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Tending-Ants Increase Survivorship and Reproductive Success of *Calloconophora pugionata* Drietch (Hemiptera, Membracidae), a Trophobiont Herbivore of *Myrcia obovata* O.Berg (Myrtales, Myrtaceae)

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Abstract

The trophic relations between ants and hemipterans are very common in the Neotropical Region, but rarely explored in dry montane ecosystems. Given the diversity of outcomes of this type of interactions influenced by variation in biotic conditions (i.e. seasonality, spatial distribution, identity of species involved), new examples in different ecosystems can provide important data for a more general understanding of their impact in communities. We investigated the outcomes (direct benefits: survivorship and reproduction) of the relationship between the trophobiont herbivore *Calloconophora pugionata* (Membracidae) and its tending ants. The interaction occurs on *Myrcia obovata* (Myrtaceae), a common tree in montane forests and rupestrian fields of southeastern Brazil, and has never been studied before. Between 2008 and 2009, we selected and manipulated (ant-exclusion) trees in a pairwise experiment performed on plant branches infested by *C. pugionata*. This Membracidae laid its eggs peculiarly on the leaf margins, a behaviour that increased egg survival even when ants were absent. All life stages of the hemipteran exhibited higher survival rates (two-fold) and increased fecundity (four-fold higher oviposition rates) when attended by ants. This study shows that this ant-hemipteran interaction occurs in dry montane biomes in a way that is similar to other tropical ecosystems in which ants protect the hemipterans against predators, thus increasing their survival and reproductive fitness.

Introduction

Nesting sites or food rewards provided by some plant species cause ants to protect it against herbivore damage (Oliveira & Freitas, 2004; Rosumek et al., 2009; Nahas et al., 2012). Ant defense is a remarkable strategy and affects herbivore density on vegetation (Janzen, 1966; Del-Claro et al., 1996; Rosumek et al., 2009; Nascimento & Del-Claro, 2010). These herbivores can be repelled or even predated by ants, and consequently are deprived of shelter, mates, food resources, nesting and oviposition sites (Crutsinger & Sanders, 2005; Rosumek et al., 2009). Nevertheless, some herbivores (Sternorrhyncha, Auchenorrhyncha and larvae of various Lepidoptera species) are able to take advantage of ant presence and increase survival and reproduction rates in their patrolling (Delabie, 2001; Rico-Gray & Oliveira, 2007).

Ants and some species of sap-sucking hemipterans (Membracidae, Cicadellidae, Psyllidae, Fulgoridae, Aphididae, Coccidae and Pseudococcidae) coevolved behavioural and morphological features to cooperate in non-obligate mutualistic interactions (Beattie, 1985; Hölldobler & Wilson, 1990; Delabie, 2001; Lach et al., 2010). These relationships are named trophobiosis and involve the release of a nutritive secretion by hemipterans (honeydew) in exchange for ant protection against natural enemies (Way, 1963; Del-Claro & Oliveira, 1999, 2000). In some cases, the ants may provide an enemy-free space for its partners and host plants around the Hemiptera aggregation (Moreira & Del-Claro, 2005).

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This type of mutualistic relationship can lead to increased reproductive fitness for ants and hemipterans (Byk & Del-Claro, 2011; Delabie, 2001; Del-Claro & Oliveira, 2000).

The interaction between plants, sap-sucking herbivores and ants are an important model for a better understanding of ecological functions and interactions in communities because it explores the effects of positive interactions for species involved and mechanisms of species coexistence and coevolution, structure of food-webs and aspects of plant defenses against herbivores (Thompson, 1999; Bluthgen et al., 2000; Del-Caro & Torezan-Silingardi, 2009; Rosumek et al., 2009). Although well known for temperate regions and agricultural systems (Delabie, 2001), the interactions between ants and hemipteran are poorly known in the tropics due to the high diversity of involved species (Moreira & Del-Claro, 2005) and also for received attention in this issue only recently (Styrsky & Eubanks, 2006; Rosumek et al., 2009). Even though basically mutualistic, the trophobiosis may vary in time and type of ecosystem, and about the identity, abundance and behavior of the species involved, which results in differences in the outcomes of the interaction (Rico-Gray & Oliveira, 2007). Studies of new examples of trophobioses can help to understand the differences found in comparisons of the trophobioses outcomes in different environments and conditions (Thompsom, 1999).

Myrcia obovata (O. Berg) Nied (Myrtaceae) is a common small tree found in Montane Atlantic Forests and Rupestrian Fields of southeastern Brazil. This species serves as host plant for the sap-sucking herbivore *C. pugionata* Drietch 1991 (Hemiptera: Membracidae, Fig 1a). A previous study has shown that *C. pugionata* interacts with at least ten ant species, with *Camponotus rufipes* Fabricius 1775 and *Camponotus crassus* Mayr 1962 as the most common ones (Fagundes et al., 2012). Infestation by *C. pugionata* occurs on branches, leaves and fruits. This treehopper lay masses of eggs on stems or leaf margins (Fig. 1b). The genus *Calloconophora* exhibits a high degree of parental care and does not usually interact with ants, which often act as predators (Lin, 2006).

In this study we investigated for the first time in a tropical montane ecosystem the interaction between ants and a Membracidae (*C. pugionata*). We evaluated the hypothesis that the interaction increases the fitness of the treehopper: reproduction and survival. We also tested the prediction that eggs laid on the margins of the leaves are less predated than those placed on twigs, since this is a particular behavior of *C. pugionata*. Finally, we evaluate the reduction in the number of predators as a mechanism of ant protection. We seek to demonstrate that the benefits of interaction with ants can be obtained by species that have low susceptibility to interact with ants, like *C. pugionata*. Furthermore, we seek to expand the distribution of the occurrence of these types of interactions by including the tropical montane ecosystems like Rupestrian Fields, providing subsidies for comparisons between different tropical ecosystems.

Figure 1. *Calloconophora pugionata* (Membracidae) aggregation in *Myrcia obovata* (Myrtaceae) highlighting the life stages of the trophobiont hemipterans. a - Aggregation, tending by *Camponotus rufipes*. b - Egg masses on twigs and leaf margins (R Fagundes, 2008).

Material and Methods

Fieldwork was conducted in Itacolomi State Park (PEIT), in the mountains of the Iron Quadrangle in Minas Gerais state, southeastern Brazil. The vegetation is composed of Atlantic Montane Forest and Rupestrian Fields (Campos rupestres). Rupestrian fields consist of tortuous trees and shrubs immersed in open fields of grasses and rocky outcrop. This ecosystem presents high diversity of endemic species (Giulietti et al., 1997) being part of Cerrado (Brazilian savannah) but related to areas with elevation higher than 1000 m, with acid soil and low nutrient availability (Ribeiro & Fernandes, 2000; Guerra et al., 2011). The experiment was performed in an area of Rupestrian fields at 1400m high (20°26'26" S, 43°30'52" W).

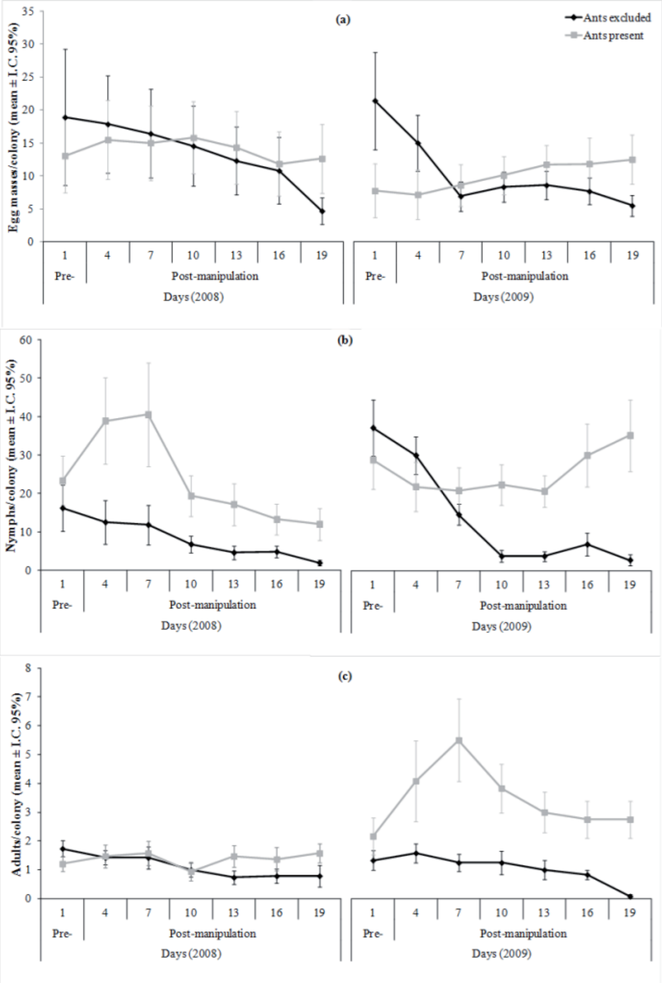

Figure 2. Repeated measures ANOVA of ant-exclusion experiments on egg masses(a), nymphs(b) and adults(c) of *Calloconophora pugionata* (Membracidae) in *Myrcia obovata* (Myrtaceae) over time.

We conducted a manipulative experiment to evaluate the effect of tending ants on *C. pugionata* survival. We used 19 *M. obovata* individuals in January 2008 and 12 in January 2009. The plants shared similar architecture (mean ± standard deviation: height = 2.65 ± 0.15 m; trunk circumference: 0.3 ± 0.02 m, crown diameter: 2.0 ± 0.15 m). We selected individuals separated by a minimum distance of 5 meters, to keep independence, but most pairs distanced more than 10 meters apart. On each tree, we selected two branches at opposite sides of the plant infested with *C. pugionata* colonies with approximately the same number of individuals. We randomly assigned the branches as control or treatment. We added a strip of tape to the base of treatment branches and covered with a nontoxic sticky resin that prevents access by ants (Tanglefoot ®). In addition, we removed nearby leaves that would get in contact with the treatment branches to ensure isolation.

The experiment consisted of monitoring the change in the number of remaining membracids in response to the absence of ants. We monitored three life stages of membracids (egg mass, nymphs and adults). On the first day of the experiment, all branches were free of manipulation and we assessed the abundance of ants and membracids. Later, at the end of the afternoon, treatment branches received the ant exclusion resin. Then, we recorded the number of remaining membracids every three days in each aggregation in control and treatment branches, totalling seven samples per experiment along 19 days.

We counted the number of intact egg masses attached to branches and leaf margins in both treatment and control groups at the beginning and the end of the experimental period to determinate the importance of oviposition site for egg permanence. It was impossible to count each egg individually due to posture in dense masses. We estimated hemipteran survival based on changes in overall number of eggs, nymphs and adults. This method may overestimate the survival of eggs, because the dead eggs are replaced by new ovipositions. However, the comparison between experimental groups, allows us to assess the fitness of the aggregation, because if the aggregation is not healthy enough to compensate losses or produce more individuals than it loses, the aggregation inevitably will perish.

We quantified the abundance of other arthropods counting those visiting the branches, treatment and control, in order to test ant repellence as a mechanism of protection. We classified the visiting arthropods as predators or herbivores by morphology and behavior with direct observation. Only arthropods observed feeding on individuals of *C. pugionata* colonies were recorded. This procedure may have underestimated the number of predators but gave us the confidence to affirm that those individuals counted definitely feed on *C. pugionata*. In addition, we recorded the abundance and species richness of tending (partners) and non-tending ants (visitors). The ants were classified as non-tending and tending by direct observation of constant care of membracids and collection of honeydew. Voucher specimens of membracids, and ants were collected at the end of the study period to confirm taxonomic identification using taxonomic keys and with the assistance of Dr. Rodrigo Feitosa, an ant's taxonomist. The specimens were deposited in the Entomological Collection of the Laboratory of Evolutionary Ecology of Canopy Insects and Natural Succession of the Federal University of Ouro Preto (UFOP). We had all authorizations required to collect and research in Brazilian natural reserves (IEF UC09/009).

Data analysis

All data were square root transformed to meet the model assumptions of normality and homoscedasticity. All graphs display non-transformed data. Statistical analysis was performed in Statistica 7.0. We used repeated measures ANOVA to compare the variation in the number of membracids over time between control and treatment branches. We considered branches as fixed factors and days as repeated measures to the response variables: number of egg masses, nymphs and adults. We used Fisher's LSD paired comparisons as post-hoc test.

We use repeated measure ANOVA to evaluate the effect of the presence of ants and oviposition site (fixed factors) on egg survival of *C. pugionata* (response variable) over time (day 1 and day 21, repeated measures) in both years. Were used ANCOVA models to assess the relative importance of the presence of ants (fixed factor) and the number of days with adults present (continuous factor) on offspring survival (response variable).

We used ANOVA models to compare the variation in the number of predators of *C. pugionata* between experimental branches (with and without ants). Values recorded on all sampling period were summed. We made comparisons using a generalized linear model, assuming a Poisson distribution and Log function.

Results

We found 36.84 ± 9.86 (n=19) membracids per aggregation in treatment branches and 37.58 ± 7.30 (n=19) in control branches in 2008, and 60.00 ± 11.42 (n=12) and 38.83 ± 7.24 (n=12) in 2009 (mean ± standard error; number of samples). Twelve ant species and morphospecies were observed tending *C. pugionata* (Table 1). The most frequent species tending *C. pugionata* were *Camponotus rufipes* (48% of ant occurrences in 2008 and 60% in 2009) and *C. crassus* (18% of occurrences in 2008 and 15% in 2009).

Table 1. Number of times an ant species was observed in different individual trees tending (Td.) *Calloconophora pugionata* (Membracidae) or just visiting (Vis.) *Myrcia obovata* (Myrtaceae).

Ants	Number of records			
	2008		2009	
	Td.	Vis.	Td.	Vis.
Formicinae				
Camponotus crassus Mayr 1862	6	4	3	1
Camponotus fastigatus Roger 1863	1	2	0	0
Camponotus novogranadensis Mayr 1870	1	3	1	0
Camponotus rufipes Fabricius 1775	16	0	12	0
Camponotus sp. 1	1	0	3	1
Camponotus sp. 2	2	1	0	0
Camponotus senex Smith 1858	1	4	0	1
Myrmicinae				
Cephalotes pusillus Klug 1824	1	0	0	0
Crematogaster sp.	1	0	0	0
Pheidole sp.	1	1	0	0
Pseudomyrmecinae				
Pseudomyrmex gracilis Fabricius 1804	1	3	1	1
Pseudomyrmex (gp *pallidus*) sp. Smith 1855	1	1	0	0

The number of hemipterans per aggregation was higher in all life stages when *C. pugionata* was tended by ants (Fig. 2). In most cases the number of individuals started higher in the treatment branch but ended lower, except for "nymphs in 2008" for which the control aggregations had higher number of individuals, in both study years (Table 2). In treatment branches the number of membracids always reduced over time. In control branches the number of membracids increased or remained constant over time. Survival of eggs and nymphs was not influenced by the presence of adult membracids (ANCOVA: Eggs: SS=0.33, df=1.41, F=1.65, p=0.21; Nymphs: SS=0.33, df=1.41, F=1.66; p=0.2).

Table 2. Repeated measures ANOVA for the number of *Calloconophora pugionata* (Membracidae) egg masses, nymphs, and adults between branch treatments (ants factor), days (repeated factor) and year in *Myrcia obovata* (Myrtaceae).

Source of variation		DF	SS	F	P
Egg mass	Ants	1	0.03	0.03	0.87
	Year	1	2.35	1.97	0.17
	Ants*Year	1	0.34	0.28	0.60
	Error	58	69.34		
	Days	6	1.41	3.32	<0.01
	Days*Ants	6	6.55	15.43	<0.01
	Days*Year	6	1.08	2.54	0.02
	Days*Ants*Year	6	1.60	3.78	<0.01
	Error	348	24.63		
Young	Ants	1	8.95	4.96	0.03
	Year	1	11.37	6.30	0.01
	Ants*Year	1	0.57	0.31	0.58
	Error	58	104.68		
	Days	6	12.57	16.39	<0.01
	Days*Ants	6	8.76	11.42	<0.01
	Days*Year	6	1.42	1.85	0.09
	Days*Ants*Year	6	8.22	10.72	<0.01
	Error	348	44.48		
Adult	Ants	1	2.75	12.56	<0.01
	Year	1	1.71	7.79	0.01
	Ants*Year	1	1.75	7.99	0.01
	Error	58	12.71		
	Days	6	1.00	5.70	<0.01
	Days*Ants	6	1.19	6.76	<0.01
	Days*Year	6	0.60	3.40	<0.01
	Days*Ants*Year	6	0.20	1.15	0.33
	Error	348	10.19		

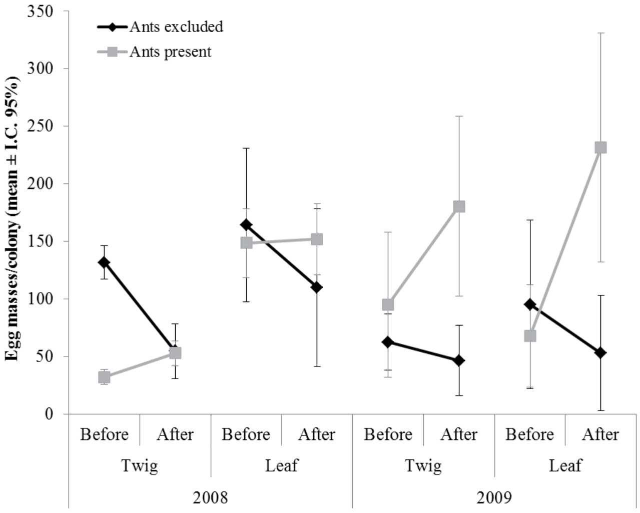

Figure 3. Number of egg masses per aggregation of *Calloconophora pugionata* before and after exclusion of ants in treatment branch for both oviposition sites and years.

The egg survival responded to the presence of ants, as well as fecundity of the female. But this response was different regarding the oviposition site and was not maintained over the years (Table 2, Fig. 2). In 2008, the number of intact eggs was lower when ants were excluded but only for those laid on the twigs. The eggs laid on the margins of the leaves did not respond to the absence of ants. In 2009, the number of intact eggs in both twigs and leaves increased in control branch but remain constant in treatment branch (Table 3). Eggs in leaf margin were always more numerous, including on ant absence (Fig. 3).

The abundance of *C. pugionata* predators was higher in treatment branches in both years (GLZ: Predators: Ants factor: Wald. $\chi^2 = 28.65$, DF=1, $p < 0.001$;). Although the overall number of predators has reduced by half in 2009, there was a difference between the experimental branches (GLZ: Year factor: Wald. $\chi^2 = 23.68$, DF=1, $p < 0.001$; Ants*Year: Wald. $\chi^2 = 0.14$, DF=1, $p < 0.001$). As a result, arthropod predators are less frequent when ants are present, mainly in 2008 (Fig. 4). Spiders (mainly Salticidae), wasps and braconid parasitoids were the most frequently observed predators of *C. pugionata*. The main type of damage to eggs was their drying out and typical opening marks from newly emerged parasitoids.

Discussion

In recent decades, many studies have been published with examples of interactions between ants and hemipteran, but few in the Neotropical region and most for the Brazilian Cerrado (Buckley, 1987; Oliveira & Del-Claro, 2005; Rosumek et al., 2009). This study presents the interaction between the ants and the Membracidae *C. pugionata* that occurs in montane ecosystems. The herbivore lives in *Myrcia obovata* where it is assisted by several ant species, but most frequently by *Camponotus crassus* and *C. rufipes*. Ant-hemipteran interactions are commonly multispecific for ant partnership which reduces the benefit of the interaction, especially when the ant species is less aggressive (Rico-Gray & Oliveira, 1987). These ant species is commonly found in association with hemipteran and plants in the Cerrado (Oliveira & Brandão, 1991; Shoereder et al., 2010) and Rupestrian fields (Guerra et al., 2011; Fagundes et al., 2012), reinforcing the importance of *Camponotus* as worldwide attendance of trophobiont insects.

In this study, the interaction with ants is beneficial at least to *C. pugionata*, which produces fewer eggs, dies or leave the plant when not associated with ants. The ants protect all hemipteran life stages against natural enemies (Way, 1963; Buckley, 1987; Del-Claro & Oliveira, 2000), increasing their life expectancy and fertility while receiving honeydew as a reward (Del-Claro & Oliveira, 2000). By becoming more aggressive while protecting the associated Hemiptera, the ants attack and drive away anyone who comes close and reduce the occurrence of species such as the predators of Hemiptera and even the host plant (Way, 1963; Del-Claro & Oliveira, 2000; Rosumek et al. 2009). This repellent effect is common to several plant-ant-Hemiptera systems (Buckley & Gullan, 1991; Cushman & Addicott, 1991; Moreira & Del-Claro 2005) but can vary with time and with the species involved in the interaction (Del-Claro & Oliveira, 2000; Billick & Tonkel, 2003). Protection against natural enemies is considered the main benefit received by arthropods interacting with ants in mutualistic relationships (Rico-Gray & Oliveira, 2007).

We did not evaluate the effects of the interaction to the ants, but such effort to collect exudate and protect the partner indicates the valuation of the resource by the ants (Styrsky & Eubanks, 2006). The honeydew is a constant source of food energy, predictable in time and space (Del-Claro & Oliveira, 1993; Blüthgen et al., 2000; Davidson et al., 2003) which may increase ant aggregation survivorship and growth, as is the case with extrafloral nectar (Davidson et al., 2003; Byk & Del-Claro, 2011). This type of resource is capable of replacing predation and scavenging, support entire colonies due to the high nutritional value and low energetic cost (Davidson, 1997). Therefore, when a kind of foraging is focused to a specific resource, such as in trophobiosis, it is defended at a high cost of energy (Stadler & Dixon, 1998a). So, it can be expected that the honeydew of *C. pugionata* is advantageous to its tending-ants. We observed in our study that *Camponotus rufipes* built its nests in the base of the stem of M. obovata, and in some cases built satellites nests made from leaves that housed aggregations *C. pugionata*. A similar observation was made for the system ants-*Guayaquila xiphias* (Oliveira et al., 2002).

These authors suggested that the shelter-nest behavior was a response to the importance of honeydew for the ants.

Table 3. Results of repeated measures ANOVA for the comparisons of the number of egg masses between branch factor (control and treatment), period factor (before vs. after ant exclusion, repeated measure) and year (2008 and 2009).

Source of variation	df	SS	F	p
Year	1	84.85	4.37	0.04
Branch	1	291.22	14.90	<0.01
Egg position	1	13.67	0.70	0.40
Year*Branch	1	211.37	10.80	<0.01
Year*Egg position	1	13.81	0.71	0.40
Branch*Egg position	1	10.59	0.55	0.46
Year*Branch*Egg position	1	4.97	0.26	0.61
Error	116	2252.10		
Period	1	1975.89	105.50	<0.01
Period*Year	1	48.88	2.70	0.10
Period*Branch	1	239.73	1.32	<0.01
Period*Egg position	1	13.67	0.75	0.39
Period*Year*Branch	1	176.23	9.70	<0.01
Period*Year*Egg position	1	13.91	0.76	0.39
Period*Branch*Egg position	1	10.59	0.58	0.45
Period*Year*Branch*Egg position	1	4.97	0.27	0.60
Error	116	2106.69		

The interaction with ants seems to be a good defense strategy for *C. pugionata*, but laying their eggs on the margins of the leaves, in addition to branches, increased egg survival at least in one studied year. Temporal variability in interactions of ants and hemipteran have already been shown in other studies (Bristow, 1984; Cushman & Whitham, 1989, 1991; Del-Claro & Oliveira, 2000). The loss of the benefits of protection by ants over the years is attributed to the fluctuations of the natural enemies of Hemiptera partners (Cushman and Addicott, 1991). Ants fail to defend or benefit is too small for hemipterans when the predator population is low. In our study the non-effect of ant exclusion in 2009 coincides with a decrease in the amount of enemies of *C. pugionata*. The strategy of laying eggs on leaf margins is uncommon for Hemiptera species (Lin, 2006). Eggs laid on leaf margins exhibited higher survival rates when compared to those placed on leaf branches, especially in the presence of ants. Eggs laid on very crowded masses or long pellets in the branches may experience higher predation and parasitism due to the reduction in search time (Tallamy & Schaefer, 1997).

The study of community ecology, focused on negative interactions between species, has led to the neglect of positive interactions and its importance in food webs (Ohgushi, 2008; Grinath et al., 2012). The effect of Hemiptera-ant interaction can cause changes in community in terms of abundance, distribution and survival of the associated arthropods (Del-Claro & Oliveira, 2000; Renault et al., 2005; Styrsky & Eubanks, 2006). On a larger scale, the effect of trophobiosis in the arthropod fauna from its host plant can affect the whole community (Wimp & Whitham, 2001; Rosumek et al., 2009). Fagundes et al. (2012) showed that the abundance of the ant species attendants of *C. pugionata*, *Camponotus crassus* and *C. rufipes*, are directly affected by the population size of *C. pugionata*, and the densification of these species in the host plant leads to reduced diversity of ants, as well as the whole arthropod fauna from the host plant.

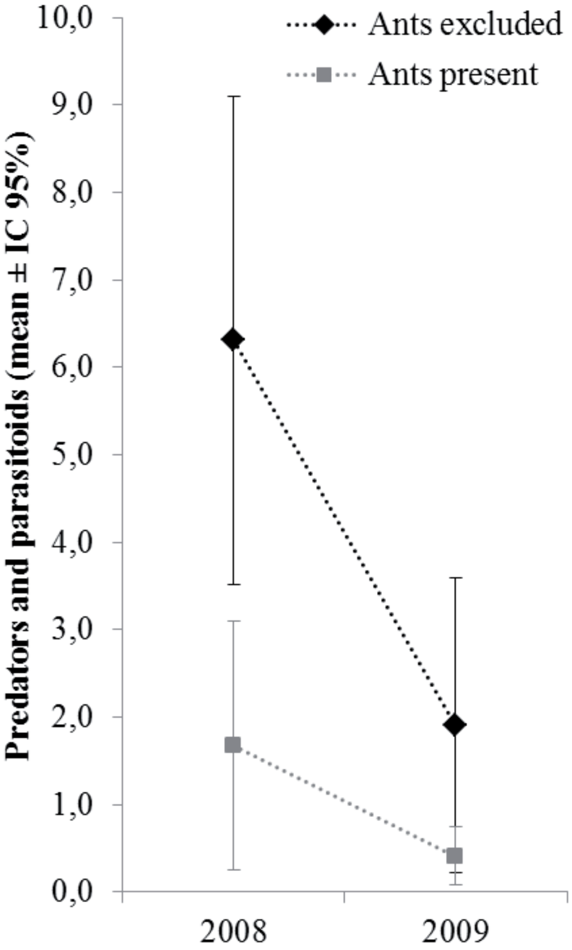

Figure 4. Mean abundance of predators of *C. pugionata* in response to ant-exclusion in each study year.

As we know from other studies in different ecosystems, ants are key for the population growth of the trophobiont hemipteran and crucial to its survival (Buckley, 1987; Holldobler & Wilson, 1990; Delabie, 2001). The sap-sucking hemipteran has many natural enemies that keep its population low in natural environment (Delabie 2001; Del-Claro, 2004). The association with ants provides an effective protection against these natural enemies in exchange for a food that has low cost to the hemipteran. The ants were important for the survival and fitness of *C. pugionata*, as is expected benefits for ants by highly energetic food provided by hemipterans. This interaction has a direct effect on the population parameters of the species involved increasing its life expectancy, growth and reproduction (Bristow, 1991; Delabie, 2001). Additionally, these populations gain advantages in negative interactions such as competition for food (Davidson, 2007; Davidson et al., 2003). This high adaptive value of trophobioses may be important for the persistence of the populations involved, directly and indirectly, and ultimately to the maintenance of diversity (Del-Claro, 2004).

Trophobioses is distributed globally (Rico-Gray & Oliveira, 2007) and occurs in almost all ecosystems (Styrsky & Eubanks, 2006; Rosumek et al., 2009), but the interaction outcomes and its ecological consequences are variable in space and time (Cushman e Addicott, 1991; Stadler & Dixon, 2005) and some ecosystems are little explored, as tropical montane ecosystems (Viana-Silva & Jacobi, 2012; Guerra et al., 2011). In some cases, the interaction can be null for both or one of the parties (Stadler & Dixon, 1998a, b), alarming as to assume that all interactions between ants and hemipteran are mutualistic. Only through studies in various conditions of space and time can predict the occurrence and degree of variability of these conditional interactions (Cushmann & Addicott, 1991). The hemipteran *C. pugionata* has a number of adaptations which suggest low interactivity with ants, as long hind legs, pigmentation for camouflage and parenting developed (Lin, 2006). This features altogether suggests low interactivity and dependence on ants as protectors (Rico-Gray & Oliveira, 2007). However, our results suggested that even in cases of low probability, the interaction can occur and have a mutualistic character.

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