Ecological Entomology

Ecological Entomology (2014), **39**, 382–390 DOI: 10.1111/een.12113

Fine-tuning of defences and counter-defences in a specialised plant–herbivore system

SERGIO RASMANN Ecology and Evolutionary Biology, University of California, Irvine, California, USA

Abstract. 1. The plant–herbivore arms race has been postulated to be a major driver for generating biological and biochemical diversity on Earth. Herbivore feeding is reduced by the production of chemical and physical barriers, but increases plant resistance against subsequent attack. Accordingly, specialisation is predicted to be an outcome of herbivores being able to circumvent plant-induced defences.

2. Using a specialised plant–herbivore system, in which adult chrysomelid beetles (*Chrysochus auratus*) feed on leaves and larvae feed on roots of dogbane (*Apocynum* spp.), this study investigated whether root latex and cardenolides are effective against the soil-dwelling larvae, and whether such defences could be circumvented by the herbivore.

3. Across two *Apocynum* species, *C. auratus* larvae were not affected by latex production or cardenolide amounts and diversity. By contrast, cardenolide apolarity was detrimental to larval growth. Yet larval feeding decreased average root cardenolide apolarity in *A. cannabinum* and larvae performed better on those plants. Finally, above-ground induction rendered the plants more toxic by increasing root cardenolide apolarity and maintaining it, even during subsequent larval herbivory.

4. Therefore, the intimate relationship and interaction between *Chrysochus* and *Apocynum* are maintained by a delicate balance of herbivore manipulation and plant chemical induction.

Key words. Cardenolide, induction, latex, plant chemical defence, polarity.

Introduction

The question of which mechanisms underlie species coexistence is central to our understanding of drivers of community composition. Plant–herbivore theory predicts that the co-evolutionary arms race between plants and the insects that consume them should lead to the escalation of defences and counter-defences (Ehrlich & Raven, 1964). Plants have evolved a multitude of defence strategies, including physical and chemical barriers, all of which can be constitutively present or, as a cost-saving strategy, induced only following herbivore attacks (Karban & Baldwin, 1997). Herbivores, in response, have evolved counter-defence strategies. Examples include choosing a particular ontogenic stage of the plant (Barton & Koricheva, 2010); deactivating physical defences, such as the ability of monarch caterpillars to trench the latex-producing veins of milkweeds (Dussourd & Eisner, 1987; Dussourd & Denno, 1991); coping with the chemical toxicity of the plant by activation of detoxification enzymes (Li *et al.*, 2002); and sequestering the plant's chemical compounds for the herbivore's own defence (Duffey, 1980). Detoxification and sequestration by specialised herbivores are particularly detrimental to plants, and to counterbalance this, plants always need to produce more toxic chemical compounds (Berenbaum & Feeny, 1981; Farrell & Mitter, 1998; Rasmann & Agrawal, 2011b).

Damage by above-ground and/or below-ground herbivores has been shown to affect the primary and secondary chemistry of the plant across all tissues, and induction of one organ of the plant can lead to positive, negative or null effects on herbivores feeding on another organ of the plant (Bezemer & van Dam, 2005; Erb *et al.*, 2008; Rasmann & Agrawal, 2008; Kaplan *et al.*, 2008a). Generally, however, an early herbivore attack induces the whole plant to become more resistant to subsequent herbivore attacks (Karban & Baldwin, 1997; Agrawal, 1998). Therefore, when the entire life cycle of the herbivore is confined to the same plant, such as insects that are foliar feeders as adults and root feeders as larvae, we should expect fine-tuning in deployment by the plant and manipulation of plant defence induction by the herbivore (Clark *et al.*, 2011). For example, we should expect foliar-feeding adults to induce increased plant resistance against subsequent root-feeding larvae. In response, root-feeding larvae should be either immune to increased levels of defences or find ways to circumvent them.

Correspondence: Sergio Rasmann, Ecology and Evolutionary Biology, School of Biological Sciences, University of California, Irvine, 321 Steinhaus Hall, Irvine, CA. 92697-2525. E-mail: srasmann@uci.edu

Dogbane plants in the genus *Apocynum* (Apocynaceae), which are eaten by specialist chrysomelid beetles of the genus *Chrysochus* (Chrysomelidae), offer an optimal opportunity to test the above/below-ground induction dilemma for specialised herbivores. Adult beetles feed on plant leaves, whereas larvae are obligate root feeders on the same host-plant. Dogbanes are well defended; they constitutively produce and are able to induce the sticky and gluey latex throughout the plant, as well as cardenolides, similar to other milkweeds (Agrawal *et al.*, 2012). Cardenolides are specific inhibitors of the ubiquitous animal enzyme Na^+/K^+-ATPase, and have evolved as a defence against herbivores (reviewed in Agrawal *et al.*, 2012). They are produced throughout all plant tissues, including the leaves, stems, roots, and flowers of plants (Rasmann *et al.*, 2009a; Manson *et al.*, 2012). The multifaceted toxicity of cardenolides is a function of the diversity, amount, and polarity of the compounds (Rasmann & Agrawal, 2011b; Agrawal *et al.*, 2012). Similarly, latex, which also contains concentrated levels of cardenolides (Malcolm, 1991; Malcolm & Zalucki, 1996), is generally produced systematically in the plant (Zalucki & Malcolm, 1999; Agrawal & Konno, 2009), with clade-specific variations. For example, species in the genus *Asclepias* (a genus closely related to *Apocynum*) have not been shown to produce latex in root tissue (Rasmann & Agrawal, 2011a), whereas preliminary field observations show that dogbanes do produce latex in root tissue (see the Results section). Both latex and cardenolide toxicity are multidimensional and complex. Latex physically impairs insects' mandibles and digestion, but also contains concentrated doses of cardenolides and other toxic molecules not found elsewhere in the plant (Farrell & Mitter, 1998). Therefore, this thick plant fluid provides the plant with a powerful physico-chemical weapon.

Adults of *Chrysochus auratus* have been shown to avoid *Apocynum* defences with behavioural and physiological adaptations. They feed between major leaf veins, accumulate a large drop of latex under their mandibles, then drag their mouthparts backwards across the leaf to eliminate latex (Williams, 1991). Moreover, *C. auratus* beetles have evolved structural modifications at the binding site of their sodium–potassium pumps to reduce cardenolide activity. Therefore, *C. auratus* beetles can sequester and store cardenolides (Dobler *et al.*, 1998). However, cardenolides and latex have been shown to be effective against other specialist herbivores. For instance, both *Tetraopes* beetles and monarch butterflies are affected by increased latex production and variations in cardenolide quantities and structural forms (Zalucki *et al.*, 1990; Malcolm & Zalucki, 1996; Zalucki *et al.*, 2001; Rasmann *et al.*, 2009b; Rasmann & Agrawal, 2011a). To date, we have little evidence that latex is an effective defence mechanism against root herbivores (Rasmann & Agrawal, 2008; Agrawal & Konno, 2009).

In this study, I investigated the mechanisms of defence and counter-defence to understand how the *Apocynum*/*Chrysochus* system is maintained, and specifically how adult feeding influences subterranean larval performance. I had the following working hypotheses: (i) *Apocynum* plants defend themselves against root herbivory using both latex and cardenolide induction; (ii) prior adult attacks will lead to increased resistance against subsequent root-feeding larvae; and (iii) root feeders have evolved tolerance or counter-defence strategies against both latex and cardenolide induction.

Material and methods

Plants and insects

During summer 2010, I conducted a field survey to measure latex production in the roots of all *Apocynum* (Apocynaceae) species in the northeastern United States. In central New York, USA, the three species – *Apocynum cannabinum* L. (Indian hemp), *Apocynum androsaemifolium* L. (spreading dogbane) and *Apocynum sibiricum* L. (the Siberian dogbane, which is classified as either a sub-species *A. cannabinum* var. *sibiricum* or a synonym of the previous (Steffey, 1983) – rarely co-exist within the same patch. *A. cannabinum* is mostly found in old, open fields, *A. androsaemifolium* is commonly found in shaded forest hedges, and *A. sibiricum* is mostly found on gravelly to sandy soils near river edges.

Dogbanes are the main source of food for specialised and aposematic chrysomelid beetles in the genus *Chrysochus*, including *C. auratus* Fabricius (Chrysomelidae: Eumolpinae), found throughout most of the USA east of the Rocky Mountains and in several localities in the Pacific Northwest (Peterson *et al.*, 2001). Adults feed and mate on leaves of the plants. A female lays several egg clutches on the leaf lamina and protects them with a faecal shield. After hatching, larvae fall to the ground and burrow into the soil in search of roots; pupation takes place during the winter and new adults emerge the following spring. For the experiments described in the following, I reared *C. auratus* larvae by collecting adults from several populations around Tompkins County, New York, and kept them under ambient conditions in plastic insect-rearing cages. Fresh *A. cannabinum* leaves were provided daily, and oviposited egg clutches were separated from leaf lamina, and kept in separated boxes until larval emergence.

Field observations

For measuring ambient latex production, soil was removed to expose the roots and rhizomes, free of soil, for all plants. When approximately 10 cm of below-ground organs were visible, a fine-mesh bag was placed around the roots, and two treatments were initiated: half of the *Apocynum* plants were inoculated with 5 *C. auratus* first-instar larvae, and the other half were left herbivore-free. From my observations it was clear that larvae are able to feed on all parts of the root system (Figure S1), but virtually no latex was observed exuding from the fine roots. Latex was therefore measured on the primary roots of the plant (Figure S1). In addition, average root diameter for both control and damaged roots was held constant (*t*-test between control and treated plants, $P > 0.05$ for all three species tested). After 7 days, the bagged roots were cut transversally on one side, and latex exudation was collected on pre-weighed filter paper until the latex stopped exuding, dried for 4 days at 60 °C, and weighed ($n = 10$ plants per treatment and per species; Figure S1).

Apocynum cannabinum *and* Apocynum androsaemifolium *experiment*

Based on the initial field survey, I measured root-feeding larval performance on two latex-producing, contrasted species: the high-latex *A. cannabinum* and the low-latex *A. androsaemifolium* (see Figure S2). Seeds of both species were collected in Tompkins County, New York, New York and pooled across five populations. After 6 months of dormancy at 5 °C, the seeds were stratified in humid paper for 2 weeks, after which the seedlings were transplanted into 15-cm-diameter plastic pots filled with a mixture of potting soil (Metro-Mix 360; Metro-Mix Sun Gro Horticulture Canada CM Ltd, Vancouver, BC, Canada) and perlite (3 : 1 parts potting soil:perlite) and grown fully randomised in a single growth chamber (LD 14:10 h, 25:16 °C day:night) ($n = 20$ for *A. androsaemifolium* and $n = 23$ for *A. cannabinum*). After 6 weeks of growth, the plants were inoculated with five newly emerged first-instar *C. auratus* larvae ($n = 12–13$) by placing them approximately 1 cm deep in the soil near the base of plant shoots. Larvae were left to feed for 1 month, after which the plants were cut at the base of the shoots, 2 cm into the soil. Latex exudation from these rhizomes was collected on filter paper and weighed after being dried at 40 °C for 5 days. Subsequently, all soil was placed in custom-made Berlese funnels (20 cm diameter, 12 cm high, with a 0.4-cm mesh grid on the bottom) for 2 weeks under the same plant growth conditions, to collect surviving root-feeding larvae. Finally, the recovered larvae, roots and shoots were weighed after being dried at 40 °C for 5 days as a measure of growth rate.

Cardenolides were measured on herbivore-treated and undamaged roots by grinding roots that had been oven-dried for 5 days at 40 °C to a fine powder using a MM300 grinder (Retsch, Haan, Germany) in 10-ml steel grinding vessels at 27 Hz for 2 min. Between 50 and 100 mg of tissue were spiked with 20 µg of digitoxin (Sigma-Aldrich, St. Louis, Missouri; CAS:71-63-6) as the internal standard and extracted with 1 ml of pure methanol in a sonicating water bath at 55 °C for 20 min. After centrifugation and filtration with a 45-µm pore size Millex filter (Millipore, Billerica, Massachusetts), high-performance liquid chromatography (HPLC) analysis was performed by injecting 15 µl of the supernatant into an Agilent 1100 series HPLC (Agilent Technologies, Santa Clara, California), and the compounds were separated on a Gemini C18 reversed phase column (3 µm, 150 mm × 4.6 mm; Phenomenex, Torrance, California). The cardenolides were eluted at a constant flow of 0.7 ml min^{-1} with an acetonitrile/0.25% phosphoric acid in water gradient as follows: 0–5 min, 20% acetonitrile; 20 min, 70% acetonitrile; 20–25 min, 70% acetonitrile; 30 min, 95% acetonitrile; 30–35 min, 95% acetonitrile. The peaks were detected using a diode array detector at 218 nm. The cardenolide concentrations were calculated using the digitoxin internal standard and initial sample mass, and total cardenolides were calculated as the sum of the individual peaks. I used the Shannon–Wiener diversity index $H = -\text{sum}(P_i \log[P_i])$ to assess cardenolide diversity, where P_i is the relative amount of a given cardenolide peak divided by the total amount of cardenolides in each individual plant. I then calculated average cardenolide polarity of the two species and across different herbivore treatments as $P = \text{sum}(P_i \text{RT}_i)$, where RT_i is the retention time of the ith peak in the species, weighted by each peak's relative amount P_i (Rasmann & Agrawal, 2011b).

Previous adult induction experiment

To measure the effect of previous adult induction on subsequent larval performance and plant responses, 30 additional *A. cannabinum* plants were germinated and grown as described earlier, but the plants were placed in an outdoor common garden to approach natural growing conditions. Six plants were set aside for measuring the constitutive defence levels, and the rest were divided into previous adult induction or no previous adult induction treatments. Adult treatments ($n = 12$ plants) were performed by caging one adult per plant on one leaf using clip cages 3 cm high × 5 cm diameter for 7 days. This treatment has the advantage of not having to mask the entire plant from sunlight. After that, all plants except the control treatment were inoculated with five first-instar larvae, which were left to feed for 3 weeks. The insect larvae, the latex exudation, and the root cardenolide toxicity indices were sampled and analysed as described earlier.

Statistical analyses

Differences in cardenolide composition among species, herbivore treatment, and the interaction in the two species experiment, as well as between spatially separate herbivore treatments in the previous adult induction experiment, were tested with a permutational ANOVA (PERMANOVA), using a distance matrix with the ADONIS function in the VEGAN package in R (Oksanen *et al.*, 2013). The Bray–Curtis metric was used to calculate dissimilarity among samples. For the two-species experiment, one-way ANOVAS were used to test the effect of plant species on field latex exudation and larval growth. Two-way ANOVAS were used to test the effects of species, larval herbivory, and their interaction on plant biomass as well as the root-to-shoot ratio, latex production, and cardenolide parameters (total amount, diversity, and polarity).

In order to underpin the mechanisms of plant resistance, average larval weight per plant was analysed using two ANCOVAS, with species and either root latex or cardenolide toxicity as main factors. To avoid inflating type I error through multiple testing, and to account for non-independence among different cardenolide dimensions of toxicity, variation in the cardenolide defensive traits (i.e. total amount, number of individual compounds, diversity, and polarity) was summarised with a principal component analysis (PCA) using PRINCOMP in R into one axis of toxicity (PC1). The first axis of the PCA separates polarity against amount and diversity of cardenolides (Figure S3). For the previous adult induction experiment, one-way ANOVAS were used to test the effect of previous above-ground induction on larval growth, latex production, and cardenolide parameters (total amount, diversity, and polarity). Variation in average larval weight per plant was analysed with two ANCOVAS, including treatment and either root latex or the first axis of the PCA (PC1) as described earlier.

Results

Field observations

During the field survey, *A. cannabinum* and *A. sibiricum* produced, on average, 17 times more latex than *A. androsaemifolium* (Figure S2, species effect, $F_{2,54} = 12.50$, $P < 0.0001$). All three species had different responses to root herbivore attack, with *A. androsaemifolium* showing no induction, and *A. cannabinum* increasing and *A. sibiricum* decreasing latex exudation after 7 days of *C. auratus* larval feeding (treatment effect, $F_{1,54} = 0.82$, $P = 0.37$; species × treatment interaction, $F_{2,54} = 10.33$, $P = 0.0002$; Figure S2).

Apocynum cannabinum *and* Apocynum androsaemifolium *experiment*

Given the species variation, it is an optimal opportunity to indirectly test the effects of latex on *C. auratus* larval performance, specifically using the high latex-producing *A. cannabinum* and the low latex-producing *A. androsaemifolium*. Overall, the two plant species differed in their growth, with *A. cannabinum* having, on average, 2.7 and four times more biomass above ground and below ground, respectively, than *A. androsaemifolium* (species effect for shoots, $F_{1,39} = 47.22$, $P < 0.0001$; for roots, $F_{1,39} = 75.41$, $P < 0.0001$). *Chrysochus auratus* larval herbivory did not affect shoot biomass but decreased root biomass, particularly for *A. cannabinum* (treatment effect for shoots, $F_{1,39} = 0.50$, $P = 0.05$, and for roots, $F_{1,39} = 4.47$, $P = 0.04$; and species × treatment interaction for shoots, $F_{1,39} = 0.09$, $P = 0.76$, and for roots, $F_{1,39} = 2.85$, $P = 0.09$). Larval herbivory decreased the root-to-shoot ratio by 60% in both species (species effect, $F_{1,39} = 15.48$, $P < 0.001$; treatment effect, $F_{1,39} = 11.80$, $P = 0.001$; interaction term, $F_{1,39} = 1.17$, $P = 0.29$).

Overall, *C. auratus* larvae grew almost three times heavier and had a 67% higher survival rate on *A. cannabinum* than on *A. androsaemifolium* (Fig. 1a, Table 1, and for survival, $F_{1,21} = 8.12$, $P = 0.001$). Confirming field observations, root latex exudation in *A. cannabinum* was almost five times greater than in *A. androsaeminifolium* (Table 1). Larval herbivory, however, did not affect latex production in the roots of either species in this experiment (Table 1). Subsequently, healthy and herbivore-damaged roots were sampled to measure several indices of cardenolide toxicity. Permutation analysis showed that the two dogbane species differed in the amount and diversity of cardenolides produced and how they were deployed after root herbivory (for species, $F_{1,36} = 25.19$, $P = 0.001$; for treatment, $F_{1,36} = 5.41$, $P = 0.003$; for the interaction, $F_{1,36} = 18.47$, $P = 0.001$). In particular, *A. cannabinum* had almost twice the concentration of cardenolides than *A. androsaemifolium*, but the cardenolide levels in the former were reduced after herbivory, whereas the cardenolide levels in the later were induced after herbivory (Table 1). Overall, *A. cannabinum* displayed 35% more cardenolide diversity, but herbivory similarly decreased cardenolide diversity in both species (Fig. 1b; see the non-significant interaction effect in Table 1). Finally, I observed that *A. cannabinum* cardenolides were generally more apolar than *A. androsaemifolium*, but larval herbivory decreased the cardenolide apolarity (Fig. 1c, Table 1). In other words, larval feeding induced *A. cannabinum*, but not *A. androsaemifolium*, to produce more polar cardenolides.

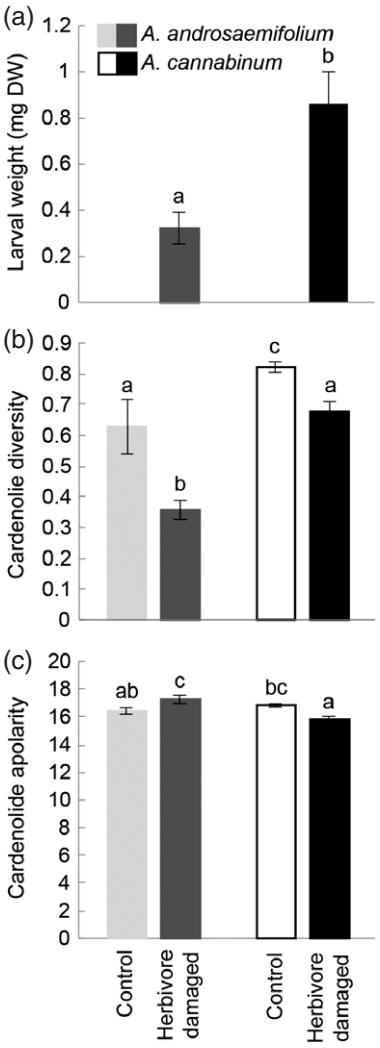

Fig. 1. Below-ground resistance and defences in *Apocynum*. All values are the averages ± 1SE. (a) *Chrysochus auratus* larval weight after 4 weeks of feeding of *Apocynum androsaemifolium* (shaded bars) and *Apocynum cannabimun* (black-and-white bars) plants; (b) root cardenolide diversity of undamaged plants (control) or plants damaged by *C. auratus* larvae; (c) root cardenolide average polarity on the same plants; higher numbers indicate more apolar cardenolides. Asterisks above the bars indicate significant differences among treatments (*t*-test, $P < 0.05$). DW, dry weight.

By dissecting the mechanism of root toxicity, I observed that larval weight showed no correlation with the latex levels in the roots (latex effect, $F_{1,19} = 0.90$, $P = 0.35$; species effect, $F_{1,19} = 3.21$, $P = 0.09$; species × latex interaction, $F_{1,19} = 2.35$, $P = 0.14$; Fig. 2a), but a positive correlation with PC1 (PC1 effect, $F_{1,18} = 5.62$, $P = 0.03$; species effect, $F_{1,18} = 0.003$, $P = 0.96$; species × PC1 interaction, $F_{1,18} = 2.16$, $P = 0.16$; Fig. 2b). These results indicate that root-feeding larval weight is positively associated with increased levels of cardenolides and

Table 1. Two-way ANOVA table for testing the effect of species (*Apocynum androsaemifolium* and *Apocynum cannabinum*), herbivore treatment (control or *Chrysochus auratus* larval induction) and their interaction, and one-way ANOVA table for testing the herbivore treatment effect (control, previous adult above-ground induction or no previous above-ground induction) for plant resistance response (measured as larval weight gain) and defence traits (latex production and cardenolide abundance, diversity and polarity).

Experiment/response variable	Effect	df numerator	df denominator	*F* ratio	*P* value
A. cannabinum versus *A. androsaeminifolium*					
Larval weight	Species (S)	1	21	10.70	**0.004**
Latex production	S	1	39	7.46	0.009
	Herbivore (H)	1	39	0.24	0.63
	S × H	1	39	0.004	0.95
Total cardenolides	S	1	36	14.88	**0.001**
	H	1	36	0.93	0.34
	S × H	1	36	11.76	**0.002**
Cardenolide diversity	S	1	36	33.19	**< 0.0001**
	H	1	36	21.66	**< 0.0001**
	S × H	1	36	2.08	0.16
Cardenolide polarity	S	1	36	4.67	**0.04**
	H	1	36	0.15	0.70
	S × H	1	36	16.31	**0.0003**
A. cannabinum only					
Larval weight	H	1	20	4.64	**0.04**
Latex production	H	2	25	2.09	0.14
Total cardenolides	H	2	25	0.16	0.85
Cardenolide diversity	H	2	25	0.73	0.50
Cardenolide polarity	H	2	25	3.99	**0.03**

Bold indicates significant values ($P < 0.05$).

their diversity, but negatively associated with increased levels of cardenolide apolarity.

Previous adult induction experiment

I next determined whether previous adult above-ground feeding influenced subsequent below-ground larval feeding using *A. cannabinum* plants. On average, larvae grew 20% less on plants that were previously attacked above ground by leaf-feeding adults, compared with previously undamaged plants (Fig. 3a, Table 1). Herbivore treatments also affected the production and deployment of root cardenolides ($F_{2,25} = 4.13$, $P = 0.003$). However, neither above- nor below-ground herbivore attacks significantly modified the latex production, total cardenolide levels, or cardenolide diversity (Table 1). Nevertheless, the direction of the response matches previous results, in which above- and below-ground feeding, on average, increased cardenolide abundance by 8% and decreased cardenolide diversity by 12%.

As shown earlier, larval feeding increased the cardenolide polarity in *A. cannabinum* (lower apolar numbers in Fig. 3b), particularly when compared with previous adult induction (Table 1). Again, I found no significant correlation between larval weight and root latex production (latex effect, $F_{1,17} = 0.03$, $P = 0.86$; treatment effect, $F_{1,17} = 2.95$, $P = 0.10$; latex × treatment interaction, $F_{1,17} = 1.90$, $P = 0.19$), but confirmed a positive correlation between larval growth and cardenolide amount and diversity, but a negative relationship with apolarity (PC1 effect, $F_{1,17} = 4.55$, $P = 0.04$; Fig. 3c), which was mediated by the larval feeding but not after previous adult feeding (treatment effect, $F_{1,17} = 6.59$, $P = 0.02$; PC1 × treatment interaction, $F_{1,17} = 2.88$, $P = 0.11$).

Discussion

In accordance with initial hypotheses, I found that *Chrysochus* beetle larvae are impaired by cardenolide toxicity in which more apolar cardenolides are more toxic, but not for measures of cardenolide abundance and diversity. However, larval feeding increases plant susceptibility by increasing cardenolide polarity in *A. cannabinum* but not in *A. androsaemifolium*. Yet *A. cannabinum* plants that have been previously eaten above ground are able to restrain subsequent larval manipulation of cardenolide polarity.

Insect induction of plant defences is mediated by the plant's ability to perceive damage and insects' secretions (Bonaventure *et al.*, 2011), and several examples have shown strong specificity of induction, which is mediated by changes in the phytohormone network, direct control of gene expression, or the redox state of the cell (Bonaventure, 2012). Specificity of induction can span variation in how much latex is produced after attack (Van Zandt & Agrawal, 2004), the amount and quality of volatile organic compounds produced (De Moraes *et al.*, 1998; Rasmann & Turlings, 2008) or the defence-related genes that are expressed (De Vos *et al.*, 2005; Erb *et al.*, 2009; Hiltpold *et al.*, 2011). So far, two major classes of insect-specific elicitors are known, including enzymes and fatty acid conjugates. Examples include the β-glucosidase, discovered in the regurgitant of *Pieris brassicae* larvae, which seems to facilitate the emission of glucosinolate breakdown

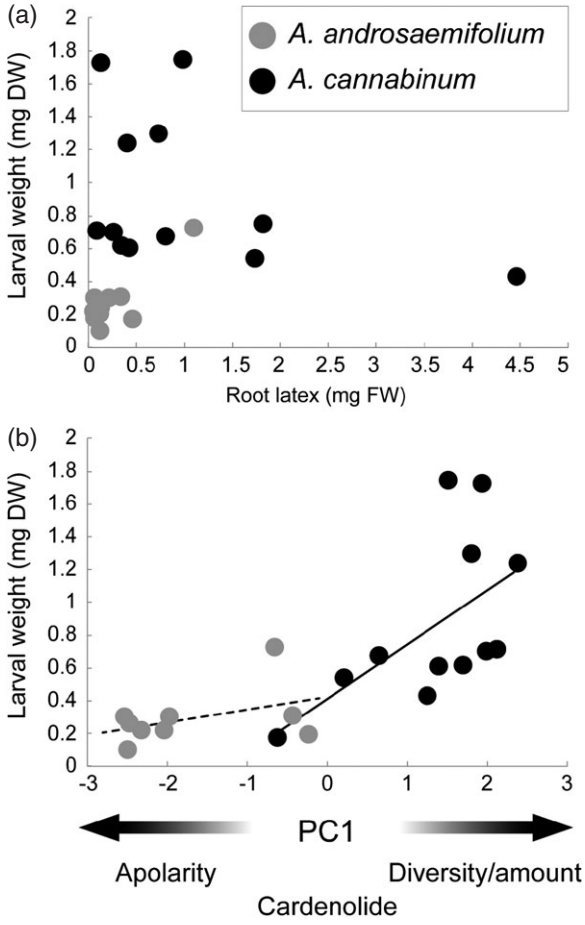

Fig. 2. *Chrysochus auratus* larval weight as a function of root latex exudation ($P = 0.07$) (a) and the first axis of the principal component analysis (PC1) (b), summarising different dimensions of cardenolide toxicity (total amount, number of individual compounds, diversity, and polarity) of herbivore-induced *Apocynum cannabinum* (black circles) and *Apocynum androsaemifolium* (shaded circles) plants. When moving along the axis of PC1 from left to right, cardenolide apolarity decreases and cardenolide diversity and amount increase. DW, dry weight; FW, fresh weight.

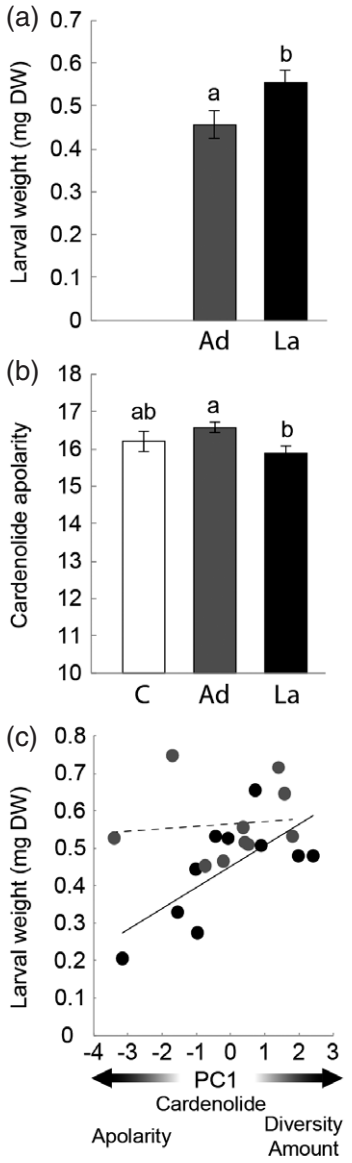

Fig. 3. Effects of previous above-ground induction on below-ground resistance and defences. All values are averages ± 1SE. (a) *Chrysochus auratus* larval growth on *Apocynum cannabinum* plants that were either previously induced by above-ground adult feeding (Ad, grey bar) or left undamaged (La, black bars). (b) Root average cardenolide polarity on the same plants and on healthy, undamaged plants (C, open bars). Higher numbers indicate more apolar cardenolides. (c) Phenotypic correlation between larval weight and the first principal component (PC1) as decribed in Fig. 2. Different letters above the bars indicate significant differences among treatments (*t*-test, $P < 0.05$). DW, dry weight.

products (Mattiacci *et al.*, 1995), and the fatty acid derivate volicitin [N-(17-hydrohylinolenolyn)-L-glutamine] and related compounds that, particularly in maize, induce the release of the full-blend volatile organic compounds normally induced by caterpillar feeding (Alborn *et al.*, 1997). I am not aware of specific elicitors or fatty acid conjugates isolated from beetles, but several studies indicate beetle-mediated specificity of inductions (Erb *et al.*, 2008; Erb *et al.*, 2012). For instance, root feeding by corn rootworm larvae on maize plants resulted in increased accumulation of 2,4-dihydroxy-7-methoxy-1,4-benzoxazine-3-one (DIMBOA) and chlorogenic acid in shoots, which is mediated by the activation of the phytohormone abscisic acid (Erb *et al.*, 2009).

Contrary to the initial hypothesis, latex had no effect on larval performance. This result might be due to the fact that root-feeding larvae are able to behaviourally circumvent latex production below ground, as was shown for above-ground feeding in this same system (Williams, 1991), or by trenching the latex exudation in other similar systems (Malcolm & Zalucki, 1996). I am not aware of any studies explicitly testing larval trenching or other behaviours for avoiding root latex, but this is an enticing hypothesis. Another plausible reason for this result is that latex production varies across the underground

system, and root herbivores display specific preferences for tissues and sites within the roots when feeding (Erwin *et al.*, 2013). Therefore, moving and feeding on different root parts (as shown in Figure S1d) will help to prevent larvae from being confronted by latex accumulation at the site of wounding, but this needs to be thoroughly tested. Finally, the slight discrepancies between latex induction observed in the field experiment and the controlled room environment suggest that there exist context-specific plant responses after herbivore induction, including different ontogenic stages, different lengths of induction, different light regimes, soil properties (Karban & Baldwin, 1997), and very likely different microbial communities that colonised the damaged roots (Pozo & Azcon-Aguilar, 2007; Pineda *et al.*, 2010).

I found that previous shoot adult herbivory was detrimental to subsequent root larval herbivory. This is in line with a similar study in which previous shoot herbivory by the noctuid moth *Spodoptera frugiperda* had a significant negative effect on colonisation by *Diabrotica virgifera* root-feeding larvae in the field and on weight gain in the laboratory (Erb *et al.*, 2011).

As a counter-example, it was shown that the caterpillars feeding on leaves of tobacco plants increased the nutritional quality of roots, thus favouring the colonisation of phytophagous nematodes (Kaplan *et al.*, 2008b). In the opposite direction, monarch (*Danaus plexippus*) caterpillars were facilitated by the presence of longhorn beetle larvae (*Tetraopes tetraophtalmus)* feeding on the roots of the same host plants, possibly an effect mediated by a reduction in latex production in the leaves when *Tetraopes* were present (Rasmann *et al.*, 2009a). Nonetheless, how the manipulation and counter-defence are mediated above and below ground by the same species is still to be completely elucidated. Future work on the role of phytohormones, particularly jasmonic acid and abscisic acid, will be informative regarding how plants respond to specialised and non-specialised attackers (Erb *et al.*, 2012).

This work supports the theory that cardenolide toxicity increases with increasing apolarity. Apolar cardenolides are more toxic, as they travel more easily through the insect membranes due to their enhanced solubility in phospholipid membranes, and they are therefore more likely to reach the Na^+/K^+ pumps on the insects' organs (Fordyce & Malcolm, 2000; Rasmann *et al.*, 2009b; Agrawal *et al.*, 2012). As a proof of concept, *D. plexippus* caterpillars that were fed with leaves painted with the highly polar cardenolide oubain grew better than those fed with leaves painted with the apolar digitoxin (Rasmann *et al.*, 2009b). Several insect species that feed on cardenolide-bearing plants have evolved molecular substitutions at the binding sites of the Na^+/K^+-ATPase (Dobler *et al.*, 2012), including *C. auratus* (Zhen *et al.*, 2012). Both plant species responded by increasing the cardenolide diversity, which was positively correlated with insect growth (Fig. 2b). These results indicate that *C. auratus* larvae do indeed suffer little from higher amounts of cardenolides; rather, they suffer from variation of cardenolide polarities. The exact mode of cardenolide toxicity is still poorly understood (Agrawal *et al.*, 2012), and future work is needed to assess the cost/benefit analysis of variation in cardenolide polarity, particularly in the light of cardenolide sequestration by larvae and adults. For instance, the costs of sequestration and storage of particularly apolar compounds might increase susceptibility to antagonists, such as entomopathogenic fungi or nematodes (Duffey, 1980; Gassmann *et al.*, 2008; Rasmann *et al.*, 2011). Indeed, because larvae suffer more when feeding on roots of a previously attacked plant, there should be selection of larvae to move in the soil to find a different host. This might expose the larvae to a higher risk of encountering soil-dwelling natural enemies. Finally, it is possible that specific individual cardenolide molecules are more toxic than others, and therefore future work needs to test the individual as well the synergistic effect of induced cardenolides on *C. auratus* larvae.

Taken together, these results show that root latex production does not explain underground herbivore performance, contrary to initial expectations; however, different dimensions of cardenolide toxicity affected herbivores differently. In particular, larval feeding has the potential to induce susceptibility in plants by increasing the overall polarity of the cardenolides. This is impaired by previous above-ground adult feeding, however, which induces the plant to increase systemic resistance below ground by increasing and maintaining cardenolide apolarity, even during subsequent larval feeding. The co-evolutionary arms race between plants and herbivores, which has been proposed to be a major driver in generating biological diversity on Earth (Farrell, 1998), is therefore balanced by the delicate fine-tuning of defence and counter-defence induction between plants and their herbivores, as shown here, and fits well within the framework of macromolecular adaptation and counter-adaptation in plant–pathogen interactions (Dangl & Jones, 2001).

Acknowledgements

I am grateful to Anurag Agrawal and the Cornell Chemical Ecology Core Facility group for providing support and infrastructure for the experiments, and to Matthias Erb, Georg Petschenk, Anurag Agrawal, and two anonymous reviewers for comments on previous versions of the manuscript. This work was supported by a postdoctoral fellowship from the Swiss National Science Foundation (PA0033-121483).

Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference:
10.1111/een.12113

Figure S1. *Apocynum* root latex and damage. (a) Latex exudation from a damaged *A. cannabiunum* root; (b) the cage that was used to bag the root-feeding larvae of the chrysomelid beetle *Chrysochus auratus* for 7 days to feed on the roots; (c) the exudation of latex after a transversal cut to the root; and (d) the visible damage done by *C. auratus* beetles on the root system of the plant.

Figure S2. Root latex production in the field. Shown is the average ± 1SE amount of latex collected from the root sections of three milkweed species (*Apocynum androsaeminifolium*, *A. cannabinum*, and *A. sibiriucum*). Plants were either left undamaged (open bars) or induced for 7 days by *Chrysocchus auratus*

(black bars). Asterisks above the bars indicate a significant difference among treatments (*t*-test, $P < 0.05$).

Figure S3. Principal component analysis (PCA) summarising different axes of cardenolide toxicity, including total abundance, number of individual peaks, diversity (H) and polarity (P) for the experiment with both species (*A. cannabinum* and *A. androsaemifolium*) (a) and the experiment with *A. cannabinum* only (b). PC1 was used for the analyses.

References

Agrawal, A.A. (1998) Induced responses to herbivory and increased plant performance. *Science*, **279**, 1201–1202.

Agrawal, A.A. & Konno, K. (2009) Latex: a model for understanding mechanisms, ecology, and evolution of plant defense against herbivory. *Annual Review of Ecology, Evolution, and Systematics*, **40**, 311–331.

Agrawal, A.A., Petschenka, G., Bingham, R.A., Weber, M.G. & Rasmann, S. (2012) Toxic cardenolides: chemical ecology and coevolution of specialized plant–herbivore interactions. *New Phytologist*, **194**, 28–45.

Alborn, H.T., Turlings, T.C.J., Jones, T.H., Stenhagen, G., Loughrin, J.H. & Tumlinson, J.H. (1997) An elicitor of plant volatiles from beet armyworm oral secretion. *Science*, **276**, 945–949.

Barton, K.E. & Koricheva, J. (2010) The ontogeny of plant defense and herbivory: characterizing general patterns using meta-analysis. *American Naturalist*, **175**, 481–493.

Berenbaum, M. & Feeny, P. (1981) Toxicity of angular furanocoumarins to swallowtail butterflies – escalation in a co-evolutionary arms-race. *Science*, **212**, 927–929.

Bezemer, T.M. & van Dam, N.M. (2005) Linking aboveground and belowground interactions via induced plant defenses. *Trends in Ecology & Evolution*, **20**, 617–624.

Bonaventure, G. (2012) Perception of insect feeding by plants. *Plant Biology*, **14**, 872–880.

Bonaventure, G., VanDoorn, A. & Baldwin, I.T. (2011) Herbivore-associated elicitors: FAC signaling and metabolism. *Trends in Plant Science*, **16**, 294–299.

Clark, K.E., Hartley, S.E. & Johnson, S.N. (2011) Does mother know best? The preference–performance hypothesis and parent–offspring conflict in aboveground–belowground herbivore life cycles. *Ecological Entomology*, **36**, 117–124.

Dangl, J.L. & Jones, J.D.G. (2001) Plant pathogens and integrated defence responses to infection. *Nature*, **411**, 826–833.

De Moraes, C.M., Lewis, W.J., Paré, P.W., Alborn, H.T. & Tumlinson, J.H. (1998) Herbivore-infested plants selectively attract parasitoids. *Nature*, **393**, 570–573.

De Vos, M., Van Oosten, V.R., Van Poecke, R.M.P., Van Pelt, J.A., Pozo, M.J., Mueller, M.J. *et al.* (2005) Signal signature and transcriptome changes of *Arabidopsis* during pathogen and insect attack. *Molecular Plant-Microbe Interactions*, **18**, 923–937.

Dobler, S., Daloze, D. & Pasteels, J.M. (1998) Sequestration of plant compounds in a leaf beetle's defensive secretion: cardenolides in Chrysochus. *Chemoecology*, **8**, 111–118.

Dobler, S., Dalla, S., Wagschal, V. & Agrawal, A.A. (2012) Community-wide convergent evolution in insect adaptation to toxic cardenolides by substitutions in the Na,K-ATPase. *Proceedings of the National Academy of Sciences of the United States of America*, **109**, 13040–13045.

Duffey, S.S. (1980) Sequestration of plant natural products by insects. *Annual Review of Entomology*, **25**, 447–477.

Dussourd, D.E. & Denno, R.F. (1991) Deactivation of plant defense – correspondence between insect behavior and secretory canal architecture. *Ecology*, **72**, 1383–1396.

Dussourd, D.E. & Eisner, T. (1987) Vein-cutting behavior – insect counterplay to the latex defense of plants. *Science*, **237**, 898–901.

Ehrlich, P.R. & Raven, P.H. (1964) Butterflies and plants – a study in coevolution. *Evolution*, **18**, 586–608.

Erb, M., Ton, J., Degenhardt, J. & Turlings, T.C.J. (2008) Interactions between arthropod-induced aboveground and belowground defenses in plants. *Plant Physiology*, **146**, 867–874.

Erb, M., Flors, V., Karlen, D., de Lange, E., Planchamp, C., D'Alessandro, M. *et al.* (2009) Signal signature of aboveground-induced resistance upon belowground herbivory in maize. *Plant Journal*, **59**, 292–302.

Erb, M., Robert, C.A.M., Hibbard, B.E. & Turlings, T.C.J. (2011) Sequence of arrival determines plant-mediated interactions between herbivores. *Journal of Ecology*, **99**, 7–15.

Erb, M., Glauser, G. & Robert, C.A.M. (2012) Induced immunity against belowground insect herbivores- activation of defenses in the absence of a jasmonate burst. *Journal of Chemical Ecology*, **38**, 629–640.

Erwin, A.C., Geber, M.A. & Agrawal, A.A. (2013) Specific impacts of two root herbivores and soil nutrients on plant performance and insect–insect interactions. *Oikos*, **122**, 1746–1756.

Farrell, B.D. (1998) "Inordinate fondness" explained: why are there so many beetles? *Science*, **281**, 555–559.

Farrell, B.D. & Mitter, C. (1998) The timing of insect/plant diversification: might *Tetraopes* (Coleoptera : Cerambycidae) and *Asclepias* (Asclepiadaceae) have co-evolved? *Biological Journal of the Linnean Society*, **63**, 553–577.

Fordyce, J.A. & Malcolm, S.B. (2000) Specialist weevil, *Rhyssomatus lineaticollis*, does not spatially avoid cardenolide defenses of common milkweed by ovipositing into pith tissue. *Journal of Chemical Ecology*, **26**, 2857–2874.

Gassmann, A.J., Carrière, Y. & Tabashnik, B.E. (2008) Fitness costs of insect resistance to Bacillus thuringiensis. *Annual Review of Entomology*, **54**, 147–163.

Hiltpold, I., Erb, M., Robert, C.A.M. & Turlings, T.C.J. (2011) Systemic root signalling in a belowground, volatile-mediated tritrophic interaction. *Plant, Cell & Environment*, **34**, 1267–1275.

Kaplan, I., Halitschke, R., Kessler, A., Rehill, B.J., Sardanelli, S. & Denno, R.F. (2008a) Physiological integration of roots and shoots in plant defense strategies links above- and belowground herbivory. *Ecology Letters*, **11**, 841–851.

Kaplan, I., Halitschke, R., Kessler, A., Sardanelli, S. & Denno, R.F. (2008b) Constitutive and induced defenses to herbivory in above- and belowground plant tissues. *Ecology*, **89**, 392–406.

Karban, R. & Baldwin, I.T. (1997) *Induced Responses to Herbivory*. The University of Chicago Press, Chicago, Illinois.

Li, X., Schuler, M.A. & Berenbaum, M.R. (2002) Jasmonate and salicylate induce expression of herbivore cytochrome P450 genes. *Nature*, **419**, 712–715.

Malcolm, S.B. (1991) Cardenolide-mediated interactions between plants and herbivores. *Herbivores: Their Interactions with Secondary Metabolites*, Vol. **I** (ed. by G. A. Rosenthal and M. R. Berenbaum), pp. 251–296. Academic Press, San Diego,California.

Malcolm, S.B. & Zalucki, M.P. (1996) Milkweed latex and cardenolide induction may resolve the lethal plant defence paradox. *Entomologia Experimentalis et Applicata*, **80**, 193–196.

Manson, J.S., Rasmann, S., Halitschke, R., Thomson, J.D. & Agrawal, A.A. (2012) Cardenolides in nectar may be more than a consequence of allocation to other plant parts: a phylogenetic study of *Asclepias*. *Functional Ecology*, **26**, 1100–1110.

Mattiacci, L., Dicke, M. & Posthumus, M.A. (1995) Beta-glucosidase: an elicitor of herbivore-induced plant odor that attracts host-searching

parasitic wasps. *Proceedings of the National Academy of Sciences*, **92**, 2036–2040.

Oksanen, J., Blanchet, F.G., Kindt, R., Legendre, P., Minchin, P.R., O'Hara, R.B. *et al.* (2013) *vegan: Community Ecology Package, Version 2.0-10* [WWW document]. URL http://vegan.r-forge.r-project.org/. [accessed on 1 December 2013].

Peterson, M.A., Dobler, S., Holland, J., Tantalo, L. & Locke, S. (2001) Behavioral, molecular, and morphological evidence for a hybrid zone between *Chrysochus auratus* and *C. cobaltinus* (Coleoptera: Chrysomelidae). *Annals of the Entomological Society of America*, **94**, 1–9.

Pineda, A., Zheng, S.-J., van Loon, J.J.A., Pieterse, C.M.J. & Dicke, M. (2010) Helping plants to deal with insects: the role of beneficial soil-borne microbes. *Trends in Plant Science*, **15**, 507–514.

Pozo, M.J. & Azcon-Aguilar, C. (2007) Unraveling mycorrhiza-induced resistance. *Current Opinion in Plant Biology*, **10**, 393–398.

Rasmann, S. & Agrawal, A.A. (2008) In defense of roots: a research agenda for studying plant resistance to belowground herbivory. *Plant Physiology*, **146**, 875–880.

Rasmann, S. & Agrawal, A.A. (2011a) Evolution of specialization: a phylogenetic study of host range in the red milkweed beetle (*Tetraopes tetraophthalmus*). *American Naturalist*, **177**, 728–737.

Rasmann, S. & Agrawal, A.A. (2011b) Latitudinal patterns in plant defense: evolution of cardenolides, their toxicity and induction following herbivory. *Ecology Letters*, **14**, 476–483.

Rasmann, S. & Turlings, T.C.J. (2008) First insights into specificity of belowground tritrophic interactions. *Oikos*, **117**, 362–369.

Rasmann, S., Agrawal, A.A., Cook, C.S. & Erwin, C.A. (2009a) Cardenolides, induced responses in shoots and roots, and interactions between above and belowground herbivores in the milkweeds (*Asclepias* spp). *Ecology*, **90**, 2393–2404.

Rasmann, S., Johnson, M.D. & Agrawal, A.A. (2009b) Induced responses to herbivory and jasmonate in three milkweed species. *Journal of Chemical Ecology*, **35**, 1326–1334.

Rasmann, S., Erwin, A.C., Halitschke, R. & Agrawal, A.A. (2011) Direct and indirect root defences of milkweed (*Asclepias syriaca*): trophic cascades, trade-offs and novel methods for studying subterranean herbivory. *Journal of Ecology*, **99**, 16–25.

Steffey, J. (1983) The dogbane family. *American Horticulturist*, **62**, 10.

Van Zandt, P.A. & Agrawal, A.A. (2004) Specificity of induced plant responses to specialist herbivores of the common milkweed *Asclepias syriaca*. *Oikos*, **104**, 401–409.

Williams, C.E. (1991) Host plant latex and the feeding-behavior of *Chrysochus auratus* (Coleoptera, Chrysomelidae). *Coleopterists Bulletin*, **45**, 195–196.

Zalucki, M.P. & Malcolm, S.B. (1999) Plant latex and first-instar monarch larval growth and survival on three North American milkweed species. *Journal of Chemical Ecology*, **25**, 1827–1842.

Zalucki, M.P., Brower, L.P. & Malcolm, S.B. (1990) Oviposition by *Danaus plexippus* in relation to cardenolide content of 3 *Asclepias* species in the Southeastern USA. *Ecological Entomology*, **15**, 231–240.

Zalucki, M.P., Brower, L.P. & Alonso, A. (2001) Detrimental effects of latex and cardiac glycosides on survival and growth of first-instar monarch butterfly larvae *Danaus plexippus* feeding on the sandhill milkweed *Asclepias humistrata*. *Ecological Entomology*, **26**, 212–224.

Zhen, Y., Aardema, M.L., Medina, E.M., Schumer, M. & Andolfatto, P. (2012) Parallel molecular evolution in an herbivore community. *Science*, **337**, 1634–1637.

Accepted 31 January 2014