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Species- and nestmate brood discrimination in the sibling wood ant species *Formica paralugubris* and *Formica lugubris*

Arnaud Maeder[1], Anne Freitag[2] & Daniel Cherix[1,2,*]

[1] *Department of Ecology and Evolution, University of Lausanne, Biology Building, CH-1015 Lausanne, Switzerland (e-mails: arnaud.maeder@unil.ch, daniel.cherix@unil.ch)*
[2] *Museum of Zoology, Palais de Rumine, Place de la Riponne 6, CH-1014 Lausanne, Switzerland (e-mails: anne.freitag@serac.vd.ch, *corresponding author: daniel.cherix@serac.vd.ch)*

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Formica lugubris and *F. paralugubris* are sympatric sibling species of wood ants, both of which are widely distributed in Switzerland. Until 1996 they were considered the same species, *F. lugubris*. To investigate whether the two species can be distinguished based on discrimination cues used by the workers we used the pupa-carrying test first introduced by Rainer Rosengren. In this test workers of discriminator colonies are faced with two kinds of pupae and their preferences for one of the types are recorded based on differential retrieval. Interspecific comparisons showed that ants preferred conspecific worker pupae to those of the sibling species regardless whether the pupae were con-colonial or hetero-colonial. Hence, this test can be used as a taxonomic tool to identify wood ants hardly distinguishable by morphological characters. In intraspecific comparisons the highly polygynous (many queens per colony) *F. paralugubris*, the polygynous form of *F. lugubris* and the monogynous (single queen per nest) to weakly polygynous form of *F. lugubris* expressed different trends in their preference behaviour (with nestmate recognition in 14%, 20% and 31% of replicates, respectively). Only *F. paralugubris* presented no significant nestmate recognition.

Introduction

Social insects have complex behavioural repertoires including discrimination between conspecific and heterospecific brood (Jaisson 1975), and recognition of nestmate brood (Lenoir 1984, Hare 1996). Species recognition allows workers to discriminate between conspecific (worker or brood) and heterospecific individuals (Carlin 1988). This ability can protect the colony from interspecific parasitism (Buschinger 1986, Lorenzi & Filippone 2000, Lenoir *et al.* 2001) and prevents maladaptive hybridization (Carlin 1988). Nestmate recognition in ants implies an ability of workers to distinguish conspecific colony members from conspecific non colony members (Vander Meer & Morel 1998, Lenoir *et al.* 1999).

Nestmate recognition is vital to ant social life because it preserves colony cohesion through territorial behaviour against potential intruders (Hölldobler & Wilson 1990, Vander Meer

& Morel 1998) and ensures that altruism is directed toward relatives (Wilson 1971, Crozier & Pamilo 1996). Compounds that may serve as recognition cues can have either endogenous and/or exogenous sources (Smith & Breed 1995, Vander Meer & Morel 1998). The former category includes compounds synthesized by the individuals and either spread from one individual to another (e.g. queen or brood pheromones; Vander Meer & Alonso 1998) or retained by the individual (e.g. cuticular hydrocarbons; Lahav *et al.* 1999, Howard & Blomquist 2005). The latter category represents compounds acquired from the environment (e.g. vegetation used as nest material and food resources; Le Moli & Mori 1989, 1990, Liang & Silverman 2000). These compounds form a "Gestalt" colony odour (Crozier & Dix 1979, Lenoir *et al.* 1999, *see* Vander Meer & Morel 1998 for alternative models). An individual learns the colony-specific odour cues soon after emergence and updates them throughout adult life (Jaisson 1975, Lenoir 1984, Smith & Breed 1995, Lenoir *et al.* 1999). Nestmate recognition in ants tends to differ depending on colony kin structure. In monogynous (one laying queen per nest) and monodomous (single nest) colonies, nestmate recognition is usually well developed, whereas in polygynous (multiple laying queens per nest) and polydomous (multi-nest) colonies nestmate recognition is less well developed (Fletcher & Blum 1983, Keller & Passera 1989, Vander Meer *et al.* 1990, Provost *et al.* 1994, Sundström 1997, Stuart & Herbers 2000). In the wood ants *F. pratensis* and *F. polyctena* nestmate recognition is based on genetic similarity and is, to a much lesser extent, environmentally influenced (Beye *et al.* 1997, 1998). Environmental cues may be less important than genetic ones in polydomous species as they may interfere with intra-colonial recognition of individuals living in distant nests (Stuart & Herbers 2000). By contrast, environmental factors may play a greater role in nestmate recognition in monodomous species. Finally, demographic variables (e.g. queen number, colony size) can influence nestmate recognition in different ways (Keller & Passera 1989, Stuart 1991, Starks *et al.* 1998, Vander Meer & Alonso 2002, Caldera & Holway 2004).

Rosengren and Cherix (1981) took advantage of both the brood-tending behaviour and the discrimination ability of ants to develop a new taxonomical tool. The pupa-carrying test is based on the inherent preferences shown by ants faced with a choice between worker pupae of their own species and pupae of an alien species. They used this test to resolve taxonomical problems within the *Formica rufa* group (Vepsäläinen & Pisarski 1981). The taxonomy of this particular group has always been controversial (Kutter 1965, 1967, Collingwood 1987, Gösswald 1989), which is partly due to the morphological similarity of the different species and their ability to form sometimes mixed (Seifert 1991, Czechowski 1996) and even hybrid colonies (Seifert & Goropashnaya 2004). In their study, Rosengren and Cherix (1981) found that although workers of Swiss *F. lugubris* do not discriminate between *F. lugubris* pupae from Switzerland and Italy, they discriminate strongly against *F. lugubris* pupae from Finland. Conversely, *F. lugubris* workers from Finland refuse to carry *F. lugubris* pupae from Switzerland or Italy. Fennoscandian and central European populations of *F. lugubris* thus behave as if they were different species. Later, an allozyme study revealed the existence of two morphologically similar sympatric genetic types of *F. lugubris* (Pamilo *et al.* 1992). In a new set of pupa-carrying tests conducted at a local scale Rosengren *et al.* (1994) confirmed the existence of the two distinct types of *F. lugubris* also with respect to behavioural discrimination. The *F. lugubris* from a supercolony in the Jura Mountains (Gris & Cherix 1977) were then described by Seifert (1996a) as a new wood ant species: *Formica paralugubris*, considered as a sibling species of *F. lugubris*. In Switzerland, *F. lugubris* and *F. paralugubris* are widely distributed, occur sympatrically and show different social forms (Cherix *et al.* 2004). *F. paralugubris* colonies are highly polygynous and polydomous (Cherix 1981, Chapuisat *et al.* 1997), whereas colonies of *F. lugubris* are either monogynous (or weakly polygynous) and monodomous, or polygynous and polydomous (Bernasconi *et al.* 2005).

The aims of this study are twofold. First we test whether workers of *F. lugubris* and *F. paralugubris* can discriminate between pupae of

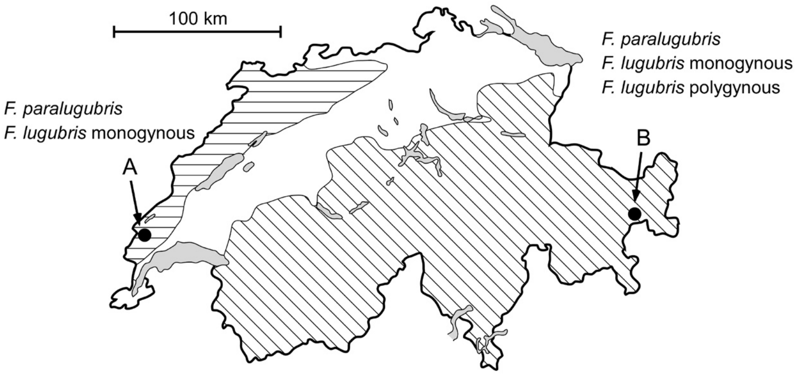

Fig. 1. Study areas (**A**: "Parc jurassien vaudois", **B**: Swiss National Park) and biogeographical regions (horizontal lines: Jura Mountains, diagonal lines: Alps).

their own and the sibling species independently of nest origin. We used material from several populations located in two Swiss biogeographical regions, where both species occur in sympatry. Second, we studied intraspecific discrimination in both species using three kinds of discrimination parameters. First we tested whether the two species differ in their nestmate discrimination patterns depending on their differences in social organisation, such that the polygynous *F. paralugubris*, as well as the polygynous form of *F. lugubris* show less nestmate discrimination than the monogynous *F. lugubris*. Second, we compared the responses towards pupae from different geographic regions, given that environmental cues change with distance. Thus, we expect workers to prefer pupae originating from the same region as they originated from. Finally, we investigated for *F. lugubris* whether workers with a different social background also differ in their degree of discrimination. Monogyny and monodomy is supposed to induce colony closure by clear colony odour signatures (Provost & Cerdan 1990, Bourke & Franks 1995, Dahbi *et al*. 1996). In addition, pupae of monogynous and monodomous societies may be less attractive to hetero-colonial workers than pupae from polygynous and polydomous societies.

Material and methods

Study species and study areas

Formica paralugubris and *F. lugubris* are abundant and widespread in the Jura Mountains and the Alps (D. Cherix unpubl. data). Both species occur syntopically in several places in the Swiss and French Jura Mountains as well as in the Swiss Alps (Cherix *et al*. 2004). *F. paralugubris* forms huge polydomous supercolonies, i.e. networks of connected polygynous nests (Cherix 1981, Chapuisat *et al*. 1997), whereas colonies of *F. lugubris* are either monogynous (or weakly polygynous) and monodomous, or polygynous and polydomous (this latter social form found at present only in the Alps; Bernasconi *et al*. 2005). Here the two social forms will be referred as monogynous *F. lugubris* and polygynous *F. lugubris*.

We collected in summer 2003 ants at two study sites 300 km apart and located in two distinct biogeographical regions (Gonseth *et al*. 2001), the Swiss Jura Mountains and the Swiss Alps (Fig. 1). In each region, we collected workers, worker pupae and nest material from two to three different sites (two to six nests per site) for *F. lugubris* and two different sites for *F. paralugubris* (three to seven nests per site) (Table 1). The collections were made at a time when worker production was at its peak (July and August). Moreover, at this time of the year nestmate recognition between workers would normally occur and consequently we assume chemical cues are used as efficient discriminators. The short duration of this study precludes seasonal variation in cuticular hydrocarbon profiles, a point that has been demonstrated in other *Formica* ant species (Nielsen *et al*. 1999, Liu *et al*. 2001).

The ants were kept in the laboratory for one week at the most to preserve the natural environmental odour profiles (mean time ± S.D. between collection and experiment was 2.9 ± 1.5 days). Workers were fed *ad libitum* with water and sugar solution (1/3 honey diluted with 2/3 water). We identified the tested species based on morphological characters (Seifert 1996a, Seifert 1996b) and by analysing cuticular hydrocarbons (A. Maeder, A.-G. Bagnères & D. Cherix unpubl. data) as the latter are used in chemotaxonomy and are involved in nestmate recognition (Bonavita-Cougourdan *et al*. 1987, Vander Meer & Lofgren 1990, Lahav *et al*. 1999, Wagner *et al*. 2000). We also used a third species, *F. rufa*, collected in one site in the Jura Mountains, as a control species. According to a recent phylogenetic reconstruction by Goropashnaya *et al*. (2004), the *Formica rufa* group comprises at least two distinct clades. The first one includes *F. polyctena* and *F. rufa* and the second one includes *F. lugubris*, *F. aquilonia* and *F. paralugubris*.

Experimental procedures

Our experiments comprised two sets of tests, the first one entailed interspecific discrimination, and the second one intraspecific discrimination. To analyse the ability of workers to discriminate between conspecific and heterospecific pupae across clades, we first tested if *F. lugubris* and *F. paralugubris* workers preferred conspecific pupae to pupae of the control species *F. rufa* (Table 2: combinations 1–2), and vice versa (Table 2: combinations 3–4). Then, we tested whether *F. lugubris* and *F. paralugubris* workers preferred conspecific pupae to those of the sibling species (Table 2: combinations 5–6). In both experiments we used equally both con-colonial and hetero-colonial pupae. In all replicates, conspecific pupae were from the same biogeographical region as workers. In the second set of experiments we analysed intraspecific brood discrimination. Workers were offered only conspecific pupae which were either (1) nestmate or non-nestmate (but always from the same biogeographical region as the workers) (Table 2: combinations 7–9), (2) from the same or the other biogeographical region (Table 2: combinations 10–11), or (3) the same or different social structure (only for the socially polymorphic *F. lugubris*; Table 2: combinations 12–13). In the last two experiments, we used about equally nestmate and non-nestmate pupae. The intraspecific discrimination ability of *F. lugubris* was analysed for both social types separately.

For the pupa-carrying test, we used the "sequence-method" experiment described in Rosengren and Cherix (1981) and Rosengren *et al*. (1994). In this design, the workers are offered a choice between two kinds of living worker pupae. We placed the workers to be tested in an artificial nest made of a plastic Petri dish (14 cm in diameter) filled with nest material from their own mound. An arena with a central hole permitting the access of the ants was placed on the artificial nest (Fig. 2A). The arena was made of a Petri dish (22 cm in diameter) with fluon-coated walls (5 cm high) to prevent ants from escaping and filled with casting plaster to a depth of 1 cm. The floor of the arena was divided into 20 numbered sectors (ten even and ten odd numbers). The

Table 1. Origin of worker ants and worker pupae used in the experiments (pupa-carrying tests). m: monogynous; p: polygynous.

Biogeographic region	Ant material	Study sites	Location	Alt. (m)	Number of nests
Alps	*F. lugubris* (m)	Zernez, Champlönch	46°40´41N, 10°10´38E	1970	4
		Zernez, Champlönch	46°40´34N, 10°10´47E	1970	2
	F. lugubris (p)	Zernez, God dal Bass	46°39´06N, 10°15´25E	1950	5
		Scuol, Val Minger	46°43´17N, 10°17´48E	1770	3
	F. paralugubris	Zernez, God la Drossa	46°39´45N, 10°12´26E	1900	3
		Zernez, Grimmels	46°40´09N, 10°10´08E	2080	6
Jura Mts.	*F. lugubris* (m)	Le Chenit, Petite Rolat	46°33´55N, 6°14´33E	1350	6
		Le Chenit, Trois Chalets	46°32´10N, 6°12´43E	1330	6
	F. paralugubris	Le Chenit, Bois des Caboules	46°32´45N, 6°11´27E	1360	4
		Le Chenit, Grande Rolat	46°33´32N, 6°14´06E	1350	7
	F. rufa (control species)	Ballens, les Bougeries	46°32´39N, 6°23´49E	680	5

Table 2. Results from pupa-carrying tests, where workers made a choice between two kinds of pupae (donor 1 and donor 2). The observed frequencies of replicates with a significant preference in either direction were tested against the expected frequencies under a binomial distribution with a probability parameter of 0.5. FPL: *Formica paralugubris*; FL: *Formica lugubris*; FR: *Formica rufa*; m: monogynous; p: polygynous. Alien species = non sibling species. *N* = number of experiments. In brackets: number of replicates involving nestmate pupae as donor 1.

Comparison	Discrimination parameters	Combination	Recipient	Donor 1	Donor 2	*N*	Significant preference for donor 1	No preference	*p* (2-tailed)
Interspecific	Alien species	1	FPL	conspecific (FPL)	alien species (FR)	12 (6)	12 (6)	0 (0)	< 0.001
		2	FL	conspecific (FL)	alien species (FR)	19 (10)	15 (8)	4 (2)	0.019
		3	FR	conspecific (FR)	alien species (FPL)	9 (3)	9 (3)	0 (0)	0.004
		4	FR	conspecific (FR)	alien species (FL)	12 (2)	11 (2)	1 (0)	0.006
	Sibling species[1]	5	FPL	conspecific (FPL)	sibling species (FL)	47 (26)	44 (25)	3 (1)	< 0.001
		6	FL	conspecific (FL)	sibling species (FPL)	44 (22)	37 (18)	7 (4)	< 0.001
Intraspecific	Nestmate[1]	7	FPL	FPL nestmate	FPL non-nestmate	22	3	19	0.001
		8	FL m	FL m nestmate	FL m non-nestmate	29	9	20	0.061
		9	FL p	FL p nestmate	FL p non-nestmate	10	2	8	0.109
	Biogeographical region	10	FPL	FPL same region	FPL other region	12 (6)	2 (2)	10 (4)	0.039
		11	FL m	FL m same region	FL m other region	14 (9)	8 (6)	6 (3)	0.791
	Social structure	12	FL m	FL monogynous	FL polygynous	14 (7)	6 (6)	8 (1)	0.791
		13	FL p	FL polygynous	FL monogynous	14 (7)	5 (3)	9 (4)	0.424

[1] Pupae are of the same biogeographical region as workers.

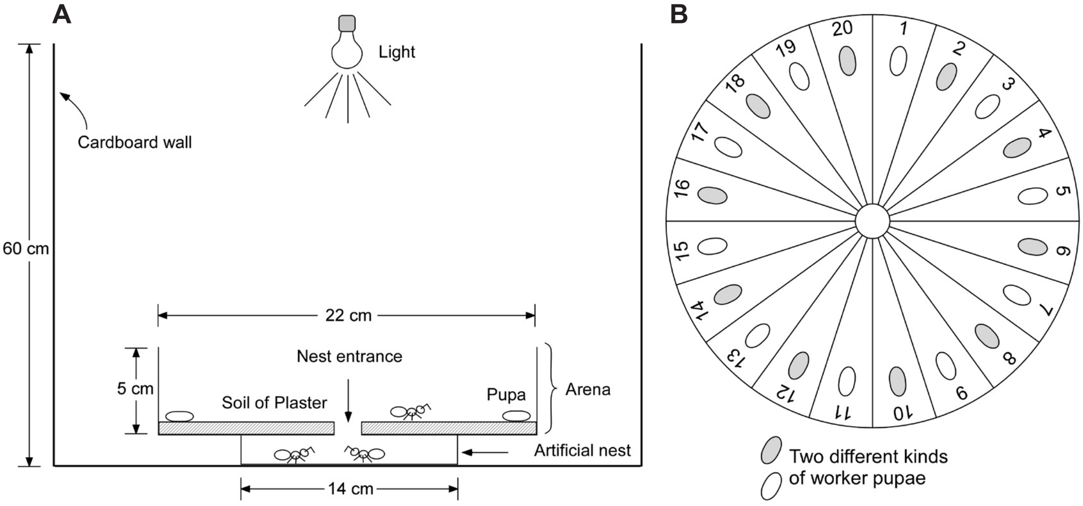

Fig. 2. — **A**: Experimental device used for the pupa-carrying test. — **B**: Top view of the arena with the 20 sectors and the ten/ten worker pupae (donor 1/donor 2) (adapted from Rosengren & Cherix 1981).

whole device was surrounded by a cardboard wall (50 cm wide and 60 cm high) and lit centrally by a 60 watt incandescent reflector bulb to prevent the ants from using visual cues for orientation.

For each experiment, we placed about 15 workers (recipient workers) in the artificial nest including a minimum of ten pupae carriers (selected according to their behaviour). To offer the workers a choice we placed 20 worker pupae of two different origins on the arena (= donors 1 and 2); ten pupae of one type were placed in odd-numbered sectors and ten pupae of the other type in the even-numbered ones (Fig. 2B). Pupae of donor 1 always shared common features with the recipient workers (same species, nestmate, same biogeographical region or same social structure) whereas pupae of donor 2 differed in this respect. If workers discriminate between the two types of pupae, they should show a preference for pupae from donor 1. Each worker was used only once a day and each pupa was used only once during the whole study. An experiment began as soon as the nest entrance was opened and ended when all pupae of one kind had been collected. If no pupae were collected within 20 minutes or fewer than five pupae collected after 45 minutes, we discarded the replicate. Between each replicate, the arena plaster was cleaned with water and the artificial nest and the experimental tools with alcohol to prevent contamination with chemical cues.

Statistical analysis

First, we analysed our results using the "worker choice test" developed by Rosengren *et al.* (1994). We constructed a matrix of the sequence in which the ants retrieved the pupae (Fig. 3). If the workers do not discriminate between the two types of pupae, most observations should fall along the diagonal of the matrix. If the ants do discriminate, most observations should deviate from the diagonal into an area of statistical significance (Fig. 3). The boundaries for the statistically significant area ($p < 0.05$) were found by simulating 100 000 experiments in which the ants did not discriminate between the two kinds of pupae (M. Elias, R. Rosengren, L. Sundström & P. Pamilo unpubl. data). Second, we used the binomial test to compare the number of experiments where workers significantly preferred one type of pupae and rejected the other one with those where workers showed no preference (i.e. they retrieved both kinds of pupae). A significant difference ($p < 0.05$) indicates that workers consistently show a clear preference in either direction. Differences between combinations were tested by Fisher's exact test and χ^2-test (with Yates correction in case of too small expected frequencies). Finally, to compare the interspecific discrimination ability of *F. lugubris* and *F. paralugubris*, we calculated a "brood preference index" (*see* Rosengren *et al.* 1994). This index represents the number of pupae of one kind collected by the workers among the ten first pupae (of either kind). This index allowed us to compare the discrimination ability of both species with the Mann-Whitney *U*-test.

Results

In a total of 301 replicates, workers successfully retrieved the pupae in 258 cases, and failed to do so in 43 cases which then were discarded. Three main kinds of failure were observed: no worker went in the arena, the workers did not carry any pupae or too few pupae were retrieved. The failures could not be attributed to a particular nest, species, social structure or biogeographical region.

When offered a choice between pupae of their own species and pupae of *F. rufa*, workers of both *F. paralugubris* and *F. lugubris* showed a statistically significant preference for conspecific pupae (Table 2: combinations 1–2). Workers of *F. rufa* expressed the same high degree of discrimination when faced with pupae of either *F. paralugubris* or *F. lugubris* (Table 2: combinations 3–4). When confronted with pupae from the sibling species versus conspecific pupae both *F. paralugubris* and *F. lugubris* showed a highly significant preference for pupae of their own species (Table 2: combinations 5–6). The replicates in which workers did not show any preference for one kind of pupae could not be attributed to the occurrence of nestmate or non-nestmate pupae as donor 1. Interestingly, *F. paralugubris* and *F. lugubris* differed in their ability to discriminate between conspecific and heterospecific pupae (Table 3). *F. paralugubris* showed significantly higher brood preference indices than *F. lugubris* when offered a choice between conspecific and *F. rufa* pupae as well as when faced with pupae of the sibling species (independently of the presence of nestmate or non-nestmate pupae).

The degree of intraspecific discrimination was significantly lower than interspecific discrimination in both species (χ^2-test on pooled data involving interspecific versus intraspecific

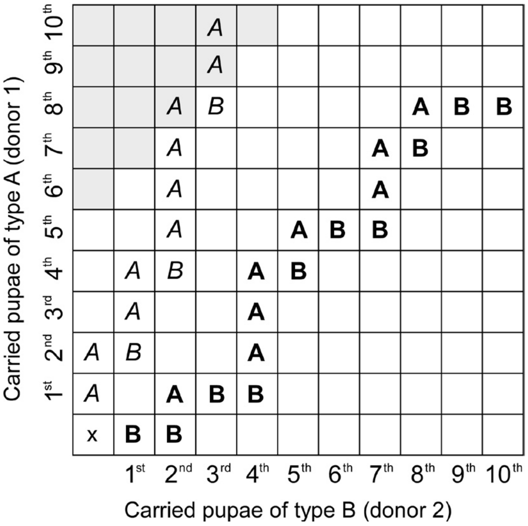

Fig. 3. Matrix used for the statistical "worker choice test". Shaded area: statistically significant area ($p < 0.05$) indicating a preference for pupae of type A (pupae of donor 1); x = starting point of the experiment. Each letter corresponds to choice event, leading to one step up in the matrix when a pupa of type A is selected, and one step to the right when a pupa of type B is selected. Bold sequence: the workers show no preference. Italic sequence: the workers show a statistically significant preference for pupae of type A (= donor 1).

Table 3. Comparison of brood preference indices of workers of *F. lugubris* (FL) and *F. paralugubris* (FPL) when offered a choice between conspecific pupae and heterospecific pupae (Mann-Whitney *U*-test). FR: *Formica rufa*; Alien species = non sibling species. *N* = number of experiments. In brackets: number of replicates involving nestmate pupae as donor 1.

Combination	Recipient	Donor 1	Donor 2	*N*	Mean Index (± SD)	Mean Rank	*U*	*p*
1	FPL	conspecific (FPL)	alien species (FR)	12 (6)	9.8 ± 0.6	20.3	62.0	0.035
2	FL	conspecific (FL)	alien species (FR)	19 (10)	8.6 ± 1.6	13.3		
5	FPL	conspecific (FPL)	sibling species (FL)	47 (26)	9.4 ± 1.2	52.3	783.0	0.019
6	FL	conspecific (FL)	sibling species (FPL)	44 (22)	8.6 ± 1.6	40.4		

discrimination: *F. paralugubris*: $\chi^2 = 57.9$, $p < 0.001$; *F. lugubris*: $\chi^2 = 23.1$, $p < 0.001$; Table 2). When offered a choice between nestmate and non-nestmate conspecific pupae, workers of *F. paralugubris* showed a lack of preference significantly more often than they showed preference (Table 2: combination 7). The trend was similar for both social types of *F. lugubris*, although not significant (Table 2: combinations 8–9). The difference between *F. paralugubris*, the monogynous *F. lugubris* and the polygynous *F. lugubris* was not significant ($\chi^2 = 2.2$, $p < 0.333$; Table 2: combinations 7–9). Similarly, when faced with the choice between pupae from the same or a different biogeographical region, workers of *F. paralugubris* showed no preference significantly more often than a preference, whereas the monogynous *F. lugubris* showed a preference in roughly half the cases (Table 2: combinations 10 and 11 respectively). With respect to social type, workers of both monogynous and polygynous *F. lugubris* showed a preference for their own type as often as not (Table 2: combinations 12–13). The strength of discrimination was of similar magnitude in the monogynous and polygynous forms of *F. lugubris* (Fisher's exact test, two-tailed: nestmate: $p = 0.693$; social type: $p = 1.000$), but the monogynous *F. lugubris* showed stronger discrimination than *F. paralugubris* when faced with pupae from distinct biogeographical regions (Fisher's exact test, two-tailed: $p = 0.051$). However, these results may also reflect nestmate versus non-nestmate discrimination. When faced with the choice between same or different region, or same or different social type, workers of monogynous *F. lugubris* preferentially retrieved nestmate pupae rather than non-nestmate pupae in 12 cases out of 16 (Table 2: combinations 11–12; data pooled, Fisher's exact test: $p = 0.006$). Unfortunately the low number of replicates precludes the corresponding tests for the polygynous *F. lugubris* and *F. paralugubris*.

Discussion

When offered the choice between conspecific and heterospecific pupae, workers of both *F. paralugubris* and *F. lugubris* preferentially retrieved conspecific pupae, independently of the origin of the nest. They showed the same high interspecific discrimination behaviour when faced with their sibling species as when faced with a species of a distinct phylogenetic clade, in this case *F. rufa*. Discrimination between the two sibling species was expressed across the entire scale of sampling (Swiss Jura Mountains and Alps). This is in accordance with the pattern of selective reinforcement, which holds that species discrimination evolves between sympatric taxa to prevent maladaptive hybridization (Noor 1999). Even if the two studied sibling species are phylogenetically close (Goropashnaya *et al.* 2004), probably leading to very similar chemical profiles retained from their common ancestor (Carlin 1988), a heritable species-specific odour and/or a brood-tending pheromone (*see* Vander Meer & Alonso 1998) probably exists. These results validate the use of the pupa-carrying test as a taxonomic tool to discriminate between sympatric sibling species of wood ants.

Interestingly, *F. paralugubris* showed higher interspecific discrimination than *F. lugubris*. This may be due to differences in social structure. *F. paralugubris* forms extensive polydomous supercolonies of highly polygynous nests, whereas *F. lugubris* is socially flexible, with both monogynous and polygynous societies. This is consistent with the predictions of Hölldobler and Wilson (1977), that workers from polygynous colonies should recognize and be more aggressive to other workers of a different species than monogynous colony workers. However, these predictions and the great majority of studies dealing with level of discrimination in ants concern worker–worker interactions (Vander Meer & Morel 1998). We suggest that in some cases interspecific brood recognition could be a side-effect of interspecific worker recognition.

Rosengren *et al.* (1994) used the pupa-carrying test to investigate the behaviour of wood ant workers of two different allozyme types, but without clear taxonomic identification. Based on this they postulated the existence of two sympatric sibling species in the Jura Mountains. Here we used for the first time the pupa-carrying test on the two clearly identified species *F. lugubris* and *F. paralugubris*. In 1994 sexual pupae were used, whereas we only used worker pupae. The present and the previous results together indicate that workers distinguish both kinds of pupae at the species level. The unexpected results obtained by Rosengren and Cherix (1981) (i.e. Swiss and Italian *F. lugubris* workers refrained from carrying pupae from Finnish *F. lugubris* and vice versa) were interpreted as the result of an intraspecific regional effect. We could recently assess that the Swiss and Italian study sites used in 1981 contained principally *F. paralugubris* colonies (Seifert 1996a, Cherix *et al.* 2004). Hence the study of Rosengren and Cherix (1981) showed a clear interspecific behaviour. This reinterpretation demonstrates the strength and the reliability of the pupa-carrying test even for populations distant by thousands of kilometres.

In a few cases the workers showed no significant preference for conspecific pupae, but they never preferred the "wrong" species. These cases could neither be attributed to particular nests nor to the speed of retrieval, but could be due to stochastic factors. Thus, one frantic or alarmed worker could randomly collect all pupae and alien ones could be later devoured within the nest (Jaisson 1975, Le Moli & Passetti 1977, 1978, Jaisson & Fresneau 1978). In a natural setting such behaviour should have minor effect within a colony comprising thousands of workers. In addition, workers may be less discriminating against pupae than against adult workers (Vander Meer & Morel 1998), or brood recognition could be transitory, so that nurse workers discriminate, whereas older workers do not (Carlin & Schwarz 1989, Fénéron & Jaisson 1992, 1995).

Intraspecific discrimination was less pronounced than interspecific discrimination in both species. In *F. paralugubris* and the polygynous *F. lugubris* nestmate recognition was virtually absent within regions. Both monogynous and polygynous *F. lugubris* workers preferentially retrieved pupae of their own social type, and in the case of monogynous workers, also their own region, in a considerable fraction of cases. However, this outcome is confounded by the fact that we used nestmate pupae in half the comparisons between regions and social types. In most cases the preferences were attributable to the retrieval of nestmate pupae, so it is likely that the discrimination detected here is due to discrimination between nestmates and non-nestmates, rather than discrimination between regions and social types.

The degree of intraspecific nestmate recognition is often associated with social structure (Morel *et al.* 1990, Vander Meer *et al.* 1990). In highly polygynous species, discrimination is less pronounced than in monogynous societies (Fortelius *et al.* 1993, Bourke & Franks 1995, Sundström 1997). Surprisingly, very few studies have focused on the influence of social structure on brood discrimination. Our study indicates a moderate recognition of nestmate pupae in the monogynous and monodomous *F. lugubris*, whereas recognition was virtually absent in the highly polygynous and polydomous *F. paralugubris*. This is consistent with the expectations for unicolonial species (e.g. *F. paralugubris*; Cherix 1981, Chapuisat *et al.* 1997), in which workers should show no or weak intraspecific discrimination (e.g. no aggression, Giraud *et al.* 2002, Brown *et al.* 2003, but *see* Chapuisat *et al.* 2004; no brood preference). In unicolonial species the exchange of adults and brood between nests leads to genetic viscosity (Chapuisat *et al.* 1997) and genetically similar nests tend to have more homogeneous recognition cues (Beye *et al.* 1997). In addition, the inter-nest exchange of adults, brood, nest materials and food (Cherix 1980) may homogenize environmental cues. As a result recognition cues will converge within colony boundaries (Astruc *et al.* 2001). Nevertheless, *F. paralugubris* showed no discrimination between biogeographical regions either, which may indicate a more extensive lack of discrimination.

Present and previous pupa-carrying studies show that *F. lugubris* and *F. paralugubris* comprise two separate but internally homogeneous genetic pools which are maintained across geographical regions. The pupa-carrying test can therefore be used as a taxonomic tool to distinguish species within the *F. rufa* group independently of social type, in addition to morphological, chemical or genetic approaches. However, an adequate replication and a precise identification of the control species are compulsory. Finally, this behavioural test can also be used to explore parameters involved in intraspecific brood discrimination (e.g. level of polygyny can affect partly the discrimination power of workers, Rosengren *et al*. 1993).

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