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**Neotropical Entomology**

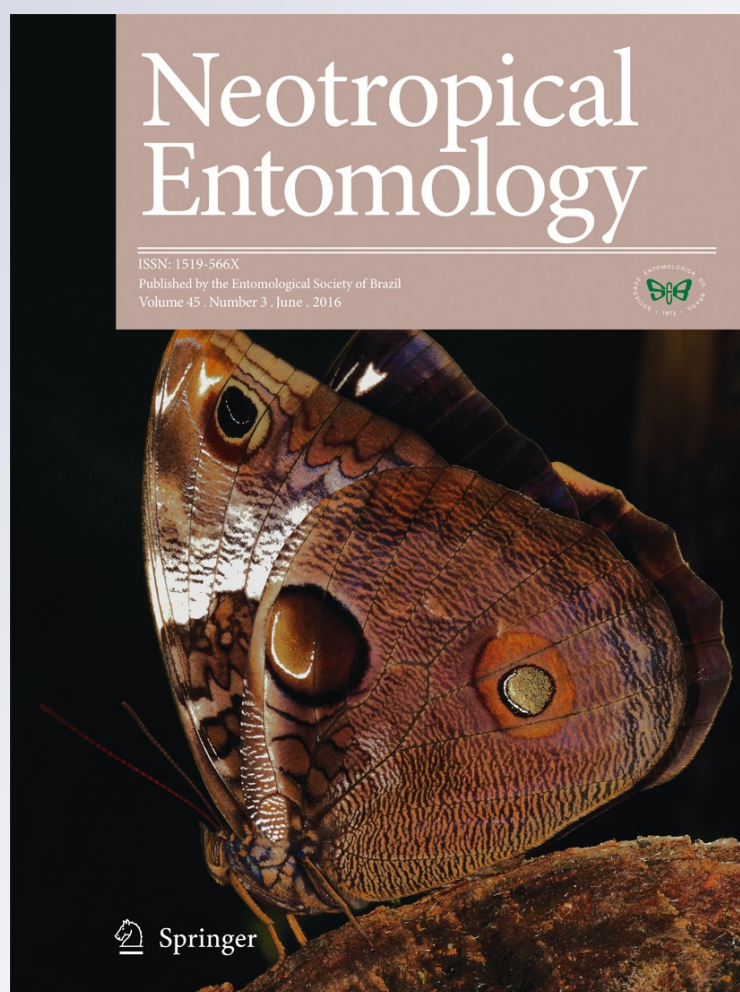
ISSN 1519-566X

Volume 45

Number 3

Neotrop Entomol (2016) 45:252-257

DOI 10.1007/s13744-016-0361-6



 Springer

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# Pyrrolizidine Alkaloids Negatively Affect a Generalist Herbivore Feeding on the Chemically Protected Legume *Crotalaria pallida*

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## Keywords

Chemical defense, herbivory, sequester, specialization

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Edited by Raúl A Laumann – Embrapa

Received 25 February 2015 and accepted 3 January 2016

Published online: 29 January 2016

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## Abstract

Plant secondary metabolites can have opposing effects on adapted specialist and non-adapted, generalist herbivores. In this study, we used *Heliothis virescens* (Fabricius) (Lepidoptera: Noctuidae) as a generalist, non-adapted model herbivore to test the possible effects of *Crotalaria pallida* (Fabaceae: Papilionoideae) defenses on herbivore performance. Neonate *H. virescens* larvae were able to consume *C. pallida* leaves and fruits and grow for a few instars, but none of them survived to pupation. We added isolated pyrrolizidine alkaloids (PAs) to an artificial diet at different concentrations, and PA concentration significantly affected the number of larvae that achieved pupation. Larval survival was not reduced at a PA concentration similar to the concentration on green seeds of *C. pallida*, but it was significantly reduced at PA concentration 5 and 100 times higher. These results suggest that PAs in isolation are not the defense responsible for the mortality in fresh *C. pallida* plants, indicating the importance of other possible defenses. The negative effect of PAs on fitness of the non-adapted, generalist herbivore is in agreement with few previous studies, but it is in clear contrast to a previous study on the effects of PAs on the adapted specialist herbivore *Utetheisa ornatrix* (L.) that were able to sequester PAs with no fitness costs.

## Introduction

Secondary metabolites are one of the most pervasive defense mechanisms against herbivores in plants (Schoonhoven *et al* 2005). Many studies have shown the negative effects of these substances on survival and fecundity of herbivores (Awmack & Leather 2002). However, herbivorous insects have evolved mechanisms to tolerate specific plant defense chemicals. Moreover, in some cases, plant defenses 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 attraction

of mates (Bowers 1992, Nishida 2002, Macel 2011, Trigo 2011). For example, the moth *Utetheisa ornatrix* (L.) (Lepidoptera: Erebidae) is able to sequester specific defensive chemicals, pyrrolizidine alkaloids (PAs), from its host plant and use it for defense and as a male mating pheromone (Eisner & Meinwald 1995). In a series of experiments in which PAs were added to an artificial diet at different concentrations, fitness components of this moth were affected only slightly or not at all by PAs, suggesting that sequestration in this species does not incur in fitness cost (Cogni *et al* 2012). In addition, larvae showed preference for feeding on diets with higher concentrations of PAs in choice experiments (Hoina *et al* 2012). These results indicate that for some specific plant defenses, specialist

herbivores can be positively affected by sequestration without any negative effect or fitness costs.

On the other hand, these plant defensive chemicals may have a negative effect on non-adapted, generalist herbivores; for example, the generalist arctiines *Estigmene acrea* (Drury) and *Grammia geneura* (Strecker) are adapted to PAs, sequestering them (Harmtann *et al* 2004); however, PAs have deterrent and toxic effects on a variety of non-adapted, generalist insect herbivores (van Dam *et al* 1995, Macel *et al* 2005, Narberhaus *et al* 2005). These findings suggest that adapted specialist herbivores can act as agents of natural selection that lower the levels of defensive chemicals in host plant populations, while non-adapted generalists may increase these levels (van der Meijden 1996). For example, introduced populations of *Senecio jacobaea* (Asteraceae) that lacked a specialist herbivore evolved increased levels of PAs and, consequently, increased resistance to generalist herbivores (Joshi & Vrieling 2005). Similarly, generalist damage was negatively correlated to glucosinolate sinigrin concentrations in *Brassica nigra* (Brassicaceae), but specialist damage was positively correlated (Lankau 2007). The increase in sinigrin concentration was favored when specialists were removed, disfavored when generalists were removed, and neutral when both generalists and specialists were present (Lankau 2007).

In this study, we tested the effects of *Crotalaria pallida* (Fabaceae: Papilionoideae) defenses on the generalist, non-specialized model herbivore *Heliothis virescens* (Fabricius) (Lepidoptera: Noctuidae). We used this herbivore as a model generalist to contrast with our previous results on the specialist *U. ornatrix* (Cogni & Futuyma 2009, Cogni *et al* 2012, Hoina *et al* 2012, Franco & Cogni 2013). *Heliothis virescens* is an economically important polyphagous pest that can develop in more than 100 plant species belonging to more than 36 families (Blanco *et al* 2008, Karpinski *et al* 2014). This species was chosen as a model because it can feed on many plant species and can consume leaves and fruits. *Crotalaria pallida* is an annual plant with current pantropical distribution that obscures its native origin (Polhill 1982). The constitutive presence of pyrrolizidine alkaloids is considered the major resistance trait in *Crotalaria* (Wink & Mohamed 2003, Hartmann 1999, Trigo 2011). Here we tested if a model generalist herbivore is able to consume and survive on the chemically protected *C. pallida*, and how the main plant defense, pyrrolizidine alkaloids, affects herbivore survival by asking the following: (1) Does *H. virescens* larva consume *C. pallida*? (2) Is *H. virescens* larva able to complete development on *C. pallida*? (3) How does different pyrrolizidine alkaloid concentrations on the diet affect *H. virescens* survival?

## Material and Methods

### Larval performance on fresh plants

*Heliothis virescens* were purchased from Bio-Serv (Frenchtown, NJ). *Crotalaria pallida* seeds were collected from two populations: Campinas, state of São Paulo, Southeastern Brazil, and Archbold Biological Station in Central Florida, USA (Cogni & Futuyma 2009). The composition of PAs of plants from these two populations was quantitatively and qualitatively similar (Cogni *et al* 2011). The quantitative analysis was performed by colorimetric method and the qualitative analysis by GC/MS (Trigo *et al* 1993, 2003, Cogni *et al* 2011). We grew plants from seeds in the greenhouse at the Life Science Building at Stony Brook University in Stony Brook, NY, under natural sunlight. Plants were watered daily and fertilized every other week as in Cogni *et al* (2011). We fed larvae on fresh leaves for the first 4 days; after that, larvae were fed with unripe seeds. This was done to simulate the wild condition for the most common herbivore of *Crotalaria* plants, *U. ornatrix*. Neonates of *U. ornatrix* first consume leaf material for some days before getting inside the pod to prey on unripe seeds (Ferro *et al* 2006, Eisner 2003). We put neonate larvae individually in 1.5-mL microcentrifuge tubes with a leaf disc for 48 h. Leaf discs were made from fresh leaves and were 1 cm in diameter. After 4 days of feeding on leaves, larvae were transferred to individual petri dishes (5-cm diameter) with a moistened filter paper and green pods. Pods were opened with a razor blade to completely expose seeds to larvae. Every other day, larvae were transferred to a clean dish and new pods were provided. The amount of pod given to each larva was 1/3 pod on days 5 and 7, 1/2 on day 9, 1 on days 11 and 13, and 2 (every other day) after day 13. We checked for larval survivorship on alternate days. All experiments were carried out in an incubator at 29°C and 12:12 photoperiod. We used 117 individual neonate larvae for the Brazilian plants and 98 for the plants from USA.

In addition to the main experiment with *H. virescens* eating on *C. pallida* plants, we carried out two controls concurrently. First, as a control for the ability of our *H. virescens* stock to grow on fresh plant material in the laboratory, we used green beans, a non-toxic host plant. Pods of organic green beans, *Phaseolus vulgaris* (Fabaceae: Papilionoideae), were purchased weekly from a local grocery store and kept refrigerated. The pods of green beans were also provided opened to the larva with the unripe seeds exposed. The same procedures described above for the *C. pallida* plants were



used. The amount of green bean pods provided each day was similar in size to the amount of *C. pallida* pods used on the first experiment. One hundred twenty-eight individual neonate larvae were used. Second, as a control for the suitability of our greenhouse grown *C. pallida* plants for herbivore development, we used the *Crotalaria* specialist herbivore *U. ornatrix*. Adults of *U. ornatrix* were collected at the same locations as the *C. pallida* seeds. *Utetheisa ornatrix* from Brazil was tested with the plants from Brazil; *U. ornatrix* from USA was tested with the plants from USA. The exactly same procedures described above for the *H. virescens* were used for *U. ornatrix*. We used 110 individual neonate larvae for the Brazilian plants and 110 for the plants from USA.

#### Larval performance on artificial diet supplemented with PAs

We measured *H. virescens* larval survival on an artificial diet based on *Phaseolus* beans (Signoretti *et al* 2008) to which we added different concentrations of PAs. PAs were extracted from leaves and flowers of *Senecio brasiliensis* (Asteraceae) as in Trigo *et al* (1993), and after crystallization with acetone, the PA extract was 100% pure (Hoina *et al* 2012). We used *S. brasiliensis* as the PA source because the yield (~4 mg/g) of these alkaloids is higher than in *C. pallida* seeds. The extracted PAs consisted of a mixture of senecionine-type PAs including approximately 4% of senecionine, 69% of integerrimine, and 27% of retrorsine. These are the same category of PAs (senecionine-type) found in unripe seeds of *C. pallida* (usaramine ~85% and integerrimine ~15%) (Ferro *et al* 2006). These PAs were identified and quantified as described earlier for the fresh plant experiment. These PAs just vary at C-12 (senecionine and integerrimine have an OH, and retrorsine and usaramine a CH<sub>2</sub>OH) and C-15 (senecionine and retrorsine are *Z* isomers, and integerrimine and usaramine *E* isomers). Other *Crotalaria* species, such as *Crotalaria incana* and *Crotalaria micans*, with integerrimine as the main PA (Flores *et al* 2009), are also used as host plant by *U. ornatrix* in the Neotropics (Cogni 2010, Sourakov 2015, J. R. Trigo, personal communication). Although structurally related PAs may differ in their effects to different generalist herbivores (Macel *et al* 2005), we have data showing no difference between the mixture of senecionine-type PAs or monocrotaline (a structurally divergent PA) in the performance of the specialist *U. ornatrix* and the generalist *H. virescens* (R Cogni and JR Trigo, personal communication). In *C. pallida*, unripe seeds have an average of 0.24 µg/mg of PAs, and leaves have only

0.054 µg/mg (Ferro *et al* 2006). Five treatments were used (concentrations represent weigh of PAs by weight of dry diet): 0% (0x) PAs added,  $4.8 \times 10^{-3}\%$  (0.2x),  $2.4 \times 10^{-2}\%$  (1x),  $1.2 \times 10^{-1}\%$  (5x), and 2.4% (100x). Sample size for each treatment was, respectively, 147, 154, 164, 162, and 162. All experiments were carried out in an incubator at 29°C and 12:12 photoperiod. Just after hatching, larvae were transferred individually to 2-mL tubes containing 0.6 mL of diet. After 3 weeks, we used a 10-mL tube with 3-mL of diet. Every week, larvae were transferred to a new tube with fresh diet. Mortality was checked every other day. 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, with a procedure analogous to the Dunnett test, using q-distribution (Zar 1999, p. 565).

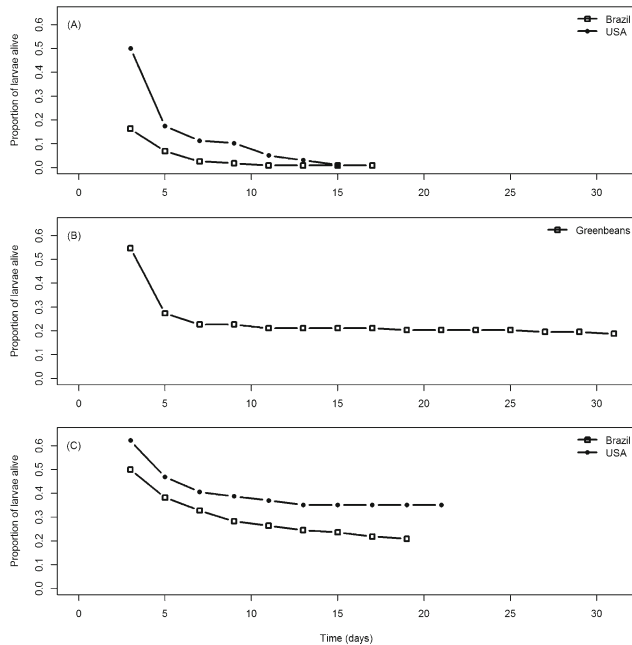
## Results

#### Larval performance on fresh plants

Neonate *H. virescens* larvae were able to consume *C. pallida* leaves and fruits. Many larvae were able to grow when eating *C. pallida* (Fig 1a), but none of the larvae survived to pupation (Fig 1a). *Heliothis virescens* larvae mortality trends were similar for the two *C. pallida* populations (Fig 1a). On the other hand, on the control group in which *H. virescens* was fed on green beans, 19% of the neonate larvae achieved pupation (Fig 1b). The *Crotalaria* specialist herbivore *U. ornatrix* was able to develop on the *C. pallida* plants used in this experiment. Twenty percent of *U. ornatrix* larvae survived to pupation on the *C. pallida* from Brazil and 35% on the *C. pallida* from USA (Fig 1c).

#### Larval performance on artificial diet supplemented with PAs

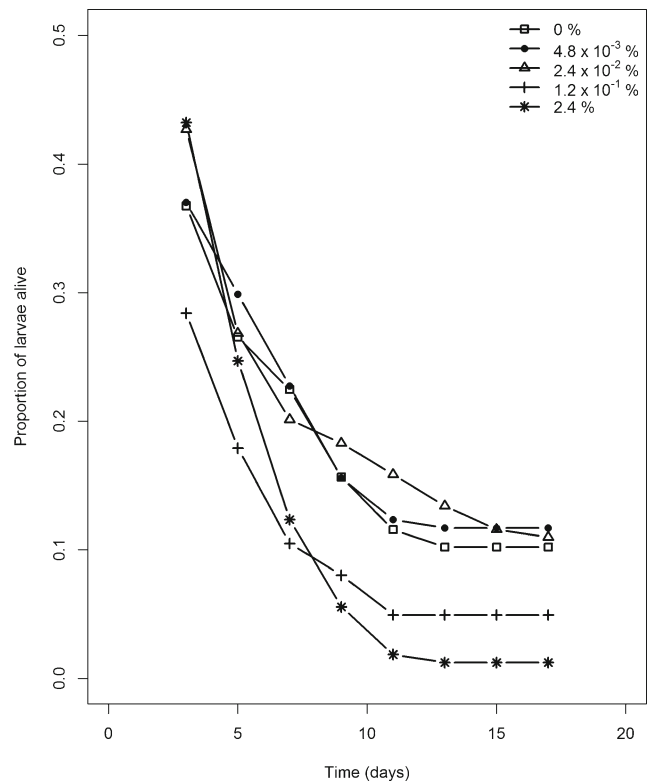
*Heliothis virescens* larvae were able to consume the artificial diet with the PAs added and survive to pupation (Fig 2). Larval survival was affected by PA concentration in the diet (0% PAs = 12.2% survival,  $4.8 \times 10^{-3}\%$  PAs = 12.3%,  $2.4 \times 10^{-2}\%$  PAs = 11.0%,  $1.2 \times 10^{-1}\%$  PAs = 4.9%, 2.4% PAs = 1.2%;  $\chi^2 = 18.42$ ,  $df = 4$ ,  $p < 0.001$ ). Larval survival was not reduced at a PA concentration similar to the concentration ( $2.4 \times 10^{-2}\%$ ) on green seeds of *C. pallida* ( $q = 0.297$ ,  $p > 0.05$ ). Survival was just significantly reduced at PA concentration 100 times higher than the concentration found on green *C. pallida* seeds ( $q = 5.123$ ,  $p < 0.05$ ).



**Fig 1** Proportion of larval survival over time for **a** *Heliiothis virescens* feeding on *Crotalaria pallida* from Brazil and from USA, **b** *Heliiothis virescens* feeding on green beans (*Phaseolus vulgaris*), and **c** *Utetheisa ornatrix* feeding on *Crotalaria pallida* from Brazil and from USA.

## Discussion

We showed a clear negative effect of PAs on fitness of a non-adapted generalist herbivore. This result is in agreement with few other studies showing negative effects of PAs on non-adapted generalists. For example, PAs of *Cynoglossum officinale* (Boraginaceae) have deterrent effects on a variety of generalist herbivores (van Dam *et al* 1995). Macel *et al* (2005) also showed that PAs have negative effects on fitness of a variety of generalist herbivores and that structurally related PAs differed in their effects to different herbivores such as thrips, aphids, and locust. In another example, PAs presented toxic effects on growth and survival of the eri silk moth, *Samia cynthia* (Drury) (Lepidoptera: Saturniidae), another generalist herbivore (Narberhaus *et al* 2005). These results are in clear contrast with our previous study on the effects of PAs on the adapted specialist herbivore *U. ornatrix* that indicates that sequestration of PAs by the specialist (Eisner & Meinwald 1995, Conner & Weller 2004, Conner 2009) does not incur in fitness costs (Cogni *et al* 2012). The lack of costs by the specialist has important implications for evolution of plant–herbivore interactions. It suggests, for example, that selection by specialist herbivores may decrease the levels of certain chemical



**Fig 2** Proportion of larval survival over time for *Heliiothis virescens* feeding on artificial diet with different concentrations of pyrrolizidine alkaloids (PA).

defenses in plant populations. In the present study, we showed that this is not the case for non-adapted herbivores, suggesting that adapted specialists and non-adapted generalists may apply opposing selection on the levels of chemical defenses, potentially maintaining genetic variation in plant populations (van der Meijden 1996, Joshi & Vrieling 2005, Lankau 2007).

Interestingly, in the experiment with controlled diet with isolated PAs, *H. virescens* survival was only negatively affected at PA concentrations 5 and 100 times higher than the concentration in the seeds of *C. pallida*. However, *H. virescens* could not survive when fed with fresh plant seeds. These results suggest that PAs are not the sole mechanism for herbivore defense in fresh plants, indicating that the possible existence of other defensive traits on the seeds may be responsible for the inability of *H. virescens* to complete development on *C. pallida*. Possible candidate defenses include isoflavonoids, non-protein amino acids, and proteinase inhibitors (Pilbeam & Bell 1979, Pilbeam *et al* 1979, Rego *et al* 2002, Wink & Mohamed 2003, Pando *et al* 2004). Another possibility is that these compounds do not affect the herbivore only in isolation but that herbivore's response is affected by interactions among two or more plant metabolites (Steppuhn & Baldwin 2007, Agrawal 2011). Finally, a third hypothesis is that artificial diets are generally rather

nutritious which could counterbalance the negative effects of PAs (see Slansky 1993).

The two main conclusions of this study open a series of questions for future investigations in the system. Our first main conclusion is that a non-adapted generalist herbivore is negatively affected by PAs, contrasting with previous results on the ability of the adapted specialist herbivore to sequester PAs with no fitness costs. In the neotropics, *U. ornatrix* is the main natural enemy of *Crotalaria* plants. In some localities, the pod-borer *Etiella zinckenella* (Treitschke) (Lepidoptera: Pyralidae) can also be found as an important herbivore (Cogni *et al* 2011). By preying on seeds, *U. ornatrix* can have a significant impact on the fitness of *Crotalaria* plants; up to 20% of *C. pallida* fruits in the field may be damaged by *U. ornatrix* (Ferro *et al* 2006, Cogni *et al* 2011, Pereira & Trigo 2013). It would be interesting to compare in the field the frequency of the adapted herbivore with a non-adapted such as the pyralid moth *E. zinckenella*, as well as PA concentrations in different plant populations. Field experiments can also be developed to measure the possible opposing selection that the adapted and the non-adapted herbivores may impose to *C. pallida*. Our second main conclusion is that PAs in isolation may not be the defense responsible for the mortality of the generalist herbivore on fresh *C. pallida* seeds. It would be interesting to test how other adapted and non-adapted generalist herbivores are affected by PAs at the concentration found on the plant. Future research can also try to isolate other possible chemical defenses on seeds of *C. pallida*. This would also be interesting because previous research suggested that patterns of local adaptation of the specialist herbivore *U. ornatrix* to its host plant *C. pallida* are not explained by variation in PAs or nutritional quality of the plants, but by possible variation on an unknown chemical defense (Cogni & Futuyma 2009, Cogni *et al* 2011).

**Acknowledgments** We are grateful to J.R.P.Parra and D. Navas for providing the artificial diet recipe for *U. ornatrix*. M.F. Pereira and A. Hoina helped in the laboratory. We thank two anonymous reviewers for suggestion on the manuscript. Thanks to FERL (Functional Ecology Research and Training Laboratory) for equipment use. We also thank IBAMA and USDA for permits to export and import live organisms. Financial support was provided by NSF (DEB 0807418) to RC, and by FAPESP (11/17708-0) and CNPq (306103/2013-3) to JRT.

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