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Chemical Deception and Structural Adaptation in *Microdon* (Diptera, Syrphidae, Microdontinae), a Genus of Hoverflies Parasitic on Social Insects

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Abstract

Various organisms, especially arthropods, are able to live as parasites in ant nests and to prey upon ant broods without eliciting any aggressive behaviour in the hosts. Understanding how these intruders are able to break the ants' communication codes in their favour represents a challenging and intriguing evolutionary question. We studied the chemical strategies of three European hoverfly species, *Microdon mutabilis* (parasitic on *Formica cunicularia*), *M. analis* (parasitic on *Lasius emarginatus*) and *M. devius* (parasitic on *L. distinguendus*). The peculiar slug-like larvae of these three species live inside ant nests feeding upon their broods. Gas chromatography-mass spectrometry analyses show that: 1) these parasites mimic the host brood rather than the ant workers, although each differs distinctly in the extent of chemical mimicry; 2) isolation experiments indicate that after 14 days the responsible cuticular hydrocarbons (CHCs) are not passively acquired but synthesized by the fly larvae. Additionally, *Microdon* larvae show an array of protective structural features, such as a thick and multi-layered cuticle, retractable head, dome-shaped tergum and a flat and strongly adhesive "foot" (sternum). This combination of protective chemical and structural features represents a successful key innovation by *Microdon* species, and one that may facilitate host switching. The results of a preliminary adoption analysis confirm that *Microdon* larvae of at least some species can readily be accepted by different species of ants.

Keywords Social parasites · Chemical mimicry · Protective structure · Host specificity · Parasitism

Introduction

The ant colony can be considered one of the most successful organizations to be found in nature. Its complex social structure has intrigued generations of scientists. Despite the fact that ant colonies are well-guarded fortresses against intruders, there are several inquiline species that are able to infiltrate the colony and exploit its resources, such as food, shelter and benign environmental conditions (Cushing 2012; Holldobler and Wilson 1990; Nehring et al. 2016; Parmentier et al. 2017; Parmentier et al. 2018; Singer 1998; von Beeren et al. 2018). These so-called myrmecophiles, obligate symbionts of ants for at least one stage of their life cycle, represent an extremely diverse assortment of taxa (Wilson 1971) that use effective and sophisticated strategies to overcome ant defences and co-exist with their hosts (Kistner 1979). Most myrmecophiles are commensals or mutualists, but about 10,000 species (Thomas et al. 2005) are ant nest parasites, which inhabit principally the brood chambers, where they feed upon larvae and pupae (Akino et al. 1999) or trick the ant workers in order to be directly fed by trophallaxis (Cammaerts 1995). Myrmecophiles overcome the ants' defences by using behavioural, chemical and/or mechanical strategies that deceive the ants and prevent them from being detected (Holldobler and Wilson 1990; Parmentier et al. 2018; von Beeren et al. 2018). For example, many myrmecophilous spiders have evolved morphological adaptations such as small size and hard

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sclerotized scuta of the abdomen which protect them from ant attacks (Cushing 2012). Furthermore, some insects, like the butterfly genus *Maculinea* (van Eecke, 1915) (Lepidoptera: Lycaenidae) and the beetle genus *Paussus* Linnaeus, 1775 (Coleoptera: Paussinae), are able to mimic sounds emitted by the different ant castes in order to break the ants' vibrational signalling (Barbero et al. 2009; Di Giulio et al. 2015). However, acoustic mimicry is not the dominant strategy since it plays a marginal role in ant society and is involved in the modulation of other signals (Sala et al. 2014). Instead chemical communication channels are those most commonly used to organize life inside an ant colony (Lenoir et al. 2001a). Cuticular hydrocarbons (CHCs) are the most abundant chemicals forming the external lipidic layer of an insect's cuticle and often occur as a mixture of saturated and unsaturated molecules with variable chain length, generally ranging from 20 to 40 carbon atoms (Akino 2006; Ginzel and Blomquist 2016). CHCs have multiple and important functions in the life of insects, such as providing a barrier against water loss and protection against infection (Lockey 1988; Gibbs 2002; Provost et al. 2008). Furthermore, many studies have demonstrated the primary role of CHCs as recognition cues (Errard et al. 2008; Hernandez et al. 2006; Nash and Boomsma 2008; Provost et al. 2008; van Zweden and d'Ettorre 2010). In social insects, especially in ants, CHCs play a key role in nest mate recognition. Differences in the cuticular profile allow recognition of enemies and elicit behavioural responses such as aggression (Greene and Gordon 2007). In the case of chemical mimicry, myrmecophiles can adopt two possible strategies to deceive their host: biosynthesis (the active synthesis of CHCs) and camouflage (acquisition of colony odour by contact/exchange with the hosts [e.g. via grooming/throphallaxis] and contact with nest material (e.g. rubbing against the nest walls) (Bagnères and Lorenzi 2010; Lenoir et al. 2001a; Nash and Boomsma 2008; van Zweden and d'Ettorre 2010). These two mechanisms often occur simultaneously, making it difficult to distinguish one from the other (Bagnères and Lorenzi 2010). A further strategy may be 'chemical insignificance', the expression of a very low CHC profile in order to be chemically undetectable inside the nest (Bagnères and Lorenzi 2010; Lenoir et al. 2001a; Nash and Boomsma 2008; Provost et al. 2008; van Zweden and d'Ettorre 2010).

The immature stages of all known species of the hoverfly genus *Microdon* (Diptera, Syrphidae, Microdontinae) are obligate parasites of ants, feeding on the ant brood. *Microdon* species are mainly Neotropical and are relatively poorly represented in temperate regions (Reemer and Stahls 2013). In Europe only six species are known: *M. analis* (Macquart, 1842), *M. major* Andries, 1912, *M. devius* (Linnaeus, 1761), *M. miki* Doczkal and Schmid, 1999, *M. mutabilis* (Linnaeus, 1758) and *M. myrmicae* Schönrogge et al., 2002 (Doczkal and Schmid 1999; Schönrogge et al. 2002; Speight 2004; Speight 2017). In 2002 the species *M. mutabilis* was split by Schönrogge et al. into *M. mutabilis* and *M. myrmicae*, cryptic species morphologically almost undistinguishable, mainly on the basis of their different host species, respectively *Formica lemani* Bondroit, 1917 and several species of *Myrmica* Latreille, 1804. Minor morphometric characters of the puparium have been considered as diagnostic as well (Schönrogge et al. 2002).

Because of their rarity and the difficulties in finding them, *Microdon* larvae are poorly known, and there are just two studies investigating their chemical strategy to infiltrate host colonies: one on *M. albicomatus* Novak, 1977, a parasite of *Myrmica incompleta* Provancher, 1881 (Howard et al. 1990a), and the other on *M. piperi* Knab, 1917, a parasite of *Camponotus modoc* Wheeler , 1910 (Howard et al. 1990b). The two studies focus on second and third instar larvae of *Microdon* and show that the hoverfly larvae possess the same CHCs as those observed in the chemical extract of their host ant larvae (Howard et al. 1990a, b), whereas *Microdon* adults show a different chemical profile. For these two parasitic species, biosynthesis seems to be the adopted chemical strategy. In a preliminary analysis, chemical insignificance was hypothesized to occur in *M. myrmicae* since the few CHCs were detected (Witek et al. 2013). However, this result was based on a small number of specimens and requires confirmation by more extensive sampling.

In the present work we investigate the different parasitic strategies of three *Microdon* species from Central Italy, *M. mutabilis*, *M. analis* and *M. devius*, by comparing the cuticular hydrocarbon profiles of their immature stages with those of larvae and workers of their hosts, respectively, *Formica cunicularia Latreille, 1798, Lasius emarginatus* (Olivier, 1792), and *L. distinguendus* (Emery, 1916). Furthermore, we discuss protective structural adaptations that supplement the chemical strategy.

Materials and Methods

Field Collection Hoverflies and ants were collected between April 2015 and October 2017 at Pisoniano (RM).

(N 41.92430, E 12.9699), Latium, Central Italy. Specimens were collected with clean soft forceps and taken back to the laboratory in sterile Petri dishes containing a small amount of nest material. We excavated one nest of *F. cunicularia* parasitized by *M. mutabilis*, two nests of *Lasius distinguendus* with *M. devius* and one *L. emarginatus* nest with *M. analis* (Table 1). *M. mutabilis* and *M. devius* were detected inside underground nests which are easily recognizable by their earth mounds, larger for *L. distinguendus*. Unlike the other two species, *M. analis* colonies are commonly located under tree bark. From each nest we analysed both ants (larvae and workers) and parasite larvae. In order to understand the chemical strategy of *Microdon* spp. (biosynthesis or camouflage),

Table 1 Samples analysed with gas chromatography-mass spectrometry

	N. samples	Sample description
Nest 1	5	Third instar *M. analis* larvae
	5	*L. emarginatus* larvae
	5	*L. emarginatus* workers
Nest 2	5	Third instar *M. devius* larvae
	5	*L. distinguendus* larvae
	4	*L. distinguendus* workers
Nest 3	5	Third instar *M. devius* larvae
	0	*L. distinguendus* larvae
	4	*L. distinguendus* workers
Nest 4	6	Third instar *M. mutabilis* larvae
	5	Third instar *M. mutabilis* isolated larvae
	2	*F. cunicularia* larvae
	4	*F. cunicularia* pupae
	6	*F. cunicularia* workers

we isolated five third instar larvae of *M. mutabilis* for 14 days, and then kept them in clean Petri dishes without feeding or contact with hosts or nest material. This procedure allows one to determine whether CHCs are actively secreted by the insect or if they are acquired passively from the ants or their nest material (camouflage). In the case of camouflage, the CHCs are generally lost within a few days (Boulay et al. 2000; Lenoir et al. 2001b).

Chemical Analyses *Microdon mutabilis*, *M. devius* and *M. analis* larvae, as well as larvae and workers of their relative ant hosts (respectively *Formica cunicularia*, *Lasius distinguendus*, *L. emarginatus*), were euthanized by freezing them for at least one hour and then immersed in pentane for cuticular hydrocarbon (CHC) extraction in the following manner. Hydrocarbons were extracted by immersing individuals (see Table 1) in pentane (HPLC grade, 99%, Sigma-Aldrich) for 10 min. The specimens were then transferred to another vial and conserved in 70% -96% ethanol and the solvent allowed to evaporate. Before chemical analysis, the extracts were re-dissolved in 50 μl of pentane, and 2 μl were injected into an Agilent Technologies 7890A gas-chromatograph (GC), equipped with a HP-5MS capillary column (30 m × 250 μm × 0.25 μm) split-splitless injector, with helium as the carrier gas at a flow rate of 1 ml/min-1. The GC was coupled with a 5975 Agilent Technologies Mass Spectrometer with 70 eV electron impact ionization. After an initial hold of one minute at 70 °C, the temperature of the column was increased to 200 °C at 30 °C/min and then to 320 °C at 5 °C/min, held for 10 min. Compounds were identified from their mass spectra, retention time and comparison with published results (d'Ettorre et al. 2002).

Statistical Analyses The abundance of each compound was calculated as its relative proportion of the total blend of all compounds of every specimen. Then CHC abundances were square-root transformed. This transformation was used to limit biasing the effect of very large peaks and was used because it preserves quantitative information and can also deal with zero values. To visualise the chemical similarities of the complete CHC profiles, a hierarchical cluster analysis was calculated from the Euclidean distances matrix of the standardized CHC quantities using the unweighted pair group method (UPGMA) algorithm and the *pvclust* function in R in pvclust package with 10,000 permutations providing two types of *p* values: AU (Approximately Unbiased) p value and BP (Bootstrap Probability) p value. Additionally, a Correspondence analysis (CA) was performed using the *dudi.coa* function in ade4 package (Fig. 2a, b). For each CHC its contribution to the total blend is reported as a percentage (Fig. 2b, Supplementary Material 1).

Also, to determine how similar the chemical profiles of the parasites and the hosts are in composition, we calculated, two by two, Jaccard's similarity coefficient (JSI). The Jaccard index is the percentage of variables (CHCs) two sets (chemical profiles) have in common out of the total number of variables.

$$JSI = a/b + c - a$$

Where:

a the number of CHCs in both samples, b = the number of CHCs in the parasite, c= the number of CHCs in the host (ant larvae or workers). If JSI= 0, the samples are completely different from each other, if JSI= 1, the samples are completely similar.

To test for differences between the CHC profiles of larvae of *M. mutabilis* that were isolated and those that were not, the significance of the CHC similarity was determined using a *PERMANOVA* calculated on Euclidean dissimilarities matrix between the standardized CHC abundances and using a maximum of 9999 permutations with Bonferroni correction.

Scanning Electron Microscopy (SEM) Ten third instar larvae of *M. mutabilis*, *M. analis* and *M. devius* were examined using a Dual-Beam (FIB/SEM) Helios Nanolab (FEI Company, Eindhoven, The Netherlands) at the L.I.M.E. (University of Roma Tre, Rome, Italy). This instrument incorporates both a focused ion beam (FIB) and a scanning electron beam (SEM) in the same microscope. However, FIB/SEM was operated only with the SEM column to acquire high resolution images.

Samples were prepared as follows: larvae were immersed in 70% ethanol and then gradually dehydrated by placing them in higher concentrations of ethanol up to 100%, with intervals of 10 min between each step. Then they were critical-point dried using a Bal-Tec CDP 030, mounted on double-sided carbon discs on standard stubs and gold sputtered using an Emitech K550 unit.

Adoption Observations Three third instar larvae of *M. analis* were placed separately in Petri dishes (5 cm diameter) with filter paper on the bottom. Eleven workers and seven larvae of *L. distinguendus* (*M. devius*'s host) were added. Behaviour was video-recorded using an Olympus OM-D camera (Movie 1). The videos were started at different intervals following the introduction of the parasite: 0 min, 15 min, 3 h. Each video lasted 7 min. A preliminary analysis was performed with the software BORIS. We recorded two different host/parasite interaction categories: a) the number of times that an ant interacts with the parasite; b) the number of times that an ant brings its own larva near or onto the parasite.

Results

Chemical Analyses

M. analis - L. emarginatus. M. analis third instar larvae possess 18 CHCs, almost all linear alkanes with carbon chain length between C_{17} and $C_{33,}$ and 2 methyl-branched alkanes, x,yMe-C_{25} and x,yMe-C_{27}. *L. emarginatus* larvae (JSI = 1) have a very similar chemical signature, whereas the ant workers (JSI = 0.17) have a richer and more complex blend that includes 44 CHCs, mainly methyl-alkanes (Table 2). In the UPGMA cluster analysis based on the Euclidean distances matrix and in the CA, a single cluster is drawn for the parasite and its host larvae, while *L. emarginatus* workers are strongly differentiated (Figs. 1 and 2a).

M. devius - L. distinguendus A similar scenario is revealed for *M. devius* and its host *L. distinguendus*. The parasite larvae express 22 CHCs, of which 5 are alkenes ($C_{18:1}$, $C_{18:1}$, $C_{20:1}$, $C_{22:1}$, $C_{22:1}$) and the others alkanes. All these compounds are shared with the host larvae which have 4 more alkenes ($C_{23:1}$, $C_{29:1}$, $C_{31:1}$, $C_{31:1}$) within a total of 26 CHCs (Table 2). The cluster analysis highlights a unique cluster for *M. devius* and *L. distinguendus* larvae with high similarity (JSI = 0.85). But surprisingly *L. distinguendus* workers have a simpler profile than that of their larvae with only 17 CHCs compared to 22 and 26 respectively for parasite and brood, lacking 7 of 9 alkenes present in the ant larvae (retaining only $C_{18:1}$, $C_{20:1}$). This simplicity has the effect of making the ant workers' CHCs cluster more closely to *M. analis* (that shows 18 total CHCs none of them alkenes) than to *M. devius* or their own larvae (Fig. 1), while the correspondence analysis (CA) shows a total overlap between the host (*L. distinguendus*) and the parasite (*M. devius*)(Fig. 2a).

M. mutabilis - F. cunicularia *Microdon mutabilis* appears to be the parasite that has the least resemblance to its host (JSI = 0.59 *M. mutabilis/F. cunicularia* pupae; JSI = 0.52 *M. mutabilis/F. cunicularia* larvae; JSI = 0.42 *M. mutabilis/F. cunicularia* workers). Third instar larvae show 14 CHCs, of which 3 are present only as traces, all linear alkanes with carbon chain length between C_{20} and C_{35} and all also observed on ant workers except for *n*-C_{31} (found in traces). In the cuticular hydrocarbon profile of *M. mutabilis* pupae, besides 10 alkanes held in common with third instar larvae, there are also two alkenes, $C_{29:1}$ and $C_{31:1}$, which are not found in the other samples (although some alkenes were present in scarcely detectable traces in some ant pupae). Of the 12 CHCs found for ant pupae, 10 are common to *M. mutabilis* larvae and pupae, and of the 17 CHCs expressed by ant larvae, 10 are present also in the cuticular profile of larvae and pupae of their parasite. These shared CHCs are all linear alkanes. *Formica cunicularia* workers show a typical rich blend of CHCs, 32 hydrocarbons including alkanes, alkenes and methyl-alkanes (Table 2). The parasites and the ant brood and workers aggregate in three separate clusters. The *M. mutabilis* cluster groups with the large *M. analis/M. devius* cluster (Fig. 1). This is most evident from the CA, in which *M. mutabilis*, *M. devius* and its *Lasius* host species form a single wholly overlapping cluster (Fig. 2a). No differences (JSI = 1; *PERMANOVA* $P = 0.28$) were found between *M. mutabilis* third instar larvae from which CHCs were extracted immediately after collection and those larvae separated from their host ants for two weeks: they cluster together.

M. analis – M. devius – M. mutabilis In general, the three parasite species of this study all express relatively simple profiles characterized by relatively few CHCs, 14 in *M. mutabilis*, 17 in *M. analis* and 22 in *M. devius.* Almost all these are linear alkanes with a very low percentage of alkenes or methyl-branched alkanes. In contrast the chemical profiles of workers of *L. emarginatus* and *F. cunicularia* are much more complex, and show a remarkable presence of methyl-branched alkanes (Fig. 2a, b). *M. analis* shows a similarity index with *M. devius* of 0.67, while with *M. mutabilis* is 0.57. The chemical profiles of *M. devius* and *M. mutabilis* have a similarity percentage of 54%.

External Morphology Third instar larvae of analysed *Microdon* species show a strongly convex body, nearly semi-circular in transverse section (Fig. 3a, b). The ventral plate is enlarged, flat and highly muscular, sticking to the substrate surface with a wet, mucous, adhesive layer (Fig. 3f). The cuticle in section appears very thick due to a multi-

Table 2 Average spectra of three European Microdon species (*M. analis*, *M. devius*, *M. mutabilis*) and their respective ant hosts (workers and larvae)

	M. analis	*L. emarginatus* larvae	*L.emaginatus* workers	*M. devius*	*L.distinguendus* larvae	*L. distinguendus* workers	*M. mutabilis*	*M. mutabilis* Isolated	*F.cunicularia* larvae	*F.cunicularia* pupae	*F.cunicularia* workers
Compounds	%(SD)	%(SD)	%(SD)	%(SD)	%(SD)	%(SD)	%(SD)	%(SD)	%(SD)	%(SD)	%(SD)
C17	2.32 (0.86)	0.99 (0.56)	–	1.98 (0.78)	1.00 (0.10)	6.06 (9.14)	–	–	–	–	–
C18:1	–	–	–	1.96 (0.74)	0.63 (0.24)	–	–	–	–	–	–
C18:2	–	–	–	2.16 (0.77)	0.79 (0.29)	1.97 (0.96)	–	–	–	–	–
C18	2.48 (0.92)	0.79 (0.23)	–	2.00 (0.62)	0.68 (0.17)	1.87 (0.55)	–	–	–	–	–
C19	2.39 (0.27)	1.69 (0.23)	0.30 (0.18)	2.16 (0.61)	1.06 (0.25)	2.73 (0.51)	–	–	–	–	–
C20:1	–	–	–	1.77 (0.71)	1.03 (0.34)	1.60 (0.48)	–	–	–	–	–
C20	2.35 (0.54)	1.76 (0.11)	0.19 (0.12)	1.99 (0.63)	0.45 (0.19)	1.82 (0.31)	2.87 (0.66)	1.09 (0.44)	0.32 (0.32)	0.20 (0.26)	0.14 (0.14)
C21	1.96 (0.51)	1.63 (0.33)	–	1.29 (0.27)	2.48 (0.58)	1.48 (0.32)	3.64 (0.50)	2.21 (0.65)	0.66 (0.66)	0.29 (0.27)	0.24 (0.16)
C22:1	–	–	–	1.20 (0.40)	0.72 (0.29)	–	–	–	–	–	–
C22:1	–	–	–	1.67 (0.74)	0.89 (0.22)	–	–	–	–	–	–
C22	3.31 (0.35)	3.21 (0.44)	0.31 (0.27)	2.61 (0.40)	2.17 (0.77)	2.62 (0.44)	4.48 (0.90)	3.18 (0.73)	0.34 (0.23)	3.17 (6.12)	0.13 (0.12)
C23:1	–	–	–	–	2.34 (0.82)	–	–	–	–	–	–
C23	6.47 (0.96)	7.28 (0.22)	0.62 (0.48)	4.56 (0.94)	5.45 (0.78)	5.62 (0.70)	6.75 (1.46)	5.30 (2.16)	2.09 (1.99)	0.99 (0.46)	1.56 (0.42)
11meC23	–	–	0.23 (0.02)	–	–	–	–	–	–	–	–
3meC23	–	–	0.34 (0.05)	–	–	–	–	–	–	–	–
5,11dimeC23	–	–	0.13 (0.03)	–	–	–	–	–	–	–	–
C25:1	–	–	–	–	–	–	–	–	–	–	3.37 (1.49)
C25	11.90 (1.89)	14.57 (1.03)	2.26 (1.49)	8.80 (2.36)	8,84 (2.46)	1148 (1.88)	12.83 (2.68)	10.77 (4.23)	15.52 (11.70)	9.20 (4.38)	10.74 (3.03)
9 + 11 + 13meC25	–	–	7.99 (1.13)	–	–	–	–	–	–	–	–
7meC25	–	–	0.21 (0.05)	–	–	–	–	–	–	–	–
5meC25	–	–	0.64 (0.18)	–	–	–	–	–	–	–	–
9,13dimeC25	–	–	5.02 (0.62)	–	–	–	–	–	–	–	–
x,y-meC25	1.41 (0.64)	2.59 (0.22)	10.77 (0.37)	–	–	–	–	–	–	–	–
5,9dimeC25 + 5,13dimeC25	–	–	2.66 (0.26)	–	–	–	–	–	–	–	–
dimeC25?	–	–	1.90 (0.94)	–	–	–	–	–	–	–	–
tridimeC25	–	–	0.76 (0.22)	–	–	–	–	–	–	–	–

Table 2 (continued)

	M. analis	*L. emarginatus* larvae	*L.emaginatus* workers	*M. devius*	*L.distinguendus* larvae	*L. distinguendus* workers	*M. mutabilis*	*M. mutabilis* Isolated	*F.cunicularia* larvae	*F.cunicularia* pupae	*F.cunicularia* workers
tridimeC25	–	–	0.38 (0.06)	–	–	–	–	–	–	–	–
9Me C25	–	–	–	–	–	–	–	–	1.34 (1.48)	–	5.92 (2.35)
3Me C25	–	–	–	–	–	–	–	–	1.32 (1.42)	–	2.57 (0.30)
C26:1	–	–	–	–	–	–	–	–	–	–	2.84 (0.63)
C26	12.05 (1.67)	12.69 (0.64)	–	8.43 (1.76)	7.99 (2.53)	10.61 (1.27)	11.71 (3.05)	10.31 (5.28)	2.47 (128)	1.87 (0.21)	0.88 (0.30)
3,7diMeC25	–	–	–	–	–	–	–	–	–	–	1.20 (0.34)
10Me C26	–	–	–	–	–	–	–	–	–	–	2.22 (0.29)
13 + 12 + 11meC26	–	–	1.80 (0.29)	–	–	–	–	–	–	–	–
4meC26	–	–	0.17 (0.03)	–	–	–	–	–	–	–	–
13,17diMeC26	–	–	0.62 (0.15)	–	–	–	–	–	–	–	–
8,14diMeC26	–	–	0.96 (0.38)	–	–	–	–	–	–	–	–
6,ydiMeC26	–	–	0.86 (0.20)	–	–	–	–	–	–	–	–
C27:1	–	–	–	–	–	–	–	–	–	–	7.05 (2.30)
C27	11.77 (1.24)	14.90 (1.47)	3.00 (1.77)	8.87 (2.45)	10.23 (1.15)	10.70 (1.35)	9.11 (1.95)	7.97 (3.90)	18.37 (5.88)	22.86 (9.83)	7.40 (2.66)
13 + 11 Me C27	–	–	–	–	–	–	–	–	2.94 (2.68)	13.91 (26.95)	11.92 (1.97)
3 Me C27	–	–	–	–	–	–	Tr	Tr	1.56 (1.61)	–	2.38 (0.67)
x,y- meC27	2.94 (2.14)	2.40 (0.23)	16.70 (4.10)	–	–	–	–	–	–	–	–
7meC27	–	–	1.03 (0.23)	–	–	–	–	–	–	–	–
5meC27	–	–	0.29 (0.05)	–	–	–	–	–	–	–	–
11,15dimeC27	–	–	4.66 (1.22)	–	–	–	–	–	–	–	–
9,13dimeC27	–	–	6.77 (0.63)	–	–	–	–	–	–	–	–
7,11dimeC27	–	–	10.61 (4.07)	–	–	–	–	–	–	–	–
5,ydimeC27	–	–	2.23 (0.82)	–	–	–	–	–	–	–	–
tridimeC27	–	–	2.63 (0.71)	–	–	–	–	–	–	–	–
dimeC27?	–	–	1.34 (0.22)	–	–	–	–	–	–	–	–
C28:1	–	–	–	–	–	–	–	–	–	–	6.72 (1.43)
C28	8.82 (1.25)	10.09 (1.02)	–	6.83 (1.15)	6.73 (1.49)	9.38 (1.55)	6.98 (1.84)	7.04 (3.75)	2.04 (0.85)	2.86 (1.01)	0.65 (0.13)
13 + 12meC28	–	–	1.34 (0.81)	–	–	–	–	–	–	–	–
8,12diMeC28	–	–	0.55 (0.24)	–	–	–	–	–	–	–	–
C29:1	–	–	–	–	2.15 (1.09)	–	–	–	–	–	–
12MeC28	–	–	–	–	–	–	–	–	–	–	2.15 (0.34)
C29	11.00 (1.40)	9.61 (0.54)	1.00 (0.78)	12.43 (5.08)	19.55 (6.14)	17.10 (2.67)	8.01 (1.56)	6.76 (2.49)	35.21 (28.85)	39.73 (24.95)	2.71 (1.17)

Table 2 (continued)

	M. analis	*L. emarginatus* larvae	*L.emaginatus* workers	*M. devius*	*L.distinguendus* larvae	*L. distinguendus* workers	*M. mutabilis*	*M. mutabilis* Isolated	*F.cunicularia* larvae	*F.cunicularia* pupae	*F.cunicularia* workers
13 + 11 Me C29	–	–	1.54 (0.35)	–	–	–	–	–	2.13 (1.84)	–	5.90 (0.80)
7MeC29	–	–	2.15 (0.27)	–	–	–	–	–	0.98 (0.38)	2.47 (3.93)	1.30 (0.94)
3meC29	–	–	0.64 (0.31)	–	–	–	–	–	–	–	–
5,17dimeC29	–	–	0.34 (0.18)	–	–	–	–	–	–	–	–
5,9,15triMeC29	–	–	0.37 (0.13)	–	–	–	–	–	–	–	–
C30:1	–	–	–	–	–	–	–	–	–	–	0.78 (0.29)
C30	6.25 (0,69)	6.24 (0.78)	–	4.46 (0.73)	4.66 (0.88)	5.84 (0.64)	2.75 (1.36)	2.94 (2.91)	–	–	Tr
C31	6.85 (1.66)	4.55 (0.44)	–	6.86 (0.98)	8.25 (1.78)	6.16 (0.94)	11.99 (3.02)	14.88 (8.14)	3.68 (1.58)	2.43 (1.10)	1.13 (0.75)
13 + 11 Me C31	–	–	–	–	–	–	–	–	7.00 (9.05)	–	4.19 (1.81)
9,13diMeC31	–	–	0.69 (0.18)	–	–	–	–	–	–	–	–
C31:1	–	–	–	–	4.47 (1.94)	–	–	–	–	–	–
C31:1	–	–	–	–	2.64 (1.42)	–	–	–	–	–	Tr
11,xdiMeC31	–	–	–	–	–	–	–	–	–	–	5.16 (2.31)
C32	2.84 (0.48)	3.00 (0.45)	–	2.61 (0.33)	2.05 (0.55)	2.97 (0.75)	Tr	Tr	–	–	Tr
C33	2.82 (1.09)	1.99 (0.45)	–	9.35 (6.81)	1,97 (0,25)	–	14,42 (9,40)	21,70 (12,86)	–	–	Tr
11 + 13 + 15meC33	–	–	0.86 (0.50)	–	–	–	–	–	–	–	–
13 + 11 MeC33	–	–	–	–	–	–	–	–	–	–	2.27 (0.87)
11,xdiMeC33	–	–	1.18 (0.46)	–	–	–	–	–	–	–	4,16 (2,03)
11,15diMeC33	–	–	0.98 (0.28)	–	–	–	–	–	–	–	–
C35	–	–	–	5.99 (4.69)	0.80 (0.30)	–	Tr	Tr	–	–	–

For all samples, the average relative area of the hydrocarbon peaks and the respective standard deviations were calculated as a percentage. Compounds with expressed less 0,1% are marked as Tr, trace

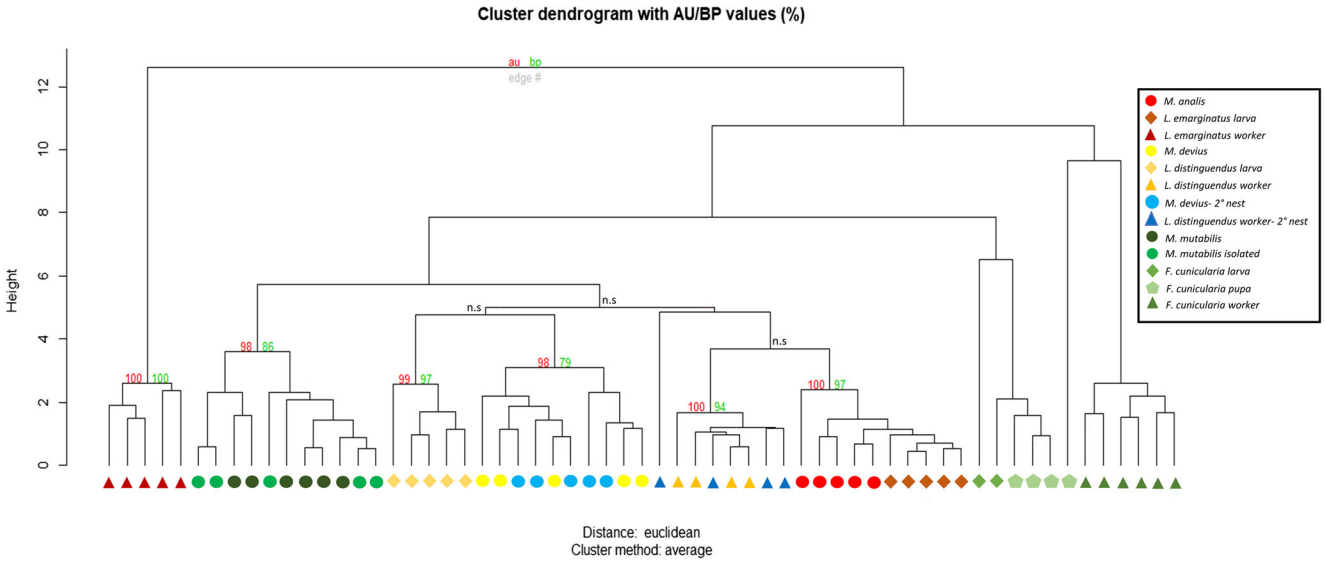

Fig. 1 Hierarchical cluster analysis of the cuticular hydrocarbon profiles of three European *Microdon* species (*M. analis*, *M. devius*, *M. mutabilis*) and the associated ant hosts (workers and larvae). Clustering was conducted with the unweighted pair group method with arithmetic mean and the Euclidean dissimilarity matrix, with 10,000 permutations. Two types of p values are provides: AU (Approximately Unbiased) *p*-value (red) and BP (Bootstrap Probability) value (green)

layered structure (Fig. 3e). The small and soft pseudocephalon is retractable, not visible when the larva is motionless. Dorsal reticulation is visible in all the three analysed species, the processes form intersecting rows that draw semi-circular or polygonal shapes (Fig. 3a–d). Each reticulation process consists of stringy, extended projections (Scarparo et al. 2017). In *M. mutabilis* the dorsal reticulation is reduced to a narrow, lateral strip along the perimeter of the abdomen (Fig. 3a, c), while *M. analis* and *M. devius* both show a strongly developed dorsal reticulation that completely covers the larval dorsum (Fig. 3b–d).

Adoption Observations In all video recordings the third instar *M. analis* larvae remain motionless or move only slightly, while the *L. distinguendus* workers are very active. The ant workers (singly or together) interact with the parasite, mostly standing on its, and approaching individual larvae an average of 39 times during the 7 min filming intervals. During these interactions no aggressive ant behaviour towards *M. analis* was observed, such as mandible opening, biting or gaster flexion, only antennal contact and grooming. Ant workers were recorded attempting to transport and place their own larvae onto the parasite 11 times (Movie 1). Other details are illustrated in Table 3.

Discussion

Myrmecophiles have evolved different strategies to be accepted by their host ants and to survive and develop in their nests. Such adaptations include: chemical and morphological mimicry; specialized feeding behaviour and ways of inducing ants to feed them; and structural modifications that allow them to avoid being attacked by ants (Thomas et al. 2005; Lachaud et al. 2013). Like other obligate myrmecophiles, the larvae of *Microdon* genus are able to successfully infiltrate into ant colonies and feed on the ant brood (Akre et al. 1973, 1988, 1990; Andries 1912; Barr 1995; Bonelli et al. 2011; Duffield 1981; Garnett et al. 1985; Remeer 2013; Scarparo et al. 2017; Speight 2017; Wolton 2011). Since these larvae live hidden in ant nests, showing complex interactions with their hosts, the study of their biology and behaviour is challenging. This is why the life cycle and the parasitic strategies of most *Microdon* species are not well known. We suggest that *Microdon* larvae have evolved and integrated two different strategies to successfully exploit the ant colony: chemical mimicry and a protective structure.

Chemical Strategies In 1990 Howard and co-authors (Howard et al. 1990a, b) studied the chemical mimicry of two North American *Microdon* species, *M. albicomatus* and *M. piperi*, highlighting the very high CHC blend similarity between parasites and host brood. The authors also suggested a possible active synthesis of CHCs by the parasites (biosynthesis strategy) (Howard et al. 1990a, b). The three species analysed in the present study exhibit chemical mimicry with the host brood (larvae) rather than with the ant workers, although each had a different degree of chemical similarity: 1) *M. analis* larvae show the highest similarity with larvae of the host *Lasius emarginatus* (100% of the CHC in common, though with different relative amounts); 2) *M. devius* displays a very high, but not complete chemical similarity with *Lasius distinguendus* larvae (85%); 3) *M. mutabilis* exhibits only a partial chemical mimicry with *F. cunicularia* larvae (52%), showing a greater similarity with the other two parasitic species than to its host (Figs. 1 and 2a). The distinct differences in

Fig. 2 Correspondence analysis performed on all the studied individuals (parasites, ant larvae and ant workers). a displays all the samples; **b** shows the relationships between CHCs (calculated as relative proportion and root-squared transformed) coloured according to their contribution to the definition of the dimensions (yellow = low; blue = medium; red = high). A detail of the percentage of contribution to the definition of the Dimension 1 and Dimension 2 for each CHC is provided by Supplementary Material 1

CHC profiles suggest that the larvae have evolved different mimicking strategies. In order to distinguish between biosynthesis or camouflage isolation experiments are generally used (see in Material and Methods paragraph). Unfortunately, due to the scarcity of available material, we could only isolate *M. mutabilis* larvae. In general, seven days are acknowledged to be a sufficient period for ant guests to lose the CHCs acquired passively with camouflage (Boulay et al. 2000; Lenoir et al. 2001b). To avoid any ambiguity, *M. mutabilis* larvae were maintained isolated for two weeks before the extraction of the CHCs. The results show that isolated *M. mutabilis* larvae keep the same hydrocarbon composition as larvae extracted immediately after collection from the ant nest. Thus we have demonstrated that the larva of this species is able to biosynthesize its CHCs actively. Concerning the other two species, *M. analis* and *M. devius*, whose CHC profiles show much higher similarity with their hosts, we are of the opinion that they actively synthesize at least the basic part of their blend (e.g. linear alkanes), but potentially acquire the more complex CHCs (e.g. alkenes and methyl-branched alkanes) by camouflage. *Microdon* parasites live in constant contact with ant larvae, inhabiting the brood chambers, so it is highly

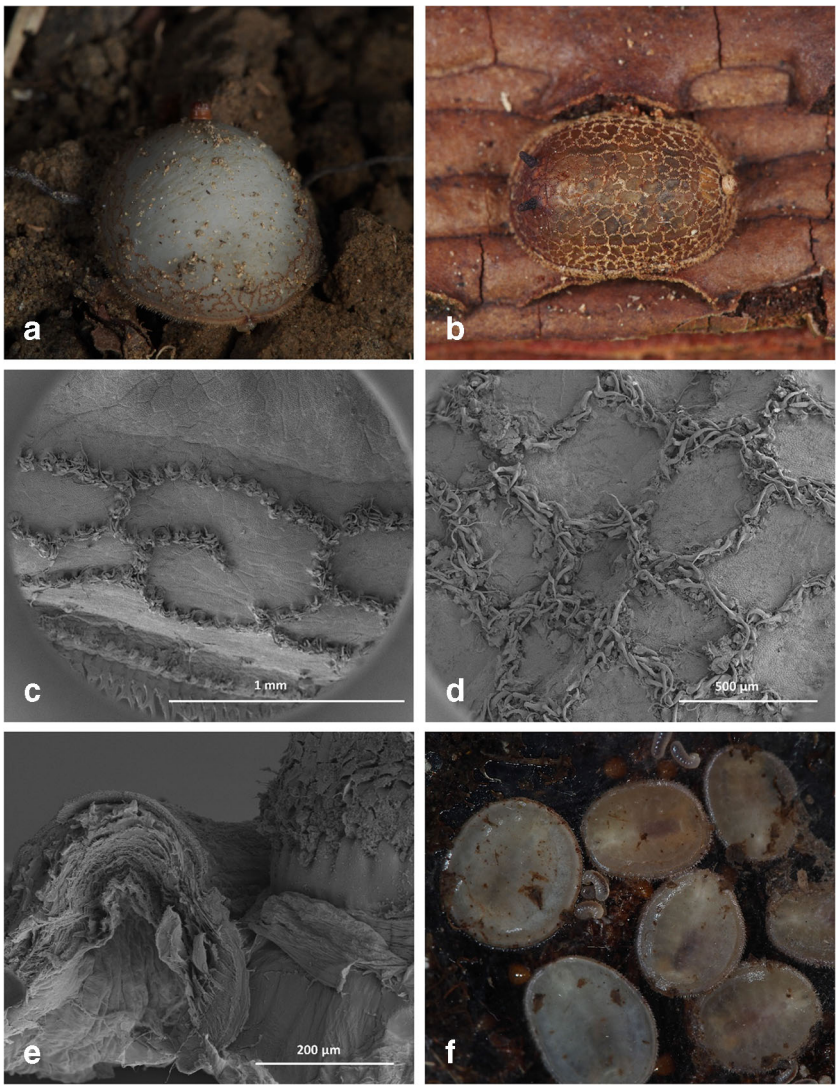

Fig. 3 **a** *M. mutabilis* third instar; **b** *M. analis* pupa; **c** scanning electron microscopy image of *M. mutabilis* dorsal reticulation; **d** scanning electron microscopy image of *M. analis* dorsal reticulation; **e** section of the thick and multi-layered *M. mutabilis* cuticle; **f** Ventral view of 7 third instar *M. myrmicae* larvae, attached with the muscular foot to the glass walls of a breeding cage

likely that they will acquire the odour of the colony passively, both by feeding on ant larvae and/or by rubbing against them or the nest material. We suspect that some structural adaptations could play a role in this respect, since the external larval morphology of the three species analysed in this work reveals significant differences. Unlike *M. mutabilis* larvae (Scarparo et al. 2017), which are almost completely smooth, *M. analis* and *M. devius* show a strongly developed dorsal reticulation (Fig. 3a–d), composed of long fibrous tufts of cuticular projections, which in these two species completely covers the larval tergum. We propose that these structures could act as brushes that gather and keep the CHCs. In other words, we hypothesize that some species, such as *M. analis* and *M. devius*, could use their dorsal reticulation like a sponge, improving the host similarity of their basic chemical profile by camouflage, matching point by point the host chemical signature. This hypothesis is in agreement with the results of the two North American species studied by Howard et al. (1990a, b). Both *Microdon albicomatus* and *Microdon piperi* exhibit dorsal reticulation similar to that of *M. analis* and *M. devius* that could allow them to acquire high chemical similarity with their hosts (Garnett et al. 1990; Howard et al. 1990a, b).

Protective Structure Although chemical mimicry is the primary strategy used by these parasites to exploit the resources of ant colonies and avoid ant defences, this strategy may not be infallible and on rare occasions the larvae could be recognized and attacked. Indeed, statistically the chemical profiles of *Microdon* parasites are clearly distinct from those of their hosts due to differences in the abundance of individual CHCs. Accordingly, as a failsafe, *Microdon* larvae show an additional strategy for their protection, a mechanical one, especially evident in third instar larvae. Their dome shaped body is dorsally characterized by a thick multi-layered cuticle, without any kind of protruding appendages except when feeding (Garnett et al. 1990; Rotheray and Gilbert 1999; Scarparo

Table 3 Adoption observations of *M. analis* larva with larvae and ants of *L. distinguendus*

			a) n° of times that an ant interacts with the parasite	b) n° of times that an ant brings his own larva near or on the parasite.
	Observations	Total length (s)		
1	t-0	420	59	0
	t-15 min	420	34	0
	t-3 h	420	21	0
2	t-0	420	39	0
	t-15 min	420	52	6
	t-3 h	420	47	2
3	t-0	420	26	0
	t-15 min	420	42	2
	t-3 h	420	29	1
			mean = 39	tot = 11

The videos were acquired at different timing from the introduction of the parasite: t0, t15 min, t3 h. Each video lasted 7 min (420 s). For each video it is reported a) n° of times that an ant interacts with the parasite; b) n° of times that an ant brings his own larva near or on the parasite

et al. 2017). The small soft head and the modified mouthparts are retractable as in many turtles (Barr 1995; Garnett et al. 1990; Scarparo et al. 2017). Additionally, the flattened strong muscular foot allows them to adhere firmly to the substrate, acting as a true sucker. *Microdon* larvae can be attacked and killed if the ventral surface is exposed to ant bites (Donisthorpe 1927). All these features make occasional attacks by the ants difficult because they cannot easily grab or bite the slug-like larvae and inflict wounds. We hypothesize that the protective structure of these peculiar fly larvae represents an effective key innovation of all *Microdon* species, and one which facilitates the exploitation of different hosts.

Host Specificity According to the scattered information present in the literature and to the results of the present study, it seems probable that *Microdon* species show considerable plasticity with respect to ant hosts, the same species being able to parasitize different species of the same or of different genera, even different ant subfamilies. Within limited geographical areas, however, they tend to be quite species-specific. A wide host range has been reported for the North American *Microdon albicomatus* which can exploit hosts of two different subfamilies, Formicinae and Myrmicinae (Howard et al. 1990b). Other studies performed on *M. piperi,* generally considered a parasite of *Camponotus modoc,* confirmed intercolonial and interspecific ease of adoption with the same genus (Howard et al. 1990a). The same species has been recorded to parasitise various species of *Formica* (Akre et al. 1988, 1990; Duffield 1981). *M. myrmicae* is an obligate parasite of several *Myrmica* species (Bonelli et al. 2011).

Microdon mutabilis was formerly considered as strictly species specific (Elmes et al. 1999; Schönrogge et al. 2002; Schönrogge et al. 2006). However, we found this species in association with a new ant host, *Formica cunicularia,* which is chemically very distant from *F. lemani* (Martin and Drijfhout 2009), the acknowledged host ant species. Furthermore, we recently found three third instar larvae of *M. mutabilis* inside a *Lasius distinguendus* nest, that is in agreement with other sporadic records of *M. mutabilis* larvae found with *Lasius* spp. ants, mostly *Lasius niger* (Linnaeus, 1758) (Andries 1912; Donisthorpe 1927; Remeer 2013; Schmid 2004; Speight 2017). However, it is not excluded that *Microdon* species known to exploit more than one host ant species, could be part of a cryptic species complex, as it was demonstrated for the sibling species *M. myrmicae* and *M. mutabilis* (Schönrogge et al. 2002).

M. devius and *M. analis* larvae share a similar chemical profile that hypothetically could allow them to switch their hosts (respectively *Lasius distinguendus* and *L. emarginatus*). The adoption observations performed with *M. analis* and *L. distinguendus* (the host of *M. devius*), suggest that the parasite is readily adopted by the new host. On more than one occasion, indeed 11 times, ants tried to transfer larvae of its own species onto the tergum of the parasite (Movie 1). This potential to switch hosts is supported by the cluster analysis where *L. distinguendus* workers aggregate with *M. analis*/*L. emarginatus* cluster rather than with *M. devius* (Fig. 1).

In conclusion, the hypothesis of strict host specificity (Elmes et al. 1999; Schönrogge et al. 2002, 2006) proposed for some *Microdon* species is possibly valid only at the local scale due to the poor dispersal ability of the adults. We hypothesize that chemical mimicry, backed up by the peculiar morphology of these fly larvae, increases their chances of survival inside the nests of ant hosts and facilitates transference among hosts.

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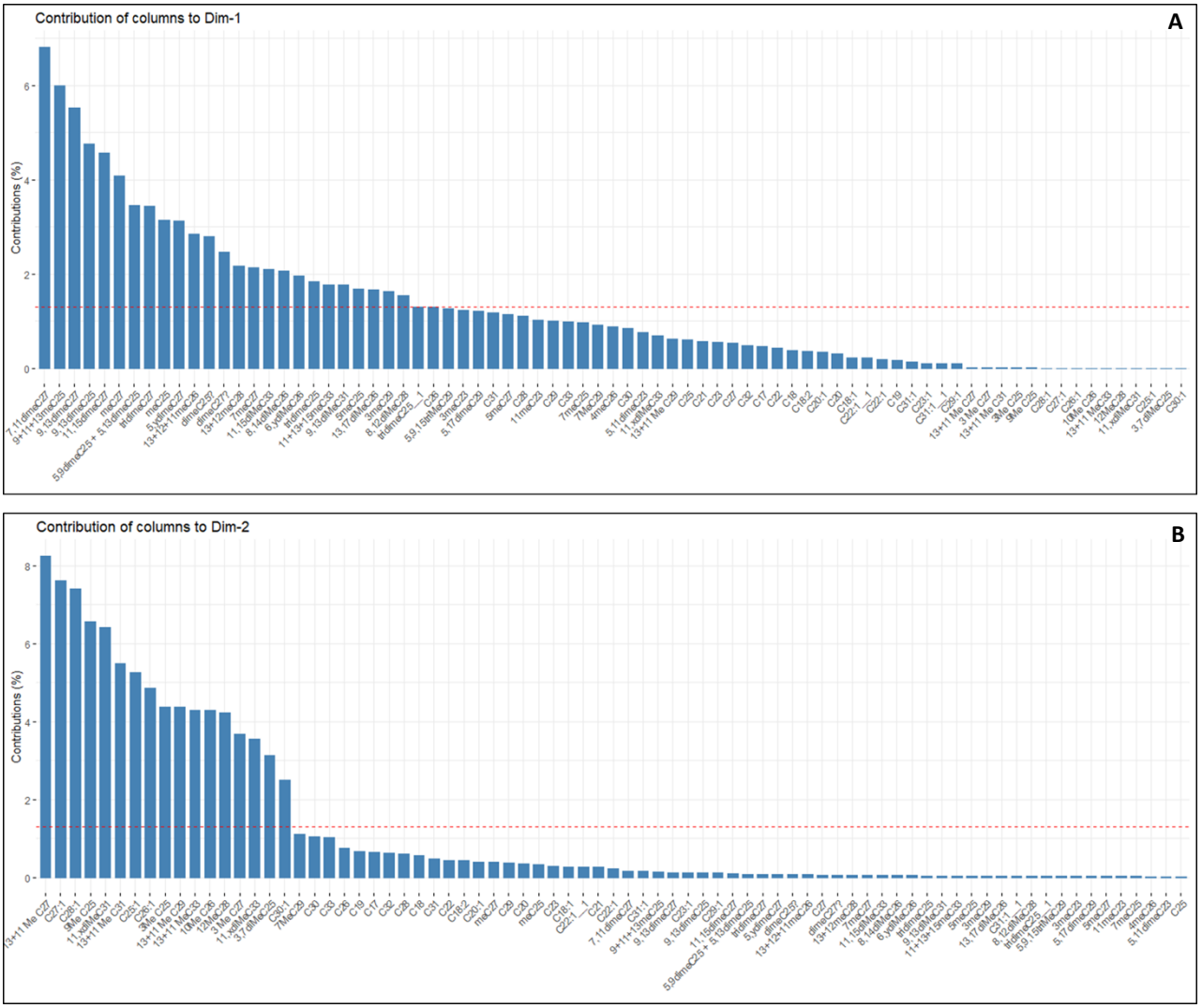
Supplementary Materials



Supplementary Material 1 Histogram of the percentage of contribution for each CHCs detected. Here Contribution of CHCs to Dimension 1(A) and Dimension 2 (B) of the correspondence analysis is shown.

Movie 1- Third instar *M. analis* larva with workers and larvae of *L. distinguendus* (*M. devius'* host).