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Biological attributes of Argentinian phorid parasitoids (Insecta: Diptera: Phoridae) of leaf-cutting ants, *Acromyrmex* and *Atta*

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Little is known about the natural history of phorid parasitoids of leaf-cutting ants, an important pest in the Neotropics. This is particularly so for phorids attacking *Acromyrmex* species. We searched for phorids ovipositing and reared them from ants collected in the field. We found that these phorids formed a guild. *Acromyrmex* phorids selected ants from all sizes available outside the nests, whereas *Atta* phorids did not parasitize the smallest workers and soldiers. Maximum parasitism by phorids was 12% for *Acromyrmex* and 35% for *Atta* hosts, percentages higher than any reported previously. Some phorid species coexisting in a site showed differences in developmental times. Only 1.1% of phorids collected over hosts were males; however, as the male : female ratios from parasitoid rearing did not differ from one, mating is suspected to occur elsewhere. Our results helped to increase the knowledge of the phorids of leaf-cutters, and highlighted their potential use for the biocontrol of these ants.

Keywords: biological control; developmental times; host–parasitoid interactions; host size selection; parasitism rate; puparia

Introduction

Leaf-cutting ants in the genera *Atta* and *Acromyrmex* are very conspicuous animals, not only because of the size of their nests and their high biomass, but also because of their striking foraging trails, full of ants carrying leaf fragments (Hölldobler and Wilson 1990). In natural environments, these ants have important effects on the soil and vegetation (Farji Brener and Silva 1996; Farji Brener and Ghermandi 2000; Sternberg et al. 2007), to the point that they are considered to be ecosystem engineers (Folgarait 1998). However, in agro-forest environments in their native range they can become important pests (Cherrett 1986). At present, biological control is not an option for suppression of leaf-cutting ants, but a potential group of natural enemies are small dipteran parasitoids in the family Phoridae (Disney 1994). These phorids, which use adult workers as hosts, are usually called ant-decapitating flies, because many species pupariate inside the head of the dead host, leading to their decapitation (Porter et al. 1995; Brown 1997). Phorid flies are currently being used for classical biological control of fire ants in the USA (Gilbert and Patrock 2002; Porter et al. 2003; Vázquez et al. 2006).

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An important first step when considering natural enemies for biological control is to know their biology in detail, as well as the interactions with their hosts (Louda et al. 1997; Schellhorn et al. 2002; Hajek 2004). Little is known yet about the biology of the phorid parasitoids of leaf-cutting ants. These phorids belong to seven genera: *Apocephalus* (Brown 1997), *Eibesfeldtphora* (previously considered a subgenus of *Neodohrniphora*, Disney et al. 2008), *Lucianaphora* (Disney et al. 2008), *Myrmosicarius* (Borgmeier 1928), *Neodohrniphora* (Disney et al. 2009), *Procliniella* and *Stenoneurellys* (Borgmeier 1931). Aside from taxonomical works mentioning the ant species over which the phorids were collected (Borgmeier 1928, 1931; Disney 1996; Brown 1997, 2001; Disney and Bragança 2000; Disney et al. 2006, 2008, 2009; Brown et al. 2010), there are few studies dealing with other aspects of their biology. Most studies of interactions between phorids and leaf-cutting ants involved only *Atta* species as hosts, focusing on one or two host species from a site (Feener and Moss 1990; Feener and Brown 1993; Tonhasca 1996; Erthal and Tonhasca 2000; Tonhasca et al. 2001; Bragança et al. 2002, 2003, 2008, 2009; Bragança and Medeiros 2006; Silva et al. 2008; Guillade and Folgarait 2011). Those studies did not specify if phorid species were using a leaf-cutting ant host other than the observed species (but see Silva et al. 2008), or even if they were using non-leaf-cutting ants. Moreover, there are hardly any studies on the interactions between *Acromyrmex* hosts and their phorid parasitoids, except for some records of phorids attacking *Acromyrmex* (reviewed in Feener and Moss 1990) and some evidence indicating that *Acromyrmex*'s parasitoids do not attack *Atta* in the field, and vice versa (Elizalde and Folgarait 2010). Remarkably, *Acromyrmex* is more species-rich than *Atta*, with c. 25 species compared with 15 species (Hölldobler and Wilson 1990). This is especially marked in subtropical and temperate South America, where most of the species of leaf-cutting ants belong to *Acromyrmex* (Fowler 1983), and many of them are important pests (Della Lucia 2003).

After finding its ant host species, the phorid needs to choose the appropriate ant size into which to insert the egg. As leaf-cutting ants are polymorphic (Hölldobler and Wilson 1990), a great range of sizes will be available for the ovipositing female. Hence, it is likely that the phorid selects particular ant sizes in which to deposit the egg. On one hand, the host ant must be sufficiently large to allow adequate larval development, but on the other it should be small enough to allow the larvae to eat all the tissue before pupariating, to avoid an infectious focus formed by putrid ant tissue (Chirino 2010). The few phorid species parasitoids of *Atta* evaluated until now showed a preference to oviposit on larger ants (Waller and Moser 1990; Feener and Brown 1993; Tonhasca 1996; Erthal and Tonhasca 2000; Bragança et al. 2002; but see Brown 1999; Tonhasca et al. 2001). By using bigger hosts, adult phorids may achieve larger body sizes, which is an important determinant of insect fitness (Kingsolver and Huey 2008). In fact, it was found that larger phorids developed from larger hosts for some species of parasitoids of *Atta* (Erthal and Tonhasca 2000; Tonhasca et al. 2001; Bragança et al. 2007; but see Guillade and Folgarait 2011). However, no such information is available for *Acromyrmex* parasitoids, and there is no comparative study about the relationship between phorid adult size and that of the hosts from which they were reared.

Natural parasitism rate, i.e. the fraction of hosts that are parasitized in the field during a certain period, is a very important aspect of host–parasitoid interactions because it measures the effect that parasitoids impose on host populations. Hence, it suggests the potential impact that those enemies may exert if used as biological control agents. Parasitism rates are known for only a few phorid species, with less than 4% for the foragers of *Atta laevigata* (Erthal and Tonhasca 2000), 2.2% for *Atta sexdens* foragers parasitized by *Eibesfeldtphora tonhascai* (Tonhasca 1996), or less than 4% for *Atta vollenweideri* (Guillade and Folgarait 2011). Low natural parasitism rates seem to be the rule in ant host–phorid parasitoid systems [e.g. *Solenopsi*s fire *ants – Pseudacteon* phorids with less than 1%, Morrison and Porter (2005); *Pheidole* big-headed *ants – Apocephalus* phorids with less than 2%, Feener (1988)].

Other key aspects of the biology of these parasitoids are developmental times and sex ratios. Only the developmental times of some phorid parasitoids of *Atta* are known (Tonhasca et al. 2001; Bragança et al. 2002, 2003; Guillade and Folgarait 2011), but nothing is known about *Acromyrmex* parasitoids. Gathering this information for many phorid species will help us to understand their population dynamics and will allow us to find efficient ways to mass rear these parasitoids in the laboratory.

The main goals of this article were to provide information on key aspects of the biology of *Acromyrmex* phorids and interactions with their hosts, to compare gathered information for *Acromyrmex* phorids with existing and new data for *Atta* phorids, and to search for interspecific differences in biological attributes of phorids coexisting in a site. Specifically, regarding the latter, (1) we asked if these phorids used non-leaf-cutting ants as hosts, and if phorids attacking *Acromyrmex* were not attacking *Atta*, and vice versa; (2) we evaluated which sizes of ants were selected by different phorid species to oviposit; (3) we tested if there was a relationship between the sizes of the ant host and the adult phorid reared from it for several phorid species; (4) we measured the natural parasitism rate of phorids on several leaf-cutting ant species; (5) we described pupariation sites and developmental times according to phorid species and gender; and (6) we investigated sex ratios of adult phorids.

Methods

Sampling for phorids and host–parasitoid interactions

To sample phorid–leaf-cutting ant interactions, we searched for phorids at the locality with the highest reported leaf-cutting ant richness, San Cristóbal in Santa Fe (30°12′ S, 61°09′ W), according to a previous sampling in 14 localities (Elizalde and Folgarait 2010). The leaf-cutting ant species present were: *Acromyrmex crassispinus*, *Acromyrmex fracticornis*, *Acromyrmex heyeri*, *Acromyrmex hispidus*, *Acromyrmex lobicornis*, *Acromyrmex lundii*, *Acromyrmex striatus* and *At. vollenweideri*. We collected phorids flying over and attacking ants in nests, foraging trails, refuse piles and cutting sites from six nests of each leaf-cutting ant species present. We aspirated the adults and put them in vials with 75% alcohol, labelled with the nest ID and site where they were found. Samplings were carried out for 20–30 days in each season throughout a year (winter 2005, spring, summer and autumn 2006). We called this sampling "Adult Parasitoid Collection" (APC).

In addition, we performed a "Larval Parasitoid Collection" (LPC) of phorids in all seasons, from winter 2005 to winter 2006. This sampling comprised the collection of all the ants crossing a point on the foraging trail 2 m away from the nest entrance during 30 min. We collected the ants using fingers, because it was more efficient than using forceps or an aspirator to capture the ants rapidly. In addition, for those ant species that took waste material outside the nest we collected ants going towards refuse piles using the same methodology. We performed the LPC in three nests of each ant species. Only in the case of *At. vollenweideri* were two people collecting simultaneously required at the same place because of the high flow of ants. Ants were kept in a dark, cool room until taken to the laboratory between the second and fifth day of collection, to rear phorids from them (see *Phorid rearing*).

To determine if leaf-cutting ants' phorids use other ants as hosts, we searched for phorids over dominant ants present in San Cristóbal, including *Camponotus rufipes*, *Camponotus* nr. *blandus*, *Pheidole* spp., *Solenopsis* spp. and *Neivamyrmex pseudops*. Upon finding a nest of any of those ants, we made a hole on it to provoke the ants to leave the nest; a sampling method used for *Solenopsis* phorids (e.g. Folgarait et al. 2007). In addition, we looked for phorids on established foraging trails.

Ant species were identified using the keys by Bonetto (1959), Kusnezov (1978), Gonçalves (1961), and Fowler (1985) and, when necessary, identifications were corroborated with museum collections and with Dr Fabiana Cuezzo, an ant taxonomist from Instituto Miguel Lillo (Tucumán, Argentina). For phorids we used the available keys (Brown 1997; Disney et al. 2006, 2009; Brown et al. 2010). Although there are no keys available to identify *Myrmosicarius* males, we were able to identify the males of *Myrmosicarius* species that parasitize *Acromyrmex* because no more than one species was found per host. The exceptions to this were *Myrmosicarius cristobalensis* and *Myrmosicarius crudelis* over *Ac. crassispinus*; however, both were discriminated by oviposition site (the latter phorid species attacked ants mainly on refuse piles, whereas *M. cristobalensis* attacked at foraging trails, Elizalde and Folgarait, in preparation). Reference specimens of both ants and phorids were deposited in the Museum Bernardino Rivadavia (Buenos Aires, Argentina).

Phorid rearing

To obtain information on other biological attributes of these phorids (host–phorid sizes, parasitism rate, pupariation site, developmental times and phorid sex ratio, see below), we maintained the ants collected through LPC in a rearing room at 22 ± 1°C and 65 ± 10% relative humidity, with 12 : 12 light : dark photoperiod. We fed them with 20% sugar solution and water. As ants died, we placed them on separate recipients, with the bottom covered with plaster and regularly moistened. A similar method was successfully used to rear phorids of fire ants (Folgarait, Bruzzone and Gilbert 2002a). Three times a week we searched for and separated newly dead ants, looked for puparia, and checked for adult emergence. When an adult fly emerged, we put it in alcohol, labelled it with the ID of the nest to which the parasitized ant belonged, and later identified it to species.

Host–phorid sizes

As a surrogate of ant size we measured ant head width below the eyes. This measure is useful because several species of phorid parasitoids of leaf-cutting ants pupariate inside the host head (see Results) and because it allows comparison with other studies that also used this measure (Morrison and Gilbert 1998; Silva et al. 2007; Guillade and Folgarait 2011). We measured all ants from which phorid puparia were reared inside the head (see Results, *Puparia*). To obtain the size distribution of ant workers (foragers or waste removers), we measured all the ants collected. However, because of the great number of workers collected, we only measured all ants from the nest with greatest number of workers for each ant species.

We used the Kolmogorov–Smirnov test to compare ant size frequency distributions selected by phorid species with size distribution of worker ants. As ant size distributions selected by phorid species did not differ among nests (all $P > 0.05$ according to Kolmogorov–Smirnov tests), we pooled those data.

We measured maximum width of mesonotum of adult phorids in dorsal view as a proxy of phorid size (Morrison and Gilbert 1998; Folgarait, Bruzzone, Patrock et al. 2002b; Guillade and Folgarait 2011). We also measured femur length of the third leg as another adult phorid size surrogate. We used linear regressions to establish the relationship between the sizes of the adult phorid and the host ant from which it was reared. We checked the assumptions of the test. As a way to evaluate if phorid species differed in the use of ant sizes, we compared regression slopes of the relationships between adult phorid size and host size among phorid species. Whenever necessary, we adjusted P-values for multiple comparisons using Bonferroni.

We used a stereo microscope (Nikon SMZ 800) with an ocular micrometer set to a resolution of 0.033 mm to measure ant head size, and 0.016 mm for phorid size measurements. We only measured non-damaged adult phorids.

Parasitism rate in the field

We estimated the percentage of parasitism in the field per nest, or parasitism rate, as the ratio of puparia from the ants collected by LPC. *Apocephalus setitarsus* can rear more than one larva per individual host (between one and three larvae; Brown et al. 2010; see Results) and we corrected for this considering that two puparia/adult were reared from one ant. In most cases we were able to obtain the percentage of parasitism contributed by each phorid species. Phorid larval mortality or premature host mortality suggest that these percentages of parasitism must be considered as underestimates of real parasitism.

Phorid development

The LPC yielded developmental times for these phorids. For each species and gender we estimated the larval period as the difference in days between date of puparium formation and date of ant collection. The pupal period was calculated as the difference in days between the date of adult emergence and the date of puparium formation. By adding larval and pupal periods we estimated total developmental times for each individual. We estimated developmental times only for phorids that reach adulthood, because it was not possible to sex the puparia.

As the ants collected in the field were already parasitized, we did not know the oviposition date so larval period and total developmental time represent underestimations of real values. In addition, because we checked for new dead ants, puparia and adult phorids every 2–3 days, developmental times have that additional underestimation. As a way to reduce that error in estimation and to eliminate the unpredictable effect of non-controlled rearing conditions experienced by the ants before they were in the laboratory (see above), we discarded the developmental times for phorids reared from ants that died before they reached the laboratory.

Interspecific differences in developmental times for phorids, as well as intraspecific differences according to collection season, were compared using Kruskal–Wallis tests because data did not adjust to the normal distribution. When we performed multiple comparisons, we adjusted *P*-values using Bonferroni's method. We evaluated whether longer developmental times were the result of bigger host size (because the larva had more food available) or bigger adult phorid size (because the larva needed more time to develop) for each phorid species using non-parametric Spearman correlations.

Male–female ratios

We obtained the proportion of male–female adult parasitoids collected over the hosts during APC. In addition, we compared male–female ratios of phorid species from LPC only for those species that yielded more than 10 individuals by season. We used binomial tests to determine if they differed significantly from one (Zar 1996).

We performed the statistical analyses using the R-environment (R Development Core Team 2010).

Results

Host–parasitoid interactions

All parasitoids collected hovering over leaf-cutting ants or reared from them belonged to the Phoridae. In addition, the phorid species that were parasitizing leaf-cutting ants were not collected over any other dominant ant species that we searched for in San Cristóbal.

We found a total of 27 interactions in San Cristóbal, 78% of which involved *Acromyrmex* hosts (Table 1). None of the phorid parasitoids collected or reared from *Acromyrmex* were collected or reared from *Atta*, and vice versa (Table 1). *Atta vollenweideri* was the species with more phorids attacking it; meanwhile most *Acromyrmex* species had three or four phorid species (Table 1). Most phorid species parasitoids of *Acromyrmex* used more than one host species (Table 1).

We reared adults of 18 phorid species using the LPC (Table 1), seven of which were not collected as adults (even after 1 year of APC sampling, resulting in 952 adult phorids). In addition, 10 of the interactions were only registered through LPC; on the other hand, only one interaction was not corroborated by LPC (Table 1). Many phorids were reared in low abundance (fewer than seven individuals), so for the following analyses we used the interactions that yielded at least 10 individuals by species (indicated by asterisks in Table 1).

Host–phorid sizes

We were able to analyse host–parasitoid size relationships only for phorid species that used the host to pupariate (i.e. *Eibesfeldtphora* and *Myrmosicarius* species), because for phorids not pupariating inside the head of the host, we could not identify, in most cases, the host they were reared from (or associate the body with the head).

Host sizes available and selected by phorids

We found no differences between the distribution of *Acromyrmex* workers and those ants oviposited by phorids. Therefore, there was a high overlap in range size

Table 1. Leaf-cutting ant species and phorid parasitoids recorded as adult females ovipositing ants in the field (phorid species name is in bold) and/or as phorid adults reared from each host (phorid species name is underlined).

Host species	Phorid species
Ac. crassispinus	***Apocephalus neivai***
	*Ap. noetingerorum**
	Myrmosicarius cristobalensis
	M. crudelis*
Ac. fracticornis	*Ap. exstriatus*
	M. cristobalensis
Ac. heyeri	*Ap. neivai*
	M. catharinensis*
	Neodohrniphora unichaeta
Ac. hispidus	*Ap. intermedius*
	Ap. neivai
	Ap. philhispidus
	M. longipalpis
Ac. lobicornis	***Ap. neivai****
	Ap. exlobicornis
	M. cristobalensis*
Ac. lundii	***Ap. neivai****
	*Ap. noetingerorum**
	Ap. penicillatus
	M. cristobalensis
Ac. striatus	*Ap. exstriatus*
Atta vollenweideri	***Ap. longisetarum***
	Ap. setitarsus*
	Ap. vicosae*
	Eibesfeldtphora trilobata*
	M. brandaoi*
	M. gonzalezae*

Note: * Denotes interactions used to gathered biological information.

distribution for workers oviposited by *Myrmosicarius catharinensis*, *M. cristobalensis* and *M. crudelis*, from available workers of *Ac. heyeri*, *Ac. lobicornis* and *Ac. crassispinus*, respectively (Figure 1). This was the case for phorids attacking foragers (*M. catharinensis* and *M. cristobalensis*) as well as for those parasitizing waste removers (*M. crudelis*).

Phorid parasitoids of *At. vollenweideri* selected mainly medium to large host ant sizes (Figure 1). The size distributions selected by *Eibesfeldtphora trilobata* and *Myrmosicarius gonzalezae* were different from the available (Figure 1), whereas the size distribution selected by *Myrmosicarius brandaoi* did not differ from the sizes

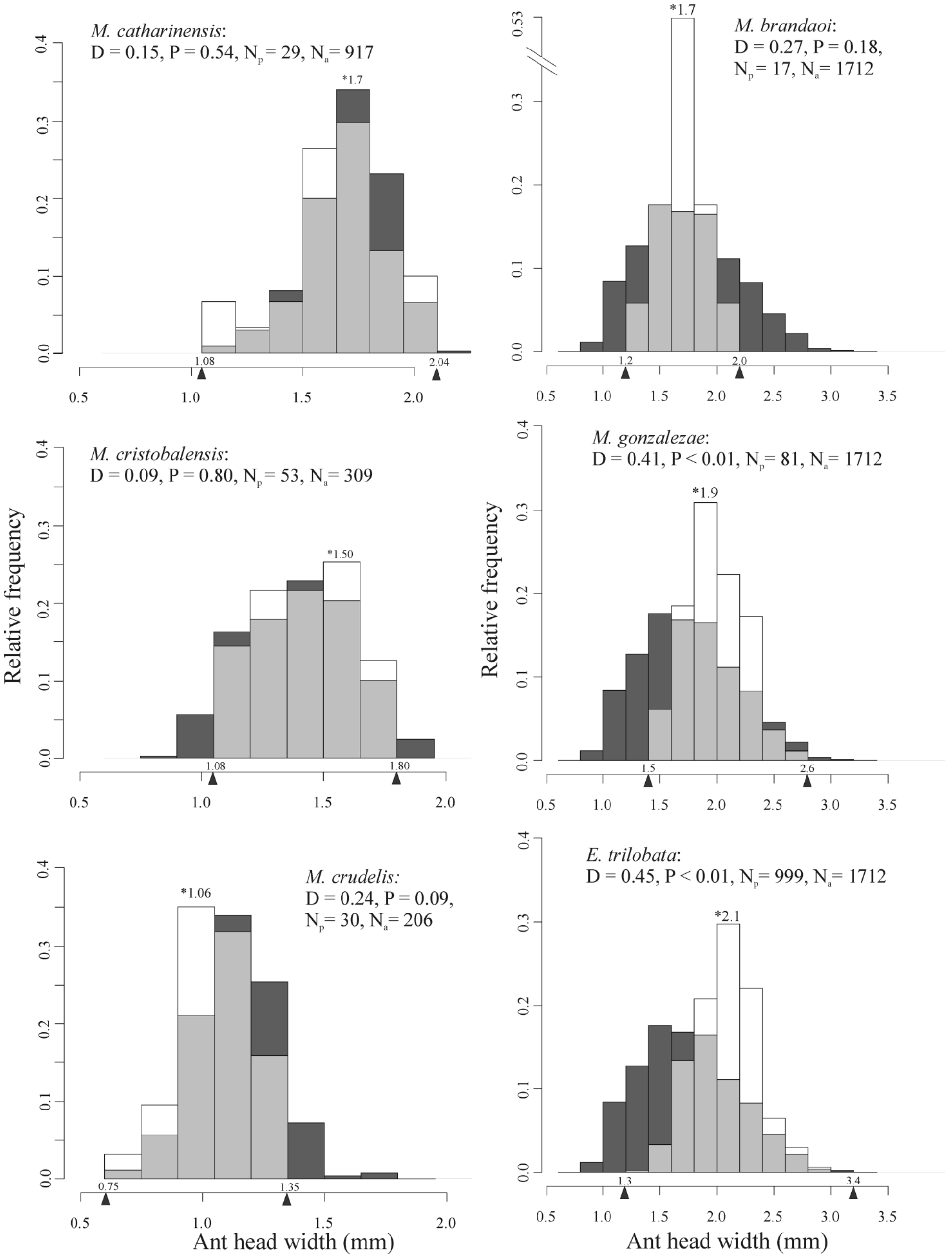

Figure 1. Ant size (head width in mm) distributions from foragers or waste removers from all nests pooled (in dark grey) discriminating those selected by different species of phorids (in white); light grey represents the intersection of both distributions). Left column, parasitoids of *Acromyrmex*: *Myrmosicarius catharinensis* selecting *Acromyrmex heyeri* foragers, *Myrmosicarius cristobalensis* selecting *Acromyrmex lobicornis* foragers, and *Myrmosicarius crudelis* selecting *Acromyrmex crassispinus* waste removers; right column, parasitoids of *Atta vollenweideri*: *Myrmosicarius brandaoi*, *Myrmosicarius gonzalezae* and *Eibesfeldtphora trilobata*. Asterisk denotes median values and black arrows indicate range of

of foragers (Figure 1). In fact, the size distribution of ants selected by these three species differed among each other (Kolmogorov–Smirnov tests with adjusted $P < 0.05$; median values differed according to the Mann–Whitney U-test, adjusted $P < 0.02$), with *M. brandaoi* using the smallest ants and *E. trilobata* using the largest (Figure 1). *Eibesfeldtphora trilobata* was the species with the broadest range of ant sizes used (almost twice those of the other species, Figure 1).

Relationship between host and adult phorid sizes

Phorid thorax width and femur length were correlated in all species (all Rho > 0.5, $P < 0.04$) and the relationship with host size was similar using both measurements. Therefore, we show the results for thorax width because is the traditional surrogate measure of phorid size.

Myrmosicarius cristobalensis showed a positive relationship between adult thorax width and *Ac. lobicornis* size (Figure 2A). *Myrmosicarius crudelis* showed a tendency for a positive relationship between parasitoid size and waste-remover workers, and *M. catharinensis* did not show any relationship (Figure 2A, statistical inference was not possible for the two later species due to the low number of reared adults).

Eibesfeldtphora trilobata and *M. gonzalezae*, parasitoids of *Atta*, exhibited a positive and significant relationship, where larger parasitoids emerged from bigger ant hosts (Figure 2B). The slopes for *E. trilobata* did not differ between gender ($P = 0.59$). In addition, median host sizes used by males did not differ from those used by females (Mann–Whitney U-test, $P = 0.3$), neither did the distribution of host ant head sizes used by both genders of *E. trilobata* (Kolmogorov–Smirnov test, $D = 0.12$, $P = 0.7$). *Myrmosicarius brandaoi* showed a tendency to increase phorid size if larger hosts were selected (Figure 2B, however, statistical inference was not possible because of the small sample size). The slopes of these regression lines did not differ between *E. trilobata* and *M. gonzalezae* ($P = 0.1$), nor compared with *M. cristobalensis*, parasitoid of *Acromyrmex* (all $P > 0.1$).

Parasitism rates in the field

Median parasitism rates in the field varied between 0.9–2.2% for *Acromyrmex* hosts and 3.8–20.2% for *Atta* hosts. Pooling data from all seasons, *Atta* showed higher parasitism than *Acromyrmex* (Mann–Whitney U-test, $P < 0.01$; median percentage parasitism for *Atta* was 6.6% and for *Acromyrmex* was 1.6%). However, for *Atta* there was great intra-seasonal and inter-seasonal variation, having similar parasitism rates than *Acromyrmex* for summer and winter 2006 (Figure 3). Remarkably, ants from both genera were parasitized by phorids during all seasons (Figures 3, 4 and 5).

The parasitism rates for *Acromyrmex* species showed variation depending on ant species, seasons and nests (Figure 4). Maximum parasitism rates were recorded during spring or autumn, depending on host species, and were 12.5% for *Ac. lundii*, 9.5% for *Ac. fracticornis*, 5.9% for *Ac. crassispinus*, 5.8% for *Ac. heyeri*, 5.3% for *Ac. hispidus*, 4.6% for *Ac. lobicornis* and only 2.7% for *Ac. striatus* (Figure 4). There were phorid

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host sizes selected used by phorids. Kolmogorov–Smirnov results from comparing the two distributions are shown. N_{p} refers to the number of puparia and N_{a} to the number of foragers or waste remover ants.

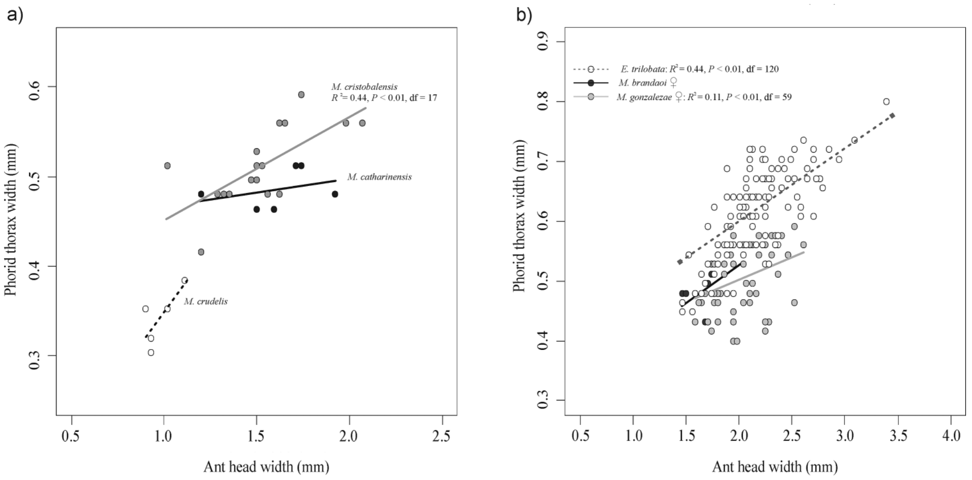

Figure 2. Adjusted least square regression lines for ant head sizes selected by phorids and the thorax width of the adult parasitoids that emerged from them. Above, parasitoids of *Acromyrmex*; below, *Atta*'s phorids; note different scales in both axes.

species that dominated the parasitism rate of their hosts, such as *M. cristobalensis* for *Ac. lobicornis* and *M. catharinensis* for *Ac. heyeri*. There was no constancy in parasitism rates by nests, as nests with the highest parasitism in a season did not have the greatest parasitism in other seasons.

Species with external refuse piles showed higher parasitism rates for waste removers than for foragers (paired Wilcoxon test: $P = 0.03$, $N = 6$, median parasitism 6.4% for waste removers and 0.8% for foragers of *Ac. lobicornis*; $P = 0.06$, $N = 5$, median parasitism 8.5% for waste removers and 3.4% for foragers of *Ac. hispidus*). However, *Ac. crassispinus* had similar percentages of parasitism for both groups of workers ($P = 0.25$, $N = 8$, median parasitism 1% for waste removers and 3.4% for foragers). The highest parasitism rate recorded for refuse removers was 12.3% for *Ac. lobicornis*, followed by *Ac. hispidus* with 8.7% and, lastly, 4.7% for *Ac. crassispinus*.

Atta vollenweideri showed the highest parasitism rate in winter 2005 for most nests (Figure 5), and those values were 1.5 to 6 times the median parasitism rates found in the other seasons. This exceptionally high percentage of parasitism, where in one nest more than 35% of the foragers were parasitized, was caused by *E. trilobata* (Figure 5). A dominance of one phorid species in the contribution to the parasitism was evident in most nests, although *E. trilobata* was not always the dominant species. Again, we did not find a coincidence in that the same nest had the highest parasitism in all seasons (Figure 5), although all nests sampled had parasitized ants throughout the year.

Phorid development

Puparia

We obtained a total of 2043 puparia of phorids. The puparia from species belonging to the same genus were similar. The puparia of *E. trilobata* occupied the ant head, and the sclerotized operculum with the respiratory horns emerging from the mouth cavity. Not all ant heads with puparia were separated from the bodies of the ants, i.e. decapitated.

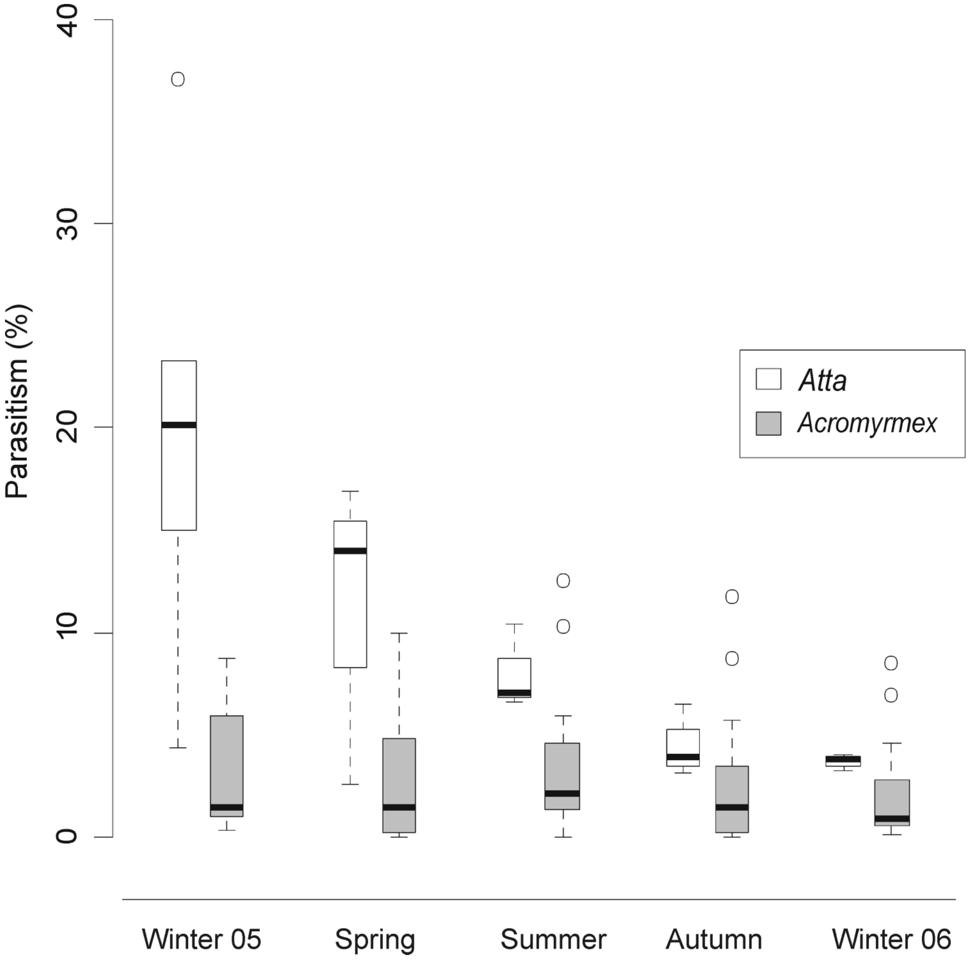

Figure 3. Seasonal parasitism rates by phorid parasitoids from of *Atta* and *Acromyrmex* ants. Boxes represent median and quartiles; circles are outliers.

The puparia of *Neodohrniphora unichaeta* reared from *Ac. heyeri* were similar to those of *Eibesfeldtphora* species.

The puparia of the six reared species of *Myrmosicarius* were inside the head, in the ventral–posterior region, under the tentorial arms. The respiratory horns were inside the empty ant head, in contrast to what happened with *Eibesfeldtphora*. All the phorid species from this genus decapitated the host.

Almost all reared *Apocephalus* species (*Ap. exstriatus*, *Ap. exlobicornis*, *Ap. intermedius*, *Ap. longisetarum*, *Ap. neivai*, *Ap. noetingerorum*, *Ap. penicillatus*, *Ap. philhispidus* and *Ap. setitarsus*) left the host body to pupariate outside. Although we did not observe any instance of the larvae leaving the host, in some dead ants there was a small hole in the mouth, between the maxillae and mandibles, from where it was possible that the larvae had left the ant body. The puparium was found at a certain distance from the dead ants, attached to the bottom or walls of the recipients. These phorids

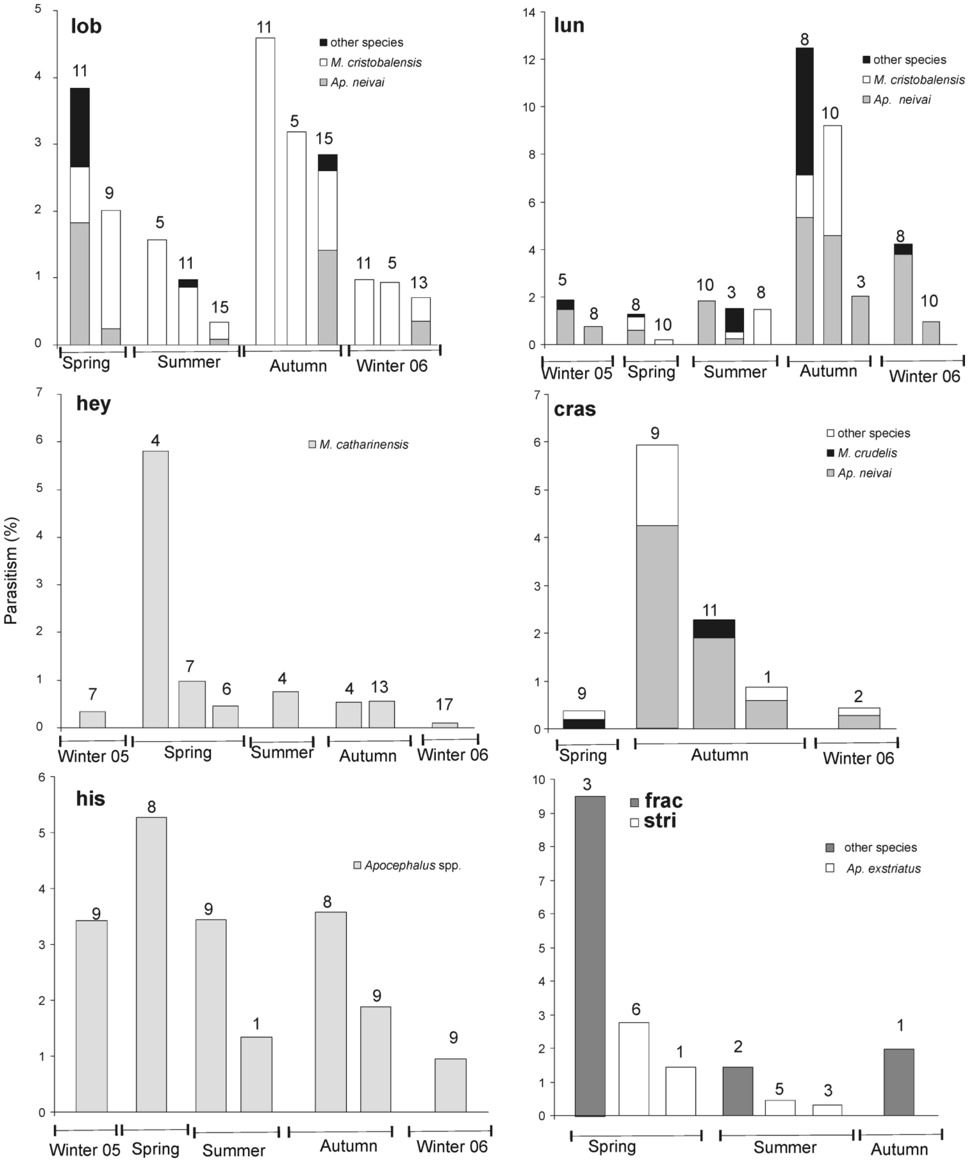

Figure 4. Natural parasitism rate by phorid species reared from ants collected from foraging trails at San Cristóbal for *Acromyrmex* phorids and discriminated by seasons. Numbers represent nest codes. Ant species names are abbreviated in the upper-left corner with the three first letters of the species epithet (lob: *Acromyrmex lobicornis*, lun: *Acromyrmex lundii*, hey: *Acromyrmex heyeri*, cra: *Acromyrmex crassispinus*, his: *Acromyrmex hispidus*, fra: *Acromyrmex fracticornis*, str: *Acromyrmex striatus*). Note that the *y* and *x* axes differ across plots, and the bottom plot from the right shows results for two ant species (*Ac. fracticornis* parasitized by "other phorids" in grey and *Ac. striatus* parasitized by *Ap. exstriatus* in white).

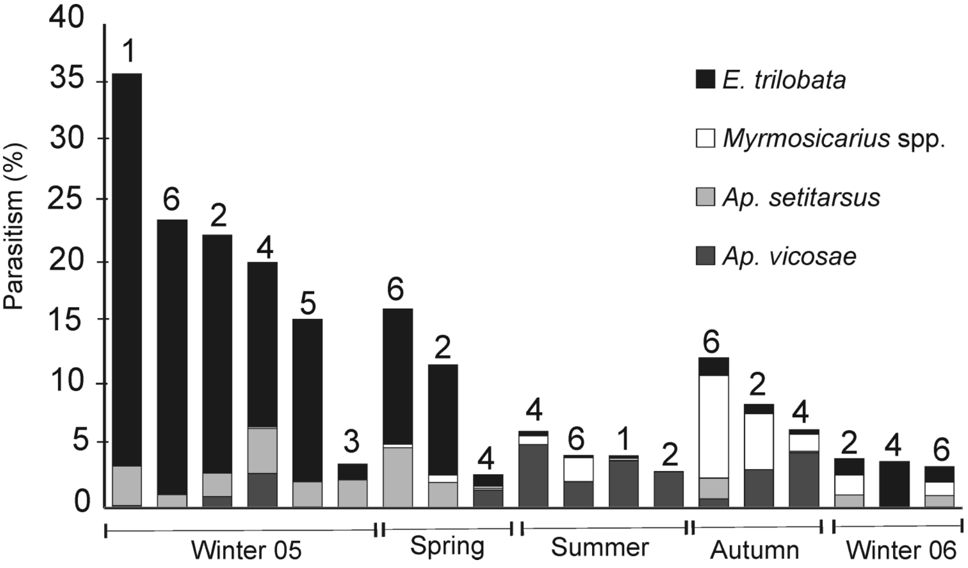

Figure 5. Natural parasitism rate by phorid species reared from *Atta vollenweideri* foragers, discriminated by seasons. The contribution of the two *Myrmosicarius* species was estimated together, as males were not possible to be identified. Numbers represent nest codes to follow parasitism through time.

did not seem to decapitate the host because dead ants in the containers where these puparia were found had the head still attached.

Apocephalus vicosae was an exception, as it used the thorax of the host ant to pupariate, with the respiratory horns peeking outside the ant body. The ants used as host by *Ap. vicosae* could have the propleura and the head separated from the rest of the body, or they could be attached by the dorsal part, leaving an exit for the protruding respiratory horns. In some cases, the gaster was detached from the rest of the body. For this species, only one individual was reared per host, however, for *Ap. setitarsus* we registered between one and three larvae per host, with males and females being reared in the same host. None of the phorids that parasitized *Acromyrmex* reared more than one individual per host.

Developmental times

We did not find differences in total developmental times for male and female phorid species. We used only female development periods for statistical comparisons of *Atta* phorids because we could not discriminate to species the male *Myrmosicarius*.

Parasitoid species of *Acromyrmex* showed differences in total developmental times ($H = 39.9$, df $= 2$, $P < 0.01$ for females and $H = 26.8$, df $= 2$, $P < 0.01$ for males; Table 2), with *M. cristobalensis* taking more time to develop (Table 2). For *Atta* phorids total developmental times were also different across species ($H = 21$, df $= 4$, $P < 0.01$).The difference was only the result of *E. trilobata*, which took more days to develop than *Ap. vicosae* (Table 2).

Parasitoids of both ant genera showed differences in the length of pupal period ($H = 41.8$, df $= 2$, $P < 0.01$ for females and $H = 20.1$, df $= 2$, $P < 0.01$ for males parasitoids of *Acromyrmex*; and $H = 34.6$, df $= 4$, $P < 0.01$, for *Atta*; see Table 2 for

Table 2. Median days (first and third quartiles between brackets) of developmental times for three phorid species parasitoids of *Acromyrmex* and five species parasitoids of *Atta* leaf-cutting ants obtained by larval parasitoid collection, discriminated by gender.

				Larval	Pupal	Total	N	emergence (%)‡
Acromyrmex hosts	*Apocephalus*	*neivai*	females	16^{a} (15–21.5)	14^{a} (14–19)	32^{a} (29.5–36)	23	90
			males	16^{a} (15.3–17.5)	14^{a} (14–16.3)	32.5^{a} (30–35)	10	
		noetingerorurm	females	14^{a} (11.3–17)	16^{a} (13–18)	30^{a} (27–30)	14	
			males	12^{a} (11.5–14.5)	18^{a} (15.5–18)	30^{a} (29.5–30)	7	
	Myrmosicarius	*cristobalensis*	females	15^{a} (13–18)	32^{b} (24–33)	48^{b} (41–49)	33	73
			males	16^{a} (13–22.5)	24^{b} (21–28)	41^{b} (37–48.3)	18	
Atta hosts	*Apocephalus*	*setitarsus*	females	$9^{a,b}$ (6–10)	24^{a} (17–31)	$34^{a,b}$ (27–37)	49	85
			males	6 (6–10)	21 (14–47)	34 (24–53)	45	
		vicosae	females	15^{c} (9–16)	14.5^{b} (12–23.5)	28^{a} (27–35)	78	60
			males	15 (12–16)	16 (12–20)	29 (28–34.8)	54	
	Eibesfeldtphora	*trilobata*	females	9^{a} (6–12)	28^{a} (21–31)	37^{b} (35–37)	128	86
			males	9 (6–11.5)	28 (21–31)	37 (32–37)	139	
	Myrmosicarius	*brandaoi**	females	$9^{b,c}$ (9–13)	$18^{a,b}$ (15–22)	$35^{a,b}$ (24–35)	10	80
		*gonzalezae**	females	$9^{a,b,c}$ (9–13.5)	25^{a} (22–28)	$35^{a,b}$ (30–37)	39	

Notes: The "Larval" column refers to the number of days from the date of collection to pupation. The "Pupal" column denotes the number of days between pupae detection and adult emergence. "Total" represents the sum of both periods. Different letters represent statistical differences among species ($P < 0.05$, adjusted for multiple comparisons) within a host genus and gender (not for male phorids reared from *Atta*) for each developmental time.

[‡] Calculated for each phorid species, but pooled for *Ap. neivai* + *Ap. noetingerorum*, and for *M. brandaoi* + *M. gonzalezae* (as it was not possible to discriminate the puparia, see Results).

[*] Information for females only because it is not possible to identify males.

multiple comparisons results). For larval developmental times there were differences for *Atta* phorids ($H = 50.1$, df $= 4$, $P < 0.01$; see Table 2 for multiple comparisons results); however, no differences were found for *Acromyrmex* phorids ($H = 4.5$, df $= 2$, $P = 0.1$ for females and $H = 6.3$, df $= 2$, $P = 0.045$ for males, but no significant differences were found for multiple comparisons among species).

We included the percentage of emergence as a way to evaluate our rearing method (Table 2). However, we were not able to estimate the percentage of emergence at the species level for *Apocephalus* reared from *Acromyrmex* and *Myrmosicarius* reared from *Atta* because it was not possible to separate the puparia belonging to the same genus up to species level. We found no correlation between larval, pupal and total developmental times with phorid adult size (all $P > 0.1$, for *E. trilobata*, *M. brandaoi*, *M. cristobalensis* and *M. gonzalezae*), nor for developmental times with host size used (all $P > 0.1$, for the same species).

Eibesfeldtphora trilobata showed differences in the total development according to the period of ant collection ($H = 173.6$, df $= 3$, $P < 0.001$). These differences were the result of phorids reared from ants collected in winter 2005, which had the longest total developmental time (median 37 days, adjusted $P < 0.01$ compared with the other seasons) and those from winter 2006, when phorids developed in the shortest time (median 27 days, adjusted $P < 0.01$ compared with the other seasons). Meanwhile, phorids reared from ants collected during spring and autumn did not differ in their total developmental period (medians 34.5 and 35 days, respectively). We only reared one individual during the summer. We did not rear enough specimens of other species for each season to make these comparisons.

Male–female ratios

Phorids collected during APC were almost all females; only 11 males were collected (representing 1.1% of all phorids collected) after 752 hours of sampling, five were *Apocephalus* and six were *Myrmosicarius* males (all collected over *Acromyrmex* hosts).

The male–female ratios obtained from LPC, however, were not significantly different from one for most phorid species reared (pooling all data for *Ap. neivai*, *Ap. setitarsus*, *E. trilobata*, *M. cristobalensis*, *M. brandaoi* and *M. gonzalezae*, because the males of these species were indistinguishable), except for *Ap. vicosae* ($P = 0.047$, male–female ratio 0.7). Nor did these ratios differ from one when estimated for each species discriminated by season (all $P > 0.05$, except for *E. trilobata* in winter 2006 with male–female ratio of 2.1, and *Ap. vicosae* during summer with a male–female ratio of 0.4).

Discussion

We report here substantial new information about the biology of phorid parasitoids of eight leaf-cutting ant species, most of them in the *Acromyrmex* genus, cohabiting in a site. First, we found that phorids parasitizing leaf-cutting ants did not use other dominant ant species as hosts and all the parasitoids that were using adult leaf-cutting ants belonged to the Phoridae. Hence, these phorids formed a well-defined guild, because they "exploit the same class of environmental resources in a similar way" (Root 1967). Although other parasitoids, such as diapriids could use the larvae of leaf-cutting ants as hosts (Loiácono 1987; Loiácono et al. 2000; Pérez-Ortega et al.

2010), it seems that phorids are the only group that have acquired the necessary adaptations to use adult leaf-cutting ant workers as hosts [but see Feener and Moss (1990)]. Furthermore, a strict definition of guilds should divide those phorids using *Atta* from those that use *Acromyrmex* species as hosts, because neither of these ant genera shared phorid species (this study; Elizalde and Folgarait 2010, with data from several localities). In the laboratory it was found that a phorid parasitoid of *Atta* could attack one of the two *Acromyrmex* species offered to oviposit (Silva et al. 2008), although it was not evaluated if the phorid could be reared from such *Acromyrmex* species.

We presented here, for the first time, the sizes of *Acromyrmex* ants susceptible to phorid parasitism. We found that almost all sizes, except for the very extremes of the range, could be used as hosts. These findings may imply that phorids of *Acromyrmex* studied here were not selecting hosts within a particular range of size, or at least, they were less selective than parasitoids of *Atta*. Even ants with a head width of 0.75 mm were used by phorids attacking *Acromyrmex*, whereas in *Atta* none of the smallest foragers, ranging from 0.70 to 1.20 mm in head width (*c*. 32% of foraging ants) were attacked. Accordingly, the defence against phorids found in *Atta*, by which the small foragers not susceptible to parasitoids continue foraging when phorids attack while the bigger ants retreat to the nest (Orr 1992; Bragança et al. 1998), seems not to be an effective defence against phorids for these *Acromyrmex* workers.

The biggest sizes of *Atta* workers (with a head width greater than 3.5 mm), represented by soldiers, were not commonly seen in foraging trails, and we did not observe any instance of phorids attacking them, nor did we ever rear a phorid from a soldier. Foragers of *At. vollenweideri* of medium size, in addition to bigger ants, were susceptible to parasitism by phorids, as was found before for *Myrmosicarius grandicornis* (Tonhasca et al. 2001). The distribution of sizes selected by two species of parasitoids attacking *At. vollenweideri*, *E. trilobata* and *M. gonzalezae*, differed from worker size distribution by selecting principally hosts from the larger end of the foragers' size range. Large foragers have been reported to be the target of most phorid species (Waller and Moser 1990; Feener and Brown 1993; Tonhasca 1996; Erthal and Tonhasca 2000; Bragança et al. 2002; but see Brown 1999). However, *E. trilobata*, although selecting the largest hosts, showed an overlap with almost all ant sizes selected by *M. brandaoi*, the species that used the smallest ant hosts. As tasks of foragers in *Atta* vary with worker size (Hölldobler and Wilson 1990), *E. trilobata* using such a wide range of sizes during our study, could have exerted a higher impact on their host than the other species.

Besides the shortage (for workers smaller than 1.20 mm in head width) or surplus (for soldiers) of food available in a host to account for host size selection for *Atta* phorids, it seems that other mechanisms might be involved, as the three phorid species frequently found attacking *At. vollenweideri* used host sizes with different medians. One such mechanism, with a possible important role in determining host size selection, could be host size segregation to reduce competition for food (Brown 1999; Tonhasca et al. 2001; Guillade and Folgarait 2011). Evidence in favour is provided by the fact that during winter 2005, when the population of *E. trilobata* showed a surprisingly high abundance, and extreme ant sizes were used with high frequency by this species, we did not rear any *M. brandaoi*, and very few individuals of *M. gonzalezae* (Figure 5 for both species together). Meanwhile, during autumn, when the abundance was evenly distributed among phorid species, *E. trilobata* used the lowest percentages of medium foragers.

Bigger phorid adults were reared from bigger *Acromyrmex* and *Atta* hosts (except *M. catharinensis*, where it is probably an artefact of low sample size), although the relationship was not very strong, accounting for a low proportion of the variation. Different responses seem to have evolved for different species of phorids of leaf-cutting ants because, for example, adults of *E. trilobata* were bigger than those of *Myrmosicarius* that attacked *Atta* workers of the same size (Figure 2B). This could be explained by a more efficient use of the available food by the phorid larvae or by the host feeding for more time or more continuously, i.e. requesting food from their nest mates more frequently – there is some evidence of increased trophallaxis for *Solenopsis* ants parasitized by *Pseudacteon* phorids, Elizalde et al. (2004). On the other hand, puparia of *Myrmosicarius* species may be constrained by space, because they are completely inside the host head, whereas the anterior part of *Eibesfeldtphora* puparia protrudes from the cephalic capsule of the ant head. In this way, *Eibesfeldtphora* can accommodate a bigger puparium than *Myrmosicarius* inside a host head of similar size.

Maximum percentages of parasitism reported here, for both *Acromyrmex* and *Atta*, were higher than any reported previously for ant phorids (Feener 1988; Erthal and Tonhasca 2000; Morrison and Porter 2005; Guillade and Folgarait 2011). For *Acromyrmex* there were no previous reports, and we found that natural parasitism could be similar to that of *Atta* depending on the season and host species. Therefore, phorids of leaf-cutting ants may exert an important direct negative effect, reducing 35% and 12% of foragers (*Atta* and *Acromyrmex*, respectively), or 12% of waste removers, affecting the worker force involved in food retrieval and colony sanitation. Maximum percentages of parasitism incorporate both host defences and the responses that parasitoids have evolved against them (Hawkins 1994), and provide an important measure of the effect that phorids could potentially have on leaf-cutting ants. This direct effect on colony mortality should complement the demonstrated indirect effects that phorids have on their hosts, such as a reduction in the foraging activity (Bragança et al. 1998), a change in daily foraging rhythms (Orr 1992) and forager size (Orr 1992; Bragança et al. 1998; Silva et al. 2007), leaf abandonment by carriers (Bragança et al. 1998), besides a halt in walking by ants to display different postures or defensive behaviours (Feener and Moss 1990; Elizalde and Folgarait in preparation).

The place where the puparia developed and the presence or absence of ant decapitation during development were distinctive for each leaf-cutting phorid genus, and so they could be used as an identification tool. The *Apocephalus* type (Feener and Moss 1990; Erthal and Tonhasca 2000; excluding *Ap. vicosae*, Bragança and Medeiros 2006; this study) is characterized by forming a puparium typical of the Phoridae (see Figure 4.1 in Disney 1994), develops outside the host body, and does not seem to produce decapitation. An *Eibesfeldtphora–Neodohrniphora* type is a puparium with the operculum emerging from the mouth of the host (Tonhasca 1996; Bragança et al. 2008; Guillade and Folgarait 2011; this study), whether the ant is decapitated or not. Finally, a *Myrmosicarius* puparium type (Tonhasca et al. 2001; Guillade and Folgarait 2011; this study) remains completely inside the head, occupying the ventral-posterior part, and always decapitates the ant host. Distinctive methods of puparia and decapitation may represent alternative ways of reducing pupal predation (Guillade and Folgarait 2011; see below) or different selective pressures for adult size (see above).

Three species of *Apocephalus* attacking *Atta* could yield more than one individual per host (*Ap. colombicus*, Feener and Moss 1990; *Ap. attophilus*, Erthal and Tonhasca

2000; *Ap. setitarsus*, Brown et al. 2010, this study), and this may have been possible because those species pupariated outside the host. A positive correlation between clutch and host size was found for one of those species (Erthal and Tonhasca 2000), suggesting that clutch size could also be influenced by the availability of host sizes. In fact, none of the *Apocephalus* phorid species using *Acromyrmex* developed more than one larva per host in this study. Probably, this relates to the size of the head of *Acromyrmex*, which is smaller than that of *Atta* (Figure 1). Much work has been devoted to investigate clutch size in hymenopteran parasitoids (reviewed in Godfray et al. 1991), and it will be interesting to compare with dipteran parasitoids.

We provide for the first time, information about the development of phorid parasitoids of *Acromyrmex* ants. Even though there was high intraspecific variation, some of these species coexisting in a locality differed in developmental times. As was suggested previously for parasitoids of *Atta* (Guillade and Folgarait 2011), differences in developmental times among phorid species could be the result of interspecific differences related to specific selective pressures. For example, the two *Apocephalus* parasitoids of *Acromyrmex* showed the shortest pupal period, which could be useful to reduce pupal predation or injury because they pupariate outside the host, while the other phorid species have their puparium protected by the empty host head (see above).

Parasitoids of *At. vollenweideri* showed differences in developmental times because of *E. trilobata* having significantly longer development than *Ap. vicosae*. Guillade and Folgarait (2011), also found that *E. trilobata*, in addition to *M. brandaoi*, had longer developmental times than *Ap. setitarsus* and *M. gonzalezae*. In that study, rearing was performed at a higher temperature than ours (26°C versus 22°C), and both intraspecific variation in developmental days and total developmental times were smaller than those reported here. Interestingly, these differences in total developmental times were the result of an increment in the pupal period but not in the larval period. Shorter development at higher temperature is a general rule for insects (Kingsolver and Huey 2008), as was found for other phorid parasitoids of ants (Folgarait, Bruzzone, Patrock et al. 2002b, Folgarait et al. 2005, 2006). The high intraspecific variation found in developmental times, both for phorids of *Acromyrmex* and *Atta*, was neither due to host size nor phorid adult size, as we found no correlation between those variables. Although part of this variation could be the result of our data collection protocol (see Methods), it will be interesting to study if host nutrition or ambient conditions during the earliest larval instars play a role to account for this variation, as suggested by the differences of developmental times found for *E. trilobata* according to different periods of ant host collection.

So far it, has been demonstrated for an *Apocephalus*, two *Eibesfeldtphora* and three *Myrmosicarius* species, that the proportion of males to females in natural populations (Guillade and Folgarait 2011) and for phorids reared in the laboratory (Tonhasca et al. 2001; Bragança et al. 2007) was close to one, and we also found similar proportions, adding another two *Apocephalus* and one *Myrmosicarius* species. However, the absence of males at oviposition sites for phorid species in five genera attacking these ants (*Apocephalus*, *Eibesfeldtphora*, *Myrmosicarius* and *Neodohrniphora* this study; *Lucianaphora*, L.E. unpublished results), suggests that mating occurs elsewhere. As these ants display aggressive behaviours against the phorids flying close to them (e.g. Feener and Moss 1990; Tonhasca 1996; Elizalde and Folgarait in preparation), mating near the ants could be very risky.

Larval parasitoid collection proved to be very useful and we would like to suggest it as an additional tool for studies of phorid parasitoids of leaf-cutting ants. First, it provides information about the presence of more species and interactions than the adult parasitoid collection, even when the adults cannot be detected in the field (we found seven species exclusively with this sampling). Second, it allows confirmation of parasitoid host ranges, as host ranges based only on adult collections over hosts miss the developmental barrier that host immune defence imposes. Third, it gives valuable information about phorids' life-history traits, such as developmental times, sex ratios in natural populations and pupariation sites. Lastly, it suggests which species can be reared easily and in high numbers in the laboratory, information useful for their use in biological control.

Finally, the information presented here highlights the potential importance of using these phorids in the biological control of leaf-cutting ants, as suggested previously by others (e.g. Borgmeier 1931; Bragança 2007). Some of these reasons include: (1) the reduced risk that they pose to non-host ants as they constitute a well-defined guild; (2) these phorids were not attacked by hyperparasitoids (in this study or in the literature, although there is evidence of superparasitism in an *Atta* phorid; Feener and Brown 1993), which are an important impediment for successful biological control programmes (Rosenheim 1998); (3) several host sizes are susceptible to parasitoid attack, for both leaf-cutting ant genera, with a potential impact on different tasks carried out by ants; (4) the high parasitism rates found indicate that increasing phorid population sizes by augmentative programmes may increase significantly direct mortality; (5) host species were attacked by phorids year round; and (6) several species were successfully reared in the laboratory and in high numbers. In addition, phorids are being used as fire ant biocontrol agents (Gilbert and Patrock 2002; Porter et al. 2003; Vázquez et al. 2006), offering a reference framework to start using them to control leaf-cutting ants, one of the most damaging pests in the Neotropics (Cherrett 1986).

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