Pyrrolizidine alkaloid variation in Jacobaea hybrids : influence on resistance against generalist and specialist insect herbivores

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Chapter 4

The influence of pyrrolizidine alkaloid variation on cinnabar moth oviposition preference in *Jacobaea* hybrids

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Specialist herbivores may use the secondary metabolites produced by their host plants for host recognition, oviposition and feeding stimulation or to their own defense against parasites and predators. Still an open question is whether specialist herbivores are a selective force in the evolution of the great diversity of plant secondary metabolites. A prerequisite for such a selective force would be that the preference and (or) performance of specialist herbivores is influenced by plant secondary metabolites.

The cinnabar moth (*Tyria jacobaeae*) is one of the main specialist herbivores of *Jacobaea vulgaris* and is adapted to pyrrolizidine alkaloids (PAs), the defense secondary metabolites in its host plants. To investigate whether oviposition preference of cinnabar moths is affected by PAs, we conducted an oviposition experiment with cinnabar moths using 40 tissue culture cloned F_2 genotypes of an artificial hybrid family of *Jacobaea vulgaris* and *Jacobaea aquatica*.

We found that the number of eggs and the number of egg batches oviposited by the cinnabar moths were dependent on plant genotypes and cinnabar moth oviposition preference was positively correlated to the concentration of tertiary amines of jacobine-like PAs and some otosenine-like PAs. Synergy was found between the effects of jacobine-like and otosenine-like PAs on oviposition preference. The PAs from the other two PA groups (senecionine- and erucifoline-like PAs) did not relate to oviposition preference. Our results suggest PAs in host plant influence the cinnabar moth oviposition preference and this insect is a potential selective agent on the concentration of some individual PAs.

Key Words: Secondary metabolites, diversity, host plant choice, specialist herbivores, chemical defense

1. Introduction

Plants produce a vast variety of structurally different secondary metabolites (De Luca and St Pierre, 2000). Secondary metabolites (SMs) mainly function as defense against antagonistic organisms and/or as signal chemicals for communication with potentially beneficial organisms. In addition they often play a role in protection against abiotic stresses (see reviews by Wink, 2003; Hartmann, 2007). Within a particular species, or an individual plant, a few major compounds are usually accompanied by several derivatives as minor components (Wink, 2003), Beside the structural diversity, SMs often show great variation in concentration. It has been demonstrated that the SM variation in regard to composition and concentration is under genetic control (Vrieling et al, 1993; van Dam and Vrieling, 1994; Kliebenstein et al, 2001, Lankau, 2007).

Herbivores are thought to play an important role in the evolution of the SM diversity in plants (Ehrlich and Raven, 1964; van der Meijden, 1996; Futuyma and Agrawal, 2009). Specialist herbivores usually adapt to a class of defense compounds in a host plant, use them as oviposition and feeding cues, and even utilize them for their own defense (Schoonhoven et al, 2005). Therefore, specialist herbivores have been regarded as being less affected by a given chemical defense than the generalist herbivores and are unlikely to be a selective force in the evolution of a group of structurally related SMs (Harvey et al, 2005; Macel et al, 2005; Arany et al, 2008), However, structurally related compounds can have different simulating effects on specialist herbivores (Macel and Vrieling, 2003) and the variation of defense chemicals in host plants may affect the specialist herbivores' preference (Nieminen et al, 2003;Leima et al, 2005). Moreover, specialist herbivores can exert selection on the concentration of defense chemicals. For instance, the field work manipulating specialist and generalist herbivores of *Brassica nigra* independently showed that specialist loads were positively correlated with increasing sinigrin concentrations in *B. nigra* and higher sinigrin concentration was favored when specialists were removed (Lankau, 2007).

Jacobaea species, formerly known as *Senecio* species, are a good model system to study the diversity of a single group of SMs in plants. 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, Joosten et al, 2009). PAs can occur in plants in two forms: tertiary amine (free base) and *N*-oxide (Rizk, 1991; Wiedenfeld et al, 2008; Chapter 3). Twenty-six different PAs (as tertiary amines) have been reported from 24 *Jacobaea* species (Pelser et al, 2005) using gas chromatography (GC). However, recently more sensitive analytical methods such as liquid chromatography-tandem mass spectrometry (LC-MS/MS) detected 37 structural PA variants even within a single species (Chapter 2-3). The effects of single PAs on PA-unadapted generalist insect herbivores and nematodes are dependent on PA structure and concentration (van Dam et al, 1995; Macel et al, 2005; Dominguez et al, 2008; Thoden et al, 2009). The two forms of the same individual PA had different deterring effect on non-adapted generalist insect herbivores from the results of *in-vitro* bioassay with isolated PAs (Dreyer et al, 1985; van Dam et al, 1995; Macel et al, 2005). The simulative effects of PAs' on oviposition and feeding of specialist were confirmed by bioassays with isolated PAs but it is still largely unknown whether PAs in host plants affect preference of specialist insects (Macel, 2011). Adapted insects are capable of *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 mainly feeds on *Jacobaea vulgaris* (syn. *Senecio jacobaea*) and a restricted number of other *Senecio/Jacobaea* species. *Tyria jacobaeae* sequesters and metabolizes PAs for its own defense (Rothschild et al, 1979; van Zoelen and van der Meijden, 1991; Lindigkeit et al, 1997; Naumann et al, 2002). Experiments with artificial leaves lined with PAs showed that PAs are oviposition stimulants for the cinnabar moth and that the stimulatory effects differ among the particular PAs (Macel and Vrieling, 2003). However, some studies showed that the adult oviposition preference and larval performance of the cinnabar moth was not related to the PAs in host plants (Vrieling and de Boer, 1999; Macel et al, 2002) and oviposition host plant choice among the plants of *J. vulgaris* were related to other factors such as sugar and nitrogen (van der Meijden et al, 1989). Macel and Klinkhamer (2010) found that the damage on *J. vulgaris* plants was mainly caused by specialist insect herbivores such as *T. jacobaeae*, *Longitarsus jacobaeae* and *Haplothrips senecionis*, and that herbivory was positively correlated to the concentration of total PAs and individual PAs (jacobine and jacobine *N*-oxide). *Jacobaea 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, the *J. vulgaris* plants in the invasive areas contain jacobine as the major PA (Joshi and Vrieling, 2005).The previous studies mentioned above seemed to be contradictory and it is not clearly yet whether plant PA variation affect cinnabar moth oviposition preference. To answer this question, we designed a controlled oviposition bioassay with cinnabar moths on the plants of different F_2 hybrid genotypes from a cross between *J. vulgaris* and *Jacobaea aquatica*. Segregating hybrid plants demonstrated greater ecological and chemical variation compared to parental species (Fritz, 1999; Orians, 2000; Kirk et al, 2011) and the various traits are expected to be independent from one another except if they are linked. Therefore they are regarded as useful tools to study the relation between 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* (Chapter 2). In this study, we address the following questions: 1) Do the cinnabar moths have an oviposition preference for certain hybrid plant genotypes? 2) Is oviposition preference affected by the concentration of total PA and of individual PAs in host plants? 3) Is oviposition preference affected by the synergistic or antagonistic effects between PAs?

2. Methods and Material

2.1. Plants grown for the oviposition bioassay

The plants used in the oviposition bioassay were from a hybrid family stored in tissue culture. The hybrid family consists of two parental, two F_1 and 102 F_2 individuals, which were cloned in order to obtain replicate individuals of a genotype for the experiments described here. Such a set of cloned individuals are referred to as 'genotypes' hereafter. The parental genotypes are a jacobine-chemotype plant of *J. vulgaris* and a *J. aquatica* plant. The *J. vulgaris* genotype is from a seed collected at Meijendel Nature Reserve (52° 7′ 54″ N, 4° 19′ 46″ E, The Netherlands) and *J. aquatica* genotype is from a seed collected at the Zwanenwater Reserve (52° 48′ 38″ N, 4° 41′ 7″ E, The Netherlands) (see more details of this hybrid system in Chapter 2). Forty F_2 hybrids genotypes were selected from the hybrid system according to PA composition and concentration in their shoot (Chapter 2). We selected genotypes with a large range in concentration of total PA and major PAs such as senecionine, jacobine and erucifoline (for both the tertiary amine and the *N*-oxide form).

The 40 F_2 genotypes were propagated by tissue culture. Plants were potted in 1.3 liter pots (ca.9 cm diameter, 9 cm high) 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, Ohio, USA). Plants were kept for six weeks in a climate room (RH = 70%, light 16h at 20°C, dark 8h at 20°C) and one week prior to the oviposition bioassay plants were placed in the greenhouse.

2.2. Cinnabar moth rearing

Last stage 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 were kept in glass tubes until pupation. The pupae were stored in cold a room (4°C) until the next season. In April and May 2010, pupae were taken out of 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 for about a week with water and honey before being released in the bioassay. Only healthy and active moths were used.

2.3. Oviposition bioassay

The bioassay was conducted in plastic cylinders (87 cm diameter, ca.1 m high) with a gauze covered top in a greenhouse in the experimental garden of the Institute of Biology in Leiden in May and June 2010 (Fig.S1a,). The cages had a wooden bottom with 20 holes to fix 20 pots with plants so that soil surface was at level with the board (Fig.S1b). Thirty virgin female and 30 virgin male cinnabar moths were released per cage. The plants were watered two or three times during the oviposition bioassay in dishes under the pots without disturbing the cinnabar moths. Cages were rotated every three days to avoid position effects on the oviposition. After ten days, the plants were harvested. The 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 (Fig.S1c). In each of the three trials 80 plants were divided over four cages. Twenty different genotypes were placed in one cage according to a random arrangement so each of the forty genotypes was represented by two replicates at each of the three trials.

2.4. PA data

We used the PA data obtained from the experiment described in Chapter 2. PA concentrations were measured by LC-MS/MS in clonal plants that were grown from the same tissue cultures, under identical conditions and consisting of the same genotypes and number of clones, as those used in the cinnabar moth bioassay. 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, because PA expression is dependent on genotypes under standard growth conditions (Chapter 2). The 37 PAs identified from the *Jacobaea* hybrids could be classified into four types, according to their structural characteristics, biosynthetic pathways and expression patterns: senecionine-, jacobine-, erucifoline- and otonecine-like PAs (Pelser et al, 2005; Chapter 2). We followed this classification in this study. The total concentration of all PAs and the amount of PAs from each structural group were calculated by summing the concentrations of the individual PAs.

2.5. Data analysis

Three variables were used to measure cinnabar moth oviposition preference among the individual plants or hybrid genotype. The variables are: number of eggs per plant; number of egg batches per plant and average egg batch size per plant (the number of eggs per plant/ the 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 mentioned above differed among trials and cages. In the three general linear models, trials and cages were defined as the fixed factors; the three indicators were defined as dependent variables, respectively; the fresh weight of the shoot was treated as a covariate (details in Table S1). The ANOVA test results of the models showed that number of eggs per plant and average egg batch size per plant was not affected by trials and cages. However, the number of egg batches per plant seemed to be affected by cages (Table S1). We did ANOVA tests of the number of egg batches per plant against cages trial by trial and found that only one cage in one trial had different number of egg batches from the other cages in the same trial (data not shown).

We also used general linear models to determine whether number of eggs per plant and average egg batch size per plant differed among the plant genotypes. In these general linear models, plant genotypes were defined as the 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 conducted to determine whether number of egg bathes per plant differed among the plant genotypes. This model differed from the two models mentioned above in that the independent variable is not the number of egg batches per plant but the residuals of the model with number of egg batches per plant against cages, because the egg batches per plant were different among the cages. Normal distributions and homogenous variances of the general linear models were confirmed by testing the residuals of the models using Shapiro tests and Bartlett tests respectively. The average egg batch size per plant appeared not to be significantly genotype-dependent (Table 1) and was not used in further analyses.

Table 1 ANOVAs of the effects of plant genotypes on the cinnabar moth oviposition preference among 40 F_2 hybrid genotypes from *Jacobaea aquatica* and *Jacobaea vulgaris*

Dependent variables	Independent variables	*df* (k-1)	*Df* (n-k-1)	*F*	*P*
The number of teggs per plant	Hybrid genotype	39	199	1.58	0.02*
	Fresh weight	1	237	4.21	0.04 *
	Error	199			
	Total	240			
The number of egg batches per plant [a]	Hybrid t genotype	39	199	1.99	< 0.001***
	Fresh weight	1	237	6.44	0.01 *
	Error	199			
	Total	240			
Average 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 the number of egg batches per plant were different among the cages.
Significance codes: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Linear multiple-regression tests were conducted to check which structural group of PAs affected the oviposition preference of cinnabar moths. 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 total concentrations of each 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 the two structural PA groups (jacobine-like PAs and otosenine-like PAs) which were related to the cinnabar moth oviposition preference according to the multiple regression tests (see result section). Since we expected positive correlations only, we used one-sided significance levels in these tests. Whether to conduct a parametric test (Pearson) or a non-parametric (Spearman rank) test depended on the distribution of the PA data. Because we performed multiple tests, the *P*-values of the tests were adjusted in sequential Bonferroni methods,

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

3. Results

3.1 Cinnabar moth oviposition preference among individual plants

The egg batches were always laid on the underside of the leaves. In total 28,323 eggs were found in 1,375 egg batches on 240 plants. On average, each plant received 118 eggs in 5.73 egg batches and on average an egg batch contained 20 eggs. Each female moth on average laid 3.8 egg batches or 78.7 eggs, 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 having between four to eight egg batches, less than 10% of the plants having more than 10 egg batches and about 5% of the plants received no egg batches (Figure 1a). The number of eggs per plant ranged from 0 to 534 and more than 50% of the plants had less than 150 eggs (Figure 1b). The number of eggs per plant differed among genotypes and 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).

3.2 Relation between cinnabar moth oviposition preference and plant PAs

Multiple-regression showed that two PA groups (jacobine- and otosenine-like PAs) positively correlated to the number of eggs per plant (Table 2). Sum 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 the number of eggs or the number of egg batches per plant. There is an interaction between the concentrations of jacobine- and otosenine-like PAs; this interaction was positively correlated to the number of egg batches and the number of eggs per plant (Table 2). This indicated that the effects of jacobine- and otosenine-like PAs on cinnabar moth oviposition preference were positive and the effect may be synergistic.

There are 9 individual PAs in the jacobine group and 7 in the otosenine group. , Strikingly, among the 9 jacobine-like PAs only the tertiary amines were positively correlated to oviposition preference, while there were no significant correlations between the corresponding *N*-oxides and the

Table 2 Results of multiple regressions of the number of eggs and the number of egg batches of the cinnabar moths 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 (the number of eggs): adjusted $R^2 = 0.37$; $F_{15,24} = 2.53$; $P = 0.020$. For model II (the number of egg batches): adjusted $R^2 = 0.33$; $F_{15,24} = 2.30$; $P = 0.033$.

	Predictors [a]	The number of eggs		The number of egg batches	
		Estimate	*t* value	Estimate	*t* value
	(Intercept)	14.8000	14.376***	5.4690	14.839***
PA groups	snt	0.0042	0.391	0.0003	0.522
	jbt	0.0286	2.186*	0.0013	2.077*
	ert	-0.0397	-0.527	-0.0011	-0.33
	otot	0.4355	2.601*	0.0138	1.779
Two-way interactions	snt:jbt	- 8.06E-06	-0.265	-1.60E-06	-1.137
	snt:ert	2.17E-05	0.174	3.10E-06	0.538
	jbt:ert	-0.0002	-1.575	-5.72E-06	-1.189
	snt:otot	3.80E-05	0.12	-3.03E-06	-0.215
	jbt:otot	0.0011	3.527**	4.87E-05	3.497**
	ert:otot	0.0012	0.443	0.0001	0.825
Three-way interactions	snt:jbt:ert	2.22E-07	0.962	1.24E-08	1.164
	snt:jbt:otot	-8.21E-08	-0.13	-2.86E-08	-1.019
	snt:ert:otot	-3.06E-06	-0.587	1.80E-08	0.075
	jbt:ert:otot	-1.30E-06	-0.335	-1.69E-07	-0.948
Four-way interaction	snt:jbt:ert:otot	-3.24E-10	-0.056	1.39E-10	0.516

[a] Snt, jbt, ert, otot are the sum concentrations of the senecionine-, jacobine- erucifoline- and otosenine-type PAs.
Significance codes: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

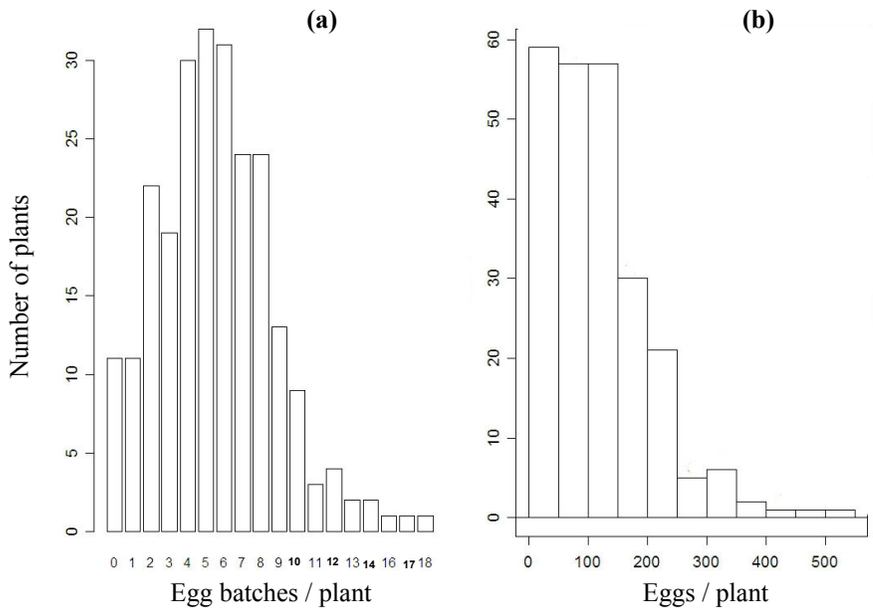

Fig. 1 Frequency distribution of the number of egg batches per plant (a), the number of eggs per plant (b), from 240 plants of 40 F_2 hybrid genotypes of a cross between *Jacobaea vulgaris* and *Jacobaea aquatica*.

Table 3 Results of the one-side Pearson/Spearman correlation tests between the number of eggs and egg batches and concentrations of jacobine- and otosenine-like PAs in the host plants of 40 F_2 hybrid genotypes from *Jacobaea aquatica* and *Jacobaea vulgaris*.

PA group	PA	Code	The number of eggs			The number of egg batchs		
			r/r_s	*P*	Adjusted *P* [a]	r/r_s	*P*	Adjusted *P* [a]
Jacobine-like PAs	jacobine	jb	0.46	**	*	0.43	**	*
	jacoline	jl	0.38	**	ns	0.40	**	ns
	jaconine	jn	0.44	**	*	0.45	**	*
	jacozine	jz	0.39	**	ns	0.48	***	*
	dehydrojaconine	dhjn [b]	0.42	**	*	0.50	***	**
	jacobine *N*-oxide	jbox	0.05	ns	ns	0.13	ns	ns
	jacoline *N*-oxide	jlox	0.08	ns	ns	0.15	ns	ns
	jaconine *N*-oxide	jnox	0.04	ns	ns	0.11	ns	ns
	jacozine *N*-oxide	jzox [c]	0.05	ns	ns	0.04	ns	ns
Otosenine-like PAs [d]	senkirkine	sk	0.25	*	ns	0.26	*	ns
	otosenine	ot	0.24	*	ns	0.12	ns	ns
	onetine	one	0.22	*	ns	0.14	ns	ns
	desacetyldoronine	desdor [c]	0.39	**	ns	0.33	*	ns
	florosenine	fs [c]	0.23	*	ns	0.14	ns	ns
	floridanine	fd [c]	0.28	*	ns	0.18	ns	ns
	doronine	dor [c]	0.19	ns	ns	0.06	ns	ns

[a] *P*-values of the correlation testes were adjusted by sequential Bonferroni method,
[b] This PA was only detected in the tertiary amine form.
[c] Spearman correlation tests were carried out for these PAs without normally distributed concentrations, while Pearson correlation tests were carried out for the other PAs with normal distribution.
[d] Only present as tertiary PAs
Significance codes: ns, $P > 0.05$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

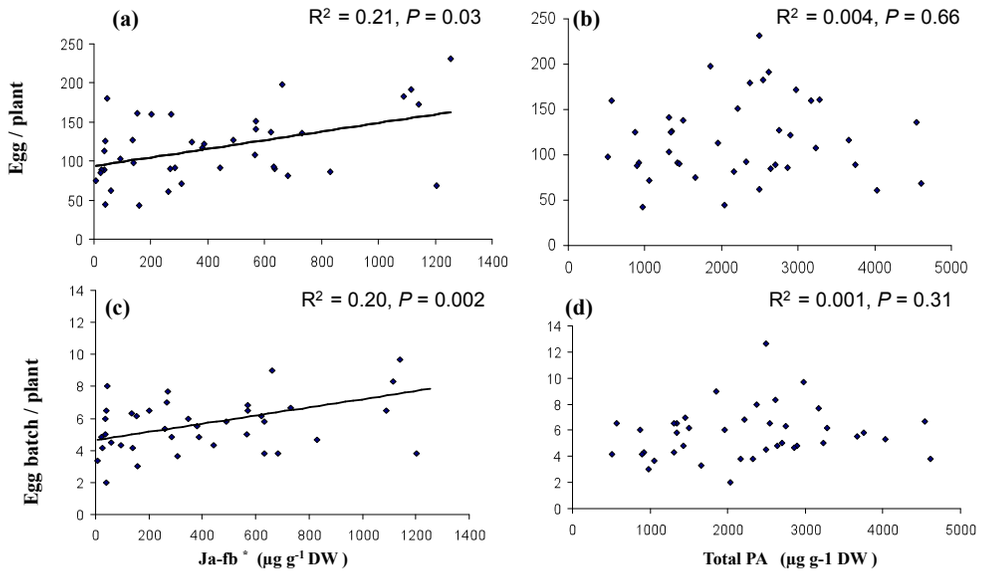

Fig. 2 Scatter graphs of the number of eggs and egg batches per plant against the sum concentration of the 5 jacobine-type tertiary amine PAs (Ja-fb) (a) and total PA (b). Ja-fb are jacobine, jacoline, jaconine, jacozine and dehydrojaconine. Data shown are the genetic mean values of 40 F_2 genotypes of a cross between *Jacobaea vulgaris* and *Jacobaea aquatica*. In both cases: *df* = 38

number of eggs or egg batches (Table 3). The tertiary amines of the five jacobine-like PAs positively correlated to the number of eggs and egg batches. After Bonferroni correction, jacobine, jaconine and dehydrojaconine were significantly correlated to the number of eggs and the number of egg batches. Jacozine was significantly correlated to the number of egg batches only. All otosenine-like PAs (except doronine) positively correlated to the number of eggs and two otosenine-like PAs (senkirkine and desacetyldoronine) positively correlated to the number of egg batches. However, none of the correlations were significant after Bonferroni correction (Table 3). The total concentration of the tertiary amines of jacobine-like PAs explained ca. 20% of the variation of the number of eggs and the number of egg batches among the hybrid genotypes (Figure 2a, c). However, this variation could not be explained by the total PA concentration (Figure 2b, d).

4. Discussion

We demonstrated that the cinnabar moth oviposition preference was affected by the host plant genotype. And we also found that at the genotype level plants with more tertiary amines of jacobine-like PAs and more otosenine-like PAs received more eggs and egg batches (Table 2 and Fig 2a, c). And there were synergistic effects between these two types of PAs. Therefore, those plants with higher levels of these PAs would suffer more damage from cinnabar moths resulting in a lower fitness in environments with abundant cinnabar moths. This indicates that cinnabar moths may potentially act as a selective force on the concentration of jacobine-like tertiary amines. If the amount of this group of PAs is closely correlated to the total PA concentration, like in jacobine-chemotype plants of *J. vulgaris*, the selective force from cinnabar moths may also act on the total amount of PAs. This conclusion agrees with the implication of the high PA concentrations in the invasive ragwort plants compared to ragwort plants in native areas where cinnabars are absent (Joshi and Vrieling, 2005). 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 the authors did not discriminate the tertiary amine and N-oxide forms of PAs and did not check the relationship between cinnabar moth oviposition preference and individual PAs.

From the view point of herbivores, we may ask why the cinnabar moths preferred host plants with more jacobine-like PAs only. The cinnabar moths used in this study were collected from Meijendel where natural-grown ragwort plants are jacobine chemotypes (Macel et al, 2004) and they may therefore have a preference for plants with jacobine-like PAs. Cinnabar moths from caterpillars collected from a population of Erucifoline chemotype plants may therefore have a preference for erucifoline-like PAs. This hypothesis needs to be tested by conducting oviposition bioassays with cinnabar moths collected from host plants belonging to different chemotypes.

Another interesting question is why significant correlations were observed between the number of cinnabar moth eggs 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 the PA concentration in the leaf tissue and that there were differences in the PA composition on the leaf surface from that of the interior (Vrieling and Derridj, 2003). In *Jacobeae* hybrid plants, the tertiary amines of jacobine-like PAs on surface and the same compounds inside the leaf

were highly correlated and the other PAs did not show such high correlations as these PAs (Cheng et al, unpublished). If female cinnabar moths can only detect PAs on the leaf surface, then the high correlation of tertiary amines of jacobine-like PAs between leaf surface and leaf interior could explain why the cinnabar moths prefer plants with more tertiary amines of jacobine-like PAs in the whole rosette. If cinnabar moths can detect PAs not only on the leaf surface but also inside the leaf, an alternative explanation for the cinnabar moth preference to the plant with more tertiary amines of jacobine-like PAs is that these PAs have a stronger stimulating effect on the cinnabar moth oviposition than other PAs. This could be tested by a cinnabar moth oviposition bioassay with isolated PAs if they are available.

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Supplementary Material

(a)

(b)

(c)

- **Fig.S1**

(a) Cinnabar moth oviposition bioassay conducted in four round plastic cages (*diameter* = 90 cm, height = 1 m) in a greenhouse

(b) Two-level boards with plants but without a cage on. The upper level had 20 holes (ca. 10 cm diameter) to hold 20 plants and on the lower level were dishes for pots. Water could be added to the dishes when the upper level board was moved.

(c) Egg batches from one plant. Purple circles indicate separated egg batches. One leaf had two single eggs but no egg batches, indicated by a blue circle.

- **Table S1** ANOVAs of the effects of trials and cages on cinnabar moth oviposition preference among 40 F_2 hybrid genotypes from *Jacobaea aquatica* and *Jacobaea vulgaris*

Dependent variables	Independent variables	*df* (k-1)	*Df* (n-k)	*F*	*P*
The number of eggs per plant	Trials	2	237	0.15	0.86
	Experimental cages	3	236	1.27	0.28
	Trials × cages	6	233	0.96	0.45
	Error	227			
	Total	240			
The number of egg batches per plant	Trials	2	237	0.91	0.40
	Experimental cages	3	236	2.76	0.04 *
	Trials × cages	6	233	1.01	0.42
	Error	227			
	Total	240			
Average egg batch size per plant	Trials	2	237	0.77	0.46
	Experimental cages	3	236	0.63	0.60
	Trials × cages	6	233	0.50	0.81
	Error	227			
	Total	240			

Significance codes: $^{*}P < 0.05$,