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Hiding in Plain Sight: Cuticular Compound Profile Matching Conceals a Larval Tortoise Beetle in its Host Chemical Cloud

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Abstract Larvae of tortoise beetles are postulated to have fecal shields as the main defensive strategy against predators. Such a device protects beetles both physically and chemically. In order to examine how larvae *Chelymorpha reimoseri* are protected against predatory ants, which frequently visit extrafloral nectaries in their host plant, the morning glory *Ipomoea carnea*, we conducted anti-predation bioassays with live 5th instars. In the field, larvae in contact with ants had survival between 40 and 73 %, independently of shield presence. In the laboratory, when exposed to *Camponotus crassus*, larvae with shields had significantly higher survival (85 %) than those without shields (64 %). In both scenarios, larval survival was significantly higher when compared with palatable *Spodoptera frugiperda* larvae, as the latter were all consumed. We also observed that when *C. reimoseri* larvae showed no movement, the ants walked on them without attacking. We hypothesized that if the larval integument has a pattern of cuticular compounds (CCs) similar to that of its host plant, larvae would be rendered chemically camouflaged. In the field and laboratory, the freeze-dried palatable larvae of *S. frugiperda* treated with CCs of 5th instar *C. reimoseri* and left on *I. carnea* leaves were significantly less removed by ants than controls without these compounds. We also found a similarity of approximately 50 % between the CCs in *C. reimoseri* larvae and *I. carnea* host leaves. Both findings provide evidence in support of the hypothesis that chemical camouflage plays an important role in larval defense, which is reported for the first time in an ectophagous leaf beetle larva.

Keywords Cassidinae · Chemical camouflage · Chemical defense · Fecal shields · Multi trait defense · Predatory ants

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Introduction

Since the Devonian period, host plants, herbivorous insects, and their natural enemies have been in a continuous evolutionary "arms race" (Labandeira 2002). The never-ending adaptations and counter-adaptations among three trophic levels are responsible for the astonishing diversity of defenses observed in both plants and their herbivorous insects (Price et al. 2011). To cope with predator attacks, insects have developed a vast array of defensive strategies, varying from avoiding detection through visual camouflage to deceiving predators by resembling unpalatable species (Ruxton et al. 2004).

To date, multi-defensive traits is an unexplored issue for herbivorous insects, but well studied in plants as they suffer attacks by several pathogens and herbivores (Walters 2011). As these herbivores are under predation pressures exerted by different types of predators, multiple defensive traits might be widespread. For example, the presence of dual defensive chemicals has been reported in Danaini, Ithomiini, and Heliconiini butterflies (Opitz and Müller 2009). The genus *Danaus* sequesters cardenolides from its larval host plants and transfers them to adults, which also sequester pyrrolizidine alkaloids. Similarly, the Ithomiini *Placidina euryanassa* sequesters tropane alkaloids as larvae, and pyrrolizidine alkaloids as adults. Some *Heliconius* species have been shown to have cyanogenic glycosides and carboline alkaloids in their tissues. Whether these chemicals show different activities against different types of predators is unknown.

Larvae of tortoise beetles are a good example of the diversity of defensive strategies. The more basal Hispini have a concealed feeding style that may protect them against predators, while more derived species demonstrate the use of a fecal shield, gregariousness with cycloalexy, or even maternal care as a defense against predator attack (Chaboo 2007; Vencl et al. 2011). Fecal shields have been reported, since Eisner's work in the 1960s (Eisner et al. 1967), as the main defense strategy against predators for tortoise beetle larvae. Since that time, a considerable number of studies using various experimental designs have reported evidence of the protective role of shields against predators (see Müller and Hilker 2004 and references therein). The shields provide a physical defense and may contain chemicals originated from the host plant, as well as derivatives of these compounds produced by the larvae (see Vencl et al. 2005, 2009 and references therein). Recently, Vencl et al. (2011) showed that together with chemical of shields, other defensive traits, such as larva gregariousness, cycloalexy, and maternal care, might enhance the weaponry of tortoise beetle larvae against predators.

Additionally, chemicals of the larval integument also act as a defense against predators. However, this issue is rarely approached. There is a single report in the literature: the larvae of *Chelymorpha reimoseri* feeding on the extrafloral nectaried morning glory *Ipomoea carnea*. In laboratory bioassays, Bottcher et al. (2009) found that larvae of this tortoise beetle species suffered low natural predation, and were protected against predation by *Ca. crassus* ants and chickens *Gallus gallus*, regardless of shield presence. Such a protection was conferred by unknown chemicals, which are present in the larval integument.

Chelymorpha reimoseri larvae might suffer a strong selective pressure by predatory ants that visit *I. carnea* extrafloral nectaries (hereafter EFNs) (Steward and Keeler 1988). This may shape the defensive mechanisms in *C. reimoseri* larvae apart from shield presence, as posed by Bottcher et al. (2009). Using this approach, we tested the hypothesis that *C. reimoseri* larvae may be protected against chemically oriented ants due to chemical camouflage (see Ruxton 2009, for a review), i.e., the larvae would have a cuticular chemical profile similar to its host plant *I. carnea*. Therefore, the ants would not be able to distinguish them from *I. carnea* leaves, the background where the larvae lie. We adopted the definitions of Vane-Wright (1976), where camouflage involves the simulation by organisms of background or uninteresting objects, or forms, i.e., the frame of reference in which the operator searches for things of importance. Larvae of *C. reimoseri* have characteristics of a model (*I. carnea* leaf chemistry) that is not of interest to the operator, predatory ants, thereby camouflaging itself against these predators. This defensive mechanism is employed by other phytophagous insects, such as treehoppers and the larvae of butterflies and moths, that defend against predation by ants (Akino et al. 2004; Akino 2005; Portugal and Trigo 2005; Silveira et al. 2010). This defensive strategy also is called chemical phytomimesis (see Akino et al. 2004; Akino 2005, 2008).

To test the above hypothesis, we first verified if ants have a negative impact on *C. reimoseri* larvae with and without shields by employing both field and laboratory bioassays. If our hypothesis were correct, we predicted that ants would not have a high impact on larval survivorship, regardless of shield presence, because the larvae would be chemically camouflaged. Next, we investigated, through field and laboratory bioassays, if the cuticular compounds of *C. reimoseri* larvae can hinder what ants perceive as prey. We predicted that palatable larvae treated with the cuticular compounds of *C. reimoseri* larvae, and placed on the host plant *Ipomoea carnea*, would not be perceived as prey by chemically oriented ants, contrarily to untreated controls, which would be detected and preyed upon. We also predicted that these treated larvae would be preyed upon when placed on non-host plants. We also tested these cuticular compounds against chickens that are not chemically oriented, and we expected that these compounds would have no anti-predatory activity. Lastly, we examined the similarity of the cuticular profile between *C. reimoseri* larvae and the leaves of its host plant, *I. carnea*, predicting that it would be higher when compared with non-host plants.

Methods and Materials

Tortoise Beetles and Host Plants The neotropical tortoise beetle *Chelymorpha reimoseri* Spaeth (Mesomphalini) (=Stolaini; the tribe designation follows Świętojańska 2009) is known to feed on the leaves of a single host plant, *Ipomoea carnea* subsp. *fistulosa* (Mart. ex Choisy) Austin (Convolvulaceae) (hereafter *Ipomoea carnea*), both as larvae and as adults (Vasconcellos-Neto 1988; Vasconcellos-Neto and Jolivet 1988) (Fig. 1a–d). Eggs are laid on both sides of the leaves, in clusters ranging from 30 to 100 eggs. The larvae are gregarious, presenting defensive cycloalexy behavior in all instars (Vasconcellos-Neto 1988; Vasconcellos-Neto and Jolivet 1988). The larvae later disperse at the end of the 5th instar to pupate on the trunk base of the host plant or on other plants near the host.

Ipomoea carnea is a perennial shrub widely distributed in South and Central America, as well as the Caribbean (Austin and Huáman 1996). In the Pantanal, South America's largest wetland located in midwest Brazil, dense stands of the 2–3-m-tall *I. carnea* occur in open non-shaded areas subject to shallow seasonal flooding lasting up to six months (Haase 1999; Heckman 1998). *Ipomoea carnea* presumably is well-defended against herbivores due to chemicals such as polyhydroxyalkaloids (Haraguchi et al. 2003) and EFNs that attract predators (Steward and Keeler 1988).

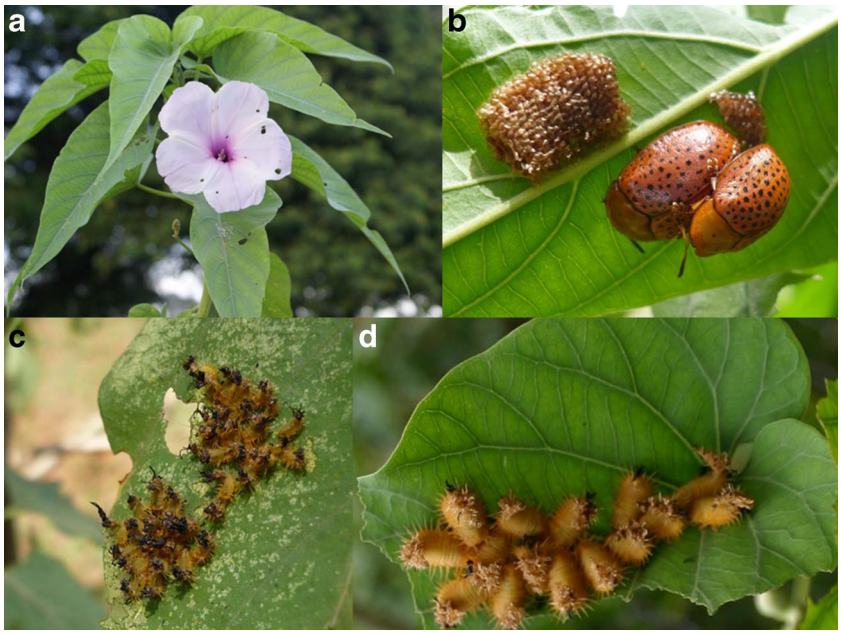

Fig. 1 *Ipomea carnea* (**a**), adults and egg clusters (**b**), gregarious 2nd instar (**c**), and 5th instars (**d**) of *Chelymorpha reimoseri*

Field Bioassays Testing Tortoise Beetle Larval Defenses against Ants We bioassayed live *C. reimoseri* 5th instars against ants at a Pantanal savannah wetland, Corumbá municipality, Mato Grosso do Sul State, midwest Brazil, in a 70 m^2 section where *I. carnea* and tortoise beetles are abundant (19°33′42.90″S 57°02′17.80 W). Although we had never previously found *C. reimoseri* in the sample area in Pantanal, it has been found near, in Argentina (Bachmann and Cabrera 2010; Borowiec 1999). We presume that this species is native to this biome because its host *I. carnea* is native to this area of the Pantanal. In addition, we conducted bioassays in a small-disturbed fragment of the Atlantic rain forest near the Department of Animal Biology at UNICAMP, in Campinas, São Paulo State, southeastern Brazil (22°49′16″S, 47° 04′08″W), where *I. carnea* was introduced approximately three decades ago and where there is an established population of *C. reimoseri* (Vasconcellos-Neto 1988; Vasconcellos-Neto and Jolivet 1988). The license for research with wild animals was given by IBAMA-ICMBio (Ministério do Meio Ambiente, Brasil).

For the bioassay at the Pantanal area, we brought *C. reimoseri* larvae from the Campinas site. We chose five adult individuals of *I. carnea* (1.0–2.0 m tall), that were found at least 3 m apart. We are not sure whether these individuals were genets or ramets from the same genet because this plant can form stolons from which new plants develop. In each individual, we assigned four branches with at least five fully expanded leaves. We assigned two branches for placing larvae with shields, and the remaining two were designated for the larvae that had their shields removed. Within these two groups of two branches, one was assigned to exclude all predators, and the other in which free access of ants only was allowed. By employing this design, we prevented interference by other flying predators, such as wasps and birds, in the survival of the herbivorous insects. We excluded ants by applying a Tree Tanglefoot Pest Barrier (Contech Enterprises, Inc., BC, Canada) around the base of each stem, as well as from flying predators by using bags made with a tulle netting fabric (2× 2 mm mesh) to cover the branches. To allow free access for ants only, we covered the branch with a net, as above, but we allowed the ants to walk freely from outside to inside the net by covering the branch with a plastic tube (7.0 cm long, 0.9 cm internal diam) tied between the branch and the net. In each treatment, we placed three 5th instars on a fully expanded leaf. We used three individuals to simulate the gregarious habit. On the following day, the number of surviving larvae was recorded in all treatments, as well as the species of patrolling ants. We identified the ant species according to Hölldobler and Wilson (1990) and Fernández (2003). Bioassays were carried out at Pantanal in March 2012, and similar procedures were used for the bioassays at Campinas municipality in April 2012, also by using five individuals of *I. carnea*.

Laboratory Bioassays Testing Tortoise Beetle Larval Defenses against Ants In the laboratory, we bioassayed 5th instar *C. reimoseri* larvae with intact and removed shields against the carpenter ant *Ca. crassus* (Formicinae), which visit *I. carnea* EFNs in both the Pantanal and Campinas areas. This ant species generally nests in live trees and in dead and

decaying logs (Kusnezov 1951). However, during the flood season, the entire colony moves upwards on *I. carnea* plants, whose workers stay together on large leaves (K.F. Massuda and J.R. Trigo, personal observations). Because other ants, like *Ca. crassus*, visit EFNs and nest on this plant, generalist predatory ants likely represent a major defense component directed against herbivores such as *C. reimorseri*.

We collected *Ca. crassus* colonies from Núcleo de Pesquisa Reserva Biológica de Mogi Guaçu, Instituto Botânico, Mogi Guaçu country, São Paulo State, Brazil (22°18′S, 47°10′W). In the laboratory, we kept the colonies in a plastic container (26×32×8, width × depth × height) in which the walls were treated with talcum powder glued to the surface with liquid soap to prevent the ants from escaping. Ant colonies were placed in a room with ambient temperature and photoperiod conditions. To provide a location for ant nesting, we placed one test tube (20 cm long, 2 cm diam) inside each container. The test tubes contained wet cotton in the bottom, and were covered with red plastic film. We fed the colonies a 20 % honey-water solution daily and one palatable noctuid moth 3rd instar *Spodoptera frugiperda* weekly. We did not control the number of ants in each colony; instead, we controlled larval removal by ants regardless of ant abundance. Two days before each bioassay began, we offered one freeze-dried 3rd instar palatable *S. frugiperda* attached to the adaxial surface of the *I. carnea* leaves with a cyanoacrylate-based fast-acting adhesive (Super Bonder®, Henkel Brasil) for each the colony. The leaf petiole was dipped into a 50-ml Erlenmeyer vial with water to prevent desication. We placed the Erlenmeyer with the leaf and freeze-dried *S. frugiperda* larva in the center of the container for 1 day, and we noted the next day whether it had been removed by the ants. In cases when the freeze-dried *S. frugiperda* larva was removed, we considered the colony active, and thus we used the corresponding colony for bioassays with *C. reimoseri* as described below. When the larva was not removed, we discarded the colony and did not use it for bioassays.

In the bioassay, we placed five living 5th instars with intact or removed shield treatments on the adaxial side of an *I. carnea* expanded leaf. The leaf was maintained in an Erlenmeyer vial with water, as previously explained. The vial was placed in the center of the colony container, as above. On the following day, we recorded the number of surviving larvae on the host plant leaf and calculated the percentage of surviving larvae for each treatment. We carried out 20 bioassays (10 for larvae with intact shields and 10 for those with removed shields).

Do Cuticular Compounds Camouflage Larvae Against Ant Attack? We predicted that cuticular compounds of *C. reimoseri* larvae would act to hinder what ants perceive as prey. To test this, we used CCs from 5th instars of *C. reimoseri* with removed shields and topically applied them on palatable prey; these larvae were bioassayed both under field and laboratory conditions for their defenses.

We extracted CCs from frozen 5th instar *C. reimoseri* with removed shields by dipping 100 larvae in 10 ml hexane for 5 min. The hexane layer was treated over anhydrous Na_2SO_4, dried on low pressure at 40 °C, and recovered with a proper volume of hexane to give a CC extract with 10 equivalents in 20 μl. We used freeze-dried 2nd instars of *S. frugiperda* as palatable prey (Portugal and Trigo 2005; Silveira et al. 2010). We dipped *S. frugiperda* larvae in 1 ml of hexane for 10 min to remove their CCs, and removed the solvent by air flux. Therefore, we topically treated them with 10 equivalents of CCs from *C. reimoseri* 5th instars diluted in 20 μl hexane (hereafter CC treated larvae). We used 10 equivalents because the hexane extract can penetrate the body of *S. frugiperda* larvae instead of remaining on the external cuticle, thus necessitating the higher amount required to compensate for this loss (Portugal and Trigo 2005; Silveira et al. 2010). We calculated the larval equivalent by the ratio of the dry weight of *S. frugiperda* larvae to the dry weight of *C. reimoseri* 5th instars, as given in Portugal and Trigo (2005). As controls, we used CC-free freeze-dried *S. frugiperda* larvae treated with 20 μl hexane (hereafter referred to as the control larvae).

In the field, we performed the bioassays in Pantanal and Campinas. We glued the CC-treated and control larvae side by side, with approximately 2 cm distance between them, on the adaxial side of a fully expanded and intact leaf of an *I. carnea* individual. One day later, we recorded the removal of CC-treated and control larvae. We also recorded the species of ants that were patrolling the plants at the beginning and end of the experiment. We replicated this bioassay 15 times for the CCs of *C. reimoseri* larvae in Pantanal, and 12 for Campinas. We did not carry out the same design used for live *S. frugiperda* larvae, which required isolating the flying predators, because wasps did not attack freeze-dried larvae, and we did not record birds foraging on *I. carnea* leaves during the bioassays with live larvae.

However, if the ants did not significantly remove the CC-treated *S. frugiperda* larvae, it would not necessarily be due to chemical camouflage; the ants could be deterred by *C. reimoseri* larvae CCs only. To assess this issue, we conducted a bioassay using the same design as above, but we glued CC-treated and control larvae on a non-host plant. We expected that ants would remove all larvae independently whether or not they were treated with *C. reimoseri* larvae CCs because the CCs of both treated and control larvae may not be similar to those of non-host plant CCs and would therefore not confer chemical camouflage. If the CCs have a deterrent effect, CC-treated larvae would be removed significantly less often than the controls. In Pantanal, we elected a non-identified legume species belonging to the Mimosoideae subfamily (Fabaceae) that had ants patrolling it due to the presence of treehoppers. We glued a CC-treated and a control

larva on the stem where the ants were walking on their way to reach the treehoppers. In Campinas, we chose *Crotalaria pallida* (Fabaceae: Papilionoideae), which possess EFNs in the base of the pedicel actively visited by ants (Guimarães et al. 2006). We glued a CC-treated and a control larva on the stem where the ants were walking to reach the EFNs. We replicated this bioassay 10 times either with Mimosoideae or *C. pallida* individuals.

In the laboratory, we bioassayed the CCs of *C. reimoseri* larvae using eight colonies of *Ca. crassus*. We glued CC-treated and control larvae on a fully expanded and intact leaf of *I. carnea*, which was dipped into a 50-ml Erlenmeyer with water to prevent the leaf from drying. We placed the Erlenmeyer containing the leaf plus both larvae in the center of the colony container for 24 h. One day later, we recorded whether the larvae were removed by the ants. Silveira et al. (2010) used the number of recruited ants in both treatments to assess chemical camouflage. However, in the present bioassays, we noticed that *Ca. crassus* ants sometimes take longer than one hour to find and recruit on either CC-treated or control larvae. Therefore, we decided to use the number of removed larvae, to assess chemical camouflage.

We also performed eight *Ca. crassus* bioassays using a non-host plant *C. pallida* where CC-treated and control *S. frugiperda* larvae were glued to test the possible deterrent role of CCs as observed in the field bioassay. A stem of *C. pallida* with unripe pods with active EFNs was dipped in water as before and left in the center of the colony. All other procedures were similar to the *Ca. crassus* bioassay described above.

Do Cuticular Compounds of Chelymorpha reimoseri Larvae Have a Deterrent Effect against Chickens? Because 5th instar *C. reimoseri* is rejected by the chicken *Gallus gallus domesticus* (Galliformes: Phasanidae) (Bottcher et al. 2009), we carried out a bioassay to evaluate if CCs are responsible for this rejection. The bioassay procedure was modified from Nogueira-de-Sá and Trigo (2005). We obtained one-d-old chicks from a commercial hatchery and brought them to the laboratory, where they were kept together in a cage of 1.5 m^3 (20 per cage). We maintained the chicks at environment temperature and natural photophase, and fed them with commercial corn-based food and ad libitum water. From day 8 to 10, we deprived chicks of food for 2 h, and trained them individually in a cage (30×30×40 cm width x depth x height). We did that by offering a single freeze-dried palatable 3rd instar *S. frugiperda* in Petri dishes. They were given 2 min to accept or reject the palatable larvae. We performed one training sessions every day. The birds that never managed to find/eat the palatable larvae were not used in the experiment. On the day following the third training day (11th day), we conducted the bioassay. We performed 40 double quantification bioassays, in which 20 control and 20 CC-treated larvae, as described previously, were individually offered to individual chickens. We deprived the chicks of food for 2 h, and then offered a control larva. When the first control was eaten, we offered immediately one CC-treated larva. We recorded the bird response in relation to the CC treated larvae as preyed, when they were consumed, and not preyed, when they were pecked and released. When the chick did not try to prey upon or attack the first control larva, we discarded the trial. After the CC-treated larvae, we immediately offered the chick a second control larva. When the bird ignored or rejected the second control larva, we discarded the trial. We never used an individual chick in more than one trial. The Ethics Committee for Animal Use of the University of Campinas approved all experimental procedures. Chicks were donated to free range farms at the end of the experiment.

Does the Chemical Similarity of CCs between the Larvae and Host Plant Explain the Bioassay Results? We extracted the CCs of 5th instar *C. reimoseri* without shields, their fecal shields alone, intact fully expanded leaves of the host plant *I. carnea*, 5 cm stems of the Mimosoideae species, and 5 cm stems of *C. pallida*. We used one larva, one shield, one leaf, or one stem per replicate; 3–5 replicate were completed. The samples were covered with hexane for 5 min, and the hexane layer was worked up as described above. We analyzed the extracts using electron impact gas chromatography/mass spectrometry in a gas chromatograph (Hewlett Packard 6890) equipped with an HP-5MS column (5 % phenyl methyl siloxane capillary 95 %, 30 m × 250 mm × 0.25 mm; Hewlett Packard) directly coupled to a mass selective detector (Hewlett Packard 5973). All analyses were performed under the following conditions: 240 °C injection temperature; the oven temperature was increased from 40 °C to 300 °C at 3 °C/min, where it was maintained for 10 min; helium 1 ml/min as the carrier gas; the ionization energy was 70 eV and a range of 40–600 amu; splitless injection mode, 1 μl injected . We calculated the retention index for each CC by co-injection with linear alkanes following van den Dool and Kratz (1963). The 9,12,15-octadecatrienoic acid and some *n*-alkanes were identified by co-injection with authentic standards (see Table 1). Linear and branched alkanes were identified using their retention indices (RIs) and mass fragmentation according to Carlson et al. (1998) and Gomes et al. (2008). The alkenes were assigned by their RIs and mass fragmentation according Gomes et al. (2008); the double bond position was identified as given by Carlson et al (1989). Primary alcohols were identified after TMS derivatization (Menéndez et al 2005). Some cuticular compounds (squalene-like, esters, and aldehydes) were tentatively assigned by using the NIST Mass Spectral Search Program (Agilent Technologies, Version 2.0 f. 2008) together with mass fragmentation interpretation as given by Budzikiewicz et al (1967). The other compounds remained as unknown.

Table 1 Relative abundance of cuticular compounds of leaves of the host plant *Ipomoea carnea* (*N*=5), 5th instars of *Chelymorpha reimoseri* without shields (*N*=5) and their respective fecal shields, stems of Mimosoideae (*N*=3), and stems of *Crotalaria pallida* (*N*=4)

Compound[1]	RI	Diagnostic ions m/z (relative abundance, %)[2]	Relative abundance (%)				
			Leaves of *Ipomoea carnea*	5th instar *Chelymorpha reimoseri*	Fecal shield of 5th instar *Chelymorpha reimoseri*	Stems of Mimosoideae	Stems of *Crotalaria pallida*
9,12,15-Octadecatrienoic acid[a]	2156	278 (M^+, 5), 108 (32), 95 (44), 93 (45), 91 (28), 81 (36), 80 (44), 79 (100) 67 (65), 55 (48), 41 (54)	3.66±0.70	0.42±0.42	0.70±0.06	–	–
9-Octadecenoic acid, ethyl ester[b]	2174	310 (M^+, 5), 264 (20), 222 (11), 180 (10), 101 (40), 88 (45), 69 (40), 55 (100), 43 (49), 41 (80)	–	–	–	1.01±0.58	–
Icosanol[c] C_{23}[d]	2300	TMS: 355 (M^+- CH_3, 100) 324 (M^+, 2)	–	–	–	–	0.89±0.15
11-Me-C_{23}[d]	2330	323 (M^+- CH_3, 2), 197 (20), 169 (18)	–	–	0.12±0.12		
9-$C_{25:1}$[d,e]	2494	350 (M^+, 2); DMD: 444 (M^+, 10), 397 (8), 271 (100), 173 (65)	–	–	–	–	0.21±0.21
Docosanol[c] C_{25}[d]	2500	TMS: 383 (M^+- CH_3, 100) 352 (M^+, 2)	–	2.94±0.45	1.45±0.07	–	2.42±0.34
Tricosanol[c] C_{26}[d]	2600	TMS: 397 (M^+- CH_3, 100) 366 (M^+, 2)	–	3.26±0.35	3.70±0.32	–	–
4-Me-C_{26}[d]	2661	380 (M^+, 1), 365 (1), 337 (10)	–	2.98±0.15	5.16±0.34	–	–
9-$C_{27:1}$[d,e]	2671	378 (M^+, 2), DMD: (M^+ 472, 18), 425 (10), 299 (100), 173 (60)	–	0.40±0.27	–	–	–
Tetracosanol[c] C_{27}[d]	2700	TMS: 411 (M^+- CH_3, 100) 380 (M^+, 2)	9.35±0.18	20.38±0.96	24.72±1.46	0.76±0.17	3.69±0.64
13-Me C_{27}[d] 11-Me C_{27}[d] 9-Me C_{27}[d]	2730	379 (M^+- CH_3, 2), 225 (5), 197 (7) 379 (M^+- CH_3, 2), 253 (5), 169 (10) 379 (M^+- CH_3, 2), 281 (18), 141 (25)	–	0.40±0.17	1.73±0.16	–	0.26±0.15
Pentacosanol[c] C_{28}[a]	2800	TMS: 425 (M^+- CH_3, 100) 394 (M^+, 1)	1.44±0.04	3.41±0.29	3.01±0.26	–	0.42±0.15
Squalene like[b]	2823	410 (M^+, 2), 81 (52), 69 (100), 41 (23)	–	2.47±1.05	2.16±0.62	2.30±0.87	1.58±0.92
Hexacosanal[b]	2835	380 (M^+, 2), 362 (M^+- H_2O, 20), 334 (6), 97 (48), 82 (80), 71 (52), 69 (50), 57 (100), 43 (88)	–	–	–	–	0.14±0.14
4-Me-C_{28}[d]	2861	408 (M^+, 1), 393 (1), 337 (9)	–	0.09±0.09	0.47±0.13	–	–
Unknown	2881	394 (3), 281 (42), 157 (37), 141 (49), 96 (28), 85 (25), 71 (87), 57 (98), 43 (100)	–	2.14±0.06	1.23±0.15	–	–
Hexacosanol[c] C_{29}[d]	2900	TMS: 439 (M^+- CH_3, 100) 408 (M^+, 1)	29.75±0.46	22.18±1.73	8.10±0.17	17.02±6.52	10.17±1.72
13-Me C_{29}[d] 11-Me C_{29}[d] 9-Me C_{29}[d]	2930	407 (M^+ - CH3), 253 (5), 197 (10) 407 (M^+ - CH3), 281 (5), 169 (5) 407 (M^+ - CH3), 309 (5), 141 (10)	–	–	–	–	0.73±0.12
Heptacosanol[c] C_{30}[d]	3000	TMS: 453 (M^+- CH_3, 100) 422 (M^+, 1)	1.69±0.04	0.32±0.20	0.32±0.14	–	0.77±0.11
Hexacosyl acetate[b]	3014		–	2.12±0.29	8.59±0.49	–	–

Table 1 (continued)

Compound[1]	RI	Diagnostic ions m/z (relative abundance, %)[2]	Relative abundance (%)				
			Leaves of *Ipomoea carnea*	5th instar *Chelymorpha reimoseri*	Fecal shield of 5th instar *Chelymorpha reimoseri*	Stems of Mimosoideae	Stems of *Crotalaria pallida*
		424 (M^+, 1), 364 (M+- $C_2H_4O_2$, 1), 97 (55), 83 (55), 69 (48), 57 (65), 43 (100)					
Octacosanal[b]	3033	418 (M^+, 1), 390 (M^+- H_2O, 20), 362 (6), 97 (48), 82 (80), 71 (52), 69 (50), 57 (100), 43 (88)	0.71±0.03	–	0.32±0.32	–	0.56±0.22
Unknown	3081	422 (3), 295 (33), 171 (30), 155 (42), 141 (10), 96 (28), 85 (25), 71 (87), 57 (98), 43 (100)	–	1.48±0.09	0.90±0.08	–	–
Octacosanol[c]	3100	TMS: 467 (M^+- CH_3, 100)	40.13±0.63	21.46±1.50	7.10±0.70	1.41±0.11	39.93±1.55
C_{31}[d]		436 (M^+, 1)					
9-Me C_{31}[d]	3130	435 (M^+ - CH_3, 1), 337 (4), 141 (10)	–	–	–	–	0.80±0.06
Nonacosanol[c]	3200	TMS: 481 (M^+- CH_3, 100)	1.44±0.04	0.14±0.14	–	–	2.20±0.10
C_{32}[a]		450 (M^+, 1)					
Octacosyl acetate[b]	3215	452 (M^+, 1), 364 (M+- $C_2H_4O_2$, 1), 97 (55), 83 (55), 69 (48), 57 (65), 43 (100)	–	4.38±0.48	15.39±1.00	–	–
Triacontanal[b]	3237	436 (M^+, 1), 418 (M^+- H_2O, 25), 390 (2), 97 (42), 82 (62), 57 (100), 43 (82)	0.28±0.12	–	0.19±0.19	–	0.54±0.18
Unknown	3217	412 (43), 394 (10), 369 (10), 351 (15), 300 (18), 271 (20), 255 (25),97 (49), 83 (65), 69 (64), 55 (100), 43 (93)	–	–	–	–	0.11±0.11
Unknown	3266	424 (15), 409 (6), 218 (100), 203 (52), 189 (20)	–	–	–	2.19±1.15	1.14±0.06
Unknown	3276	414 (58), 398 (25), 396 (34), 381 (27), 329 (33), 303 (36), 273 (20), 255 (25), 231 (19), 213 (30), 43 (100)	0.73±0.05	2.11±0.75	4.03±0.56	–	1.14±0.06
Unknown	3283	424 (28), 409 (40), 205 (40) 204 (42), 189 (60), 177 (100)	–	–	–	19.29±3.68	–
Triacontanol[c]	3300	TMS: 495 (M^+- CH_3, 100)	9.66±0.18	4.70±0.41	2.65±0.27	–	10.42±1.03
C_{33}[d]		464 (M^+, 1)					
Unknown	3304	426 (23), 411 (24), 204 (65), 189 (74), 177 (67), 59, 43 (100)	–	–	–	1.82±0.30	–
Unknown	3309	424 (10), 409 (7), 218 (100), 203 (20), 189 (18), 71 (50), 57 (80), 43 (66)	0.16±0.10	0.43±0.43	2.35±0.19	–	–
Unknown	3320	424 (43), 409 (25), 313 (34), 245 (29), 218 (26), 205 (100), 189 (41), 109 (80), 95 (75), 81 (66)	–	–	1.39±0.13	36.11±2.89	11.55±0.57
Unknown	3340	426 (6), 411 (25), 315 (18), 218 (58), 203 (50), 189 (99), 135 80), 121 (84), 109 (89), 95 (100), 81 (94), 69 (81)	–	–	–	3.37±0.34	3.13±0.28
Unknown	3343	426 (6), 408 (8), 393 (9), 274 (93), 259 (96), 95 (78), 69 (100), 57 (96), 43 (88)	0.91±0.18	–	–	–	–
Unknown	3347	426 (8), 218 (100), 203 (2), 189 (19)	–	0.37±0.37	0.34±0.21	–	–
Unknown	3379	424 (20), 409 (48), 257 (100)	–	–	–	0.31±0.31	–
Unknown	3406	424 (87), 409 (28), 391 (17), 218 (41), 205 (70), 189 (71), 161 (51), 95 (94), 81 (82), 69 (93)	–	–	–	1.22±0.22	–
Unknown	3407	412 (63), 397 (10), 370 (24), 289 (27), 229 (57), 124 (100)	–	–	–	–	1.58±0.31

Table 1 (continued)

Compound[1]	RI	Diagnostic ions m/z (relative abundance, %)[2]	Relative abundance (%)				
			Leaves of *Ipomoea carnea*	5th instar *Chelymorpha reimoseri*	Fecal shield of 5th instar *Chelymorpha reimoseri*	Stems of Mimosoideae	Stems of *Crotalaria pallida*
Triacontyl acetate[b]	3421	480 (M^+, 1), 420 (M+- $C_2H_4O_2$, 1), 97 (55), 83 (55), 69 (48), 57 (65), 43 (100)	–	1.42±0.15	2.54±0.22	–	–
Unknown	3432	426 (20), 412 (25), 273 (30), 246 (25), 229 (25), 123 (70), 109 (77), 95 (86), 81 (64), 69 (100), 55 (86), 41 (76)	–	–	–	–	0.23±0.13
Unknown	3438	410 (100), 395 (20), 275 (40), 174 (38), 160 (50), 136 (59), 95 (62), 43 (77)	–	–	–	–	0.10±0.10
Unknown	3442	426 (3), 408 (8), 393 (4), 274 (100), 259 (95), 245 (12), 231 (24), 152 (28), 134 (52)	–	–	–	10.25±1.50	–
Dotriacontanal[b]	3442	464 (M^+, 1), 446 (M+- H_2O, 20), 418 (2), 97 (42), 82 (62), 57 (100), 43 (82)	0.09±0.09	–	–	–	1.17±0.19
Unknown	3456	424 (20), 409 (72), 271 (21), 257 (100), 245 (15), 137 (29), 95 (36)	–	–	–	1.68±0.26	–
Unknown	3485	426 (40), 411 (100), 259 (82), 241 (37), 137 (55), 95 (63)	–	–	–	0.85±0.18	4.11±0.65
Unknown	3500	424 (25), 205 (30), 109 (100), 95 (57), 69 (75)	–	–	–	0.41±0.24	–
13-Me-C_{35}[d] 11-Me-C_{35}[d]	3530	491 (M^+ - CH_3, 1), 337 (2), 197 (18) 491 (M^+ - CH_3, 1), 365 (1), 169 (20)	–	–	1.31±0.08		

Data are presented as mean±SE. *RI* retention index

1: [a.] identified by co-injection with authentic standards; [b.] tentatively assigned by using the NIST Mass Spectral Search Program (Agilent Technologies, Version 2.0 f. 2008) together with mass fragmentation interpretation as given by Budzikiewicz et al. (1967); [c.] Primary alcohols eluted together with *n*-alkanes and were identified after TMS derivatization according Menéndez et al. (2005); [d.] identified by retention index and mass fragmentation according Carlson et al. (1998) and Gomes et al. (2008); [e.] double bond position identified according Carlson et al (1989) after DMD derivatization

2: For methyl-branched alkanes, ion clusters occur as even/odd mass pairs depending on the branching point (Nelson et al. 1972). For brevity, only the higher fragment of each pair is listed. TMS: trimethylsilyl derivative. DMD: Dimethyl disulfide derivative

We calculated the percentage of absolute abundance of the compounds found in the cuticular extracts by taking the most abundant compound as 100 %. We discarded CCs with an absolute abundance <1 %. From these data, we calculated the relative abundance, that is, the quantity of each separate compound expressed as a percentage of the total occurrence of the class of substance. We predicted, based on the bioassays that tested the activity of CCs of *C. reimoseri* in camouflage against ant attack (see Results), that the CCs of *C. reimoseri* larvae would be more similar to the CCs of leaves of its host plant *I. carnea* than to the non-host plants Mimosoideae and *C. pallida*. To test this prediction, we compared the percentage of similarity of Renkonen (Krebs 1999) among the CCs of *C. reimoseri* and the host and non-host plants (N=25 for the host *I. carnea*, N=15 for non-host Mimosoideae, and N=20 for non-host *C. pallida*). The Renkonen index of similarity is expressed as a range between 0 (no similarity) and 1 (total identity). We also calculated the Renkonen index of similarity between CCs of five larvae and their respective fecal shields.

Statistical Analysis In the field bioassays testing *C. reimoseri* larvae against ants, we verified the difference in the number of surviving individuals in the two areas (Pantanal and Campinas) and two scenarios (ant and shield treatments) by a three-way generalized linear model, since the data did not meet the ANOVA assumptions. We used Poison distribution, log function link, and deviance correction coefficient to correct for overdispersion (McCullagh and Nelder 1989, p. 98; Statistic 7.0, StatSoft, Inc. 2004). We considered each treatment on an *I. carnea* individual as independent of each other, since the branches stayed at least 1 m apart.

For laboratory bioassays testing larval defenses against ants, we compared the number of surviving larvae between shield treatment (intact or removed) by a Wilcoxon paired sample test, since the data did not meet the normality assumptions (Quinn and Keough 2002). We carried out a paired test because the same colony was used for the bioassay with larvae with intact and removed shields.

We used the paired Cochran Q test (Quinn and Keough 2002) in all bioassays where we compared the removal frequencies for *S. frugiperda* treated or not with CC of larvae of *C. reimoseri* (CC-treated and control larva). For bioassay with chicks, we used the same test for comparison among the proportion of rejected larvae among the CC-treated, first and second control larvae.

For comparison of percentage of similarity among the CCs of *C. reimoseri* and the host and non-host plants, we used the Kruskal-Wallis non-parametric analysis followed by Dunn multiple comparison tests, since the data did not meet the ANOVA assumptions (Quinn and Keough 2002). The factor was the plant species where the bioassay was carried out (the host-plant *I. carnea*, and the non-host plants Mimosoideae and *C. pallida*).

Results

Field and Laboratory Bioassays Testing Tortoise Beetle Larval Defenses Against Ants In both field bioassays (Pantanal and Campinas sites), the presence of ants resulted in a significant decrease in survival of 5th instar *C. reimoseri* in comparison with ant exclusion treatment (Wald Statistic=36.303, P<0.001, Fig. 2). An interaction between sites and ant exclusion was significant, with a higher mortality in Campinas (Wald Statistic=9.719, P=0.002, Fig. 2). There was no significant difference in the survival with respect to shield treatment, and all larvae survived when ants were excluded, independently of study site (Fig. 2). When ants were present, 73.3 % survived in Pantanal and 40 % survived in Campinas. In both areas, we always found at least one ant species per individual plant. In Pantanal, we observed *Ca. crassus*, *Cephalotes* sp., and *Paratrechina* sp., and in Campinas, we observed *Ca. crassus*, *Cephalotes* sp., *Crematogaster* sp., and *Pseudomyrmex* sp. We also observed that ants frequently seemed to not perceive larvae of *C. reimoseri* as prey, walking on them without any attack; this outcome occurred mainly if the larvae were immobile (Fig. 3). This ant behavior is independent of the presence of a shield.

In the laboratory bioassay, we found that the number of surviving larvae was influenced by the shield treatment. When the shield was present, the number of larvae that survived ant attack was higher than when the shield was removed (Wilcoxon test, Z=2.293, df=9, P=0.022, Fig. 4). Again, we observed that when *C. reimoseri* larvae showed no movement, the ants walked on them without attacking, independently of the presence of a shield. When *Ca. crassus* killed the *C. reimoseri* larvae, they disposed them in the waste heap at the boundary of the nest.

Cuticular Compounds Camouflage Larvae against Ant Attack In field bioassays, the factor that influenced the removal of *S. frugiperda* larvae by predators on *I. carnea* leaves was the treatment applied to them. CC-treated larvae were removed significantly less often than the corresponding controls, independently of the site where the bioassay was carried out (Cochran's Q test, Q=8, df=1, P=0.005, N=15 for Pantanal, and Q=9, df=1, P=0.003, N=12 for Campinas; Fig. 5). Both CC-treated and control larvae were totally removed by ants when placed on the non-host plants Mimosoideae or *C. pallida*. *Camponotus crassus*, *Crematogaster* sp., and *Solenopsis* sp. ants were found foraging in the bioassays in Pantanal, and *Ca. crassus* and *Crematogaster* sp. were found in Campinas.

In the laboratory bioassay with *Ca. crassus*, all control larvae were removed, and all CC-treated larvae were left intact when they were placed on *I. carnea* leaves. When the larvae were placed on the non-host *C. pallida* stems, both CC-treated and control larvae were totally removed.

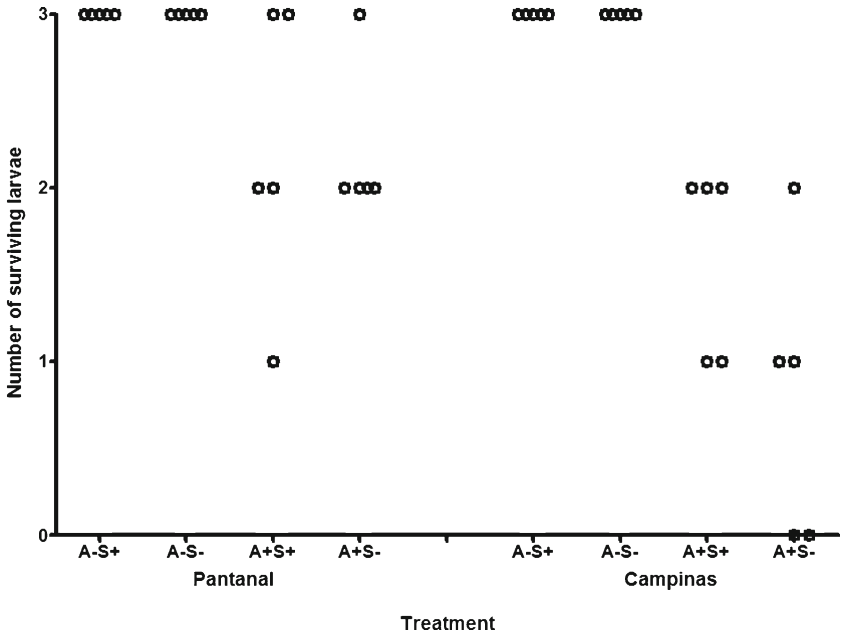

Fig. 2 Surviving 5th instars of *Chelymorpha reimoseri* on its host *Ipomoea carnea* in Pantanal and Campinas, when ants were either excluded (A-) or allowed to forage on the host (A+). For both localities and ant treatments, fecal shields were either intact (S+) or removed (S-). For statistics, see Results

Do the Cuticular Compounds of Chelymorpha reimoseri Larvae Have a Deterrent Effect against Chickens? All 20 larvae of *S. frugiperda* treated with CCs of 5th instars of *C. reimoseri* were preyed upon by chickens, as were the 20 first and second control larvae.

Does the Chemical Similarity of CCs between the Larvae and Host Plant Explain the Bioassay Results? The cuticular pattern of larvae of *C. reimoseri*, leaves of *I. carnea* and stems of *C. pallida* showed as main compounds the *n*-alkanes C_{27}, C_{29}, C_{31}, and C_{33} eluting together with the primary alcohols *n*-tetracosanol, *n*-hexacosanol, and *n*-octacosanol, respectively. There are slight quantitative differences among these three groups (Table 1). The CCs of Mimosoideae stems mainly contained unknown compounds at RIs 3283, 3320, and 3442 and the *n*-alkane C_{29} (Table 1). When comparing the percentage similarity among the CCs of *C. reimoseri* larvae and the CCs of the plants used in the bioassays (*I. carnea* host, and Mimosoideae and *C. pallida* non-hosts), we found that the similarity

Fig. 3 Workers of *Camponotus crassus* walking on 5th instar *Chelymorpha reimoseri* on leaves of *Ipomoea carnea*, without attacking them

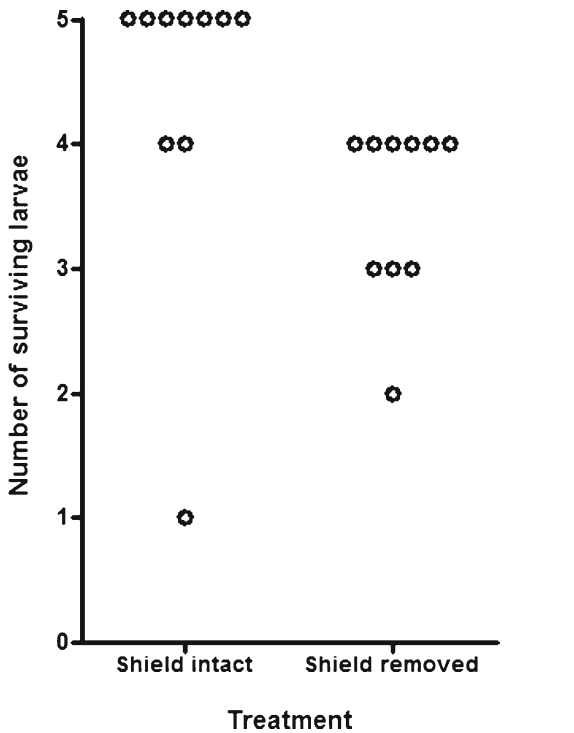

Fig. 4 Surviving 5th instars of *Chelymorpha reimoseri* on its host *Ipomoea carnea* during the bioassay using *Camponotus crassus*, where fecal shields were either intact or removed. For statistics, see Results

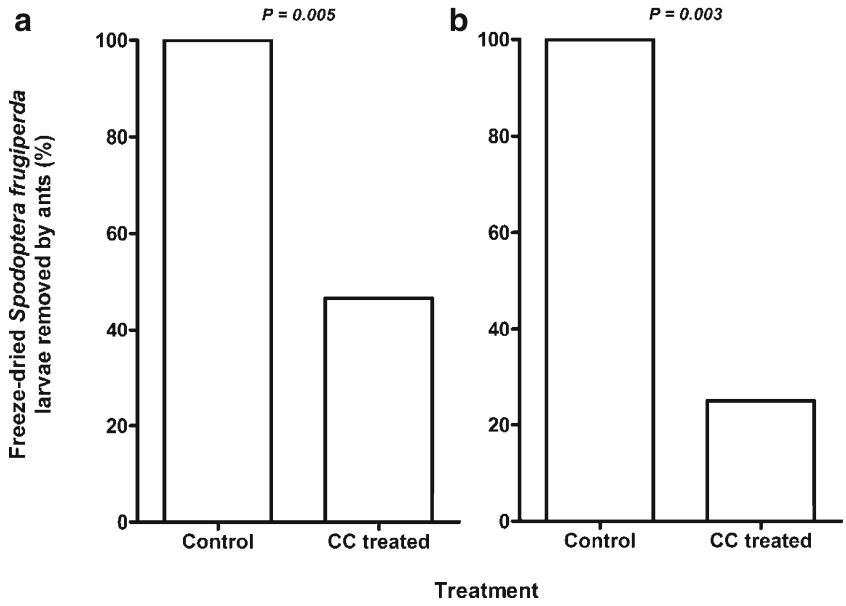

Fig. 5 Proportion of freeze-dried *Spodoptera frugiperda* larvae that were attacked and removed by ants on *Ipomoea carnea* in Pantanal (**a**) and Campinas (**b**). The larvae were treated (CC treated) or not (control) with cuticular compounds of 5th instars of *Chelymorpha reimoseri*. The significant difference was given above the bars (Cochran's *Q* test)

between *C. reimoseri* and *I. carnea* was significantly higher than the similarity of *C. reimoseri* and the non-hosts (Kruskal-Wallis statistic, $H=49.36$, $P<0.01$, Fig. 6). We found a CC similarity of 63±1 % (mean± SE) between larvae and their fecal shields.

Discussion

Based on previous research on *C. reimoseri* larvae (Bottcher et al. 2009), we postulated that the presence of a shield is unimportant for defense against predation. As predatory ants are ubiquitous patrolling vegetation (Davidson et al. 2003), defenses against this predator guild should be widespread in herbivorous insects. Besides, *I. carnea* possess EFNs, which may enhance the ants patrolling on this plant. For that reason, we suggested that chemical camouflage against ant predation would be an important defensive trait in *C. reimoseri* larvae. Therefore, we assumed that ants would not have a high impact on larval survival, independently of shield presence. However, our results did not fully match what Bottcher et al. (2009) reported, and did not support such predictions. The comparison with Bottcher's results suggests either a variation in ant aggressiveness or defensive chemistry of larvae. With respect to our prediction, in field bioassays we saw that the presence of ants decreased the survival of 5th instar *C. reimoseri*, independently of shield presence. Moreover, in laboratory bioassays with *Ca. crassus* ants, we observed the survival of larvae without shields was lower than that of shielded larvae. Nevertheless, when we take into account the survival of a palatable prey without any chemical defense, in both field and laboratory scenarios, we observed that palatable prey, such as *Spodoptera frugiperda* larvae, were 100 % removed by ants in these two bioassays. A survival of 40–73 % when ants were present in the field bioassay and 70 % when shields were removed in the laboratory bioassay suggests that *C. reimoseri* larvae are somehow defended against predatory ants.

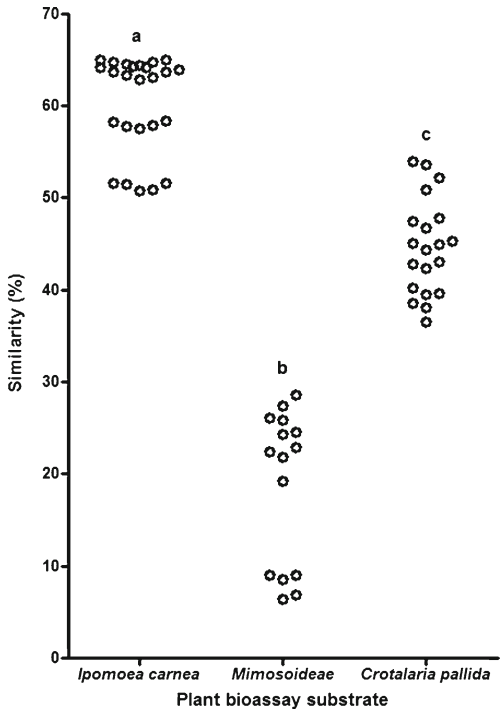

Fig. 6 Similarity of cuticular compounds (CCs) of *Chelymorpha reimoseri* larvae in relation to the CCs of different plant species on which the chemical camouflage bioassays were carried out (leaves of the host-plant *Ipomoea carnea*, and stems of the non-hosts (an unknown Mimosoideae and *Crotalaria pallida*). Different letters above bars indicate significant differences at the 1 % level (Dunn multiple comparison test)

Generally, no defensive trait is 100 % effective against predation. Chemical defenses can vary enormously in wild populations of a prey species. The differential aggressiveness, naiveté, and hunger threshold of predators also could explain why some well-defended prey are attacked and killed. Even fecal shields do not bestow efficient protection against tortoise beetle predators. Bacher and Luder (2005) have shown that larval shields of *Cassida rubiginosa* did not deter predation by the paper wasp *Polistes dominulus*, but they were highly effective against the endoparasitoid wasp *Foersterella reptans*. Furthermore, shields can be used as cues by predators to detect tortoise beetle larvae. Müller and Hilker (1999) showed that larvae of *Cassida denticollis* and *Cassida stigmatica* with intact shields were bitten and dragged significantly more often by *Myrmica rubra* ants than were larvae without shields. The authors suggested that the shield volatiles derived from the composite host plant *Chrysanthemum vulgare* were responsible for attracting ants.

However, even with some predation of ants on *C. reimoseri* larvae, the use of chemical camouflage as a defense in tortoise beetle larvae is strongly suggested by the *Ca. crassus* ant behavior observed in our research. When the larvae were immobile, the ants walked over them without disturbing or attacking them. This behavior does not depend on shield presence. Portugal and Trigo (2005) showed that *Ca. crassus* ants do not seem to recognize the ithomiini *Mechanitis polymnia* larvae as prey and walk over them without showing any aggressive behavior. Further, these authors demonstrated that these butterfly larvae were chemically camouflaged.

Through field and laboratory bioassays with predatory ants, we demonstrated the presence of a chemical camouflage defense mechanism in *C. reimoseri* larvae. Cuticular compounds extracted from 5th instar *C. reimoseri* prevent the removal by ants of palatable larvae treated with these compounds and placed on the host plant *I. carnea*. However, CC-treated palatable larvae were removed by ants when placed on non-host plants, similarly to the corresponding control. Nogueira-de-Sá (2004) and Nogueira-de-Sá and Trigo (2005) had already proposed such a defense mechanism for *Plagiometriona falvescens* larvae because both larvae and host plant showed similar CC patterns. Other herbivorous insects unrelated to tortoise beetle beetles also may exhibit chemical camouflage against chemically oriented ants: larvae of the geometrid moth *Biston robustum* (Akino et al. 2004; Akino 2005), larvae of the ithomiine butterfly *Mechanitis polymnia* (Portugal and Trigo 2005), the codling moth larvae *Cydia pomonella* (Tortricidae) (Piskorski et al. 2010), and *Guayaquila xiphias* treehopper nymphs (Silveira et al. 2010). However, this type of defense has been demonstrated only in laboratory bioassays with predatory ants only for *M. polymnia* and *G. xiphias*.

As the development of chemical defenses can be costly (Mappes et al. 2005; Nishida 2002), we suggest that chemical camouflage may be widespread in herbivorous insects due to its presumably low cost. CCs may have a primary physiological function in the insect integument in the regulation of permeability and the protection against water loss (Howard 1993), and a further exaptation as defense could be easily selected for. Nevertheless, the efficacy of this defense may be restricted to chemically oriented predators such as some ant species.

Because chemical camouflage worked with the CCs of *C. reimoseri* larvae on the *I. carnea* host plant and did not work with these CCs on the non-hosts *C. pallida* and Mimosoideae, we expected that the chemical similarity between the CCs of larvae and the host plants would be high and that between larvae and non-hosts would be low. The study confirmed this hypothesis. This result matches the result obtained by Silveira et al. (2010), where the similarity between the insect CCs and host plant CCs was higher than that of the insect and non-host plant.

As reported here, chemical camouflage represents an additional defensive trait for tortoise beetle larvae. In larvae of this taxon, concealment and internal feeding in the basal hispini, the presence of a physical and chemical barrier via a fecal shield (which is mobile and enhanced with host-derived chemicals), gregariousness, and maternal care are well-described strategies against predation (see Vencl et al. 2011 and references therein). These multiple defensive trait interactions may increase the effectiveness of the defenses (Vencl et al. 2011), and chemical camouflage must be considered in this scenario. Besides, shield presence may not interfere with chemical camouflage, since shields showed a similar CC profile with larva (around 63 %).

When we focus on the multiple defensive traits in herbivorous insects or other animals or plants, one important question comes to mind: does the number or the diversity of predators that a species has drive defensive displays to become increasingly complex? Rowe and Halpin (2013) reviewed this subject and proposed several hypotheses to explain the evolution of multimodal warning displays. We followed the logic proposed in the above review in the context of multiple defenses of tortoise beetle larvae. First, in the perceptual variability hypothesis, Rowe and Halpin (2013) claim that attention should be paid to the high variability of the perceptual abilities of different species of predators to locate their prey, and the consequent warning signal of prey that may adapt to the predators' sensory systems. Similarly, the defensive chemistry of herbivorous insects could have evolved towards a greater complexity due to the diversity of predators. For example, birds and ants may be deterred by distasteful compounds from the fecal shield or the integument of tortoise beetle larvae (Bottcher et al. 2009; Müller and Hilker 2004), but only ants may be affected by cuticular compounds that camouflage the insects against the host plant. The CCs of *C. reimoseri* had no deterrent effect on chickens.

Rowe and Halpin (2013) also support the increased detection hypothesis in which detection can be enhanced by the use of multiple components that reduce a predator's reaction time to a warning signal. In this manner, multiple defensive traits would enhance defenses in tortoise beetle larvae. That is, the presence of a shield plus cuticular compounds would defend the larvae better against ants than one defense alone. The role of gregariousness and the sequestration of deterrent compounds from the host plant, which were not taken into in account in this work, may also be part of the arsenal of defenses of some tortoise beetle larvae. Gregariousness was comprehensively approached by Vasconcellos-Neto (1988), Vasconcellos-Neto and Jolivet (1988), and Vencl et al. (2011). However, defensive chemistry present in the larval integument has been studied rarely for tortoise beetle larvae (e.g., Bottcher et al. 2009). Massuda and Trigo (unpublished) have observed an example of this type of defense, where the alkaloid swainsonine is sequestered from *I. carnea* by *C. reimoseri* larvae and adults, and may be responsible for the defense against predators. A phylogenetic approach, incorporating chemical camouflage and deterrent compounds, in a model similar to those reported by Vencl et al. (2011) and Vencl and Srygley (2013) would help to clarify whether there is a continuous escalation or rather a shift of defenses through the evolutionary history of tortoise beetles.

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