

# Iridoid Glycoside Variation in the Invasive Plant Dalmatian Toadflax, *Linaria dalmatica* (Plantaginaceae), and Sequestration by the Biological Control Agent, *Calophasia lunula*

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**Abstract** Invasive plant species can have significant ecological and economic impacts. Although numerous hypotheses highlight the importance of the chemical defenses of invasive plant species, the chemical ecology of many invasive plants has not yet been investigated. In this study, we provide the first quantitative investigation of variation in iridoid glycoside concentrations of the invasive plant Dalmatian toadflax (*Linaria dalmatica*). We examined variation in chemical defenses at three levels: (1) variation within and among populations; (2) variation due to phenology and/or seasonal differences; and (3) variation among plant parts (leaves, flowers, and stems). Further, we examined two biological control agents introduced to control *L. dalmatica* for the ability to sequester iridoid glycosides from this invasive plant. Results indicate that *L. dalmatica* plants can contain high concentrations of iridoid glycosides (up to 17.4% dry weight of leaves; mean =  $6.28 \pm 0.5$  SE). We found significant variation in iridoid glycoside concentrations both within and among plant populations, over the course of the growing season, and among plant parts. We also found that one biological control agent, *Calophasia lunula* (Lepidoptera: Noctuidae), was capable of sequestering antirrhinoid, an iridoid glycoside found in *L. dalmatica*, at levels ranging from 2.7 to 7.5% dry weight. A second biological control agent,

*Mecinus janthinus* (Coleoptera: Curculionidae), a stem-mining weevil, did not sequester iridoid glycosides. The demonstrated variation in *L. dalmatica* chemical defenses may have implications for understanding variation in the degree of invasiveness of different populations as well as variation in the efficacy of biological control efforts.

**Keywords** Iridoid glycosides · Antirrhinoid · Linarioside · *Linaria dalmatica* · Sequestration · *Calophasia lunula* · *Mecinus janthinus* · *Junonia coenia* · Chemical defenses · Invasive plant · Biological control · Lepidoptera · Noctuidae

## Introduction

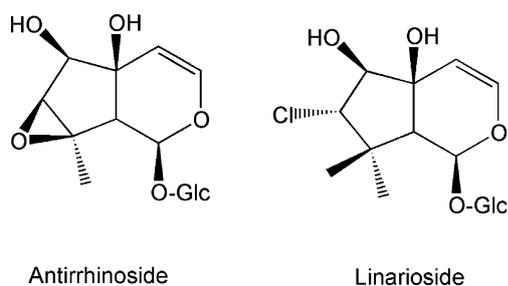
The invasion of non-native plant species has affected ecosystems throughout the world and represents a major threat to native biodiversity (Wilcove et al. 1998; Levine et al. 2003; Myers and Bazely 2003; Mitchell et al. 2006). Numerous hypotheses have been proposed to elucidate the mechanisms that underlie plant invasions. Many of these hypotheses, including the enemy release, evolution of increased competitive ability, novel weapons, and novel chemistry hypotheses, highlight the importance of plant defensive chemistry in the success and invasion dynamics of introduced species (Blossey and Notzold 1995; Keane and Crawley 2002; Callaway and Ridenour 2004; Cappuccino and Arnason 2006; Inderjit et al. 2006). Moreover, plant secondary compounds are known to play an important role in mediating species interactions, including plant-plant, plant-pathogen, and plant-herbivore interactions. Thus, research that examines the chemical ecology of invasive plants may contribute greatly to understanding factors that facilitate invasion success as well as factors that influence management and biological control efforts.

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In this paper, we present the first quantitative investigation of the chemical defenses of Dalmatian toadflax, *Linaria dalmatica* (L.) P. Mill. (Plantaginaceae), which has been identified as both an ecologically and economically important invasive plant species (Duncan et al. 2004). This species contains iridoid glycosides (Handjieva et al. 1993; Ilieva et al. 1993; Franzyk et al. 1999), but its iridoid content has not been quantitatively examined. Iridoid glycosides are a group of cyclopentanoid monoterpenoid-derived compounds found in approximately 50 plant families (Bobbitt and Segebarth 1969; Jensen et al. 1975; El-Naggar and Beal 1980; Boros and Stermitz 1990). These compounds mediate plant interactions with both specialist and generalist herbivores, acting as deterrents and toxins for generalists and as attractants and feeding stimulants for certain specialists (review in Bowers 1991). Further, these compounds can be sequestered by some specialist insect herbivores and provide protection against their natural enemies (Bowers 1991; Rimpler 1991; Nishida 2002).

We quantified the two major iridoid glycosides, antirrhinoside and linarioside (Fig. 1), found in *L. dalmatica*, and examined three levels of variation in iridoid glycoside concentrations: (1) variation within and among populations; (2) variation due to phenology and/or seasonal differences; and (3) variation among plant parts (leaves, flowers, and stems). Additionally, we examined two introduced biological control agents, the toadflax defoliator, *Calophasia lunula* Hufnagel (Lepidoptera: Noctuidae) and the stem-mining weevil, *Mecinus janthinus* Germar (Coleoptera: Curculionidae), for their ability to sequester iridoids. We were interested especially in examining *C. lunula* because the bright coloration of larvae (white, black, and yellow) suggested aposematism and because a number of lepidopteran species are known to sequester iridoid glycosides that provide protection against natural enemies (Bowers 1991; Nishida 2002). For comparison, we also analyzed larvae of the common buckeye butterfly, *Junonia coenia* Hübner (Lepidoptera: Nymphalidae), for its ability to sequester iridoid glycosides from *L. dalmatica* because it is known to sequester other iridoid glycosides (Bowers and Collinge 1992) and will feed on *L. dalmatica* (pers. obs.).



**Fig. 1** Structures of the iridoid glycosides antirrhinoside and linarioside

## Methods and Materials

**Study System** *Linaria dalmatica* is a perennial species native to Eurasia that escaped cultivation in North America during the early 1900's and since has become a land management concern throughout the western United States and Canada (Vujnovic and Wein 1997; Wilson et al. 2005). Synonyms of this species include *L. dalmatica* ssp. *dalmatica* and *L. genistifolia* ssp. *dalmatica* (Vujnovic and Wein 1997; Weber and Wittmann 2001; USDA 2009). *Linaria dalmatica* is designated as a noxious weed in 12 states throughout the western United States and has been reported to occur in at least 33 states (USDA 2009). Once established, *L. dalmatica* can be a strong competitor and may reduce the abundance of native grasses and forbs (Carpenter and Murray 1998).

Several iridoid glycosides have been identified in *L. dalmatica* and other *Linaria* species (Ilieva et al. 1992, 1993; Handjieva et al. 1993; Franzyk et al. 1999). *Linaria dalmatica* is reported to contain five iridoid glycosides: antirrhinoside, linarioside, isolinarioside, 5-O-glucosylantirrhinoside, and 5-O-allosylantirrhinoside (Handjieva et al. 1993; Ilieva et al. 1993; Franzyk et al. 1999). To date, the iridoid content of *L. dalmatica* has not been investigated quantitatively; however, one study has examined other closely related *Linaria* species. Nikolova-Damyanova et al. (1994) used quantitative thin-layer chromatography (TLC) to estimate iridoid glycosides from the dried ground aerial plant tissue of *L. vulgaris*, *L. simplex*, *L. pelliseriana*, and *L. genistifolia*, and they found that antirrhinoside and linarioside represent the two major iridoids found in these species. In *L. genistifolia*, antirrhinoside represented approximately 60–70% of the total iridoid content and linarioside represented 10–20% (Nikolova-Damyanova et al. 1994).

Management of *L. dalmatica* by using chemical and mechanical control often is unsuccessful due to waxy leaves that protect the plant from herbicides as well as long taproots and the ability to reproduce vegetatively from root buds and fragments (Carpenter and Murray 1998; Wilson et al. 2005). Consequently, biological control may be the most effective management strategy for managing invasive populations of this species. Seven specialist herbivores of *L. dalmatica* have been introduced into North America, either intentionally as biological control agents or accidentally (Wilson et al. 2005). Iridoids are likely to play a role in mediating interactions between *L. damatica* and these specialist herbivores; however, little is known about the iridoid content and chemical ecology of this invasive plant.

*Mecinus janthinus* is a stem-mining weevil native to Eurasia, which was approved for release as a biocontrol agent in Canada and the US in the early 1990s (Wilson et

al. 2005). *Mecinus janthinus* larvae develop from early to mid-summer within the stems of *L. dalmatica*. Larvae pupate during late summer and overwinter in dead stems. Adults emerge the following spring and feed on the leaves and stems of the plant (Jeanneret and Schroeder 1992). Because *L. dalmatica* population growth appears to be limited by interspecific competition rather than seed availability, Grieshop and Nowierski (2002) suggest that biocontrol agents that reduce the competitive ability of plants by attacking stems and roots are most effective at reducing population densities. Furthermore, research by Peterson et al. (2005) demonstrated that *M. janthinus* larval injury had a significant deleterious effect on the primary physiology of *L. dalmatica*, whereas *Calophasia lunula* larval injury did not result in any significant physiological responses. Accordingly, *Mecinus janthinus* seems to be the most promising biological control agent for management of *L. dalmatica* populations.

*Calophasia lunula* is a defoliating lepidopteran, also native to Eurasia, which was introduced into North America in the early 1960's as a biocontrol agent for *L. dalmatica* and *L. vulgaris* (Wilson et al. 2005). Adult moths emerge in May, and caterpillars can be found feeding on *L. dalmatica* from late May through August. Depending on weather and the length of the growing season, *C. lunula* will complete one to three generations per year. This specialist herbivore is found throughout the range of *L. dalmatica* and its occurrence is widespread in the United States. Caterpillars can cause severe defoliation, but larval injury is likely only significant at the seedling stage, not at the adult stage of *L. dalmatica* (Wilson et al. 2005).

*Junonia coenia* is a lepidopteran native to North and Central America and is not a specialist on *L. dalmatica*, but it is a specialist on plants that contain iridoid glycosides (Bowers 1984). Common host plants of this species include native and cultivated snapdragons, *Antirrhinum majus* (Plantaginaceae) (Robinson et al. 2002). Moreover, larvae also will feed on *L. dalmatica* (pers. obs.). Snapdragons are known to contain antirrhinoside and other iridoid glycosides (Beninger et al. 2007). Although *J. coenia* larvae have been shown to sequester the iridoid glycosides aucubin and catalpol (Bowers and Collinge 1992), this species has not been examined for its ability to sequester antirrhinoside.

**Sample Collection** Plant samples were collected from three field sites in the northwestern region of Boulder County, Colorado, USA, in the spring and summer of 2006. Two field sites were located on Boulder County Parks and Open Space property: Rabbit Mountain (40° 14' 13" N; 105° 12' 53" W) and Hall Ranch (40° 12' 42" N; 105° 17' 20" W). The third site was located on private property in Lefthand Canyon (40° 7' 14" N; 105° 19' 26" W). Field sites ranged from 1675–2075 m in elevation and were characterized as

dry and rocky foothills grassland habitat with vegetation dominated by mixed grasses, forbs, and shrubs. Plant samples were collected on three dates that spanned the major phenological stages of *L. dalmatica*: pre-flowering (late-May), peak flowering (mid-July), and during fruiting and seed set (mid-September). We collected plant samples from 10 patches at each field site ( $N=10$  samples per date/site). Each sample was comprised of three ramets collected from a distinct patch. Because *L. dalmatica* reproduces vegetatively and from seed, it was not possible to determine whether ramets come from a single or multiple individuals without genetic analyses. Although *L. dalmatica* can hybridize with *L. vulgaris* in natural habitats (Ward et al. 2009), we are confident that our samples are pure *L. dalmatica* because *L. vulgaris* is not present at our study sites and the sampled plants in these populations clearly showed the morphology of *L. dalmatica*.

*Mecinus janthinus* adults ( $N=10$ ) were collected from Lefthand Canyon in early June 2006. *Calophasia lunula* samples came from a lab culture started from eggs obtained from the Colorado Department of Agriculture Insectary (Palisade, CO). Larvae were reared in a growth chamber (model Percival 36-LLVL; interior volume = 0.84 m<sup>3</sup>) with a 16:8 h L/D photoperiod and temperatures set to 25°C day and 20°C night. Larvae were fed *L. dalmatica* plants collected from a field population located behind the University of Colorado 30th St. Greenhouse, Boulder, CO during August 2006. Larvae were selected for sample preparation at three stages: newly molted 4th and 5th instars and midway through the 5th instar (larvae were starved for 12–18 h to empty gut contents). Male and female pupae, newly emerged adults, and eggs also were prepared for iridoid analyses. The sample size for each stage was  $N=10$  individuals, except in the case of eggs, which were analyzed as 4 samples of 50–100 eggs. In addition to caterpillars, pupae, and eggs, we examined *C. lunula* hemolymph collected from 5th instar larvae ( $N=4$ ) for the presence of iridoid glycosides. Hemolymph was collected with a 10 µl capillary tube from an incision made on a larval proleg. *Junonia coenia* larvae were obtained from a laboratory culture maintained at the University of Colorado, and were reared mid-August through mid-September 2006 from hatching until they molted to the 5th instar on either field collected *L. dalmatica* or garden collected *Antirrhinum majus* ( $N=10$  individuals per host plant). For *J. coenia*, we analyzed iridoid glycoside concentrations for 5th instars only.

**Sample Preparation** Plant tissues were separated into leaves, stems, and flowers, oven-dried at 50°C to a constant mass, weighed to the nearest 0.01 g, ground into a fine powder, and then 25–30 mg of each plant sample was prepared for chemical analysis. Sample preparation

methods have been described previously (Gardner and Stermitz 1988; Bowers and Stamp 1993). Briefly, sample preparation involves extracting plant material in methanol, filtering out plant material from the methanol extract, evaporating extracts to dryness, and then partitioning the dried extract between water and ether to remove hydrophobic compounds.

*Calophasia lunula* and *J. coenia* samples were weighed fresh, killed by freezing at  $-40^{\circ}\text{C}$ , ground and extracted fresh in methanol, and then prepared for iridoid analyses by using the same method as plant tissues. For *C. lunula* and *J. coenia* samples, a separate set of individuals at each life stage ( $N=5$  per life stage) was weighed fresh, oven dried at  $50^{\circ}\text{C}$  to constant mass, reweighed, and data were used to calculate wet to dry weight conversion factors for the various life stages. *Calophasia lunula* eggs ( $N=4$  composite samples) were weighed and extracted fresh (no conversion factors calculated). The 10 *M. janthinus* adults were combined into a single composite sample, weighed, and extracted fresh (no conversion factors calculated). These samples were then prepared for iridoid analysis as described above.

**Chemical Analyses** In this study, we compared two methods for examining iridoid glycosides: gas chromatography (GC) and high performance liquid chromatography (HPLC). Standards of antirrhinoside and linarioside were provided by Søren Jensen. Methods for analyzing iridoids using GC with phenyl- $\beta$ -D-glucopyranoside (PBG) as an internal standard have been described previously (Gardner and Stermitz 1988; Bowers and Stamp 1993). For GC analysis, iridoid glycosides were derivatized to the corresponding trimethylsilyl derivatives by using Tri-sil Z<sup>TM</sup> (Pierce Chemical Company). GC analyses were performed on a Hewlett-Packard (HP) 5890A system (Agilent Technologies), and data were processed with HP ChemStation software (version A.03.34).

HPLC analyses were performed on a Hewlett-Packard 1090 system (Agilent Technologies) equipped with a diode array detector (DAD) and Apex (Jones Chromatography, U.K.) ODS 5  $\mu\text{m}$  reverse phase C-18 column ( $250 \times 4.6$  mm o.d.) protected with a guard column of the same material ( $7.5 \times 4.6$  mm). HPLC data were processed with HP ChemStation software (version A.10.02) that included a DAD spectral evaluation module. Our method for HPLC analysis was modified from a previous method described in Høgedal and Mølgaard (2000). Specifically, we used an isocratic method with a sample injection volume of 20  $\mu\text{l}$ , mobile phase consisting of 3% acetonitrile in water (0.01% phosphoric acid added to bring pH to 4.0), flow rate of 1 ml/min, analysis time of 60 min, and spectrophotometric detection at 205 nm. Calibration curves were made using standard solutions

with concentrations of 0.125–3.5 mg/ml. Standards included antirrhinoside, linarioside, and phenyl- $\beta$ -D-glucopyranoside (PBG), which was the internal standard. The HPLC method was validated carefully and system suitability standards ( $<5\%$  C.V for standard solution injections) were verified for each group of sample runs.

**Statistical Analyses** Iridoid glycosides were analyzed as proportions of dry weight (concentration). Data were arcsine square root transformed to meet assumptions of normality. Simple linear correlation analyses using Pearson correlation coefficients were performed to examine correspondence between iridoid glycosides measured with GC and HPLC methods and to determine if antirrhinoside and linarioside concentrations measured in plant tissues were correlated.

Because antirrhinoside and linarioside are potentially correlated, we used multivariate analysis of variance (MANOVA) to examine variation in iridoid glycoside concentrations due to (1) site and/or population differences, (2) date of sample collection and/or phenology, and (3) the part of the plant from which tissues were analyzed (flowers, leaves, or stems). We examined the main effects of date and plant part in separate analyses due to the unbalanced nature of our sampling design (i.e., iridoid glycoside concentrations from flowers and stems were measured only on one sampling date). The first MANOVA model, which examined iridoid glycoside concentrations of leaves only, included site, date, and a site by date interaction term. In the second MANOVA model, site, plant part, and a site by plant part interaction term were the main factors. MANOVAs were performed by using the general linear model (GLM) procedure in SYSTAT (version 11). When significant effects were detected by MANOVA, we followed up with univariate repeated-measures ANOVAs for each dependent variable (antirrhinoside and linarioside). Variation in iridoid glycoside concentrations (% dry weight) and total amount of iridoid glycosides (mg) of different life stages of *Calophasia lunula* were examined by ANOVA. For all univariate ANOVAs, Tukey's *post hoc* multiple comparisons tests were used to examine pairwise differences among groups when significant differences were detected. All statistical analyses were performed in SYSTAT (version 11).

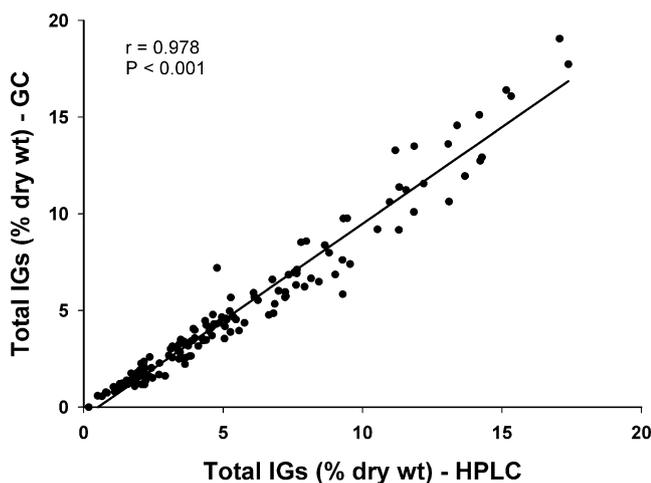
## Results

**Comparison of GC and HPLC Methods** Derivatized antirrhinoside and linarioside co-eluted when analyzed by GC, and the mean retention time for both compounds was 7.4 min. Derivatized PBG had a mean retention time of 4.1 min. With HPLC methods, separation of antirrhinoside

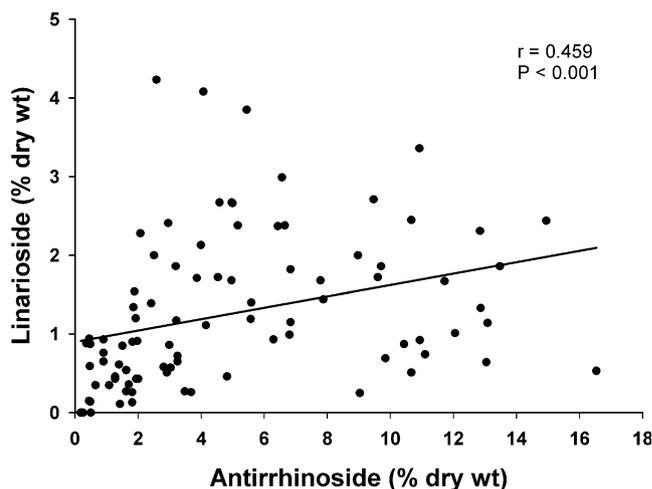
and linarioside peaks was achieved. Mean retention times for standard compounds were 20.1 min for antirrhinoside, 37.1 min for PBG, and 42.6 min for linarioside. Calibration curves indicated good linearity with standard solution concentrations between 0.125–3.5 mg/ml. The correlation coefficients for calibration curves were 0.995, 0.976, 0.995 for antirrhinoside, linarioside, and PBG, respectively. There was a highly significant positive relationship between GC and HPLC data (i.e., combined iridoid glycosides) ( $r=0.978$ ;  $P<0.001$ ; Fig. 2).

**Iridoid Glycoside Variation in *Linaria dalmatica*** Combined iridoid glycoside (antirrhinoside + linarioside) concentrations ranged from as high as 17.4% dry weight to as low as 0.2% dry weight of leaves (mean =  $6.28 \pm 0.5$  SE). We found antirrhinoside concentrations up to 16.5% (mean =  $5.02 \pm 0.4$  SE) dry weight of leaves and linarioside concentrations up to 6.7% (mean =  $1.26 \pm 0.1$  SE). The simple linear correlation analysis revealed a significant positive relationship between concentrations of antirrhinoside and linarioside ( $r=0.459$ ;  $P<0.001$ ; Fig. 3). On average, antirrhinoside represented 75.7% of the combined iridoid glycosides measured in leaves.

In our analysis of leaf tissue collected from plants on three sampling dates, we found significant variation in iridoid glycoside concentrations due to both site (*Wilks'*  $\lambda=0.566$ ,  $F_{4,160}=13.19$ ,  $P<0.001$ ) and date (*Wilks'*  $\lambda=0.378$ ,  $F_{4,160}=25.07$ ,  $P<0.001$ ). There was no site by date interaction effect (*Wilks'*  $\lambda=0.958$ ,  $F_{8,160}=0.43$ ,  $P=0.900$ ). Iridoid glycoside concentrations varied significantly both within and among populations (Fig. 4). Univariate ANOVAs revealed that antirrhinoside and linarioside

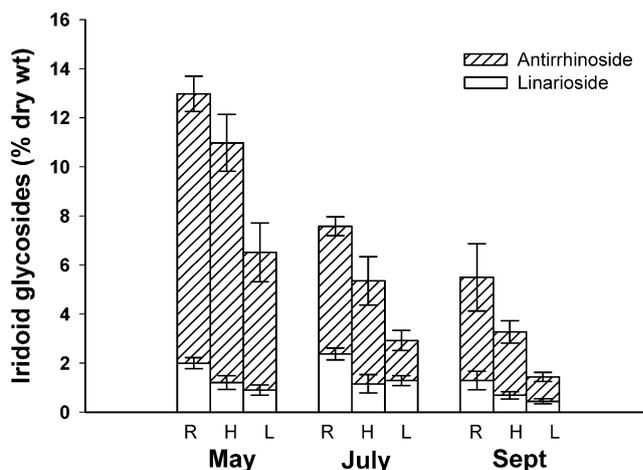


**Fig. 2** Comparison of combined iridoid glycosides (antirrhinoside + linarioside) measured in *Linaria dalmatica* using GC and HPLC methods. Data are reported as % dry weight of leaf tissues. Antirrhinoside and linarioside co-eluted when analyzed by GC. Separation of compounds was achieved by HPLC



**Fig. 3** Relationship between antirrhinoside and linarioside concentrations (% dry weight) measured in *Linaria dalmatica* leaves

showed similar patterns of variation among populations (site effect) and over the course of the growing season (date effect) (Table 1). Of the three populations, plants collected from Rabbit Mountain had the highest iridoid glycoside concentrations (Tukey's *post-hoc* pairwise comparisons:  $P<0.05$  in both cases). In this one population, combined iridoid glycoside concentrations of leaf tissues ranged from 17.4 to 9.5% (mean =  $12.97 \pm 0.7$  SE) in May and from 14.2 to 0.5% (mean =  $5.50 \pm 1.6$  SE) in September, showing an average decline of 40% in the concentrations of these defense compounds over the course of the peak growing season. Across all field sites, iridoid glycoside concentrations were highest early in the growing season and declined over time (Fig. 4).



**Fig. 4** Pattern of variation in iridoid glycoside concentrations of *Linaria dalmatica* leaves. Plant samples were collected in late-May, mid-July, and mid-September from three populations located in Boulder County, Colorado (USA): Rabbit Mountain (R), Hall Ranch (H), and Lefthand Canyon (L). Data reported are mean % dry weight  $\pm$  SE ( $N=10$  per site per date)

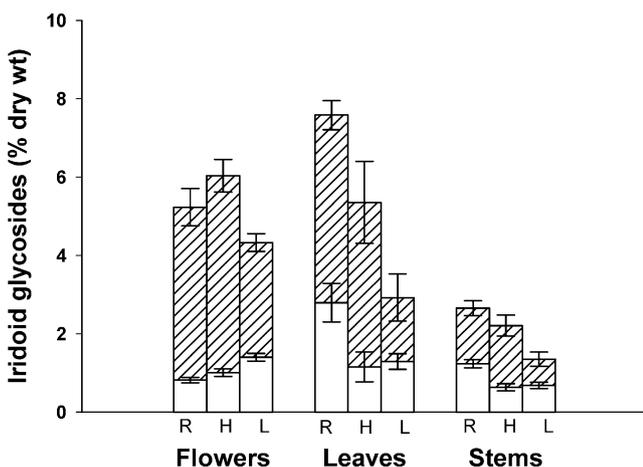
**Table 1** ANOVA results summarizing the effects of site and date on iridoid glycoside concentrations of *Linaria dalmatica* leaves

Source	Antirrhinoside		Linarioside	
	F (df)	P	F (df)	P
Between Subjects				
Site <sup>a</sup>	18.18 (2)	<0.001	10.81 (2)	<0.001
Error	(27)		(27)	
Within Subjects				
Date <sup>b</sup>	52.33 (2)	<0.001	11.51 (2)	<0.001
Date x Site	0.17 (4)	0.953	0.67 (4)	0.615
Error	(54)		(54)	

<sup>a</sup> Field sites (Rabbit Mountain, Hall Ranch, Lefthand Canyon) were located in Boulder County, Colorado (USA)

<sup>b</sup> Samples were collected in May, July, and September of 2006 ( $N=10$  per site per date)

Results of the second MANOVA, which examined iridoid glycoside concentrations of flowers, leaves, and stems, indicated effects of site (*Wilks'*  $\lambda=0.506$ ;  $F_{4,160}=16.25$ ;  $P<0.001$ ) and plant part (*Wilks'*  $\lambda=0.358$ ;  $F_{4,160}=26.90$ ;  $P<0.001$ ) as well as a site by plant part interaction effect (*Wilks'*  $\lambda=0.673$ ;  $F_{8,160}=4.38$ ;  $P<0.001$ ; Fig. 5). Again, univariate ANOVAs revealed that antirrhinoside and linarioside demonstrated similar response patterns, this time in response to the effects of site, plant part, and the interaction of these two factors (Table 2). Across field sites, combined iridoid glycoside concentrations of flowers (mean =  $5.20\pm 0.3$  SE) and leaves (mean =  $5.29\pm 0.5$  SE) were more than double the concentrations found in stems (mean =  $2.07\pm 0.2$ ). At Hall Ranch and Lefthand Canyon, flowers had similar iridoid glycoside



**Fig. 5** Iridoid glycoside concentrations of flowers, leaves, and stems of *Linaria dalmatica*. Plant samples were collected in July 2006 from three populations located in Boulder County, Colorado (USA): Rabbit Mountain (R), Hall Ranch (H), and Lefthand Canyon (L). Data reported are mean % dry weight  $\pm$  SE ( $N=10$  per site)

**Table 2** ANOVA results summarizing the effects of site and plant part on iridoid glycoside concentrations of *Linaria dalmatica* plants

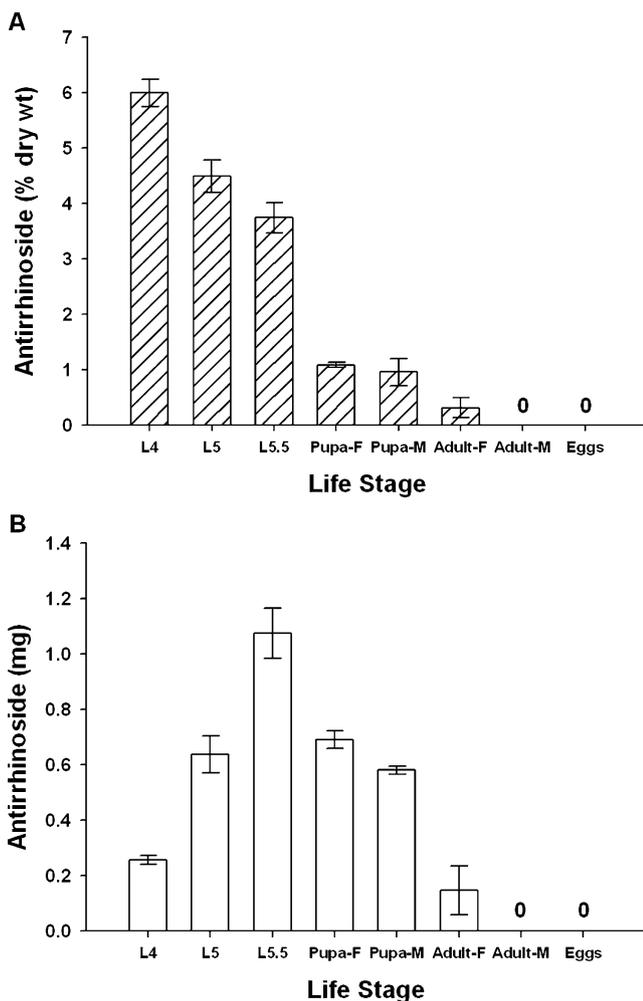
Source	Antirrhinoside		Linarioside	
	F (df)	P	F (df)	P
Between Subjects				
Site <sup>a</sup>	15.10 (2)	<0.001	7.19 (2)	0.003
Error	(27)		(27)	
Within Subjects				
Plant part <sup>b</sup>	71.53 (2)	<0.001	26.77 (2)	<0.001
Plant part x Site	3.08 (4)	0.024	9.68 (4)	<0.001
Error	(54)		(54)	

<sup>a</sup> Field sites (Rabbit Mountain, Hall Ranch, Lefthand Canyon) were located in Boulder County, Colorado (USA)

<sup>b</sup> Tissues from leaves, flowers, and stems were analyzed for samples collected in July of 2006 ( $N=10$  per site)

concentrations compared to leaves (Tukey's *post-hoc* pairwise comparisons:  $P>0.05$  in all cases); however, at Rabbit Mountain, flower tissues contained slightly higher concentrations of iridoid glycosides compared to leaves ( $P<0.05$ ). Site-specific differences in iridoid glycoside concentrations of flowers compared to leaves resulted in the observed plant part site by interaction effect (Table 2).

**Iridoid Glycoside Sequestration by Herbivores** We found no evidence of iridoid sequestration by *M. janthinus*. However, results indicated that both *C. lunula* and *J. coenia* sequestered antirrhinoside from *L. dalmatica*. Antirrhinoside was found in larvae, pupae, and adult females of *C. lunula*, but was not found in eggs or adult males. In adult females, only three of the 10 samples contained antirrhinoside. Both the concentrations (% dry weight) and total amounts (mg) of antirrhinoside varied significantly among different life stages ( $P<0.001$  in both cases; Fig. 6). The highest levels of antirrhinoside were found in larvae, with concentrations ranging from 2.7 to 7.5% dry weight (mean =  $4.8\pm 0.3$  SE), and total amounts equal to  $0.64\pm 0.07$  mg (mean  $\pm$  SE) in the three larval stages analyzed. Antirrhinoside amounts in hemolymph ranged from 1.5 to  $5.5\ \mu\text{g}/\mu\text{l}$ , indicating that hemolymph is one important site of iridoid storage in *C. lunula* larvae. *Junonia coenia* also sequestered antirrhinoside. For 5th instars reared on *L. dalmatica*, we found antirrhinoside concentrations as high as 15.7% dry weight (mean =  $12.1\pm 0.5$  SE), and total antirrhinoside amounts were  $2.72\pm 0.17$  mg (mean  $\pm$  SE). For 5th instar larvae reared on *A. majus*, antirrhinoside represented 9.6% ( $\pm 0.5$  SE) of the larval dry weight, and total amounts were  $2.06\pm 0.15$  mg (mean  $\pm$  SE). We found no evidence for sequestration of linarioside by either *C. lunula* or *J. coenia*, despite the presence of this iridoid glycoside in *L. dalmatica* host plants.



**Fig. 6** Sequestration of antirrhinoside by *Calophasia lumula* (Noctuidae) at various life stages (a) concentration (% dry weight) (b) total amount (mg).  $N=10$  individuals for each larval (L) stage, pupae, and adults. For eggs,  $N=4$  samples each with 50–100 eggs

## Discussion

Of the many plants introduced into novel habitats, few become established, and even fewer become invasive (Williamson and Fitter 1996). The novel chemistry and novel weapons hypotheses propose that some introduced plant species are successful invaders because natural enemies in the introduced range are not adapted to their unique chemical profiles (Callaway and Ridenour 2004; Cappuccino and Arnason 2006). Although many invasive plant species are thought to be phytochemically unique in their introduced range (Cappuccino and Arnason 2006), often little is known about the quantitative variation of defense compounds and the chemical ecology of plant-herbivore interactions for these species. Our research provides the first investigation of quantitative variation in the chemical defenses of the invasive plant *Linaria dalmatica*.

**Comparison of GC and HPLC Methods** In this study, we compared two different methods for analyzing iridoid glycosides found in *L. dalmatica*. We found that GC and HPLC methods were comparable when considering combined iridoid glycosides (antirrhinoside + linarioside), as demonstrated by a strong correlation between results from the two methods. Thus, for studies interested in overall levels of defense compounds either method is appropriate. However, for studies interested in quantifying levels of individual iridoid glycosides, analysis by HPLC is required because GC methods do not provide separation of antirrhinoside and linarioside. We suspect that these compounds co-elute in GC analyses due to the production of the same derivatization product when these two compounds are derivatized using Tri-sil Z (Pierce Chemical Company). Nevertheless, the GC method may be preferred when examining iridoid glycosides of *L. dalmatica* because this method produces less chemical waste and allows for more rapid analysis. Furthermore, our results indicate that antirrhinoside and linarioside concentrations are correlated, and that these compounds demonstrate similar patterns of variation among populations, over the course of the growing season, and among plant parts.

**Iridoid Glycoside Variation in *Linaria dalmatica*** We found that *L. dalmatica* contains high levels of iridoid glycosides, up to 17% dry weight with a mean of 6%. These results suggest that the species is well defended against generalist natural enemies, in particular naïve species. However, different generalist species can demonstrate varying responses to iridoid glycosides. For example, Beninger et al. (2008) found that gypsy moth larvae (*Lymantria dispar*: Lymantriidae) avoided leaves of cultivated snapdragon, *Antirrhinum majus* (Plantaginaceae), which contain antirrhinoside, and larval growth was reduced when fed a diet with 3.3% antirrhinoside concentrations. By contrast, cabbage looper (*Trichoplusia ni*: Noctuidae) larvae readily fed on *A. majus*, and larval growth actually increased on diets with antirrhinoside. The overall defensive nature of *L. dalmatica* iridoid glycosides against generalist herbivores requires further investigation.

Our study indicates that *L. dalmatica* plants are both spatially and temporally variable in levels of iridoid glycosides and that plant parts have varying concentrations of iridoid glycosides. Similar patterns of variation in iridoid glycoside concentrations have been documented for other plant species containing iridoid glycosides (e.g., Darrow and Bowers 1997). Although in contrast to *L. dalmatica*, Darrow and Bowers (1997) found that iridoid glycoside concentrations of *Plantago lanceolata* (Plantaginaceae) increased over the course of the growing season. Also, iridoid glycoside concentrations found in *P. lanceolata* were lower in general than those observed in *L. dalmatica*.

The results of our study suggest that there may be opportunities for generalist herbivores to avoid the deterrent or toxic properties of these compounds. For example, generalist herbivores, including both insects and grazing animals, may be able to utilize *L. dalmatica* as a resource in certain populations where plants produce lower levels of iridoid glycosides and at certain times of the year such as the later part of the growing season when levels are low. Also, stems are not well defended compared to leaves and flowers. Consequently, *L. dalmatica* may be especially susceptible to stem-mining or boring insects in the introduced range. It should be noted, however, that it is unknown whether iridoid glycosides found in *L. dalmatica* are inducible, as demonstrated in other plants that contain iridoids (e.g., Darrow and Bowers 1999; Fuchs and Bowers 2004).

Our research indicates that a number of factors, including genotype, ontogeny, phenology, and site differences may influence iridoid glycosides concentrations found in *L. dalmatica*. Genetic, ontogenetic, biotic, and abiotic environmental factors have all been shown to contribute to quantitative variation in iridoid glycosides in *Plantago* spp. (Bowers et al. 1992; Bowers and Stamp 1993; Adler et al. 1995; Darrow and Bowers 1999; Marak et al. 2002; Fuchs and Bowers 2004; Barton and Bowers 2006; Barton 2007; Wurst et al. 2008). Furthermore, such quantitative variation in these compounds has been shown to influence the degree to which individual plants are defended against generalist natural enemies, including pathogens and herbivores (e.g., Biere et al. 2004; Harvey et al. 2005).

In addition to influencing generalists, quantitative variation in iridoid glycosides of *L. dalmatica* also may affect specialists, including biological control agents used to manage *L. dalmatica* populations. Iridoid glycosides have been shown to act as feeding stimulants and oviposition cues, as well as to affect the performance of specialist insect herbivores (reviewed in Bowers 1991). In general, iridoid glycosides have been positively associated with both oviposition preference and performance of specialist insect herbivores (Bowers 1984; Pereyra and Bowers 1988; Bowers and Puttick 1989; Klockars et al. 1993; Nieminen et al. 2003; Harvey et al. 2005; Prudic et al. 2005; Saastamoinen et al. 2007; Reudler Talsma et al. 2008). Thus, iridoid glycosides may influence interactions between *L. dalmatica* and its biological control agents, potentially affecting host plant choice of ovipositing females as well as performance of offspring. Interestingly, we found that stems contain much lower levels of iridoid glycosides than flowers and leaves, which may have implications for biological control efforts, in particular for the use of *Mecinus janthinus*, which is a stem-miner. Gaining an understanding of the factors that contribute to variation in

iridoid glycoside content of *L. dalmatica* may contribute to improving the management of this species.

**Iridoid Glycoside Sequestration by Herbivores** This study is the first to document sequestration of iridoid glycosides by *Calophasia lunula*. We found that *C. lunula* sequesters intermediate levels of antirrhinoside, on average about 5% of the caterpillar dry weight. At these concentrations, sequestered iridoid glycosides have been shown to be deterrent or toxic to a wide variety of predators, including birds, spiders, stink bugs, wasps, and ants (Bowers 1980; De la Fuente et al. 1994; Dyer and Bowers 1996; Strohmeier et al. 1998; Theodoratus and Bowers 1999; Stamp 2001; Rayor and Munson 2002). We were surprised to find low levels of antirrhinoside in a few female adult moths; however, moths were frozen soon after emergence and may have retained some meconium, which can contain iridoid glycosides (Bowers and Puttick 1986; Bowers unpublished data). Variation in iridoid glycoside content of host plants has been shown to influence levels of iridoids found in sequestering species (e.g., Theodoratus and Bowers 1999; Prudic et al. 2005). Thus, factors that influence iridoid glycoside concentrations of *L. dalmatica* may also affect the degree to which *C. lunula* is defended against its natural enemies.

Compared to *Junonia coenia*, *C. lunula* is less efficient at sequestering antirrhinoside, as *J. coenia* larvae sequestered approximately twice as much antirrhinoside as *C. lunula* larvae. In general, *J. coenia* sequesters iridoid glycosides at high levels, with observed amounts up to 25% dry weight (Theodoratus and Bowers 1999). Larvae of three other lepidopteran species (*Meris paradoxa* Rindge (Geometridae), *Lepipolys perscripta* Gn (Noctuidae), and an undescribed species of *Lepipolys*) have been shown to sequester antirrhinoside at levels as high as 9.6 mg or 11.2% dry weight (Boros et al. 1991). Larvae of all three species feed on *Maurandya antirrhiniflora* (Plantaginaceae), which contains antirrhinoside in very high concentrations, up to 36% dry weight (Boros et al. 1991). Like *C. lunula*, these larvae are warningly colored; *M. paradoxa* is black, white, and yellow and the two *Lepipolys* species are gray with yellow, black, and white markings.

Plant defensive chemistry has been linked to the invasion success of numerous introduced species (Callaway and Aschehoug 2000; Callaway and Ridenour 2004; Cappuccino and Arnason 2006). Defense compounds are known to be of key importance in protecting plants from attack by generalist enemies and in host-plant selection by specialist enemies. However, as found in our study, plant defense compounds can demonstrate significant variation at multiple levels, including at the individual plant and population levels as well as over time. Such variation will ultimately influence the degree to which plants are

defended against generalist herbivores. Moreover, the influence of variation in plant chemical defenses on insect host plant selection and performance may contribute to variable success in biological control efforts. Thus, future studies that examine variation in the chemical defenses and chemical ecology of *L. dalmatica* may prove useful for understanding factors that influence the invasion success, impacts, and management of this species.

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