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Plant-mediated effects of soil nitrogen enrichment on a chemically defended specialist herbivore, *Calophasia lunula*

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Abstract. 1. Nitrogen enrichment is an important driver of environmental change. In the present study, plant-mediated effects of increased nitrogen on a specialist herbivore, *Calophasia lunula* Hufnagel, which sequesters antirrhinoside, an iridoid glycoside produced by its host plants, were examined.

2. Caterpillars were reared on *Linaria dalmatica* plants grown under low or high N treatments, and *C. lunula* performance traits and antirrhinoside levels were measured. Additionally, a bioassay was conducted with predatory ants to examine their response to antirrhinoside.

3. Nitrogen enrichment increased larval biomass and decreased larval antirrhinoside concentrations, but had no effect on plant iridoid glycoside concentrations or larval antirrhinoside content. Thus, differences in larval antirrhinoside concentrations were evidently a consequence of increased larval biomass. Additionally, nitrogen treatment had no effects on pupal performance or defence traits.

4. Bioassay results demonstrated a deterrent effect of antirrhinoside, but additional tests are necessary to evaluate the defensive role of this compound in insects.

5. Surprisingly, this study demonstrated little effect of a six-fold increase in nitrogen availability on *L. dalmatica* iridoid glycoside concentrations or sequestration by *C. lunula.* Moreover, the results suggested that changes in plant primary chemistry were more important than secondary chemistry for this specialist herbivore, and that some insects may be insensitive to N enrichment.

Key words. Antirrhinoside, *Calophasia lunula*, *Formica rufa,* iridoid glycoside, *Linaria dalmatica*, nitrogen enrichment, sequestration, tritrophic interactions.

Introduction

Human activity, in particular agriculture and industry, has led to dramatic changes in the global nitrogen cycle (reviewed in Galloway *et al*., 2004). Anthropogenic increases in soil nitrogen availability have direct and indirect biotic effects at multiple levels, including individual organisms, populations, communities, and ecosystems (reviewed in Vitousek *et al*., 1997; Fenn *et al*., 2003; Throop & Lerdau, 2004). Plant responses to increased soil nitrogen (N) availability include increased foliar N concentrations, photosynthetic rates, growth rates, and reproduction (Padgett & Allen, 1999; Aerts & Chapin, 2000; Leith *et al*., 2000; Magill *et al*., 2000). Additionally, increasing soil nitrogen availability can influence plant allelochemistry (e.g., Fajer *et al*., 1992; Höft *et al*., 1996; Wilkens *et al*., 1996; Hättenschwiler & Schafellner, 1999; Cipollini *et al*., 2002; Osier & Lindroth, 2004), although the direction and magnitude of change varies greatly among different plant species and types of allelochemical compounds. Such direct effects of nitrogen enrichment on plants may indirectly affect insect herbivores by modifying the phenology, quantity, and quality of host plants, which can alter herbivore behaviour, physiology, and chemical defences (Throop & Lerdau, 2004; Prudic *et al*., 2005). Such changes may also affect higher trophic levels by altering the availability and suitability of the herbivores as food for predators and parasitoids;

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however, little is known about how these higher trophic levels may be impacted. The aim of the present study was to explore the indirect effects of soil nitrogen enrichment on a specialist herbivore as mediated by the plant allelochemical response.

In comparison to plants, insects show greater constraints (i.e. fixed stoichiometric requirements) in the carbon–nitrogen balance of their tissues and often adjust feeding rates or differentially excrete elements in response to the C : N ratios of their food sources in order to maintain homeostasis (White, 1993, 2005; Moe *et al*., 2005). Nitrogen fertilisation can alter insect herbivore consumption rates, development, and survivorship; however, similar to plant allelochemical responses, the direction and magnitude of change is often species and context specific (Mattson, 1980; Kerslake *et al*., 1998; Power *et al*., 1998; Hättenschwiler & Schafellner, 1999; Throop & Lerdau, 2004; White, 2005). However, in general, there is a positive relationship between foliar N concentration and insect survivorship, development, growth, and reproduction (reviewed in Mattson, 1980; White, 1993, 2005; Throop & Lerdau, 2004).

Although increases in plant-available nitrogen may increase the nutritional quality of plant tissues (by increasing foliar N concentration), the effect of soil nitrogen enrichment on the overall quality of plant tissues for herbivores also depends on changes in plant allelochemicals. For specialist insect herbivores, host plant allelochemistry is known to play an important role in oviposition and feeding preferences as well as performance (Bernays & Chapman, 1994; Schoonhoven *et al*., 2005). Moreover, some insect herbivores have evolved the ability to sequester plant allelochemicals for their own protection against natural enemies (Bowers, 1991; Nishida, 2002; Opitz & Müller, 2009). For these specialist herbivores, the effects of soil nitrogen enrichment on plant allelochemistry may lead to changes in their ability to defend themselves against predators and other natural enemies.

In this study, we conducted a set of greenhouse experiments to examine the influence of soil nitrogen enrichment on the growth, defensive chemistry, and development rate of *Calophasia lunula* Hufnagel (Noctuidae) as mediated by changes in the allelochemistry of its host plant, *Linaria dalmatica* (L.) Mill. (Plantaginaceae). *Calophasia lunula* is a specialist herbivore of *Linaria* spp. and sequesters antirrhinoside, an iridoid glycoside produced by *L. dalmatica* (Jamieson & Bowers, 2010). Several previous studies involving other iridoid glycoside producing plants, such as *Plantago* spp., have demonstrated a decrease in plant iridoid glycoside concentrations with increased nutrient availability (Fajer *et al*., 1992; Darrow & Bowers, 1999; Jarzomski *et al*., 2000; Marak *et al*., 2003; Prudic *et al*., 2005; Jamieson & Bowers, 2012). Moreover, research by Prudic *et al*. (2005) showed an associated decrease in iridoid glycosides sequestered by *Junonia coenia* Hübner larvae. Except in the case of Jamieson and Bowers (2012), these previous studies focused on nutrient enrichment [using either a complete nutrient or nitrogen:phosphorous:potassium (N : P : K) addition] and thus did not isolate the effects of increased nitrogen. However, soil nitrogen enrichment can ultimately lead to phosphorous limitation (Vitousek *et al*., 2010) and thus may yield different plant responses compared with N : P : K enrichment.

In a previous greenhouse study, we found that increased soil N availability can lead to a decrease in *L. dalmatica* iridoid glycoside concentrations (Jamieson & Bowers, 2012). In the present study, we were interested in examining potential plant-mediated effects of anthropogenic increases in nitrogen availability on the performance and defence of a sequestering specialist herbivore and how such variation might be important for potential natural enemies of this caterpillar. Accordingly, we reared *C. lunula* larvae on *L. dalmatica* plants grown under variable soil nitrogen environments, specifically low and high N availability. Through a series of experiments, we tested the following predictions: (i) increased soil nitrogen availability will have a positive effect on *C. lunula* performance (i.e. growth and development rates) because plants will have a higher nitrogen content and thus be more nutritious; (ii) soil nitrogen enrichment will negatively affect *C. lunula* defensive chemistry owing to a decrease in iridoid glycoside concentrations of *L. dalmatica*; and (iii) antirrhinoside is a deterrent to putative *C. lunula* predators.

Materials and methods

Study system

Linaria dalmatica is a perennial plant native to Eurasia that escaped cultivation in North America during the early 1900s and is now a problematic invasive species in the United States and Canada (Vujnovic & Wein, 1997; Wilson *et al*., 2005). This species produces iridoid glycosides (Handjieva *et al*., 1993; Franzyk *et al*., 1999), which are a group of carbon-based monoterpenene derived compounds found in over 50 plant families (Boros & Stermitz, 1990, 1991). Iridoid glycosides are feeding deterrents and/or toxins for some generalist insect herbivores and plant pathogens as well as oviposition attractants and feeding stimulants for some specialist insect herbivores (Bowers, 1991; Marak *et al*., 2002; Beninger *et al*., 2008; Reudler Talsma *et al*., 2008). Moreover, these compounds are sequestered by a number of specialist insect herbivores for their own protection against natural enemies (Bowers & Puttick, 1986, Boros *et al*., 1991; Bowers, 1991). Levels of host plant iridoid glycosides can influence levels of iridoid glycosides stored in insect tissues, as demonstrated by a positive relationship between plant and insect iridoid glycoside concentrations (Camara, 1997a; Prudic *et al*., 2005; Lampert *et al*., 2011). Also, iridoid glycosides are positively associated with the preference and performance of insects that specialise on plants containing these compounds (Bowers, 1984; Pereyra & Bowers, 1988; Bowers & Puttick, 1989; Klockars *et al*., 1993; Nieminen *et al*., 2003; Harvey *et al*., 2005; Prudic *et al*., 2005; Saastamoinen *et al*., 2007).

Calophasia lunula is a defoliating lepidopteran, also native to Eurasia, which was introduced into North America in the early 1960s as a biocontrol agent for *L. dalmatica* and *L. vulgaris* (Wilson *et al*., 2005). This specialist herbivore is found throughout the range of *L. dalmatica* and its occurrence is widespread in the United States and Canada. *Calophasia lunula* larvae are warningly coloured and sequester antirrhinoside from *L. dalmatica* at levels ranging from 2.7% to 7.5%

caterpillar dry weight (Jamieson & Bowers, 2010). Although antirrhinoside has been shown to act as a defence against some generalist herbivores (Beninger *et al*., 2008), it has not been tested for defensive properties against natural enemies (i.e. predators, parasites, and pathogens) of sequestering insects. However, the demonstration of antirrhinoside sequestration in other specialist, aposematic larvae suggests that this compound functions in defence. Similar to *C. lunula*, larvae of *Meris paradoxa* Rindge (Geometridae), and an undescribed *Lepipolys* sp. (Noctuidae), which fed on *Antirrhinum majus* L. and *Maurandya antirrhiniflora* Willd., sequestered antirrhinoside at concentrations ranging from 3% to 11% dry weight (Boros *et al*., 1991). In order to evaluate the defensive properties of antirrhinoside, we performed a bioassay using a predatory ant species (see below).

Greenhouse experiment

Linaria dalmatica plants were grown from seeds collected from three populations in Boulder County, CO, U.S.A.: Lefthand Canyon (40°7′14″N; 105°19′26″W), Hall Ranch (40°12′42″N; 105°17′20″W), and Rabbit Mountain (40°14′13″N; 105°12′53″W). These seeds were collected from many plants (>15 per population) and were mixed together, which provided a random sample of offspring from many genetic families. Seeds were sown in flats filled with a sterile, nutrient-poor growing medium (Fafard Mix #2 – 70% peat plus equal parts perlite and vermiculite, manufactured by Conrad Fafard, Agawam, MA) and were maintained in the greenhouse during germination. Seedlings were transplanted into 8 l pots in a growing medium composed of equal parts sterilised sand, Fafard Mix #2, and Turface MVP (Turface Athletics, Buffalo Grove, IL). Seedlings were randomly assigned to one of two N addition treatments with supply rates approximating either 2 or 12 g m^{-2} $year^{-1}$ (referred to as low and high N treatments, respectively). These levels of N addition were selected because the low level falls within the range of nitrogen availability found in the introduced range of *L. dalmatica* and the high level just above that range, thus simulating an elevated nitrogen environment. Specifically, average annual nitrogen mineralisation rates across the Great Plains of the U.S.A. have been estimated to range from 1.5 to 10.5 g m^{-2} (Burke *et al*., 1997). Nitrogen treatments were added weekly in a 200 ml aqueous NH_4NO_3 solution. In addition to N treatments, 200 ml of a complete nutrient (quarter-strength Hoagland's solution minus N) solution was added approximately every 2 weeks (see Logan *et al*., 1999 for a description of the nutrient solution).

Greenhouse experiments were conducted in the summer/fall of 2007 and 2009. In both experiments, seeds were sown in late May. In 2007, seedlings were transplanted and experimental treatments began in late August. In 2009, seedlings were transplanted and experimental treatments began in mid-July. Thus, plants in the first experiment were approximately 5 weeks older at the start of the experiment. In both experiments, plants were grown for 10 weeks with N treatments before placing caterpillars on plants (initial $n = 25$ plants/treatment for each experiment). The average daytime greenhouse temperature was 25 °C and an average LD 14:10 h photoperiod was maintained with supplemental lighting. Before caterpillars were added to the plants, approximately 20 randomly selected leaves were clipped from each plant for chemical analysis of plant iridoid glycoside concentrations.

Calophasia lunula eggs were obtained from the Colorado Department of Agriculture Insectary (Palisade, CO, U.S.A.). Caterpillars were initially reared on field-collected plants to the third instar in a growth chamber (model Percival 36-LLVL) with a LD 16 : 8 h photoperiod and temperatures set to 25 °C day and 20 °C night. Upon reaching the third instar, caterpillars were weighed to the nearest 0.1 mg and placed on greenhouse plants (one individual per plant), which were then completely enclosed in Remay® bags to prevent the caterpillar from escaping. After 10 days of feeding, caterpillars were removed from plants, starved in Petri dishes for 10–12 h to empty the gut contents, weighed, and prepared for chemical analysis. In 2007 and 2009, we collected data on larval growth and chemical defences. In 2009, a second experiment was performed in which an additional set of caterpillars was added to plants 1 week after the first group was removed in order to also examine the effects of N treatment on development time and pupal chemical defences. These caterpillars were also put on plants as third instars (one individual per plant) and weighed prior to placement. Caterpillars were allowed to develop through pupation. We checked on caterpillars twice a day, recorded the time to complete development (number of days to pupation), weighed pupae to the nearest 0.1 mg, and then prepared samples for chemical analyses.

Sample preparation and chemical analyses

Plant samples were oven-dried at 50 °C to a constant mass, weighed to the nearest 0.01 g, leaf tissues were ground into a fine powder, and then 25–30 mg of each sample was prepared for chemical analysis. For *C. lunula*, larvae and pupae were weighed fresh, killed by freezing at −40 °C, macerated and extracted in methanol, and then prepared for chemical analyses. Methods for preparing and analysing samples by gas chromatography have previously been described (Gardner & Stermitz, 1988; Bowers & Stamp, 1993). Briefly, sample preparation involved extracting plant or insect material in methanol, filtering the methanol extract, evaporating the extract to dryness, and then partitioning the dried extract between water and ether to remove hydrophobic compounds (see Jamieson & Bowers, 2010 for further details).

Iridoid glycosides were analysed using gas chromatography on a Hewlett-Packard (HP) 5890A system (Agilent Technologies Inc., Santa Clara, CA, USA) and data were processed with HP ChemStation software (version A.03.34). Standards of antirrhinoside and linarioside were provided by Søren Jensen (Organic Chemistry, Lyngby, Denmark). Plant iridoid glycosides are reported as concentrations (% dry weight) of combined iridoid glycosides (antirrhinoside + linarioside). A previous study (Jamieson & Bowers, 2010) showed that *C. lunula* only sequesters antirrhinoside, and may convert

linarioside to antirrhinoside, although the fate of ingested linarioside has not been investigated. In this study, we report *C. lunula* antirrhinoside concentrations (% dry weight) and content (total mg) because both concentration and the total amount of this compound may be important to natural enemies. For example, predators ingesting small portions of prey tissues or haemolymph may be affected by concentrations; whereas for those ingesting entire individuals, the absolute amount may be more important. Finally, to facilitate comparisons between *L. dalmatica* and *C. lunula* iridoid glycosides, *C. lunula* antirrhinoside concentrations were calculated using wet to dry weight conversion factors (see Jamieson & Bowers, 2010).

Antirrhinoside bioassay

To test for deterrence of antirrhinoside and its potential function as a defence against predators, we conducted a bioassay in which laboratory ant colonies of *Formica rufa obscuripes* Forel were offered the choice of a control sugar solution or treatment sugar solution containing antirrhinoside (5 mg ml^{-1}). This ant species is a voracious predator and feeds on lepidopteran caterpillars (Weber, 1935; Hölldobler & Wilson, 1990). Ants were collected from 10 field colonies at the University of Colorado Mountain Research Station in late September 2009. They were maintained as 10 distinct colonies in the laboratory in rectangular plastic bins (35 cm × 25 cm × 15 cm) with Fluon® applied to prevent the ants from escaping. Colonies were maintained on a diet consisting of water and a 20% sugar solution. This sugar source was removed for 12–18 h prior to bioassay trials. Then, ants were presented with the choice of a control and treatment (antirrhinoside) sugar solution that each consisted of 20% sucrose. These sugar solutions were presented to ants as 100-µl drops on top of a two small Parafilm® squares (5 cm^2), which were placed side by side. We observed ant visitations for 2 h and recorded individual feeding bouts by ants (i.e. total consecutive time spent at each drop by individual ants). Although our original experimental plan was to use live caterpillars, preliminary experiments with protein baits, *C. lunula* larvae, and artificial diet-reared *Manduca sexta* larvae, which are palatable to predatory ants (Cornelius and Bernays, 1995), were unsuccessful for several reasons, including poor recruitment to these food sources.

Statistical analyses

Plant and caterpillar iridoid glycosides were analysed using mixed model ANOVA (analysis of variance) with N treatment as a fixed effect and year as a random effect. Treatment by year interaction effects were not significant in preliminary analyses and were not included in our final statistical models. For these analyses, we used linear mixed model procedures with the REML (restricted maximum likelihood) method in JMP® Pro, Version 9 (SAS Institute Inc., Cary, NC). Iridoid glycoside concentrations were analysed as proportions of dry weight and were arcsine square root transformed to meet assumptions of normality. For pupal chemistry data, we used one-way ANOVA to examine N treatment effects, as these data were collected only in 2009. Because *C. lunula* antirrhinoside levels were analysed in two ways (concentration and content), statistical significance was evaluated at a Bonferroni adjusted α-level of 0.025. Finally, we conducted a regression analysis to examine the relationship between plant and caterpillar iridoid glycoside concentrations.

We examined *C. lunula* performance traits (larval weight, pupal weight, and development time) using ANCOVA (analysis of covariance) with initial larval weight as a covariate and N treatment as the main effect. For the response variable larval weight, year was additionally included as a random effect. As in the analyses above, we used the REML method for linear mixed models. To test for heterogeneity of slopes, we included a covariate by treatment interaction according to the methods described in Raubenheimer and Simpson (1992). Non-significant interaction terms were removed from the final statistical model.

A non-parametric Wilcoxon test was used to examine differences in ant feeding time at control versus antirrhinoside sugar solutions. All statistical analyses were performed in JMP® Pro version 9.0.2 (SAS Institute Inc., 2010).

Results

Linaria dalmatica *and* Calphasia lunula *chemical defenses*

We examined leaf chemistry for plants that served as hosts for developing *C. lunula* larvae. Because a number of larvae died or escaped during the feeding experiments, the final sample size was $n = 75$ for both plant and larval data. In 2007, sample sizes were $n = 17$ and $n = 14$ for low and high N treatments, respectively, and in 2009, sample sizes were $n = 23$ and $n = 21$. Although plant iridoid glycoside concentrations decreased with increased N availability in both years (Fig. 1), the effect of N treatment was not significant ($F_{1,72.01} = 2.1836$, $P = 0.1438$). Overall, concentrations were higher in 2009 compared with 2007 (Fig. 1), and this difference may have been related to the older age of plants in the 2007 experiment. There was no significant difference in mean

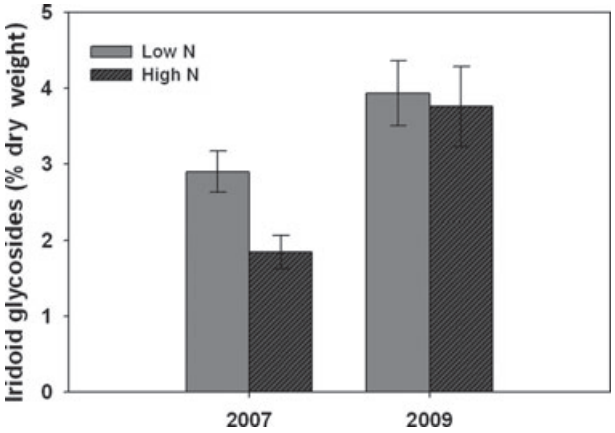

Fig. 1. Effect of nitrogen enrichment on iridoid glycoside concentrations (mean ± SE) of *Linaria dalmatica* leaf tissues.

Table 1. Effect of nitrogen enrichment on chemical sequestration by *Calophasia lunula.*

Response variables*	Df	*F*	*P*
Larvae ($N = 75$)			
Antirrhinoside content	1,72	0.8110	0.3708
Antirrhinoside concentration	1,72	5.8046	**0.0185**
Pupae ($N = 35$)			
Antirrhinoside content	1,33	0.180	0.6740
Antirrhinoside concentration	1,33	3.305	0.0782

*Significance was evaluated at a Bonferroni adjusted α level of 0.025 because antirrhinoside data were analysed in two ways (content and concentration). Significant effects are in bold type.

larval antirrhinoside content (mg per individual) between the two N treatment groups (Table 1; Fig. 2a), but there was a significant decrease in larval antirrhinoside concentrations (% dry weight) with nitrogen enrichment (Table 1; Fig. 2b). The overall mean concentration of antirrhinoside for larvae grown on plants in the high N treatment was 2.53% ± 0.163 SE ($n = 40$) compared with 2.96% ± 0.163 SE ($n = 35$) for the low N treatment. Larvae had higher levels of antirrhinoside (concentrations and content) in 2009 compared with 2007, which corresponded to the variation in plant iridoid glycoside concentrations observed in those years. However, plant iridoid glycoside concentration was a weak predictor of larval antirrhinoside concentration ($r^2 = 0.097$, $P = 0.0067$, Fig. 3). For pupae, nitrogen treatments had no effect on antirrhinoside content or concentration (Table 1; Fig. 4).

Calophasia lunula *performance traits*

ANCOVA indicated significant effects of N treatment ($F_{1,71.03} = 6.4065$, $P = 0.0136$) and initial size ($F_{1,71.97} = 5.3969$, $P = 0.0230$) on final larval weight. Larvae developing on plants grown in the high N treatment group were approximately 25% larger (153.37 mg ± 8.11 SE; $n = 40$) than larvae in the low N treatment (122.40 mg ± 7.56 SE; $n = 35$; Fig. 5). After 10 days of feeding on experimental plants, all caterpillars were in their fifth instar in both years. However, in 2007, most caterpillars were recently molted; whereas, caterpillars developed faster in 2009 and were mostly in the middle of the fifth instar. Accordingly, larvae were larger in 2009 compared with 2007. There was no significant effect of N treatment ($F_{1,33} = 1.4210$; $P = 0.2417$) or initial larval weight ($F_{1,33} = 0.0110$; $P = 0.9173$) on pupal weight.

The mean development time to pupation was 11.2 days ± 0.4 SE ($n = 17$) for larvae in the high N treatment and 12.8 days ± 0.5 SE ($n = 19$) for those in the low N treatment. ANCOVA results showed significant effects of N treatment ($F_{1,32} = 7.871$, $P = 0.0085$), initial larval weight ($F_{1,32} = 14.7475$, $P = 0.0005$), and N treatment by initial weight ($F_{1,32} = 15.051$, $P = 0.0005$). The significant treatment by covariate interaction revealed heterogeneous slopes, indicating that the relationship between initial weight and development time differed between N treatment groups (Fig. 6). Specifically, there was a negative linear relationship between initial weight and development time under the low N treatment (estimated slope =-309.55; $P < 0.0001$), but no relationship under the high N treatment (estimated slope = 1.58; $P = 0.9795$).

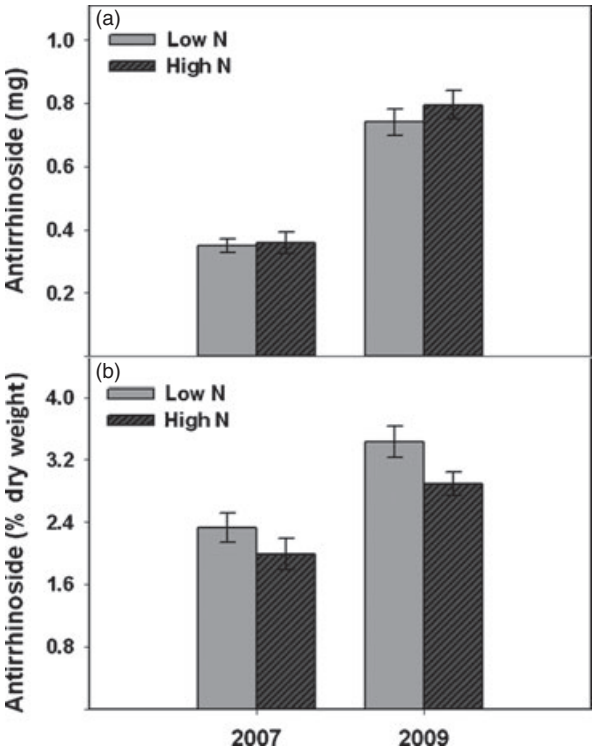

Fig. 2. Effect of nitrogen enrichment on *Calophasia lunula* larval antirrhinoside (a) content and (b) concentration (mean ± SE).

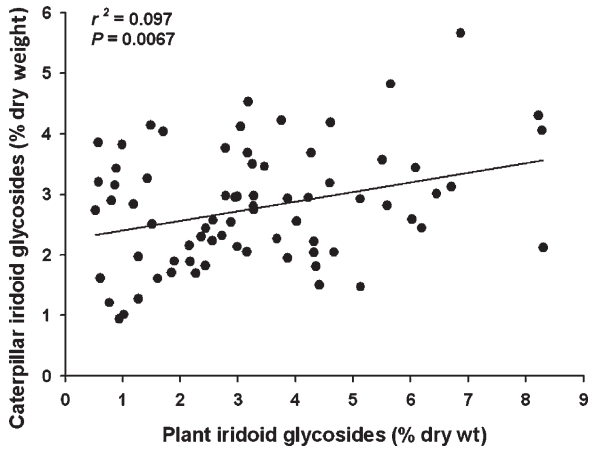

Fig. 3. Relationship between iridoid glycoside concentrations of *Linaria dalmatica* plants and *Calophasia lunula* caterpillars.

Antirrhinoside bioassay

Ants showed a clear preference for the control sugar solution ($n = 38$) over the treatment sugar solution containing

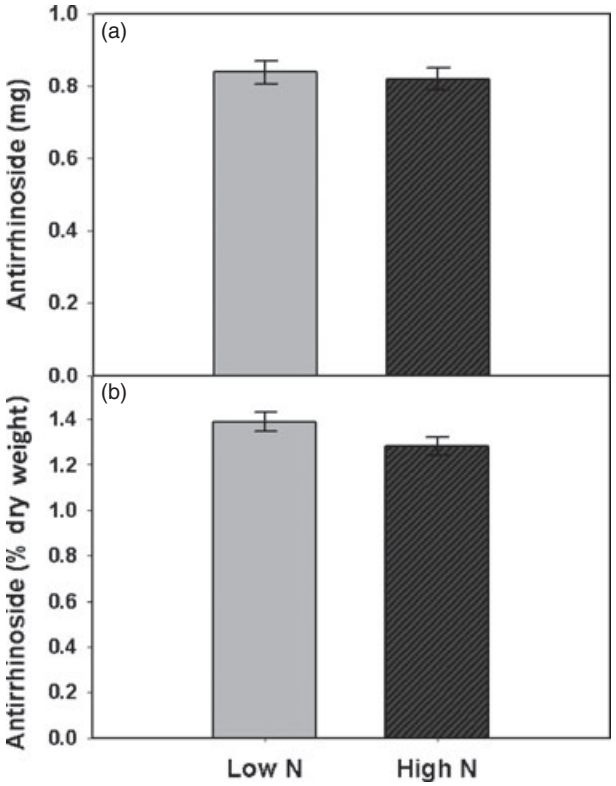

Fig. 4. Effect of nitrogen enrichment on *Calophasia lunula* pupal antirrhinoside (a) content and (b) concentration (mean ± SE).

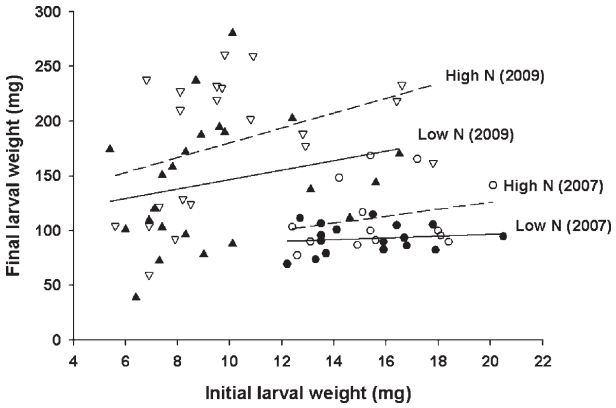

Fig. 5. Effect of nitrogen enrichment on *Calophasia lunula* larval weight.

antirrhinoside ($n = 16$) as demonstrated by greater mean feeding time spent at control solutions (Wilcoxon $P = 0.0004$; Fig. 7). Moreover, ants were observed demonstrating aggressive behaviour (e.g. tucked gaster indicating a sting posture) at drops containing antirrhinoside on numerous occasions. Ants were also observed exhibiting a grooming behaviour (e.g. cleaning antennae and mouth parts) after tasting the antirrhinoside sugar solution but not after tasting the control sugar solution.

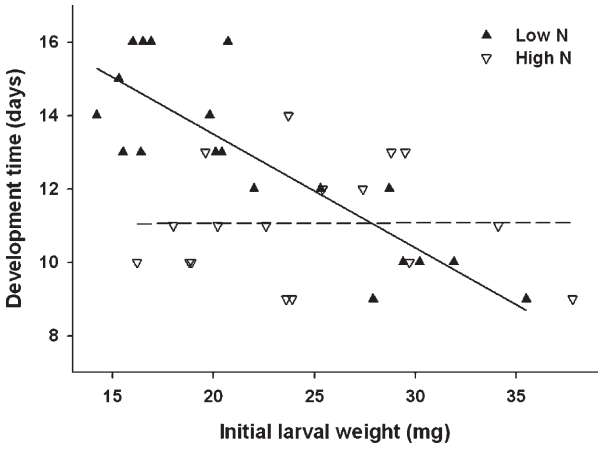

Fig. 6. Relationship between *Calophasia lunula* development time (days to pupation) and initial caterpillar size (the covariate) for low and high nitrogen treatment groups.

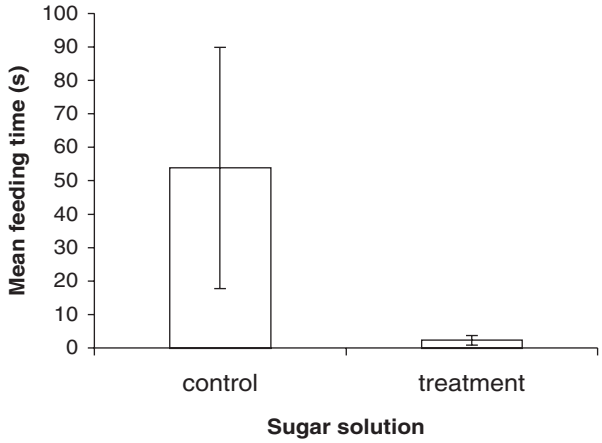

Fig. 7. Ant bioassay of antirrhinoside. Bars are mean ± SE time spent feeding (in seconds) per feeding bout by foraging individuals of *Formica rufa obscuripes*. The control sugar solution was 20% sucrose in water, and the treatment solution was 20% sucrose plus antirrhinoside at a concentration of 5 mg ml^{-1}.

Discussion

In the present study, we found little to no effect of nitrogen enrichment on either plant iridoid glycoside concentrations or sequestration by *C. lunula*. Overall, there were no changes in total antirrhinoside content for *C. lunula* larvae or pupae. There was a significant decrease in *C. lunula* larval antirrhinoside concentrations with nitrogen enrichment. However, this effect was small and apparently a consequence of increased larval biomass (a dilution effect), as plant iridoid glycoside concentrations explained less than 10% of the variation in larval defences. Thus, our results suggest that changes in plant primary chemistry were more important than changes in plant secondary chemistry for this specialist herbivore. Although plant nutritional quality was not measured in this study, numerous previous studies have documented increases in plant nutritional

quality with increased soil N availability as well as positive effects of increased nutritional quality on insect performance (Mattson, 1980; Awmack & Leather, 2002; Throop & Lerdau, 2004).

Past studies have shown that soil nitrogen enrichment can decrease plant iridoid glycoside concentrations, similar to the trend observed in this study during 2007 (Prudic *et al*., 2005; Jamieson & Bowers, 2012). However, the present study reveals that this response is not consistent and may depend on a number of factors, such as the amount and rate of N addition, plant age, and soil nutrient (e.g. phosphorous) limitation. In this study, we found no effect of a six-fold increase in nitrogen availability (2–12 g m^{-2}) on *L. dalmatica* iridoid glycoside concentrations. However, in a previous study (Jamieson & Bowers, 2012), there was an approximate 30% decrease in iridoid glycoside concentrations when soil nitrogen was increased from 2 to 8 g m^{-2}. In that study, nitrogen treatments were applied over 5 weeks compared with 10 weeks in this study. Similarly, Prudic *et al*. (2005) showed a 50% decrease in iridoid glycoside concentrations of fertilised *Plantago lanceolata* L., and a corresponding decrease in iridoid glycoside sequestration by *J. coenia* larvae. In contrast to the present study, plants in that study received an N : P : K nutrient enrichment treatment equivalent to a rate of 8.9 g m^{-2} $year^{-1}$ and this application was made over 8 weeks.

Although we attempted to simulate a realistic rate of increase and to isolate the effects of N enrichment, this study may underestimate the potential plant-mediated effects of N enrichment on *C. lunula* and other specialist herbivores that sequester iridoid glycosides. In these experiments, *L. dalmatica* plants demonstrated lower levels of iridoid glycosides as well as less variation compared with plants from field populations (Jamieson & Bowers, 2010, 2012). Moreover, *C. lunula* antirrhinoside concentrations were approximately 30–50% lower compared with concentrations found in a previous study (Jamieson & Bowers, 2010). However, we also found a weak relationship ($r^2 = 0.097$) between plant and larval iridoid glycosides, suggesting that changes in plant iridoid glycoside concentrations may only marginally affect levels of sequestered antirrhinoside. Research on other iridoid glycoside containing plants and sequestering herbivores have shown a wide range in the relationship found between plant and caterpillar iridoid glycosides: Lampert *et al*. (2011) found a similarly weak relationship ($r^2 = 0.14$), whereas Prudic *et al*. (2005) demonstrated a much stronger relationship ($r^2 = 0.44$).

In this study, the observed discrepancy in plant and larval defences may reflect a lack of knowledge about how *C. lunula* processes iridoid glycosides, in particular linarioside. Alternatively, overall plant iridoid glycoside concentrations may not be indicative of the concentrations that larvae consume. In our experiments, larvae foraged freely on individual plants and were observed feeding on both leaf and floral tissues, which may differ in iridoid glycoside levels (Jamieson & Bowers, 2010). Furthermore, leaf age may influence iridoid glycoside levels (Beninger *et al*., 2007). Thus, larvae could be selecting tissues with varying levels of iridoid glycosides that are not necessarily reflective of overall concentrations. Such foraging behaviour may also mitigate plant-mediated effects of N fertilisation.

Iridoid glycosides, namely aucubin and catalpol, have been shown to play an important defensive role against both vertebrate and invertebrate predators, with some predators showing a dose-dependent response (Bowers, 1991; De La Fuente *et al*., 1994/1995; Dyer & Bowers, 1996; Camara, 1997b). Results from our ant bioassay demonstrated deterrent properties for a different iridoid glycoside, antirrhinoside. Considering the warning colouration of *C. lunula* caterpillars and other antirrhinoside sequestering caterpillars (e.g. Boros *et al*., 1991), in addition to the results and observations in this study, antirrhinoside probably acts as a defence compound for this species and other insect herbivores that sequester this compound. However, further experiments are needed to examine the defensive nature of antirrhinoside as well as the importance of quantitative variation in this compound for natural enemies.

Host plant quality, both nutritional components and defence compounds, can have important direct and indirect effects on insect herbivores (Price *et al*., 1980; Awmack & Leather, 2002; Ode, 2006). Relatively little attention has been given to investigating the plant-mediated effects of soil N enrichment on indirect effects, in particular, those involving higher trophic levels (Throop & Lerdau, 2004). Such investigations may reveal complex effects of nitrogen addition on sequestering species. For example, as shown here, increased herbivore performance (i.e. larval biomass) may be associated with decreased chemical defences (i.e. larval antirrhinoside concentrations). However, our study also revealed that N enrichment (even a six-fold increase) had little effect on plant iridoid glycoside concentrations, indicating that secondary chemistry may be limited by other controlling factors, such as additional plant nutrients or resources. Moreover, these results suggest that changes in plant primary chemistry can potentially influence both herbivore performance and chemical defences, although insect herbivores may be resilient to small changes in host plant quality.

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