Variability in the behavioural responses of three generalist herbivores to the most abundant coumarin in *Daphne laureola* leaves

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Abstract

We evaluated the responses of three generalist herbivores (Lepidoptera: Noctuidae) to the most abundant coumarin in young leaves of spurge-laurel, *Daphne laureola* L. (Thymelaeaceae), a perennial shrub consistently fed upon by several noctuid species. *Pseudenargia ulicis* Staud and *Noctua janthe* Borkhausen are natural herbivores of the species in southeastern Spain, whereas *Spodoptera littoralis* Boisduval is a model species frequently used to address the antifeedant role of secondary compounds. Discrimination between control and coumarin-coated *D. laureola* leaves was investigated by short-term choice experiments. We found species-specific behavioural responses to the treatment: *P. ulicis* preferred the control leaves, whereas *S. littoralis* did not discriminate between leaf treatments, although it clearly avoided coumarin when incorporated into an artificial diet, and *N. janthe* preferred the coumarin-coated leaves. Furthermore, *N. janthe* larvae reduced consumption when the proportion of treated leaves ingested increased and consumption of *S. littoralis* larvae was also reduced in coumarin-containing diet under no-choice conditions. Our results highlight that different herbivore species feeding simultaneously on the same host plant respond differently to a single chemical defence compound, likely constraining a directional response of the plant to selection.

Introduction

Insect herbivores use chemical cues in the process of host selection in an attempt to reduce the cost of information processing (Bernays, 2001) and to obtain a balance between nutrient intake and plant secondary compounds dilution (Hägele & Rowell-Rahier, 1999; Simpson & Raubenheimer, 2001; Behmer et al., 2002). Although insect herbivores are usually described as specialized consumers, they show a wide variation in host ranges (Fox & Morrow, 1981; Bernays & Chapman, 1994; Bernays & Minkenberg, 1997; Zangerl & Berenbaum, 2003), with a weak evolutionary tendency towards specialization within families (Nosil, 2002). The Noctuidae stand out among the most generalist lepidopteran families, and include many pest species such as armyworms, cutworms, and stem borers (Merzheevskaya, 1989). Although noctuid species are frequently used as model insects in toxicological studies, their natural host range, host selection behaviour, and responses to natural variation of secondary compounds of their host species remain largely unknown (Niemelä et al., 1981; Merzheevskaya, 1989; Tune & Dussourd, 2000). In this paper, we analysed the behavioural responses of *Noctua janthe* Borkhausen and *Pseudenargia ulicis* Staud larvae, two species of noctuids feeding on spurge-laurel, *Daphne laureola* L. (Thymelaeaceae), and of *Spodoptera littoralis* Boisduval, a highly polyphagous noctuid pest species not naturally found on it, to the most abundant coumarin in young leaves of spurge-laurel.

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Daphne laureola is a long-lived evergreen shrub, distributed throughout the Palaearctic region, and generally found in the understory of coniferous and mixed montane forests in the Mediterranean area. In the Natural Park of Sierras de Cazorla, Segura y Las Villas (Jaén province, southeastern Spain, 37°56′N, 2°52′W), where the study was conducted, there is a rich fauna of moths with more than 280 species of Noctuidae, and up to 40 reported for a single locality and year (Yela & Herrera, 1993). In this region, *D. laureola* is consumed by four noctuid caterpillars, *Trigonophora flammea* Esper, *Noctua fimbriata* Schreber, *N. janthe*, and *P. ulicis* (Alonso & Herrera, 1996), whose relative abundance varies from year to year. Like most noctuids, these species are generalist consumers that also feed on a variety of other plant species in the study area [e.g., *Helleborus foetidus* L., *Paeonia broteroi* Boissieri & Reuter, *Phillyrea latifolia* L., *Primula vulgaris* Hudson, and *Sorbus torminalis* (L.) Crantz]. Population defoliation levels of *D. laureola* averaged less than 5% of the leaf area, and individual defoliation ranged between 0.9 and 12.8% (Alonso & Herrera, 1996, 2003). Intraspecific variation in defoliation levels has been related to plant architecture (Alonso & Herrera, 1996) and leaf nutrient composition (Alonso & Herrera, 2003). The influence of secondary metabolites on this plant-herbivore system is still unknown.

Coumarins, a group of phenolic compounds, are remarkable among the species secondary metabolites for being toxic to a wide variety of organisms, including bacteria, fungi, invertebrates, and vertebrates (Berenbaum, 1991). Three glycosides of 7-methoxy-coumarin have recently been isolated from *D. laureola* leaves (Alonso et al., 2005). Hence, we analysed the effect of variations in the concentration of the most abundant coumarin in young leaves of *D. laureola*, the 5-O-β-D-glucosyl-7-methoxy-8-hydroxy coumarin (hereafter referred to as coumarin). Our aim was to evaluate the defensive role of this compound under natural conditions. We expected that all species would prefer the untreated leaves if the coumarin functions as an antifeedant against generalist herbivores. Alternatively, only the naïve species *S. littoralis* would respond to the treatment if the coumarin is interpreted by the natural plant consumers as a cue to find a suitable host, as reported for some specialized herbivores feeding on plants characteristically rich in glucosinolates (Hopkins et al., 2009) or furanocoumarins (Berenbaum, 1991). We also analysed larval frass to study coumarin degradation through the larval midgut as a potential mechanism to reduce its toxicity that could vary between natural herbivores and the model pest species studied. Implications of our findings for plant responses to selection by multiple herbivores are discussed.

Materials and methods

Coumarin extraction and quantification

As none of the coumarins present in *D. laureola* leaves is commercially available, we isolated the most abundant one, 5-O-β-D-glucosyl-7-methoxy-8-hydroxy coumarin, from *D. laureola* leaves collected during the previous season and stored it at −80 °C. The procedure to analyse coumarins is detailed in Alonso et al. (2005). Briefly, leaves were extracted with methanol (80%), filtered, and concentrated to dryness under reduced pressure. The residue was dissolved in water and cleaned by sequential decantation with chloroform. Coumarin was purified from the aqueous phase by liquid chromatography with a LiChroprep C_{18} column (Loβar 40–63 μm; Merck, Darmstadt, Germany) and a mixture of water:methanol:glacial acetic acid (84.8:14.2:1) as mobile phase. Coumarin was re-purified by an HPLC (Waters 2690, 996 PDA; Waters Cromatografía, Barcelona, Spain) with a μ-Bondapack C_{18} column (Waters; 7.8 × 300 mm; 10 μm) and the same mobile phase to finally obtain coumarin at a > 95% purity level. Purified coumarin was lyophilized and stored at −20 °C.

Larval frass was collected after each experimental night, weighed, and processed to identify and quantify coumarins. Frass was extracted 24 h with methanol (80%), the extract was concentrated under reduced pressure, and the residue dissolved in 0.5 ml water and centrifuged for 10 min at 10 483 *g*. Solid-phase extraction of 250 μl of supernatant was conducted on packed MFE C_{18} 3/500 columns (Análisis Vínicos, Tomelloso, Spain) prior to HPLC analysis [see Alonso et al. (2005) for details].

Feeding experiments with natural herbivores

Discrimination by noctuid larvae between control and coumarin-coated *D. laureola* leaves was determined by short-term choice experiments during the 2004 feeding season. On 12 May 2004, noctuid caterpillars (n = 17 *N. janthe* and 18 *P. ulicis*) were collected at sunset from *D. laureola* plants in the study area and placed individually into 15-cm labelled Petri dishes without food. We could not find sufficient individuals of *T. flammea* or *N. fimbriata* to be included in this experiment. The age of the naturally-collected larvae could not be determined. Leaves were collected simultaneously from five hermaphroditic plant individuals. Trials started the following night, following the normal activity pattern of noctuid larvae, and the same individual larvae were tested for two consecutive nights to check for consistency in behaviour.

In order to minimize other sources of natural variation in leaf characteristics that could interfere with our specific treatment, individual larvae were tested using a single

current-season leaf cut in halves; one half was coated with methanol and the other with a coumarin solution at a concentration of 15 mg ml^{-1}. We aimed for an increase in coumarin concentration of 30 mg g^{-1} dry mass, to maintain the concentration of treated leaves within the natural range of variation [see Alonso et al. (2005) for details]. Following Ayres et al. (1997), the volume of coumarin solution applied was calculated so that it was proportional to the estimated leaf dry mass, following the equation:

$$V \ (\mu l) \ \times \ 15 \, \mu g \, \mu l^{-1} = [\text{fresh mass (mg)} \ \times \\ (1 \ - \ \text{water content})] \times 30 \, \mu g \, mg^{-1}.$$

Fresh mass of each experimental half-leaf was determined to the nearest 0.01 mg, and a set of 18 leaves was dried in an oven and used to estimate the average water content (0.77) of the tested leaves. The coumarin solution was pipetted and spread evenly on the upper leaf surface with a fine brush previously dipped in methanol, and left to dry before offering leaves to larvae. The effectiveness of the treatment was subsequently checked by an HPLC at the end of the experiment and this revealed a difference between control and treated leaves of ca. 20 mg g^{-1} coumarin.

Caterpillars were placed in the centre of the dish and equidistant from the two leaf halves, which were alternately placed right and left from the observer viewpoint. A piece of wet filter paper was placed into every dish to reduce leaf desiccation. Larvae were weighed at the beginning of each trial and allowed to feed overnight (ca. 9 h; temperature 9–11 °C), then they were reweighed and left in the dish without food until the next night, when another choice between control and coumarin coated half-leaves was offered. At the end of each experimental night frass was collected, leaf area consumed was estimated by using a graph paper, and leaves were reweighed. Ten control dishes, without caterpillars, were added to the experiment and handled in the same way in order to estimate water loss rate of leaves during the experimental period and final coumarin concentration. For each date, final leaf weight was regressed without intercept on initial leaf weight and the regression coefficients obtained were used to compute the expected post-experiment mass of leaves without consumption. The difference between real and expected mass was used as a quantitative estimate of consumed biomass. The relationship between consumed leaf area and consumed biomass was linear, statistically significant (P<0.0001), and tight (R^2>0.80) in all cases. Thus, only consumed biomass was used in further analyses.

Feeding experiments with *Spodoptera littoralis*

Spodoptera littoralis larvae used in the experiments came from a laboratory strain maintained at the Unidad de Protección Vegetal, ETSI Agrónomos, Universidad Politécnica, Madrid, Spain. Preference between control and coumarin-coated *D. laureola* leaves was tested twice as described above. In each choice test, all leaves offered to *S. littoralis* belonged to a single *D. laureola* plant. Although larvae and leaves were weighed as mentioned above, in this case the results based on leaf area consumed were more accurate due to reduced consumption. In addition, choice behaviour, amount of biomass consumed, and growth of sixth instars feeding on standard artificial diets (Poitout & Bues, 1974) containing 1 mg g^{-1} of coumarin were investigated by choice (n = 20) and no-choice (n = 20 and 17, for control and coumarin-containing diet, respectively), experiments lasting 24 h. Approximately 2 g (fresh mass) of the corresponding diet were offered in Petri dishes to larvae previously deprived of food for 2 h.

Data analysis

Unless otherwise stated, analyses were performed using the SAS statistical package (SAS Institute, 1996), and values are reported as mean ± SD. Differences in total consumption between noctuid species were analysed by using repeated measures ANOVA (Proc MIXED; SAS Institute, 1996), including larval initial body mass as a covariate, modelling 'plant' as a random effect, and defining individuals as subjects. The proportion of control leaf consumed within each Petri dish was used as an estimate of the effect of treatment on individual choice behaviour. Differences between species in the proportion of control leaf consumed were analysed by Generalized Linear Models (glimmix macro; Littell et al., 1996), with binomial distribution, Pearson scale and probit link function, including 'plant' as a random effect and defining individual larvae as subjects.

The consequences for larvae of their feeding behaviour were explored by using path analysis (Proc CALIS), based on the variance–covariance matrix of the standardized variables, and estimating parameters by Generalized Least Squares. The initial theoretical model (Figure 1) considered that variation in the percentage of control leaf consumed (i.e., concentration of coumarin ingested) could affect larval growth either directly via efficiency, or indirectly through consumption. Additionally, larval body size directly determined consumption, and consumption in turn would determine growth. Model goodness-of-fit was assessed by the χ^2 test and Normed Fit Index (NFI) (Hatcher, 1994).

Results

Antifeedant effects of coumarin on natural herbivores

The size of the larvae of the two noctuid species that were collected in the field was similar (404.2 ± 137.3 mg and
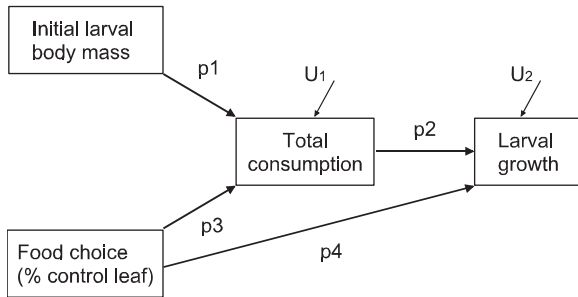
Figure 1 Path diagram showing the expected causal relationships between food choice and performance of the larvae. Single-headed arrows indicate direct relations and have a path coefficient associated (p1, …, p4). Each of the residual variables (U$_1$, U$_2$) represents the unmeasured factors that affect a given dependent variable and that are unexplained by the path diagram.

382.2 ± 134.4 mg for *N. janthe* and *P. ulicis*, respectively; $F_{1,33} = 0.23$, $P = 0.63$). Total leaf consumption per individual depended on larval size ($F_{1,34} = 20.62$, P<0.0001) and differences between species were also statistically significant, with *N. janthe* larvae consuming less biomass than *P. ulicis* larvae ($F_{1,34} = 7.66$, $P = 0.009$; Figure 2). Choice behaviour in response to leaf treatment did not depend on larval size ($F_{1,33} = 0.01$, $P = 0.93$) and on average, the percentage of control leaf consumed by *N. janthe* larvae (38.4%) was lower than that consumed by *P. ulicis* (63.3%; $F_{1,33} = 8.05$, $P = 0.008$). Based on confidence intervals estimated by bootstrap with 10 000 iterations, choice of both species differed significantly (P<0.05) from the 50% consumption expected with random choice. *Noctua janthe* larvae preferred treated leaves, whereas *P. ulicis* larvae preferred the control leaves (Figure 2). Both species showed a broad individual variation in preference as shown by the high coefficients of variation recorded, 86.9 and 80.4 for *N. janthe* and 53.4 and 48.8 for *P. ulicis* on the first and second study night, respectively. In addition, individual choice behaviour on consecutive nights was not significantly correlated ($r_s = 0.11$ and 0.38, P>0.10, for *N. janthe* and *P. ulicis*, respectively).

Antifeedant effects of coumarin on *Spodoptera littoralis*

Three different bioassays were conducted with *D. laureola* leaves on different dates. The first time *D. laureola* leaves were offered to *S. littoralis* third instars, 28 out of 30 larvae bit them but only consumed a minuscule portion (3.4 mm^2). After 4 days with lettuce and *D. laureola* leaves simultaneously offered as food, seven out of 10 larvae that were allowed to choose between control and coumarin-coated *D. laureola* half-leaves did not consume any leaf, whereas the other three only consumed control leaves (3 mm^2). In the third trial, conducted after 2 weeks, all
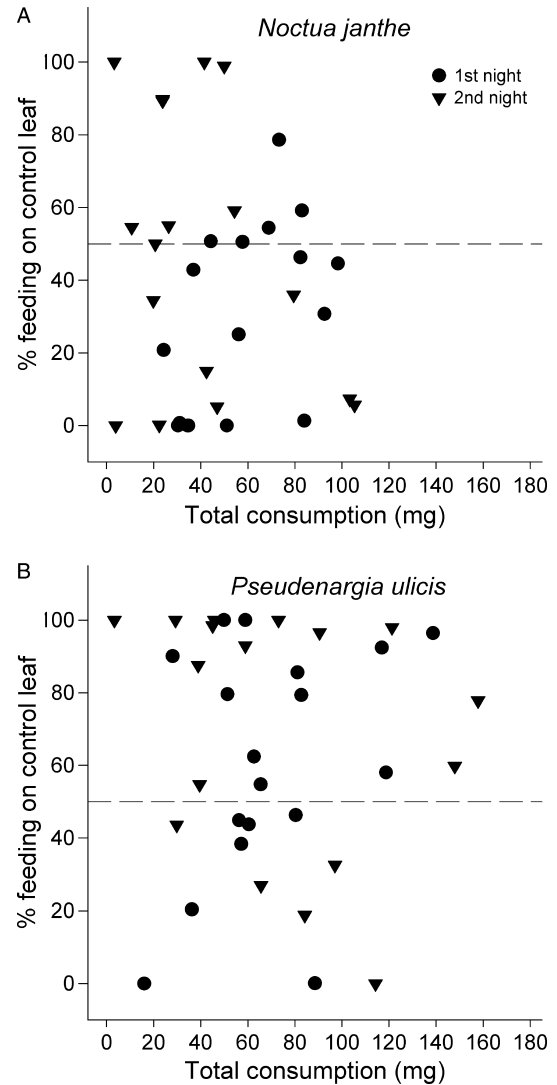

Figure 2 Discrimination between control and coumarin-coated *Daphne laureola* half-leaves by (A) *Noctua janthe* and (B) *Pseudenargia ulicis* larvae. Each dot symbolizes an individual, and dots and triangles were used to represent the 1st and 2nd night results, respectively.

fifth instars assayed (n = 15) consumed *D. laureola* (67.7 ± 12.01 mm^2 leaf consumed). Leaf consumption in the third trial depended significantly on larval size ($F_{1,14} = 8.34$, $P = 0.01$) but did not differ between treatments ($F_{1,14} = 1.14$, $P = 0.30$). Percentage of feeding on control leaves averaged 50.8%, and a broad individual variability in choice behaviour was observed (coefficient of variation = 77.8; Figure 3A).

Furthermore, choice as well as no-choice experiments were conducted with sixth instars feeding on control and coumarin-enriched artificial diets. Under choice
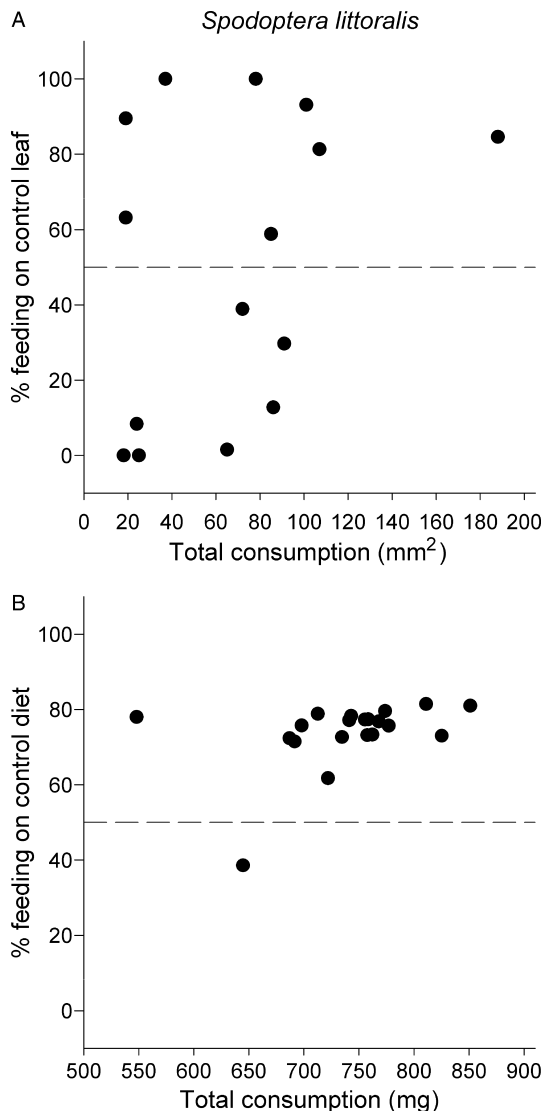
A

B

Figure 3 Food consumption of *Spodoptera littoralis* larvae under choice conditions with (A) control vs. coumarin-coated *Daphne laureola* half-leaves, and (B) standard artificial diet (control) vs. diet containing 1 mg g^{-1} of coumarin. Each dot symbolizes an individual.

conditions, the larvae clearly preferred control diet, with 73.7 ± 9.3% of the total biomass consumed, and choice behaviour was not so variable as in leaf bioassays (coefficient of variation = 12.7; Figure 3B). Total consumption depended on larval body mass ($F_{1,53} = 13.25$, P = 0.0006) and varied between choice and no-choice experiments ($F_{2,53} = 47.48$, P<0.0001), with consumption of the coumarin-containing diet offered alone being the lowest (577.2 ± 13.1 mg), the choice-experiment consumption the highest (742.0 ± 12.1 mg), and consumption of the

control diet offered alone (617.4 ± 12.1 mg) being intermediate and significantly different from the other two trials ($t_{1,53} = 7.26$, P<0.0001; $t_{1,53} = 2.26$, P = 0.03; contrast with choice and coumarin-containing diet, respectively).

Consequences of choice behaviour for larval consumption and growth

The consequences of the choice feeding behaviour of all three species of larvae were explored by path analysis. *Spodoptera littoralis* was characterized by the results of the choice experiment conducted with artificial diet due to limited consumption recorded with *D. laureola* leaves. Goodness-of-fit analyses indicated that the initially-proposed model (Figure 1) was improved by dropping the path between choice and growth (p4) in both natural herbivores and *S. littoralis* (Table 1), suggesting that differences in the concentration of coumarin ingested at the doses studied here did not modify growth efficiency in any of the three species. Only in *N. janthe*, food choice significantly affected consumption (Table 1, p3), and the relationship was positive indicating that total consumption increased when the proportion of control leaf ingested also increased. For all three species, the positive causal relationship between size and consumption (Table 1, p1) and between consumption and growth (Table 1, p2) were consistently statistically significant. On an average, relative consumption was similar between the two natural herbivores (0.15 ± 0.05 and 0.18 ± 0.05 g leaf per g larva, for *N. janthe* and *P. ulicis*, respectively). The same was true for larval relative growth (0.08 ± 0.06 and 0.05 ± 0.05). As expected, relative consumption (4.23 ± 0.36 g diet per g larva) and growth (1.14 ± 0.14) of *S. littoralis* larvae feeding on an artificial diet were much higher. In contrast, the relative consumption (0.04 ± 0.04 g leaf per g larva) and growth (0.02 ± 0.06) of *S. littoralis* larvae feeding on *D. laureola* leaves were the lowest recorded. Differences between species in the strength of the causal relationships established (Table 1) were not statistically significant (analysis not shown).

Coumarins in larval frass

Analysis of larval frass before and after feeding on diets enriched with coumarin allowed us to determine whether this metabolite was partially modified in the larval midgut or not. Samples of *N. janthe* and *P. ulicis* were pooled before analyses because of low mass, hence the possible effects of species or individual variation could not be tested. Larvae collected in the field produced frass that contained the same three glycosides of 5,8-dihydroxy-7-methoxy coumarin present in *D. laureola* leaves differing only in the nature and position of the glycoside

Table 1 Model goodness-of-fit and path coefficients (see Figure 1 for details) estimated for *Noctua janthe* (n = 17), *Pseudenargia ulicis* (n = 18), and *Spodoptera littoralis* (n = 20)

Goodness-of-fit	*N. janthe*	*P. ulicis*	*S. littoralis* (diet)
Initial model (Figure 1)	$\chi^2 = 3.29$, d.f. = 1	$\chi^2 = 3.68$, d.f. = 1	$\chi^2 = 3.93$, d.f. = 1
	P = 0.07	P = 0.055	P = 0.047
	NFI = 0.73	NFI = 0.71	NFI = 0.66
Improved model (p4 excluded)	$\chi^2 = 3.30$, d.f. = 2	$\chi^2 = 3.76$, d.f. = 2	$\chi^2 = 3.94$, d.f. = 2
	P = 0.19	P = 0.15	P = 0.14
	NFI = 0.73	NFI = 0.71	NFI = 0.66
Parameters			
p1	**0.58**	**0.82**	**0.66**
p2	**0.83**	**0.93**	**0.89**
p3	**0.46**	0.21	0.04

Non-significant χ^2 tests and a Normed Fit Index (NFI) >0.9 indicate good fit of data to model.
Coefficients marked in boldface differed significantly from zero.

substituent: a single glucose at position 5 in the coumarin assayed, a disaccharide at position 5 in 'coumarin 2', and a single glucose at position 8 in 'coumarin 3'. However, the relative proportion of the three compounds (denoted as coumarin 1%: coumarin 2%: coumarin 3%) was different in leaves and frass. The coumarin assayed was the most abundant one in leaves with a relative percentage of 70:6:24, but not in frass, in which the relative concentrations of these compounds were 40:15:45, respectively. After the 1st night of choice experiments, frass was slightly enriched in coumarin (45:13:42) and even more so after the second study night (60:4:36), although the relative concentration of coumarin 3 also apparently increased.

Frass of *S. littoralis* fed on standard artificial diet did not contain coumarins. After feeding for 24 h on the artificial diet containing 1 mg g^{-1} of coumarin, both the coumarin ingested and coumarin 3 appeared in the frass in similar proportions (59:0:41), supporting the idea that noctuid larvae partially modify the ingested coumarin, transferring the glucose from position 5 to position 8 of the coumarin ring.

Discussion

Daphne laureola seems to be a well-defended plant. In southern Spanish populations in which Noctuidae diversity is high (Yela & Herrera, 1993), *D. laureola* is only consumed by four species and herbivory levels are moderate to low (Alonso & Herrera, 1996, 2003). Field observations indicate that simultaneous consumption by larvae of the four species is frequent in this region, the relative abundance of different herbivore species varying between plant individuals and years (Alonso & Herrera, 1996 and unpubl.). Despite the fact that simple 7-hydroxycoumarins tend to be less toxic to generalist herbivores than

furanocoumarins (Berenbaum, 1991), our experiments with the highly polyphagous *S. littoralis* demonstrated rejection of *D. laureola* leaves at early stages of larval development; the most abundant coumarin in young leaves also efficiently decreased consumption by late instars, thus supporting a defensive role of this compound against generalist herbivorous insects.

Experiments aiming to evaluate the defensive role of any secondary compound under natural conditions should consider the true diet of the herbivore (e.g., leaves) and compound concentration (e.g., Bernays & Cornelius, 1992; Ayres et al., 1997; Cianfrogna et al., 2002), because repellence and toxicity of secondary compounds may also depend on epistatic interactions with other compounds, including the primary ones (e.g., Behmer et al., 2002; Kester et al., 2002; Zangerl & Berenbaum, 2004). Our experimental design minimized variability on the food side by using leaf halves differing only in the concentration of coumarin, within the range of natural variation observed in the study region. Our results demonstrated that differences of 20 mg g^{-1} of coumarin are detected by the natural herbivores of *D. laureola*, and that the behavioural response to such variation is species specific. The compound had antifeedant properties against *P. ulicis*, and also against the inexperienced *S. littoralis* larvae, both of which tended to avoid the food with the higher concentration of coumarin. In contrast, *N. janthe* larvae tended to prefer the leaves with increased coumarin concentration; however, such choice has further negative consequences for this species because total consumption was inversely related to the proportion of treated leaves ingested.

Noctua janthe seems to be more specialized on *D. laureola* than *P. ulicis*. All *N. janthe* larvae collected from woody plants in the same locality although in a different year were found on *D. laureola*, whereas 61.5% of *P. ulicis* larvae were

found on another woody plant species (CM Herrera, pers. comm.). These results suggest that deterrence of the secondary metabolites present in a host plant could be higher in less-specialized consumers. Although such results contrast to the greater taste sensitivity recorded by specialist consumers when assayed for several non-host secondary metabolites (Bernays et al., 2000), they correspond to a token stimulus function similar to that reported for glucosinolates and some furanocoumarins in species specialized in feeding on plants characteristically rich in those compounds (Berenbaum, 1991; Hopkins et al., 2009).

Natural selection could act on an animal's behaviour such that its diet best fits its nutritional needs only if food choice behaviour is variable among individuals (Arsenos et al., 2000), an aspect highlighted by our results (Figure 2). Unfortunately, most studies in which preference assays have been conducted with insect herbivores included no reference to the extent of individual variability (e.g., Bernays & Cornelius, 1992; Cianfrogna et al., 2002; Viswanathan et al., 2005), although some authors controlled for genetic variability by using siblings (e.g., Salminen & Lempa, 2002). The choice responses to variation in coumarin concentration of field-collected herbivores were highly variable (Figure 2) and unrelated to individual size and total leaf consumption. Conversely, response of the model species *S. littoralis* tested on artificial diets was more homogeneous, as larvae consistently avoided the diet containing coumarin (Figure 3B). The responsiveness of the insect taste cells is dependent on developmental and nutritional stage, time of day, and experience, and can vary between closely-related species (Karowe, 1989; van Loon, 1996; Bernays, 2001; Schoonhoven & van Loon, 2002). Again, most of these factors were taken into account in our design because all field-collected larvae had spent at least 16 h without food before the assay and were tested simultaneously. Furthermore, preference was not correlated to larval body mass (as a surrogate of larval age), suggesting that the observed variation showed individual differences. However, individual choice behaviour was not consistent between successive nights, also suggesting that short-term adjustment of behaviour and previous experience could greatly modify individual choice.

At the post-ingestion level, the short-term physiological responses to coumarin were apparently similar in the three species. The absence of a direct influence of the amount of coumarin consumed on growth efficiency shown by the path analyses suggested a lack of toxicity that could be explained by molecule modification occurring in the midgut. Both the natural herbivores and *S. littoralis* showed increased concentrations of coumarin in frass after the experiments but they also partially transformed it by

transferring the glucose from position 5 to position 8 of the coumarin ring. Thus, the noctuid species apparently differed more in pre- than post-ingestion responses to this particular coumarin, suggesting that in this case repellence and toxicity are not directly related (see also Bernays & Cornelius, 1992).

Altogether, this study confirms a defensive role of *D. laureola* coumarin against generalist insect herbivores, although behavioural responses are species specific. Thus, the interaction between *D. laureola* and its noctuid herbivores would constitute an example in which diffuse evolution of plant defences is expected due to simultaneous consumption by multiple herbivores responding differentially to the same defensive trait (Strauss et al., 2005, Criterion 2). Variability in the identity of the herbivore assemblage between individual plants and years would also constrain a directional plant response to selection, and consequently maintain natural variation in coumarin concentration.

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