



Chemical profiles in *Iberoformica subrufa* and *Formica frontalis*, a new example of temporary host–parasite interaction

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Abstract

The aim of this paper is to describe the relationship between two ant species, *Formica frontalis* and *Iberoformica subrufa*, found together in shared nests. Therefore, we obtained data from dug nests and outdoor activity in two sympatric populations and investigated the cuticular hydrocarbons (CHCs) in both sympatric populations and in 10 *I. subrufa* allopatric populations to unravel whether the relationship becomes tuned between both species. We also determined the CHCs of two sympatric *Serviformica* species (*F. cunicularia* and *F. lemani*). Our results showed that the ant *F. frontalis* is a temporary parasite of *I. subrufa* which facultatively forms mixed colonies complying with a loose form of the Emery's rule. Alkanes and methylalkanes are the most abundant compounds found in *F. frontalis* and *I. subrufa* CHCs, respectively, but esters were only abundant in *I. subrufa*. As far as the CHC similarity is concerned, the sympatric free-living hosts were chemically closer to the parasite, albeit not identical, whereas the allopatric *I. subrufa* populations always maintained a separate CHC composition. We provide different potential hypotheses to explain this similarity of cuticular profiles only in the two geographically distant sympatric populations.

Keywords Emery's rule · Esters · Environmental hypothesis · Different species hypothesis · Host-tolerance hypothesis · *F. lemani* · *F. cunicularia*

Introduction

The presence of two different ant species within the same nest is not an uncommon phenomenon, although the strict recognition systems operate in ant societies. In fact, an extensive classification exists which establishes differences for parasitic (mixed nests) or non-parasitic relationships (compound nests) ranging from a close vicinity (pleisiobiosis) to a strict and obligate parasitism (slavery, inquilinism)

(Hölldobler and Wilson 1990; Buschinger 2009). Amongst the compound nests, the term pleisiobiosis includes species nesting very closely but without interactions, cleptobiosis occurs when one species steals food from the stored food of another species, lestobiosis when one species predares their brood from another species, and parabiosis when the species share the nest and outdoor trails but raises their own brood separately (Buschinger 2009).

On the other hand, social parasitism is the coexistence of two social insect species in the same nest, one of which is parasitically dependent upon the other. Different types of social parasitism occur, including slavery, inquilinism, xenobiosis, and temporary parasitism (Hölldobler and Wilson 1990; Buschinger 2009).

Temporary parasitism is considered a basic type of social parasitism, in which the parasitic species only depends on a host species for a short time, during the foundation of the colony. The parasitic queen, after the nuptial flight, penetrates a host colony, and kills and replaces the original host queen and lays eggs, which are initially raised by the workers of the host species. Over time, the proportion of the host workers declines, so the colony gradually comprises only

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individuals from the temporary social parasite, which now cares for their own queen's brood. From this point onwards, the colony continues to function independently as a founded colony would (Hölldobler and Wilson 1990). This kind of parasitism appears in several species in the sub-families Formicinae, Myrmicinae and, at a lesser frequency, Dolichoderinae (Buschinger 2009).

It has been assumed in the bibliography that ants of the genus *Formica* are frequently temporary parasites. This behaviour has been reported in three of the four *Formica* sub-genera (*Formica* s. str., *Raptiformica*, and *Coptoformica*), commonly using different species of the *Serviformica* basal group of species as hosts (Buschinger 1986, 2009; Romiguier et al. 2018). Occasionally, other different hosts from the subgenus *Formica* s. str. have been found (Table 1 and references therein). However, little of the biology of these hosts and parasites has been proved, due to the difficulties in finding the mixed nests in the field (Pisarski and Czechowski 1994). Thus, the frequent temporary parasitism in *Formica* genera is an assumed rather than a proven phenomenon (e.g., Collingwood 1979; Goropashnaya et al. 2012). Nevertheless, some authors have generalized that

temporary parasitism is rather common for monogynous and monodomous wood-ant species in the subgenus *Formica* s. str. (Martin et al. 2011 and references there in).

The existence of mixed species nests in ant social parasites while overcoming the strict mechanisms of nest-mate recognition has been attributed to a strong phylogenetic relationship between both species (Emery 1909; Le Masne 1956). Emery's rule postulates that host and parasites generally derive from closely related species, rather than distant random species. However, most evidence supports a loose version of Emery's rule in which parasites and hosts are close relatives, e.g., belonging to the same species group (Buschinger 2009), but are not necessarily the closest possible taxa (Lopez-Osorio et al. 2015). For instance, most of the dulotic species parasitize species from a different genus but which still belongs to the same tribe (Huang and Dornhaus 2008). Notwithstanding, a strict application of Emery's rule is used for most temporary parasites, because around 60% of them parasitize hosts of the same genus (Huang and Dornhaus 2008).

The pacific coexistence of a social parasite and its host in mixed nests, whether phylogenetically close or distant, is

Table 1 Data about parasitism in the genus *Formica* in the bibliography

Genus <i>Formica</i>	Parasitism type	Hosts	References
<i>Subgenus Formica</i> s.str	Temporary parasitism	<i>Serviformica</i> species	Buschinger (1986, 2009) and Goropashnaya et al. (2012)
<i>F. rufa</i>	Temporary parasitism	<i>Serviformica</i> species	Collingwood (1979) and Ito and Higashi (1990)
<i>F. truncorum</i>	Temporary parasitism	<i>Serviformica</i> species	Collingwood (1979)
	Temporary parasitism	<i>F. lemani</i>	Chernenko et al. (2011) and Higashi (1983)
	Temporary parasitism	<i>F. fusca</i>	Chernenko et al. (2011), Forel (1874, 1886, 1913)
	Temporary parasitism	<i>F. pratensis</i>	Forel (1874, 1886, 1913)
	Temporary parasitism	<i>F. rufibarbis</i>	Forel (1874, 1886, 1913)
	Temporary parasitism	<i>F. sanguinea</i>	Forel (1874, 1886, 1913)
<i>F. polycтена</i>	Temporary parasitism	<i>Serviformica</i> species	Collingwood (1979; Ito and Higashi 1990)
<i>F. yesensis</i>	Temporary parasitism	<i>Serviformica</i> species	Ito and Higashi (1990)
<i>F. lugubris</i>	Temporary parasitism	<i>Serviformica</i> species	Collingwood (1979 and Ito and Higashi (1990)
<i>F. pratensis</i>	Temporary parasitism	<i>F. fusca</i>	Forel (1900)
	Temporary parasitism	<i>Polyergus rufescens</i>	Forel (1900)
	Temporary parasitism	<i>F. sanguinea</i>	Czechowski and Godzinska (2015)
<i>Subgenus Raptiformica</i>			
<i>F. sanguinea</i>	Slavery	<i>Serviformica</i> species	Buschinger (1986, 2009), Ito and Higashi (1990 ref. included)
	Slavery	<i>F. japonica</i>	Ito and Higashi (1990)
	Slavery	<i>F. lemani</i>	Ito and Higashi (1990)
	Slavery	<i>Formica</i> s.str. species	
	Slavery	<i>F. pratensis</i>	Forel (1886)
	Slavery	<i>F. rufa</i>	Włodarczyk (2011)
	Slavery	<i>F. fusca</i> , <i>F. cunicularia</i>	Mori et al. (2001)
<i>F. subnuda</i>	Slavery	<i>F. polycтена</i>	Włodarczyk and Szczepaniak (2014)
	Slavery	<i>F. fusca</i> , <i>F. cinerea</i>	Czechowski and Godzinska (2015)
	Slavery	<i>F. pratensis</i>	Czechowski and Godzinska (2015)
	Slavery	<i>F. podzolica</i>	Czechowski and Godzinska (2015)
<i>Subgenus Coptoformica</i>	Temporary parasitism	<i>Serviformica</i> species	Buschinger (1986, 2009)

generally possible due to at least partial congruency of their cuticular hydrocarbons (CHCs) (Lenoir et al. 2001). Accordingly, aggression amongst non-nest-mate ants correlates positively with CHC differences, whether involved in a host–parasite system (Zamora-Muñoz et al. 2003) or not (Martin et al. 2012). Moreover, the diversity of CHCs in the hosts may be prompted as well as maintained by the presence of parasites, especially for those compounds clearly related with nest-mate recognition (Martin et al. 2011). In general, it is considered that the *Formica* species have evolved two paths of species-specific CHCs related with nest-mate recognition: the elevated production of Z-9-alkenes and alternatively the production of various dimethylalkanes [dimethylpentacosanes in *F. fusca* or C27–C35 dimethylalkanes for *F. truncorum* and other species of the genus (Martin et al. 2008)]. This information is relevant, because *F. truncorum* is the closest species to *F. frontalis* (Goropashnaya et al. 2012).

During a field study on *F. frontalis* (subgenus *Formica* s. str), we found some nests composed of *F. frontalis* and *I. subrufa*. To clarify the relationship between both species, we studied different biological and chemical traits in this pair.

Formica frontalis is an endemic and scarce species distributed throughout the high mountains of the Iberian Peninsula, with the exception of the north-west region, and is generally linked to coniferous forests and open areas nearby (Tinaut and Martínez Ibañez 1998; Tinaut et al. 2015; Stockan et al. 2016). In southern Spain, it shows a more restricted distribution, in contrast to what was proposed by Stockan et al. (2016). *Formica frontalis* is an aggressive and dominant species (Tinaut, Silvestre, unpublished data), as are the other species from the subgenus *Formica* s. str. (Pisarski and Czechowski 1994).

The host species, *I. subrufa*, was formerly included in the genus *Formica* and the subgenus *Serviformica*, but has been reclassified, according to molecular markers, in a new and phylogenetically distant genus (Muñoz-López et al. 2012). *Iberoformica subrufa* is endemic in the Iberian Peninsula with a wide distribution living in *Quercus ilex* or *Q. rotundifolia* habitats and even in the vegetation where these forests have disappeared at different altitudes (sea level to 1900 m a.s.l.) (Tinaut 1990). This species was classified as a subordinate species (Arnan et al. 2007), having brachypterous queens (Tinaut and Ruano 1992).

The objective of this study was to explore the *F. frontalis*–*I. subrufa* relationship and CHC correlates, which permit the coexistence of these unrelated species in the same nests. We also included two additional sympatric *Formica* species from the *Serviformica* group of species (*F. lemani* and *F. cunicularia*) in the study, because most of temporary parasites from the *Formica* s. str. subgenus parasitize *Serviformica* species. Thus, the study of these sympatric species which could potentially serve as hosts for *F. frontalis*, should improve our knowledge about the relationship between *F. frontalis* and *I. subrufa*.

Materials and methods

Studied populations

In this study, only the two larger populations of *F. frontalis*, out of three currently active locations in Andalusia (southern Spain), were included: El Purche (Sierra Nevada, SN thereafter) and Cerro Quintana (Sierra de Baza, SB thereafter) (Tinaut and Martínez Ibañez 1998; Tinaut et al. 2015). These two populations are sympatric with *I. subrufa*.

Biological data for mixed nests

To study the interaction and possible aggressive behaviour between the two species, the outdoor activities of two mixed nests, six of free-living *F. frontalis* and six of *I. subrufa*, were followed in the field for 4 days between April and August 2016 (5 min/nest every 2 h between 10 and 18 h).

In addition, we excavated a mixed nest in April 2016 and another in July 2018 that were both installed in the laboratory, and we followed the behaviour of both species.

Cuticular hydrocarbons

To obtain geographical representation of CHC variation for sympatric and allopatric *I. subrufa*, we studied the chemical profiles of *F. frontalis* and *I. subrufa* from either mixed (three nests) or sympatric free-living colonies (10 *F. frontalis* and 10 *I. subrufa* nests), in the two Andalusian *F. frontalis* populations (60 km apart, sites 1 and 2, Fig. 1), as well as 15 allopatric nests of *I. subrufa* from 10 different localities (sites 3–12, Fig. 1). We also included two populations of the *Serviformica* species *F. lemani* (sites 1 and 8, 4 nests, Fig. 1) and *F. cunicularia* (sites 13 and 14, 2 nests, Fig. 1). Distances between the sites ranged from 9 to 350 km (See Fig. 1; Table 2 and Suppl. Table 1 for a complete view and list of sampled populations and their altitude).

Chemical analysis

Five workers from each of the studied colonies were collected and killed by freezing. All the collected ants were individually weighed (fresh weight) and immersed in 1 ml of hexane for 60 min, after which the ants were retrieved from the vials and the solvent evaporated. The samples were kept frozen at –20 °C until chemical analyses. For analysis, the samples were re-dissolved in 50 µl of hexane containing 400 ng of eicosane (C20) as an internal standard. Two microliters of each extract were injected into a Perkin–Meyer GC–MS functioning at 70 eV with a source temperature of 230 °C and equipped with a ZB-5HT column (30 ml × 0.25 mm ID × 0.252 µm *df*; 5% phenyl

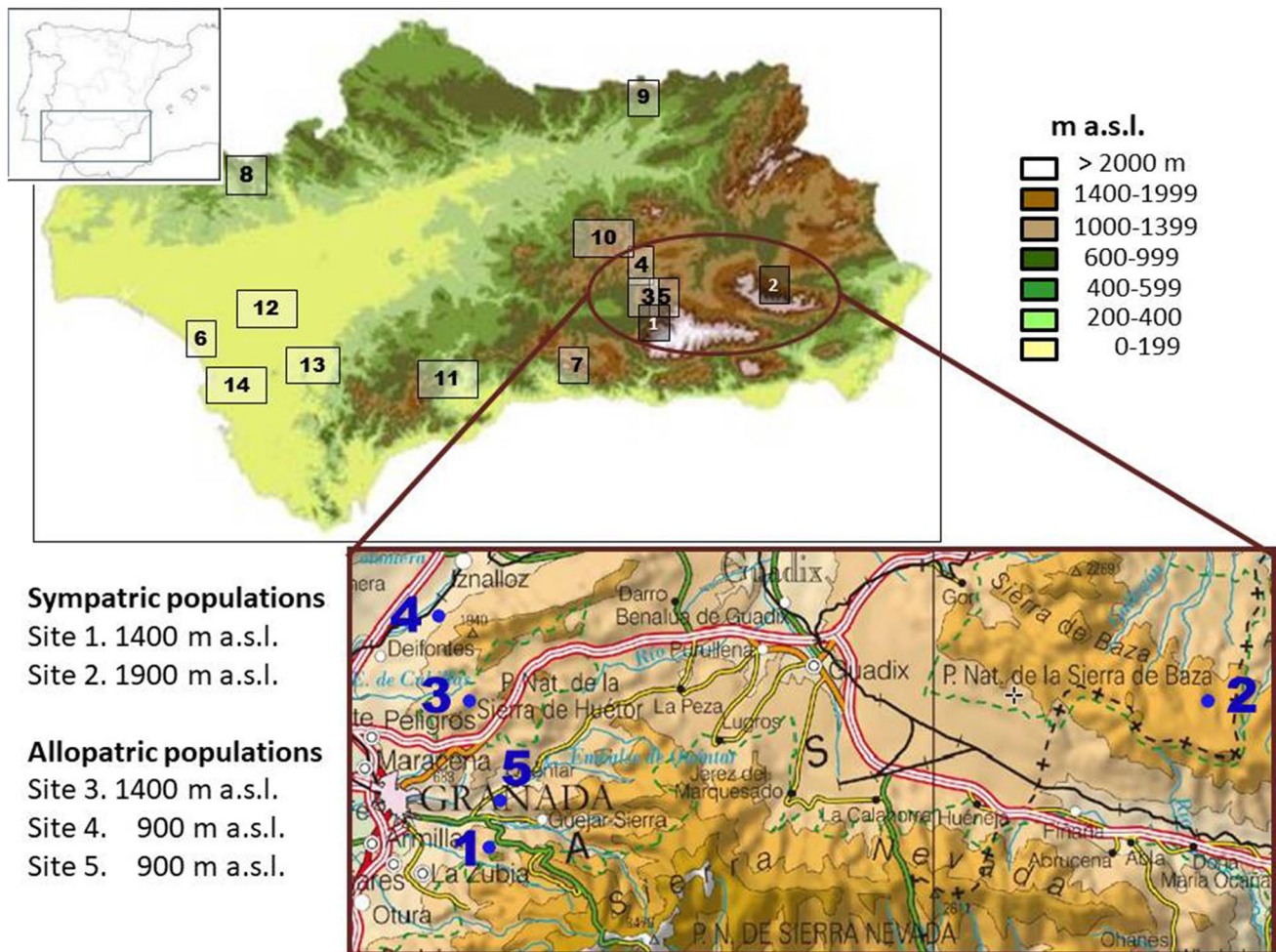


Fig. 1 Above, sampled populations (numbers as in Table 2). In black squares, the two main Andalusian localities for *F. frontalis*, sympatric with *I. subrufa*. Below, a detail of the sympatric populations 1 and 2

and the closest allopatric ones is shown. The altitude of each of those populations is also given

– 95% dimethylpolysiloxane). The temperature program was 2 min at 150 °C, and then increasing at 5 °C/min to 320 °C, and 5 min hold at 320 °C (total 41 min). The gas vector was helium at 2.0 ml min⁻¹. Compounds were identified by their fragmentation pattern, compared to standard alkanes, library data, and Kovats retention indices. All the measurements in the text are always provided as mean ± standard error. For each sample, we calculated the percentage of each substance per individual worker from the total content of its respective category. Since there are considerable weight differences between the species (*F. frontalis* 10.97 mg ± 0.54, *n* = 56 vs *I. subrufa* 4.98 mg ± 0.15, *n* = 66), we also calculated the quantities in ng per mg of fresh weight (ng/mg FW). As esters were found only in *I. subrufa*, *F. lemani*, and *F. cunicularia*, which are of similar size, we only calculated the quantities per ant.

The chemical profile data were calculated using the mean of 3–5 workers for each colony. The data were analyzed using the cluster analysis on Euclidian distances and the Ward method (using Statistica 8.0 program).

Dufour's glands of 3 *I. subrufa* workers were also extracted and their content analyzed as described above, to pinpoint the origin of extracted cuticular compounds and for comparison with the known content of Dufour's glands in other *Formica* species (Bagnères et al. 1991).

Results

Biological data on mixed nests

Mixed nests were very infrequent (one mixed nest/ 42 *F. frontalis* nests in SN and three mixed nests from 18 *F. frontalis* nests in SB populations in 2016).

Table 2 List of sampled populations

	Localities	Abbreviation	Sampled species	Mean altitude (m a.s.l.)
1	Sierra Nevada, El Purche	(SN)	<i>F. frontalis</i> <i>I. subrufa</i> <i>F. lemni</i>	1439
2	Sierra Baza, Cerro Quintana	(SB)	<i>F. frontalis</i> <i>I. subrufa</i>	1807
3	Sierra Alfaguara	(Alfa)	<i>I. subrufa</i>	1424
4	Deifontes	(Deif)	<i>I. subrufa</i>	972
5	Aguas Blancas	(Agu)	<i>I. subrufa</i>	991
6	Doñana	(Don)	<i>I. subrufa</i>	7.5
7	Sierra Tejada	(ST)	<i>I. subrufa</i>	1102
8	Sierra Morena, Dehesa San Francisco	(Deh)	<i>I. subrufa</i> <i>F. lemni</i>	467
9	Sierra Morena, Santa Helena	(SM)	<i>I. subrufa</i>	753
10	Sierra Morena, Huelma	(Hue)	<i>I. subrufa</i>	683.5
11	Sierra de Ronda	(Ron)	<i>I. subrufa</i>	760
12	Aracena	(Ara)	<i>I. subrufa</i>	603
13	Sevilla	(Sev)	<i>F. cunicularia</i>	21
14	Sanlúcar de Barrameda	(San)	<i>F. cunicularia</i>	85

During the observations of the outdoor activity of the mixed nests, we did not observe any signs of aggression between the workers of *F. frontalis* and *I. subrufa*, which were active outside the mixed nests for several hours every day.

The excavated nest in April 2016 was composed of one queen of *F. frontalis*, accompanied by 39 *I. subrufa* and 28 *F. frontalis* workers. The excavated nest in July 2018 was composed of one queen of *F. frontalis*, 145 males and 358 workers of *I. subrufa*. Moreover, once nests were installed in the laboratory, both species shared the given space and none showed any patent agonistic behaviour towards the *F. frontalis* queens (Fig. 2). Moreover, between-species trophallaxis and reciprocal allogrooming were detected.

Chemical profiles of host and parasite

In the sympatric populations (SN and SB), there was a significant difference in the total CHC amounts in body washes of *F. frontalis* and *I. subrufa* in monospecific colonies as well as in the mixed colonies (Kruskal–Wallis test, $H = 24.82$, $p < 0.001$; multiple comparisons Ff vs. MxFf and Is vs. MxIs $p > 0.05$; all other $p < 0.02$; Fig. 3). *Formica frontalis* always had a significantly greater amount of CHC than the *I. subrufa* workers in monospecific colonies (Ff vs Is in Fig. 3; Kruskal–Wallis test $p < 0.001$) and in mixed colonies (Kruskal–Wallis test, $p = 0.02$). On the other hand, the amounts of CHCs in *F. frontalis* workers and their *I. subrufa* hosts in mixed colonies were not significantly different from

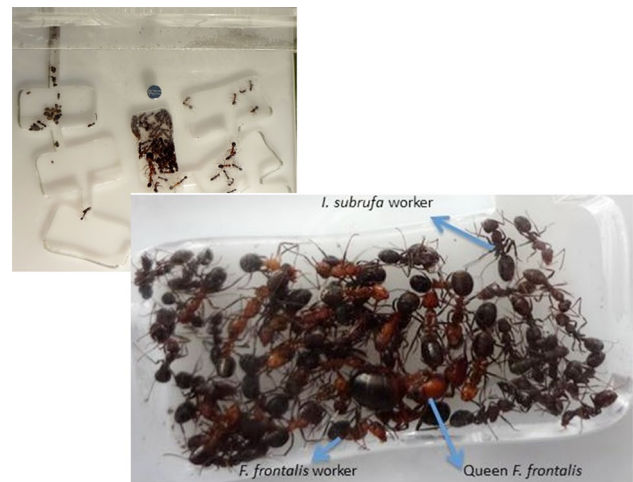


Fig. 2 Mixed nest dug in 2016 in the laboratory, showing workers of both species together with the queen of *F. frontalis* with a general view of the artificial nest (up)

their counterparts in free-living colonies (Kruskal–Wallis test, $p > 0.05$).

As expected and probably due to exchanges between host and parasite, the CHC composition of individuals of both *F. frontalis* and *I. subrufa* from mixed colonies was more diverse (high number of compounds), when compared with free-living nests of both species. The parasite in monospecific nests showed an intermediate complexity, greater than the host *I. subrufa* whether sympatric or allopatric

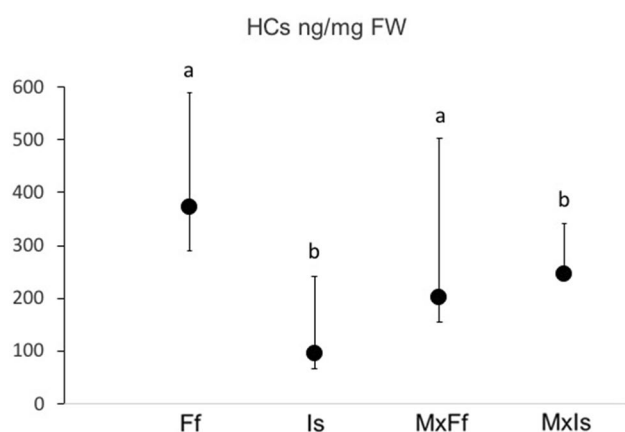


Fig. 3 Total quantities of hydrocarbons (ng/mg fresh weight) in body washes. The black circles are the means and the whiskers upper and lower quartiles. Ff *F. frontalis* and Is *I. subrufa* monospecific colonies; MxFf and MxIs *F. frontalis* and *I. subrufa* in mixed colonies. Different letters indicate significant differences (see text for statistical details)

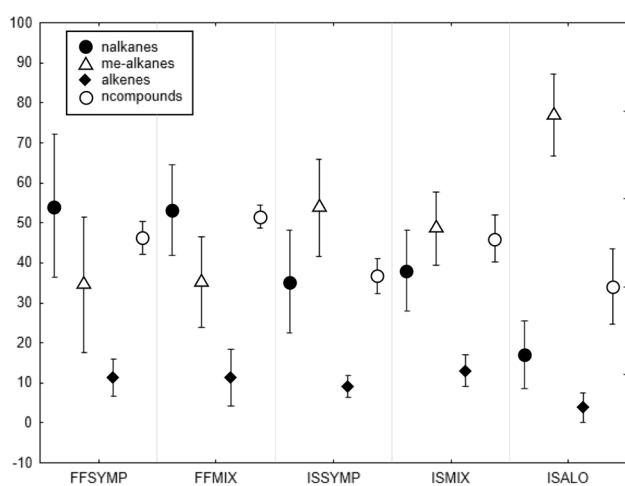


Fig. 4 Mean number of compounds (white circles) \pm SD and mean percentage \pm SD of *n*-alkanes (black squares), Methylalkanes (white triangles), and alkenes (black diamonds) in CHCs of each species and populations: FFSYMP (sympatric *F. frontalis*), FFMIX (*F. frontalis* from mixed nests), ISSYMP (sympatric *I. subrufa*), ISMIX (*I. subrufa* from mixed nests), and ISALO (allopatric *I. subrufa*)

(Kruskal–Wallis test, $H = 87.47$, $p < 0.001$; multiple comparisons $p < 0.001$; Fig. 4).

The carbon chain length in *F. frontalis* extends to nonatriacontane (C39) compared to its host in allopatric populations, *I. subrufa*, for which the heaviest hydrocarbon was pentatriacontane (C35). However, in *I. subrufa* from mixed species colonies, the CHC composition also extended to C39, like its co-inhabiting parasite and sharing most of these added compounds (Suppl. Table 2).

A comparative analysis of the percentage of *n*-alkanes, methyl-branched alkanes, and alkenes of all the species and populations revealed significant differences in all the compounds (Kruskal–Wallis test, $H = 87.87$, $p < 0.002$; $H = 94.7$, $p < 0.001$; $H = 57.8$, $p < 0.0001$, respectively, and Fig. 4). *Ieroformica subrufa* from allopatric populations always showed a very different and distinctive proportion of *n*-alkanes and methyl-branched alkanes, with the latter being the most abundant. *Ieroformica subrufa* from sympatric populations and from mixed nests, together form a similar intermediate group showing methyl-alkanes only slightly more abundant than the alkanes. Finally, *F. frontalis*, whether from free-living or mixed nests, formed another extreme group with a high proportion of alkanes (Fig. 4). The abundance of alkenes was similar for both species in sympatric and mixed nests, with the exception of the allopatric *I. subrufa* that had significantly lower quantities of alkenes (Kruskal–Wallis test, multiple comparisons $p < 0.007$, Fig. 4).

Ieroformica subrufa possessed five species-specific compounds, methyl- and dimethylalkanes of C23, C25, C26, C27, and C30 chain length, but all the six species-specific compounds found in *F. frontalis* were shared by *I. subrufa* in mixed nests, having a wide chain length (C30, C35, C36, C37, and C39) (Table 3).

Another interesting difference between host and parasite species was the presence of abundant esters in body washes of *I. subrufa*, both in allopatric and sympatric populations, from either free-living or mixed nests (Kruskal–Wallis test, $H = 98.51$, $p < 0.0001$; Table 4). In sympatric free-living colonies, the most abundant esters were decyl dodecanoate ($51.2\% \pm 2.31$, $n = 51$) and nonyl 4-methyldodecanoate ($19.73\% \pm 1.74$, $n = 51$). In mixed colonies, workers of *I. subrufa* possessed lesser amounts of esters than their free-living conspecific, but the difference was not significant (Multiple comparisons Is/MxIs $p = 1$; Ff/MxFf $p = 0.27$; see Fig. 5). In allopatric populations, we also detected a remarkable between-sites' quantitative variation of esters (e.g. SB: 528.2 ± 134.8 ng/worker, $n = 26$; SN: 815.4 ± 297.8 ng/w, $n = 25$; Doñana: 159.6 ± 89.3 ng/w, $n = 6$). Nevertheless, there were no significant differences in ester concentration among localities (Kruskal–Wallis test, $H = 1.22$, $p = 0.54$). In *F. frontalis* from either free-living or mixed nests, only traces of esters occurred (Fig. 5). Esters were also found in the body washes of the *Serviformica* species analyzed (*F. lemani* and *F. cunicularia*), but in different relative quantities. Due to the small number of samples and the large quantitative variation, we did not further analyze these data (Table 4).

Analysis of the Dufour's gland secretion of *I. subrufa* revealed the presence of wax-type esters similar to body washes, straight chain as well as branched, of medium chain length [m.w. ranging from 312 (nonyl undecanoate) to 368

Table 3 Species-specific compounds found in the species sampled involved in host–parasite interactions

Species/populations	Species-specific compounds
<i>I. subrufa</i> (all populations and mixed nests)	9,11-Dimethyltricosane 5,11- + 5,13-Dimethylpentacosane 8-Methylhexacosane 5,9-Dimethylheptacosane Triacontene
<i>F. frontalis</i> SB and SN + <i>I. subrufa</i> (mixed nests)	10,12- + 10,14- + 12,14- + 12,16-Dimethyltriacontane 11- + 13-Methylnonatriacontane
<i>F. frontalis</i> SB + <i>I. subrufa</i> (mixed nests)	7-Methylpentatriacontane 10- + 12-Methylhexatriacontane 3,x-Dimethylheptatriacontane <i>n</i> -Heptatriacontane

Table 4 Mean composition of esters and SE

Esters	<i>I. subrufa</i> cuticle		<i>I. subrufa</i> Dufour glands		<i>F. lemani</i>		<i>F. cunicularia</i>	
	Mean (%)	SE	Mean (%)	SE	Mean (%)	SE	Mean (%)	SE
C9/C11 Nonyl undecanoate	0.58	0.07	0.17	0	0	0	0	0
C8/C12 + C10/C10 Octyl dodecanoate + decyl decanoate	0.82	0.12	0.20	0	10.30	2.66	0	0
C10/4-meC10 Decyl 4-methyl decanoate	2.46	0.51	0.90	8.09	7.75	3.25	12.79	8.09
C9/C12 Nonyl dodecanoate	7.87	0.87	15.26	5.60	4.37	1.25	18.23	5.60
C9/4meC12 Nonyl 4-methyl dodecanoate	4.88	0.46	8.33	1.61	3.71	1.30	2.55	1.61
C9/C13 Nonyl tridecanoate	0.76	0.10	1.45	6.47	1.48	0.83	12.80	6.47
C10/C12 Decyl dodecanoate	45.61	2.47	37.24	1.99	30.32	8.34	16.78	1.99
C8/4,xdimeC12 Octyl 4,x dimethyl dodecanoate	8.60	1.72	9.91	1.53	9.42	4.66	11.32	1.53
C10/4meC12 Nonyl 4-methyl dodecanoate	18.94	1.46	17.04	1.23	10.26	0.77	18.19	1.23
C10/C13 Decyl tridecanoate	3.62	0.36	3.29	0.33	0.77	12.74	1.35	0.33
C11/C12 Undecyl dodecanoate	1.95	0.19	3.52	0.22	19.99	0.68	3.53	0.22
C10/4-meC13 Decyl 4-methyl tridecanoate	1.90	0.19	0.82	0.36	0.68	0.94	1.79	0.36
C11/4meC12 Undecyl 4-methyl dodecanoate	1.61	0.15	1.67	0.42	0.94	0	0.67	0.42
C11/C13 Undecyl tridecanoate	0.41	0.16	0.20	0	0	0	0	0
Total	100	<i>n</i> =51	100	<i>n</i> =3	100	<i>n</i> =5	100	<i>n</i> =2

(dodecyl dodecanoate)], but in different relative abundance. Esters comprised over 99.5% of the gland secretion with a mean total content of 606.5 ± 171.7 ng/gland, ($n=3$). There were only negligible amounts ($\sim 0.5\%$) of hydrocarbons identical to those found in the body washes.

A global cluster analysis of cases based on CHC composition of the four-studied species and 14 populations showed striking findings (Fig. 6 and Suppl. Table 2). Foremost, the two *I. subrufa* populations sympatric with the temporary parasite, although separated between them by a large valley (Fig. 1), clustered together with their temporary parasite in a distinct clade that was clearly distant from the other ten allopatric *I. subrufa* populations, including the closest populations to site 1 (sites 3, 4, 5). Interestingly, both the sympatric host and the

parasite were also more similar to the allospecific *Serviformica* sp. than to the allopatric *I. subrufa* populations (Fig. 6).

A comparison of the CHC profiles of *I. subrufa* workers collected from mixed colonies and their sympatric, but free-living conspecific colonies showed compositional changes in the former which rendered them even more similar to the parasite *F. frontalis* chemical profile (Fig. 7).

Discussion

All the signs found in our study pointed to a new case of temporary parasitism: (1) the two species and the brood shared the same chamber in the artificial nest dug in 2016 with only one *F. frontalis* queen without any signs of aggression

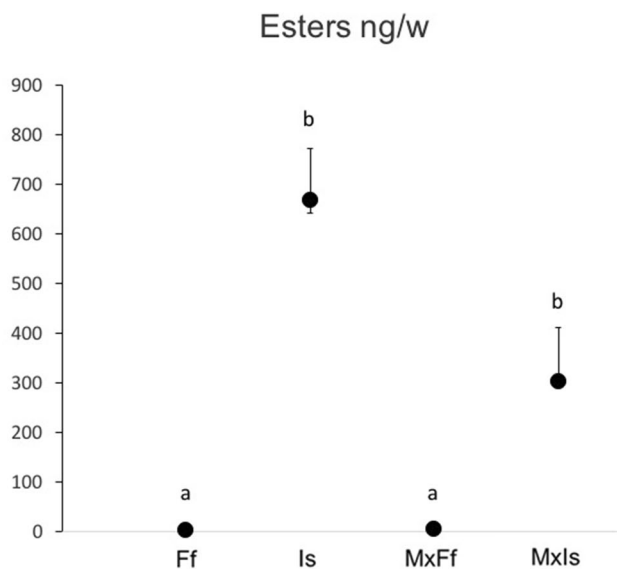
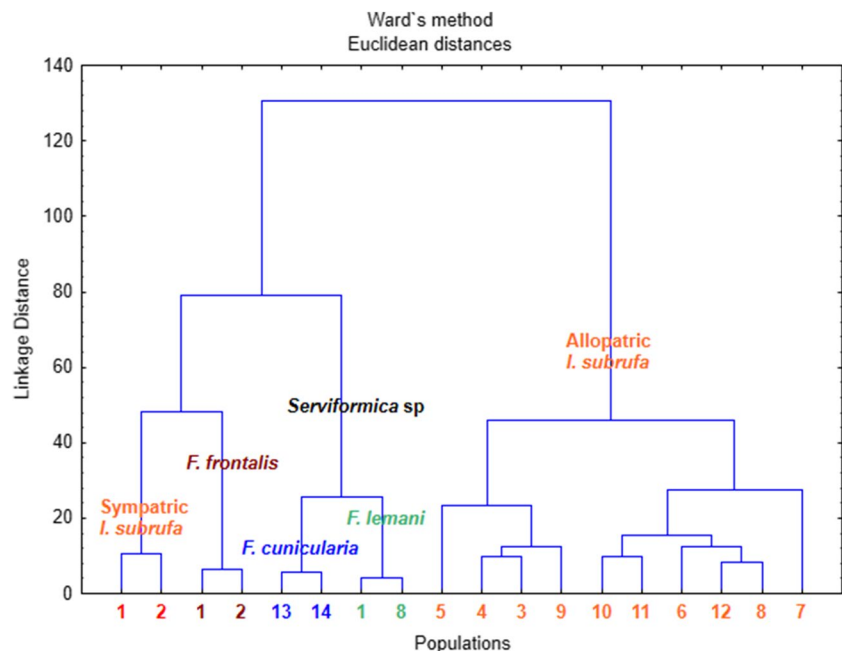


Fig. 5 Total quantity of esters in body washes. Black circles are the means and the whiskers are the upper and lower quartiles. Ff: *F. frontalis* and Is: *I. subrufa* monospecific colonies; MxFf and MxIs: *F. frontalis* and *I. subrufa* in mixed colonies. Different letters indicate significant differences (see text for statistical details)

between individuals belonging to both species, (2) the second nests found with only one queen of *F. frontalis* did not show either the queen of *I. subrufa* or the brood, and (3) the cuticular profile of *I. subrufa* and *F. frontalis* were tuned in the sympatric populations. Thus, in this study, a new example of temporary host–parasite system is reported in which the *F. frontalis* queen invades the *I. subrufa* nest and the *I. subrufa* queen disappears.

Fig. 6 Dendrogram of *I. subrufa*, *F. frontalis* (monospecific nests), and *F. lemani* and *cunicularia* hydrocarbons from the different collection sites referred to with a number as in Fig. 1 (populations 1–14)

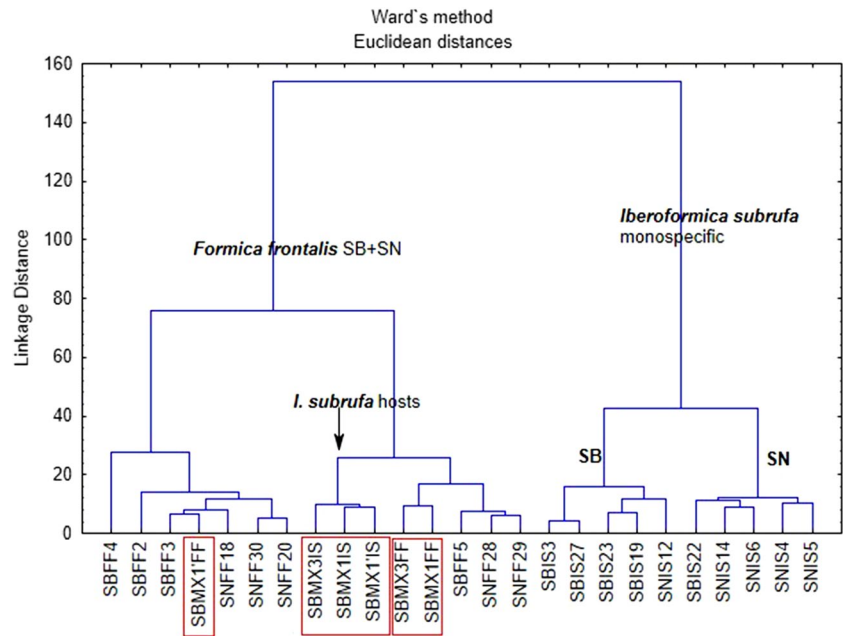


In the field, individuals of both species belonging to the same nests also exhibited peaceful outdoor activity. However, most of the *F. frontalis* nests observed in both populations were apparently monospecific colonies, judging from the fact that only the *F. frontalis* workers were observed to be active outside the nest during many hours of observation. This was also the case in the other distribution localities of this species in the Iberian Peninsula where *I. subrufa* is not present (Tinaut 1990; Tinaut and Martínez Ibáñez 1998; Tinaut et al. 2015). Taking into account all these biological data, we can determine that *F. frontalis* is a temporary and probably facultative parasite of *I. subrufa* (Wilson 1971; Savolainen and Deslippe 1996) because of the very low ratio of mixed nests related with the total monospecific *F. frontalis* nests in each population and the fact that both species distributions only overlap in a narrow stripe.

The above-described host–parasite system does not follow the strict interpretation of Emery’s rule (the host *I. subrufa* is not the closest species to the parasite *F. frontalis*; the closest species were the species belonging to the *Serviformica* group of species (Muñoz-López et al. 2012; Romiguier et al. 2018), but they still comply with the relaxed interpretation of the rule: host and parasite belong to different genera, but are still classified to the same group of closely related species (within the same tribe) (Buschinger 2009; Huang and Dornhaus 2008). Nevertheless, Huang and Dornhaus (2008) concluded that most (60%) of the temporary parasites should follow the strict application of Emery’s rule; therefore, our species must belong to the remaining 40%.

In this new host–parasite interaction, some cuticular peculiarities arose. We found the parasite showed the highest diversity of cuticular compounds, then not only the host

Fig. 7 Dendrogram of all the colonies from SB and SN. In red circles, the profiles of individuals of *F. frontalis* and *I. subrufa* involved in mixed nests are shown



increased its CHC diversity driven by the presence of the parasite, as demonstrated by Martin et al. (2011). Thus, our study demonstrates that the presence of both hosts and parasites could produce a diversification of cuticular hydrocarbons in sympatric populations.

Martin et al. (2008, 2011) signaled two alternative paths of nest-mate recognition in ants from the genus *Formica*: the elevated production of Z-9-alkenes and alternatively the production of various dimethylalkanes. Our results point to the first possibility in *F. frontalis*, and the second for *I. subrufa*. Amongst the abundant alkanes in *F. frontalis* there might be the species-specific compounds that could be responsible for nest-mate recognition in this species. On the other hand, the *I. subrufa*, species-specific compounds were methyl- or dimethylalkanes, but with a wide chain length (C23, C25, C26, and C27) pointing to a larger set of compounds that could be good candidates to be involved in nest-mate recognition in this species, following Martin et al. (2011).

Another particular characteristic of *I. subrufa* exocrinology, shared by *F. lemani* and *F. cunicularia*, is the presence of esters in a high quantity in their body washes. In *I. subrufa*, Dufour's gland secretion appeared to be the source of the cuticular ester content, because the gland mainly contained these compounds (99% of the gland content). On the other hand, *F. frontalis* completely lacked esters. According to Bagnères et al. (1991) and Pherobase (2017), the *Formica* species are divided into two groups: those that produce almost exclusively hydrocarbons in the Dufour's gland (*F. fusca*, *F. lemani*, and *F. selysi*) and those that produce additionally large amounts of oxygenated compounds such as acetates and other esters (*F. rufibarbis*). *F. frontalis* appears to belong to the first group, while, according to our results,

F. lemani and *F. cunicularia* belong to the second and *I. subrufa* should be added to the latter group of species.

The role of esters and whether they participate in host–parasite interactions remains to be investigated. The fact that they are only present in traces in the *F. frontalis* from mixed colonies, and abundantly in the host *I. subrufa* and the potential hosts from the *Serviformica* group *F. lemani* and *F. cunicularia*, seems to preclude their role in host–parasite interactions, but they may have other functions in the biology of the host. Some of the non-species-specific compounds and the low quantity of esters appearing in the *F. frontalis* cuticle from mixed nests might be exchanged with the host during trophallaxis and host–parasite cleaning interactions. Esters and acetates are also found in Dufour's glands of some *Cataglyphis* (Gökçen et al. 2002), acetates were reported as propaganda chemicals produced by the slave-maker *F. subintegra* (Regnier and Wilson 1971) and some esters are also found in the cuticle of the harvester ant *Pogonomyrmex barbatus*, probably produced in the Dufour's glands. Nevertheless, the function of this group of compounds needs to be investigated.

With respect to the cuticular profile study in both species of the pair, we found that *I. subrufa* showed a very different CHC profile only in both separated sympatric populations and different to the allopatric ones. This striking result is difficult to explain, because both populations are geographically distant more than 60 km but, more importantly, separated by a valley in which *I. subrufa* is not distributed (Fig. 2). Moreover, some populations close to the sympatric SN site 1 (sites 3, 4, and 5) showed very different profiles clustering with all the other *I. subrufa* allopatric populations.

Three possible hypotheses could be taken into account to explain this result:

1. *The different species hypothesis.* A unique species of *Iberoformica* might be distributed in Sierra Nevada (SN site 1) and Sierra de Baza (SB site 2), but different species to that found in the allopatric populations. Nevertheless, it is difficult to find some cause of isolation of population of SN site 1, from those other close populations found in the surrounding sites 3, 4, and 5, and at the same time connection with the distant sympatric population SB in site 2. Nevertheless, a study about more populations surrounding site 2 and on genetic diversity and relatedness of different populations should clarify this point.
2. *The environmental hypothesis.* Both sympatric populations might share some environmental trait conditioning the cuticular profile such as the high altitude (1400 m a.s.l. for site 1 and 1900 m a.s.l. in site 2). Nevertheless, we sampled another allopatric population (site 3) with a similar altitude (1424 m a.s.l.) whose cuticular profile grouped together with the other allopatric populations (Fig. 7). On the other hand, the possibility that environmental factors significantly affect CHCs is not totally applicable, and thus, Martin et al. (2008) in *Formica* species did not find a clear association between the cuticular profile and the habitat-type; meanwhile, other authors pointed to slight changes in cuticular profiles due to environmental differences (diet), but most of the CHC variation was attributable to heritable traits (Van Zweden et al. 2009); as a consequence, CHCs have been even proposed as a taxonomic tool for most insects (Kather and Martin 2012). An exception is the invasive ant species, in which the diet changed the CHCs (Liang and Silverman 2000; Buczkowski et al. 2005). This trait could be responsible for promoting unicoloniality, an uncommon trait attributable only to some invasive ants (Buczkowski et al. 2005).
3. *The host-tolerance hypothesis.* Host tolerance has been reported as a possible outcome of selective pressures imposed by enemies pertaining to very different taxa (pathogens, parasites, and competitors for a mate or a territory) when resistance is too costly for the host-victim (Svensson and Råberg 2010). Tolerance results in the parasite's acceptance by the host, including some adjustments in the host's natural history to avoid parasite aggression (Kilner and Langmore 2011). In our study system, the presence of the parasite might induce changes in the host's cuticular profile to become more congruent to those of the parasite in mixed or sympatric free-living conspecific nests thus avoiding parasite aggression. This hypothesis should be tested to find the way that permits tolerant traits (i.e. changing CHC pro-

files) to be fixed in sympatric populations, even though the host's reproductive queen is killed. From inferences in the published references in other well-studied social host–parasite systems, some possibilities arise of recuperating some host fitness even when the host queen dies, such as worker reproduction (Cini et al. 2014), worker revolts (Czechowski and Godzinska 2015), or kin selection in structured populations (Pamminger et al. 2014). The latter is a trait expected to be found in *I. subrufa* due to their brachypterous founder females (Tinaut and Ruano 1992).

Exhaustive comparative studies on more populations close to the sympatric ones, including population genetics and nest relatedness, together with testing the possibility of worker reproduction should be carried out in the future to clarify the causes of this striking result of host–parasite cuticular congruence only found in sympatric populations.

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