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A free lunch? No cost for acquiring defensive plant pyrrolizidine alkaloids in a specialist arctiid moth (*Utetheisa ornatrix*)

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Abstract

Many herbivorous insects sequester defensive chemicals from their host plants. We tested sequestration fitness costs in the specialist moth *Utetheisa ornatrix* (Lepidoptera: Arctiidae). We added pyrrolizidine alkaloids (PAs) to an artificial diet at different concentrations. Of all the larval and adult fitness components measured, only development time was negatively affected by PA concentration. These results were repeated under stressful laboratory conditions. On the other hand, the amount of PAs sequestered greatly increased with the diet PA concentration. Absence of a detectable negative effect does not necessarily imply a lack of costs if all individuals express the biochemical machinery of detoxification and sequestration constitutively. Therefore, we used qPCR to show that expression of the gene used to detoxify PAs, pyrrolizidine-alkaloid-*N*-oxygenase (*pno*), increased 41-fold in our highest PA treatment. Nevertheless, fitness components were affected only slightly or not at all, suggesting that sequestration in this species does not incur a strong cost. The apparent lack of costs has important implications for our understanding of the evolution of ecological interactions; for example, it implies that selection by specialist herbivores may decrease the levels of certain chemical defences in plant populations.

Keywords: adaptation, arms-races, co-evolution, fitness costs, plant-herbivore interaction, specialization

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Introduction

Plants produce a great variety of defensive chemicals that make them unpalatable to herbivores. However, during evolutionary adaptation to their food plants, herbivorous insects have developed specific mechanisms to tolerate specific plant defence chemicals. In some cases, the specific plant defences can be used as a cue by specialist herbivores to find their host plants or be used as phagostimulants (Bernays & Chapman 1994). Many specialist herbivorous insects can also sequester these defensive chemicals and use them as protection against predators or for attracting mates (Bowers 1992; Trigo 2000, 2011; Nishida 2002; Conner & Weller 2004; Kuhn *et al.* 2004; Després *et al.* 2007; Opitz & Müller 2009; Macel 2011). Sequestration is considered an important adaptation of herbivorous insects to the plant host's defences (Rausher 2001; Karban & Agrawal 2002) and has evolved in a diversity of insect lineages (Bowers 1992; Dobler 2001; Nishida 2002; Opitz & Müller 2009). Although specialist herbivores can tolerate and take advantage of some plant defensive chemicals, they may also be negatively affected by these compounds (Camara 1997; Agrawal & Kurashige 2003; Fordyce & Nice 2008). Contrasting with the multitude of studies showing the advantages of sequestration (reviewed by Nishida 2002), very few studies have addressed whether or not the herbivores are negatively affected by the defensive chemicals and if sequestration incurs a fitness cost (Bowers 1992).

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There are two main reasons for this lack of studies. First, for many systems, it is methodologically difficult to isolate plant chemicals as the only factor varying among different diets and to directly measure fitness components under controlled conditions (Bowers 1992). The second, and more challenging, issue is how to access fitness costs if the adaptations to sequester the plant chemicals lack variation, and if all individuals pay a fixed constitutive cost of expressing the biochemical machinery of detoxification and sequestration. However, it is becoming increasingly possible to determine whether these mechanisms are constitutively expressed or induced, even in nonmodel organisms.

The specialist arctiid moth *Utetheisa ornatrix* acquires pyrrolizidine alkaloids (PAs) as larvae, mainly from unripe seeds of the host plants *Crotalaria* spp. (Fabaceae: Papilionoideae) that constitutively produce PAs (Fig. 1A; Eisner & Meinwald 1995; Conner & Weller 2004; Ferro *et al.* 2006; Guimarães *et al.* 2006; Cogni & Futuyma 2009; Cogni 2010a). By preying on the seeds, *U. ornatrix* can have a significant impact on the fitness of *Crotalaria* plants (Cogni *et al.* 2011). PAs sequestered by the larvae are maintained in the pupal and adult stages (Eisner & Meinwald 1995; Conner & Weller 2004). These compounds are transmitted from males to females as nuptial gifts, and from the female to eggs; in all stages of the life history, the PAs provide protection against vertebrate and invertebrate predators (Eisner & Meinwald 1995; Conner & Weller 2004). *Utetheisa ornatrix* males also modify PAs into courtship pheromones, and the amount of pheromone produced by a male correlates with systemic levels of PAs, the quantity of alkaloid transmitted to the female at mating, and male body size (Eisner & Meinwald 1995; Conner & Weller 2004). This species is amenable to experimentation because adult and larval fitness components can be directly measured in the laboratory, using a chemically controlled diet (Cogni & Futuyma 2009; Cogni 2010b).

Fig. 1 (a) *Utetheisa ornatrix* larva on the fruit of its main host plant, *Crotalaria pallida*, in Central Florida. (b) Chemical structure of the senecionine-type pyrrolizidine alkaloids (PAs) used in the experiments.

In addition, the interaction between PA-containing plants and arctiid moths is one of the few systems in which the mechanism by which an herbivorous insect detoxifies chemical defences from its host plants is known (see Brückmann *et al.* 2000; Li *et al.* 2003 and Wheat *et al.* 2007 for other examples). In larvae of the PA-specialist arctiid *Tyria jacobaeae*, these alkaloids are absorbed as tertiary bases in the gut, *N*-oxidized in the hemolymph by a flavin-dependent monooxygenase enzyme (pyrrolizidine-alkaloid-*N*-oxygenase *pno*) and stored in the tissues (Lindigkeit *et al.* 1997; Naumann *et al.* 2002). The gene for *pno* in *T. jacobeae*, which is highly specific for PAs, was recruited from an insect-specific flavin-dependent monooxygenase gene family of unknown function (Naumann *et al.* 2002). Sehlmeyer *et al.* (2010) found the same enzyme in other PA-feeding arctiid species and that the Lepidoptera has three gene families of flavin-dependent monooxygenases. A gene duplication early in the arctiid lineage produced the pyrrolizidine-alkaloid-*N*-oxygenase that enables these moths to feed on PA-containing plants and to successfully accumulate these compounds in the tissue (Sehlmeyer *et al.* 2010).

We purified large amounts of macrocyclic pyrrolizidine diesters from plant material and added this purified mixture of PAs at different concentrations to an artificial diet. We fed larvae during their entire development on this chemically controlled diet with different PA concentrations. We used qPCR to measure expression of the *pno* gene used to detoxify PAs at the different treatments. We asked the following questions: (i) Does diet PA concentration affect larval and adult fitness components and the amount of PAs sequestered in adult moths? (ii) Are the effects of PAs more pronounced under more stressful laboratory conditions?

(iii) Is the biochemical machinery used to detoxify PAs constitutively expressed or is it induced depending on diet PA concentration?

Material and methods

General design and larval performance

Pyrrolizidine alkaloids were extracted from leaves and flowers of *Senecio brasiliensis* (Asteraceae) as in Trigo *et al.* (1993). Plant material was homogenized in MeOH and separated by vacuum filtration, and the extract was evaporated under low pressure at low temperature and redissolved in 2 N H_2SO_4, followed by a three times extraction with $CHCl_3$. The acid aqueous solution was reduced with Zn dust for 3 h, alkalinized with NH_4OH and extracted three times with $CHC1_3$-MeOH (3:1) and once with pure $CHC1_3$. The combined organic extracts were dried over anhydrous Na_2SO_4 and evaporated. We used *S. brasiliensis* as the PA source because the yield of these alkaloids is higher than in *C. pallida* seeds. We used approximately 7 kg of plant material, and the yield was *c.* 4 mg/g. The extracted PAs consisted of a mixture of senecionine-type PAs including approximately 4% of senecionine, 69% of intergerrimine and 27% of retrorsine (Fig. 1B). These are the same category of PAs (senecionine-type) found in unripe seeds of *C. pallida* (usaramine *c.* 85% and intergerrimine *c.* 15%) (Ferro *et al.* 2006), the most common *U. ornatrix* host. These PAs just vary at one position (OH or H) or are *cis-trans* isomers of each other (Fig. 1B). Other *Crotalaria* species, such as *C. incana* and *C. micans*, with integerrimine as the main PA (Flores *et al.* 2009), are also used as host plant by *U. ornatrix* in the Neotropics (Cogni 2010a, J. R. Trigo, personal communication).

We used an artificial diet based on *Phaseolus* beans (Signoretti *et al.* 2008) to which we added 20 mL of soybean oil to dissolve the PAs. The PAs dissolved in the oil were added to the diet at 60 °C and mixed in a blender. Based on the average concentration of PAs in unripe seeds of *C. pallida* (0.024% dry weight) published by Ferro *et al.* 2006; we used five treatment concentrations: 0.024% (1×) PAs added, 0.0048% (0.2×), 0.12% (5×), 2.4% (100×) and a control without PA (0×). When we later expanded the sampling to other populations, we found that the concentration of PAs in unripe seeds of *C. pallida* can vary up to 10 times among individuals and populations (Cogni 2010b; J. R. Trigo personal communication), a range of variation comparable to our 0.024–0.12% treatments. In addition, in some localities, *U. ornatrix* can use alternate uncommon hosts with a higher PA concentration; for example, unripe seeds of *C. spectabilis* have an average of 4.6% (SD = 0.4; $N = 9$) (J. R. Trigo & R. Cogni, unpublished data). Therefore, although our higher concentration treatment represents around 100 times the average concentration of hosts in the population where the moths used in this experiment were collected (Cogni *et al.* 2011), it is likely that there has been selection in the past to use such high concentrations in other hosts.

A large moth stock was maintained in the laboratory, initiated by *U. ornatrix* adults that were collected at Archbold Biological Station in central Florida, United States. Larvae were fed on an artificial diet based on *Phaseolus* beans as above, with no PAs added. Adults were kept in paper cages (*c.* 3.2 litres) where 5% honey solution was provided. To avoid maternal and paternal effects (because eggs are endowed with PAs), eggs used in the experiments were from adults that had been in the laboratory on a PA-free diet for at least one generation.

Experiments were carried out in an incubator at 29 °C. Just after hatching, larvae were transferred individually to 2-mL microcentrifuge tubes containing 0.6 mL of diet. Every week, the larvae were transferred to a new tube with fresh diet. After 3 weeks, we used a 10-mL test tube with 3 mL of diet. We measured larval survival to pupation, larval weight (after 3 weeks), weight of diet consumed during the fourth week, larval development time (from egg hatching to pupation), pupal weight (7 days after pupation), adult dry weight (after freeze-drying), and we determined adult sex (according to Travassos 1946). Pupal weight correlates with adult fitness in *U. ornatrix*; larger females lay more eggs, and large males attract more females to mate (Iyengar & Eisner 1999). The numbers of larvae used per treatment were 102 for 0%, 108 for 0.0048%, 110 for 0.024%, 113 for 0.12% and 150 for 2.4%.

Sequestered PAs quantification

Twenty adults per treatment were sexed and saved for quantification of PAs sequestered. PAs were extracted from these freeze-dried adults. Adults were individually homogenized in ethanol three times using powdered glass. The ethanol extracts were centrifuged; the supernatants were recovered and combined and evaporated to dryness under vacuum at 45 °C before resuspension in 1.5 mL of ethanol. Total PAs were quantified by a colorimetric method as in Trigo *et al.* (1993). Three replicate readings were performed for each individual, and the average was used as the dependent variable. Dixon's *Q*-test was used to detect possible outliers among the three replicated spectrophotometer readings (Rorabacher 1991). Retrorsine isolated from *Senecio brasiliensis* was used for the calibration curve. Absorbance values lower than 0.020 (representing <2 μg of PA per replicate reading) were considered as no PA detected, because of the limit of sensitivity of the instrument.

We did not detect PAs in 16 individuals that fed on the diet with the two lowest PA concentrations (0% and 0.0048%). We calculated the total amount of PAs sequestered and the concentration in each moth (dividing the total amount by dry weight). To establish the average level of sequestration in the field, 16 adults were collected at Archbold Biological Station in November 2010, and their PA content was quantified as above.

Adult fitness

To test the effect of larval feeding on the highest PA concentration diet on adult fitness components (male and female longevity, fecundity and egg viability), we fed larvae, from hatching to pupation, on two diet types: the 0.024% PA dry weight (1×) and the 2.4% (100×). After these larvae pupated, emerging adults were divided into four treatments in which one male and one female were paired: (i) 0.024% PA male with 0.024% PA female, (ii) 0.024% PA male with 2.4% PA female, (iii) 2.4% PA male with 0.024% PA female and (iv) 2.4% PA male with 2.4% PA female. Sixteen pairs were used per treatment. Each pair was kept in a paper cage (*c.* 3.2 litres) for their entire adult life. We provided 5% honey solution in each cage. We checked daily for deaths and on alternate days for eggs laid. Eggs were transferred to translucent plastic cups (1.25 oz.) and checked on alternate days for hatching. Adult longevity was defined as the number of days from adult emergence to adult death. Fecundity represents the total number of eggs laid per individual female through the entire lifetime. Egg viability is defined as the proportion of eggs laid by individual females through the entire lifetime that successfully hatched.

Larval performance under more stressful laboratory conditions

Because under ideal laboratory conditions costs are less likely to be detected, we also tested larval performance under more stressful conditions. We repeated the experiment with similar conditions (except that we did not used the 0.0048% PA concentration) used in the larval performance described above (control) (N = 56–60 larvae/concentration), with high larval competition (10 larvae per vial for the two-first weeks and five larvae per vial during the third week) (N = 160 total larvae/concentration), and a stress treatment in which once a week the larvae were kept without food and at lower temperature (20 °C instead of 29 °C) and lower humidity (30% instead of 60%) for 23 h and at 7 °C and 20% for 1 h (N = 59–61 larvae/treatment). In this experiment, we used the first generation of larvae from field-collected adults.

pno gene expression level

Larvae were raised on diets with four PA concentrations (0%, 0.024%, 0.12% and 2.4%) as above (N = 6–8 per treatment). At 3 weeks after hatching, they were immersed in liquid nitrogen and stored at −80 °C. For the 2.4% treatment, we also sampled three additional larvae 4 weeks after hatching. RNA was extracted with QIAGEN RNeasy kit using the anterior third of the larva, because the *pno* gene is expressed in the fat body and head (Sehlmeyer *et al.* 2010). No DNase treatment was performed, but controls with no reverse-transcriptase confirmed the lack of genomic DNA amplification in the qPCRs. RNA extraction quality and integrity were tested with a NanoDrop (Thermo) and a Bioanalyser (Agilent Technologies). qPCR was performed in two steps using QIAGEN QuantiTect Reverse Transcription Kit and QuantiFast SYBR Green PCR Kit, according to the manufacturer's protocol. We designed primers based on sequences of related species (Sehlmeyer *et al.* 2010) and used them to sequence a segment of the *pno* gene in *U. ornatrix*. Our sequence fragment (accession number JX514176) aligned with mRNA for *pno* in *Tyria jacobeae* (AJ420233.1) with a score of 141 bits, e-value of 1^{-30} and 83% identity, at the protein level the score was 88.2 bits, e-value of 2^{-19} and 89% identity. We used this sequence to design qPCR primers with Primer3 Plus with qPCR parameters. We used *β-actin* as a house-keeping control gene. The primers used in the qPCRs were qPNO_F: 5-AACTTGGGTGCAACGGATAG-3, qPNO_R: 5-CGACAACAAAGTCACATGCTTC-3 and qBAC6-F: 5-TCGAGTTGTAAGTGGTCTCGTG-3, qBAC6-R: 5-AA CGAACGATTCCGTTGC-3. For each sample, three replicated qPCRs were carried out for the *pno* gene and the control gene, and the three replicated Ct values were averaged. With dilution series, we determined the amplification efficiency of the two genes and used the primer efficiency to estimate expression level for each gene. The normalized *pno* expression level was calculated by dividing *pno* expression by expression of β-actin.

Statistical analyses

Larval survival on the PA concentration treatments was compared by the test to compare more than two proportions (Zar 1999, p. 562), followed by a comparison of each proportion to the proportion of survived larvae on the control (Zar 1999, p. 565). We tested the effect of diet PA concentration, moth sex and interaction on each response variable (weight of diet consumed, larval weight, development time, pupal weight, adult weight, total PAs sequestered and adult PA concentration) with fixed model ANOVAs. If the concentration effect was

Table 1 Effect of diet pyrrolizidine alkaloid (PA) concentration on *Utetheisa ornatrix* fitness components

PA concentration in diet	Diet consumed (mg)	Larval weight (mg)*	Development time (days)*	Pupal weight (mg)	Adult dry weight (mg)
0(%)	361 ± 430	80 ± 30[a]	43 ± 5[a]	83.1 ± 34.7	16.9 ± 11.8
0.0048(%)	413 ± 445	78 ± 32[a]	42 ± 3[a]	93.4 ± 35.9	18.3 ± 11.3
0.024(%)	360 ± 461	71 ± 30[a]	44 ± 5[a]	80.7 ± 31.2	16.0 ± 10.2
0.12(%)	303 ± 357	73 ± 28[a]	44 ± 5[a]	71.9 ± 28.3	14.5 ± 9.7
2.4(%)	347 ± 273	46 ± 27[b]	50 ± 11[b]	84.3 ± 29.6	15.7 ± 6.7

Larvae were fed from hatching to pupation on artificial diet with five different pyrrolizidine alkaloid (PA) concentrations. Values are means ± SD. *Indicates variables that significantly varied among the treatments (effect of diet PA concentration on ANOVA tests). [a] and [b] represent differences in *post hoc* Tukey tests.

significant, we compared pairwise differences with Tukey *post hoc* tests. For the adult fitness experiment, we tested the effect of the diet that the adult was raised on, the diet that the partner was raised on, and the interaction on each response variable (male longevity, female longevity, fecundity and egg viability) with fixed model ANOVAs. We used a log transformation for fecundity and arcsine transformation for egg viability to achieve a normal distribution. For the stressful condition experiment, we tested the effect of condition (control, competition or stress), diet PA concentration and the interactions on larval survival with a logistic regression. The effect of condition, diet PA concentration, moth sex and the interactions on larval weight, development time, pupal weight and adult weight were tested with fixed model ANOVAs. The absolute *pno* gene expression was compared among treatments with a fixed model ANOVA, after log transformation to achieve a normal distribution.

Results

Larval performance

Larval survival was affected by the PA concentration in the diet (0% PAs = 38% survival, 0.0048% PAs = 30%, 0.024% PAs = 67%, 0.12% PAs = 41%, 2.4% PAs = 53%; $\chi^2 = 38.6$, d.f. = 4, $P < 0.0001$); survival was significantly higher than control on the 0.024% and the 2.4% diets (0.024%: $q = 4.27$, $P < 0.01$; 2.4%: $q = 2.35$, $P < 0.01$). Diet consumption was not affected by PA concentration (Tables 1 and 2). Three weeks after hatching, larvae eating the diet with the highest PA concentration (2.4%) were smaller than the larvae eating diets with lower PA concentrations (Tables 1 and 2). Larvae eating the highest PA concentration also took longer to pupate (Tables 1 and 2). On the other hand, pupal and adult weights were not affected by PA concentration (Tables 1 and 2). Development time was longer for males than for females (Table 2). The other response variables were not different between the sexes (Table 2), and there was no significant interaction between moth sex and PA concentration on

Table 2 Effect of diet pyrrolizidine alkaloid (PA) concentration, moth sex, and the interaction on diet consumed, larval weight, development time, pupal weight, adult weight, total PAs sequestered by adult moths and PA concentration on adult moths

Source	d.f.	*F*-ratio	*P*
Diet consumed			
PA concentration	4	0.209	0.933
Sex	1	0.955	0.330
PA concentration × Sex	4	0.666	0.616
Error	145		
Larval weight at week 3			
PA concentration*	4	8.719	<0.001
Sex	1	0.002	0.968
PA concentration × Sex	4	0.504	0.733
Error	155		
Development time			
PA concentration*	4	15.343	<0.001
Sex*	1	6.524	0.012
PA concentration × Sex	4	0.695	0.597
Error	155		
Pupal weight			
PA concentration	4	1.421	0.230
Sex	1	2.260	0.135
PA concentration × Sex	4	0.747	0.561
Error	155		
Adult dry weight			
PA concentration	4	0.651	0.627
Sex	1	0.014	0.906
PA concentration × Sex	4	0.973	0.424
Error	153		
Total PAs in adults			
PA concentration*	2	46.125	<0.001
Sex	1	0.998	0.322
PA concentration × Sex	2	1.011	0.371
Error	56		
PAs concentration in adults			
PA concentration*	2	71.275	<0.001
Sex	1	0.863	0.357
PA concentration × Sex	2	0.893	0.415
Error	56		

Utetheisa ornatrix was fed from hatching to pupation on artificial diet with five different pyrrolizidine alkaloid (PA) concentrations. *Indicates factors with significant effect.

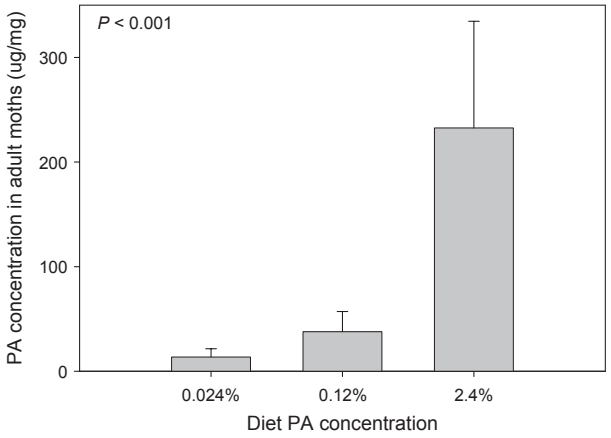

Fig. 2 Effect of diet concentration of PAs on PA concentration in adult *Utetheisa ornatrix*. Bars represent mean values + SD. *P* value indicates the effect of diet PA concentration in an ANOVA test.

any of the variables measured (Table 2). Pupal mortality was low and did not differ among treatments (0% PAs = 4.9%, 0.0048% PAs = 2.8%, 0.024% PAs = 1.8%, 0.12% PAs = 1.8%, 2.4% PAs = 3.3%).

Sequestered PAs quantification

The amount of PAs sequestered, and the PA concentration in adult moths, greatly increased with increasing PA concentration in the diet (Fig. 2, Table 2). Field-collected adults had a PA concentration similar to our 0.024% (1×) treatment (average ± SD = 8.8 ± 3.4 μg/mg for males, and 11.7 ± 5.9 for females).

Adult fitness

Male and female longevity did not depend on the diet that the larva was reared on or that of its partner (Tables 3 and 4). Likewise, neither fecundity nor egg viability were affected by the diet that the female or the male was reared on (Tables 3 and 4).

Table 3 Effect of larval diet pyrrolizidine alkaloid (PA) concentration on *Utetheisa ornatrix* fitness components

Larval diet		Male longevity (days)	Female longevity (days)	Fecundity (# eggs)	Egg viability
Female	Male				
0.024(%)	0.024(%)	34 ± 12	31 ± 15	133 ± 113	0.82 ± 0.12
0.024(%)	2.4(%)	33 ± 10	30 ± 12	103 ± 95	0.82 ± 0.13
2.4(%)	0.024(%)	35 ± 11	26 ± 12	103 ± 126	0.85 ± 0.14
2.4(%)	2.4(%)	30 ± 12	29 ± 13	120 ± 100	0.86 ± 0.07

Values are means ± SD.

Table 4 Effect of diet pyrrolizidine alkaloid (PA) concentration that males were reared on and that females were reared on, and the interaction on male longevity, female longevity, fecundity, and egg viability

Source	d.f.	*F*-ratio	*P*
Male longevity			
Male diet	1	1.046	0.331
Female diet	1	0.097	0.757
Male diet × Female diet	1	0.583	0.466
Error	60		
Female longevity			
Male diet	1	0.060	0.807
Female diet	1	0.756	0.388
Male diet × Female diet	1	0.516	0.475
Error	60		
Fecundity			
Male diet	1	0.263	0.610
Female diet	1	0.876	0.354
Male diet × Female diet	1	1.841	0.181
Error	52		
Egg viability			
Male diet	1	0.000	0.994
Female diet	1	0.930	0.339
Male diet × Female diet	1	0.145	0.705
Error	52		

Larval performance under more stressful laboratory conditions

The effect of diet PA concentration on larval performance was similar in the different laboratory raising conditions (Tables 5 and 6). Larval survival was greatly decreased under the competition treatment, but independently of PA concentration (Table 5) (logistic regression Wald tests: concentration $\chi^2 = 2.20$, $P = 0.53$; condition $\chi^2 = 141.17$, $P < 0.0001$; concentration × condition: $\chi^2 = 1.37$, $P = 0.96$). Larvae under the stress treatment presented longer development time and higher pupal and adult weight compared with the control and competition treatments (Tables 5 and 6). In all treatments, development time was longer when eating the 2.4% PA diet (Tables 5 and 6).

pno gene expression level

pno gene expression level in the larvae was influenced by the PA concentration of the diet (Fig. 3; ANOVA *F*-ratio 16.80, d.f. = 3, $P < 0.0001$). Expression level was about eight-fold higher at the 0.12% PA diet and 41-fold higher at the 2.4% PA diet (Fig. 3), compared with the control. In the 2.4% PA treatment, expression remained high in the third and the fourth week of larval development (Fig. 3).

Discussion

Of all the fitness components measured, only development time was negatively affected by diet PA concentration. At

Table 5 Effect of laboratory condition (control, competition or stress), and diet pyrrolizidine alkaloid (PA) concentration, on *Utetheisa ornatrix* fitness components

Response variable	PA concentration	Control	Competition	Stress
Larval survival	0(%)	29(%)	8(%)	38(%)
	0.024(%)	29(%)	6(%)	42(%)
	0.12(%)	34(%)	8(%)	49(%)
	2.4(%)	33(%)	6(%)	48(%)
Larval weight (mg)	0(%)	107 ± 40	99 ± 40	117 ± 25
	0.024(%)	106 ± 35	102 ± 26	115 ± 35
	0.12(%)	124 ± 32	99 ± 44	116 ± 33
	2.4(%)	114 ± 28	101 ± 28	123 ± 25
Development time (days)	0(%)	26 ± 8	25 ± 4	25 ± 4
	0.024(%)	25 ± 3	25 ± 4	28 ± 6
	0.12(%)	26 ± 4	25 ± 4	27 ± 3
	2.4(%)	29 ± 3	30 ± 4	30 ± 4
Pupal weight (mg)	0(%)	140 ± 35	151 ± 22	159 ± 38
	0.024(%)	127 ± 33	144 ± 42	166 ± 37
	0.12(%)	132 ± 42	137 ± 47	148 ± 46
	2.4(%)	134 ± 36	137 ± 42	150 ± 38
Adult dry weight (mg)	0(%)	25.6 ± 9.4	30.7 ± 5.3	29.9 ± 10.3
	0.024(%)	22.2 ± 7.5	27.5 ± 6.6	31.9 ± 10.7
	0.12(%)	24.6 ± 10.7	27.4 ± 9.3	28.9 ± 9.9
	2.4(%)	27.4 ± 9.8	27.7 ± 12.0	30.8 ± 12.0

Larvae were fed from hatching to pupation on artificial diet with four different pyrrolizidine alkaloid (PA) concentrations and under three different laboratory conditions (control, competition and stress). Values are means ± SD.

the highest PA concentration, larvae grew slower, but due to a longer development time these larvae achieved the pupal stage at similar sizes as larvae feeding at the lower PA concentrations, and adults showed similar longevity, fecundity and egg viability. Even under more stressful laboratory conditions, there was no significant effect of PAs on any performance trait other than development time. On the other hand, the amount of PAs sequestered greatly increased with increasing diet PA concentrations; sequestration was around 3.7 times higher than field conditions at our focal location in the 5 × treatments and 21.9 times higher in the 100 × treatment. It is important to notice that in the field site where we collected the moths, the main host plant is *Crotalaria pallida*, a species with relatively low PA concentration (similar to our 1 × treatment). In other localities, alternative hosts with PA concentrations similar to our 100 × treatment (such as *C. spectabilis*) can be occasionally used. A few previous studies have reported no negative effects of sequestration in arctiid moths (Kelley *et al.* 2002; Del Campo *et al.* 2005; Hartmann *et al.* 2005) and some other insects (Rowell-Rahier & Pasteels 1986; Bowers 1988; Fordyce 2001; Kearsley & Whitham 1992). Other studies provide some evidence for negative effects (Cohen 1985; Bjorkman & Larsson 1991; Bowers & Collinge 1992; Camara 1997; Fordyce & Nice 2008). However, in many of these pioneer studies, it was not possible to isolate the specific plant metabolite in a chemically controlled diet, or to measure both larval and adult fitness components, as performed here. Although cases are known in which an herbivore's response is affected by interactions among two or more plant metabolites (e.g. Steppuhn & Baldwin 2007), most of the research in this field has assumed and tested for single-compound effects (Bowers 1992; Bernays & Chapman 1994) as have we. Additionally, because of our relatively large sample sizes, it is unlikely that the lack of negative effect (in all, but one of the fitness components measured) is attributable to lack of statistical power.

Absence of a detectable negative effect of a plant chemical in an herbivorous insect does not necessarily imply a lack of costs, if all individuals express the biochemical machinery of detoxification and sequestration constitutively. If, however, the mechanism is inducible, and if it incurs a fitness cost, the herbivore performance would be reduced at higher concentrations. In our high PA treatments, larvae showed elevated expression of the *pno* gene, suggesting increased levels of the enzyme used to detoxify PAs. Compared with our control diet, *pno* expression was eightfold higher at the 5 × diet and 41-fold higher at the 100 × diet. Nevertheless, fitness components were affected only slightly or not at all, suggesting that sequestration in this species does not incur a strong cost. The induction of detoxifying mechanisms may be common in other specialist herbivores; the best studied example is the black swallowtail,

Table 6 Effect of diet pyrrolizidine alkaloid (PA) concentration, laboratory condition (control, competition or stress), moth sex and the interactions on larval weight, development time, pupal weight and adult weight

Source	d.f.	*F*-ratio	*P*
Larval weight at week 3			
PA concentration	3	0.462	0.709
Treatment	2	2.286	0.105
Sex*	1	6.176	0.014
PA concentration × Treatment	6	0.742	0.617
PA concentration × Sex	3	1.324	0.269
Treatment × Sex	2	2.362	0.098
PA concentration × Treatment × Sex	6	0.818	0.558
Error	138		
Development time			
PA concentration*	3	9.137	<0.001
Treatment*	2	3.937	0.021
Sex*	1	18.199	<0.001
PA concentration × Treatment	6	0.952	0.460
PA concentration × Sex	3	0.303	0.823
Treatment × Sex	2	0.934	0.395
PA concentration × Treatment × Sex	6	0.891	0.503
Error	156		
Pupal weight			
PA concentration	3	0.977	0.405
Treatment*	2	5.955	0.003
Sex*	1	34.364	<0.001
PA concentration × Treatment	6	0.586	0.741
PA concentration × Sex	3	0.461	0.710
Treatment × Sex	2	0.241	0.786
PA concentration × Treatment × Sex	6	1.294	0.263
Error	158		
Adult dry weight			
PA concentration	3	0.413	0.744
Treatment*	2	4.828	0.009
Sex*	1	14.511	<0.001
PA concentration × Treatment	6	0.492	0.814
PA concentration × Sex	3	0.459	0.712
Treatment × Sex	2	0.727	0.485
PA concentration × Treatment × Sex	6	1.673	0.131
Error	160		

Utetheisa ornatrix larvae were fed from hatching to pupation on artificial diet with four different pyrrolizidine alkaloid (PA) concentrations, and under three different laboratory conditions (control, competition and stress). *Indicates factors with significant effect in ANOVA tests.

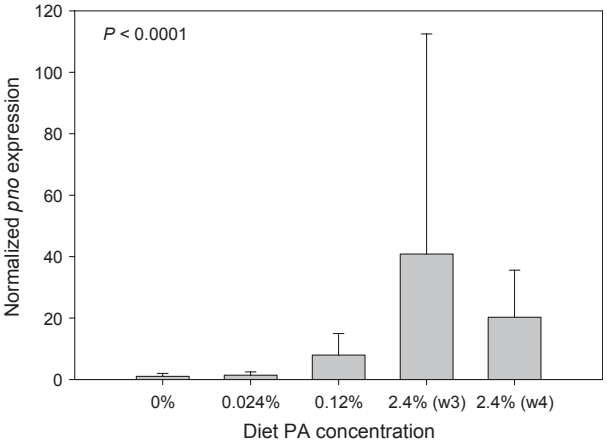

Fig. 3 Effect of diet concentration of PAs on expression level of pyrrolizidine *N*-oxygenase (*pno*) in larval *Utetheisa ornatrix*. The normalized *pno* expression level was calculated by dividing *pno* expression by expression of the house-keeping gene β-actin. Expression at the control (0% PAs) was set to one; therefore, values represent fold increase in expression. At the 2.4% concentration, larvae were sampled at weeks three and four. Bars represent mean values + SD. *P* value indicates the effect of diet PA concentration in an ANOVA test.

Papilio polyxenes, which detoxifies the furanocoumarins of its host plant by a P450 monooxygenase (Ma *et al.* 1994; Prapaipong *et al.* 1994; Hung *et al.* 1995).

That chemical sequestration in herbivorous insects has only slight costs has significant implications. First, it challenges a basic assumption of the plant-herbivore literature (and more broadly, the literature of ecology, evolution and behaviour) namely a trade-off in organisms' investments in defence, reproduction and growth (Andersson 1994; Koricheva 2002). Second, lack of strong costs for the herbivore may cause asymmetry in co-evolution. Meta-analysis of the plant-herbivore literature shows unequivocally that for plants the production of defensive chemicals has a cost (Koricheva 2002). Third, theoretical models of co-evolution, including local adaptation, geographical mosaic, arms-races and Red Queen hypotheses, assume costs of parasites' adaptations to overcome host defenses (Bergelson *et al.* 2001). Future studies can address if the lack of costs is also common in other host-parasite interactions, and how these models would behave in the absence of costs.

Fourth, our results bear on how specialist herbivores act as agents of natural selection on the levels of chemical defences of their host plants. PAs and many other plant compounds have toxic and deterrent effects on generalists (van Dam *et al.* 1995; Macel *et al.* 2005; Narberhaus *et al.* 2005). [We have found that this also holds for the generalist herbivore *Heliothis virescens* (in prep.).] As a rule, specialist herbivores are less affected by plant chemical defences than generalists (van der Meijden 1996). However, many specialists are not fully adapted to their host plants' compounds and can also be strongly affected by them (Agrawal & Kurashige 2003 and references therein). *Utetheisa ornatrix*, however, displays an unusually weak negative effect of PAs, together with a strong advantage in sequestering higher amounts of these compounds. It therefore appears advantageous for this herbivore to use individual plants with higher PA concentrations and consequently to act

as an agent of natural selection for a lower level of chemical defence in populations of its host plant. Indeed, the lack of a specialist herbivore on introduced populations of the weed *Jacobaea vulgaris* (formerly *Senecio jacobaea*) resulted in the evolution of higher levels of PAs and consequently increased resistance to generalist herbivores (Joshi & Vrieling 2005). Therefore, the balance of selective pressure from specialist and generalist herbivores must be considered an important factor that might maintain genetic variation for resistance in natural plant populations (van der Meijden 1996; Lankau 2007). Under this scenario, no escalation of plant defenses is expected (Vermeij 1994); in fact, specialist herbivores may cause phylogenetic decline in defences. Interestingly, cardenolides, which are sequestered by specialist herbivores, show phylogenetic decline in milkweeds, while phenolic compounds, which are not sequestered, show escalation (Agrawal & Fishbein 2008; Agrawal *et al.* 2009). In another example, derived species of *Aristolochia* have lost aristolochic acids that are present in basal clades and instead produce labdanoic acids; specialist Troidini butterflies sequester aristolochic acids, but are negatively affected by labdanoic acids (Brown *et al.* 1995). The occurrence of only weak costs associated with the sequestration of plant chemical defences by herbivorous insects has important implications for our understanding of the evolution of ecological interactions.

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RC, JRT and DJF designed research, RC performed research, JRT contributed analytical tools, RC analysed data, RC wrote the paper with inputs from DJF and JRT.

Data accessibility

PNO fragment sequence: Genbank accession JX514176. Larval and adult fitness experiments, stress experiments, PA content in adult moths and *pno* expression: DRYAD doi:10.5061/dryad.14fd6.