

Host specificity in the host-seeking larva of the dipteran parasitoid *Mallophora ruficauda* and the influence of age on parasitism decisions

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Abstract

Larvae of the robber fly *Mallophora ruficauda* are ectoparasitoids of white grubs and adults are an important apiculture pest in Argentina. Females oviposit on tall grasses and the second instar larva actively searches and locates hosts. There are nine potential hosts in the distribution area of this parasitoid and *Cyclocephala signaticollis* (Coleoptera: Scarabaeidae) is the most parasitized in the field. However, *M. ruficauda* has a certain degree of behavioural flexibility towards different host species, and not being a strict specialist. The conditions under which the parasitoid orientates and accepts different hosts' species are unknown. We studied the host specificity of *M. ruficauda* towards three species of *Cyclocephala* genus and we determined whether this specificity depends on larval age. We also evaluated whether larva orientation towards *Cyclocephala* species changes with chemical cue concentration. We assessed host specificity measuring the orientation and acceptance behaviours towards kairomones extracts and live individuals of *Cyclocephala* species using *M. ruficauda* larvae of low and high life expectancy (i.e., young and aged second instar larvae). We observed that young larvae orientated only towards *C. signaticollis* chemical stimulus, whereas aged larvae orientated also towards *C. modesta*, and the same was observed with increasing stimuli's concentration. Both young and aged *M. ruficauda* larvae orientate towards live *C. signaticollis* and *C. putrida* species and rejected *C. modesta*. Also, we found that larvae accepted all *Cyclocephala* hosts. In conclusion, our results indicate that specificity in the laboratory, observed through host orientation and host acceptance behaviours, depends not only on the availability of host species, but also on the nature of the host's stimuli combined with parasitoid age.

Keywords: Asilidae, host location, life expectancy, Scarabaeidae

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Introduction

Successful parasitism by parasitoids requires a sequence of hierarchical steps including host habitat location, host location, acceptance and suitability (van Alphen & Vet, 1986; Brodeur & Boivin, 2004). There are numerous works that relate the parasitism success for each of these hierarchical steps to external factors, such as host availability and host

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quality, and internal factors, such as egg load, life expectancy or energy reserves (Javoiš & Tammaru, 2004; Mohamed *et al.*, 2006; Wajnberg *et al.*, 2006; Canale & Benelli, 2012; Benelli *et al.*, 2013a). Most of these studies were done on Hymenoptera, where the adult female carries out most of the behavioural steps of the parasitism process (Godfray, 1994). In a few Hymenoptera (Eucharitidae) and in other orders of parasitoids, such as Diptera (Tachinidae, Bomyliidae), Lepidoptera (Epipyropidae), Neuroptera (Mantispidae) and most Coleoptera (Staphylinidae), location and selection of the host are performed by the first instar parasitoid larva (Eggleton & Belshaw, 1992, 1993; Yeates & Greathead, 1997; Brodeur & Boivin, 2004; Stireman *et al.*, 2006; Torrén, 2013). Nevertheless, relatively few studies have focused on the behavioural ecology of immature parasitoids (Royer *et al.*, 1999; Brodeur & Boivin, 2004; Crespo & Castelo, 2009). This might be because the factors influencing host acceptance have been considered irrelevant since host-seeking larvae have a lower frequency of host encounter due to their limited dispersal ability (Feener Jr. & Brown, 1997). However, for parasitoid species whose hosts are spatially aggregated, host discrimination might be advantageous for the larvae since they may encounter more than one host in their life (Royer *et al.*, 1999; Vet *et al.*, 2002; Brodeur & Boivin, 2004).

In parasitoids where the host-searching task is performed by the first instar larva, its entire fitness comes from a single host (Royer *et al.*, 1999; Brodeur & Boivin, 2004). Then, fitness can be directly related to host quality, which varies according to host species, age, sex, size, parasitism degree, host defences and nutritional state, and assessing host quality is of crucial importance for parasitoids (Godfray, 1994; Brodeur *et al.*, 1996; Brodeur & Boivin, 2004; Castelo & Crespo, 2012). However, even when parasitoids can assess host quality efficiently, low-quality hosts are not always rejected (Heimpel *et al.*, 2003). Physiological state could influence the acceptance of low-quality hosts (Fletcher *et al.*, 1994; Sirot *et al.*, 1997; Javoiš & Tammaru, 2006), and how the animal modifies its behavioural decisions throughout its life in response to its physiological and reproductive states is known as state-dependency (Mangel & Clark, 1988; Clark & Mangel, 2000; Roitberg & Bernard, 2007; Bernstein & Jervis, 2008).

Another important aspect for the successful parasitism is the capacity of parasitoids to attack one or more host species. The host range, i.e., the number and taxonomic diversity of species in which the parasitoid is able to develop, defines the host specificity (Futuyma & Moreno, 1988; Desneux *et al.*, 2009). This specificity is affected by the physiological state and nutritional value of the host, behavioural host defences and presence of natural enemies (Slansky, 1986; Brodeur *et al.*, 1996). As host acceptance represents the last step of parasitoid host-searching, it might be used as a reliable indicator of parasitoid host-specificity (Brodeur *et al.*, 1996). However, the influence of internal factors such as parasitoid age, on the behavioural steps during parasitoid host-searching could influence the parasitism success due to the effects of the state-dependency on the process.

The intense selection pressure on parasitoids to locate hosts is well illustrated by the variety of cues and strategies used in host searching (Wang *et al.*, 2010; Joyce *et al.*, 2011; Benelli *et al.*, 2013b, c; Colazza *et al.*, 2013; Uefune *et al.*, 2013). The stimuli sources used by parasitoids may be direct, as the host itself, or indirect, as the microhabitat of the host or a cue associated with its activity (Vet & Dicke, 1992; Steidle & van Loon, 2003;

Colazza *et al.*, 2013; Uefune *et al.*, 2013). In turn, these cues can be acoustic or visual but chemical cues seem to be the most frequent method of host location (Godfray, 1994; Rutledge, 1996; Feener Jr. & Brown, 1997; Cournoyer & Boivin, 2004; Vet *et al.*, 2002; Gray *et al.*, 2007). Since pheromones mediate the communication between conspecifics, they might be an important source of information for predators and parasitoids that can benefit from exploiting the host pheromones as kairomones (Dicke & Sabelis, 1988; Zuk & Kolluru, 1998; Ruther *et al.*, 2002; Wertheim, 2005; Wertheim *et al.*, 2005). Kairomones are composed by a particular qualitative blend of substances (Chapman, 1998; Greenfield, 2002). The responses of parasitoids to host pheromones as kairomones appear to be host-specific, since they respond to pheromones of natural hosts, but not to those of a more distantly related non-target host species (Yong *et al.*, 2007). Thus, the quantitative blend composition of a cue, and the concentration in which it is released, could inform parasitoids about the identity of a host species among species belonging to the same genus.

Among dipteran parasitoids the most common strategy to locate hosts is the exploitation of the host's communication system, mainly through the detection of chemical cues (Feener Jr. & Brown, 1997; Groba & Castelo, 2012). Most dipteran parasitoids, which have a split host location strategy with an active larval stage performing the final location and parasitism of the host, must use reliable cues, such as pheromones, to find them efficiently given their reduced mobility and the potential time-limitation (Eggleton & Belshaw, 1992, 1993; Stowe *et al.*, 1995; Feener Jr. & Brown, 1997; Brodeur & Boivin, 2004; Stireman *et al.*, 2006). The use of host-reliable cues enhances the efficiency in host finding and consequently increases the fitness on time-limited parasitoids (Vet *et al.*, 1991; Wajnberg *et al.*, 2006).

Mallophora ruficauda Wiedemann (Diptera: Asilidae) is a robber fly endemic to the Pampas region of Argentina that inhabits open grasslands near apiaries (Rabinovich & Corley, 1997). This fly presents a biological duality; as an adult it is a predator that feeds mainly on honey bees and as a larva it is a solitary koinobiont ectoparasitoid of scarab beetle larvae (Coleoptera: Scarabaeidae), commonly known as white grubs. In this parasitoid, host searching is shared by adults and immature stages. During the summer, females *M. ruficauda* oviposit on tall grasses maximizing larvae dispersal by the wind (Castelo & Corley, 2004; Castelo *et al.*, 2006). After hatching, first instar larvae fall to the ground and rapidly bury themselves. Seven days later they moult into the second instar larva using their own reserves (Crespo & Castelo, 2010). In this instar, the larvae acquire the ability to orientate towards the host through chemical cues originating in the hosts' hindgut (Castelo & Lazzari, 2004; Crespo & Castelo, 2008; Groba & Castelo, 2012). Crespo & Castelo (2010) estimated under laboratory conditions the median duration of the second instar larvae was 32 days in absence of the host and 109 days after they parasitized the host. Moreover, this larval instar is capable of discriminating the parasitism status of the host by means of chemical cues (Crespo & Castelo, 2009). Once *M. ruficauda* larva locates the host and parasitism takes place, the larva remains attached to its host during the winter as a second instar. Then, at the end of the winter, when temperature slowly increases, the larva grows rapidly and one month later it completes its development, by consuming the host and pupating (Crespo & Castelo, 2010). The successful parasitic relationship occurs between the

second instar larva of *M. ruficauda* and the third instar scarab larva.

Scarabaeidae larvae are rhyzophagous and live in the soil during the winter (March to August) (Remedi de Gavotto, 1964; Alvarado, 1983; Potter, 1998). There are nine species of scarab beetles within the *M. ruficauda* distribution area (Alvarado, 1980). Castelo & Corley (2010) described the field specificity of *M. ruficauda* towards the species of white grubs and they found that *M. ruficauda* selects *Cyclocephala signaticollis* Burmeister among several scarab species because its relative frequency of parasitism towards this species is the highest (86.60%). In other species of the same genus, the relative frequencies of parasitism are smaller, being 6.70% for *C. modesta* Burmeister and 1.44% for *C. putrida* Burmeister. For *Heterogeniates bonariensis* Ohaus the frequency is 0%. The relative frequencies for the other species vary between 3.35 and 0%. However, this shows *M. ruficauda* to have a certain degrees of behavioural flexibility towards the acceptance of different host species and is not a strict specialist of *C. signaticollis*. However, it still remains poorly understood that if this parasitoid larva has the ability to orient itself towards different species of white grub in different scenarios according to its own physiological state.

This work seeks to determine the host orientation and host acceptance behaviours of the second instar larva of *M. ruficauda* for different white grub host species, as a measure of host's specificity. Furthermore, we test whether the larvae's host specificity is affected or modulated by larval life expectancy and concentration of host stimuli.

Materials and methods

Insects

Larvae of *M. ruficauda* were obtained from egg clutches collected from the grasslands in Moreno (34°46'S, 58°93'W), a locality associated with apiaries in Buenos Aires province, Argentina, between January and March 2009, 2010 and 2011. The egg clutches were individualized in Falcon type tubes and were observed daily to register hatching. When the eggs hatched, the larvae were separated individually in Eppendorf type tubes of 1.5 ml with a piece of filter paper as a substrate sowed with mineral water. Tubes were stored in complete darkness between 24 and 26°C until they were used in the experiments. Before initiating any experiment every larva was checked to have moulted to the second instar (LII).

Hosts were collected in Moreno, Pilar (34°28'S, 58°55'W), General Rodríguez (34°27'S, 58°57'W), Escobar (34°20'S, 58°49'W) and Mercedes (34°65'S, 59°43'W), localities from Buenos Aires province and the Experimental Field of Ciudad Universitaria (Nuñez, Buenos Aires city, 34°32'S, 58°26'W) between March and August 2008–2011. White grubs were collected digging the soil to a depth of 0.3 m (López *et al.*, 1994; Castelo & Corley, 2010). At the laboratory, each individual was identified up to species level using a taxonomic key (Alvarado, 1980). The hosts, third instar larvae of white grubs, were maintained individually at room temperature in black tubes filled with soil and were fed weekly with fresh pieces of carrots until they were exposed alive in the experiments or used for preparing the stimuli extracts offered to the parasitoid larvae in the orientation experiments.

General considerations

M. ruficauda larvae used in all the experiments differed in their age and thus on their life expectancy. First, *M. ruficauda* larvae of 17–28 days old were considered 'young larvae'. These larvae have recently moulted to the second instar and had a high life-expectancy, i.e., 86–94% (Crespo & Castelo, 2010). Then, 'old larvae' were those with an age of more than 58 days old, where the risk of mortality was higher and the probability of survival decreased to 55% or less (Crespo & Castelo, 2010).

Specificity experiments were performed to determine if host selection is determined by behavioural flexibility related to parasitoid age. We used as treatments chemical cues extracts and live white grubs of three species of *Cyclocephala* (*C. signaticollis*, Cs; *C. modesta*, Cm and *C. putrida*, Cp) tested on both young and old parasitoid larvae. The first species is the naturally most selected in the field and the other species have a relative low parasitism frequency. In all the experiments with live white grubs or with chemical cues extracts, individuals of *H. bonariensis* (Hb) (orientation and acceptance behaviour, tables 1 and 2, respectively) were used as a negative control due to their null parasitism frequency in the field (Castelo & Capurro, 2000; Castelo & Corley, 2010). This species was used to evaluate the selectivity towards other species tested. The control species allowed us to compare the response of parasitoid larvae against the experimental groups and also rule out any age effect on the larvae's mobility, as indicator of larvae's health.

Specificity measured as orientation behaviour

In order to determine the degree of specificity of the LIIs, measured through the orientation behaviour towards host chemical cues extracts and live hosts, and if this specificity changes with the age of the parasitoid larva, we performed two laboratory dual-choice experiments in two rectangular static air two-way olfactometers. Since the natures of stimuli offered were different, the olfactometers used on each experiment differed in size (see details below). However, the principle was the same in both devices.

In both olfactometers, the arena was divided into three equal size zones (one middle and two laterals) along the long axis. On each lateral zone either the stimulus or control was placed. At the beginning of each trial, an individual *M. ruficauda* larva was released on the central area of the arena, and after 90 min, each device was examined to record the larva's position. Three possible responses could be obtained: choice for the stimulus (S), for the control (C) or no decision (ND) if the larvae remained in the middle zone. Replicates where *M. ruficauda* larva died during the assay were excluded from the statistical analysis. Because environmental factors can influence patch-time exploitation (Amat *et al.*, 2006; Crespo & Castelo, 2012), all the experiments of dual-choice were done between 10:00 and 17:00 h, under laboratory conditions (25.1 ± 3.7°C, 52 ± 22% relative humidity) and in complete darkness. Additionally, olfactometry experiments were conducted only on sunny days with barometric pressure values between 1008 and 1021 hPa, since it has been shown that both a low and a sudden drop in barometric pressure has dramatic effects on patch-time exploitation and host-seeking behaviour in insects (Roitberg *et al.*, 1993; Crespo & Castelo, 2012).

Table 1. Experimental design to determine the degree of specificity of the second instar larvae of *M. ruficauda* measured as orientation behaviour towards host chemical cues and live hosts, and if the specificity changes with the age of the parasitoid larva. This experimental series were performed to evaluate the responses of *M. ruficauda* larva to chemical cues and live hosts in olfactometer assays. *N*, number of replicates. Between brackets the total number of individuals that made a choice (left, stimulus; right, solvent/empty). The difference between the number of replicates and number of parasitoids that chose any side of the arena are called the 'No decision' larvae.

Larval age	Stimulus (species)	Experiment (nature of stimulus)	<i>N</i>	χ^2	df	<i>P</i>
Young	<i>Cyclocephala signaticollis</i>	Chemical cue	132 (63–42)	4.200	1	0.040*
		Live host	51 (23–10)	5.121	1	0.023*
	<i>Cyclocephala modesta</i>	Chemical cue	129 (45–59)	1.885	1	0.169
		Live host	62 (22–13)	2.314	1	0.128
	<i>Cyclocephala putrida</i>	Chemical cue	133 (52–45)	0.505	1	0.477
		Live host	49 (25–10)	6.428	1	0.011*
	<i>Heterogeniates bonariensis</i>	Chemical cue	140 (50–52)	0.039	1	0.84
		Live host	57 (17–19)	0.111	1	0.739
	Control	Chemical cue (hexane)	139 (56–54)	0.036	1	0.85
		No host	66 (16–16)	0	1	>0.999
Old	<i>Cyclocephala signaticollis</i>	Chemical cue	137 (64–41)	5.038	1	0.024*
		Live host	81 (31–5)	18.777	1	<0.001*
	<i>Cyclocephala modesta</i>	Chemical cue	197 (77–49)	6.222	1	0.012*
		Live host	152 (21–15)	1.000	1	0.317
	<i>Cyclocephala putrida</i>	Chemical cue	168 (53–59)	0.321	1	0.571
		Live host	89 (27–10)	7.811	1	0.005*
	<i>Heterogeniates bonariensis</i>	Chemical cue	189 (65–72)	0.357	1	0.55
		Live host	157 (19–18)	0.027	1	0.869
	Control	Chemical cue (hexane)	138 (51–56)	0.223	1	0.636
		No host	58 (18–12)	1.200	1	0.273

Table 2. Experimental design performed to evaluate the degree of specificity of the second instar larva of *M. ruficauda* regarding to age as the acceptance behaviour to different white grub species through no-choice tests (artificial parasitism). *N*, number of replicates. Between brackets the individuals found attached to the host (left) and number of missing larvae (right).

Larval age	Host species	<i>N</i>	Parasitism rate (%)
Young	<i>Cyclocephala signaticollis</i>	48 (41–7)	100
	<i>Cyclocephala modesta</i>	43 (34–8)	97.14
	<i>Cyclocephala putrida</i>	39 (35–4)	100
	<i>Heterogeniates bonariensis</i>	40 (0–5)	0
Old	<i>Cyclocephala signaticollis</i>	42 (26–16)	100
	<i>Cyclocephala modesta</i>	44 (16–26)	88.88
	<i>Cyclocephala putrida</i>	39 (21–17)	95.45
	<i>Heterogeniates bonariensis</i>	40 (0–16)	0

Orientation to chemical cues

For assays with chemical cues extracts we used a 9 cm × 6 cm × 1 cm olfactometer, which is the same experimental arena used by Castelo & Lazzari (2004) and Crespo & Castelo (2008). The stimuli were odour extracts from the four species of white grubs. They were offered a piece of filter paper (2 cm × 1 cm) at each lateral zone containing either 10 µl of chemical cue or solvent. Experimental design and replicates for each treatment are detailed in table 1.

To obtain the stimuli extracts from the hosts with kairomones, the posterior body part of third instar larvae was homogenized using hexane as solvent (Castelo & Lazzari, 2004; Groba & Castelo, 2012). We used experimental solutions equivalent to 2.5 white grubs per ml, being more than double the attractive concentration used by Castelo & Lazzari (2004), guaranteeing the occurrence of behavioural responses.

Orientation to live hosts

For live hosts we used an arena of 15 cm × 4 cm × 4 cm divided equally in three areas with a plastic mesh preventing the movement of the white grub outside the lateral zone but allowing the parasitoid larva to move freely. An individual host was placed in one of the lateral zones, whereas the other remained empty. Control series for assays with live hosts were performed without hosts in both sides. Experimental design and replicates for each treatment are detailed in table 1. These series allowed us to detect any possible asymmetry effect inherent to the experimental device.

Specificity measured as host acceptance behaviour

In order to evaluate the degree of specificity of the LII, measured through the acceptance behaviour towards different white grub species, and if the acceptance changes with the age of the parasitoid larva, we carried out no-choice tests consisting in artificial parasitism. For each assay, an individual host was placed in a 30 ml black tube filled with soil and food and with an LII. Each replicate, 39–49 per host species and parasitoid age treatment, was run for 1 week. After this period, the hosts were inspected to verify if the larva was attached to their cuticle, and had become a parasitized host. If the parasitism did not occur, we proceeded to register the soil to detect whether the larva was dead or remained free in the tube unattached to the host. Parasitism rate was calculated for each white grub species as the ratio between the number of parasitized hosts and the total number of hosts used in the experiment. Replicates where the white grub or the *M. ruficauda* larva died unattached to the host during the assay were not taken into account for calculating parasitism rate nor for the statistical analysis. Treatments and controls that were carried out for each white grub species and for each larval age are shown in table 2. Also, we registered when larvae got lost (i.e., not found neither attached to the host

Table 3. Experimental design series carried out in olfactometer assays to evaluate the degree of orientation (specificity) of the second instar larva of *M. ruficauda* to increasing concentrations of *Cyclocephala* hosts extracts according to the age of the parasitoid larva. *N*, number of replicates. Between brackets the total number of individuals that made a choice (left, stimulus; right, solvent).

Larval age	Host species	Extract concentration	<i>N</i>	χ^2	df	<i>P</i>	
Young	<i>Cyclocephala signaticollis</i>	5	113 (53–34)	4.149	1	0.041*	
		7.5	139 (61–37)	5.877	1	0.015*	
		10	178 (60–66)	0.285	1	0.593	
	<i>Cyclocephala modesta</i>	5	116 (50–49)	0.010	1	0.920	
		7.5	129 (58–43)	2.227	1	0.135	
		10	142 (58–43)	2.227	1	0.135	
	<i>Cyclocephala putrida</i>	5	117 (49–40)	0.910	1	0.340	
		7.5	136 (60–49)	1.110	1	0.292	
		10	123 (47–40)	0.563	1	0.453	
	Old	<i>Cyclocephala signaticollis</i>	5	184 (61–37)	5.133	1	0.023*
			7.5	149 (46–30)	3.368	1	0.066
			10	143 (45–27)	1.032	1	0.309
<i>Cyclocephala modesta</i>		5	133 (48–29)	4.688	1	0.030*	
		7.5	146 (43–36)	0.620	1	0.431	
		10	146 (34–50)	3.047	1	0.080	
<i>Cyclocephala putrida</i>		5	133 (38–32)	0.514	1	0.473	
		7.5	142 (42–31)	1.657	1	0.198	
		10	197 (28–22)	0.720	1	0.396	

nor free in between the soil) to calculate the missing rate, as an indirect measure of the larvae physiological state (Crespo & Castelo, 2008), and evaluate if there are any differences between treatments.

Orientation behaviour towards increasing concentrations of Cyclocephala chemical cues

To determine whether the specificity is influenced by host stimuli concentration in the *Cyclocephala* species we observed the orientation behaviour of the parasitoid larvae towards host chemical extracts with ascendant quantities of host cues. The aim of this experiment was to elucidate in a behavioural context whether the chemical cue that results attractive to *M. ruficauda* larvae is the same in all *Cyclocephala* species, if different host extracts promote the same orientation response, and if the parasitoid's interpretation of the host identity among species is due to differences in the cue concentration to which the parasitoid is exposed. We performed one experiment in the laboratory that is detailed in table 3. We used as the experimental arena a 9 cm × 6 cm × 1 cm static air two-way olfactometer, the same experimental arena and methodology as in 'Specificity measured as orientation behaviour' section. Three increasing concentrations of each host species chemical extracts were used: 5, 7.5 and 10 white grubs per m hexane. They were prepared using the same protocol as in 'Orientation to chemical cues' section. We performed experiments combining host species, extract concentration and parasitoid age (18 treatments). Experimental design and number of replicates for each treatment are shown in table 3.

Statistical analysis

In dual-choice experiments where the preference of *M. ruficauda* larvae for either side of the arena was tested against a random distribution, the data were analysed by means of chi-square (χ^2) tests of goodness of fit (Zar, 2010). To evaluate the influence of larval age, host species and nature of stimuli on *M. ruficauda* motivation to initiate movements related to host-searching behaviour, a three-way analysis

of variance (ANOVA) was performed using *M. ruficauda* larval age, host species and cue nature as factors (Zar, 2010). These data were checked for normality using a Shapiro–Wilk test. Previously, data from dual-choice experiments were randomized and the proportion of non-decision larvae was calculated every 16 observations. These proportions were used as response variable and were arcsin transformed and subjected to three-way ANOVA (Zar, 2010). *Post hoc* comparisons for all treatments were performed using a Tukey–Kramer multiple comparison test (Zar, 2010). When host specificity was measured through the host acceptance behaviour, the differences between parasitism rates for each treatment were analysed with a generalized lineal model analysis (GLM) with a logit link function, where the number of larvae successfully established to their respective host was defined as response variable (Hardy, 2002). Complementarily, data were transformed with a modification of the Freeman and Tukey transformation and a multiple comparison procedure analogous to the Tukey or Dunnett test was applied for non-parametric comparison (Zar, 2010). Moreover, to assess any differences in the missing larvae along both the larval ages and host species, the ratio of missing larvae was tested with a GLM analysis, with a logit link function (GLM, GenStat 11.1) (Hardy, 2002).

Results

Specificity measured as orientation behaviour

Mallophora ruficauda young second instar larvae orientated significantly towards *C. signaticollis* chemical stimulus (fig. 1, table 1). This result is coincident with the one already found by Crespo & Castelo (2008). On the contrary, when young larvae were exposed to *C. modesta* and *C. putrida* stimuli, they distributed randomly in the experimental arena. The control series with *H. bonariensis* stimulus larvae also showed a random distribution (fig. 1, table 1).

Like the young larvae, the old larvae oriented towards *C. signaticollis* stimulus, but also larvae orientates significantly towards *C. modesta* stimulus (fig. 1, table 1). When larvae were

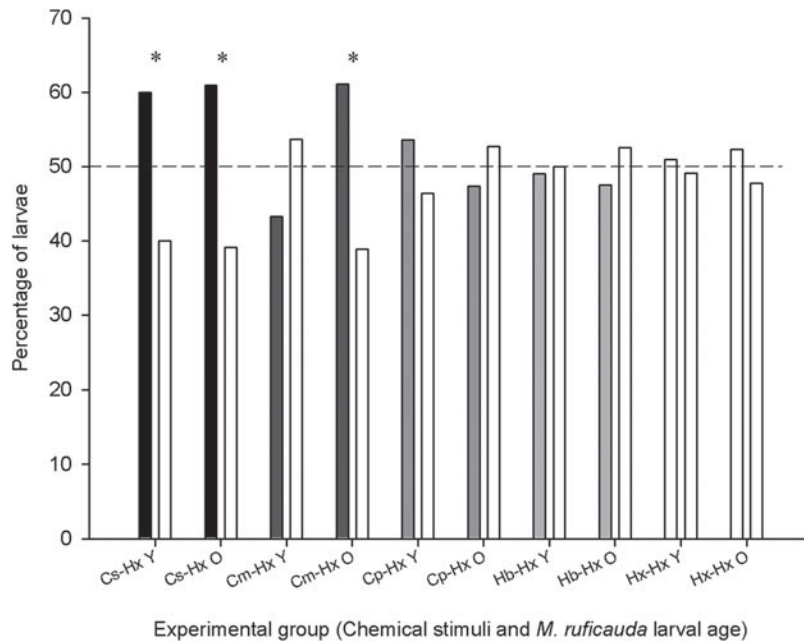


Fig. 1. Orientation responses of *M. ruficauda* second instar larvae of different ages to extracts of *Cyclocephala* hosts species. Y, young larvae; O, old larvae; Hx, hexane (control); Cs, extract of *C. signaticollis*; Cm, extract of *C. modesta*; Cp, extract of *C. putrida*; Hb, extract of *H. bonariensis*; *, statistically significant differences (χ^2 test, $P < 0.05$).

exposed to *C. putrida* stimulus, they distributed randomly in the experimental arena. Similarly, larvae showed a random distribution when tested against to *H. bonariensis* extract (fig. 1, table 1). These results suggest that aged second instar larvae of *M. ruficauda*, with a high risk of mortality, are less selective and orientate towards other near potential host species as *C. modesta*.

When live white grubs were offered as stimuli the results were strikingly different (fig. 2, table 1). Both young and old larvae oriented not only towards *C. signaticollis* as we expected, but also oriented to *C. putrida*. When young larvae were exposed to *C. modesta*, they distributed randomly in the arena. Unlike the chemical stimulus experiments, where old *M. ruficauda* larvae were exposed to live *C. modesta*, they distribute randomly. When young and old larvae were exposed to live *H. bonariensis*, in both cases they distributed randomly in the lateral sides of the experimental arena (fig. 2, table 1).

Motivation of LII to initiate host-searching movements were analysed for young and old larvae stimulated with both chemical and live host stimuli from the different host species used in this work. Only the double interaction between larval age and cue nature was significant (ANOVA: $F_{1,116} = 10.78$, $P = 0.0014$), while other interactions were not statistically significant (ANOVA: *M. ruficauda* larval age * host species * cue nature: $F_{4,116} = 2.35$, $P = 0.0587$; host species * *M. ruficauda* larval age: $F_{4,116} = 2.14$, $P = 0.0806$; host species * cue nature $F_{4,116} = 1.76$, $P = 0.1425$). Furthermore, host species was not statistically significant ($F_{4,116} = 2.33$, $P = 0.0601$). *Post hoc* comparison showed that hosts' chemical cues elicit a stronger response to initiate host-searching behaviour than live hosts, in both young and old larvae. Moreover, when experiments were performed with live host as stimuli, young larvae showed a higher motivation than old larvae. When young

and old larvae exposed to chemical cues were compared, despite there is slightly higher values for young larvae than old ones, no significant differences were found (fig. 3).

Specificity measured as host acceptance behaviour

When the specificity of *M. ruficauda* was analysed through the acceptance behaviour between the larval ages and among host species, it was found that both young and old second instar larvae were attached, in high proportion, to the three species of *Cyclocephala*. For *H. bonariensis* the proportion of attached larvae drastically decreased to zero (fig. 4, table 2). In particular, we found that 100% of young larvae were attached both to *C. signaticollis* and *C. putrida*, 95.6% to *C. modesta* and 0% to *H. bonariensis*. Similar values were found in the experiments with old larvae, being 100% to *C. signaticollis* and *C. putrida*, 93.7% to *C. modesta* and 0% to *H. bonariensis*. Responses were significantly different according to the host, being higher to species belonging to genus *Cyclocephala* (GLM: deviance=207.8, $P < 0.001$; Hb-Cs $q_{0.05, \infty, 4} = 23.523$; Hb-Cm $q_{0.05, \infty, 4} = 19.153$; Hb-Cp $q_{0.05, \infty, 4} = 21.073$; Cs-Cm $q_{0.05, \infty, 4} = 3.027$; Cs-Cp $q_{0.05, \infty, 4} = 1.525$; Cm-Cp $q_{0.05, \infty, 4} = 1.476$). The effect of the interaction between larval age and host species was not significant (GLM: deviance=3.17, $P = 0.075$), neither larval age (GLM: deviance=1.09, $P = 0.297$).

When the percentage of missing larvae in the arena was analysed for different hosts and *M. ruficauda* larval ages, no host species effect was found (GLM: deviance=0.049, $P = 0.824$), neither the interaction between host species and parasitoid larval age (GLM: deviance=0.321, $P = 0.571$). For larval age, old larvae showed significantly more 'disappearance' rate than young larvae (GLM: deviance=43.75,

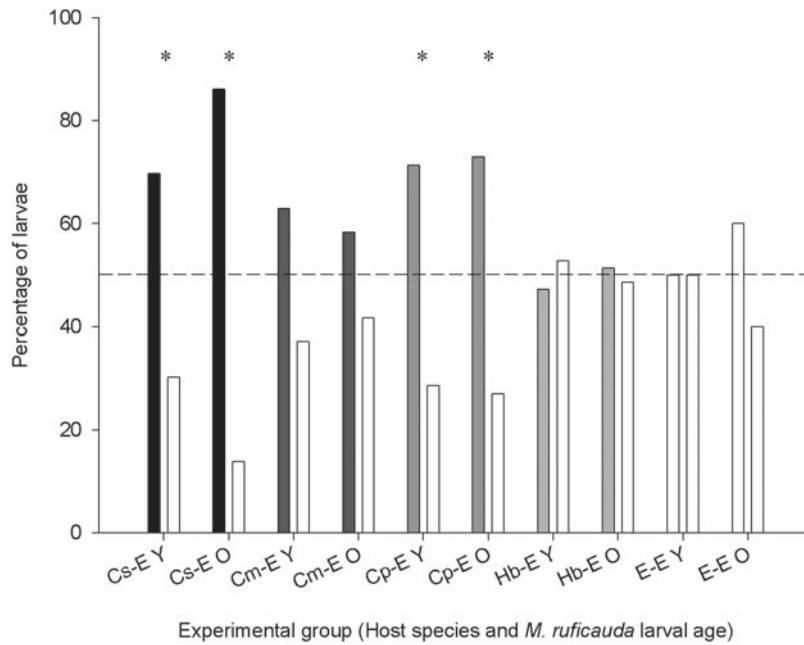


Fig. 2. Orientation responses of *M. ruficauda* second instar larvae of different ages to live *Cyclocephala* host species. Y, young larvae; O, old larvae; E, empty (control); Cs, live *C. signaticollis*; Cm, live *C. modesta*; Cp, live *C. putrida*; Hb, live *H. bonariensis*; *, statistically significant differences (χ^2 test, $P < 0.05$).

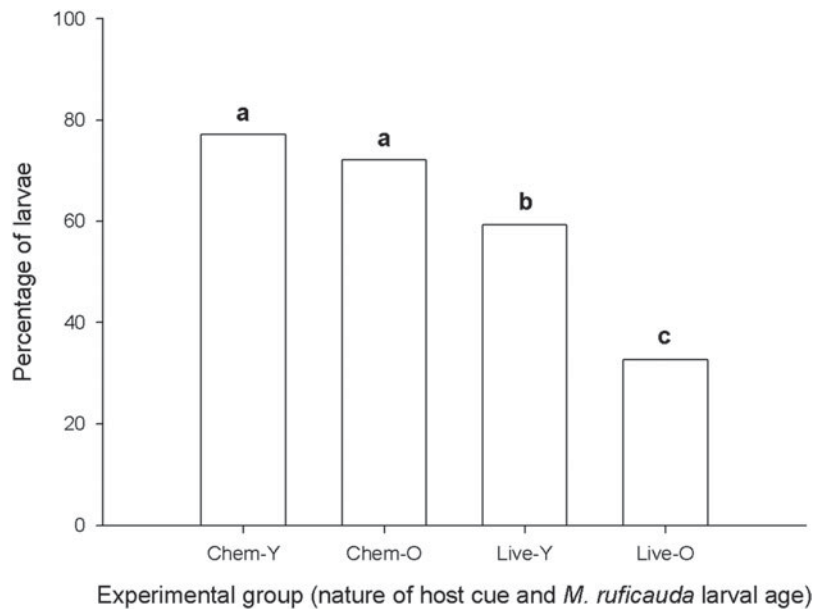


Fig. 3. Motivation of young and old second instar *M. ruficauda* larvae to initiate movements related to host-searching behaviour, when they are exposed to host's chemical cues and live hosts as stimuli. Chem, chemical host stimuli; Live, live host; Y, young larvae; O, old larvae. Different letters indicate significant differences (ANOVA, Tukey-Kramer test, $P < 0.05$).

$P < 0.001$). The number of dead larvae in acceptance experiments was four in a total of four replicates and the mortality rate was insignificant among the host species assays, being zero for *C. signaticollis*, three for *C. putrida*, and one for *C. modesta*, and they were not considered in the analysis.

Orientation behaviour towards increasing concentrations of *Cyclocephala* chemical cues

When orientation to increasing chemical cues was evaluated, young larvae oriented to *C. signaticollis* concentrations

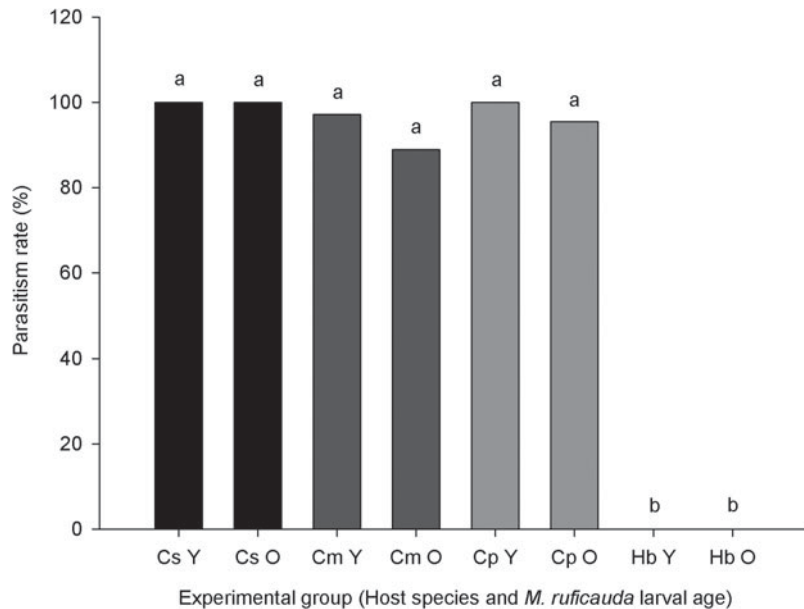


Fig. 4. Specificity of young and old second instar larvae of *M. ruficauda* measured as acceptance behaviour towards different *Cyclocephala* hosts species. Y, young larvae; O, old larvae; Cs, live *C. signaticollis*; Cm, live *C. modesta*; Cp, live *C. putrida*; Hb, live *H. bonariensis*. Different letters indicate significant differences (χ^2 test, $P < 0.05$).

of 5 and 7.5 white grubs per ml extract, but the distribution in the arena was at random in the 10 white grubs per ml concentration experimental series (fig. 4, table 3). When young larvae were exposed to each of these concentrations of *C. modesta* and *C. putrida* extracts, they distributed randomly in the experimental arena in all the experimental series (fig. 5, table 3).

When host orientation was tested on old larvae with *C. signaticollis* increasing concentrations, the larvae only oriented significantly to the host extract of 5 white grubs per ml, and distributed randomly in the experimental arena with 7.5 and 10 white grubs per ml concentrations. Similar results were observed with *C. modesta*. Old larvae oriented to host extract of 5 white grubs per ml, but not to 7.5 and 10 white grubs per ml concentrations. No preference for either side of the arena was registered to any concentration of *C. putrida* extract, and the larvae distributed randomly in both sides of the arena (fig. 5, table 3). In every case, i.e., each combination of host species and larval age, *M. ruficauda* did not orientate to extracts of 10 white grubs per ml.

Discussion

Our results indicate that host orientation changes with the host species, type of cues and parasitoid age; however, host acceptance is only influenced by the host species. Moreover, we found that increasing concentrations of the host stimuli might impair the parasitoid capability to distinguish the host identity.

As in previous works, *M. ruficauda* shows a high preference for parasitizing *C. signaticollis* and detects its chemical cues originating in the posterior half of the host's body (Castelo & Lazzari, 2004; Groba & Castelo, 2012). However, our work suggests that orientation to potential hosts of the genus *Cyclocephala* can show some flexibility depending on the

nature of the cue detected by the parasitoid and its age, and this behaviour is modulated by the different chemical cues released by the potential hosts. Particularly, besides the expected response to *C. signaticollis* chemical cues, only old *M. ruficauda* larvae orientated to *C. modesta* chemical cues, suggesting that *C. modesta* odours are attractive to larvae with low life expectancy. The fact that life expectancy can influence and decreases parasitoids selectivity thresholds has been suggested many times for female adult parasitoids and, sometimes, for host-seeking larvae (Mangel, 1987; Royer *et al.*, 1999; Javoiš & Tammaru, 2004). In *Aleochara bilineata* Gyllenhal (Coleoptera: Staphylinidae), a solitary parasitoid of puparia of some species of Diptera, host acceptance is influenced by their life expectancy and the host condition, and was observed that the degree of acceptance of suboptimal hosts (parasitized hosts) increases significantly with the age of the larvae (Royer *et al.*, 1999).

The positive attraction of the aged parasitoid to *C. modesta* host odours is irrespective of the concentration used. Differences observed in young larvae orientation pattern towards *C. modesta* and *C. signaticollis* could be due to the quantities of attractive cue contained in the extract since they differ in size (one *C. signaticollis* equals 2.13 *C. modesta* in weight, Castelo & Crespo, 2012). In this scenario, we expect the response changes when young larvae are exposed to a higher concentration of *C. modesta* extract. Given that orientation patterns in both larval age groups are similar when we increased the extract concentration, quantity of cue contained in extracts cannot explain the observed differences. The behavioural responses of *M. ruficauda* larva suggest that *C. signaticollis* and *C. modesta* might have similar cues because the larvae orientate to both hosts but in different conditions. These cues may share chemical components but vary in their proportions, or differ in their components, while close structural similarities; these similarities are likely a result of

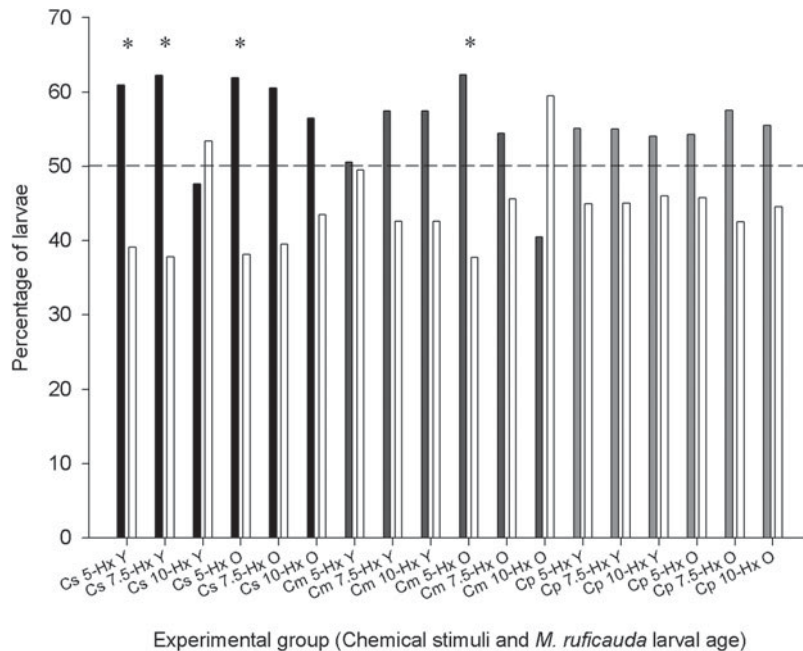


Fig. 5. Orientation responses of *M. ruficauda* second instar larvae of different ages to extracts of *Cyclocephala* hosts species of increasing concentrations. Y, young larvae; O, old larvae; Hx, hexane (control); Cs, extract of *C. signaticollis*; Cm, extract of *C. modesta*; Cp, extract of *C. putrida*; Hb, extract of *H. bonariensis*; 5, 7.5 and 10 correspond to concentrations of white grubs per ml; *, statistically significant differences (χ^2 test, $P < 0.05$).

the phylogenetic relationship between them (Tillman *et al.*, 1999; Guerrieri *et al.*, 2005; Yong *et al.*, 2007; Félix *et al.*, 2011).

Given that when high concentration stimuli were used no orientation was observed, it might suggest that there was no odour plume because the olfactometer was odour saturated. Another possible explanation involves saturation of chemoreceptor structures. Crespo *et al.* (2011) suggested that *M. ruficauda* larva orientates by means of klinotaxis by the successive comparison of stimulus concentration during the insect movement. If the parasitoid's chemoreceptors were saturated, there is an impossibility to detect and to orient through the odour gradient. At intermediate concentration, only young larvae show a similar orientation pattern to the two lower concentrations, but the older larvae do not choose any side of the experimental arena, even when *C. signaticollis* extract was used as stimulus. The parasitoid age might be influencing the sensitivity of chemoreceptors and old larvae chemoreceptors might become saturated at lower odour concentrations than younger ones, and cannot detect changes in stimulus strength (Blaney *et al.*, 1986). So, we found that old larvae fail to discriminate the odours of alternative hosts when they are concentrated. On the contrary, young larvae only failed to discriminate them at the highest concentration.

Some works show that parasitoids can exploit a multiplicity of cues during the host orientation process (Fischer *et al.*, 2001; Wang *et al.*, 2010). In this work, when orientation to live white grubs was tested in young and old parasitoid larvae, we found that they orientated not only towards *C. signaticollis* but also towards *C. putrida*, and in no case towards *C. modesta*, as it happened when stimulated with chemical cues. This fact suggests that *M. ruficauda* may be using another type of cue to detect and orientate towards the hosts, such as mechanical

cues, due to the vibrations caused by hosts on the substrate, as observed in other insects (Laumann *et al.*, 2007; Wang *et al.*, 2010). If this were the case, differences between the species tested could be explained given the differences and similarities between sizes of host species. *C. signaticollis* and *C. putrida* are similar in size as *C. modesta* and *H. bonariensis*, but the first pair is considerably larger than the second one (Castelo & Crespo, 2012). So, it is possible that *M. ruficauda* cannot discriminate among different species of live hosts with similar size, because mechanical cues originated by the movement of the host could be similar between *C. signaticollis* and *C. putrida*. Moreover, according to *C. modesta*, it is possible that the parasitoid larva detects a chemical cue similar to the other *Cyclocephala* species, but may recognize this species as not the preferred one by detecting other cues such as movement pattern, size, stridulation pattern, other odours, as was observed in other parasitoids. In addition, Alvarado (1980) has made an exhaustive morphological description of the four species tested in this work and found that these scarab larvae have structures for stridulation in their mandibles and maxillae. These structures might generate a detectable and recognizable cue for *M. ruficauda* larva as other parasitoids do (Schmidt & Smith, 1987a,b; Laumann *et al.*, 2007; Flores-Prado & Niemeyer, 2012).

Regarding the orientation experiments, we observed that motivation to initiate exploratory movements depended on the stimulus nature and the larva's age used in the experiments and not on the host species. Chemical stimulus might imply a more reliable indicator of host presence as opposed to mechanical stimulus. However, when we offered live hosts chemical stimuli are present. The difference in larvae's motivation might be due to a higher concentration of the

attractive odours in the chemical stimulus than in the live host. Also, the olfactometer used with live white grubs was higher in size than that used with chemical solutions. Thereby, differences in olfactometers size could reinforce the difference in attractive odour concentrations. Young larvae showed greater motivation than older larvae, but only when live hosts are offered. As observed in previous works, second instar of young and old ages have a similar motivation to initiate locomotive movements to chemical stimulus (Crespo & Castelo, 2008).

Unlike the field studies, no-choice experiments allow us to find out whether the parasitoid attempts to attack unsuitable hosts or conversely, whether potentially suitable hosts are not attacked at all (Morehead & Feener Jr., 2000; Desneux *et al.*, 2009). In this case, our laboratory experiments showed that *H. bonariensis* is not attacked by *M. ruficauda*, but *C. modesta* and *C. putrida* are attacked even though the parasitoid cannot develop successfully, i.e., parasitoid cannot complete its development until adulthood or the emerged adult was of lower weight or malformed in relation to parasitoids emerged from optimal host (unpublished data). These aspects have been observed also in other parasitoid species (Godfray, 1994; Desneux *et al.*, 2009). Differences between results from orientation and acceptance behaviours may be understood as a response of *M. ruficauda*, in absence of optimal hosts, accepting other *Cyclocephala* species available as hosts, like occur in other insect species (Stephens & Krebs, 1986; Janssen, 1989; Ellers *et al.*, 1998).

Finally, the physiological condition of parasitoids is crucial on the host location process and on the subsequent parasitism success. In the experiments where we measured the acceptance of hosts, we also registered when larvae were not found. We found that the proportion of missing larvae was significantly higher with increasing *M. ruficauda* age. Only in some cases, dead larvae were found in the soil, but in almost all cases they were not found. Dead larvae were probably degraded quickly due its minute size. The death of larvae may be because of natural causes (i.e., the mortality rate proper of each parasitoid age) or larvae might have suffered some kind of injury derived from mechanical host defences when the parasitoid larva was trying to parasitize the white grubs (Castelo & Crespo, 2012). Old larvae could have probably been attacked more due to their low mobility and their poor physiological condition. However, the difference in the death proportion between young and old larvae could be due to the specific mortality rate of each age, being higher in older parasitoids.

Throughout this work we attempted to show the complexity of the chemical ecology in the *Cyclocephala* species host-*M. ruficauda* parasitoid model. Although *M. ruficauda* could be considered a specialist host, it has been demonstrated that the host selection is not completely rigid, showing certain flexibility to choose other potential hosts. Under particular conditions, hosts can be considered as suboptimal for the parasitoid, such as a limited availability in the environment or decreased larval survival probability. The time-limited survival probability, as an example of state-dependency, has been largely addressed with adult parasitoid models. In the models where the larvae perform host location, this topic was addressed from host-discrimination, where aged larvae select a previously parasitized host, and this mechanism also seems to occur in *M. ruficauda*, suggesting an age-dependency host selection in this dipteran host-seeking larva.

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