Pyrrolizidine alkaloid composition influences cinnabar moth oviposition preferences in jacobaea hybrids

Cheng, D.; Meijden, E. van der; Mulder, P.P.J.; Vrieling, K.; Klinkhamer, P.G.L.

J Chem Ecol (2013) 39:430–437
DOI 10.1007/s10886-013-0257-4

Pyrrolizidine Alkaloid Composition Influences Cinnabar Moth Oviposition Preferences in *Jacobaea* Hybrids

Dandan Cheng · Eddy van der Meijden · Patrick P. J. Mulder · Klaas Vrieling · Peter G. L. Klinkhamer

Received: 21 August 2012 / Revised: 25 November 2012 / Accepted: 2 December 2012 / Published online: 24 February 2013

Abstract Plants produce a variety of secondary metabolites (PSMs) that may be selective against herbivores. Yet, specialist herbivores may use PSMs as cues for host recognition, oviposition, and feeding stimulation, or for their own defense against parasites and predators. This summarizes a dual role of PSMs: deter generalists but attract specialists. It is not clear yet whether specialist herbivores are a selective force in the evolution of PSM diversity. A prerequisite for such a selective force would be that the preference and/or performance of specialists is influenced by PSMs. To investigate these questions, we conducted an oviposition experiment with cinnabar moths (*Tyria jacobaeae*) and plants from an artificial hybrid family of *Jacobaea vulgaris* and *Jacobaea aquatica*. The cinnabar moth is a specialist herbivore of *J. vulgaris* and is adapted to pyrrolizidine alkaloids (PAs), defensive PSMs of these plants. The number of eggs and egg batches oviposited by the moths were dependent on plant genotype and positively correlated to concentrations of tertiary amines of jacobine-like PAs and some otosenine-like PAs. The other PAs did not correlate with oviposition preference. Results suggest that host plant PAs influence cinnabar moth oviposition preference, and that this insect is a potential selective factor against a high concentration of some individual PAs, especially those that are also involved in resistance against generalist herbivores.

Keywords Plant secondary metabolites · Diversity · Host-plant choice · Specialist herbivores · Chemical defense

Introduction

Plant secondary metabolites (PSMs) function as defenses against antagonistic organisms and/or as signals for communication with potentially beneficial organisms. In addition, they often play a role in protection against abiotic stresses (Hartmann, 2007). Within a particular species, or an individual plant, a few major compounds usually are accompanied by several structural analogs in lower concentrations (Wink, 2003). PSM variation in composition and in concentration is under genetic control (Vrieling et al., 1993; van Dam and Vrieling, 1994; Kliebenstein et al., 2001; Orians et al., 2010).

Herbivores are thought to play a role in the evolution of PSM diversity in plants (Ehrlich and Raven, 1964; van der Meijden, 1996; Futuyma and Agrawal, 2009). Specialists may adapt to a class of defense compounds in a host plant, use them as oviposition and feeding cues, and utilize them for their own defense (Schoonhoven et al., 2005). Therefore,

Electronic supplementary material The online version of this article (doi:10.1007/s10886-013-0257-4) contains supplementary material, which is available to authorized users.

D. Cheng
School of Environmental Studies, China University of Geosciences, Lumo Road 388, 430074, Wuhan, China

D. Cheng (✉) · E. van der Meijden · K. Vrieling · P. G. L. Klinkhamer
Plant Ecology & Phytochemistry, Institute of Biology, Leiden University, Sylviusweg 72, P.O. Box 9505, 2300 RA, Leiden, The Netherlands
e-mail: dan-d-cheng@163.com

P. P. J. Mulder
RIKILT-Institute of Food Safety, Wageningen University and Research Centre, P.O. Box 230, 6700 AE, Wageningen, The Netherlands

specialists have been regarded as being less affected by chemical defenses than generalists, and are thought unlikely to be a selective force in the evolution of a group of structurally related PSMs (Harvey et al., 2005; Macel et al., 2005; Arany et al., 2008). However, structurally related compounds can have different stimulating effects on specialists (Macel and Vrieling, 2003), and the variation of defense chemicals in host plants may affect specialist preference (Nieminen et al., 2003; Leimu et al., 2005). For instance, field work that manipulated specialist and generalist herbivores of *Brassica nigra* showed that feeding by the specialist increased with increasing sinigrin concentrations in *B. nigra*, while damage by the generalist decreased with increasing concentrations (Lankau, 2007). Both preference of specialist herbivores (Macel et al., 2002; Nieminen et al., 2003; Leimu et al., 2005), and PSM variation are related to host plant genotype. This makes it is possible to explore the relationship between the two parameters at the genotype level of the host plants.

Jacobaea, formerly known as *Senecio*, is a good model system to study the diversity of a single group of PSMs. These species contain a diverse but structurally related group of PAs that play a role in interactions between plants and their herbivores and pathogens (Hol and van Veen, 2002; Macel et al., 2005; Kowalchuk et al., 2006). Pyrrolizidine alkaloids can occur in plants in two forms: tertiary amines (free base) or *N*-oxides (Rizk, 1991; Wiedenfeld et al., 2008; Joosten et al., 2011). Using a sensitive analytical technique such as liquid chromatography-tandem mass spectrometry (LC-MS/MS) 37 structural PA analogs have been detected within a single species (Cheng et al., 2011a; Joosten et al., 2011).

The effects of single PAs on generalist insect herbivores and nematodes that are not adapted to PAs are dependent on both PA structure and concentration (Macel et al., 2005; Dominguez et al., 2008; Thoden et al., 2009). The two forms of the same individual PA have different deterring effects on non-adapted generalists, as demonstrated by an *in vitro* bioassay with isolated PAs (Dreyer et al., 1985; Macel et al., 2005). The stimulating effects on oviposition and feeding of specialists have been confirmed by artificial diet bioassays with isolated PAs, but have not been evaluated *in vivo* (Macel, 2011). Adapted insects carry out *N*-oxidation of tertiary amines formed in the gut and subsequently store these PA *N*-oxides in their body (Hartmann, 1999).

The cinnabar moth (*Tyria jacobaeae*) is a specialist arctiid moth that feeds mainly on *Jacobaea vulgaris* (syn. *Senecio jacobaea*) and a restricted number of other *Jacobaea* species. *Tyria jacobaeae* sequesters and metabolizes PAs and uses these substances for its own defense (Rothschild et al., 1979; Lindigkeit et al., 1997; Naumann et al., 2002). Experiments with artificial leaves coated with PAs have shown that PAs are oviposition stimulants for the cinnabar moth and that the stimulatory effects differ among the particular PAs (Macel and Vrieling, 2003). However, other studies have failed to demonstrate such a relation between adult oviposition preference and larval performance of the cinnabar moth and PAs in host plants (Vrieling and de Boer, 1999; Macel et al., 2002). Oviposition choice among the plants of *J. vulgaris* rather were related to other factors such as sugar and nitrogen levels (van der Meijden et al., 1989). Macel and Klinkhamer (2010) found that the damage on *J. vulgaris* plants was caused mainly by insect specialists such as *T. jacobaeae*, *Longitarsus jacobaeae*, and *Haplothrips senecionis*, and that herbivory was positively correlated to the concentration of total PAs and some individual PAs (jacobine and jacobine *N*-oxide). However, *J. vulgaris* in invasive areas (where it is free of specialist insect herbivore attack) contained higher amounts of PAs compared to those in native areas. In addition, *J. vulgaris* plants in the invasive areas contain jacobine as the major PA (Joshi and Vrieling, 2005).

The studies mentioned above seem to be contradictory, and it is not clear yet whether plant PA variation as can be found within *J.spp* affects cinnabar moth oviposition preference. To answer this question, we designed a controlled oviposition bioassay with adults of the cinnabar moth using plants of different F_2 hybrid genotypes from a cross between *J. vulgaris* and *Jacobaea aquatica*. Segregating hybrid plants demonstrate greater ecological and chemical variation compared to parental species (Fritz, 1999; Orians, 2000), and the various traits are expected to segregate independent of one another unless they are linked. Therefore, hybrids are useful tools to study the relation among different traits in plants (Hochwender et al., 2000; Orians, 2000; Lexer et al., 2003; Orians et al., 2010). We found in a previous study that PA composition and concentration varied widely between the F_2 hybrids of *J. vulgaris* and *J. aquatica* (Cheng et al., 2011a). Here, we addressed the following questions: 1) Does the cinnabar moth have an oviposition preference for certain hybrid plant genotypes? 2) Does total PA concentration or that of individual PAs in host plants influence oviposition preference? 3) Can (groups of) PAs exert synergistic or antagonistic effects on oviposition preference?

Methods and Materials

Plants Grown for the Oviposition Bioassay

Plants used in the oviposition bioassay were from a hybrid family stored in tissue culture. The hybrid family consisted of two parental lines, two F_1 lines, and 102 F_2 lines, which can be cloned in order to obtain replicate individuals of any

of the lines. Each set of cloned individuals is referred to hereafter as a 'genotype'. One parental genotype is a jacobine-chemotype plant of *J. vulgaris*. The other is a *J. aquatica* plant, with PA composition dominated by senecionine *N*-oxide and seneciphylline *N*-oxide (Cheng et al., 2011a). The *J. vulgaris* genotype is from a seed collected at Meijendel Nature Reserve (52° 7′ 54″ N, 4° 19′ 46″ E, The Netherlands) and the *J. aquatica* genotype is from a seed collected at the Zwanenwater Reserve (52° 48′ 38″ N, 4° 41′ 7″ E, The Netherlands) (see details of this hybrid system in Cheng et al., 2011a). For this experiment, 40 F_2 hybrid genotypes were selected from the 102 F_2 genotypes based on their PA composition and concentration in shoots (Cheng et al., 2011a). We selected genotypes displaying a large range of concentrations of total PA and major PAs such as senecionine, jacobine, and erucifoline.

The 40 F_2 genotypes were propagated by tissue culture, and 6 individual plants were grown from each of these. Plants were potted in 1.3 L pots (diam 9 cm, height 9 cm) filled with a mixture of 95 % sandy soil from Meijendel, 5 % potting soil (Slingerland Potgrond company, Zoeterwoude, The Netherlands) and 1.5 g/l Osmocote slow release fertilizer (N:P:K=15:9:11; Scott®, Scotts Miracle-Gro, Marysville, OH, USA). Plants were kept for 6 weeks in a climate room (RH=70 %, light 16 h at 20 °C, dark 8 h at 20 °C) and they were placed in the greenhouse 1 week prior to the oviposition experiment.

Cinnabar Moth Rearing

Full-grown fifth instar caterpillars of the cinnabar moth were collected from plants of *J. vulgaris* in Meijendel Nature Reserve (52° 7′ 54″ N, 4° 19′ 46″ E, The Netherlands) in July 2009 and kept in glass tubes until pupation. The pupae were stored in a cold room (4 °C) until the next season. In April and May 2010, pupae were removed from the cold room in three different batches and placed in transparent plastic cages (70×70×50 cm) under room temperature and natural light. Moths emerged 2–3 weeks later, and they were fed on water and honey for about 1 week before being released in the bioassay. Only healthy and active moths were used.

Oviposition Bioassay

The bioassay was conducted in three trials using plastic cylinders (87 cm diam,1 m height) with gauze covered tops in a greenhouse in the experimental garden of the Institute of Biology in Leiden in May and June 2010 (Supplementary Fig.1a). The cages had a wooden floor with 20 holes to insert 20 pots with plants so that soil surface was at level with the floor (Supplementary Fig. 1b). Thirty virgin female and 30 virgin male moths were released per cage. Plants were watered two or three times during the experiment in dishes under the pots without disturbing the moths. Cages were rotated every 3 days to avoid position effects on

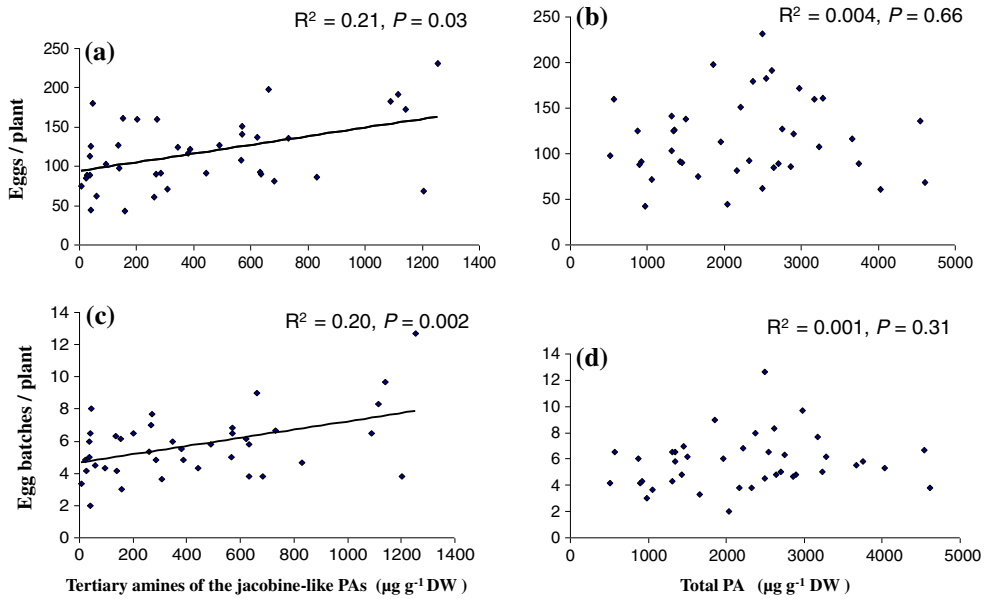

Fig. 1 Scatter plots showing the relationship between the oviposition preference of cinnabar moth and the pyrrolizidine alkaloids (PAs) in the shoots of the host plants (*Jacobaea* hybrid plants): **a** the number of cinnabar moth eggs per plant against the sum concentration of the tertiary amines of the jacobine-like PAs and **b** against the total PA concentration per plant ; **c** the number of egg batches per plant against the sum concentration of the tertiary amines of the jacobine-like PAs and **d** against total PA concentration per plant. The 5 jacobine-like PAs are: jacobine, jacoline, jaconine, jacozine and dehydrojaconine. Each individual dot represents the genetic mean values of one of the 40 F_2 genotypes of a cross between *Jacobaea vulgaris* and *Jacobaea aquatica*. DW=shoot dry weight

oviposition. After 10 days, plants were harvested. Fresh weight was measured for each plant. Digital photographs were taken of all leaves with eggs. The numbers of egg batches per leaf and eggs per egg batch were counted from these photographs (Supplementary Fig. 1c). In each of the three trials, 80 plants were divided over 4 cages. Twenty different genotypes were placed per cage according to a random arrangement such that each of the 40 genotypes was represented by two replicates at each of the three trials.

PA Data

We used the PA data obtained by Cheng et al., (2011a). In that study, PA concentrations were measured by LC-MS/MS in 3–6 clonal replicates of plants that were grown from the same tissue culture, under identical conditions and consisting of the same genotypes and number of clones, as those used in the cinnabar moth bioassay described here. We averaged the concentration of each PA across all replicates of each genotype, and these genotypic mean concentrations were used in the analyses presented here. These methods also were applied in the analysis of our bioassay of western flower thrips (Cheng et al., 2011b). The PA expression in *Jacobaea* hybrid plants was determined strongly by genotypes. The 37 PAs identified from the *Jacobaea* hybrids were classified into four groups, according to their structural characteristics, biosynthetic pathways, and expression patterns: senecionine-, jacobine-, erucifoline- and otosenine-like PAs (Cheng et al., 2011a). Total PA concentration and the concentration of each PA group were calculated by summing the concentrations of the individual PAs.

Data Analysis

To measure cinnabar moth oviposition preference among the individual plants or hybrid genotypes, three variables were used: number of eggs per plant; number of egg batches per plant; and average egg batch size per plant (number of eggs per plant/ number of egg batches per plant).

The experiment was not a full three factorial design. Therefore, we first checked the effects of trials and cages by two-way ANOVA, and then checked the effect of genotype by one-way ANOVA. We used general linear models to determine whether the three selected indicator variables differed among trials and cages. In the three models, trials and cages were defined as the fixed factors; the three indicators were defined as dependent variables, and the fresh weight of the shoot was treated as a covariate. The ANOVA test showed that the number of eggs per plant and average egg batch size per plant was not affected by trials and cages. However, number of egg batches per plant seemed to differ among cages (Supplementary Table 1). For each of the three trials we used ANOVA to test for differences in the number of egg batches per plant between cages. In one cage in one of the trials, the number of egg batches differed from the other cages in that trial.

We also used general linear models to determine whether the number of eggs per plant and the average egg batch size per plant differed among plant genotypes. In these models, plant genotype was defined as random factor, number of eggs per plant and average egg batch size per plant were defined as dependent variables, and fresh weight of the shoot as a covariate. A similar general linear model was applied to determine whether the number of egg batches per plant differed among the plant genotypes. This model differed from the other two models in that the independent variable was not the number of egg batches per plant but the residuals of the model with cage as factor and the number of egg batches per plant as dependent variable, because the number of egg batches per plant differed among the cages.

Normal distributions and homogenous variances of the general linear models were confirmed by testing the

Table 1 ANOVA results on the number of eggs per plant, number of egg batches per plant and egg batch size per plant in an oviposition experiment with the cinnabar moth and 40 hybrid genotypes from *Jacobaea aquatica* and *Jacobaea vulgaris*

Dependent variables	Independent variables	*df* (k-1)	*Df* (n-k-1)	*F*	*P*
Number of eggs per plant	Hybrid genotype	39	199	1.58	0.02
	Fresh weight	1	237	4.21	0.04
	Error	199			
	Total	240			
Number of egg batches per plant[a]	Hybrid genotype	39	199	1.99	<0.001
	Fresh weight	1	237	6.44	0.01
	Error	199			
	Total	240			
Egg batch size per plant	Hybrid plant genotype	39	199	1.00	0.48
	Fresh weight	1	237	1.28	0.26
	Error	199			
	Total	240			

[a]Residuals of the model with the number of egg batches per plant against cages, because number of egg batches per plant differed amongst cages

residuals of the models using Shapiro tests and Bartlett tests, respectively. Average egg batch size per plant was not significantly related to genotype (Table 1) and was not used in further analyses.

In hybrid plants, the concentrations of individual PAs belonging to the same structural groups were closely correlated, thus making it difficult to investigate the interactions among individual PAs. However, the PAs from different structural groups generally were expressed independently. The PA sum concentrations of the four groups were not correlated. We used a multiple regression model to test for interactions between the effects of different PA structural groups on oviposition preference. The regression was completed in a linear model, in which genotypic mean number of eggs and number of egg batches were selected as independent variables. The genotypic mean concentrations of the four PA groups were used as dependent variables in these models, and interactions between the independent variables were included. This model was conducted in R (R Development Core Team, 2009).

One-tailed Pearson or Spearman rank correlation tests were conducted between the genotypic mean number of eggs, number of egg batches, and concentrations of individual PAs from two PA groups (jacobine- and otosenine-like PAs). These were related to cinnabar moth oviposition preference, as shown by the multiple regression tests. Since we expected positive correlations only, we used one-sided significance levels in these tests. Whether to conduct a parametric (Pearson) or a non-parametric (Spearman rank) test depended on the distribution of the PA data. Because multiple tests were performed, the *P*-values of the tests were adjusted according to the sequential Bonferroni method.

All analyses except the linear model for multiple regressions (conducted in R) were conducted in SPSS 17.0.

Results

Cinnabar Moth Oviposition Preference among Individual Plants

Egg batches were always laid at the underside of the leaves. In total, 28,323 eggs were counted in 1,375 egg batches on 240 plants. On average, each plant received 118 eggs in 5.73 egg batches, and the mean egg-batch size was 20 eggs. Each female moth on average laid 78.7 eggs in 3.8 egg batches, assuming that all females laid eggs.

The number of egg batches per plant ranged from 0 to 18, with more than 50 % of the plants receiving between 4–8 egg batches, less than 10 % of the plants having more than 10 egg batches, and about 5 % of the plants having no egg batches (Supplementary Fig. 2a). The number of eggs per plant ranged from 0 to 534, and more than 50 % of the plants had fewer than 150 eggs (Supplementary Fig. 2b). The number of eggs per plant differed among genotypes, but was not different among trials and cages. The number of egg batches differed among genotypes and cages, but the average egg batch size per plant did not differ among the plant genotypes (Table 1, Supplementary Table 1).

Relationship between Cinnabar Moth Oviposition Preference and Plant PAs

Multiple-regression analysis showed that two PA groups (jacobine- and otosenine-like PAs) positively correlated to the number of eggs per plant (Table 2). The total concentration of jacobine-like PAs also positively correlated to the number of egg batches. The other two PA groups (senecionine- and erucifoline-like PAs) were not correlated to either number of eggs or number of egg batches per plant. There was an interaction between the concentration of jacobine- and otosenine-like PAs, and this interaction was positively correlated to the number of eggs and egg batches per plant (Table 2). This indicates that the effects of jacobine- and otosenine-like PAs on cinnabar moth oviposition preference are positive, and the effect may be synergistic.

There are 9 individual PAs in the jacobine group and 7 in the otosenine group. Of the first group, jacobine, jaconine, and dehydrojaconine showed the strongest correlation to both the number of eggs and number of egg batches. Jacozine showed a significantly positive correlation to the number of egg batches only. Of the second group senkirkine and desacetyldoronine positively correlated to the number of egg batches (Table 3). The total concentration of the tertiary amines of jacobine-like PAs explained approx. 20 % of the variation of the number of eggs and number of egg batches among the hybrid genotypes (Fig. 1a, c). However, there was no significant correlation with total PA concentration (Fig. 1b, d).

Discussion

We demonstrated that cinnabar moth oviposition preference was affected by host plant genotype. At the genotype level, plants with higher concentrations of tertiary amines of jacobine-like PAs and higher levels of otosenine-like PAs received more eggs and egg batches (Table 2 and Fig. 1a, c). This might indicate synergistic effects between these two PA groups. Thus, plants with higher levels of these PAs might suffer more feeding damage from cinnabar moth and this could result in a lower fitness in environments with abundant cinnabar moths. This suggests that cinnabar moths may potentially act as a selective force to decrease the concentration of jacobine-like tertiary amines.

Table 2 Results of multiple regressions of number of eggs and number of egg batches of the cinnabar moth against the sum concentration of the four structural groups of pyrrolizidine alkaloids (PA, μg/g dw) in the host plants of 40 F_2 hybrid genotypes from *Jacobaea aquatica* and *Jacobaea vulgaris*. For model I (number of eggs): adjusted R^2=0.37; $F_{15,24}$=2.53; P=0.020. For model II (number of egg batches): adjusted R^2=0.33; $F_{15,24}$=2.30; P=0.033

	Predictors[a]	Number of eggs		Number of egg batches	
		Estimate	*t* value	Estimate	*t* value
	(Intercept)	14.8000	14.376***	5.4690	14.839***
PA groups	Sn	0.0042	0.391	0.0003	0.522
	Jb	0.0286	2.186*	0.0013	2.077*
	Er	−0.0397	−0.527	−0.0011	−0.33
	Ot	0.4355	2.601*	0.0138	1.779
Two-way Interactions	Sn:Jb	- 8.06E-06	−0.265	−1.60E-06	−1.137
	Sn:Er	2.17E-05	0.174	3.10E-06	0.538
	Jb:Er	−0.0002	−1.575	−5.72E-06	−1.189
	Sn:Ot	3.80E-05	0.125	−3.03E-06	−0.215
	Jb:Ot	0.0011	3.527**	4.87E-05	3.497**
	Er:Ot	0.0012	0.443	0.0001	0.825
Three-way interactions	Sn:Jb:Er	2.22E-07	0.962	1.24E-08	1.164
	Sn:Jb:Ot	−8.21E-08	−0.135	−2.86E-08	−1.019
	Sn:Er:Ot	−3.06E-06	−0.587	1.80E-08	0.075
	Jb:Er:Ot	−1.30E-06	−0.335	−1.69E-07	−0.948
Four-way interaction	Sn:Jb:Er:Ot	−3.24E-10	−0.056	1.39E-10	0.516

[a] *Sn*, Jb, Er, Ot are the sum concentrations of senecionine-, jacobine- erucifoline- and otosenine-type PAs.

* P<0.05, ** P<0.01, *** P<0.001.

In previous studies, no significant positive correlations between cinnabar moth oviposition preference and PA variation were found in *Jacobaea* plants (Vrieling and de Boer, 1999; Macel et al., 2002). The lack of significant correlations might be due to the fact that previous studies used natural genotypes of different populations and species for

Table 3 Results of the one-sided Pearson/Spearman correlation tests between number of eggs and egg batches of cinnabar moths and concentrations of jacobine- and otosenine-like pyrrolizidine alkaloids (PAs) in the 40 F_2 hybrid genotypes from *Jacobaea aquatica* and *Jacobaea vulgaris*.

PA group	PAs	Number of eggs		Number of egg batches	
		r/r_s	P[a]	r/r_s	P[a]
Jacobine-like PAs	jacobine	0.46	*	0.43	*
	jacoline	0.38	Ns	0.40	Ns
	jaconine	0.44	*	0.45	*
	jacozine	0.39	Ns	0.48	*
	dehydrojaconine [b]	0.42	*	0.50	**
	jacobine *N*-oxide	0.05	Ns	0.13	Ns
	jacoline *N*-oxide	0.08	Ns	0.15	Ns
	jaconine *N*-oxide	0.04	Ns	0.11	Ns
	jacozine *N*-oxide [c]	0.05	Ns	0.04	Ns
Otosenine-like PAs[d]	senkirkine	0.25	Ns	0.26	Ns
	otosenine	0.24	Ns	0.12	Ns
	onetine	0.22	Ns	0.14	Ns
	desacetyldoronine	0.39	Ns	0.33	Ns
	florosenine [c]	0.23	Ns	0.14	Ns
	floridanine [c]	0.28	Ns	0.18	Ns
	doronine [c]	0.19	Ns	0.06	Ns

[a] P-values of the correlation testes were adjusted by the sequential Bonferroni method.

[b] PA was only detected in the tertiary amine form.

[c] Spearman correlation tests were carried out for PAs with not normally distributed concentrations

[d] Only present as tertiary PAs

Ns P>0.05, * P<0.05, ** P< 0.01

studying oviposition preference, and that differences in other plant traits among populations and species may potentially have masked the effects of PAs. Our study used F_2 hybrids, which allowed us to examine the effects of a large variety of PAs against a similar genetic background. This approach may more readily reveal relationships between specialist preference and PA content.

The cinnabar moth individuals used in this study were collected from Meijendel where native ragwort plants are jacobine chemotypes (Macel et al., 2004), and they might, therefore, have a preference for plants with jacobine-like PAs. By analogy, cinnabar moths collected from a population of erucifoline chemotype plants might have a preference for erucifoline-like PAs. This hypothesis needs to be tested by conducting oviposition bioassays with moths collected from host plants belonging to different chemotypes.

We do not know why significant correlations were observed between cinnabar moth egg numbers and the jacobine-like tertiary amines but not with the corresponding *N*-oxides of these PAs. A previous study showed that the PA concentration on the leaf surface was marginally correlated to PA concentration in the leaf tissue, and that there were differences in PA composition on the leaf surface from that of the interior (Vrieling and Derridj, 2003). In the *Jacobaea* hybrid plants, there was a strong correlation between the tertiary amines of jacobine-like PAs on the surface and in the leaf tissue, but not for the other PA groups (Cheng et al., unpublished). If female cinnabar moths can detect only PAs on the leaf surface, then the correlation between tertiary amines of jacobine-like PAs on the leaf surface and in the leaf interior could explain why the moths prefer plants with more tertiary amines of jacobine-like PAs in the whole rosette. Alternatively, these PAs may have a stronger stimulating effect on cinnabar moth oviposition than other PAs. This could be tested by a cinnabar moth oviposition bioassay with individual PAs.

We focused on the relationship between PAs in *Jacobaea* hybrid plants and the oviposition preference of moths. We tried to uncouple the effects of alkaloid concentration from other factors by using plants from a single hybrid family for our oviposition bioassay. Pyrrolizidine alkaloid variation accounted for a relatively small amount of the variation in moth oviposition (Fig. 1). Some other features of the plants that may affect attractiveness to the moth include plant physical characteristics such as size, which was found to be a significant covariate in this study (Table 1), and metabolites such as sugar and nitrogen (van der Meijden et al., 1989). Whether these factors are confounded with the effects of PAs on oviposition preference of cinnabar moth among the plants remains to be explored.

In summary, our results showed that variation in PA concentration and composition in *Jacobaea* hybrid plants was related to the cinnabar moth oviposition preference among host plants, which suggests that PAs might be important to plant fitness in relation to herbivory from the moth. Our study complements earlier research that showed that the cinnabar moth is attracted by PAs to oviposit but that not all PAs have an equal effect (Macel and Vrieling, 2003), Our study adds evidence to the hypothesis that specialist herbivores may play a role in the evolution of a group of PSMs in plants.

Acknowledgments Dandan Cheng thanks the China Scholarship Council (CSC) of the Ministry of Education for financial support. We thank Cilke Hermans, Karin van der Veen-van Wijk, Natasha Schidlo, Henk Nell, Christian Burbea, Jelle Zandveld, and Jun Rong for technical assistance in plant growth, cinnabar moth bioassays, and plant harvesting.

References

ARANY, A. M., DE JONG, T. J., KIM, H. K., VAN DAM, N. M., CHOI, Y. H., VERPOORTE, R., and VAN DER MEIJDEN, E. 2008. Glucosinolates and other metabolites in the leaves of *Arabidopsis thaliana* from natural populations and their effects on a generalist and a specialist herbivore. *Chemoecology* 18:65–71.

CHENG, D., KIRK, H., MULDER, P. P. J., VRIELING, K., and KLINKHAMER, P. G. L. 2011a. Pyrrolizidine alkaloid variation in shoots and roots of segregating hybrids between *Jacobaea vulgaris* and *Jacobaea aquatica*. *New Phytol.* 192:1010–1023.

CHENG, D., KIRK, H., VRIELING, K., MULDER, P. P. J., and KLINKHAMER, P. G. L. 2011b. the relationship between structurally different pyrrolizidine alkaloids and western flower thrips resistance in F_2 hybrids of *Jacobaea vulgaris* and *Jacobaea aquatica*. *J. Chem. Ecol.* 37:1071–1080.

DOMINGUEZ, D. M., REINA, M., SANTOS-GUERRA, A., SANTANA, O., AGULLO, T., LOPEZ-BALBOA, C., and GONZALEZ-COLOMA, A. 2008. Pyrrolizidine alkaloids from Canarian endemic plants and their biological effects. *Biochem. Syst. Ecol.* 36:153–166.

DREYER, D. L., JONES, K. C., and MOLYNEUX, R. J. 1985. Feeding deterrency of some pyrrolizidine, indolizidine, and quinolizidine alkaloids towards pea aphid (*acyrthosipon pisum*) and evidence for phloem transport of indolizidine alkaloid swainsonine. *J. Chem. Ecol.* 11:1045–1051.

EHRLICH, P. R. and RAVEN, P. H. 1964. Butterflies and plants - a study in coevolution. *Evolution* 18:586–608.

FRITZ, R. S. 1999. Resistance of hybrid plants to herbivores: Genes, environment, or both? *Ecology* 80:382–391.

FUTUYMA, D. J. and AGRAWAL, A. A. 2009. Macroevolution and the biological diversity of plants and herbivores. *Proc. Natl. Acad. Sci. USA*. 106:18054–18061.

HARTMANN, T. 1999. Chemical ecology of pyrrolizidine alkaloids. *Planta* 207:483–495.

HARTMANN, T. 2007. From waste products to ecochemicals: Fifty years research of plant secondary metabolism. *Phytochemistry* 68:2831–2846.

HARVEY, J. A., VAN NOUHUYS, S., and BIERE, A. 2005. Effects of quantitative variation in allelochemicals in *Plantago lanceolata* on development of a generalist and a specialist herbivore and their endoparasitoids. *J. Chem. Ecol.* 31:287–302.

HOCHWENDER, C. G., FRITZ, R. S., and ORIANS, C. M. 2000. Using hybrid systems to explore the evolution of tolerance to damage. *Evol. Ecol.* 14:509–521.

HOL, W. H. G. and VAN VEEN, J. A. 2002. Pyrrolizidine alkaloids from *Senecio jacobaea* affect fungal growth. *J. Chem. Ecol.* 28:1763–1772.

JOOSTEN, L., CHENG, D., MULDER, P. P. J., VRIELING, K., VAN VEEN, J. A., and KLINKHAMER, P. G. L. 2011. The genotype dependent presence of pyrrolizidine alkaloids as tertiary amine in *Jacobaea vulgaris*. *Phytochemistry* 72:214–222.

JOSHI, J. and VRIELING, K. 2005. The enemy release and EICA hypothesis revisited: incorporating the fundamental difference between specialist and generalist herbivores. *Ecol. Lett.* 8:704–714.

KLIEBENSTEIN, D. J., KROYMANN, J., BROWN, P., FIGUTH, A., PEDERSEN, D., GERSHENZON, J., and MITCHELL-OLDS, T. 2001. Genetic control of natural variation in *Arabidopsis* glucosinolate accumulation. *Plant Physiol.* 126:811–825.

KOWALCHUK, G. A., HOL, W. H. G., and VAN VEEN, J. A. 2006. Rhizosphere fungal communities are influenced by *Senecio jacobaea* pyrrolizidine alkaloid content and composition. *Soil Biol. Biochem.* 38:2852–2859.

LANKAU, R. A. 2007. Specialist and generalist herbivores exert opposing selection on a chemical defense. *New Phytol.* 175:176–184.

LEIMU, R., RIIPI, M., and STAERK, D. 2005. Food preference and performance of the larvae of a specialist herbivore: variation among and within host-plant populations. *Acta Oecol.* 28:325–330.

LEXER, C., RANDELL, R. A., AND RIESEBERG, L. H. 2003. Experimental hybridization as a tool for studying selection in the wild. *Ecology* 84:1688–1699.

LINDIGKEIT, R., BILLER, A., BUCH, M., SCHIEBEL, H. M., BOPPRE, M., and HARTMANN, T. 1997. The two faces of pyrrolizidine alkaloids: The role of the tertiary amine and its *N*-oxide in chemical defense of insects with acquired plant alkaloids. *Eur. J. Biochem.* 245:626–636.

MACEL, M., KLINKHAMER, P. G. L., VRIELING, K., and VAN DER MEIJDEN, E. 2002. Diversity of pyrrolizidine alkaloids in *Senecio* species does not affect the specialist herbivore *Tyria jacobaeae*. *Oecologia* 133:541–550.

MACEL, M. and VRIELING, K. 2003. Pyrrolizidine alkaloids as oviposition stimulants for the cinnabar moth, *Tyria jacobaeae*. *J. Chem. Ecol.* 29:1435–1446.

MACEL, M., VRIELING, K., and KLINKHAMER, P. G. L. 2004. Variation in pyrrolizidine alkaloid patterns of *Senecio jacobaea*. *Phytochemistry* 65:865–873.

MACEL, M., BRUINSMA, M., DIJKSTRA, S. M., OOIJENDIJK, T., NIEMEYER, H. M., and KLINKHAMER, P. G. L. 2005. Differences in effects of pyrrolizidine alkaloids on five generalist insect herbivore species. *J. Chem. Ecol.* 31:1493–1508.

MACEL, M. and KLINKHAMER, P. G. L. 2010. Chemotype of *Senecio jacobaea* affects damage by pathogens and insect herbivores in the field. *Evol. Ecol.* 24:237–250.

MACEL, M. 2011. Attract and deter: a dual role for pyrrolizidine alkaloids in plant–insect interactions. *Phytochem. Rev.* 10:75–82.

NAUMANN, C., HARTMANN, T., and OBER, D. 2002. Evolutionary recruitment of a flavin-dependent monooxygenase for the detoxification of host plant-acquired pyrrolizidine alkaloid-defended arctiid alkaloids in the moth *Tyria jacobaleae*. *Proc. Natl. Acad. Sci. USA.* 99:6085–6090.

NIEMINEN, M., SUOMI, J., VAN NOUHUYS, S., SAURI, P., and RIEKKOLA, M. L. 2003. Effect of iridoid glycoside content on oviposition host plant choice and parasitism in a specialist herbivore. *J. Chem. Ecol.* 29:823–844.

ORIANS, C. M. 2000. The effects of hybridization in plants on secondary chemistry: implications for the ecology and evolution of plant-herbivore interactions. *Am. J. Bot.* 87:1749–1756.

ORIANS, C. M., HOCHWENDER, C. G., FRITZ, R. S., and SNALL, T. 2010. Growth and chemical defense in willow seedlings: trade-offs are transient. *Oecologia* 163:283–290.

R DEVELOPMENT CORE TEAM 2009. R: A Language and Environment for Statistical Computing. Austria, Vienna.

RIZK, A. M. 1991. Naturally Occurring Pyrrolizidine Alkaloids. CRC Press, Boca Raton.

ROTHSCHILD, M., APLIN, R. T., COCKRUM, P. A., EDGAR, J. A., FAIRWEATHER, P., and LEES, R. 1979. Pyrrolizidine alkaloids in arctiid moths (Lep) with a discussion on host plant relationships and the role of these secondary plant-substances in the Arctiidae. *Biol. J. Linn. Soc.* 12:305–326.

SCHOONHOVEN, L. M., VAN LOON, J. J. A., and DICKE, M. 2005. Insect-Plant Biology. Oxford University Press, Oxford.

THODEN, T. C., BOPPRE, M., and HALLMANN, J. 2009. Effects of pyrrolizidine alkaloids on the performance of plant-parasitic and free-living nematodes. *Pest Manag. Sci.* 65:823–830.

VAN DAM, N. M. and VRIELING, K. 1994. Genetic-variation in constitutive and inducible pyrrolizidine alkaloid levels in *Cynoglossum officinale* L. *Oecologia* 99:374–378.

VAN DER MEIJDEN, E. 1996. Plant defence, an evolutionary dilemma: Contrasting effects of (specialist and generalist) herbivores and natural enemies. *Entomol. Exp. Appl.* 80:307–310.

VAN DER MEIJDEN, E., VAN ZOELEN, A. M., and SOLDAAT, L. L. 1989. Oviposition by the cinnabar moth, *Tyria jacobaeae*, in relation to nitrogen, sugars and alkaloids of ragwort, *Senecio jacobaea*. *Oikos* 54:337–344.

VRIELING, K., DE VOS, H., and VAN WIJK, C. A. M. 1993. Genetic analysis of the concentrations of pyrrolizidine alkaloids in *Senecio jacobaea*. *Phytochemistry* 32:1141–1144.

VRIELING, K. and DE BOER, N. 1999. Host-plant choice and larval growth in the cinnabar moth: do pyrrolizidine alkaloids play a role? *Entomol. Exp. Appl.* 91:251–257.

VRIELING, K. and DERRIDJ, S. 2003. Pyrrolizidine alkaloids in and on the leaf surface of *Senecio jacobaea* L. *Phytochemistry* 64:1223–1228.

WIEDENFELD, H., ROEDER, E., BOURAUEL, T., and EDGAR, J. 2008. Pyrrolizidine Alkaloids: Structure And Toxicity. V&R unipress GmbH, Bonn.

WINK, M. 2003. Evolution of secondary metabolites from an ecological and molecular phylogenetic perspective. *Phytochemistry* 64:3–19.