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Host gut microorganisms' cues mediate orientation behaviour in the larva of the parasitoid *Mallophora ruficauda*

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Abstract

The robber fly *Mallophora ruficauda* is one of the most important apicultural pests in the Pampas region of Argentina. This species is a parasitoid of scarab beetle larvae. Females lay eggs away from the host, and the larvae perform active search behaviour toward *Cyclocephala signaticollis* third instar larvae, parasitoid's preferred host. This behaviour is mediated by host-related chemical cues produced in hosts' fermentation chamber. Also, *C. signaticollis* larvae are attracted to fermentation chamber extracts. As scarab larvae have microbe-rich fermentation chamber, it has been suggested that microorganisms could be involved in the production of these semiochemicals. The aims of this work were first to ascertain the presence of microorganisms in the fermentation chamber of *C. signaticollis* larvae and second to determine the role of microorganisms in the orientation response of parasitoid and host larvae. We found that microorganisms-free *C. signaticollis* larvae showed deterioration in their development and did not produce the attractive semiochemicals. Therefore, we isolated fermentation chamber microorganisms of host larvae by means of different cultures media, and then, assayed different microorganisms' stimuli by binary choice tests. We were able to isolate microorganisms and determine that *M. ruficauda* larvae are attracted to semiochemicals from protein degradation in the fermentation chamber. However, *C. signaticollis* larvae were not attracted to any semiochemicals associated with microorganisms' activity in the fermentation chamber. Although we were unable to elucidate the exact role of gut microorganisms in host behaviour, we discuss their relevance in parasitoid host-seeking behaviour and host conspecific interaction in *M. ruficauda*–*C. signaticollis* system.

Keywords: Asilidae, host location, microorganisms, parasitoid; Scarabaeidae, semiochemicals

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Introduction

Interaction between phytophagous insects and their natural enemies, i.e., predators and parasitoids, involves several sensory modalities (Eggleton & Belshaw, 1992; Godfray, 1994; Stowe *et al.*, 1995; Feener Jr. & Brown, 1997; Zuk & Kolluru, 1998; Greenfield, 2002). For natural enemies, chemical cues play an especially important role in location of phytophagous insects. Chemical cues (i.e., semiochemicals) are either produced directly by insects or indirectly by their host plants and/or by organisms associated with insects (e.g., microbes, symbionts, fungi, etc.) (Lewis & Martin, 1990; Vet & Dicke, 1992; Godfray, 1994; Stowe *et al.*, 1995; Rutledge, 1996; Davis *et al.*, 2013). In natural enemies' seeking

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behaviour, semiochemicals produced by plants offer relevant cues due to their high detectability. In turn, cues produced directly by phytophagous insects are also widely used by natural enemies but their detectability is lower. However, they are the most reliable cues that can inform the presence, identity, density, availability and suitability of individuals (Vet & Dicke, 1992; Steidle & Van Loon, 2003; Afsheen *et al*., 2008).

Exploitation of hosts' communication systems by parasitoids has been widely registered (Eggleton & Belshaw, 1993; Godfray, 1994; Stowe *et al*., 1995; Hoffmeister & Gienapp, 1999; Wertheim *et al*., 2003). In hymenopteran parasitoids, this behaviour has been observed in several species that attack concealed hosts (Wiskerke *et al*., 1993; Hoffmeister & Gienapp, 1999; Steidle & Van Loon, 2003), or in females that use semiochemicals produced by a host at a more detectable stage than the one they actually parasitize (*infochemical detour*) (Vet *et al*., 1991; Stowe *et al*., 1995; Steidle & Van Loon, 2003; Wertheim *et al*., 2003). In Diptera, species of Tachinidae and Phoridae families have been reported as they use pheromones produced by host adults to locate the host immature stages to parasitize (Aldrich, 1995; Feener Jr. & Brown, 1997; Stireman III *et al*., 2006; Mathis *et al*., 2011). Particularly, exploitation of pheromones as semiochemicals would be more relevant in those dipteran parasitoids that have a split host location strategy, where a female parasitoid seeks a host habitat and then at an active larval stage, performs the final location and parasitism of the host (Eggleton & Belshaw, 1992, 1993; Godfray, 1994; Feener Jr.& Brown, 1997; Brodeur & Boivin, 2004). Therefore, given their low mobility and potential time-limitation, immature parasitoids must use reliable cues (e.g., pheromones) to find hosts efficiently (Brodeur & Boivin, 2004). Use of pheromones enhance efficiency in host finding and consequently increase the fitness of the time-limited parasitoids (Vet *et al*., 1991; Wajnberg *et al*., 2006). However, there are few studies dealing with the origin of pheromones used as cues by the active larval stage in the host-seeking behaviour of parasitoids.

In insects, pheromone biosynthesis and production is related to glandular tissue or associated microorganisms (Hoyt *et al*., 1971; Dicke, 1988; Hunt & Borden, 1990; Tillman *et al*., 1999; Dillon *et al*., 2000; Ma & Ramaswamy, 2003; Wyatt, 2003; Ganter, 2006; Davis *et al*., 2013). Pheromone production by means of glandular tissue has been reported by several studies (Leal, 1998; Tillman *et al*., 1999; Ma & Ramaswamy, 2003; Wyatt, 2003). In contrast, insect–to–insect interactions involving semiochemical production by their associated microorganisms have been less studied (Dillon & Dillon, 2004). The location of these microorganisms in the host varies according to the species they are associated with (Pontes *et al*., 2009; Clark *et al*., 2010; Feldhaar, 2011; Minard *et al*., 2013). In most of the works that have studied the role of microorganisms, it was observed that microorganisms living in the insect digestive tract produce sexual or aggregation pheromones (Hunt & Borden, 1990; Dillon *et al*., 2000; Dillon & Dillon, 2004; Ganter, 2006; Davis *et al*., 2013). Particularly in coleopteran species, gut microbiota is capable of producing compounds that are used as pheromones by its host (Hunt & Borden, 1990; Ganter, 2006; Davis *et al*., 2013). However, the few available studies focus on adults and there are no reports where microorganisms' semiochemicals mediate larval conspecific interactions.

There are many reports showing that insect's larvae respond to chemical cues produced by their conspecifics (Greenfield, 2002; Matthews & Matthews, 2010). Authors suggest that conspecific interactions, such as aggregation behaviour elicited by chemical cues, are indirectly beneficial to organisms living in groups. Several benefits have been reported such as an increase in survival and growth, improvement of individual development (Despland & Le Huu, 2006; Jumean *et al*., 2009), efficiency enhancement in the exploitation of food, improvement of defensive skills against natural enemies (Capinera, 1980; Tsubaki & Shiotsu, 1982; Deneubourg *et al*., 1990), and even a decrease in the mate-searching period (Duthie *et al*., 2003). In all cases, cues that trigger aggregation behaviour are pheromones produced by individuals through active secretion, cuticle deposition or in the faeces (Flowers & Costa, 2003; Despland & Hamzeh, 2004; Singh & Johnson, 2012; Farine *et al*., 2014; Mota *et al*., 2014). However, no studies have determined whether these pheromones are the result of the association between insects and microorganisms; or whether natural enemies are capable to exploit these semiochemicals.

Mallophora ruficauda Wiedemann (Diptera: Asilidae) is a robber fly endemic to the Pampas region of Argentina that inhabits open grasslands near bee farms (Rabinovich & Corley, 1997). As adult, *M. ruficauda* feeds mainly on foraging honeybees, and as larva is an ectoparasitoid of soil-dwelling Scarabaeidae larvae (commonly named white grubs), with a noticeable preference for third instar larvae of *Cyclocephala signaticollis* Burmeister (Copello, 1922; Castelo & Capurro, 2000; Castelo & Corley, 2010; Barrantes & Castelo, 2014). This parasitoid has a split host seeking behaviour. Females oviposit on tall grasses or artificial supports, such as wire fences, laying egg-clutches covered by albumin (Copello, 1922; Castelo & Corley, 2004; Castelo *et al*., 2006). After hatching, larvae are dispersed by the wind, and fall to the ground, where they start digging and searching for their host (Castelo & Capurro, 2000; Castelo *et al*., 2006). Particularly, it is the second instar larva of *M. ruficauda* that performs the active searching of the hosts (Crespo & Castelo, 2008). Previous studies have shown that the source of semiochemicals involved in active host-seeking behaviour is associated with the digestive tube of the third instar larva of *C. signaticollis* (Castelo & Lazzari, 2004; Crespo & Castelo, 2008). In addition, Groba & Castelo (2012) determined that fermentation chamber of hindgut of the host is the anatomical location of these chemical cues. Moreover, in that work, third instar larvae of *C. signaticollis* also showed a positive orientation towards extracts of this portion of the gut. This fact might indicate the presence of an aggregation cue present in white grub gut involved in communication of *C. signaticollis*, which could also be exploited by larvae of *M. ruficauda* (Groba & Castelo, 2012).

Cyclocephalla signaticollis have a typical scarab alimentary tract with a modified expanded portion of the hindgut, i.e., the fermentation chamber. Scarab larvae have a microbe-rich alimentary tract with most microorganisms concentrated in a fermentation chamber, which play an important role in digestion of plant material (Egert *et al*., 2005; Zhang & Jackson, 2008; Huang *et al*., 2010; Zheng *et al*., 2012). However, the presence of microorganisms in fermentation chamber of *C. signaticollis* still has not been confirmed; or whether they are involved in production of semiochemicals related to both intraspecific communication of third instar larvae of *C. signaticollis* and host seeking behaviour of second instar larvae of *M. ruficauda*.

The aims of this work were to determine: (i) the presence of associated microorganisms in the fermentation chamber of third instar larvae of *C. signaticollis*, and (ii) whether these potential microorganisms associated with the fermentation chamber of *C. signaticollis* produce semiochemicals that are

attractive to both *M. ruficauda* and *C. signaticollis* larvae. In this way, we will have a better understanding of the chemical cues involved in both parasitoid host-seeking strategy and host conspecific interaction. Furthermore, the results of this survey will allow us to discuss the potential role of microorganisms in the trophic interactions and behavioural ecology of insects.

Material and methods

Experimental conditions

Orientation behaviour experiments with the parasitoid were conducted during January–March 2011–2013 under laboratory conditions [27.5 ± 6.3°C, 57.0 ± 12% relative humidity (RH)], on days with barometric pressure between 1008 and 1019.2 mbar. Experiments with hosts were made in July–August 2011–2012 under laboratory conditions (24.2 ± 2.1°C, 37.0 ± 11% RH) with barometric pressure values between 1009.6 and 1024.1 mbar.

Since environmental conditions influence insect behaviour (Roitberg *et al.*, 1993; Amat *et al.*, 2006), *M. ruficauda* and *C. signaticollis* experiments were performed under barometric pressure and temperature conditions where insects had shown orientation to hosts or conspecifics, respectively, in previous experiments (Castelo & Lazzari, 2004; Crespo & Castelo, 2008, 2012; Groba & Castelo, 2012). In order to keep experimental conditions similar to natural ones, all experiments were carried out in darkness, as both instars larvae species live underground.

Insects

Larvae of *M. ruficauda* were reared in the laboratory from egg-clusters collected from January to March 2011–2013 on grasslands in Pilar (34°28′S, 58°55′W) and Moreno (34°46′S, 58°93′W), two localities with a presence of this species, in Buenos Aires province, Argentina. In the field, egg-clusters were carefully cut off from their support and were kept individually in Falcon-type tubes until the larvae hatched. After hatching, the neonate larvae were kept individually in Eppendorf-type tubes with a moistened piece of filter paper as substrate to keep humidity inside the tube at 100%. Tubes were stored in darkness and at room temperature (25–28°C) under controlled photoperiod (14:10 L:D). When larvae reached second instar and were 20–50 days old, they were used to perform the experiments.

Scarab larvae were collected at soil depth of 0.30 m in grasslands of Pilar, Moreno and Mercedes (34°40′S, 59°26′W) in Buenos Aires province, Argentina, from May–August 2011–2012. Third instar larvae of *C. signaticollis* were identified using the taxonomic key of Alvarado (1980). *Cyclocephalla signaticollis* individuals were maintained individually in the laboratory at room temperature (18.6–29.8°C) in black tubes (30 ml) filled with soil, and were fed weekly with pieces of fresh carrots.

Presence assessment and determination of the role of microorganisms associated with C. signaticollis

Firstly, in order to assess the presence of microorganisms in fermentation chamber of *C. signaticollis* and, secondly, to determine whether these potential microorganisms are involved in the production of semiochemicals associated with the orientation behaviour of both parasitoid and host larvae, we performed different experiments. To begin with, we assessed indirectly the presence of microorganisms in *C. signaticollis* larvae gut by rearing third instar individuals with a broad-spectrum antibiotic. In addition, we ascertained the presence of microorganisms in the fermentation chamber of host larvae by means of different culture media. Secondly, we tested extracts from individuals treated with antibiotic by means of binary choice tests in order to determine whether the presence of microorganisms in *C. signaticollis* gut larvae is involved in production of these semiochemicals. Then, we performed assays with extracts of different culture media to ascertain whether semiochemicals are derived from microorganisms' activity in the fermentation chamber.

Stimuli extract of microorganisms-free C. signaticollis *larvae*

Larvae of *C. signaticollis* were maintained for 7 days in black tubes (30 ml) filled with soil with tetracycline at 0.1% w/w concentration. Then, white grubs' stimuli extraction was obtained following the protocol outlined in Castelo & Lazzari (2004) and Crespo & Castelo (2008). Each larva of *C. signaticollis* was anaesthetized and posterior body half of the larva was dissected and homogenized using hexane as solvent, extracting nonpolar compounds. In order to guarantee the occurrence of behavioural responses, we used experimental extracts equivalent to 2.5 hosts per ml for *M. ruficauda* larvae experiments; while for *C. signaticollis* larvae experiments one white grub per ml was used (Groba & Castelo, 2012).

As controls we carried out two experimental series. We tested the orientation behaviour of parasitoid and host larvae toward antibiotics using an extract of 20 mg ml^{-1} tetracycline in hexane. For the other control series, we tested an extract of 1 mg ml^{-1} sterile soil in hexane to assess a possible orientation towards sterile soil that resulted from the protocol manipulation.

Stimuli extracts of semiochemicals derived from the activity of microorganisms associated with the fermentation chamber

We made three simple cultures with different types of nutrients to isolate fermentation chamber microorganisms: starch, cellulose and protein. For the three different nutrients we performed four series of cultures, one experimental series and three control series that ensure that semiochemicals were from microorganisms' activity. These series were medium inoculated with fermentation chamber microorganisms (culture – *C*); medium without inoculation (medium – *M*); medium plus tetracycline inoculated with fermentation chamber microorganisms (culture + tetracycline – *C* + *T*); and medium plus tetracycline without inoculation (medium + tetracycline – *M* + *T*) (Table 1). For *C* and *M* series, we prepared laboratory test tubes (25 ml) with 10 ml of medium and then were sterilized by autoclave. For *C* + *T* and *M* + *T* series a laboratory test tube with 9 ml of medium were sterilized by autoclave, and then medium with tetracycline was added to 10 ml volume, obtaining a tetracycline concentration of 0.1 mg ml^{-1}. This protocol ensures no degradation of tetracycline by heat (Mitscher, 1978).

In order to isolate and culture fermentation chamber microorganisms, the digestive tube of third instar larva of *C. signaticollis* was dissected. Once the fermentation chamber was located, we circumscribed the chamber tying it with a thread soaked in tetracycline solution (1 mg ml^{-1}), to ensure no foreign contamination occurred. Subsequently in sterile conditions, we soaked the fermentation chamber in tetracycline

Table 1. Behavioural response of *M. ruficauda* and *C. signaticollis* larvae to cues associated with microorganisms of third instar larvae of *C. signaticollis*. Numbers show the replicates for experiments with *MR* and *CS* larvae. Between brackets, the total number of individuals that made a choice (left: stimulus, right: control solvent) in the experimental arena.

Experiment (stimulus – control)	*MR*	*CS*	Description	*MR* χ^2; *P*	*CS* χ^2; *P*
CS + T – Hx	150 (50–50)	64 (29–26)	Extract of third instar larvae of *C. signaticollis* treated with tetracycline (microorganisms-free individuals)	0; 1	0.16; >0.68
T – Hx	200 (67–68)	64 (25–23)	Extract of tetracycline in control solvent (hexane)	0.01; >0.93	0.08; >0.77
Soil – Hx	150 (38–51)	64 (25–26)	Extract of sterile soil in control solvent (hexane)	1.90; >0.16	0.02; >0.89
St C – Hx	200 (62–69)	64 (32–21)	Extract of starch culture of fermentation chamber microorganisms	0.37; >0.54	2.28; >0.13
St M – Hx	150 (55–44)	64 (31–24)	Extract of starch medium	1.22; >0.27	0.89; >0.34
St C + T – Hx	150 (60–44)	–	Extract of starch medium plus tetracycline inoculated with fermentation chamber extract	2.46; >0.12	–
St M + T – Hx	150 (52–45)	–	Extract of starch medium plus tetracycline	0.5; >0.47	–
Ce C – Hx	200 (85–64)	64 (22–23)	Extract of cellulose culture of fermentation chamber microorganisms	2.96; >0.08	0.02; >0.88
Ce M – Hx	200 (68–77)	64 (26–24)	Extract of cellulose medium	0.56; >0.45	0.08; >0.77
Ce C + T – Hx	150 (66–51)	–	Extract of cellulose medium plus tetracycline inoculated with fermentation chamber extract	1.92; >0.16	–
Ce M + T – Hx	150 (45–52)	–	Extract of cellulose medium plus tetracycline	0.5; >0.47	–
Pt C – Hx	150 (79–46)	64 (29–23)	Extract of protein culture of fermentation chamber microorganisms	8.71; <0.004	0.69; >0.40
Pt M – Hx	150 (63–57)	64 (20–24)	Extract of protein medium	0.3; >0.58	0.36; >0.54
Pt C + T – Hx	150 (55–45)	–	Extract of protein medium plus tetracycline inoculated with fermentation chamber extract	1; >0.31	–
Pt M + T – Hx	150 (61–48)	–	Extract of protein medium plus tetracycline	1.55; >0.21	–
Ag C – Hx	150 (54–56)	–	Extract of agar culture of fermentation chamber microorganisms	0.04; >0.84	–

MR, *M. ruficauda*; *CS*, *C. signaticollis*; *CS* + T, *C. signaticollis* individuals reared with tetracycline; T, tetracycline; Soil, sterile soil; St C, starch culture of fermentation chamber microorganisms; St M, starch medium; St C + T, starch medium plus tetracycline inoculated with *C. signaticollis* fermentation chamber extract; St M + T, starch medium plus tetracycline; Ce C, cellulose culture of fermentation chamber microorganisms; Ce M, cellulose medium; Ce C + T, cellulose medium plus tetracycline inoculated with *C. signaticollis* fermentation chamber extract; Ce M + T, cellulose medium plus tetracycline; Pt C, protein culture of fermentation chamber microorganisms; Pt M, protein medium; Pt C + T, protein medium plus tetracycline inoculated with *C. signaticollis* fermentation chamber extract; Pt M + T, protein medium plus tetracycline; Hx, hexane (control solvent).

solution (1 mg ml^{-1}), and then we homogenized it using sterile distilled water to get a solution equivalent to one white grub per ml (henceforth *fermentation chamber extract*). For *C* and *C* + *T* series we inoculated with 1 ml of fermentation chamber extract meanwhile for *M* and *M* + *T* series we inoculated with 1 ml of sterile distilled water as control. Cultures without tetracycline were incubated at 25–28°C for 7 days, while cultures with tetracycline were incubated for 2 days due to the stability of the antibiotic (Kühne *et al.*, 2000). After incubation, we extracted semiochemicals associated with the cultures and media by means of a solvent extraction using a separating funnel. This technique allowed us to separate semiochemicals from cultures, due to the non-miscibility of the solvent extraction (hexane) and the aqueous media. As they are nonpolar compounds (Groba & Castelo, 2012), semiochemicals were extracted in hexane. Stimuli were obtained using 10 ml hexane from four lab test tube cultures. In the case of cellulose media series we used 7 ml hexane for funnel extraction and 3 ml hexane for homogenization of filter paper strip (see below), then both fraction were added and used as stimuli in behavioural experiments.

Presence of microorganisms in cultures can be determined indirectly from degradation of nutrients in the medium and/or subsequent verification of the presence of secondary metabolic compounds derived from their activity. Lyses or breakdown of macromolecules by microorganisms in the cultures performed are amylolysis, cellulolysis and proteolysis. We assessed lyses of macromolecules by means of protocols used in soil microbiology (Pochon & Tardieux, 1965) to confirm the isolation of microorganisms. Metabolic activity was ascertained for each of the four series of cultures above described. Media were modified from Pochon & Tardieux (1965), Bailón Lira *et al.* (2003) and Sánchez Colin (2004) by adjusting pH to resemble conditions in the larvae fermentation chamber (Lemke *et al.*, 2003).

- *Starch medium (St).* Modified from Pochon & Tardieux (1965). Per 100 ml distilled water medium contain 5 ml Winogradski standard saline solution [5 g PO_4HK_2, 2.5 g SO_4Mg, 2.5 g ClNa, 0.05 g $(SO_4)_3Fe_2$ and 0.05 g SO_4Mn per L of distilled water], 0.1 ml trace element solution [0.05 g MoO_4K_2, 0.05 g $B_4O_7Na_2$, 0.05 ml Cl_3Fe, 0.05 g $(NO_3)_2Co$, 0.05 g SO_4Cd, 0.05 g SO_4Cu, 0.05 g SO_4Zn and 0.05 g SO_4Mg per L of distilled water], 0.1 g NO_3NH_4 and 0.15 g corn starch. Starch lysis was detected by means of addition of Lugol's iodine to 0.5 ml of the medium. When lysis of medium has occurred, the solution turns yellow-amber colour (Pochon & Tardieux, 1965).
- *Cellulose medium (Ce).* Modified from Pochon & Tardieux (1965). Per 100 ml distilled water medium contain 5 ml Winogradski standard saline solution, 0.1 ml trace element solution and 0.1 g NO_3NH_4. For each lab test tube we added a filter paper strip of 1 × 7 cm as cellulose sources. Microorganisms' presence assessment and lysis of cellulose

was observed by the presence of coloured spots and degradation on non-submerged filter paper strip.

- *Protein medium (Pt).* Modified from Pochon & Tardieux (1965), Bailón Lira *et al.* (2003) and Sánchez Colin (2004). Per 100 ml distilled water medium contain 5 ml Winogradski standard saline solution, 0.1 ml trace element solution, 0.5 g beef extract powder, 0.5 g bacteriological peptone (Oxoid Limited, England) and 1.5 g agar. Microorganisms' presence assessment and degradation of the substrate were detected by observation of the liquefaction of the medium. As to the fact that microorganisms can degrade agar (Brock *et al.*, 1987), we performed a control experiment with a medium of agar 1.5%w/v without protein nutrients (agar culture – *Ag C*) inoculated with fermentation chamber extract. This allowed us to determine whether semiochemicals were obtained from proteolysis or agar degradation.

Orientation behaviour experiments

Evaluation of behavioural responses of insects toward stimuli was performed using similar experimental arenas as in Castelo & Lazzari (2004). We divided the arenas into three equally sized zones (one in middle and two laterals) along the long axis. On each lateral zone of the arena, a piece of filter paper impregnated with a volume of either stimulus extract or control solvent (hexane) was placed. At the beginning of each trial, an individual was released at the centre of the arena and allowed to move freely. After a fixed experimentation time, its position in the arena was registered. Three possible responses could be obtained: choice for stimulus (S), for control (C) or no decision (ND) if the individual remained in the middle zone. After every trial, each individual was discarded and the arena was cleaned with soap and water, and then dried with an air current in order to eliminate possible larval odours. Experimental design and number of replicates for each experiment are detailed in Table 1.

Orientation behaviour of M. ruficauda *to host stimuli*

Behavioural experiments with *M. ruficauda* were carried out in an arena of 9 × 6 × 1 cm using a piece of filter paper of 1 × 2 cm impregnated with 10 µl of either the stimulus extract or hexane. Each trial, a larva was released as experimental individual at the centre of the arena, and after 90 min, its position in the arena was recorded (Table 1).

Orientation behaviour of C. signaticollis *to conspecific stimuli*

For behavioural experiments with *C. signaticollis*, we carried out trials with an arena of 13 × 8 × 2 cm. On each lateral side of the experimental arena, was presented a filter paper of 2 × 3 cm impregnated with 40 µl of stimulus extract or hexane. In each trial a larva was released at the centre of the arena; and, after 45 min, its position in the arena was recorded (Table 1).

Statistical analysis

In the experiments, we analysed the influence of stimuli tested on the orientation behaviour of both *M. ruficauda* and *C. signaticollis* larvae. In both behavioural experiments, orientation of insects to the stimulus was tested against a random distribution by means of χ^2 tests of goodness-of-fit (one-way contingency table analysis: Rosner, 1995; Zar, 2010). Individuals that remained in the middle zone of the arena (ND response) were excluded from the analysis. Statistical analyses were done using the software package R version 3.1.3 (R Core Team, 2015, Viena, http://www.R-project.org/).

Results

After 7 days of treatment, individuals reared with tetracycline showed deterioration and an inactive behaviour. Starch, cellulose and protein media inoculated with fermentation chamber extract showed degradation activity of the media, revealing the presence of microorganisms. On the contrary, media inoculated with sterile distilled water, showed no microorganisms' metabolic activity, verifying that there was no contamination during manipulation. Finally, cultures treated with tetracycline and inoculated with fermentation chamber extract showed no microorganism metabolic activity.

Orientation behaviour of M. ruficauda *to host stimuli*

When tested with *C. signaticollis* microorganisms-free larvae extract, larvae of *M. ruficauda* showed a random distribution in the experimental arena (Table 1). Furthermore, experiments that tested the behavioural responses of *M. ruficauda* larvae to semiochemicals associated with metabolic activity of fermentation chamber microorganisms proved that the behavioural response varies according to the stimulus. Larvae showed a positive orientation toward protein culture (*Pt C*) extract (Table 1). In order to determine whether semiochemicals come from protein or agar degradation, we performed a culture and control medium with agar only. We observed that microorganisms of *C. signaticollis* degraded agar. However, larvae were not attracted toward stimuli of agar degradation (Table 1). Moreover, semiochemicals derived from protein culture with tetracycline (*Pt C + T*) were not attractive to experimental individuals (Table 1).

Also, we observed a *M. ruficauda* larvae tendency to orientate toward cellulose culture (*Ce C*) extract (Table 1). To determine whether this attraction is random or if it is in fact a positive orientation towards cellulose culture semiochemicals, we tested the larvae response to a solution two times more concentrated than in previous extracts. This experiment showed a random distribution of tested individuals between stimulus and control solvent in the arena as well ($\chi^2 = 1.44$, $N = 150$, $n_{\text{STIMULUS}} = 56$, $n_{\text{CONTROL}} = 44$, $P > 0.23$). This result indicates that products of cellulose degradation by microorganisms are not attractive to *M. ruficauda* larvae.

When offered tetracycline culture and control media extracts, larvae of *M. ruficauda* showed random distribution in the experimental arena (Table 1). These results suggest that metabolic activity of fermentation chamber microorganisms is needed to produce semiochemicals involved in positive orientation of *M. ruficauda* larvae toward its host.

Orientation behaviour of C. signaticollis *to conspecific stimuli*

We found that third instar larvae of *C. signaticollis* show a random distribution in the experimental arena when we offered conspecific microorganisms-free larvae extract (Table 1). For all cultures and control media extracts tested, *C. signaticollis* larvae showed no positive orientation toward stimulus or hexane (Table 1). However, we observed a trend of *C. signaticollis* individuals to orient toward starch culture

stimuli (Table 1). In order to determine whether this result was due to a low concentration of stimulus, we conducted behavioural experiments increasing two times (2*x*) and four times (4*x*) the concentration of starch culture and media stimuli. For both experiments *C. signaticollis* larvae distributed randomly in the arena (2*x St C*: $\chi^2 = 0.49$, $N = 64$, $n_{STIMULUS} = 28$, $n_{CONTROL} = 23$, $P > 0.48$; 2*x St M*: $\chi^2 = 0.18$, $N = 64$, $n_{STIMULUS} = 27$, $n_{CONTROL} = 24$, $P > 0.67$; 4*x St C*: $\chi^2 = 0.67$, $N = 64$, $n_{STIMULUS} = 24$, $n_{CONTROL} = 30$, $P > 0.41$; 4*x St M*: $\chi^2 = 0.07$, $N = 64$, $n_{STIMULUS} = 27$, $n_{CONTROL} = 29$, $P > 0.79$).

Discussion

In the present work, we performed different protocols to assess the presence of microorganisms in fermentation chamber of *C. signaticollis* and whether they are involved in production of semiochemicals that mediate orientation behaviour in *M. ruficauda* and *C. signaticollis* larvae. Microorganism-free individuals of third instar larvae of *C. signaticollis* revealed a close relationship between gut microorganisms and this species of white grub. Removal of microorganisms with tetracycline, a broad-spectrum antibiotic, showed a deterioration of white grubs. We observed dehydration and an inactive behaviour of individuals, as was observed in other insects with a similar treatment (Douglas, 1988, 1989). This suggests that microorganisms have a relevant role in the development of *C. signaticollis*. Previous researches have proposed that persistence of the relationship between microorganisms and insects should be for mutual benefit. Particularly for insects, the microorganisms provide nutrients that are limited or lacked in their diet (Moran *et al.*, 2003; Dillon & Dillon, 2004; Zhang & Jackson, 2008; Ben-Yosef *et al.*, 2010; Grünwald *et al.*, 2010; Huang *et al.*, 2010; Feldhaar, 2011; Zheng *et al.*, 2012; Huang & Zhang, 2013; Oliver & Martinez, 2014). By means of three simple culture media, we could isolate fermentation chamber microorganisms and determine their ability to degrade carbohydrates, cellulose and proteins in aerobic media. Throughout dissections of guts, we observed several tracheae reaching the fermentation chamber suggesting the requirement of an aerobic environment for the microorganisms. Several insect species show tracheae infiltrating into gut compartment that harbours microorganisms. This observation suggests that gut microorganisms are either obligate aerobes or facultative anaerobes (Billen & Buschinger, 2000; Woolfolk, *et al.*, 2004; Bution & Caetano, 2008). Regarding isolation of fermentation chamber microorganisms, the three control series performed allowed us to verify that isolation was carried out without contamination. Therefore, these results ensure that behavioural response of both *M. ruficauda* and *C. signaticollis* larvae toward those stimuli tested are due to microorganisms in the fermentation chamber and their metabolic activity.

Behavioural experiments revealed interesting results related to host location of immature parasitoid *M. ruficauda*. Second instar larvae did not show orientation behaviour toward semiochemicals of microorganisms-free *C. signaticollis* larvae. This suggests that microorganisms in the digestive tract of the host have a relevant role in the production of cues involved in host location. Insect associated microorganisms have been revealed to be a source of kairomone production for natural enemies (Madden, 1968; Pettersson *et al.*, 2001; Sullivan & Berisford, 2004; Boone *et al.*, 2008; Leroy *et al.*, 2011). Our experiments show that *M. ruficauda* larvae are oriented only towards protein extracts cultures of *C. signaticollis* fermentation chamber microorganisms. Therefore, these results suggest that microorganisms' activity in the fermentation chamber may be related to the production of semiochemicals involved in host-seeking behaviour of the parasitoid. Moreover, based on *C. signaticollis* larvae diet (i.e., plants' roots and tubers), we expected that orientation behaviour of parasitoid larvae is triggered by semiochemicals originated of microorganisms' activity over cellulose or starch media. Nonetheless, for some species of parasitoids proteins and amino acids have proven to have a kairomonal role in host acceptance and oviposition behaviour (see review Rutledge, 1996). As proteins are essential in individual development, we could consider that proteins or metabolic protein by-products would be reliable indicators of nutritional state of insects. In addition, several works show that vitamins and essentials amino acids are provided by insects' associated microorganisms from metabolism of proteins and other nutrients that hosts consume (Cruden & Markovetz, 1987; Douglas, 1988, 1998; Sasaki & Ishikawa, 1995; Bernays & Klein, 2002; Moran *et al.*, 2003; Dillon & Dillon, 2004; Woolfolk *et al.*, 2004; Chandler *et al.*, 2008; Oliver & Martinez, 2014). Then, microorganisms provide their hosts with these essential nutrients for development and growth, which are scarce in phytophagous insects' diet (Bernays & Klein, 2002; Moran *et al.*, 2003; Thompson & Simpson, 2003; Woolfolk *et al.*, 2004; Klowden, 2007). Therefore, second instar of *M. ruficauda* larvae detecting semiochemicals related to protein metabolism of *C. signaticollis* larvae, could be an indicator of the identity and nutritional status of the host. This ability of *M. ruficauda* larvae was expected, as previous works have shown that they can identify and differentiate between optimal and suboptimal hosts by means of chemical cues (Barrantes & Castelo, 2014; Crespo *et al.*, 2015). As such, the use and detection of protein-like semiochemicals by *M. ruficauda* parasitoid larvae could be associated with the predatory entomophagous nature of Asilidae family larvae (Clausen, 1940; Wood, 1981; Dennis *et al.*, 2013).

Regarding *C. signaticollis* behavioural experiments, white grubs were not attracted to extracts of microorganisms-free larvae. Previous studies have shown that extracts derived from gut were attractive to host larvae (Groba & Castelo, 2012). Therefore, microorganisms' activity in digestive tube could be evoking the conspecific aggregation behaviour. Nevertheless, we could not dismiss that antibiotic treatment affected emission/production of volatiles involved in conspecific behaviour, because this treatment could have been a stress factor. Moreover, experimental individuals did not respond to the cultures' extracts, suggesting that the determination of the role of fermentation chamber's microorganisms in larvae aggregation behaviour is more complex than expected. The absence of orientation toward the stimuli could be related to the fact that concentration tested was under the threshold detection of *C. signaticollis* larvae. It is clear that behavioural or physiological response to any stimulus occurs only if the concentration exceeds the detection threshold of the receiver (Wigglesworth, 1982; Greenfield, 2002; Klowden, 2007; Chapman, 2013). Therefore, new experiments with larger concentrations for all stimuli might be needed to confirm this hypothesis. On the other hand, whether a pheromone is involved in the aggregation behaviour of *C. signaticollis*, it would be expected to be a blend of compounds (Greenfield, 2002; Wyatt, 2003). Then, isolation of microorganisms by means of three different types of cultures media may alter the composition of the pheromone. This could be because we isolated the microorganisms in the fermentation chamber in an unrepresentative way, or because the isolation used a culture media with a

single type of nutrient that modified the by-products of microorganisms. Groba and Castelo (2012) showed that extracts obtained by dissection of fermentation chamber in content and epithelium, were unable to elicit a behavioural response of *C. signaticollis* third instar larvae. Moreover, a preliminary work showed the presence of a glandular tissue in *C. signaticollis* fermentation chamber (Groba, 2014) that could be relevant in semiochemicals production. Both the previous works and the results reported here are indicative that it is necessary to have the fermentation chamber as a whole unit to produce chemical cues involved in conspecific behaviour of *C. signaticollis*.

Although we were unable to determine the exact role of microorganisms in the *C. signaticollis* larvae' behaviour, our results showed that presence of gut's microorganisms is decisive in individual's orientation toward conspecific's stimuli. This evidence as well as aggregation in field and the previous behavioural experiments in the laboratory (Castelo & Capurro, 2000; Castelo & Corley, 2010; Groba & Castelo, 2012), indicate a probable communication system between immature individuals of *C. signaticollis* by means of microorganisms' semiochemicals. Several surveys have determined that microorganisms produce pheromones involved in aggregation behaviour and mate's searching. Also, there is evidence that microorganisms mediate resources location as oviposition sites and host plant selection (Wertheim *et al.*, 2005; Witzgall *et al.*, 2012; Davis *et al.*, 2013). Furthermore, microorganisms play a relevant role between phytophagous insects and their pathogens, parasite and natural enemies, as well as our model system (Chaves *et al.*, 2009; Feldhaar, 2011; Davis *et al.*, 2013). Our results showed that microorganisms could be involved in *M. ruficauda* host-seeking behaviour by producing reliable cues, thus increasing parasitoid's fitness. As other studies have shown, the interactions between microorganisms, phytophagous insects and their natural enemies could shape relationships among trophic levels (Feldhaar, 2011; Oliver & Martinez, 2014). Hence, microorganisms prove to be relevant in ecology, behaviour and evolution of insects, due to they might have consequences over insects' fitness, contribute to evolutionary diversification in resource's selection and have an effect on the food webs (Feldhaar, 2011; Witzgall *et al.*, 2012; Davis *et al.*, 2013; Oliver & Martinez, 2014). Thereby, the present work contributes to the research on insect-microorganisms association which it shows to be relevant in insect's biology studies.

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