2000				
Multivariate	H	8.05	2, 7	0.015
Aucubin	H	4.41	1, 8	0.07
Catalpol	H	10.4	1, 8	0.012

[a] Panel = panels as in Figure 3; test statistic = F test results from univariate ANOVA (Bonferroni-adjusted critical P value is 0.025), if no other test statistic is mentioned, or multivariate ANOVA (= Pillai test results).
[b] All samples.
[c] Random plants (in early and late summer only random plants could be sampled).

the differences, as only patches 119 (highest mean concentration) and 1051 (second lowest mean concentration) differed from each other, but not from patches 565, 22, or 1377 (Figure 1). Catalpol concentrations also varied among habitat patches but without any spatial pattern (Table 3; Figure 3C). Only patch 565 (highest concentration) differed from patches 1051 (third lowest mean concentration) and 22 (lowest mean concentration), while the other pairwise differences were not significant.

Hosts Used for Oviposition. Thirty-six egg batches or groups of newly hatched larvae were found on *P. lanceolata* and 18 on *V. spicata* in 10 habitat patches (Table 1). *Plantago lanceolata* individuals that were used for oviposition by *M. cinxia* in the field had significantly higher aucubin concentrations than random plants and neighboring plants (Table 3; Figure 3E). Furthermore, oviposition and random plants had higher catalpol concentrations than neighboring plants.

Fresh *P. lanceolata* individuals had higher aucubin and catalpol concentrations than somewhat wilted individuals (Table 3; Figure 3F). Eggs did not appear to be laid preferentially on either fresh or wilted plants (Fisher's exact test: oviposition plants versus neighboring plants $P = 0.30$, oviposition plants versus random

plants $P = 0.54$). In a two-factor ANOVA on ranks, the effects of plant category and freshness on aucubin concentrations were significant, but their interaction was not. Only the effect of plant category on catalpol concentrations was significant in a two-factor ANOVA on ranks. Therefore, female butterflies appear to select higher iridoid glycoside concentrations independently of the freshness of *P. lanceolata* individuals.

Iridoid glycoside concentrations did not differ significantly between fresh and somewhat wilted *V. spicata* plants (Table 3; Figure 3F). Neither plant type, freshness, nor their interaction was significant in two-factor ANOVAs. Fresh plants were used for oviposition in proportion to wilted plants (Fisher's exact test: oviposition plants versus neighboring plants $P = 0.36$, oviposition plants versus random plants $P = 1.0$).

Small *P. lanceolata* individuals had higher aucubin concentrations than large ones (size measure was square-root transformed maximum diameter of the individual host plant; coefficient $= -0.252$, Student's $t = -3.29$, $P = 0.001$, $R^2 = 0.08$). Plants used for oviposition were larger than neighboring plants, but random plants did not differ from oviposition or neighboring plants ($F = 3.24$, $df = 119$, $P = 0.043$; Figure 3G). Aucubin concentrations were higher in oviposition plants independent of plant size, as their interaction was not significant in an analysis of covariance. Plant size was not associated with catalpol concentrations (coefficient $= -0.064$, Student's $t = -1.00$, $P = 0.32$, $R^2 = 0.01$).

There were no significant associations between iridoid glycoside concentrations and the hairiness of leaves or the flowering status of individuals in either of the species.

Larval Samples. The average aucubin and catalpol concentrations in fourth instar larvae in August 1999 were 0.26 and 1.16% of dry weight in *P. lanceolata*-feeding larvae ($N = 14$), and 0.22 and 0.81% in *V. spicata*-feeding larvae ($N = 7$). The differences in iridoid glycoside concentrations were not significant (Table 3; Figure 3H). The average aucubin and catalpol concentrations in the last instar *P. lanceolata*-feeding larvae were 0.16 and 0.27% of dry weight in May 1999 ($N = 5$), and 0.53 and 1.36% in May 2000 ($N = 5$). The difference was different only for catalpol (Table 3; Figure 3H), but due to the small sample size, the results are suspect.

Parasitism Rate. Plants on which larval groups of *M. cinxia* occurred ($N = 189$) were sampled in the autumn of 1999 and 2000. During the subsequent winters, 62 of these larval groups died. Twenty of the remaining 124 groups were parasitized by *C. melitaearum*. Parasitism occurred most frequently in larval groups that were feeding on plants with a low concentration of catalpol (logistic regression, effect of catalpol concentration $F_{1,178} = 4.43$; $P = 0.04$, coefficient $= -0.23$; Wald's $\chi^2 = 26.29$, $P < 0.001$; Figure 3I). This was true irrespective of year, population, and host plant species. We found no association between parasitism and aucubin concentration.

DISCUSSION

Chemical Analyses. The concentrations of aucubin and catalpol in *Plantago lanceolata* and *Veronica spicata* were reliably and quickly determined by MECC. Hot water extraction used for the isolation of the compounds was reproducible and simple. In addition, it avoided the use of organic solvents, which burden the environment. Because the amounts of iridoid glycosides isolated from plant samples were so high, the limit of detection did not cause any problems. In fact, most extracts had to be diluted so that they could be reliably quantified.

Iridoid Glycoside Concentrations in Plants. Aucubin and catalpol concentrations have been intensively studied especially in *P. lanceolata*, which allows comparisons of our results from Finland with studies mainly performed in the United States. For example, the biomass and iridoid glycoside content of *P. lanceolata* were analyzed four times during one growing season in five different populations by Darrow and Bowers (1997). They found no correlation between the iridoid glycoside content and biomass, but both biomass and iridoid glycoside concentration varied among sampling dates and among populations (concentrations increased during the season). Our results showed differences in aucubin concentration among populations in mid-summer. The differences in the mid-summer may be important for ovipositing females, as well as for newly hatched larvae, which are vulnerable to various adverse environmental effects.

In several American studies on *P. lanceolata*, the average content of catalpol in the leaves has been lower than that of aucubin (Darrow and Bowers, 1997, 1999; Stamp and Bowers, 2000), although in one study the reverse was observed (Bowers, 1991). In our data from Finland, catalpol concentrations were higher than aucubin concentrations in mid- and late summer and overall. In both American studies in which plants were sampled from the field, the aucubin concentration was higher [ca. 1.6–2.7% in Bowers (1991), ca. 0.5–5% in Darrow and Bowers (1997)] than in Finland (ca. 0.6–2.2% in this study). Catalpol concentrations were similar to our results in those studies [ca. 0.4–3.6% in Bowers (1991), ca. 0.2–2.2% in Darrow and Bowers (1997), ca. 0.7–2.0% in this study]. Interestingly, in the two American studies in which laboratory-reared plants were used, catalpol concentrations were extremely low (ca. 0–0.6%) (Darrow and Bowers, 1999; Stamp and Bowers, 2000). The iridoid glycoside concentrations in *V. spicata* have not been studied previously. We found that the iridoid glycoside concentrations did not differ over the season, but aucubin did differ among populations.

Stamp and Bowers (1994) found that iridoid glycoside concentrations in *P. lanceolata* increase over the growing season in dry years and decrease in wet years. Therefore, temporal variation in catalpol concentrations in our data could be related to the wet summer of 1998, with low iridoid glycoside concentrations in host plants remaining in the following spring, and to the dry summer of 1999, with high iridoid glycoside concentrations remaining in the next spring. The mid-summer

of 1999 was extremely dry, and many host plants were either completely dry or at least wilted during the oviposition period of *M. cinxia*. However, fresh plants were not used for oviposition more often than wilted ones, even though fresh plants contained more aucubin. As wilting of host plants is detrimental for the newly hatched larvae, it would be beneficial to avoid such plants for oviposition. It is, of course, possible that the host plants wilted after oviposition, but before our sampling.

Role of Iridoid Glycosides in Oviposition. Melitaea cinxia females lay eggs more frequently on *P. lanceolata* with high iridoid glycoside concentrations than on individuals with low concentrations. Females of the butterfly *Junonia coenia* are known to select for *P. lanceolata* with high aucubin and catalpol concentrations for oviposition (Pereyra and Bowers, 1988), and our results suggest the same for *M. cinxia* searching for *P. lanceolata* as the host plant. However, there was no difference in iridoid glycoside concentration among *V. spicata* individuals used for oviposition and random *V. spicata*. Both spatially and temporally, *V. spicata* had more stable iridoid glycoside concentrations than *P. lanceolata*, and the aucubin and catalpol concentrations were also significantly lower. These characteristics may make the identification of high iridoid glycoside plants among *V. spicata* individuals difficult for ovipositing females. There may also be some as yet undetected iridoids, which would explain the host selection, and we are currently searching for them in these plant species.

There is also the possibility that butterflies do not choose iridoid glycoside-rich *P. lanceolata* but that the presence of herbivore eggs induces increased production of iridoid glycosides by the plant. We do not yet have any evidence for this, but iridoid glycoside production can apparently be induced by herbivory in *P. lanceolata* (Darrow and Bowers, 1999; Stamp and Bowers, 2000), and there are examples of induction of plant defense by eggs in other plant herbivore systems (Agrawal, 2000). If this were the case, then perhaps the iridoid glycoside concentrations of neighboring plants, which one might expect to be similar to the oviposition plants, have lower iridoid glycoside concentrations because they have not been induced. Yet another possibility is that the iridoid glycoside levels in the chosen plants are high due to attacks by *M. cinxia* larvae or some other herbivores in the spring or previous summer, which again may cause induced defense.

Larvae of some *Euphydryas* species (close relatives to *M. cinxia*) and *Junonia coenia* sequester catalpol selectively from *P. lanceolata* (Bowers and Puttick, 1986; Bowers and Collinge, 1992), and *E. anicia* even metabolizes another iridoid glycoside into catalpol (Gardner and Stermitz, 1988). Catalpol is especially important to the chemical defense of larvae because it is more toxic to generalist herbivores than aucubin (Bowers and Puttick, 1986, 1988; Bowers, 1991, 1992). *Melitaea cinxia* larvae appear also to sequester catalpol more efficiently than aucubin, as the catalpol–aucubin ratio in *P. lanceolata*-feeding larvae in late summer of 1999 was higher in larvae (6:1) than in the plants (2:1; Figure 3). Interestingly,

M. cinxia larvae appear not to sequester catalpol more efficiently than aucubin from *V. spicata*, as the catalpol–aucubin ratio in *V. spicata*-feeding larvae in late summer of 1999 was the same in larvae (4:1) as in the plants (4:1; Figure 3). This finding requires further research because, as we have seen, plant iridoid glycoside concentration is variable, and the plants used for iridoid glycoside determinations were different individuals from those fed upon by the larvae.

The average catalpol concentration in the *P. lanceolata*-feeding fourth instar larvae was only 1.2% of dry weight, and the maximal concentrations detected in *M. cinxia* larvae so far are ca. 2% of dry weight (Suomi et al., 2001), as opposed to the maximum of 9.3% in *P. lanceolata* in our study. Bowers and Collinge (1992) observed notably higher average catalpol concentrations for larvae of *J. coenia* with a maximum of 3% dry weight in fifth instars. However, the iridoid glycoside concentrations in larvae and adult butterflies seem highly variable. For example, Gardner and Stermitz (1988) found mean catalpol concentrations of 3.1% and 0.8% dry weight in *Euphydryas anicia* males in successive years, and Suomi et al. (2001) noticed that the average catalpol concentrations in last instar *M. cinxia* larvae were 0.3% and 1.4% of dry weight in the springs of 1999 and 2000. These results call for further research, as we have so far not sampled a large number of larvae.

Role of Iridoid Glycoside in Parasitism. As shown above, *M. cinxia* females appear to use *P. lanceolata* with higher iridoid glycoside concentrations for oviposition than is expected by random choice of plant individuals. Moreover, larvae effectively sequester catalpol from *P. lanceolata*, which is probably related to their defense against natural enemies. It has been suggested that natural enemies (predators in this case) are the driving force for the oviposition host preference by female *Junonia coenia*, a species with high iridoid glycoside concentrations in larvae (Camara, 1997b). Van Nouhuys and Hanski (1999) showed that *Cotesia melitaearum*, a specialist parasitoid of *M. cinxia* in Åland, successfully parasitizes larval groups feeding on *V. spicata* more often than larval groups, which feed on *P. lanceolata*. They suggested several possible explanations for this difference, one of which was plant chemistry, but as olfactory cues to locate host larvae. It may be, as iridoid glycosides are used both in plant and herbivore defense against their respective natural enemies, that high concentrations in larvae may function as deterrents to ovipositing *C. melitaearum* or may cause mortality of parasitoids developing within relatively well defended hosts. *Cotesia melitaearum* are specialist parasitoids, only using hosts feeding on iridoid producing plants (Lei et al., 1997; van Nouhuys and Hanski, 2003), so one may expect them to be adapted to high iridoid concentrations. Indeed, laboratory studies using artificial diet suggest that high iridoid glycoside concentration does not reduce acceptance of hosts by *C. melitaearum* adults or the survival of developing *C. melitaearum* larvae (Lei and Camara, 1999).

Nevertheless, our result, that parasitoids are found in *M. cinxia* larval groups feeding on plants with low catalpol concentration, lends support to the latter

hypothesis. A low rate of successful parasitism of larvae feeding on especially high iridoid glycoside *P. lanceolata* might explain why female *M. cinxia* select the latter plants for oviposition. This does not happen with *V. spicata* as the host plant, perhaps because the iridoid concentrations may not be high enough to deter parasitoids and because larvae feeding on *V. spicata* do not appear to be able to accumulate catalpol as efficiently as those feeding on *P. lanceolata*. If such selectivity by the ovipositing butterfly would reduce parasitism by *C. melitaearum* on larvae feeding on *P. lanceolata*, it might also reduce consumption by generalist invertebrate predators and decrease competition with generalist herbivores.

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