Coevolution at the population level: empirical studies in an insect-plant interaction

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Abstract of the Dissertation

Coevolution at the population level: empirical studies in an insect-plant interaction

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Coevolution, the reciprocal evolutionary change in interacting species driven by natural selection, has an enormous importance on ecological and evolutionary theory. However, there are very few empirical studies demonstrating coevolution. In particular for plant-herbivore interactions, even though there are many adaptations that seem to be the result of the interaction, just a few empirical evidences on the processes leading to these adaptations exist. I used a highly integrative approach to study coevolution in an ideal plant-herbivore system: the alkaloid bearing legume *Crotalaria pallida* and its seed predator, the arctiid moth *Utetheisa ornatrix*. To unravel coevolutionary dynamics and to demonstrate reciprocal coevolutionary selection a through story of the specific interaction is necessary. In my dissertation I started to develop a through story of the coevolution between *U. ornatrix* and *C. pallida*. First, I investigated how the history of a host introduction may affect coevolutionary dynamics by showing that the native herbivore *U.*

ornatrix has much higher fitness on the introduced host *C. pallida* than on a native congeneric *C. incana*. Second, I used a geographic approach to investigate patterns of local adaptation. I showed local adaptation of the moth to its host plant populations at a continental scale (populations from Brazil and Florida), but not at a local scale (populations ca. 150 Km apart). Third, I investigated how genetic differentiation among plant and moth populations may affect the patterns of local adaptation. I found genetic differences among populations in plant defense traits and moth differentiation on neutral microsatellite loci even at the local scale. Finally, because a thoroughly understanding of coevolutionary dynamics depends on a careful examination of the adaptations related to the interaction for both interacting species, I studied the counteradaptation of the moth to the host-plant defensive alkaloids. I combined chemical ecology techniques with extensive laboratory experiments to show that sequestration of alkaloids has no fitness costs. I discuss how these unexpected and novel results have many important implications to our understanding of coevolution.

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Chapter 1

Introduction

Almost all living species on Earth depend on interactions with other species for survival and reproduction. These interactions are molded by coevolutionary processes. Coevolution is reciprocal evolutionary change between interacting species driven by natural selection (Thompson 2005). The adaptations that have evolved by the interaction among species have enhanced the diversity of life on earth and have had profound effects on the structure of ecological communities (Futuyma 2005). The concept of coevolution encompasses several patterns and processes occurring at different scales of biological organization. In this sense the integration of different disciplines is necessary to empirically unravel coevolutionary dynamics. In my dissertation I used a highly integrative approach to unravel coevolution in an ideal plant-herbivore system.

Ehrlich and Raven, in their seminal paper in 1964, were among the first to describe coevolution. They compiled the host plants used by caterpillars of different butterfly groups, and proposed a very controversial coevolutionary scenario in which plants diversify by increased cladogenesis in enemy-free space while herbivores diversify in competitor-free space. After the popularization of the term by Ehrlich and Raven, the word coevolution has been used in a very broad sense, meaning the adaptation of one species to the characters of other species with which it interacts. During the 1960s and 1970s the term coevolution started to become "*synonymous of anything having to do with interactions between species*" (Thompson 1994). Janzen (1980) and Futuyma and Slatkin (1983) advocated the restricted use of the term for just reciprocal impact of evolution in each of two or more species on the evolution of features of the other. Later, Thompson (1989) classified the several related concepts named coevolution as follows: (1) *pairwise (specific) coevolution* – the adaptive response of two (or a few species) to evolutionary changes in each other. This mode also includes two other concepts: a *gene to gene* correspondence between the traits evolving in the two species and the *coevolutionary arms race* in which there is an escalation of traits in one species and counter response in the other; (2) *diffuse (guild) coevolution* – reciprocal adaptation of a group of ecological similar species to selection imposed by another group of species; (3) *escape-and-radiate coevolution* – the Ehrlich and Raven ideas described above; (4) *cospeciation* – coincident speciation between two interacting species; (5) *parallel cladogenesis* – due to cospeciation phylogenetic relationships of a species will be mirrored in phylogenetic patterns of an interacting species (Futuyma and Keese 1992, Strauss and Irwin 2004). These concepts represent different patterns and processes occurring at different scales, but they all relate to the idea of reciprocity, meaning that the interacting species affect the evolution of each other.

Thompson (1994, 1997, 2005, 2009) proposed a change in the scale of coevolutionary studies. He argued that studies including complete geographical ranges of interacting species would be preferred over studies of local populations or phylogenetic patterns. Thompson introduced the geographic mosaic theory based on three processes acting across geographic space: (1) selection mosaics, the variation between habitats in the trajectories of natural selection; (2) coevolutionary hot spots, the occurrence of reciprocal selection in only some communities; and (3) a constantly changing genetic landscape by gene flow and other evolutionary forces. These processes would lead to three main patterns: (1) interpopulational differences in traits related to the interaction; (2) combinations of traits matching and mismatching among populations; and (3) few species-level coevolved traits. Some empirical data support parts of this theory (reviewed by Thompson 2005).

From now on I will restrict the meaning of coevolution to reciprocal selection among interacting species at the population level (microevolutionary scale). At this scale, evidence for coevolution comes from studies showing herbivore adaptations to overcome plant resistance traits and evidence that herbivores can act as selective agents on plant resistance traits. Rausher (2001) summarizes some of this evidence, which includes, for the herbivores, the cytochrome P-450 detoxifying enzyme of *Papilio polyxenes* against toxic furanocoumarins (Cohen, Schuler & Berenbaum 1992), the modified tRNA synthetase of the bruchid *Caryedes brasiliensis* against the toxic amino acids of the seeds of *Dioclea megacarpa* (Rosenthal *et al*. 1976), the silk scaffolding of the caterpillar *Mechanitis isthmia* against trichomes of their host plant *Solanum* (Rathcke & Poole 1975), the leaf-vein-cutting by monarch butterfly larvae against latex of *Asclepias* (Dussourd & Eisner 1987), the elongated mouthparts of *Jadera* bug species against enlarged fruits of Sapindales plant species (Carroll & Loye 1987), and the shelter construction by the larvae of a gelechiid *Polyhymno* sp. against predaceous ants on *Acacia* trees (Eubanks *et al*. 1997). Most of the evidence that plants also adapt in response to herbivore attack comes from field studies on the patterns of selection on resistance traits in the presence and absence of herbivores (Rausher 2001). These studies have demonstrated that herbivores can impose selection on glucosinolate content and trichome density in *Arabidopsis* (Mauricio & Rausher 1997), alkaloid content in *Datura*

(Schonle & Bergelson 2000), fungal pathogen resistance in *Silene* (Biere & Antonovics 1996, Rausher 1996), and insect resistance and tolerance in *Ipomoea* (Simms & Rausher 1989, Tiffin & Rausher 1999). Most of these studies do not show the two processes, herbivores adapting to the plants and plants adapting to herbivores, occurring on the same system. The best evidence of coevolution comes from studies of the wild parsnip (*Pastinaca sativa*) and the parsnip webworm (*Depressaria pastinacella*) in North America. Studying different populations, Berenbaum and Zangerl (1998) found a remarkable degree of correspondence between parsnip resistance (furanocoumarins content) and the parsnip webworm virulence (ability to metabolize furanocoumarins). In addition a previous study in the system has shown that the parsnip webworm imposes selection on the plant content of furanocoumarins (Berenbaum *et al*. 1986).

I studied coevolutionary dynamics in an ideal plant herbivore system: the rattlebox moth, *Utetheisa ornatrix* (Lepidoptera: Arctiidae), and its main host plant, the rattlebox *Crotalaria pallida* (Fabaceae: Papilionoideae). *U. ornatrix* is a neotropical species specialized on the genus *Crotalaria*. *C. pallida* is a species native to Africa that was introduced in America and now occurs from South Brazil to the southeastern US. There are many native *Crotalaria* species in the New World (Flores, 2004), but *C. pallida* is now the main host of *U. ornatrix* (Eisner 2003, Ferro 2001). *U. ornatrix* sequesters pyrrolizidine alkaloids (PAs) from *Crotalaria* and uses them as defense against predators and as male pheromone (Eisner 2003, Conner 2009). I chose this system because of the important impact that each species has on the fitness of the other (Ferro 2001, Eisner 2003), as well as the wealth of available natural history information. In addition, in several places throughout their distribution both *C. pallida* and *U. ornatrix* occur at high density, and several investigations have already demonstrated the suitability of this system to ecological and evolutionary studies. *C. pallida* can be grown in the greenhouse and seeds are readily available. The moth has a short generation time (larvae develop in few weeks) and can be maintained in the laboratory, readily mating and laying eggs, and developing on an artificial diet suitable for manipulations of pyrrolizidine alkaloids (PAs) levels. In addition, microsatellite markers have been developed for both *C. pallida* and *U. ornatrix* (Bezzerides *et al*. 2004b, Wang *et al*. 2006).

To unravel coevolutionary dynamics and to demonstrate reciprocal coevolutionary selection a thorough history of the specific interaction is necessary. In this dissertation my aim is to start to develop a thorough history of the coevolution between *U. ornatrix* and *C. pallida* using an integrative approach.

First, I investigate how the history of a host introduction may affect coevolutionary dynamics. Because the most common host of *U. ornatrix*, *C. pallida*, is introduced into the New World, I compared the fitness of *U. ornatrix* on a native host (*C. incana*) and on the introduced host (*C. pallida*). In chapter 2, I show that *U. ornatrix* caused much higher seed predation rates on the introduced host than on the native host in the field. In addition, females preferred to oviposit on the introduced over the native host, and larvae feeding on the introduced host had higher fitness (higher pupal weight) than larvae feeding on the native host. I discuss these results in terms of the evolution of this particular interaction, and the implications to plant invasion.

Second, I attempted to unravel coevolutionary dynamics with a geographic approach investigating patterns of local adaptation. In chapter 3, I studied local adaptation of the moth to different populations of its host plant. Local adaptation is an important mechanism in many theories within evolutionary biology. In host-parasite interactions such as plant-herbivore interactions, parasites are expected to exhibit local adaptation (Kawecki & Ebert 2004). However, there is great variation on the outcome of empirical studies on local adaptation (Greischar & Koskella 2007, Hoeksema & Forde 2008). My research has demonstrated local adaptation of the moth to its host plant populations at a continental scale (populations from Brazil and Florida), but not at a local scale (populations ca. 150 km apart). However, my results indicate that both the moth and plant populations were genetically differentiated even at the local scale.

Third, I further investigate how genetic differentiation among plant and moth populations may affect coevolutionary dynamics. In chapter 4, I investigate differences among populations in factors that may be responsible for the patterns of local adaptation at different scales that were reported on chapter 3. I investigate geographical differences in herbivore pressure in the field, genetic differences among populations in plant resistance traits, and population structure and patterns of gene flow of the moth population. I found differences among populations in specialist and generalist herbivore pressure. I also found genetic differences among populations in plant defense traits, and that the presence of pyrrolizidine alkaloids, the main plant defense trait, is not the trait responsible for the local adaptation patterns of this specialist herbivore. Finally, my microsatellite data showed weak but significant differentiation among moth populations in 2005, but no significant differentiation in 2008. These results suggest that temporal changes in *U. ornatrix*'s population structure are the most likely cause for the lack of local adaptation at the regional scale.

Finally, a thorough understanding of coevolutionary dynamics depends on a careful examination of the adaptations related to the interaction for both interacting species. In this way, to complement the studies on plant defense traits, I studied the counteradaptation of the moth to the host-plant defensive alkaloids. *U. ornatrix* is able to sequester the alkaloid from the plant and to use it as a sex pheromone and as protection against predators (Gonzáles *et al.* 1999). Several studies have shown the advantages of sequestration in many herbivorous insects (Nishida 2002). However, there is no evidence if this adaptation incurs fitness costs. In chapter 5, I combined chemical ecology techniques with extensive laboratory experiments to test for possible fitness costs of sequestration behavior. My results show that larvae are able to sequester 100-fold higher alkaloids than normal without any significant negative effects on fitness. This is the first study to clearly show a lack of costs in sequestration of a plant chemical defense by an herbivorous insect. I discuss how these unexpected and novel results have many important implications to our understanding of coevolution.

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Chapter 2

Resistance to plant invasion? A native specialist herbivore shows preference for and higher fitness on an introduced host

ABSTRACT

The response of native herbivores to the introduction of a new plant to the community has important implications for plant invasion. Under the Enemy Release Hypothesis introduced species become invasive because of reduced enemy control in the new range, while under the New Association Hypothesis introduced species lack effective defenses against native enemies because they do not share an evolutionary history. I tested the response of a native South-American specialist herbivore *Utetheisa ornatrix* (Lepidoptera: Arctiidae) to a native (*Crotalaria incana*) and an introduced host (*C. pallida*) (Fabaceae: Papilionoideae). I compared seed predation rates between the two hosts in the field, and I tested preference and performance traits with common garden experiments. *Utetheisa ornatrix* caused much higher seed predation rates on the introduced host than on the native host. Females also preferred to oviposit on the introduced over the native host. Additionally, larvae feeding on the introduced host had higher fitness (higher pupal weight) than larvae feeding on the native host. I discuss how the response of this specialist herbivore to this introduced host plant contradicts the predictions of the Enemy Release Hypothesis and support the New Association

Hypothesis. This study shows that the New Association Hypothesis can also be true for specialist herbivores.

INTRODUCTION

The introduction of non-native species threatens native biodiversity by altering ecosystem structure and function (Pimentel 2002). However, only a small fraction of exotic species have become major pests or have formed monocultures and displaced native species (Rejmánek & Richardson 1996). Therefore, it is crucial to understand what determines the success of introduced species. One of the most accepted hypotheses attributes the difference in success of introduced species to natural enemies, the Enemy Release Hypothesis (Keane & Crawley 2002). These introductions of species to new communities also present a valuable opportunity to understand coevolution. Species coevolve with their natural enemies, and, in plant–herbivore interactions, plants are expected to evolve defense traits and herbivores are expected to evolve virulence traits (*i.e.*, traits that overcome the plant defense trait; Thompson 1982). With the introduction of a new species a mismatch between the plant defense traits and the herbivore virulence traits is likely to occur (Callaway & Maron 2006). In the new community, the native herbivores may not be able to consume the exotic plant or, alternatively, a native plant may not be well defended against the new herbivores.

The response of the native natural enemies to the introduced community member is a key aspect to determine whether they will facilitate or prevent invasiveness. The Enemy Release Hypothesis posits that introduced plant species become invasive because they are less affected by consumers in the new range (Darwin 1859, Elton 1958). Under this hypothesis, the introduced species will be less affected by both specialist and generalist herbivores; specialists on the exotic plant species will not be present in the new range and the native generalists will preferentially consume native plants instead of the exotics (Maron & Vila 2001, Mitchell *et al.* 2006). The release from enemies will contribute to the success of the introduced species. However, an understudied alternative possibility is that introduced plants may be poorly adapted to avoid the native herbivores. Because exotic plants do not share an evolutionary history with the native herbivores, they have not experienced selection from these consumers and may lack effective defenses. This alternative possibility is called the Increased Susceptibility Hypothesis or New Association Hypothesis (Hokkanen & Pimentel 1989, Parker & Hay 2005). Under this scenario, natural enemies will limit the success of introduced species. However, these two hypotheses are not mutually exclusive as they address two ends of the spectrum of introduced species invasiveness.

Responses of a herbivore to an introduced plant depend on features such as behavior (preference for host plants), phenology, and physiological adaptations to features of the plant that affect the herbivore's growth, survival, and reproduction (performance). While some studies have reported correlations between preference and performance (Singer *et al.* 1988), in other cases these traits are independent (Forister 2005). In addition, the response to a new host is likely to differ between generalist and specialist herbivores because plants may employ different defense traits against generalists and specialists (van der Meijden 1996, Joshi & Vrieling 2005).

In this study I investigate the response of a native specialist herbivore to the introduction of an exotic plant to the community. I used the specialist herbivore *Utetheisa ornatrix* L. (Lepidoptera: Arctiidae) feeding on a native (*Crotalaria incana* L.) and an introduced host (*C. pallida* Aiton) (Fabaceae: Papilionoideae). First, I compared damage caused by this specialist herbivore on the introduced and the native host plant in the field. Second, I used a common garden experiment to address the mechanisms responsible for the differences in seed predation rates. I tested how the introduction of the new host affects the herbivore preference and performance. Specifically, the following questions were investigated: (1) Are seed predation rates in the field different between the two hosts? (2) Do ovipositing females discriminate between the two hosts? If so, which host is preferred? (3) How do host species affect larval performance?

METHODS

STUDY SYSTEM.—*Crotalaria* is a pantropical genus of weeds with approximately 600 species (Polhill 1982). Most species are native to Africa and Asia and about 70 species occur in the neotropics. In Brazil, for example, 42 species occur, of which 31 are native and 11 introduced (Flores 2004). Two *Crotalaria* species that are currently widely distributed in the New World and Old World tropics were used in this study: the native (in the New World) *C. incana* and the introduced *C. pallida*. *C. incana* is native to the neotropics, while *C. pallida* is native to Africa and is now considered an invasive weed in the neotropics (Lorenzi 2000, Flores 2004). In the New World both species are distributed from Argentina to the southern United States. Both species are abundant in sandy soils, near rivers, and in human disturbed habitats, especially road sides (Flores 2004, Fonseca *et al.* 2006). There is no clear evidence about when *C. pallida* was introduced in the New World, but it is likely that it was transported from Africa during slavery trade in the sixteenth century (Polhill 1982). The two species contain pyrrolizidine alkaloids with very similar chemical structures and extrafloral nectaries that attract ants that may prey on herbivores (Flores 2004, Guimarães *et al.* 2006). *Utetheisa ornatrix* is a neotropical moth species that specializes on the genus *Crotalaria* (Eisner 2003). Females lay eggs on the plant leaves, and the larvae eat leaves for a few days before entering into the fruit and preying on green seeds (Ferro *et al.* 2006).

FIELD SITE.—Field work was carried out in Campinas (22°54′20″ S, 47°03′39″ W), São Paulo State, southeastern Brazil. The site consists of grassland where three *Crotalaria* species co-occur: the native *C. incana* and two introduced species, *C. pallida* and *C. lanceolata* E.Mey. The moth uses all three species as hosts in this area (Ferro 2001). Adult moths were collected in the site where the three species co-occur. Larvae were observed on the three hosts at the time of the collection. Seeds were collected in October 2005; moths were collected in January and July 2006. I collected seeds from at least 25 different individuals for each species. Thirty-six adult moths were collected in January and an additional 20 adults were collected in July.

HERBIVORE EFFECT ON PLANTS IN THE FIELD.—The use of the native *C. incana* and the introduced *C. pallida* by *U. ornatrix* and the impact that *U. ornatrix* cause on each host were quantified in the field. In December 2008, I collected all the fruit pods of 26 *C. incana* individuals and 28 *C. pallida* individuals that were distributed in an area of *ca* 0.5 km^2. Plants were randomly chosen, were at least 1 m from each other, and had approximately similar sizes. *Crotalaria* fruits stay on the plant for several weeks before autochoric dispersion. I avoided plants that had already started seed dispersion to be able to calculate the entire seed set of individuals and to restrict sample to plants that have been exposed to *U. ornatrix* at approximately the same time. Each fruit pod was classified as attacked by *U. ornatrix* or unattacked. Pods attacked by *U. ornatrix* can be easily identified by the characteristic opening that the larvae make to enter the pod (Pereira 2008). The only other herbivore attacking *C. incana* and *C. pallida* seeds was *Etiella zinckenella* Treit. (Pyralidae). However, this herbivore does not leave the characteristic opening on the pod as *U. ornatrix* (Ferro, 2001) and it occurred at extremely low frequency (seven of 2239 pods). I calculated the proportion of pods attacked by *U. ornatrix* per individual plant as the number of pods attacked divided by the total number of pods. I counted the number of seeds on unattacked pods (up to 10 pods/plant) to calculate the average number of seeds per pod. The total number of seeds per individual plant was estimated by multiplying the number of pods by the average number of seeds per pod. For each damaged pod, I counted the number of seeds that was not attacked. The number of seeds attacked was estimated by subtracting the number of seeds not attacked from the average number of seeds per pod. Finally, the proportion of seed set consumed was calculated by dividing the estimated number of seeds attacked by the estimated total number of seeds per plant. Differences in the proportion of attacked pods and the proportion of seed set consumed were compared between the two hosts by Mann-Whitney tests.

ORGANISM MAINTENANCE.—In May 2006, I placed seeds on Petri dishes with water-soaked filter paper and kept them in an incubator with 24 h light (four 32W fluorescent lamps) at 26°C. After germination and the emergence of leaves, seedlings were transferred to trays (*ca* 650 ml volume) filled with standard potting soil (Sunshine Mix #1 by Sun Gro Horticulture, Canada). After 3 wk, I transferred seedlings to large pots (*ca* 7.6 l). Seedlings and plants were kept at a greenhouse at Stony Brook University, New York, under natural sunlight. Plants were watered daily with the minimum water amount to avoid wilting; standard fertilizer (15N:5P:15K at 300 ppm) was added weekly. These plants produced fruits from September to November, when all the experiments were carried out. Forty-five plants of each species were grown. I kept a large colony of moths (with > 25 adults at any single time) in the laboratory. I fed non-experimental larvae on an artificial diet based on *Phaseolus* beans (Signoretti *et al.* 2008), to avoid maternal and paternal effects, and to avoid selection for the use of host plant available in captivity. I kept adults in paper cages (*ca* 3.2 l) where 5 percent honey solution was provided (as in Cogni & Futuyma 2009). All experiments were carried out in an incubator at 26°C.

PREFERENCE: OVIPOSITION EXPERIMENT.—An experiment was designed to test if females discriminate between a native (*C. incana*) and an exotic (*C. pallida*) host species for egg-laying. A single female and two males were kept in a paper cage (*ca* 3.2 l) for 48

h. All females used were 1–2 wk old and had previous contact with males. In each cage 5 percent honey solution was provided. I placed two leaves (with three leaflets each) from each host species in the cage. The leaves were cut from the plants and the petiole was immediately inserted in a vial with water. The leaves remained fresh during the 48-h period. Leaves from each host were chosen to match in size. I counted the total number of eggs laid on the leaves of each host species after 48 h. The assay was repeated 43 times, each time with different females and different individual plants. The average number of eggs laid on each host species was compared by a paired *t*-test, after log (x + 1) transformation of the data.

PERFORMANCE: LARVAE EXPERIMENT.—Another experiment was designed to test if host plant affects fitness components of *U. ornatrix* larvae, such as survival, development time, and pupal weight. I fed larvae on each host plant from hatching to pupation. One hundred and forty larvae were reared on *C. pallida* and 130 on *C. incana*. Larvae were fed on fresh leaves for the first 4 days; after that, the larvae were fed with green fresh fruit. This was done to simulate condition in the wild, where neonates first consume leaf material for some days before entering the fruit pod to prey on seeds (Ferro 2001). Neonate larvae were put individually in a 1.5-ml microcentrifuge tube with a leaf-disc for 48 h. Leaf-discs were made from fresh leaves and were 1-cm diam. After 4 days of eating leaves, larvae were transferred to individual Petri dishes (5-cm diam) with a moistened filter paper and green fruit. The fruits were opened with a razor blade to completely expose the seeds. On alternative days, I transferred the larvae to a clean dish and provided new fruit. The amount of fruit given to each larva was: 1/3 fruit on days 5 and 7, 1/2 on day 9, 1 on days 11 and 13, and 2 (every other day) after day 13. Each fruit was weighed (to the nearest 0.001g) before and after each 48-h period to calculate the total weight of seeds consumed by individual larva. Fruits lost about 20 percent of weight due to water loss during the 48 h under the conditions used; however, water loss did not differ between the two host species. I recorded larval development time as the number of days each larva took from egg hatching to pupation. I recorded larval survival as the percent of neonate larvae that survived to pupation. Pupal weight was measured 5 days after pupation. Pupal weight is directly related with adult fitness in *U. ornatrix* (see discussion section). I calculated efficiency by dividing pupal weight by the weight of fruits consumed. Growth rate was calculated by dividing pupal weight by development time. The proportion of larvae that survived to pupal stage was compared between the two hosts by a χ^2 test. Larval performance (pupal weight, larval development time, total weight of seeds consumed, efficiency, and growth rate) was compared by two-factor analyses of variance, with host species and sex as factors.

RESULTS

HERBIVORE EFFECT ON PLANTS IN THE FIELD.—The introduced host *C. pallida* suffered much higher levels of herbivore damage caused by *U. ornatrix* than the native *C. incana* in the field (Fig. 1). Seventy-nine percent of *C. pallida* individuals were damaged, while for *C. incana* only 38 percent of individuals were damaged. The percent of pods attacked by *U. ornatrix* was higher for *C. pallida* than for *C. incana* (U = 139, N_1= 28, N_2 = 26, $P < 0.001$; Fig. 1A). The percent of the plant seed set that was consumed by *U. ornatrix* was also higher for *C. pallida* than for *C. incana* (U = 154, N_1= 28, N_2 = 26, $P <$ 0.001; Fig. 1B).

OVIPOSITION PREFERENCE IN THE LABORATORY.—Females preferred to oviposit on the introduced *C. pallida* plants over the native *C. incana* (Fig. 2). The mean number of eggs laid on *C. pallida* was more than double the number of eggs laid on *C. incana* (t = 2.70, df = 42, $P = 0.01$). Twenty-four females laid a higher number of eggs on *C. pallida* and just seven females laid more eggs on *C. incana*.

LARVAE PERFORMANCE IN THE LABORATORY.—The percentage of larvae that survived to pupation on *C. pallida* (15.7%) was not significantly different from *C. incana* (23.8%; $\chi^2 = 2.82$, df = 1, $P = 0.09$). Larvae that fed on the introduced *C. pallida* had higher pupal weight than those that fed on the native *C. incana* (Table 1A; Fig. 3A). Larvae feeding on *C. pallida* took on average one day longer to pupate than larvae feeding on *C. incana* (Table 1B; Fig. 3B). The total weight of fruits consumed by larvae, larval efficiency (pupal weight/weight of fruits consumed) and growth rate (pupal weight/development time) did not differ between the two hosts (Table 1 C–E). Neither sex nor the interaction between sex and host species had a significant effect on any of the response variables (Table 1).

DISCUSSION

Seed predation rates in the field were much higher for the introduced host than the native host. The common garden experiment indicates that this difference in herbivory rate is caused by differences in the herbivore preference and performance. *Utetheisa ornatrix* showed higher larval performance on the introduced host *Crotalaria pallida* than on the native host *C. incana*. Even though survival did not differ between the two hosts, larvae feeding on *C. pallida* achieved higher pupal weight. Pupal weight in this species is a reliable correlate of adult body weight, and adult body weight is directly related to fitness (Iyengar & Eisner 1999). Larger females lay more eggs, and larger males copulate with more females because females prefer larger males (Iyengar & Eisner 1999). The small difference in development time (average of just one day) between the two hosts may not affect overall fitness significantly. Although longer development time may result in higher chance of attack by natural enemies (Schoonhoven *et al.* 1998), this small difference might not be biologically meaningful. *Utetheisa ornatrix* larvae are protected against predators by two mechanisms, mechanically by feeding inside the pod (Ferro *et al.* 2006), and chemically by the sequestration of pyrrolizidine alkaloids (Eisner 2003).

Additionally, parasitoids are not an important mortality factor. Other researchers and I have collected thousands of eggs and larvae in the field to rear at the laboratory and we rarely found parasitoids (R. Cogni and J. R. Trigo pers. comm.). Nonetheless, this difference in development time may be the explanation for larger pupae on the introduced host. Growth rate and efficiency did not differ between the two hosts, but, due to a longer development time, larvae feeding on the introduced host consume slightly more seeds (although not statistically significant), resulting in significantly larger pupae.

Utetheisa ornatrix females also showed preference to oviposit on the introduced host (*C. pallida*) over the native host (*C. incana*). There are two possible explanations for the preference and performance results. It could be the result of rapid evolution (see for example Carrol & Boyd 1992). Alternatively, preference and performance traits may be the result of a 'pre-adaptation' in the sense that the introduced host and the moth possess traits that evolved independent of the interaction, and incidentally those traits led to oviposition preference and higher larval performance when these species first encountered each other. As an example, Thomas *et al.* (1987) showed that all the studied populations of the butterfly *Euphydryas editha* were able to grow and survive in the introduced host *Plantago lanceolata*, but oviposition preference for the new host evolved only in communities where the introduced plant occurs.

Support to the Enemy Release Hypothesis or the New Association Hypothesis depends on the level of invasiveness of the species studied. For the species pair used in this study, the results contradict the predictions of the Enemy Release Hypothesis and support the New Association Hypothesis. It is possible that the introduced host lacks effective defenses against the native herbivore because they do not share an evolutionary history. *Crotalaria pallida* is considered an invasive species in the neotropics (Fonseca *et al.* 2006). It is widely distributed and abundant in several localities; however, this species does not form monocultures and does coexist with native *Crotalaria* species. It is likely that host range expansion of the specialist herbivore *U. ornatrix* makes *C. pallida* less invasive. In the neotropics *Utetheisa ornatrix* is the main natural enemy of *Crotalaria* species and other herbivores or pathogens are rarely found in the field (Ferro 2001, Pereira 2008, Cogni & Futuyma 2009, chapter 3). Future studies can address how the differences in seed predation affect demography of both host species. Such a study is necessary to confirm if the great reduction in seed set caused by *U. ornatrix* makes *C. pallida* less invasive.

The Enemy Release Hypothesis has been challenged recently. For example, a recent study examining a large variety of plant taxa and diverse enemies reported a limited potential role for consistent enemy release in the success of introduced species (Agrawal *et al.* 2005). In addition, recent studies have reported that exotic plants are more palatable than native plants to generalist herbivores, and that introduced plant species are especially susceptible to novel native generalist herbivores that they have not been selected to resist (Agrawal & Kotanen 2003, Parker & Hay 2005, Parker *et al.* 2006). My study shows that the New Association Hypothesis may also be true for a specialist herbivore. A host-range expansion of native specialist herbivores may be common when the introduced host has close relatives with similar chemical defenses in the native flora. A few other studies in natural environments have reported preference of specialist herbivores to introduced hosts (Thomas *et al.* 1987; Solarz & Newman 1996, 2001; Trowbridge & Todd 2001; Trowbridge 2004). Furthermore, in a meta-analysis study Strauss *et al.* (2006) found that introduced plants that were less phylogenetic related to community members were more invasive. The main mechanism responsible for such a pattern may be host shifts of native specialist herbivores and the lack of effective resistance traits on the introduced host, as reported here. My study shows the importance of considering host-expansion of native specialist herbivores as a resistance mechanism to plant invasion.

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TABLE 1. Effect of host plant and moth sex on (A) pupal weight, (B) larval development time, (C) weight of seeds consumed, (D) larval efficiency, and (E) larval growth rate. *Utetheisa ornatrix* larvae were fed with fruits of *Crotalaria pallida* and *C. incana* in the laboratory. * indicates significant effects

Source	df	*F*-ratio	*P*
(A) Pupal weight			
Host plant*	1	7.29	0.009
Sex	1	0.02	0.88
Host plant × Sex	1	0.84	0.36
Error	49		
(B) Development time			
Host plant*	1	10.13	0.003
Sex	1	0.32	0.57
Host plant × Sex	1	0.86	0.36
Error	49		
(C) Weight of seeds consumed			
Host plant	1	0.68	0.41
Sex	1	0.00	0.96
Host plant × Sex	1	0.62	0.43
Error	49		
(D) Larval efficiency			
Host plant	1	0.02	0.89
Sex	1	0.02	0.88
Host plant × Sex	1	0.10	0.75
Error	49		
(E) Larval growth rate			
Host plant	1	1.42	0.24
Sex	1	0.03	0.87
Host plant × Sex	1	0.09	0.76
Error	49		

FIGURE 1. Herbivore damage caused by *Utetheisa ornatrix* on the native host *Crotalaria incana* and the introduced host *C. pallida* in the field. (A) Proportion of pods attacked per plant. (B) Proportion of the seed set consumed by larvae per plant. Median, 10th, 25th, 75th and 90th percentiles. $P < 0.05$ = difference significant in Mann-Whitney U test. $N = 26$ for *C. incana* and 28 for *C. pallida*.

FIGURE 2. Mean (+SE) number of eggs laid by *Utetheisa ornatrix* on leaves of the native host *Crotalaria incana* and the introduced host *Crotalaria pallida* in laboratory choice tests. $P < 0.05$ = difference significant in paired *t*-test. $N = 43$.

FIGURE 3. Differences in *Utetheisa ornatrix* larval performance on the native host *Crotalaria incana* and the introduced host *Crotalaria pallida*. Larvae were reared on leaves for the first 4 days and on green fruits from day 5 until pupation. (A) Pupal weight. (B) Development time. $P < 0.05$ = difference significant in ANOVA tests. $N = 31$ for *C. incana* and 22 for *C. pallida*.

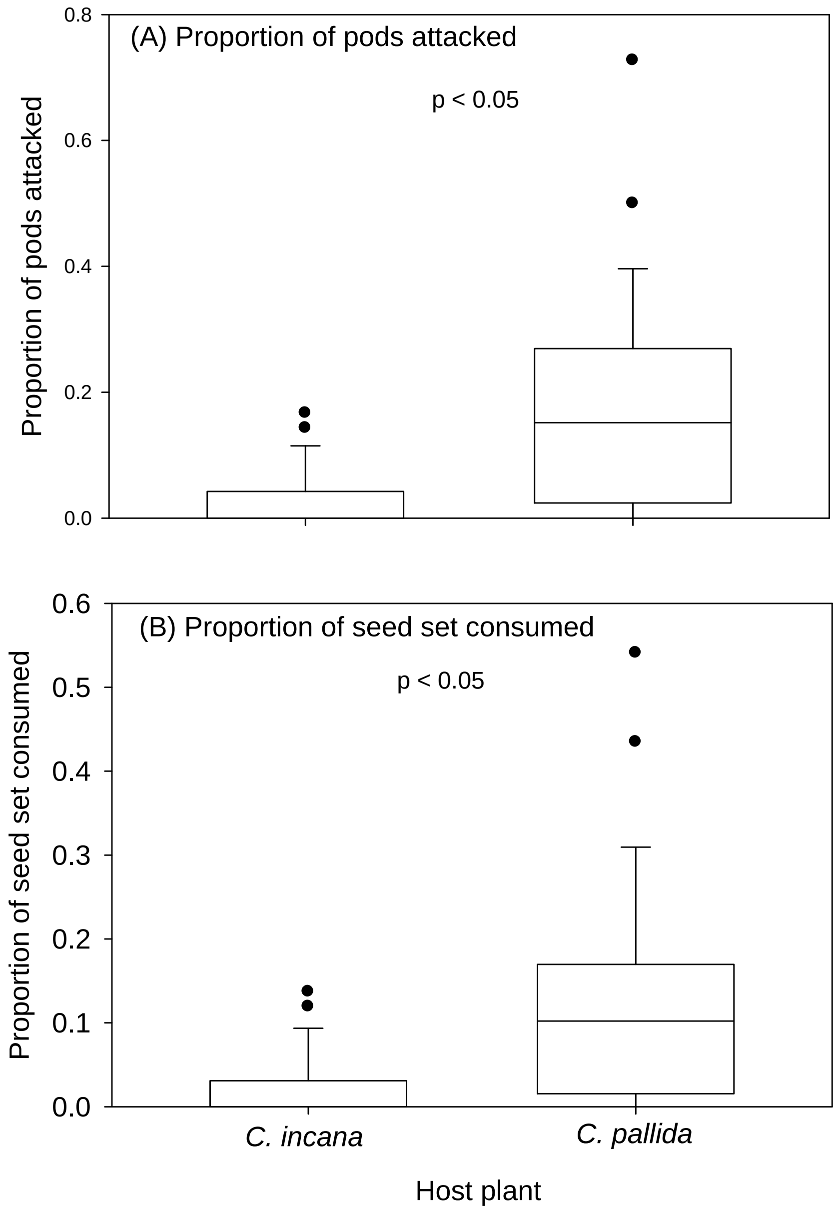
(A) Proportion of pods attacked
p < 0.05
Proportion of pods attacked
0.0
0.2
0.4
0.6
0.8
(B) Proportion of seed set consumed
p < 0.05
Proportion of seed set consumed
0.0
0.1
0.2
0.3
0.4
0.5
0.6
C. incana
C. pallida
Host plant

Figure 1

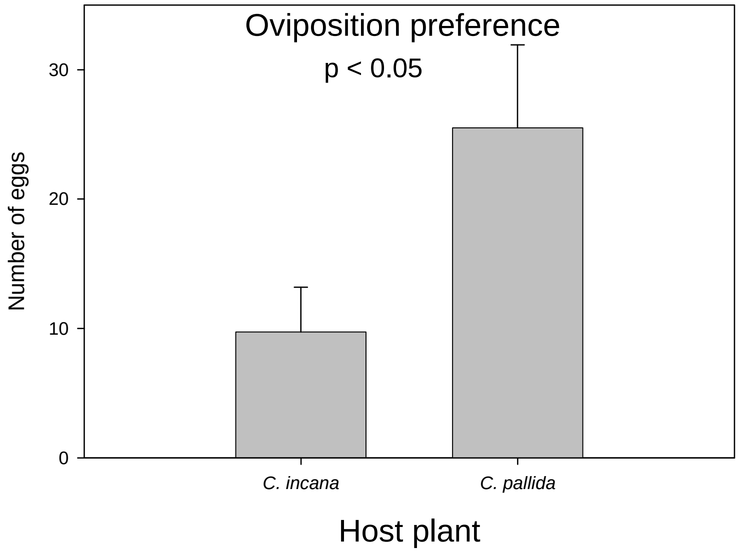
Oviposition preference
p < 0.05
Number of eggs
0
10
20
30
C. incana
C. pallida
Host plant

Figure 2

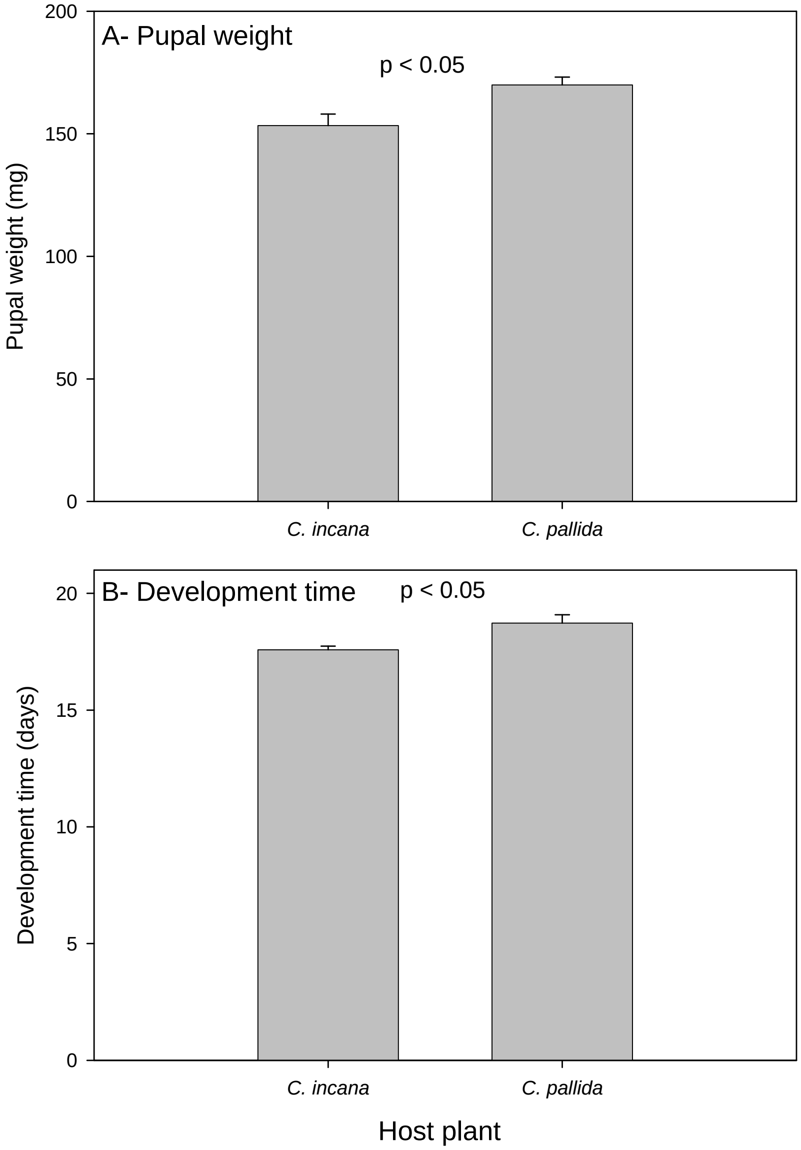
A- Pupal weight
p < 0.05
Pupal weight (mg)
0
50
100
150
200
C. incana
C. pallida
B- Development time
p < 0.05
Development time (days)
0
5
10
15
20
C. incana
C. pallida
Host plant

Figure 3

Chapter 3

Local adaptation in a plant herbivore interaction depends on the spatial scale

INTRODUCTION

Coevolution, the reciprocal evolutionary change in interacting species driven by natural selection, is a central theme in studies of interactions among species (Thompson 2005). The adaptations that have evolved by the interactions among species have enhanced the diversity of life and have affected the structure of ecological communities (Futuyma & Slatkin 1983). Studies of coevolutionary dynamics can benefit from spatial variation in the interactions (Thompson 2005). In antagonistic interactions, the reciprocal selection imposed by coevolving species leads to a constant shift in the adaptive peaks (Peters & Lively, 2007), and this can result in local adaptation (Kawecki & Ebert 2004). In parasite-host interactions, such as herbivorous insects eating plants, the parasites are expected to exhibit local adaptation owing to their larger population sizes, shorter generation times and higher mutation rates (Ebert 1994, Lively 1999, Dybdahl & Storfer 2003). Local adaptation is revealed by a higher mean fitness of parasites on local vs. foreign hosts or by a higher mean fitness of local parasites than foreign parasites on local hosts (Williams 1966, Kawecki & Ebert 2004). This dynamic nature of local adaptation among coevolving species is an important mechanism in many theories in evolutionary biology, including explanations for the maintenance of genetic variation, maintenance of sexual reproduction and the processes of parapatric and sympatric speciation (reviewed by Kawecki & Ebert 2004).

Empirical studies of local adaptation in host-parasite interactions show highly variable outcomes. Although many studies detected parasite local adaptation, others failed to detect local adaptation, or even found parasites to be locally maladapted (Greischar & Koskella 2007, Hoeksema & Forde 2008). This variation is expected because local adaptation can be influenced by several factors. First, migration rates of both the host and the parasite can influence local adaptation. Theoretical models show that if the migration rate of parasite is higher than that of the host, the parasite will be locally adapted; but if the host has higher migration, the host will be locally adapted (Gandon 2002). Indeed, this prediction was confirmed by two recent meta-analyses of the empirical studies (Greischar & Koskella 2007, Hoeksema & Forde 2008). This seems counterintuitive because gene flow can swamp local adaptation. However, factors that increase the evolutionary potential of the parasite increase its ability to track local host allele frequencies (reviewed by Dybdahl & Storfer 2003).

Local adaptation can also vary across spatial scales. Many negative results on local adaptation studies may be the consequence of coevolutionary process occurring at either larger or smaller spatial scales (Thrall *et al.* 2002). Assuming that physical distance corresponds with genetic differences among populations, the distance among populations is predicted to be negatively correlated with adaptation (Gandon *et al.* 1996). Therefore, parasites are predicted to be more adapted to hosts from nearby populations than to those that are further away (Kaltz & Shykoff 2002). A few empirical studies confirm the prediction that local adaptation is more likely to be detected at a larger than a smaller scale (Hanks & Denno 1994, Burdon & Thompson 1995, Thrall *et al.* 2002, Stenberg *et al.* 2006, Stenberg & Axelsson 2008). However, very few studies of local adaptation investigate populations at different scales. Most studies of local adaptation just include populations less than 1000 kilometers apart (Hoeksema & Forde 2008), and there is no investigation on local adaptation at very large scales, such as continental comparisons with populations several thousand kilometers apart.

In this study, we investigated local adaptation in the interaction between the alkaloid-bearing legume *Crotalaria pallida* (Fabaceae: Papilionoideae) and its seed predator, the arctiid moth *Utetheisa ornatrix* (Lepidoptera: Arctiidae). We did a common-garden experiment with reciprocal combinations of host and herbivore populations at a regional and a continental scale, and demonstrated that local adaptation depends on the geographical scale.

METHODS

Study system

Crotalaria is a pantropical genus of weeds with *ca*. 600 species (Polhill 1982). *Crotalaria pallida* is a species native from Africa that now occurs at high densities from southern Brazil to southeast United States. There is no clear evidence about the New World introduction; possibly it was transported from Africa during slavery trade in the sixteenth century (Polhill 1982). *Crotalaria pallida* constitutively produces pyrrolizidine alkaloids (PAs) (Wink & Mohamed 2003). PAs are assumed to serve as protective chemicals against herbivores (Hartmann 2004), and the toxic effect of PAs on a generalist herbivore has recently been shown (Narberhaus *et al.* 2005). *Crotalaria pallida* also has extrafloral nectaries that attract ants, which positively affect plant fitness by decreasing herbivore activity (Heil & McKey 2003, Ferro *et al.* 2006, Guimarães *et al.* 2006).

Utetheisa ornatrix is a neotropical species that specializes on the genus *Crotalaria*. It originally fed on native *Crotalaria* species, but now *C. pallida* is its main host in several locations (Ferro 2001, Eisner 2003). In all the areas included in this study *Utetheisa ornatrix* just occasionally uses the other *Crotalaria* species that occur at low densities. *Utetheisa ornatrix* sequester PAs from the host plant. The PAs not only protect larvae and adults, but they are also transmitted from the female (and from males through nuptial gift) to eggs (Eisner 2003). Males also modify the PAs into a courtship pheromone (Dussourd *et al.* 1991, Iyengar & Eisner 1999a,b). *Utetheisa ornatrix* can have a significant impact on the fitness of *Crotalaria* plants. Even though the larvae are mobile and can walk about the plant and feed on leaves, most of the larvae are found inside the fruit preying on the seeds (Ferro *et al.* 2006). Up to 20% of *C. pallida* fruits in the field may be damaged by *U. ornatrix* (Ferro 2001).

Populations studied and organism maintenance

In May 2005, we collected *C. pallida* seeds and moths from three populations in São Paulo State, Southeast Brazil: Campinas (22°47’S; 47°04’W; 680m a.s.l.); Juquiá (24°19’S; 47°38’W; 25m a.s.l.); and the district of Vitoriana in Botucatu (22°47’S; 48°24’W, 578 a.s.l.). These populations will be referred as CA, JU and BO. JU is 200 Km from BO, and 186 Km from CA. CA is 160 Km from BO. In April 2006, we collected from another population at Archbold Biological Station (27°15’N; 81°21’S; 30m a.s.l.), in central Florida, US. For each population we collected seeds from at least 30 individuals and a minimum of 40 adult moths.

We kept a large colony of moths from each population (with more than 25 adults at any single time) in the laboratory. We fed non-experimental larvae on an artificial diet based on *Phaseolus* beans (Signoretti *et al.* 2008) to avoid maternal and paternal effects, and to avoid selection for the use of host plant population available in captivity. We kept adults in paper cages (*ca.* 3.2 liters) where 5% honey solution was provided.

We carried out two sets of experiments: in 2005 we studied the three populations from Brazil and in 2006 we studied one population from Brazil (CA) and the population from Florida. We grew plants from seeds in a common environment. Seeds were germinated in an incubator at 26°C and 24 hours of light. Seedlings were transferred to trays (*ca.* 650 ml volume) filled with standard potting soil. After 3 weeks, we transferred seedlings to large pots (*ca.* 7.6 liters). Seedlings and plants were kept at a greenhouse under natural sunlight. Plants were watered daily and standard fertilizer (15N:5P:15K at 300 ppm) was added weekly. In the 2005 experiment we used the greenhouse of the Instituto de Biologia at Universidade Estadual de Campinas in São Paulo State, Brazil without temperature control. In the 2006 experiment we used the greenhouse at the Life Science Building at Stony Brook University in Stony Brook in New York State with approximate temperature of 28°C (day) and 24°C (night). Even if it is very likely that the conditions of the greenhouses in the two different countries differed, such differences did not affect our results. When we compared the same treatment carried out in 2005 and 2006 (moths from CA eating plants from CA), there was no significant effect of the greenhouse (2005-Brazil *vs*. 2006-Stony Brook) on any of the response variables (Pupal weight: F = 2.77, d.f. = 1, p = 0.10; Development time: F = 2.25, d.f. = 1, p = 0.14; Total number of seeds consumed: F = 2.98, d.f. = 1, p = 0.09; Weight of seeds consumed: F = 3.4, d.f. = 1, p = 0.07).

Experiment details

In 2005 we started the plants in May and carried out the experiments from September to December. In 2006 we started the plants in April and carried out the experiments from August to November. We carried out all experiments on an incubator at 26^{o}C. We fed larvae from each population from hatching to pupation on each host population. In 2005 we studied three populations from Brazil (BO, CA, JU), so there were 9 treatments (each of the three moth populations feeding on each of the three plant populations). In 2006 we studied one population from Brazil (CA) and one population from Florida, so there were four treatments. Sample sizes for each treatment are given on Tables 1 and 3. We fed larvae on fresh leaves for the first 4 days and on green fresh fruits after that. This simulates wild condition, where neonates consume leaf material before getting inside the fruit (Ferro 2001, Eisner 2003). We put neonate larvae individually in a 1.5ml microcentrifuge tube with a leaf-disc. We provided a new leaf-disc after 48 hours. Leaf-discs were made from fresh leaves and were 1 cm in diameter. After 4 days of eating leaves, the larva was transferred to individual Petri dishes (5cm diameter) with a moistened filter paper and green fruit. We opened fruits with a razor blade to completely expose the seeds. Every other day we transferred the larva to a clean dish and provided new fruit. The amount of fruit given to each larva was: 1/3 fruit 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 weighed each fruit (to the nearest 0.001g) before and after each 48-hour period. Fruits lost about 20% of weight due to water loss during the 48 hours; however, water loss did not differ among the host populations. We also counted the number of seeds on each fruit given to each larva and the number of seeds not eaten during each two days period to calculate the total number of seeds consumed per larva. We recorded larval development time as the number of days each larva took from egg hatching to pupation. We recorded larval survival as the percent of neonate larvae that survive to pupation. We measured pupal weight 5 days after pupation. Pupal weight correlates with adult weight and adult fitness in *U. ornatrix* (Iyengar & Eisner 1999b); larger females lay more eggs and large males attract more females to mate.

Statistical analyses

We tested the effect of moth population, plant population and the interaction of plant and moth population on larval survival with a nominal logistic model (Hosmer & Lemeshow 1989). We tested the effect of plant population, moth population, sex and all possible interactions on each response variable (pupal weight, larval development time, total number of seeds consumed per larva and total weight of seeds consumed per larva) with ANOVA tests. We define local adaptation as a significant interaction between the herbivore source population and the host source population, in which individual herbivores on average exhibit higher fitness on its native host population than on hosts from allopatric populations.

RESULTS

Three populations from Brazil (2005)

No evidence of local adaptation was detected in this experiment (no significant interaction between plant population and moth population). Moths from JU showed lower survival than the moths from the other two populations, and survival was not affected by plant population or the interaction between plant population and moth population (Table 1; Effect Likelihood Ratio Tests in a Nominal Logistic Model: moth population $\chi^2 = 21.54$, d.f. = 2, $p < 0.0001$; plant population $\chi^2 = 1.32$, d.f. = 2, $p = 0.51$; plant population*moth population $\chi^2 = 3.23$, d.f. = 4, $p = 0.52$). Moths from JU also had lower pupal weight and longer development time than the other two moth populations (Table 2A). The weight of seeds consumed was slightly higher for larvae from CA (Table 2A). Pupal weight was higher for males than for females (Table 2A). Interactions between plant population and moth sex on herbivore performance indicate that plant quality varies among populations. Pupal weight, development time and the number of seeds consumed were significantly affected by the interaction of plant population and sex (Table 2A; Figure 1), i.e. the sexes showed different responses to differences in plant population. Although males showed higher pupal weight on plants from CA, females had the lowest pupal weight on this plant population (Figure 1A). Females took longer to develop and ate more seeds when feeding on the JU plants, while males had the lowest development time and lowest seed consumption on this plant population (Figure 1B-C).

Brazil vs. Florida (2006)

Local adaptation of *U. ornatrix* to its host plant was detected in the experiment with populations from Brazil and Florida; pupae from each population were heavier when reared on the sympatric than the allopatric host population (Table 2B, Figure 2). Pupal weight was higher for males than for females (Table 2B). Moths from Florida showed lower survival than moths from Brazil, and survival was not affected by plant population or the interaction between plant population and moth population (Table 3, Effect Likelihood Ratio Tests in a Nominal Logistic Model: moth population $\chi^2 = 17.34$, d.f. = 1, $p < 0.0001$; plant population $\chi^2 = 0.24$, d.f. = 1, $p = 0.62$; plant population*moth population $\chi^2 = 0.0002$, d.f. = 1, $p = 0.99$). Development time and the weight of seeds consumed did not significantly vary among the treatments (Table 2B). A higher number of seeds were consumed for the Brazilian plants than from the plants from Florida (Table 2B).

DISCUSSION

We did not find local adaptation at a regional scale, but we could detect both moth and plant population differences at this scale. First, one moth population showed consistently lower performance than the other two. Second, we find significant interactions between herbivore sex and plant population on several performance traits of the herbivore. These interactions indicate that the plant populations are not uniform at this scale. Some previous studies have reported differential responses of male and females to host quality (Tikkanen *et al.* 2000, Jormalainen *et al.* 2001, Asshoff & Hättenschwiler 2005), and these differences have been attributed to sexual selection and differences in optimal reproductive strategies. Assuming sequestration is costly at high levels (Bowers 1992), *U. ornatrix* males and females are likely under stabilizing selection to acquire enough PAs for protection, but males may also be under runaway sexual selection to sequester higher amounts of PAs for courtship (Iyengar & Eisner 1999a). Future studies will explore this hypothesis by investigating differences in PA content among plant populations, and possible differences in the ability of the sexes to sequester PAs.

Even though we found both moth and plant population differences at a regional scale, we were unable to detect local adaptation at this level. In other plant herbivore systems, empirical studies have found herbivore local adaptation, herbivore local maladaptation or evidence for neither local adaptation or maladaptation (Karban 1989, Zangerl & Berenbaum 1990, Strauss 1997, Roy 1998). For our system, why is local adaptation expected even at a regional scale and why we did not detect it? First, we used the best design for detection of local adaptation: a common-garden with reciprocal design and large sample sizes (Thrall *et al.* 2002, Blanford *et al.* 2003, Kawecki & Ebert 2004, Laine 2007). We chose a common-garden design because it tests the spatial covariance between the genotype frequencies of the interacting species. This reflects how parasites track their host over evolutionary time - Red Queen coevolution (Kawecki & Ebert 2004, Nuismer & Gandon 2008). However, common-garden designs do not test for local adaption of the host and the parasites to their sympatric ecological environment, such as abiotic conditions and interactions with other species (Ridenhour & Nuismer 2007, Nuismer & Gandon 2008). This may be important when adaptations to the host plant quality are also mediated by adaptation to other ecological variables (Laine 2008).

Second, local adaptation depends on the strength of selection due to the interaction (Nuismer *et al.* 2000), for instance the best example of local adaptation in a plant-insect interaction is the parsnip webworm that imposes strong selection on the wild parsnip by feeding exclusively on the host's reproductive structures (Berenbaum *et al.* 1986, Berenbaum & Zangerl 1998). In our system the reciprocal selection due to the interaction is also believed to be strong. The moth depends on the plant for food and PAs (Eisner 2003), and *U. ornatrix* feed mostly on the fruits, greatly decreasing the plant seed set (Ferro 2001). Third, the level of specificity on the interaction can also influence local adaptation (Gandon 2002). In the interaction between the parsnip and the parsnip webworm there are relatively few other herbivores and a rare alternative host plant (Zangerl & Berenbaum 2003). In our system there is also a high level of specificity. *U. ornatrix* is the main herbivore on *C. pallida* plants and *C. pallida* is the main *U. ornatrix*'s host throughout the neotropics (Ferro 2001, Eisner 2003).

Fourth, demography can influence the patterns of local adaptation. High levels of migration can prevent local adaptation (Gandon *et al.* 1996), as occurs in the pinyon needle scale (Cobe & Whitham 1998). In our system both species are spatially differentiated at the regional scale. Theoretical models also predict that in the arms-race between the host and the parasite, the one with higher migration rate is expected to be locally adapted (Gandon 2002). *Crotalaria pallida* is an annual plant with limited dispersal; this species is partially selfing and bee pollinated, and lacks any mechanism for long-distance seed dispersal. Short distance differentiation can evolve in a few generations for plants with such traits (Heywood 1991, Linhart & Grant 1996). On the other hand, flying insect herbivores have higher dispersal abilities; therefore we expect local adaptation by the herbivore in our system. We are currently investigating genetic variation and population structure of *C. pallida* and *U. ornatrix* with molecular markers. Additionally, future studies can address if local adaptation is influenced by demographic stochasticity such as genetic drift, founder effects and meta-population extinction, and by temporal variability in natural selection, and if it is constrained by the lack of genetic variation, and the genetic architecture of traits relevant to the interaction (Kawecki & Ebert 2004).

Our study shows that local adaptation depends on the geographical scale; while we did not find local adaptation at a regional scale, we found evidence of local adaptation at a continental scale. We expect the host plant to be more differentiated at a continental scale. For example, several salt marsh plants have evolved latitudinal differences in palatability to herbivores along North America East coast (Salgado & Pennings 2005). Another possible explanation for the scale dependence of our results might be that when individual parasite populations are ephemeral, local adaptation may only be found at larger geographical scales (Thrall & Burdon 1997, Burdon & Thrall 2000). Indeed, *C. pallida* has a patchy distribution and occurs in habitats where fire and other human disturbances that can cause local moth extinction are common.

A few other studies report similar results in which local adaptation was detected just at larger scales (Ebert 1994, Hanks & Denno 1994, Burdon & Thompson 1995, Thrall *et al.* 2002, Stenberg *et al.* 2006, Stenberg & Axelsson 2008, but see Imhoof & Schmid-Hempel 1998 for a counter example). However, Lively (1989) found local adaptation even between nearby populations and Laine (2005) found local adaptation to sympatric host populations, and that local adaptation may extend to the scale of the sympatric host metapopulation. Our results also show the importance of studying coevolution and local adaptation at different geographical scales, otherwise coevolutionary dynamics occurring at either larger or smaller scales can be missed.

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Tables

Table 1. Percent survival of *Utetheisa ornatrix* larvae eating *Crotalaria pallida* in a laboratory experiment. Sample sizes (number of neonate larvae) are giving in parenthesis. Moth and plant populations are from three localities in São Paulo State, SE Brazil. * moths from JU showed a significant lower survival on a nominal logistic model.

		Plants from		
		BO	CA	JU
Moths from	BO	*0.24 (128)*	0.24 (133)	0.20 (133)
	CA	0.21 (131)	*0.29 (139)*	0.30 (129)
	JU*	0.12 (108)	0.16 (134)	*0.16 (140)*

Table 2. Effect of plant source population, moth source population, sex, and all possible interactions on pupal weight, larval development time, total number of seeds consumed, and weight of seeds consumed. *Utetheisa ornatrix* larvae were fed with fruits of *Crotalaria pallida* in the laboratory. (A) Moth and plant populations are from three localities in Sao Paulo State, SE Brazil. (B) Moth and plant populations are from Campinas in Sao Paulo State in SE Brazil and Central Florida in the USA.

	(A) Three population from Brazil (2005)			(B) Brazil *vs.* Florida (2006)		
Source	**df**	**F-ratio**	**P**	**df**	**F-ratio**	**P**
Pupal weight						
Plant pop	2	0.88	0.415	1	0.14	0.709
Moth pop	2	14.82	0.000*	1	0.29	0.589
Sex	1	18.26	0.000*	1	9.26	0.003*
Plant pop*Moth pop	4	0.10	0.984	1	11.51	0.001*
Plant pop*Sex	2	6.47	0.002*	1	0.70	0.402
Moth pop*Sex	2	0.45	0.634	1	0.20	0.651
Plant pop*Moth Pop*Sex	4	2.96	0.020*	1	0.34	0.561
Error	252			163		
Development time						
Plant pop	2	0.21	0.808	1	0.01	0.938
Moth pop	2	3.12	0.046*	1	1.43	0.233
Sex	1	2.73	0.100	1	0.40	0.525
Plant pop*Moth pop	4	1.13	0.341	1	2.32	0.130
Plant pop*Sex	2	4.41	0.013*	1	1.39	0.241
Moth pop*Sex	2	0.15	0.862	1	0.46	0.501
Plant pop*Moth Pop*Sex	4	1.51	0.201	1	2.44	0.120
Error	252			163		
Number of seeds consumed						
Plant pop	2	1.57	0.211	1	43.67	0.000*
Moth pop	2	1.71	0.184	1	0.001	0.980
Sex	1	1.20	0.274	1	1.11	0.294
Plant pop*Moth pop	4	1.54	0.190	1	0.004	0.949
Plant pop*Sex	2	3.54	0.031*	1	0.25	0.873
Moth pop*Sex	2	0.33	0.718	1	0.22	0.636

Plant pop*Moth Pop*Sex	4	1.87	0.116	1	0.57	0.450
Error	252			163		
Weight of seeds consumed						
Plant pop	2	0.02	0.984	1	0.65	0.422
Moth pop	2	4.34	0.014*	1	0.29	0.593
Sex	1	0.75	0.389	1	0.13	0.719
Plant pop*Moth pop	4	1.22	0.303	1	0.089	0.766
Plant pop*Sex	2	2.53	0.082	1	0.142	0.706
Moth pop*Sex	2	1.05	0.351	1	0.052	0.821
Plant pop*Moth Pop*Sex	4	0.48	0.747	1	0.821	0.366
Error	252			163		

Table 3. Percent survival of *Utetheisa ornatrix* larvae eating *Crotalaria pallida* in a laboratory experiment. Sample sizes (number of neonate larvae) are giving in parenthesis. Moth and plant populations are from Campinas (CA) in São Paulo State in SE Brazil and Central Florida (FL) in the US. * moths from Florida showed a significant lower survival on a nominal logistic model.

		Plants from	
		CA	FL
Moths from	CA	*0.33 (160)*	0.34 (179)
	FL*	0.18 (164)	*0.18 (162)*

Figure legends

Figure 1. Interaction between sex and plant population on (A) pupal weight (B) development time and (C) total number of seeds consumed for larvae of *Utetheisa ornatrix* feeding on *Crotalaria pallida* fruits in a laboratory experiment. Moth and plant populations are from three localities in São Paulo State, SE Brazil: BO = Botucatu, CA = Campinas and JU = Juquiá. Average ± SE.

Figure 2. Pupal weight of *Utetheisa ornatrix* feeding on *Crotalaria pallida* fruits in a laboratory experiment. Moth and plant populations are from Campinas (CA) in São Paulo State in SE Brazil and Central Florida (FL) in the US. Average ± SE.

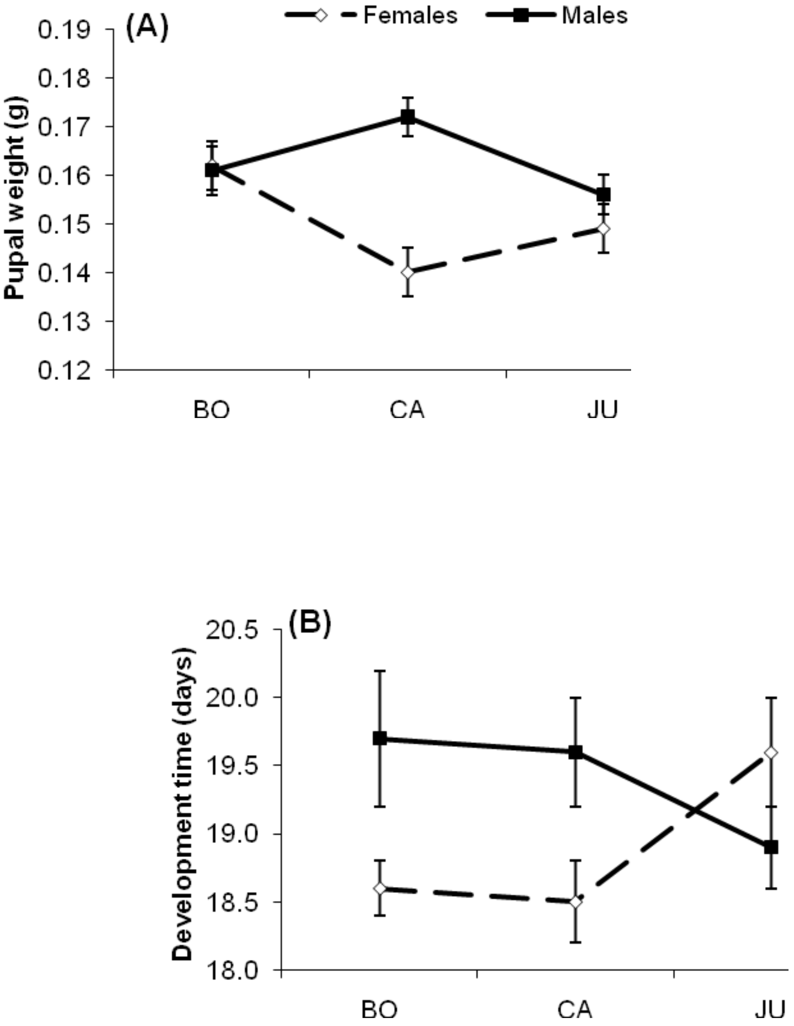
(A)
Females
Males
Pupal weight (g)
0.19
0.18
0.17
0.16
0.15
0.14
0.13
0.12
BO
CA
JU
(B)
Development time (days)
20.5
20.0
19.5
19.0
18.5
18.0
BO
CA
JU

Figure 1 A B

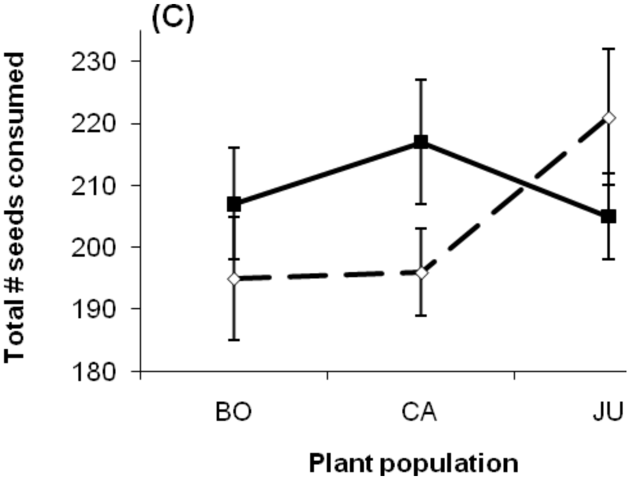
(C)
Total # seeds consumed
230
220
210
200
190
180
BO
CA
JU
Plant population

Figure 1C

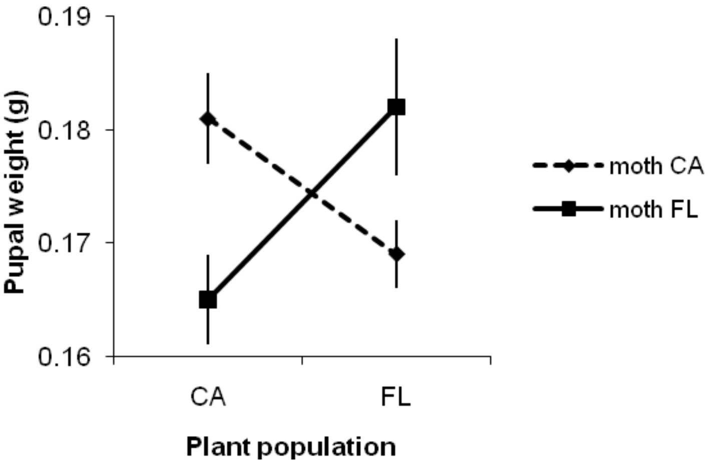
0.19
0.18
0.17
0.16
Pupal weight (g)
CA
FL
Plant population
moth CA
moth FL

Figure 2

Chapter 4

The genetic scenario of coevolution: population structure and geographic differences in a plant herbivore interaction

INTRODUCTION

Coevolution, the reciprocal evolutionary changes in interacting species driven by natural selection, has enhanced the diversity of life on earth and has had profound effects on the structure of ecological communities (Ehrlich & Raven 1964, Futuyma & Slatkin 1983, Thompson 2005). Coevolution is a dynamic process that continually reshapes interactions among species across ecosystems, creating geographic mosaics over timescales sometimes as short as thousands or even hundreds of years (Thompson 2009). Coevolution is important in many theories within evolutionary biology, including evolution of ecological interactions, explanations for the maintenance of genetic variation, maintenance of sexual reproduction, and the processes of parapatric and sympatric speciation (Kawecki & Ebert 2004). Spatial variation in the interactions among coevolving species is a key component to understand coevolutionary dynamics (Thompson 2005). The Red Queen hypothesis predicts spatial variation in host resistance and parasite virulence traits (Van Valen 1973). The geographical mosaic theory also predicts spatial differences in traits related to the interaction (Thompson 2005). In both theories, spatial differences evolve as local adaptations by selection patterns that vary geographically, resulting in genetic differences among populations on the traits important to the ecological interaction.

Several mathematical models have predicted that local adaptation is more likely when migration is low and selection due to the interaction is high (Lively 1999, Nuismer et al. 2000, Gandon 2002, Gandon & Michalakis 2002, Sisterson & Averill 2004). Interestingly, these models show that if the migration rate of parasite is higher than that of the host, the parasite will be locally adapted, but if the host has higher migration than the parasite, the host will be locally adapted (Gandon 2002). This seems counterintuitive because gene flow can swamp local adaptation. However, factors that increase the evolutionary potential of the parasite increase its ability to track local host allele frequencies and vice versa (reviewed by Dybdahl & Storfer 2003). Therefore, studies on population structure with neutral markers and estimations of migration rates are key components to understand coevolutionary dynamics.

Another key component to understand coevolutionary dynamics is the spatial scale that the coevolutionary patterns and processes are occurring (Kaltz & Shykoff 1998). Studies that do not incorporate different geographical scales can miss coevolutionary dynamics occurring at either larger or smaller scales (Thompson 2005). The larger the distance among the populations the larger is the expected difference in the traits related to the ecological interaction (Thrall *et al.* 2002, Gandon *et al.* 1996, Cremieux *et al.* 2008). This is the consequence of two factors. First, the larger the distance the more likely the sites would differ in both abiotic factors, such as soil nutrients and temperature, and biotic factors, such as pressure from natural enemies (Laine 2009). Second, gene flow among populations also depends on the geographical scale. In general, larger distance prevents higher levels of gene flow that can homogenize the populations.

In a previous study I investigated local adaptation in the alkaloid-bearing legume *Crotalaria pallida* and its main seed predator, the arctiid moth *Utetheisa ornatrix* (Cogni & Futuyma 2009, chapter 3). The study was designed in a common-garden to test for genetic differences among populations. The herbivore showed local adaptation to its host plant population just at a continental scale (populations from Brazil and Florida), but not at a regional scale (populations *ca.* 150 Km apart). However, at the regional scale I found interpopulational differences in herbivore performance, and a significant interaction between herbivore sex and plant population. These results indicate that both the moth and plant populations were genetically differentiated also at the regional scale.

In the present study I investigate factors that may be responsible for the patterns of local adaptation at different scales that were previously reported (Cogni & Futuyma 2009). I investigate geographical differences in herbivore pressure in the field, genetic differences among populations in plant resistance traits, and population structure and patterns of gene flow for the moth. First, I surveyed herbivore pressure in the field to estimate the frequency of attack by generalist herbivores and the specialist *U. ornatrix.* Second, I used common-garden studies to test for genetic differences on plant resistance traits. Specifically, I tested if pyrrolizidine alkaloids concentration and carbon and nitrogen content in the seeds, trichome density on the leaves, and attractiveness of ants to extrafloral nectaries differ among populations, and if this variation depends on the spatial scale. Third, I investigated population structure of the moth with a microsatellite study. Specifically, I tested for population differentiation in space and time, and I estimated gene flow values.

METHODS

Study system

Crotalaria is a pantropical genus of weeds with more than 600 species (Polhill 1982). *Crotalaria pallida* is native from Africa and currently occurs at high densities from southern Brazil to the southeastern United States. There is no clear evidence about the New World introduction; possibly it was transported from Africa during slavery trade in the sixteenth century (Polhill 1982). *C. pallida* is an annual plant with limited dispersal; this species is partially selfing and bee pollinated, and lacks any mechanism for long-distance seed dispersal. In the neotropics, *Utetheisa ornatrix* is the main natural enemy of *Crotalaria* plants. *U. ornatrix* originally fed on native *Crotalaria* species, but currently *C. pallida* is its main host in several locations (Ferro 2001, Eisner, 2003, Cogni 2010, chapter 2). By preying on the 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 2001, Ferro *et al*. 2006).

The constitutive presence of pyrrolizidine alkaloids (PAs) is considered to be the major resistance trait in *Crotalaria* plants. PAs encompass a group of about 360 chemical structures with restricted occurrence in higher-plant taxa (Hartmann 1999). PAs have deterrent and toxic effects on a variety of generalist herbivores (van Dam *et al.* 1995, Macel *et al.* 2005, Narberhaus *et al.* 2005). *U. ornatrix* is able to sequester PAs from the host plant. The PAs not only protect larvae and adults, but they are also transmitted from the female (and from males through nuptial gift) to eggs (Trigo 2000, Eisner 2003, Conner 2009). Males also modify the PAs into a courtship pheromone (Dussourd *et al.* 1991, Iyengar & Eisner 1999 a and b, Conner 2009).

I investigated another resistance trait occurring in some species of *Crotalaria,* the presence of extra-floral nectaries (EFNs). EFNs are sugar-producing plant structures not directly related to pollination. Ants are the most frequent visitors to these glands, and by exhibiting aggressive behavior towards herbivores, EFN-gathering ants can positively affect plant fitness by decreasing herbivore damage (Heil & McKey 2003). *C. pallida* has EFNs located on the base of the peduncle. The EFNs remain active from the early development of flowers to formation of mature fruits. Ants attracted to EFNs frequently patrol the fruit pods, expelling *U. ornatrix* larvae that are outside the fruit (Ferro *et al.* 2006, Guimarães *et al.* 2006).

I also investigated two other plant traits: carbon and nitrogen content of the seeds and trichome density on the leaves. Relative nitrogen content is important in nutritional quality for herbivores (White 1978, 1993). Herbivores prefer plants with higher nitrogen content over plants with low nitrogen content (Vince *et al.* 1981, Denno *et al.* 1986, Bowdish & Stiling 1998, Gratton & Denno 2003). I also measured leaf trichomes because it it affects leaf herbivores (Bjorkman *et al.* 2008), and it is an easy trait to measure. Trichomes may also affect *U. ornatrix* neonate larvae (because neonate larvae eat leaves before entering the plant fruit to prey on the seeds).

Populations studied

In May 2005, I collected *C. pallida* seeds and moths from three populations in São Paulo State, Southeast Brazil: CAvi05, BOvi05 and JU05 (Table 1; Figure 1). In April 2006, I collected from another population at Archbold Biological Station in central Florida, US (Table 1). These populations were used in a previous study of local adaptation (Cogni & Futuyma 2009) and in the results described here investigating population differences in plant resistance traits in a common-garden. For each population I collected seeds from at least 30 individual plants and used a minimum of 40 adult moths. I also collected 16 adult moths per population for the microsatellite study (Table 1). Field studies for the quantification of herbivore pressure were carried out in January 2007 in the three Brazilian populations and in November 2009 for the population from Central Florida. In April 2008, I performed additional collections for the microsatellite study. I collected a larger number of individuals (22-29 moths) in the populations previously studied (except the JU05 population, which was destroyed by fire), and I also collected from four additional populations (Table 1). In two of these additional populations moths were collected in an alternative host plant of *U. ornatrix*, *Crotalaria trichotoma* (Table 1). The number of individuals collected in each population is given in Table 1.

Herbivore pressure in the field

I evaluated herbivore pressure in the field. In January 2007 I sampled three populations from Southeastern Brazil (BOvi, CAvi, JU – the same populations used in the previous local adaptation study). In November 2009, I sampled the population in Central Florida, US. The plants sampled were at least 5 meters from each other. In each population I examined 30 plants for the presence of herbivores. For each of these plants I also collected all fruits. The fruits were opened in the laboratory in the search for internal feeding herbivores. I recorded the proportion of pods that were attacked by the two most common herbivores - *U. ornatrix* and *Etiella zinckenella* Treit. (Pyralidae). *Crotalaria* fruits stay on the plant for several weeks before autochoric dispersion. I avoided plants that had already started seed dispersion and plants with only young fruits, to restrict sampling to plants that had been exposed to herbivores at approximately the same time. Pods attacked by *U. ornatrix* can be easily identified by the characteristic opening that the larva makes to enter the pod (Pereira 2008). *E. zinckenella* does not leave the characteristic opening on the pod that *U. ornatrix* makes (Ferro 2001). The average number of each herbivore species per plant and the proportion of pods attacked per plant were compared among the populations by a Kruskal-Wallis test because the data was not normally distributed even after transformations.

Plant resistance traits

Common environment conditions - I grew plants using seeds from different populations in a common environment. Seeds were germinated in an incubator at 26^{o}C and 24 hours of light. Seedlings were transferred to trays (ca. 650 ml volume) filled with standard potting soil. After 3 weeks, I transferred seedlings to large pots (ca. 7.6 liters). Seedlings and plants were kept at a greenhouse under natural sunlight. Plants were watered daily and standard fertilizer (15N:5P:15K at 300 ppm) was added weekly. In the 2005 experiment (populations CAvi05, BOvi05 and JU05) I used the greenhouse of the Instituto de Biologia at Universidade Estadual de Campinas in São Paulo State, Brazil without temperature control. In the 2006 experiment (populations CAvi05 and FL06) I used the greenhouse at the Life Science Building at Stony Brook University in Stony Brook (NY) with approximate temperature of 28°C (day) and 24°C (night). In 2005, I started the plants in May and collected the samples for chemical analyses and trichome counts in November. In 2006, I started the plants in April and collected the samples in October. Although the conditions of the greenhouses in the two different countries differed, I have data showing that any such difference did not affect my results (Cogni & Futuyma 2009). For the EFNs experiment I used a set of plants grown at the Brazilian greenhouse in 2005. The plants were started in May and the experiment was carried out in October. For the EFNs experiment comparing Brazil and Florida I grew plants in 2008 from the same set of seeds used in the 2006 experiments. The plants were grown under the same conditions described above, except that they were grown on the outside, in the city of Mogi Mirim (SP, southeastern Brazil). I did not observe any attack by herbivores on the plants on the outside that could have influenced the results. The plants were started in August 2008 and the experiments carried out in February and March 2009.

PAs concentration – I collected approximately 10 green fruits per individual common-garden plant in ethanol. All the fruits were at the same developmental stage as offered to larvae in my local adaptation study (Cogni & Futuyma 2009, chapter 3). Sample size varied from 15 to 16 individuals per population. The samples were triturated and PAs were extracted in ethanol three times. The extractions were evaporated to dryness under vacuum at 45°C before resuspension in 2.0 ml of ethanol. Total PAs was quantified by the colorimetric method as in Trigo *et al.* (1993). Four replicates were performed for each individual. I used Dixon's Q-test to detect possible outliers among the four replicated spectrophotometer readings. A calibration curve was constructed using retrorsine extracted from *Senecio brasiliensis* (Asteraceae). Because I initially collected and analyzed the entire fruit, and *Utetheisa ornatrix* larva just eats the seeds, I later analyzed only seeds. I did a correction curve to estimate the PA concentration in the seeds from the PA concentration in the entire fruit. The curve was constructed from 20 samples in which I separated the green seeds from the other parts of the fruit before quantifying the PAs as described above. Therefore, the PAs concentration in the seeds was estimated as $y = 2.1105x - 0.0003$ ($R^2 = 0.934$), where x is the total concentration of PAs in the entire fruit. GC/MS analyses confirmed that all the populations studied have the same PAs in the green seeds: usaramine (*ca.* 85%) and intergerrimine (*ca.* 15%).

Carbon and Nitrogen content – Fruits were collected and dried at 60^oC for 72 hours. The seeds were separated and ground to a fine powder with liquid nitrogen and further dried at $60\,^oC$. About 6 mg per sample was analyzed in a CHN elemental analyzer. Sample size varied from 13 to 16 individuals per population.

Trichome density – I estimated trichome density on the lower (abaxial) surface of leaves. Thirty individuals per population were sampled at the regional scale. At the continental scale 68 individuals were sampled from the Brazilian population and 69 from the Florida population. For each individual the central leaflet of the third fully developed leaf from the shoot tip was collected. A leaf-disc was cut at a central position and, by using a compound microscope, the number of trichomes was counted using a grid of 10x10 $1mm^2$. For each leaf-disc nine $1mm^2$ squares were counted avoiding squares with major veins. The 9 counts were averaged for an estimate of the number of trichomes per mm^2.

Extra-floral nectaries (EFNs) – For the regional comparison in 2005, forty-two plants from each of the 3 populations (CAvi05, BOvi05 and JU05) grown in a greenhouse in Campinas- SP were transferred to the borders of the Santa Genebra conservation unit (in Campinas – SP). One plant from each population was placed in each block (42 blocks of 3 plants each). All plants were in the reproductive stage (flowers or young fruits), and plants in the same block had similar size and phenological stage. Blocks were 10 meters apart. Ten days after the plants were transferred, I checked for the presence of ants visiting the EFNs and counted the total number of ants per plant during a 30 second interval. To estimate ant aggressiveness, a termite worker (*Nasutitermes*) was glued on a reproductive stem of each plant and observed for 10 minutes if it was attacked by ants (a standard procedure in ant-plant studies, see Barton 1986, Cogni *et al.* 2003). The ants visiting the EFNs during the experiment were *Camponotus* sp., *Brachymyrmex* sp., *Pheidole* sp. and *Crematogaster* sp. For the continental comparison, 25 plants from each population (CAvi05 and FL06) grown in a common environment in Mogi Mirim-SP were transferred to a grass field at the Mogi Mirim City Zoo (Mogi Mirim – SP). The same procedures described above were used. The observed ants visiting the EFNs were *Brachymyrmex* sp., *Camponotus* (3 species), *Crematogaster* sp., *Pheidole* (2 species) and *Pseudomyrmex* sp.

Statistical analyses – At the regional scale, differences among the three populations for each plant resistance trait were tested with one-factor ANOVAs. At the continental scale differences between the two populations were tested with t-tests. PAs concentration and trichome density variables were log transformed to obtain normal distribution. For the EFNs experiments in 2005, the number of ants visiting plants from each population was compared by a Kruskal-Wallis test because the data was not normally distributed even after transformations. For the 2009 EFNs experiment the mean number of ants visiting plants from each population was compared by a paired t-test. The percent of termites attacked by ants was compared by χ^2 tests.

Genetic structure - Microsatellites study

DNA extraction and microsatellite amplification – Moth genomic DNA was extracted with Qiagen DNeasy tissue kit; adult moths preserved in alcohol were ground in liquid nitrogen after the removal of wings and the abdomen. Five microsatellite loci developed by Bezzerides *et al.* (2004) were amplified for *U. ornatrix*. PCR conditions were similar to Bezzerides *et al.* (2004), with the exception that GoTaq polymerase and dNTPs from Promega were used and the final volume of the reactions was 25 μL. Amplifications that failed on individual samples were repeated one time. Fragments were analyzed with an ABI3730 DNA Analyzer with the size standard LIZ 500 (Applied Biosystems). Allele sizes were estimated using GENEMAPPER 3.0 (Applied Biosystems) and verified by eye.

Data analyses – I used GENEPOP (vers. 4.0, Rousset 2008) to test loci for linkage disequilibrium (1,000 dememorisations; 10,000 batches; 10,000 iterations per batch). To contrast genetic diversity within the populations I used GENEPOP to calculate number of alleles, and the expected and observed number of heterozygotes for each locus. I used GENEPOP to test for deviations from Hardy-Weinberg equilibrium (exact test; 1,000 dememorisations; 100 batches; 1,000 iterations per batch) with the sequential Bonferroni correction for multiple testing (Rice 1989). Pairwise population differentiation was tested with exact G tests in GENEPOP (genic differentiation) for each locus and across all loci (assuming statistical independence across loci). This procedure tests the null hypothesis that alleles are drawn from the same distribution in the different populations. Pairwise F_{st} values were calculated in GENEPOP by the "weighted" analysis of variance for each locus and across all loci. This method uses ANOVA mean sum of squares (for gametes, individuals and populations) to estimate F statistics (Cockerham 1973, Weir & Cockerham,1984). The estimation across all loci is a modification of the method using a weighted sum of each locus (Weir & Cockerham 1984, Weir 1996) that gives higher weight to loci with larger sample sizes (Rousset 2008). I estimated the number of migrants using private alleles (Barton & Slatkin 1986). This method assumes an island model and quasi-equilibrium between drift and migration. These assumptions are unlikely to be met in my system (see results below), but I cautiously used it as a preliminary estimate of this important parameter in coevolution models.

RESULTS

Geographical differences in herbivore pressure

Only three species of herbivores were observed feeding on *C. pallida* (Table 3): *U. ornatrix*, *Thianta perditor* (Hemiptera: Pentatomidae), and *Etiella zinckenella* (Lepidoptera: Pyralidae). The same species were observed in all populations sampled, except for the pentatomid *T. perditor*, which was observed in the three Brazilian populations, but not in the population from Florida. The average number of each herbivore species found per plant differed among the populations (Table 3; *U. ornatrix*: H

$= 35.62$, $p < 0.0001$, $N = 120$; *T. perditor*: $H = 39.29$, $p < 0.0001$, $N = 120$; *E. zinckenella*: $H = 25.60$, $p < 0.0001$, $N = 120$). The proportion of pods attacked by each herbivore was also different among the populations (Table 4; attacks by *U. ornatrix*: $H = 37.2$, $p < 0.0001$, $N = 120$; attacks by *E. zinckenella*: $H = 22.22$, $p < 0.0001$, $N = 120$).

There were differences in herbivore pressure even at the regional scale. Even when only the three Brazilian populations were compared the differences in herbivore number were significant. A higher number of the specialist arctiid *U. ornatrix* was found on the CA population (Table 3; $H = 36.229$; $p < 0.0001$; $N = 90$). A higher number of the generalist pentatomid *Thianta perditor* was found in the JU population (Table 3; $H = 21.704$; $p < 0.0001$; $N = 90$). On the other hand, the average number of the generalist pyralid *E. zinckenella* was similar among the three Brazilian populations (Table 3; $H = 4.371$; $p = 0.112$; $N = 90$). The proportion of pods attacked in the field by the two major herbivores also differed in the comparison of just the three Brazilian populations (Table 4). A larger proportion of pods were attacked by *U. ornatrix* in the CA population (Table 4; $H = 14.644$; $p = 0.001$; $N = 90$). The proportion of pods attacked by *E. zinckenella* was higher in the BO population (Table 4; $H = 11.687$; $p = 0.003$; $N = 90$).

Plant resistance traits

At the local scale, the three populations from Brazil differed in the concentration of PAs in the seeds (Figure 2A; Table 5A). There were no differences among the populations in the seeds' carbon and nitrogen content, or in the trichome density in leaves (Figures 2 B-D; Table 5B-D). Ant attractiveness to EFNs differed among the populations;

there was a small difference in the average number of ants per plant ($H = 6.305$; $p = 0.043$; $N = 126$) and a difference in the percent of termite baits attacked by ants ($\chi^2 = 8.32$; d.f. = 2, $p = 0.016$) (Figures 2 E-F).

At the continental scale, there was no difference in the PAs' concentration in seeds between the population from Florida and the Brazilian population (Figure 3A; $t = 0.213$, d.f. = 30, $p = 0.833$). There was no difference between the populations in the seeds' carbon content (Figure 3B; $t = 1.383$, d.f. = 27, $p = 0.178$). On the other hand, nitrogen content differed between the two populations; the seeds from Brazil have higher nitrogen content than the seeds from Florida (Figure 3C; $t = 2.18$, d.f. = 28, $p = 0.037$). Trichome density in the leaves also differed between the two populations; plants from Brazil have more trichomes than plants from Florida (Figure 3D; $t = 2.82$, d.f. = 135, $p = 0.006$). Ant attractiveness to EFNs was similar between the two populations; the average number of ants per plant ($t = 0.099$, d.f. = 24, $p = 0.922$) and the percent of termite baits attacked by ants ($\chi^2 = 0.00$; d.f. = 2, $p > 0.99$) did not differ between the two populations (Figures 3 E-F).

Moth genetic structure

None of the loci were in linkage disequilibrium (only one of the 100 tests within populations was individually significant at $\alpha = 0.05$). Pairs of loci tested across all populations were not significant either (Table 6). Allele frequency for each locus on each population is given at Appendices 1. Table 7 describes genetic diversity for each locus on each population. Locus Utor 2 deviated from Hardy-Weinberg equilibrium in all populations (Table 7), suggesting the presence of null alleles. The three populations collected in 2005 showed significant differentiation (Table 8). In contrast, the populations collected in 2008 were not significantly differentiated (Table 9). For the two populations sampled in both 2005 and 2008 (BOvi and CAvi), there was significant pairwise differentiation in 2005 and no differentiation in 2008. The test of 2005 *vs.* 2008 for each of these two localities revealed significant temporal differentiation (Table 10). In 2005, the estimate for the number of migrants was 1.43 (mean sample size = 14.6; mean frequency of private alleles = 0.078). In 2008, the estimated number of migrants was 7.67 (mean sample size = 20.2; mean frequency of private alleles = 0.027). The host-plant species present in each population did not influence the genetic structure of the *U. ornatrix*'s populations sampled. The two populations occurring on the alternative host *C. trichotoma* (CAia08 and PI08) were not differentiated from localities where only the most common host, *C. pallida*, was present (Table 9). At the continental scale, the same populations used in the local adaptation study (CAvi05 and Fl06) were compared. There was significant differentiation between these populations (genic differentiation exact G test: $P < 0.0001$). Even at this large geographical scale, the pairwise F_{st} value between the two populations was small (0.038), presumably due to relatively recent introduction and disjunction.

DISCUSSION

My results show that there are genetic differences in resistance traits among *C. pallida* populations. Several studies have shown that natural populations vary in defensive traits such as resistance to natural enemies (Burdon & Thrall 1999, Brodie Jr *et al.* 2002, Siska *et al.* 2002, Lewis *et al.* 2006, Toju & Sota 2006 a and b, Brenes-Arguedas *et al.* 2008, Hanifin *et al.* 2008, Tewksbury *et al.* 2008). Indeed, in the best studied empirical example of plant herbivore coevolution, Berenbaum and colleagues showed extensive variation in the levels of furanocoumarins in populations of the wild parsnip from different regions and continents (Berenbaum & Zangerl 2006, Zangerl *et al.* 2008). This variation correlates with the presence and absence, or intensity of attack, of the plant main herbivore, the parsnip webworm. In my study, the common-garden design indicates that the differences in resistance traits are indeed genetic (excluding the alternative and unlikely hypotheses that all differences were due to maternal effects) and not plastic responses to different environmental conditions. This provides support for findings in several other studies that present evidence of genetic differences in plant resistance traits (Dirzo & Harper 1982, Schappert & Shore 1995, Salmore & Hunter 2001 a and b, Salgado & Pennings 2005, Bacom & Mauricio 2008, Bidart-Bouzat & Kliebenstein 2008, Cremieux *et al.* 2008, Barbour *et al.* 2009, Gerson *et al.* 2009).

I showed that geographical variation in *C. pallida*'s resistance traits depends on the spatial scale. While trichome density and nitrogen content differed at the continental scale, PAs and ant attractiveness to EFNs just varied at the regional scale. My results showing that PAs and EFNs vary at the regional scale confirm my earlier evidence that the plant populations are differentiated even at this small scale (Cogni & Futuyma 2009). These differences may be caused by drift or selection. There is evidence in other systems that selection trajectories in ecological interactions may diverge even among local neighbor populations (Laine 2009, Vos *et al.* 2009). A larger number of populations would be preferred to confirm the generality of the patterns observed at the continental scale (Kawecki & Ebert 2008), but my study just included one population from Florida and one population from Brazil. The reason was the difficulty in finding an additional population in Florida that would be distant enough from Archbold that would represent an independent population (to avoid pseudo-replication). In addition, constraints in time and greenhouse and laboratory space limited the total number of populations included in the local adaptation study. This kind of study requires a very high number of moths from each population to be reared on each plant population.

The patterns of variation of PAs among *C. pallida* populations suggest that PAs are not an effective defense against its main herbivore, the specialist *U. ornatrix*. PAs are considered the main resistance mechanism of *Crotalaria* species (Wink & Mohamed 2003, Ferro *et al*. 2006). Therefore, I initially believed that *U. ornatrix* local adaptation observed at the continental scale was driven by differences in PAs between Florida and Brazilian *C. pallida* populations (Cogni & Futuyma 2009, chapter 3). My present results clearly exclude this possibility since *C. pallida* populations from Florida and from Campinas (Brazil) present similar concentrations of PAs in the green seeds. In addition, the PAs are similar qualitatively; usaramine (*ca*. 85%) and intergerrimine (*ca*. 15%) were the main PAs in both populations. The local adaptation by *U. ornatrix* may be the response to the differences in nitrogen content reported here, but this explanation in unlikely because there was no difference in the amount of seeds consumed for plants from Florida and Campinas (Cogni & Futuyma 2009, chapter 3). More likely, other possible resistance traits not yet measured may be responsible to the local adaptation results. *Crotalaria* plants may also have isoflavonoids, non-proteic amino acids and proteinase inhibitors (Pilbeam & Bell 1979, Pilbeam *et al.* 1979, Rego *et al.* 2002, Wink & Mohamed 2003, Pando *et al.* 2004). Future studies should investigate if any of these resistance traits varies among *C. pallida* populations and how different *U. ornatrix* populations may be affected by these possible differences. I also have direct evidence that PAs do not affect the specialist herbivore; in another study I have shown that PAs do not negatively affect *U. ornatrix* performance and that sequestration of PAs has no fitness cost (see chapter 5). I suggest, indeed, that specialist herbivores can act as natural selection agents that decrease the level of chemical defenses in plant populations, and that PAs may be effective against some generalist herbivores (Cogni *et al. in prep.*, Macel *et al.* 2005, van Dam *et al.* 1995). As an example, populations of the weed *Senecio jacobaea* introduced to areas where a specialist is absent evolved higher levels of PAs and consequently become more resistant to generalists (Joshi & Vrieling 2005). In my system, my results suggest that the herbivore pressure by generalists and the specialist varies among the populations. Even though the same species were present in all populations, the frequency of these herbivores varies in space. Therefore, the geographical variation in the concentration of PAs reported here is likely the result of spatial difference in a balance of selective pressures from specialist and generalist herbivores.

Since *C. pallida* is not native to the neotropics, there are two possible explanations for the patterns of variation in resistance reported here. First, it could be the result of rapid evolution; differentiation may have evolved since this plant’s introduction (possibly 500 years ago). Alternatively, it may be the result of multiple introductions of individuals from divergent populations in the native range. In the future, I intend to use molecular markers on *C. pallida* populations from the native and introduced range to discern between these two alternatives. I also intend to study the genetic structure of *C. pallida*'s populations used in this study and compare it with the structure of the moth populations reported here. These will permit the comparison of migration rates between the plant and the moth. Theoretical models of coevolution show that if the migration rate of parasite is higher than that of the host, the parasite will be locally adapted, but if the host has higher migration than the parasite, the host will be locally adapted (Gandon 2002). These predictions have been confirmed in experimental laboratory studies with phage and bacteria (Forde *et al*. 2004, Morgan *et al.* 2005). In natural populations these predictions have been supported by correlations between local adaptation and natural history information on migration rates on a variety of host-parasite interactions (Greischar & Koskella 2007, Hoeksema & Forde 2008). However, none of these studies has provided more reliable estimations of migration rates in the field by molecular markers (but see Brandt *et al.* 2007, Keeney *et al.* 2009), despite the common use of molecular markers to estimate migration in ecology and evolutionary biology (Broquet & Petit 2009).

Since there is differentiation in plant resistance traits and on herbivore pressure at the regional scale, why does local adaptation by *U. ornatrix* not occur (Cogni & Futuyma 2009, chapter 3)? The most likely explanation for the lack of local adaptation at this scale is *U. ornatrix*'s population structure. My microsatellite results from 2008 indicate a lack of population differentiation at the regional scale, with very high migration rates among populations. High levels of dispersal can have a detrimental effect on genetic variation and thereby adaptive potential (Garant *et al.* 2007). High rates of dispersal can cause ‘genetic swamping’ by replacing locally adapted alleles with locally maladapted alleles common in the metapopulation as whole (Alleaume-Benharira *et al.* 2006). High rates of dispersal can also homogenize genetic variation among patches, reducing the supply of novel variation attainable through dispersal (Gandon & Michalakis 2002). As empirical examples, gene flow reduced adaptation between parsnips and webworms (Zangerl & Berenbaum 2003), and prevented local adaptation of the scale *Matsucoccus acalyptus* to pinyon pines (Cobe & Whitham 1998). Contrary to the 2008 results, samples collected in 2005 showed some level of differentiation among populations and much lower migration rates. Under this scenario local adaptation is likely to evolve. However, the comparison of 2005 and 2008 samples shows that this differentiation is not stable over time. These results suggest a pattern of local population extinction and recolonization. Indeed, *C. pallida* has a patchy distribution and occurs in habitats where fire and other human disturbances are common, and these can cause local moth extinctions. When individual parasite populations are ephemeral, local adaptation may only be found at larger geographical scales (Thrall & Burdon 1997, Burdon & Thrall 2000). In addition, recolonization may occur by moths originating from populations where alternative host plants are present. Alternative hosts may decrease the level of adaptation to the main host (Zangerl & Berenbaum 2003). My results show no differentiation among populations occurring on the main host, *C. pallida*, and on the alternative host *C. trichotoma*, suggesting the possibility of recolonization of populations from individuals occurring on alternative hosts. In conclusion temporal change in *U. ornatrix*’s population structure is the most likely cause for the lack of local adaptation at the regional scale.

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Table 1. Populations used in the study.

Locality	Year collected	Coordinates	Altitude (m)	Acronym	# individuals *C. pallida*	# individuals *U. ornatrix*
Campinas (Village)**	2005	22°45’12’’S 47°03’20”W	605	CAvi05	16	16
Campinas (Village)	2008	22°45’12’’S 47°03’20”W	605	CAvi08	23	27
Campinas (IAC)	2008	22°51’21”W 47°04’27”S	629	CAia08	0*	25
Botucatu (Vitoriana)**	2005	22°46’45”S 48°24’14”W	540	BOvi05	16	16
Botucatu (Vitoriana)	2008	22°46’45”S 48°24’14”W	540	BOvi08	30	29
Botucatu (Bairro)	2008	22°52’07”S 48°26’24”W	785	BOba08	20	23
Juquiá**	2005	24°19’55”S 47°38’15”W	21	JU05	16	16
Limeira	2008	22°36’18”S 47°21’45”W	569	LI08	30	24
Piracicaba	2008	22°44’29”S 47°31’17”W	518	PI08	0*	22
Florida (Lake Placid)**	2006	27°15’N 81°21’W	30	FL06	16	16

* *U. ornatrix* collect on the alternative host *Crotalaria trichotoma*. **Populations used on the plant resistance study.

Table 2. Microsatellite loci used in the *Utetheisa ornatrix* population structure study.

Locus	Primer sequence (5’-3’) forward	reverse	No. of alleles	Size range
Utor2	TCAACTGTTATTCTTTAAATGTTTG	TCATATCTACGTATAGCTGGTG	16	217-277
Utor7	TGCTAAGAACGTGTATATTGTAGGAAC	ATATGTGACTCAGAGAAGAAATACAAAG	10	233-273
Utor10	TCGAGAGCCCCTGTCTGTAAC	CGGGATAAAACATAGCCTATAACC	7	234-274
Utor28	GGAGAATTGAGGTGCCTCTG	TGGTCACCCATCCATATAATG	13	199-275
UtorTac	GTTTTGCGTGGGTAATTATAA	AGCTGAAGAGTTTGTTTGTTTG	12	198-250

Table 3. Field differences among populations in the mean number of herbivores per plant. Populations are from São Paulo State, Southeastern Brazil, and Central Florida, US. Values are mean ± SD.

	Population			
	SE Brazil			US
	BO	CA	JU	FL
Utetheisa ornatrix	0.17 ± 0.38	1.37 ± 1.03	0.13 ± 0.35	0.77 ± 1.04
Thianta perditor	0.33 ± 0.48	0.07 ± 0.25	0.70 ± 0.60	0
Etiella zinckenella	0.87 ± 1.00	0.43 ± 0.73	0.40 ± 0.50	3.13 ± 3.59

Table 4. Field differences among populations in the proportion of pods attacked by *Utetheisa ornatrix* and *Etiella zinckenella* per plant. Populations are from São Paulo State, Southeastern Brazil, and Central Florida, US. Values are mean ± SD.

	Population			
	SE Brazil			US
	BO	CA	JU	FL
Utetheisa ornatrix	0.028 ± 0.052	0.132 ± 0.220	0.015 ± 0.030	0.189 ± 0.196
Etiella zinckenella	0.081 ± 0.101	0.020 ± 0.046	0.022 ± 0.043	0.094 ± 0.099

Table 5. Effect of population origin on *Crotalaria pallida* resistance traits at a regional scale. (A) pyrrolizidine alkaloids (PAs) in green seeds, (B) carbon content of green seeds, (C) nitrogen content of green seeds, and (D) trichome density on leaves. Plants were grown in a common greenhouse environment and are from three localities in São Paulo State, SE Brazil. * indicates significant differences.

Source	**d.f.**	**Mean squares**	**F-ratio**	**P**
(A) PAs concentration *				
Population	2	1.310	4.32	0.019
Error	43	0.303		
(B) Carbon content				
Population	2	0.578	1.246	0.298
Error	43	0.464		
(C) Nitrogen content				
Population	2	0.278	2.378	0.105
Error	43	0.117		
(D) Trichome density				
Population	2	0.163	1.594	0.209
Error	87	0.102		

Table 6. Test of linkage disequilibrium for pairs of *Utetheisa ornatrix'*s microsatellite loci tested across all populations.

Locus pair	**X^2**	**d.f.**	**p**
Utor 10 & Utor 28	0.00	10	1.00
Utor10 & Utor 2	3.05	6	0.80
Utor28 & Utor2	8.06	20	0.99
Utor10 & Utor7	0.00	8	1.00
Utor28 & Utor7	14.70	20	0.79
Utor2 & Utor7	8.82	20	0.98
Utor10 & UtorTAC	0.00	8	1.00
Utor28 & UtorTAC	8.14	20	0.99
Utor2 & UtorTAC	15.71	20	0.73
Utor7 & UtorTAC	18.40	20	0.56

Table 7. Genetic diversity for each *Utetheisa ornatrix*'s microsatellite locus on each population. Number of alleles (A), expected number of heterozygotes (H_e) and observed number of heterozygotes (H_o). * indicates significant deviation from Hardy-Weinberg equilibrium after correction for multiple testing.

Population		*Utor2*	Utor7	Utor10	Utor28	*UtorTAC*
CAvi05	A	26	28	0	30	30
	H_e	11.4	10.96	-	1.93	10.28
	H_o	4*	12	-	2	8
CAvi08	A	50	50	16	52	52
	H_e	21.39	19.26	6.20	7.37	18.39
	H_o	12*	19	4	6	12*
CAia08	A	48	50	8	50	50
	H_e	21.11	19.43	3	11.96	19.96
	H_o	11*	15	2	6*	17
BOvi05	A	30	30	0	32	32
	H_e	12.76	10.28	-	3.61	11.97
	H_o	4*	7	-	4	13
BOvi08	A	50	48	12	58	58
	H_e	22.65	17.87	5.09	9.12	23.95
	H_o	15*	16	2*	7	20
BOba08	A	40	46	12	46	46
	H_e	17.28	17.69	3.36	8.69	18.38
	H_o	10*	10*	2	6	16
JU05	A	28	32	10	28	24
	H_e	12.30	12.68	3.11	9.78	9.48
	H_o	4*	10	2	5*	8
LI08	A	42	48	14	44	48
	H_e	16.83	18	5.46	7.23	18.55
	H_o	12*	10*	1*	5	17
PI08	A	38	42	8	44	44
	H_e	17.08	16.78	2.85	7.05	16.12
	H_o	11*	12	0	4	12
FL06	A	24	28	6	30	28
	H_e	10.48	10.63	2.4	2.9	11.56
	H_o	3*	10	0	2	8

Table 8. *Utetheisa ornatrix* population differentiation at the regional scale in 2005. Above diagonal: p value for genic differentiation (exact G test) for each population pair, for each locus and across all loci (significant values in italic). Below diagonal: pairwise F_{st} values. Locus Utor 10 was not included because it did not amplify in any individual of BOvi05 and CAvi05 populations.

Utor 2			
	BOvi05	**CAvi05**	**JU05**
BOvi05	-	*<0.001*	0.079
CAvi05	0.031	-	*0.01*
JU05	- 0.030	0.012	-
Utor7			
	BOvi05	**CAvi05**	**JU05**
BOvi05	-	*<0.001*	*0.008*
CAvi05	0.068	-	*0.01*
JU05	0.080	0.028	-
Utor28			
	BOvi05	**CAvi05**	**JU05**
BOvi05	-	0.06	*0.004*
CAvi05	0.035	-	*<0.0001*
JU05	0.110	0.165	-
UtorTAC			
	BOvi05	**CAvi05**	**JU05**
BOvi05	-	*0.009*	*<0.0001*
CAvi05	0.057	-	*<0.001*
JU05	0.101	0.037	-
Across all loci			
	BOvi05	**CAvi05**	**JU05**
BOvi05	-	*<0.0001*	*<0.0001*
CAvi05	0.050	-	*<0.0001*
JU05	0.060	0.049	-

Table 9. *Utetheisa ornatrix* population differentiation at the regional scale in 2008. Above diagonal: p value for genic differentiation (exact G test) for each population pair, for each locus and across all loci (significant values in italic). Below diagonal: pairwise F_{st} values.

Utor2

	BOba08	BOvi08	CAia08	CAvi08	LI08	PI08
BOba08	-	0.668	0.144	0.348	0.169	*0.*304
BOvi08	-0.008	-	0.145	0.245	0.*009*	0.429
CAia08	-0.002	0.000	-	0.069	0.080	0.359
CAvi08	-0.015	0.009	-0.008	-	0.515	0.263
LI08	-0.013	0.029	0.001	-0.020	-	*0.*060
PI08	-0.010	-0.014	-0.006	0.007	0.018	-

Utor7

	BOba08	BOvi08	CAia08	CAvi08	LI08	PI08
BOba08	-	0.080	0.471	0.209	0.648	*0.*937
BOvi08	0.005	-	0.166	0.927	0.736	0.710
CAia08	-0.007	-0.015	-	0.107	0.736	0.815
CAvi08	0.012	-0.016	-0.008	-	0.657	0.897
LI08	-0.012	-0.022	-0.022	-0.011	-	*0.*962
PI08	-0.019	-0.019	-0.022	-0.018	-0.026	-

Utor10

	BOba08	BOvi08	CAia08	CAvi08	LI08	PI08
BOba08	-	0.180	0.259	0.230	0.*026*	*0.017*
BOvi08	0.020	-	1.00	0.297	0.215	0.622
CAia08	-0.040	-0.154	-	0.375	0.121	0.536
CAvi08	0.030	-0.045	-0.058	-	0.779	0.*028*
LI08	0.118	-0.041	-0.016	-0.082	-	*0.012*
PI08	0.155	-0.117	-0.130	-0.005	0.052	-

Utor28

	BOba08	BOvi08	CAia08	CAvi08	LI08	PI08
BOba08	-	0.493	0.255	0.399	0.*05*	*0.012*
BOvi08	-0.011	-	0.655	0.872	0.517	0.*038*
CAia08	-0.010	-0.003	-	0.810	0.248	0.287
CAvi08	-0.006	-0.016	0.001	-	0.796	0.317
LI08	-0.003	-0.016	-0.004	-0.019	-	*0.027*
PI08	0.001	0.001	-0.007	-0.014	-0.006	-

UtorTAC

	BOba08	BOvi08	CAia08	CAvi08	LI08	PI08
BOba08	-	0.555	0.687	0.357	0.762	*0.*128
BOvi08	0.004	-	0.448	0.234	0.395	0.197
CAia08	0.006	-0.010	-	0.537	0.607	0.330
CAvi08	0.026	0.004	-0.008	-	0.650	0.894
LI08	-0.008	-0.006	-0.014	-0.008	-	*0.*088
PI08	0.037	0.001	-0.012	-0.018	-0.001	-

Across all loci

	BOba08	BOvi08	CAia08	CAvi08	LI08	PI08
BOba08	-	0.294	0.315	0.280	0.051	*0.008*
BOvi08	0.000	-	0.449	0.573	0.089	0.216
CAia08	-0.004	-0.014	-	0.201	0.188	0.588
CAvi08	0.007	-0.005	-0.009	-	0.948	0.251
LI08	0.000	-0.004	-0.018	-0.019	-	*0.003*
PI08	0.012	-0.015	-0.017	-0.005	0.001	-

Table 10. Temporal differentiation in population structure of *Utetheisa ornatrix.* Two localities (BOvi and CAvi) were sampled in 2005 and 2008, and differentiation between years was tested with exact G tests for genic frequencies. F_{st} values were calculated for each locality between the years for each locus and across all loci. Locus Utor 10 was not included because it did not amplify in any individual of BOvi05 and CAvi05 populations.

Locality		**Utor2**	**Utor7**	**Utor28**	**UtorTAC**	**Across all loci**
BOvi	p value (G test)	*0.001*	*0.003*	0.484	0.117	< 0.*0001*
	F_{st}	0.036	0.079	-0.008	0.0003	0.032
CAvi	p value (G test)	*0.007*	*0.018*	0.550	0.465	0.*008*
	F_{st}	0.030	0.021	-0.002	0.016	0.021

Figure legends

Figure 1. Map of populations included in this study from São Paulo State, SE Brazil.

Figure 2. Among population variation on *Crotalaria pallida* resistance traits at a regional scale. (A) pyrrolizidine alkaloids (PAs) in green seeds, (B) carbon content of green seeds, (C) nitrogen content of green seeds, (D) trichome density on leaves, (E) number of ants attracted to extrafloral nectaries (EFNs), and (F) percent of termites bites attacked by ants. Boxes represent median, 10th, 25th, 75th and 90th percentiles, dots are outliers. Plants were grown in a common greenhouse environment and are from three localities in São Paulo State, SE Brazil: BO = Botucatu, CA = Campinas and JU = Juquiá. P values and ns (non-significant) indicate the effect of population on ANOVA tests (A-D), Kruskal-Wallis (E) or χ^2 test (F).

Figure 3. Population variation on *Crotalaria pallida* resistance traits at a continental scale. (A) pyrrolizidine alkaloids (PAs) on green seeds, (B) carbon content of green seeds, (C) nitrogen content of green seeds, (D) trichome density on leaves, (E) number of ants attracted to extrafloral nectaries (EFNs), and (F) percent of termites bites attacked by ants. Boxes represent median, 10th, 25th, 75th and 90th percentiles, dots are outliers. Plants were grown in a common environment and are from Campinas in São Paulo State in SE Brazil (Br) and Central Florida (Fl) in the US. P values and ns (non-significant) indicate between population differences on t tests (A-E) or χ^2 test (F).

Figures

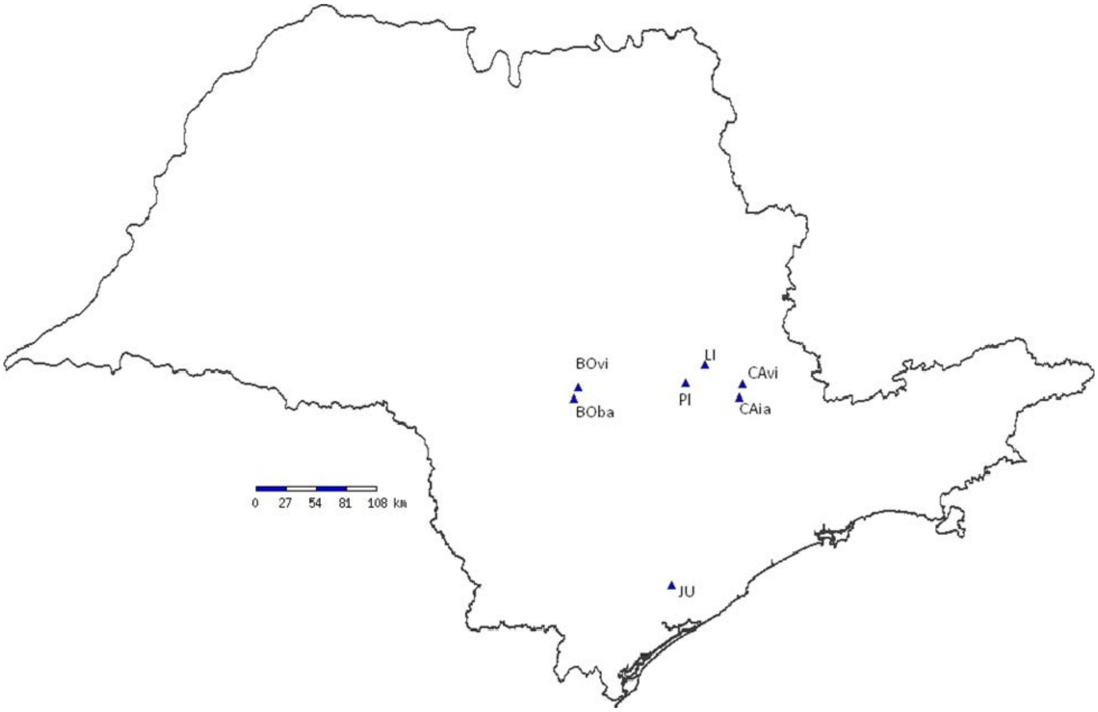


Fig. 1

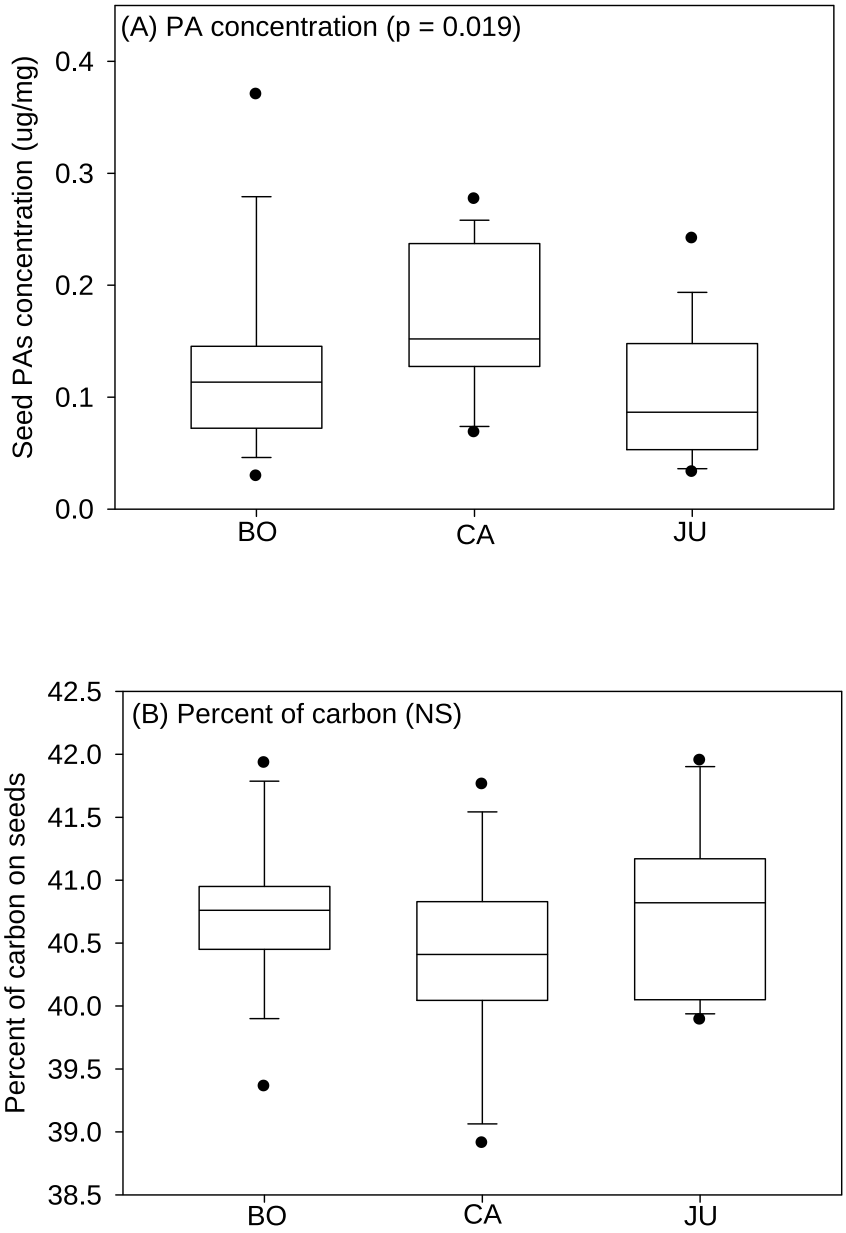
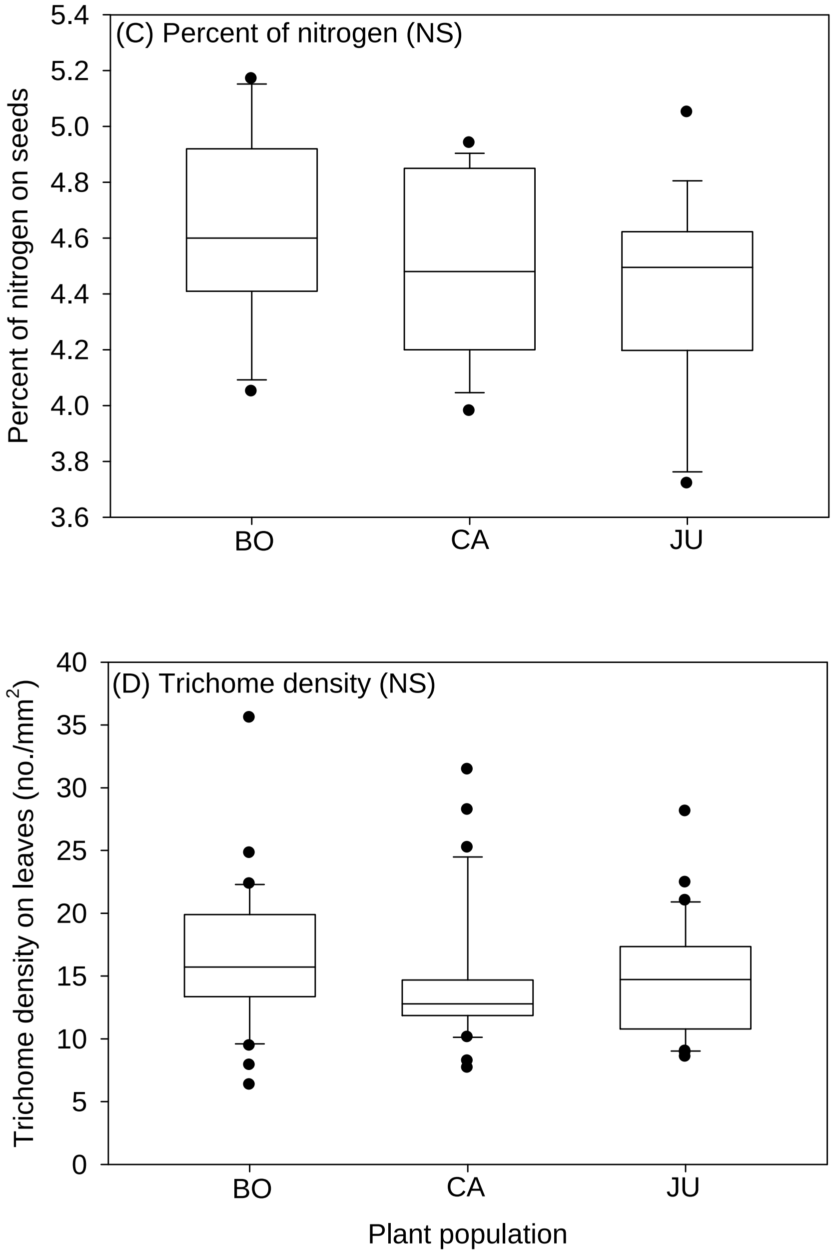

(A) PA concentration (p = 0.019)
Seed PAs concentration (ug/mg)
0.0
0.1
0.2
0.3
0.4
BO
CA
JU
(B) Percent of carbon (NS)
Percent of carbon on seeds
38.5
39.0
39.5
40.0
40.5
41.0
41.5
42.0
42.5
BO
CA
JU

Fig. 2 A and B



(C) Percent of nitrogen (NS)
Percent of nitrogen on seeds
5.4
5.2
5.0
4.8
4.6
4.4
4.2
4.0
3.8
3.6
BO
CA
JU
(D) Trichome density (NS)
Trichome density on leaves (no./mm^2)
40
35
30
25
20
15
10
5
0
BO
CA
JU
Plant population

Fig 2 C and D

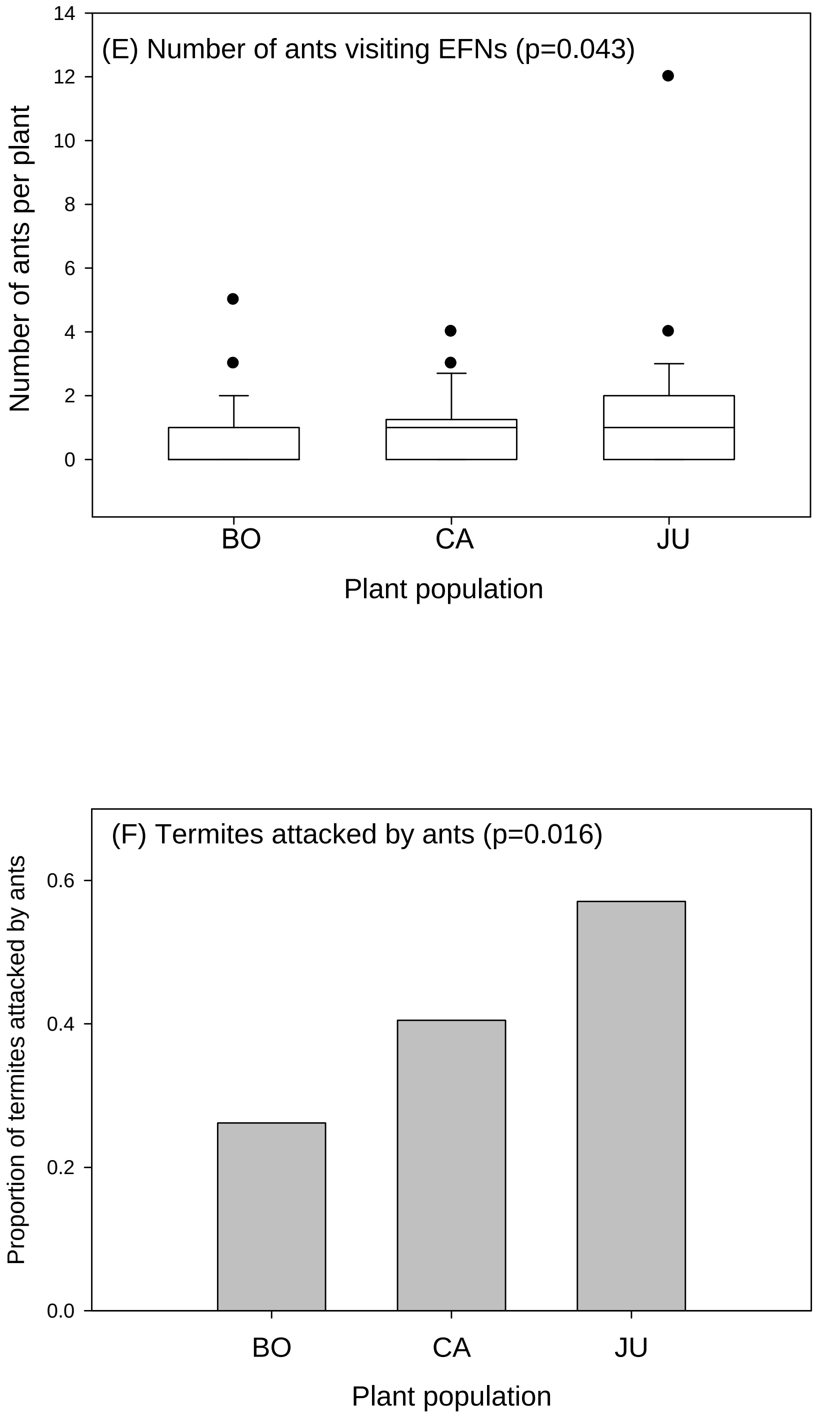

(E) Number of ants visiting EFNs (p=0.043)
Number of ants per plant
14
12
10
8
6
4
2
0
BO
CA
JU
Plant population
(F) Termites attacked by ants (p=0.016)
Proportion of termites attacked by ants
0.6
0.4
0.2
0.0
BO
CA
JU
Plant population

Fig 2 E and F

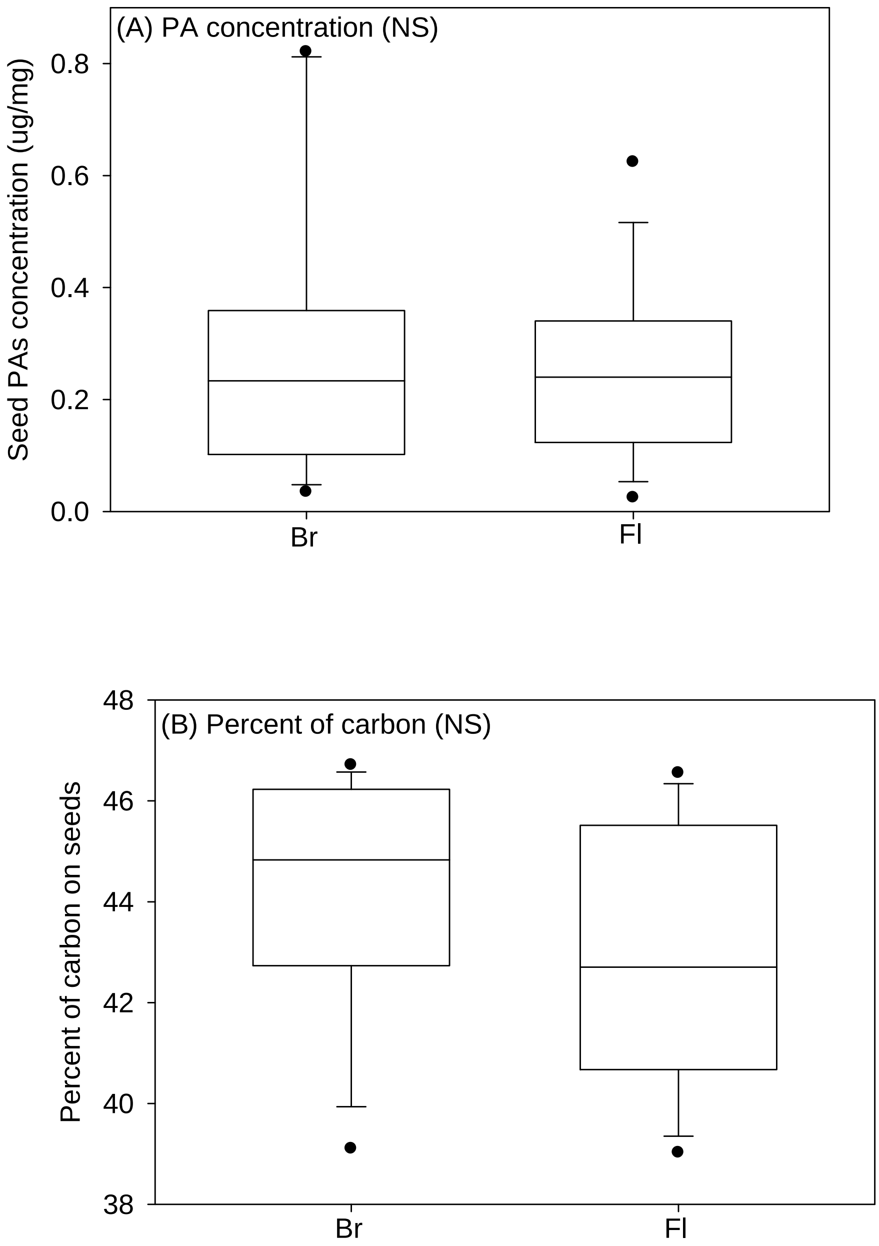

(A) PA concentration (NS)
Seed PAs concentration (ug/mg)
0.0
0.2
0.4
0.6
0.8
Br
Fl
(B) Percent of carbon (NS)
Percent of carbon on seeds
38
40
42
44
46
48
Br
Fl

Fig 3 A and B

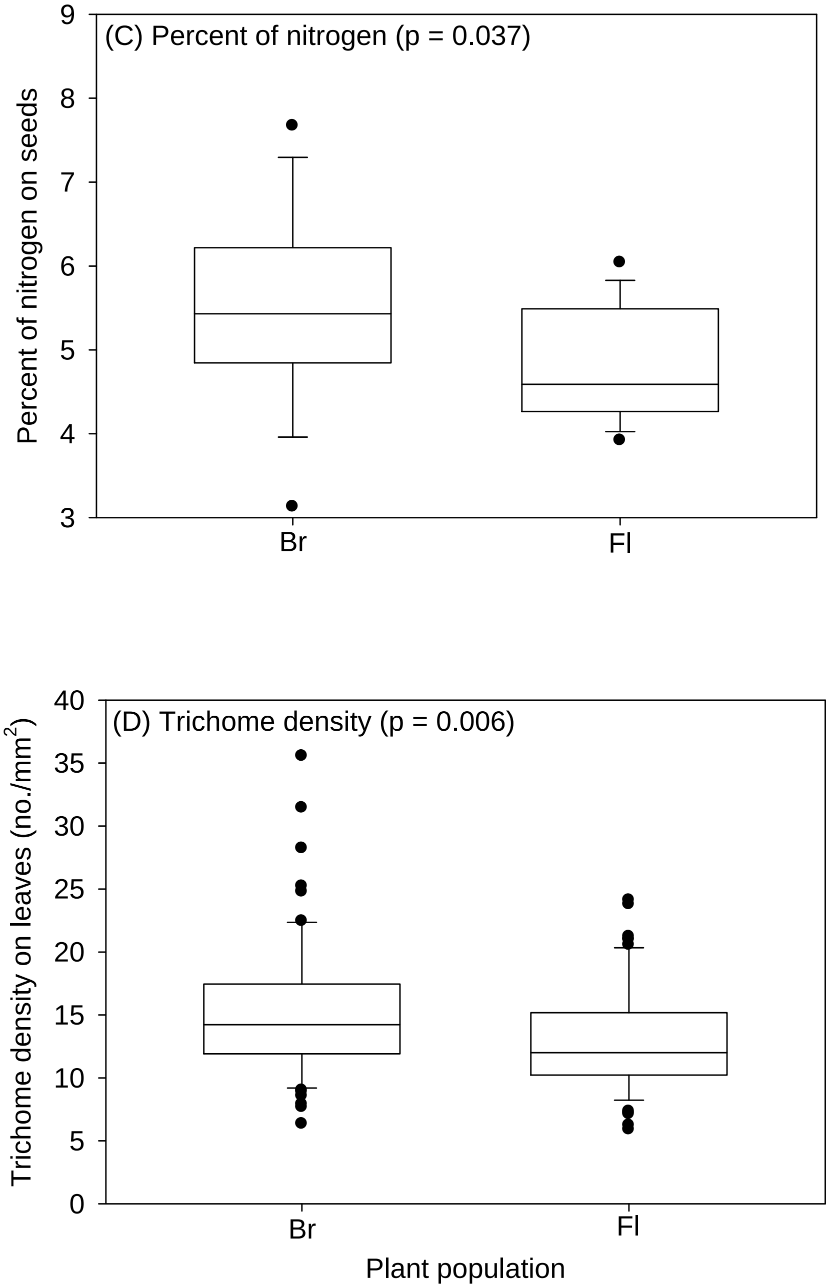

(C) Percent of nitrogen (p = 0.037)
Percent of nitrogen on seeds
9
8
7
6
5
4
3
Br
Fl
(D) Trichome density (p = 0.006)
Trichome density on leaves (no./mm^2)
40
35
30
25
20
15
10
5
0
Br
Fl
Plant population

Fig 3 C and D

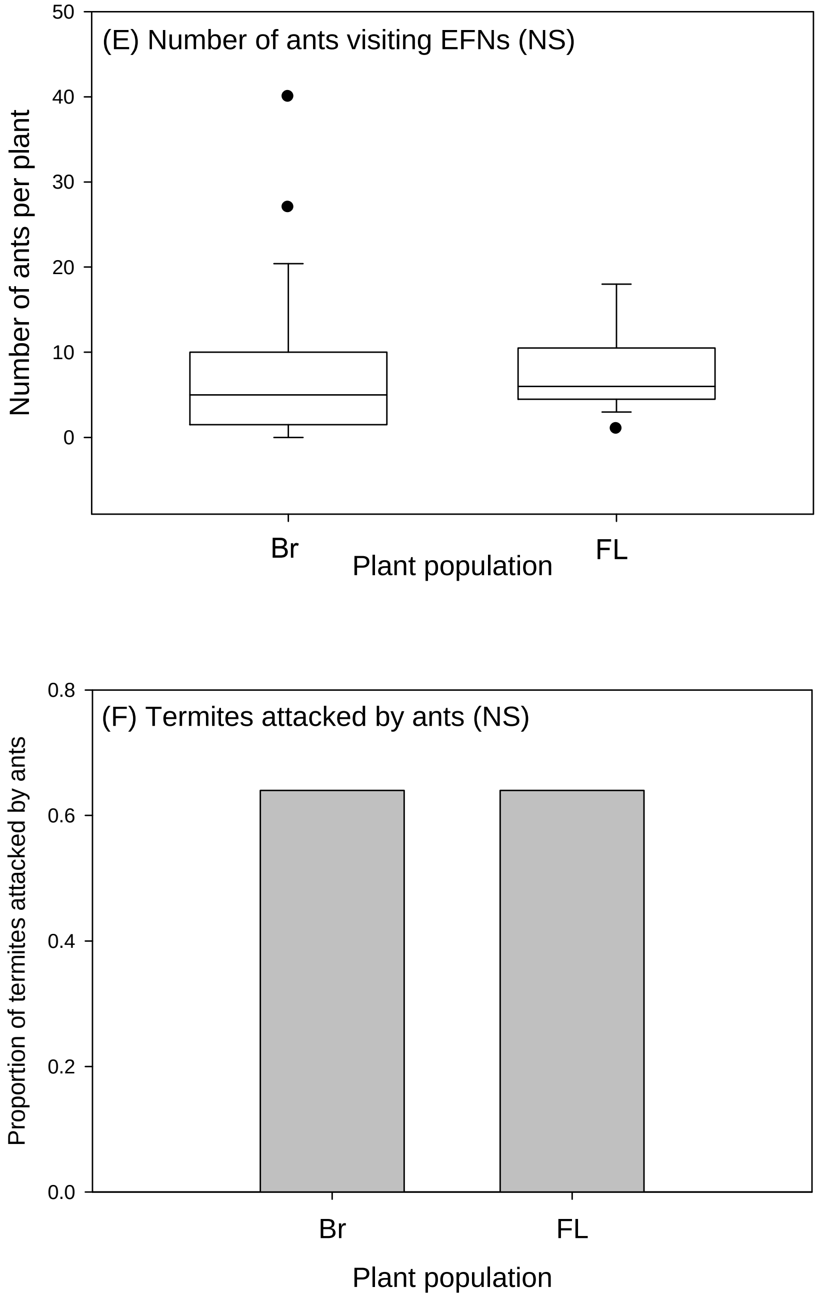
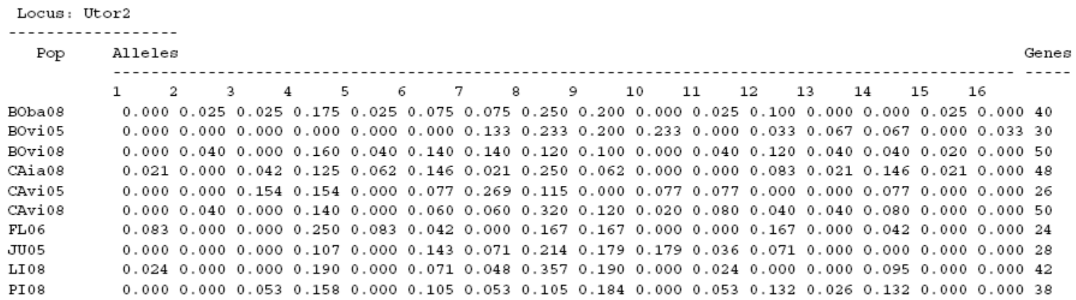
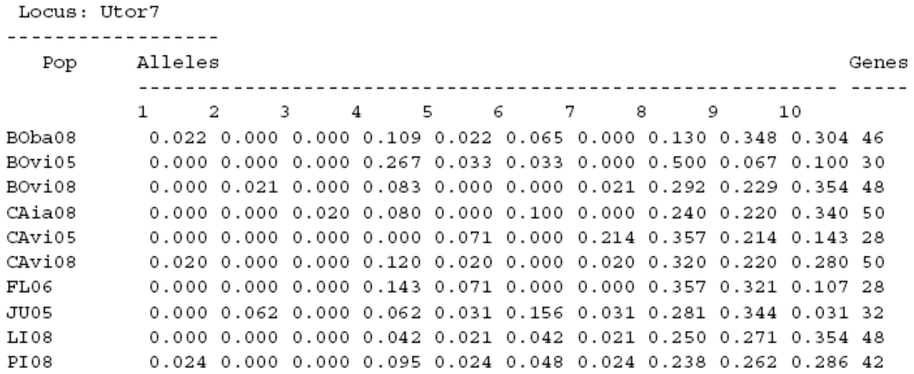


Figure 3 E and F

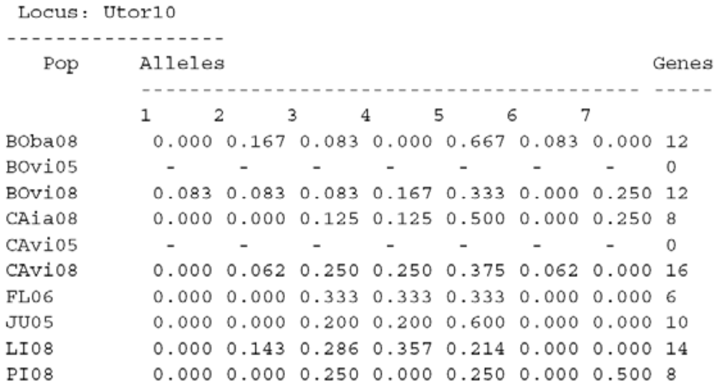
Appendices 1. Allele frequencies for each locus on each population for 5 microsatellite loci amplified in *Utetheisa ornatrix.*



```
 Locus: Utor2
------------------
   Pop     Alleles                                                                                         Genes
           ---------------------------------------------------------------------------------------------- -----
           1      2      3      4      5      6      7      8      9      10     11     12     13     14     15     16
BOba08      0.000 0.025 0.025 0.175 0.025 0.075 0.075 0.250 0.200 0.000 0.025 0.100 0.000 0.000 0.025 0.000 40
BOvi05      0.000 0.000 0.000 0.000 0.000 0.000 0.133 0.233 0.200 0.233 0.000 0.033 0.067 0.067 0.000 0.033 30
BOvi08      0.000 0.040 0.000 0.160 0.040 0.140 0.140 0.120 0.100 0.000 0.040 0.120 0.040 0.040 0.020 0.000 50
CAia08      0.021 0.000 0.042 0.125 0.062 0.146 0.021 0.250 0.062 0.000 0.000 0.083 0.021 0.146 0.021 0.000 48
CAvi05      0.000 0.000 0.154 0.154 0.000 0.077 0.269 0.115 0.000 0.077 0.077 0.000 0.000 0.077 0.000 0.000 26
CAvi08      0.000 0.040 0.000 0.140 0.000 0.060 0.060 0.320 0.120 0.020 0.080 0.040 0.040 0.080 0.000 0.000 50
FL06        0.083 0.000 0.000 0.250 0.083 0.042 0.000 0.167 0.167 0.000 0.000 0.167 0.000 0.042 0.000 0.000 24
JU05        0.000 0.000 0.000 0.107 0.000 0.143 0.071 0.214 0.179 0.179 0.036 0.071 0.000 0.000 0.000 0.000 28
LI08        0.024 0.000 0.000 0.190 0.000 0.071 0.048 0.357 0.190 0.000 0.024 0.000 0.000 0.095 0.000 0.000 42
PI08        0.000 0.000 0.053 0.158 0.000 0.105 0.053 0.105 0.184 0.000 0.053 0.132 0.026 0.132 0.000 0.000 38
```



```
 Locus: Utor7
------------------
   Pop     Alleles                                                     Genes
           ----------------------------------------------------------- -----
           1      2      3      4      5      6      7      8      9      10
BOba08      0.022 0.000 0.000 0.109 0.022 0.065 0.000 0.130 0.348 0.304 46
BOvi05      0.000 0.000 0.000 0.267 0.033 0.033 0.000 0.500 0.067 0.100 30
BOvi08      0.000 0.021 0.000 0.083 0.000 0.000 0.021 0.292 0.229 0.354 48
CAia08      0.000 0.000 0.020 0.080 0.000 0.100 0.000 0.240 0.220 0.340 50
CAvi05      0.000 0.000 0.000 0.000 0.071 0.000 0.214 0.357 0.214 0.143 28
CAvi08      0.020 0.000 0.000 0.120 0.020 0.000 0.020 0.320 0.220 0.280 50
FL06        0.000 0.000 0.000 0.143 0.071 0.000 0.000 0.357 0.321 0.107 28
JU05        0.000 0.062 0.000 0.062 0.031 0.156 0.031 0.281 0.344 0.031 32
LI08        0.000 0.000 0.000 0.042 0.021 0.042 0.021 0.250 0.271 0.354 48
PI08        0.024 0.000 0.000 0.095 0.024 0.048 0.024 0.238 0.262 0.286 42
```



```
 Locus: Utor10
------------------
   Pop     Alleles                                  Genes
           ---------------------------------------- -----
           1      2      3      4      5      6      7
BOba08      0.000 0.167 0.083 0.000 0.667 0.083 0.000 12
BOvi05        -     -     -     -     -     -     -   0
BOvi08      0.083 0.083 0.083 0.167 0.333 0.000 0.250 12
CAia08      0.000 0.000 0.125 0.125 0.500 0.000 0.250 8
CAvi05        -     -     -     -     -     -     -   0
CAvi08      0.000 0.062 0.250 0.250 0.375 0.062 0.000 16
FL06        0.000 0.000 0.333 0.333 0.333 0.000 0.000 6
JU05        0.000 0.000 0.200 0.200 0.600 0.000 0.000 10
LI08        0.000 0.143 0.286 0.357 0.214 0.000 0.000 14
PI08        0.000 0.000 0.250 0.000 0.250 0.000 0.500 8
```

```
Locus: Utor28
------------------
   Pop     Alleles                                                                  Gene
           ------------------------------------------------------------------------ ----
           1     2     3     4     5     6     7     8     9     10    11    12    13
BOba08      0.000 0.109 0.065 0.043 0.783 0.000 0.000 0.000 0.000 0.000 0.000 0.000 0.000 46
BOvi05      0.000 0.125 0.000 0.000 0.875 0.000 0.000 0.000 0.000 0.000 0.000 0.000 0.000 32
BOvi08      0.034 0.052 0.017 0.034 0.828 0.017 0.017 0.000 0.000 0.000 0.000 0.000 0.000 58
CAia08      0.020 0.040 0.040 0.040 0.740 0.080 0.000 0.000 0.020 0.020 0.000 0.000 0.000 50
CAvi05      0.000 0.000 0.000 0.067 0.933 0.000 0.000 0.000 0.000 0.000 0.000 0.000 0.000 30
CAvi08      0.019 0.019 0.058 0.038 0.846 0.019 0.000 0.000 0.000 0.000 0.000 0.000 0.000 52
FL06        0.033 0.000 0.000 0.033 0.900 0.033 0.000 0.000 0.000 0.000 0.000 0.000 0.000 30
JU05        0.143 0.071 0.000 0.000 0.536 0.036 0.000 0.000 0.036 0.000 0.071 0.036 0.071 28
LI08        0.068 0.000 0.045 0.045 0.818 0.000 0.000 0.023 0.000 0.000 0.000 0.000 0.000 44
PI08        0.000 0.000 0.114 0.000 0.818 0.068 0.000 0.000 0.000 0.000 0.000 0.000 0.000 44
```

```
 Locus: UtorTAC
------------------
   Pop     Alleles                                                               Genes
           ------------------------------------------------------------------ -----
           1     2     3     4     5     6     7     8     9     10    11    12
BOba08      0.043 0.304 0.043 0.152 0.000 0.304 0.065 0.022 0.000 0.022 0.022 0.022 46
BOvi05      0.156 0.375 0.000 0.219 0.000 0.250 0.000 0.000 0.000 0.000 0.000 0.000 32
BOvi08      0.086 0.293 0.034 0.241 0.017 0.138 0.121 0.017 0.034 0.000 0.000 0.017 58
CAia08      0.060 0.300 0.060 0.300 0.000 0.160 0.020 0.020 0.000 0.000 0.020 0.060 50
CAvi05      0.000 0.233 0.000 0.500 0.000 0.167 0.000 0.000 0.000 0.000 0.067 0.033 30
CAvi08      0.019 0.423 0.019 0.327 0.000 0.135 0.019 0.000 0.019 0.019 0.019 0.000 52
FL06        0.000 0.286 0.036 0.179 0.000 0.143 0.250 0.036 0.071 0.000 0.000 0.000 28
JU05        0.000 0.000 0.042 0.375 0.000 0.250 0.125 0.000 0.125 0.083 0.000 0.000 24
LI08        0.000 0.333 0.042 0.271 0.000 0.229 0.042 0.042 0.021 0.000 0.000 0.021 48
PI08        0.045 0.341 0.000 0.386 0.045 0.114 0.023 0.000 0.000 0.023 0.023 0.000 44
```

Chapter 5

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

ABSTRACT

Several specialist herbivorous insects have evolved adaptations to acquire protection against predators and attractiveness to mates by sequestration of defensive chemicals from their host plants. We used the specialist moth *Utetheisa ornatrix* feeding on plant defensive alkaloids to test for fitness costs associated with sequestration. We added alkaloids purified from plant material to an artificial diet at different concentrations. Larval and adult performance was not affected by alkaloid concentration in the diet, but the amount of alkaloids sequestered by individuals increased linearly with the diet alkaloid concentration. By showing that larvae are able to sequester 100-fold higher alkaloid concentration than normal without any significant negative effects on fitness, we can conclude that sequestration has no cost. A lack of costs has important implications for our understanding of the evolution of ecological interactions; for example, it implies that specialist herbivores may decrease the levels of chemical defenses on plant populations.

INTRODUCTION

The evolution of any particular trait is subject to a balance between the fitness advantages and the costs associated with it. It is usually assumed that there is a fitness cost for any trait that confers fitness advantages to an organism, such as protection against natural enemies or attractiveness to mates (Andersson 1994, Koricheva 2002, Lahti *et al.* 2009). In some specialist herbivorous insects, protection against predators and attractiveness to mates are due to sequestration of defensive chemicals from their host plants (Bowers 1992, Trigo 2000 and 2008, Nishida 2002, Conner & Weller 2004, Kuhn *et al.* 2004, Després *et al.* 2007). Sequestration is considered an important adaptation of herbivorous insects against the host defenses (Rausher 2001, Karban & Agrawal 2002) and has evolved in several insect lineages (Dobler 2001, Nishida 2002). Several studies have shown the advantages of sequestration (reviewed by Bowers 1992, Nishida 2002). However, to fully understand the evolution of this adaptation it is necessary to investigate possible fitness costs.

Previous attempts to investigate fitness costs of sequestration were limited by methodological challenges. First, it may be difficult to isolate plant chemicals as the only factor varying among different insect diets (Bowers 1992 and references therein). Second, in many systems it is difficult to directly measure fitness components (Camara 1997, Fordyce & Nice 2008). In this study we used a system suitable to overcome all these limitations: the arctiid moth *Utetheisa ornatrix* feeding on pyrrolizidine alkaloids (PAs).

Utetheisa ornatrix is a specialist moth that acquires PAs as larvae mainly from unripe seeds of the host plant *Crotalaria* spp. (Fabaceae: Papilionoideae) (Conner & Weller 2004, Ferro *et al.* 2006; Guimarães *et al.* 2006; Cogni & Futuyma 2009, chapter 3). PAs sequestered by the larvae are maintained in the pupal and adult stages (Del Campo *et al.* 2005). In the PA-specialist arctiid larvae *Creatonotos trunsiens* these alkaloids are absorbed as tertiary base in the gut, N-oxidized in the haemolymph and stored in the tissues; similar mechanisms may occur in other PA-specialist arctiids (Lindigkeit *et al.* 1997). In *U. ornatrix* PAs are transmitted from males to females as nuptial gifts, and from the female to eggs; the different ontogenetic stages use the PAs as protection against vertebrate and invertebrate predators (Del Campo *et al*. 2005). *U. ornatrix* males also modify PAs into courtship pheromone (Iyengar & Eisner 1999). 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 (Iyengar & Eisner 1999).

In this study our goal is to look for possible fitness costs of PA sequestration by *U. ornatrix*. We purified large amounts of PAs from plant material and added these purified PAs at different concentrations to an artificial diet. We fed larvae during their entire development on the different PA concentrations and measured larval and adult fitness components, and the amount of PAs sequestered by individual moths. We found no negative effect in performance even at high levels of sequestration. To the best of our knowledge, this is the first study to clearly show a lack of costs in sequestration of a plant chemical defense by an herbivorous insect.

METHODS

General design and larval performance

PAs were extracted and identified from leaves and flowers of *Senecio brasiliensis* (Asteraceae) as in Trigo et al. (1993) and presented 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 *ca.* 85% and integerrimine *ca.* 15%), the most common *U. ornatrix* host. Other *Crotalaria* species, such as *C. incana*, with integerrimine as the main PA are also used as a host plant by *U. ornatrix* in the Neotropics (J.R. Trigo pers. comm.). We used *S. brasiliensis* as a PA source since the yield of these alkaloids is higher than in *C. pallida* seeds. 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^{o}C and mixed in a blender. The average concentration of PAs in green seeds of *C. pallida* is 0.024% dry weight (Ferro *et al.* 2006). We used five concentrations in treatments: 0.024% (1X) PAs added, 0.0048% (0.2X), 0.12% (5X), 2.4% (100X) and a control without PA (0X). *U. ornatrix* adults were collected at Archbold Biological Station in central Florida, US. We kept large moth colonies in the laboratory. Larvae were fed on an artificial diet based on *Phaseolus* beans as above with no PAs added. Adults were kept in paper cages (*ca.* 3.2 liters) where 5% honey solution was provided. To avoid maternal and paternal effects (because eggs are endowed with PAs) all *U. ornatrix* eggs used in the experiments were from adults that had been in the laboratory on a PA-free diet for at least one generation. Sample size for each treatment was 102 for 0%, 108 for 0.0048%, 110 for 0.024%, 113 for 0.12%, and 150 for 2.4%.

All 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 three 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. Pupal weight correlates with adult weight and adult fitness in *U. ornatrix*; larger females lay more eggs and large males attract more females to mate (Iyengar & Eisner 1999).

PA quantification

Twenty adults per treatment were 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 recovered and combined, and evaporated to dryness under vacuum at 45°C before resuspension in 1.5ml of ethanol. Total PAs were quantified by the colorimetric method as in Trigo *et al*. (1993). Three replicate readings were performed for each individual. Dixon's Q-test was used to detect possible outliers among the three replicated spectrophotometer readings. Retrorsine extracted from *Senecio brasiliensis* was used for the calibration curve. Absorbance values lower than 0.020 (representing less than 2µg of PA per replicate, 6µg per individual moth) were considered as no PA detected. For *U. ornatrix* we could not detect PAs (absorbance values greater than 0.02) on 16 individuals that fed on the diet with the two lower PAs 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).

Adult fitness

We carried out this experiment to test the effect of larval feeding on the higher 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 (1X), and the 2.4% (100X). After these larvae pupated, emerging adults were divided into four treatments in which one male and one female were paired: (1) 0.024% PA male with 0.024% PA female, (2) 0.024% PA male with 2.4% PA female, (3) 2.4% PA male with 0.024% PA female, and (4) 2.4% PA male with 2.4% PA female. Sixteen couples were used per treatment. Each couple was kept in a paper cage (*ca.* 3.2 liters) 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.

Statistical analyses

The proportion of larvae that survived on the PA concentration treatments was compared as in Zar (1996, p. 562), followed by a comparison of each proportion to the proportion of survived larvae on the control (Zar 1996, p. 565). We tested the effect of dietary PA concentration, moth sex, and their 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 ANOVA 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.

RESULTS

Larval survival was affected by the PAs 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 1). The other response variables were not different between the sexes (table 1), and there was no significant interaction between moth sex and PAs concentration on any of the variables measured (table 1).

The amount of PAs sequestered by adult moths was greatly influenced by the PA concentration on the diet (fig. 1). We were unable to detect PAs on the moths that fed on the two lower PA concentrations (0%, 0.0048%). For the other three concentrations the amount of PAs sequestered, and the PA concentration of adult moths greatly increased with increasing PA concentration on the diet (fig. 1, table 1).

Male and female longevity did not depend on the diet that the larva was raised on or on the diet that the partner was raised on (tables 3 and 4). In addition, fecundity and egg viability were not affected by the diet that the female or the male was raised on (tables 3 and 4).

DISCUSSION

By showing that larvae are able to sequester 100-fold higher PAs than normal without any significant negative effects on fitness, we can conclude that sequestration in this species has no cost. The only negative effect of PAs occurred at the 100-fold PAs with larvae growing slower, but due to a longer development time these larvae achieved the pupal stage at similar sizes as larvae feeding at the lower PAs concentrations. However, this difference is unlikely to significantly affect *U. ornatrix'*s fitness; this species mates around the year and larvae are protected against predators mechanically by feeding inside the pod and chemically by the sequestration of PAs (Ferro *et al.* 2006). Moreover, parasitoids are extremely rare in the field.

The lack of costs reported here may be related to a key innovation of arctiid moths: the biochemical mechanisms of PAs detoxification. Arctiid moths that sequester PAs can avoid the formation of toxic products by oxidizing the absorbed pro-toxic free base PAs into the non-toxic N-oxide (Hartmann 2004). This reaction is performed by a NADPH-dependent mono-oxygenase enzyme present in the hemolymph (Lindigkeit *et al.* 1997; Naumann *et al.* 2002). Our results suggest that this enzyme is able to oxidize much higher amounts of PAs than normally encountered in nature.

A few previous studies have reported evidence of no costs 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 costs (Cohen 1985, Bjorkman & Larsson 1991, Bowers & Collinge 1992, Camara 1997, Fordyce & Nice 2008). However, some studies do not isolate the plant chemical from the food provided to the insects (Cohen 1985, Bjorkman & Larsson 1991, Bowers & Collinge 1992, Kelley *et al.* 2002, Del Campo *et al*. 2005), do not control for maternal effects through the endowment of sequestered plant chemicals from the female to the eggs (Fordyce & Nice 2008), or do not measure fitness directly (Camara 1997, Fordyce & Nice 2008). To fully address possible fitness cost associated with sequestration it is necessary to isolate the plant chemical and provide it at different concentrations in the herbivore diet, feed the insect at the different concentrations during the entire development (because insects grow exponentially and neonate larvae may be more susceptible to plant defenses), quantify the amount of the chemical sequestered by the insect, and then measure several larval and adult fitness components, as done here.

The lack of costs for chemical sequestration in herbivorous insects has important implications. First, a basic assumption of the plant-herbivore literature, and more broadly the ecology, evolution and behavior literature, is a trade-off in organism's investments in defense, reproduction and growth (Andersson 1994, Koricheva 2002). However, if herbivores can sequester protective chemicals from the host plant without a cost, as reported here, this trade-off is not expected. Second, this lack of cost for the herbivore causes an important asymmetry in coevolution. 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 coevolution, including local adaptation, geographical mosaic, arms-races and Red Queen hypotheses assume costs of parasites' adaptations to overcome host defenses (Bergelson *et al.* 2001). However, if an absence of costs is common in other host-parasite interactions, this fact must be incorporated into these models.

Fourth, our results have important implications for how specialist herbivores act as agents of natural selection on the levels of chemical defenses of their host plants. As a rule, specialist herbivores are less affected by plant chemical defenses than generalists (van der Meijden 1996, Cornell & Hawkins 2003). For example, PAs have toxic and deterrent effects on non-adapted generalists (van Dam *et al.* 1995, Macel *et al.* 2005, Narberhaus *et al.* 2005). Additionally, we have results (to be submitted elsewhere) demonstrating that PAs affect survival of the generalist herbivore *Heliothis virescens*. However, specialists can also be strongly affected by plant chemicals to which they are adapted (Agrawal & Kurashige 2003 and references therein). In this sense our results are unexpected because we clearly show no negative effect of PAs for this specialist herbivore. In addition, our results are remarkable because the sequestration of PAs has a clear advantage for the herbivore. Therefore, the herbivore is not only unaffected by a plant chemical, but takes advantage of it without cost. Because there is a cost-free advantage in sequestering higher amounts of plant chemicals, it is advantageous for the herbivore to use individual plants with higher PAs concentrations. As a result, specialist herbivores can act as natural selection agents that decrease the level of chemical defenses in plant populations. Indeed, the lack of a specialist herbivore on introduced populations of the weed *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). Fifth, under this scenario no escalation of plant defenses is expected (Vermeij 1994); in fact, specialist herbivores may cause phylogenetic decline in defenses. 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 on basal clades; these acids are sequestered by specialist Troidini butterflies (Brown *et al.* 1991). Thus, cost-free sequestration of plant chemical defenses by herbivorous insects has important implications for our understanding of the evolution of ecological interactions.

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Tables

Table 1. Effect of diet pyrrolizidine alkaloids (PAs) concentration, moth sex and the interaction on diet consumed, larval weight, development time, pupal weight, adult weight, total PAs sequestered by adult moths, and PAs concentration on adult moths.

Source	df	F-ratio	P
Diet consumed			
PA concentration	4	0.209	0.933
Sex	1	0.955	0.330
PA concentration x 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 x Sex	4	0.504	0.733
Error	155		
Development time			
PA concentration *	40	15.343	<0.001
Sex *	1	6.524	0.012
PA concentration x 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 x 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 x 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 x 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 x Sex	2	0.893	0.415
Error	56		

Note: *Utetheisa ornatrix* were fed from hatching to pupation on artificial diet with five different pyrrolizidine alkaloid (PAs) concentrations. * indicates factors with significant effect.

Table 2. Effect of diet pyrrolizidine alkaloids (PAs) concentration on *Utetheisa ornatrix* larval fitness components.

PAs concentration on diet	**Diet consumed (g)**	**Larval weight (g)***	**Development time (days) ***	**Pupal weight (mg)**	**Adult dry weight (mg)**
0%	0.36 ± 0.43	0.08 ± 0.03	43.5 ± 4.8	83.1 ± 34.7	16.9 ± 11.8
0.0048%	0.41± 0.45	0.08 ± 0.03	42.0 ± 3.5	93.4 ± 35.9	18.3 ± 11.3
0.024%	0.36 ± 0.46	0.07 ± 0.03	44.2 ± 4.7	80.7 ± 31.2	16.0 ± 10.2
0.12%	0.30 ± 0.36	0.07 ± 0.03	43.9 ± 5.3	71.9 ± 28.3	14.5 ± 9.7
2.4%	0.35 ± 0.27	0.05 ± 0.03	50.2 ± 11.0	84.3 ± 29.6	15.7 ± 6.7

Note: Larvae were fed from hatching to pupation on artificial diet with five different (PAs) concentrations. Values are means ± SD. * indicates variables that significantly varied among the treatments (effect of diet PAs concentration on ANOVA tests).

Table 3. Effect of diet pyrrolizidine alkaloids (PAs) concentration that males were raised on, that females were raised on, and the interaction on male longevity, female longevity, fecundity, and egg viability.

Source	df	F-ratio	P
Male longevity			
Male diet	1	1.046	0.331
Female diet	1	0.097	0.757
Male diet x 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 x 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 x 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 x Female diet	1	0.145	0.705
Error	52		

Table 4. Effect of larval diet pyrrolizidine alkaloids (PAs) concentration on *Utetheisa ornatrix* adult fitness components.

Larval diet of the couple		**Male longevity (days)**	**Female longevity (days)**	**Fecundity (# eggs)**	**Egg viability**
Female	**Male**				
0.024%	**0.024%**	33.8 ± 11.8	31.3 ± 15.4	133 ± 113	0.82 ± 0.12
0.024%	**2.4%**	32.9 ± 10.4	29.8 ± 12.3	103 ± 95	0.82 ± 0.13
2.4%	**0.024%**	34.9 ± 10.8	26.1 ± 11.8	103 ± 126	0.85 ± 0.14
2.4%	**2.4%**	30.0 ± 11.9	29.3 ± 13.2	120 ± 100	0.86 ± 0.07

Note: Values are means ± SD.

Figure legends

Figure 1. Effect of diet concentration of pyrrolizidine alkaloids (PAs) on PAs concentration in adult *Utetheisa ornatrix*. Bars represent mean values + SD. P value indicates the effect of PAs concentration on ANOVA test.

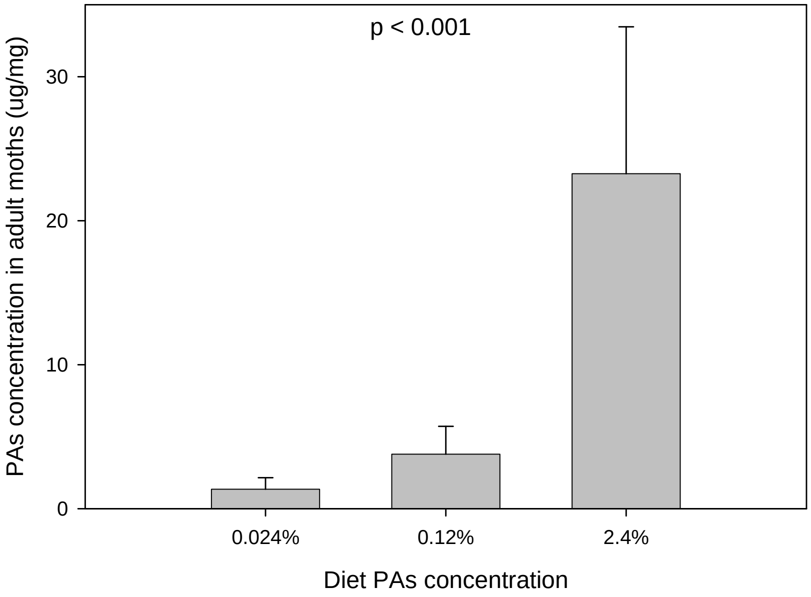
p < 0.001
PAs concentration in adult moths (ug/mg)
0
10
20
30
0.024%
0.12%
2.4%
Diet PAs concentration

Figure 1

Chapter 6

General conclusions

Coevolution is a central concept in evolutionary theory (Thompson 2005), and the interactions between plants and herbivorous insects have been used extensively in empirical studies of coevolution (Ehrlich & Raven 1964, Rausher 2001). Although there are many studies showing that herbivores have adaptations to overcome plant defense traits, and that herbivores represent an important selection pressure on plant defense traits, most of these studies do not show the two sides of the processes occurring on the same system (Rausher 2001). Additionally, several studies were based on the incorrect assumption that correlations indicate coevolution (Nuismer *et al.* 2010). To unravel coevolutionary dynamics and to demonstrate reciprocal coevolutionary selection a thorough story of the specific interaction is necessary. In this dissertation my aim was to start to develop a thorough story of the coevolution between *U. ornatrix* and *C. pallida* using an integrative approach. My studies of this interaction confirmed the suitability of the system to integrative multidisciplinary approaches. In addition, my results have important implications to our understanding of coevolution, and provide a foundation for future studies aiming to develop a complete story of coevolutionary dynamics in the system.

First, I investigated how the history of a host introduction may affect coevolutionary dynamics. In chapter 2, I showed that *U. ornatrix* caused much higher seed predation rates on the introduced host (*C. pallida*) than on the native host (*C.*

incana) in the field. Females also preferred to oviposit on the introduced over the native host, and larvae feeding on the introduced host had higher fitness (higher pupal weight) than larvae feeding on the native host.

Second, I investigated patterns of local adaptation at different geographical scales. In chapter 3, I used greenhouse experiments and I demonstrated local adaptation of the moth to its host plant populations at a continental scale (populations from Brazil and Florida), but not at a local scale (populations ca. 150 km apart). My results also indicated that even at the local scale both the moth and plant populations were differentiated.

Third, in chapter 4 I investigated differences among populations in factors that may be responsible for the patterns of local adaptation at different scales reported on chapter 3. I found differences among populations in specialist and generalist herbivore pressure. I also found genetic differences among populations in plant defense traits, such as such as the concentration of alkaloids and the attractiveness of defensive ants to extra-floral nectaries. These differences were observed even at the local scale in Brazil, confirming the first indication of population differentiation found in chapter 3. I also used microsatellite markers to investigate population structure of the moth. I found weak but significant differentiation among moth populations in 2005, but no significant differentiation in 2008. These results suggest that temporal changes in the population structure of *U. ornatrix* are the most likely cause for the lack of local adaptation at the regional scale reported in chapter 3.

An important result of this dissertation is that PAs are not an effective defense against the specialist *U. ornatrix*. I initially believed that *U. ornatrix* local adaptation observed at the continental scale was driven by differences in PAs between Florida and Brazilian *C. pallida* populations. However, in chapter 4 I showed that PAs did not vary between Brazil and Florida. In addition, there is variation in PA concentration within Brazil, where no local adaptation was found. Additionally, in chapter 5 I showed that PAs do not negatively affect *U. ornatrix* performance, and that sequestration of PAs has no fitness cost. Therefore, the patterns of local adaptation are driven by other possible resistance traits not yet measured. Possible candidates include isoflavonoids, non-proteic amino acids, and proteinase inhibitors. Future studies should investigate what resistance traits are responsible for the patterns of local adaptation.

Fourth, in chapter 5 I combined chemical ecology techniques with extensive laboratory experiments to study the counteradaptation of the moth to the host-plant defensive alkaloids. Specifically, I tested for possible fitness costs of alkaloid sequestration. I showed that larvae are able to sequester 100-fold higher alkaloids than normal without any significant negative effects on larval or adult fitness. These results indicate a lack of costs in sequestration of a plant chemical defense by an herbivorous insect. These unexpected and novel results have important implications to our understanding of coevolution. First, plant production of chemical defenses has a cost, causing an important asymmetry in coevolution. Second, theoretical models in coevolution assume costs of parasites' adaptations to overcome host defenses. Third, because for the herbivore there is a cost-free advantage in sequestering higher amounts of the chemical defense, specialist herbivores can act as natural selection agents that decrease the level of chemical defenses in plant populations. Fourth, this selection for decreased chemical defenses may lead to patterns of phylogenetic decline in defenses, and not escalation, as generally assumed in the coevolution literature (Agrawal & Fishbein 2008). And finally, the lack of costs may be an important factors favoring specialization in herbivorous insects and contributing to the diversification of the most species rich group of animals on Earth.

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