I see where you walked – how chemical cues influence movement decisions in ants

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Abstract

Interactions between animals are not restricted to direct encounters. Animals can also sense the proximity of others through indirect cues. For example, prey species use predator cues to assess predation risk. However, in contrast to predator-prey interactions, the role of indirect cues in interactions among competitors has been little investigated to date. Ant communities are usually structured by aggressive interactions between competing species. Responding to cues of others should be beneficial - an ant may avoid competitors or discover new food sources. In ants and other insects, such cues include chemical footprints, which they unintentionally leave while walking. We therefore investigated how different ant species responded to footprints of other ants. Footprints were obtained by from workers walking on paper for 5 min or 1 h. We then confronted laboratory colonies with footprints of other colonies or species, and let them choose between cue-bearing and cue-free areas. Moreover, we determined the chemical composition of footprints, and compared the absolute quantities of footprint and cuticular hydrocarbons. *Lasius niger* ants avoided footprints of non-nestmate conspecifics, and tended to avoid footprints of two other species. We suggest that they avoided encounters with competitors to reduce costly fights. In contrast, *Myrmica rubra, Formica polyctena* and *Tetramorium caespitum* often followed heterospecific footprints. This behaviour may represent eavesdropping in order to find food resources discovered by other species. Furthermore, all four species followed cues of nestmates. Footprints of non-nestmate conspecifics were either followed to a lower degree, or antennated more intensely. The chemical composition of footprints was species-specific and largely congruent to cuticular hydrocarbons. Footprint quantities left by one worker after 5 min worker represented 1/170 to 1/64 of the quantity of its cuticular hydrocarbons. We showed that chemical footprints represent an important cue for behavioural decisions in ants. Even footprints left after 5 min walking time provided sufficient cues so that ants could detect the comparatively minor differences between nestmates and non-nestmate conspecifics. The ability to identify and respond to chemical footprints may represent an important strategy for insects to cope with competing species in their habitat.

Key words

behavioural trait; chemotactile cues; competitor avoidance; cuticular hydrocarbons; eavesdropping; Formicidae; interspecific interaction; interspecific recognition

Introduction

Interactions between organisms are not restricted to direct encounters. Frequently, animals detect another species' presence through chemical, i.e. olfactory cues (Gonzalo et al. 2008, Zöttl et al. 2013, Cisterne et al. 2014) or e.g. spider silk (Rypstra and Buddle 2013). Their responses have largely been studied in predator-prey interactions. Certain predator species use chemical cues to locate prey (Hughes et al. 2010, Cárdenas et al. 2012, Clavijo McCormick et al. 2012). However, prey also use predator cues to detect predation risk. The effects caused by predator cues ('non-consumptive' or 'trait-mediated' effects) include morphological and chemical defense mechanisms such as spine structures in *Daphnia* (Tollrian 1990, Walls et al. 1991) or secondary plant compounds (Zangerl and Rutledge 1996). In animals, behavioral changes to reduce predation risk (antipredator behaviour) represent by far the most frequent non-consumptive effects. Antipredator behaviour includes reduced feeding activity, changes in microhabitat or emigration from areas with high predation risk, and has been described in terrestrial, marine and freshwater ecosystems (Kats and Dill 1998, Persons and Rypstra 2001, Persons et al. 2002, Preisser et al. 2005, Preisser and Bolnick 2008, Bowler et al. 2013, Hill and Weissburg 2013, Hill and Heck 2015). Effects of antipredator behaviour can even exceed those of direct consumption on prey demography (Werner and Peacor 2003, Preisser et al. 2005). They can also cascade down to lower trophic levels such as herbivores (Schmidt-Entling and Siegenthaler 2009) or plants (Schmitz et al. 1997). Consequently, non-consumptive effects in predator-prey interactions play an important role in shaping ecosystem functions (Schmitz et al. 2008).

In contrast to predator-prey interactions (Persons and Rypstra 2001, Binz et al. 2014a), the role of cue use among competitors has received less attention so far. For this research question, ants represent an ideal study system. They show high levels of interference competition within and between species, and foragers often aggressively displace other individuals from food sources (Parr and Gibb 2010, Cerdá et al. 2013, Binz et al. 2014b). Thus, there should be pressure to detect, and respond to cues of other species to reduce the costs of competition. In ants and other insects, a particularly important source of such potential cues are chemical footprints. Every walking insect inevitably leaves footprints behind (Devigne and Detrain 2006). They consist of hydrocarbon droplets and stem from a tarsal adhesive structure (Geiselhardt et al. 2010), called 'arolium'. The arolium is covered with a thin film of liquid, which generates attachment forces when the insect climbs or walks (Drechsler and Federle 2006). During walking, the arolium leaves liquid droplets behind (Federle et al. 2002). The droplets contain hydrocarbons largely congruent to cuticular hydrocarbons (Akino and Yamaoka 2005, Geiselhardt et al. 2010, 2011), but also small quantities of hydrophilic substances largely unknown to date (liquid A in Federle *et al.,* 2002).

Cuticular hydrocarbons (CHC) are ubiquitous in insects and have been intensely studied during the last decades (Blomquist and Bagnères 2010). They serve a dual function - as desiccation barrier and as cues for intra- and interspecific communication (Gibbs 1998, Menzel et al. 2008a, van Zweden and

d'Ettorre 2010, Lang and Menzel 2011). Cuticular hydrocarbon composition usually differs between species, and (in eusocial insects) also between conspecific colonies. Insects use them as recognition cues to distinguish between species, colonies, or even castes within a colony (van Zweden and d'Ettorre 2010).

If footprint hydrocarbons (FHC) provide similar cues as cuticular hydrocarbons, they should similarly enable recognition of its bearer's species and colony membership and hence allow differential responses. In studies on intracolonial footprint use, footprints of nestmates were used for 'homerange' or 'territory' marking, and their presence influenced foraging decisions of workers (Depickère et al. 2004, Devigne et al. 2004, Saleh et al. 2007, Lenoir et al. 2009, Rottler et al. 2013). Although potentially of high ecological relevance, however, the use of footprints from *other* colonies or species has been little investigated to date. As described above, a behavioural response to allocolonial or allospecific footprint cues could prevent encounters of potential competitors and thus be highly beneficial: it would reduce costs from competition. Indeed, Binz *et al.* (2014b) recently showed that subordinate ant species avoided cuticular hydrocarbons of dominant species. Ants might also actively approach heterospecific footprints in order to obtain access to food sources discovered by others ('eavesdropping', Nieh, Barreto & Contrera 2004; Menzel *et al.* 2010). These effects should also depend on the cue quantity an individual encounters – low cue quantities may reflect footprints of few foraging ants, while high cue quantities should indicate that the nest of the other species is closeby. However, to our knowledge, the quantity of footprint hydrocarbons relative to those present on an insect's cuticle is unknown so far.

Here, we investigated the importance of footprint cues for behavioural decisions in ants. We tested whether ants followed footprints of con- and heterospecific individuals, or whether they preferred cue-free areas. We expected that firstly, ants showed differential responses to footprints of other species. They should either avoid potential competitors to reduce costly fights, or approaching footprints of others to discover food sources detected by the other species (Binz et al. 2014b). In a similar reasoning, ants should secondly distinguish between intraspecific footprints of the same and a foreign colony, and approach those of their own colony more frequently. These two responses should be stronger at higher footprint concentrations (Oliver et al. 2008). Thirdly, we analysed the chemical composition of cuticular and footprint hydrocarbons, testing whether footprint hydrocarbons were as different between species as those from the cuticle. Finally, we determined the absolute quantities of footprint hydrocarbons and compared them to cuticular hydrocarbons of the same colony. We expected that footprint quantities increased with the duration of ants walking on the substrate.

Material & Methods

Species studied. We studied five species: *Formica polyctena*, *Formica rufibarbis, Lasius niger* (all three Formicinae), *Myrmica rubra* and *Tetramorium caespitum* (both Myrmicinae). All species are widespread in Central Europe and have overlapping habitats and food sources (Seifert 2007). *Myrmica*

rubra is polygynous with ca. 15 queens and 1000 workers per nest. *Formica polyctena* is a territorial ant species common in Central European forests. Its colonies contain multiple queens and often comprise multiple mounds. *Lasius niger, F. rufibarbis* and *T. caespitum* were collected on a bush land meadow near Mainz-Drais, ca. 1.5 km from the first site. Nests of all three species were located closely together, often with nests of several species within 1 m². *Lasius niger* and *T. caespitum* are monogynous, while *F. rufibarbis* is allegedly polygynous. *Formica polyctena* and *M. rubra* were collected in a mixed forest near Mainz, Germany (49°57'43" N / 8°10'51" E). Since *F. polyctena* is protected by law in Germany, we only collected worker groups of this species (200-500 individuals/colony; permit: Gestattungsvertrag 11.4.2014, local nature conservation authority and Forstamt Rheinhessen).

For all other species, we collected whole colonies including brood. Despite considerable efforts, we did not always succeed in obtaining the queen. No queens could be obtained for the *L. niger* nests, whereas a single queen was obtained for 2/12 *F. rufibarbis* and 3/12 *T. caespitum* nests. In *M. rubra,* nests with ≥ 5 queens could be obtained for all colonies. However, we argue that even the nests without queens showed normal foraging behaviour, because previous studies indicated that queen presence or absence did not affect foraging behaviour as long as brood was present (Binz et al. 2014b). Furthermore, all experiments were conducted within 1-2 months weeks after nest collection; *F. polyctena* behavioural assays were conducted within 1-2 weeks after collection.

After two weeks in the lab, the *F. rufibarbis* workers became rather inactive and did not approach our experimental mazes (despite presence of brood and queen in the nest). Therefore, this species was only used as a footprint donor and for the chemical analyses, but not for behavioral experiments. In a previous study, *F. polyctena* and *L. niger* were dominant in interspecific comparisons, while *F. rufibarbis, T. caespitum* and *F. rufibarbis* were submissive (Binz et al. 2014b). After collection, the ants and part of the nest material were transferred to plastic nest boxes (24 x 18 x 9.8 cm) with a plaster floor and fluon-coated walls, and kept at room temperature, including daily fluctuations in temperature and light. They were fed twice a week with water, honey and dead crickets *ad libitum.* The behavioral experiments were performed in summer from June to August 2014.

Behavioural experiments: experimental design. We analysed behavioural reactions of workers towards conspecific and heterospecific footprints. In all assays, the workers were presented a Y maze with one footprint-free arm and one arm containing footprints. In the conspecific setup, workers were confronted with footprints of nestmates and those of a non-nestmate conspecific colony; each in separate assays. In the heterospecific assays, workers were confronted with footprints of four other species. Furthermore, each footprint type was presented in two concentration levels (low and high). We tested 12 colonies for each of four species: *M. rubra, T. caespitum, L. niger* and *F. polyctena*, and each colony was tested in two conspecific and four heterospecific treatments (total *N*: 6 treatments * 2

concentrations * 4 species * 12 colonies = 576). *Formica rufibarbis* was included as a donator of footprints, but we did not test their behavioural responses since they were highly reluctant to enter the Y maze voluntarily and thus could not be tested with this same setup. Footprints were obtained from 20 ant workers that were walking around on a defined area for 5 min (low concentration) or 1 h (high concentration) (Fig. S1). To analyze whether ants react to footprints of ants which walked on the y-maze before and whether they have a side preference (possibly due to laboratory conditions), we conducted experiments where ants were presented a Y maze without any footprints, where we analysed whether ants would follow the previous ant.

Behavioural experiments: setup of the assays. The ants were presented paper Y mazes which were placed on a wire rack directly on their nest (Fig. S1). Prior to the assays, we obtained footprints, by placing fluon-coated PVC boxes without floor (7.0 x 1.8 cm; height 2.0 cm), onto each arm of the Y maze. We then gently placed 20 foragers into one of the boxes, such that they would walk around and leave footprints. The workers stayed there for 5 min (low footprint concentration) or 1 h (high footprint concentration). Afterwards, they were removed and the Y maze used for an assay. The side of the Y maze containing footprints was alternated randomly. The wire rack allowed the ants to enter the Y maze, but they were not forced to do so. Using this construction, it was easy to place the y-maze such that only one ant after another could enter it. For the first 15 workers entering the Y maze, we recorded whether they chose the footprint-covered or the footprint-free arm. Each decision was considered final if the ant had crossed at least 20 mm (approximately 1/3) of the footprint-covered area of the Y maze arm, or the analogous position on the footprint-free arm. When reaching this point, the ant was collected and put into a separate box until the end of the experiment. If an ant stopped and antennated the paper floor containing footprints for at least 1 s with both antennae before entering a Y arm, this behaviour was recorded as 'antennation'. Antennation was recorded at maximum once per ant individual. The colonies and footprint treatments were tested in random order. We always wore gloves to minimise effects of human contamination.

Behavioural experiments: statistics. For each assay, we calculated the number of ants following the footprint. The numbers of following vs. non-following workers were then compared between treatments using generalised linear mixed-effects models with binomial error distribution (GLMM; R command g*lmer*, package *lme4*, followed by *Anova*, package *car*). First, the response to heterospecific footprints was analysed. We tested, (i) whether ants significantly followed each of the heterospecific footprints, i.e. whether the number of ants approaching footprint cues from each species differed from random expectation. In addition, (ii) we tested whether the tendency to approach footprints differed between footprints of different species. Secondly, we analysed the response to intra- and allocolonial footprints of conspecific individuals. As above, we separately tested whether the response to intra- and allocolonial footprints differed from random expectation, and subsequently analysed whether the two responses differed from each other. (iii) In addition to footprint following, we also analysed whether

ants antennated intra- and allocolonial footprints in different frequencies, using generalised linear mixed-effects models with poisson distribution. Antennation of heterospecific cues was not analysed because there was no clear *a priori* expectation, and because different species leave different footprint quantities. Hence, different antennation frequencies between species could be due to species-specific differences, but also to different footprint quantities. However, we tested whether, for each species, antennation frequencies differed between cue-free mazes, low-concentration and high-concentration footprints, in order to assess whether the ants distinguished between footprint quantities. To this end, we used GLMMs with poisson distribution, 'cue concentration' as fixed factor and 'colony ID', 'cue species' and 'cue colony' as random factors. Finally, we tested whether ants followed the footprints laid by the previous ant, which might have biased our results. All statistical analyses were conducted in R version 3.2.0 (R Core Team 2015).

Chemical analyses: sample preparation. We analysed and compared composition and absolute quantity of cuticular hydrocarbons, footprint hydrocarbons after 5 min, and footprint hydrocarbons after 1 h. Since the footprints of *T. caespitum* and *M. rubra* did not yield substances in analysable quantities, we concentrated on *F. rufibarbis*, *F. polyctena* and *L. niger*. We collected one sample per colony for each of the three treatments, resulting in 36 extracts per ant species. Footprint hydrocarbons were collected from 20 workers per sample, which were placed in a fluon-covered, floor-less box (y x z cm) on a glass petri dish. After 5 min or 1 h (respectively), the ants were removed, and the petri dish was rinsed with hexane. This setup is similar to those used by Akino & Yamaoka (2005), Eltz (2006) and Wilms & Eltz (2008)**.** Cuticular hydrocarbons were obtained from one individual per sample, which was frozen to death and then immersed in hexane for 10 min. As internal standard, 100 ng of *n*-C18, solved in 10 µl *n*-heptane (Fulka, Sigma-Aldrich Co., Germany) were added to each sample. The extracts were concentrated under nitrogen flow and analysed using GC-MS (GC 7890A; MSD 5975; Agilent Technologies, Santa Clara CA, USA). Of every sample, 2 µl were injected at a temperature of 250 °C in splitless-mode. Helium was used as carrier gas with a flow rate of 1.2 ml per minute. The stationary phase was a capillary column (Zebron Inferno DB5, 30 m x 0.25 mm, coating 0.25 µm; Phenomenex). The temperature program started at 60 °C for 2 min, followed by a ramp of 60 °C per minute up to 200 °C, and a second ramp of 4 °C per minute up to 320 °C, where temperature remained constantly for ten minutes. The ionization current was 70 eV. Molecular fragments were detected in a scan range of 40-550 m/z. Data were analysed using the software MSD Chem Station E.02.02 1431 (Agilent Technologies, Santa Clara CA, USA).

Chemical analyses: statistics. We identified all hydrocarbons between C20 and C50 based on retention index and diagnostic ions; non-hydrocarbon substances were omitted from the analysis. Quantitative substance composition was analysed with a two-factorial PERMANOVA (factors 'species' and 'cue type') using the software PRIMER (PRIMER 6, 64-bit, PRIMER-E Ltd., Ivybridge UK). In addition, an NMDS ordination was performed in R (package *vegan*).

Absolute hydrocarbon quantities were calculated based on the internal *n*-C18 standard (Mas et al. 2009). Absolute quantities of cuticular hydrocarbons were compared between species using a GLM on log-transformed data. Furthermore, we calculated the ratios footprint quantities after 5min and after 1h to cuticular hydrocarbons. Footprint quantities were divided by 20 to obtain an estimate of the quantity left by a single individual. The ratios were compared between species using GLMs on log-transformed data. As above, all statistical analyses were conducted in R 3.2.0.

Results

Response to heterospecific cues. *Myrmica rubra* approached footprints of all four species (Fig. 1a). Footprints of *F. polyctena* were approached slightly more often than those of *T. caespitum* and *L. niger* (GLMM: $\chi^2_3 = 8.0$, $p = 0.045$). The workers' tendency to follow was higher at high footprint quantities (GLMM: $\chi^2_1 = 5.2$, $p = 0.023$). In contrast, *Formica polyctena* workers approached footprints of two species only, i.e. *F. rufibarbis* and *M. rubra* (Fig 1b) ($\chi^2_3 = 9.0$, $p = 0.029$). The responses did not differ between high and low footprint quantities ($\chi^2_1 = 1.3$, $p = 0.26$). In *Lasius niger*, none of the approaching reactions to heterospecific footprints differed from random expectation (Fig. 1b). However, this species showed different responses to high and low footprint concentrations (interaction quantity:species: $\chi^2_3 = 10.5$, $p = 0.015$), and marginally differentiated between footprints of different species at high, but not at low quantities (high: $\chi^2_3 = 6.7$, $p = 0.081$; low: $\chi^2_3 = 4.2$, $p = 0.24$). Footprints of *F. polyctena* and *M. rubra* were marginally avoided at high quantities, while at low quantities, those of *T. caespitum* were marginally avoided. Finally, *Tetramorium caespitum* approached footprints of 3 out of 4 species, but did not significantly differentiate between species ($\chi^2_3 = 2.1$, $p = 0.54$) nor footprint quantity ($\chi^2_1 = 1.2$, $p = 0.27$).

In three of the four species, the ants antennated 1 h footprints more frequently than 5 min footprints, and the 5 min footprints more often than cue-free Y mazes (data pooled for all cue species). The difference between high concentration, low concentration and empty Y mazes was significant for *M. rubra, L. niger* and *T. caespitum* (GLMM: all three $\chi^2_2 > 55$, $p < 0.0001$), but not for *F. polyctena* ($\chi^2_2 = 4.3$, $p = 0.12$) (Fig. S2).

Response to conspecific cues. *Myrmica rubra* frequently followed conspecific footprints (GLMM: both $p < 0.0025$), irrespective of whether they were intra- or allocolonial (GLMM: $\chi^2_1 = 0.00$, $p = 0.99$; Fig. 2a). Allocolonial cues were antennated more frequently ($\chi^2 = 5.50$, $p = 0.019$; Fig. 2b). *Formica polyctena* followed intracolonial cues ($p = 0.040$), but not allocolonial ones ($p = 0.12$), although the difference between the two was not significant ($\chi^2_1 = 0.14$, $p = 0.71$). Allocolonial cues of both concentrations were antennated more intensely than those of the own colony ($\chi^2_1 = 8.55$, $p = 0.0035$). *Lasius niger* was the only species that actually avoided all allocolonial footprints, but approached those of their own colonies (difference intra/allocolonial: GLMM, $\chi^2_1 = 20.0$, $p < 0.0001$; difference from random: $p = 0.017$ and $p = 0.00025$). Finally, *T. caespitum* approached footprints of their own

252 colony ($p = 0.0032$), but ignored those of foreign, conspecific colonies ($p = 0.34$) (difference: GLMM
253 $\chi^2_1 = 2.09$, $p = 0.14$). Neither *L. niger* nor *T. caespitum* antennated allocolonial and intracolonial cues
254 in different frequencies (both $\chi^2_1 \leq 0.27$, $p \geq 0.61$). Concentration effects were found in *M. rubra* and
255 *F. polyctena*, where cues of higher concentrations were approached significantly more often (*M.*
256 *rubra*: $\chi^2_1 = 10.0$, $p = 0.0015$; *F. polyctena*: $\chi^2_1 = 4.1$, $p = 0.044$).

257 During the Y maze assays, an individual's decision might have been influenced by the footprints of
258 previous ants on the maze. To assess this, we tested whether, on cue-free mazes, the choice of the
259 second ant was influenced by that of the first ant. However, this was not the case for any of the four
260 species (binomial GLM: $z \leq 1.13$, $p \geq 0.26$ for all four species, n=12 per species). Furthermore, we
261 analysed whether the ants were influenced by the decision of the one directly before, summing up
262 following decisions of the 14 ants after the first one per assay. Again, no influence was detected for
263 any of the four species (binomial GLM: all four $z \leq 1.7$, $p \geq 0.091$).

264 **Chemical analyses.** For the three formicines *Formica rufibarbis, Formica polyctena* and *Lasius niger*,
265 footprint profiles were highly similar to the cuticular hydrocarbon profiles of the respective species
266 (Table A1). In contrast, footprints of the two myrmicines *Tetramorium caespitum* and *Myrmica rubra*
267 contained only few overall substances, and mostly in quantities unsufficient for analyses. For this
268 reason, we focused our analysis on the three formicine species.

269 **Hydrocarbon composition of cuticle and footprints.** As expected, hydrocarbon composition
270 strongly differed between the three species (PERMANOVA: pseudo-$F = 65.85$, $df = 2$, $p < 0.0001$).
271 This effect was much larger than differences between cuticular hydrocarbons, 5 min and 1 h footprints
272 (pseudo-$F = 19.67$, $df = 2$, $p < 0.0001$), and between colonies (pseudo-$F = 2.64$, $df = 33$, $p < 0.0001$).
273 Thus, footprint profiles were as species-specific as CHC (Fig. 3). In all three species, hydrocarbons
274 from the cuticle, from 5 min footprints and from 1 h footprints all differed from each other. The
275 differences of CHC to 5 min or 1 h footprints were largest in *F. polyctena* (pair-wise $t = 6.1$ and 7.1 in
276 *F. polyctena* compared to 2.7-5.0 in *L. niger* and *F. rufibarbis*). This may account for the significant
277 interaction of species and extract type (pseudo-$F = 16.74$; $df = 4$; $p < 0.0001$).

278 **Absolute quantities of cuticular and footprint hydrocarbons.** Individual *L. niger* ants carried
279 significantly less CHC on their cuticle (1.68 ± 0.45 µg) than the two *Formica* species (*F. rufibarbis*:
280 8.58 ± 1.59 µg, *F. polyctena:* 7.40 ± 0.79 SE µg) (GLM: $\chi^2_2 = 50.0$, $p < 0.0001$; Fig. 4a). The average
281 hydrocarbon quantity in 5 min footprints per individual was 27 ± 7.4, 50 ± 7.5, and 56 ± 8.1 ng
282 (respectively), which corresponds to 1/170 – 1/64 of an individual's cuticular hydrocarbons. Despite a
283 12 times longer timespan, however, the mean footprint quantity after 1 h was only 0.8 to 3.4 times as
284 high as after 5 min, and corresponded to 1/163 to 1/25 of the cuticular hydrocarbons. The quantity
285 ratios of 1 h footprint and CHC differed between all three species (GLM: $\chi^2_2 = 28.6$, $p < 0.0001$),
286 suggesting that the proportion of footprint hydrocarbons deposited while walking is species-specific

287 (Fig. 4b). Similarly, the quantity ratio of 5 min footprints to CHC was significantly higher in *L. niger*
288 but did not differ between the two *Formica* species (GLM: $\chi^2_2 = 6.54$, p = 0.038; Fig. 4c).

289 Discussion

290 The present study showed that ant species detect and respond to footprints. While some species
291 followed cues of other colonies or species, others avoided unknown cues. However, all ant species
292 differentiated between cues of different species, and/or between cues of nestmate and non-nestmate
293 conspecifics. This indicates that chemical footprints represent an ecologically important cue that ants
294 use for their movement decisions, even if their relevance seems to differ between species. The
295 chemical composition of footprint hydrocarbons resembled those on the insect cuticle and was
296 species-specific. Their quantities were only a fraction of those of an individual ant's cuticular
297 hydrocarbons (factor 25-170), but they were sufficient for ants to differentiate between nestmates and
298 non-nestmates. It has been shown before that intracolonial footprints (home-range markings) can
299 stimulate foraging and encourage foragers to leave the nest (Devigne and Detrain 2002, Devigne et al.
300 2004, Lenoir et al. 2009). However, our results show that ants also use cues from other colonies or
301 even species, which has been little studied to date.

302 Footprint recognition and behavioural response

303 Our results showed that footprints left by 20 workers after 5 min were sufficient for the ants to
304 discriminate between intra- and allocolonial cues. All four species we tested significantly followed
305 cues of nestmates. This is consistent with earlier reports that ants use nestmate footprints as an
306 indication of their home-range (Devigne and Detrain 2002, 2006, Lenoir et al. 2009). All four species,
307 however, differentiated between intra- and allocolonial footprints. Two species did not follow
308 allocolonial cues (*F. polyctena*, *T. caespitum*), and *L. niger* even avoided them. *Myrmica rubra* and *F.*
309 *polyctena* antennated allocolonial cues more than intracolonial ones, indicating that they perceived
310 them differently. More frequent antennation, however, does not necessarily indicate that allocolonial
311 footprints were recognized as non-nestmate, but may also show recognition uncertainty (Menzel et al.
312 2008b) such that ants antennated more to perceive a more precise signal.

313 The ants often showed stronger responses to 1 h footprints compared to those obtained after 5 min.
314 Since hydrocarbons beyond C20 are little volatile, it is likely that footprint hydrocarbons accumulate
315 in the surroundings of nests and/or food sources. Thus, for an ant, high footprint concentrations may
316 indicate nest proximity or food resources constantly exploited over a longer timespan (e.g.
317 trophobioses), whereas lower concentrations may indicate foraging trails or the presence if single
318 foragers. The differential effects of 1 h and 5 min footprints suggests that the ants adjusted their
319 response to the perceived cue quantities. Interestingly, chemical analysis showed that the quantities of
320 footprint hydrocarbons after 1 h were not twelve times higher than after 5 min, as one might expect.
321 This non-linear increase may be firstly because during 1 h, the ants rested longer and walked relatively

less than during 5 min, thus leaving fewer footprints per time. Secondly, the substrate for extraction (glass) may have become satiated with footprint hydrocarbon droplets. On a more adsorbent natural substrate such as soil or dry leaves, more footprints may have been deposited.

Using footprints to avoid competitors

For a foraging insect, responding to footprints of others should be highly beneficial. Bumblebees, for example, detect conspecific footprints on flowers and use them to assess whether the flowers are depleted or still worth visiting (Eltz 2006, Wilms and Eltz 2008). Similarly, foraging ants may use cues of other colonies or species to avoid competition, e.g. by avoiding areas frequented by other ants. Our experiments were designed to test those individuals who left the colony deliberately, i.e. who would be most likely to encounter them in nature. Evidence of competitor avoidance was found in one of the species we tested: foragers of *Lasius niger* avoided footprints of non-nestmate conspecifics, and tended to avoid those of *Myrmica rubra* and *Formica polyctena* at high concentrations. This seems surprising at first glance, since *L. niger* is a dominant species, and usually superior to *M. rubra* colonies, or to worker groups of *F. polyctena* (Binz et al. 2014b). However, by avoiding areas with cues of unknown opponents, *L. niger* might efficiently invest its resources, and only risk fights if necessary. Originating from a meadow with *T. caespitum* and *F. rufibarbis*, our *L. niger* colonies may have habituated to these competitively inferior species, but may have been unfamiliar to *M. rubra* and *F. polyctena*, and thus unaware of their competitive ability.

In a previous study (Binz et al. 2014b), the subordinate *T. caespitum* avoided cuticular hydrocarbon extracts of *L. niger*, while this species did not avoid *L. niger* footprints in this study. Since these two species often co-occur in close proximity, where nests of both species can be found within 1 m², we suggest that *T. caespitum* only avoids high concentrations of *L. niger* cues. Such high concentrations should indicate the proximity of their nest, where footprint concentrations on the ground should easily reach those of cuticular hydrocarbon extracts. In contrast, footprint concentrations should be lower in less frequented parts of a colony's foraging range. We suggest that ants adjust their behavioural response to the perceived risk of encountering the other species, as is consistent with the threat-sensitive avoidance hypothesis (Helfman 1989).

It remains to be studied to what degree footprint responses are genetically determined, or shaped by experience. In particular, future studies should address whether the propensity to follow footprints varies consistently between individuals and/or colonies, and thus could be viewed as a personality trait (Wolf and Weissing 2012). It seems plausible that footprint following is connected to explorative behaviour, which is regarded as a personality trait in various species (Modlmeier et al. 2012, Bengston and Dornhaus 2014). However, like other behavioural traits (Van Wilgenburg, Clémencet & Tsutsui 2010), footprint following may also be shaped by experience. Ants may habituate to footprints of harmless species, or learn to avoid cues of competitors after aggressive encounters. Such intraspecific variation in response, be it due to heritable factors or to experience, may substantially affect the fitness

of different colonies in different contexts, with considerable consequences for population dynamics of competing species (Bolnick et al. 2003, Sih et al. 2012). At the same time, the ability to adjust responses to previous experience should reduce costly fights and may thus drastically enhance colony fitness.

Using footprints to eavesdrop on non-nestmates

Tetramorium caespitum, Formica polyctena and especially *Myrmica rubra* often followed heterospecific cues. While the dominant *F. polyctena* was expected to follow footprints of others, this was unexpected for the subordinate *M. rubra* and *T. caespitum* (Binz et al. 2014b). We believe that this reflects an adaptive response to the relatively low footprint intensities. Compared to high quantities in the surroundings of a nest, low quantities may indicate only few workers of the other species, and hence a lower risk of being attacked (Helfman 1989). While following allocolonial cues may have been due to mis-recognition, heterospecific cues clearly belonged to non-nestmates. We suggest that following these footprints is a form of eavesdropping, i.e. they intercepted signals intended for nestmates in order to gain access to their food resources. Eavesdropping of intraspecific signal has been shown in a variety of contexts, such as predators that locate prey (Zuk and Kolluru 1998), parasites or parasitoids that find hosts (Clotfelter 1998, Gray et al. 2007, Fatouros et al. 2008), mutualists (Menzel et al. 2010a, 2014) or among competing species (Nieh et al. 2004, Menzel et al. 2010a, 2010b). However, it is unusual that this behaviour was common even among species that did not originate from the same habitat. Further studies are needed to assess whether footprint-following among non-associated species does result in the take-over of food resources in the field.

Chemical composition of footprints

In three of the five species, *L. niger, F. rufibarbis* and *F. polyctena*, footprints could be obtained in analysable quantities, and closely resembled each species' cuticular hydrocarbon profile whereas no analysable footprint extracts could be obtained from *M. rubra* and *T. caespitum*. The congruence of footprint and cuticular hydrocarbons confirms previous studies in different insect taxa (Akino and Yamaoka 2005, Geiselhardt et al. 2010). Interestingly, the quantity of footprint HC was not a constant fraction of the CHC quantity. Instead, this ratio differed between the three ant species. We tentatively assume that this is because the secreted footprint quantity depends on the species-specific tarsus anatomy. The size of the tarsal pad (arolium) differs between species, e.g. depending on whether they are arboreal or terrestrial (Orivel et al. 2001), since these pads are important to ensure tarsal adhesion on smooth surfaces (Drechsler and Federle 2006). It seems possible that smaller pads release fewer footprints, resulting in a lower FHC/CHC ratio. This might also account for the low footprint quantities in *M. rubra* and *T. caespitum.*

Despite the general congruence, footprint hydrocarbons still differed from CHC in chemical composition. These differences are probably related to compound-specific viscosities. For example, *n*-alkanes are more viscous than most other hydrocarbons (Gibbs and Pomonis 1995) and thus less likely

to be released in footprints. Indeed, footprints of the two *Formica* species we analysed (but not those of *Lasius*) possessed less *n*-alkanes than their respective cuticular hydrocarbons (data not shown). However, more analyses are necessary to further investigate the composition of footprints.

Conclusion

Using indirect cues while foraging should enable an organism to avoid competitors or predators, and thus reduce costs from aggressive encounters. Thus, the ability to discriminate between cues of different organisms should be highly beneficial. Our study showed that ants indeed use indirect cues - chemical footprints - of nestmates, non-nestmate conspecifics and other species for their movement decisions. However, ant species differed in cue use. *Lasius niger* showed different reactions among heterospecific cues, and was the only species to significantly avoid cues of foreign, but conspecific colonies. In contrast, other species such as *Myrmica rubra*, showed less differentiation, but rather approached all cues. We suggest that avoidance of other cues may be an important mechanism to reduce intra- and interspecific competition, and thus facilitate local species coexistence (stabilising coexistence mechanism *sensu* Chesson 2000). Similarly, approaching cues may represent an efficient mechanism to find resources discovered by other species, or to prey on them. Compared to trail pheromones, footprints are less volatile, and constantly deposited by walking ants. Thus, they provide more accurate and probably quantitative information on the local presence of other species, which explains why footprints evoke more reactions by other species than trail pheromones (Binz et al. 2014b).

Interspecific differences in the degree and the direction of footprint responses may translate to fitness differences between species, enabling some species to exploit resources or avoid competitors more effectively than others. We believe that footprint responses represent a highly fitness-relevant trait, which influences foraging dynamics. Our results open up intriguing questions in two directions: firstly, future studies should address how footprint use affects an ant colony's performance compared to competing colonies or species. Secondly, it remains open how the propensity to follow footprints is affected by experience and by heritable factors. Further studies are hence needed to elucidate the role of learning in the response to previously unknown footprints.

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582

Figure legends

Fig. 1: Response to heterospecific footprints: Footprint following. The plots show the mean number (± SE) of ants (out of 15) choosing the footprint arm of the Y-maze minus those choosing the cue-free arm (n = 360 per cue donor). Zero demarcates the random expectation of 7.5 ants. The plots show data pooled for both footprint concentrations (A: *Myrmica rubra, Formica polyctena, Tetramorium caespitum*), and separate for high and low concentrations where the interaction of concentration and cue species was significant pooled for both. Asterisks show if each treatment significantly differed from random expectation (binomial GLMM): °p < 0.1, *p < 0.05, **p < 0.01, ***p < 0.001. Plots with the same letters are not significantly different.

Fig. 2: Response to conspecific footprints. (A) Footprint following. The plots show the mean number (± SE) of ants (out of 15) choosing the footprint arm of the Y-maze minus those choosing the cue-free arm. Zero demarcates the random expectation of 7.5 ants. The plots show data pooled for both footprint concentrations. Asterisks indicate if each treatment significantly differed from random expectation, and whether: *p < 0.05, **p < 0.01, ***p < 0.001. (B) Number of ants (out of 15) that antennated the footprints. Asterisks indicate if intra- and allocolonial cues evoked significantly different responses according to a GLMM with poisson error distribution. (B) Antennation frequency; antennation behavior of the ants walking on the y-maze. Each ant was counted once when antennated the footprint covered area. Maximum value is 15.

Fig. 3: NMDS ordination of cuticular and footprint hydrocarbons of *Formica polyctena, Formica rufibarbis* and *Lasius niger*. Different colors represent different species and different symbols represent the different hydrocarbon origins. Each data point represents the chemical profile of the footprint assay or the CHC of one Individual per colony. (n=12 per treatment)

Fig. 4: Absolute FHC and CHC quantities. (A) CHC quantities per individual. (B) Ratio of footprint quantity per individual after 1 h and cuticular hydrocarbon quantity. (C) Ratio of footprint quantity per individual after 5 min and cuticular hydrocarbon quantity. The plot for *L. niger* marginally differs from *F. polyctena* and *F. rufibarbis* (Wilcoxon tests: W = 38, p = 0.052 and W = 41, p = 0.078). All plots show mean ± SE; those with same letters are not significantly different according to pair-wise Wilcoxon tests.

Fig. 1

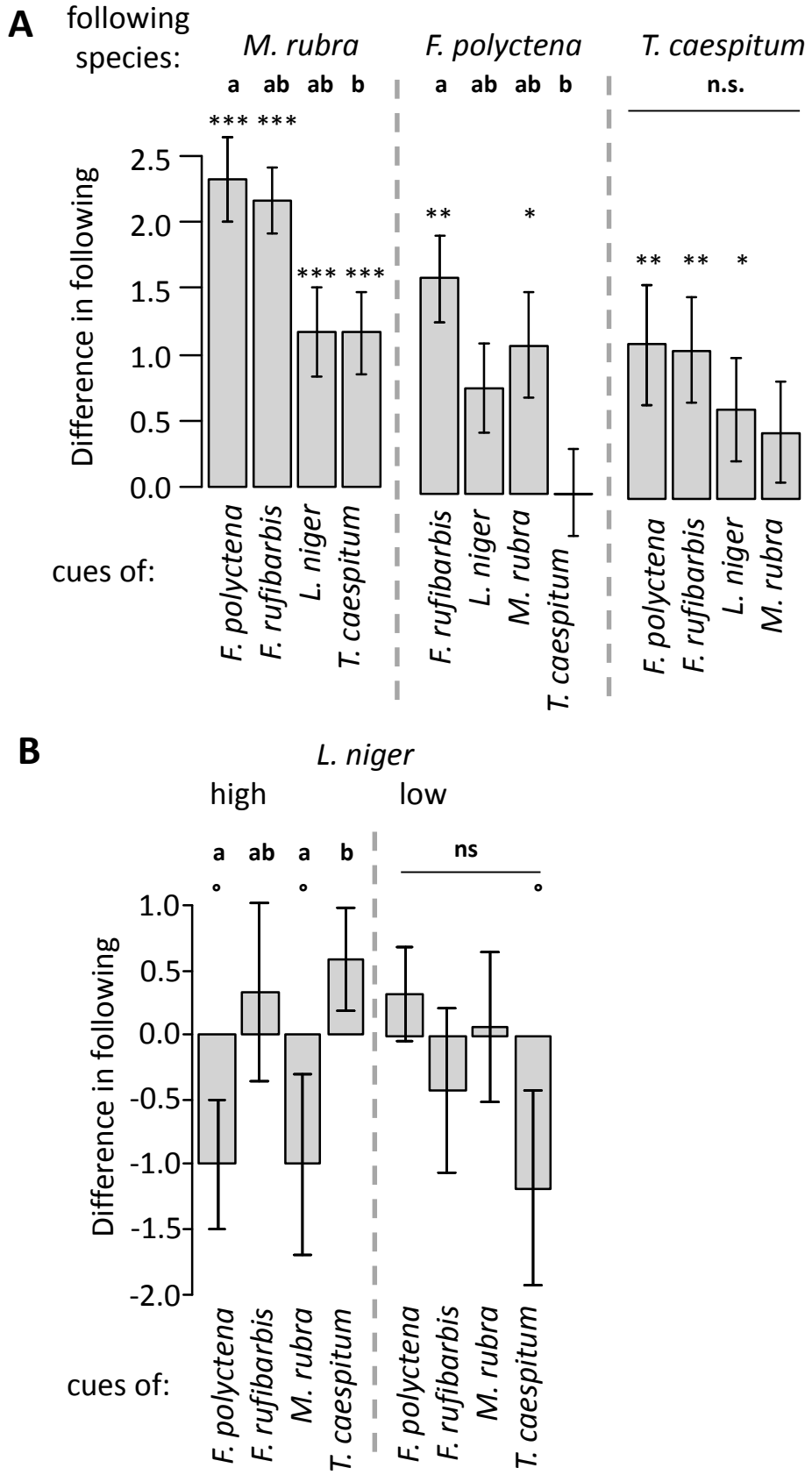

A
following species:
M. rubra
F. polyctena
T. caespitum
a ab ab b
a ab ab b
n.s.
*** ***
*** ***
**
*
** ** *
Difference in following
2.5
2.0
1.5
1.0
0.5
0.0
cues of:
F. polyctena
F. rufibarbis
L. niger
T. caespitum
F. rufibarbis
L. niger
M. rubra
T. caespitum
F. polyctena
F. rufibarbis
L. niger
M. rubra
B
L. niger
high
low
a ab a b
ns
°
°
°
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
Difference in following
cues of:
F. polyctena
F. rufibarbis
M. rubra
T. caespitum
F. polyctena
F. rufibarbis
M. rubra
T. caespitum

Fig. 2

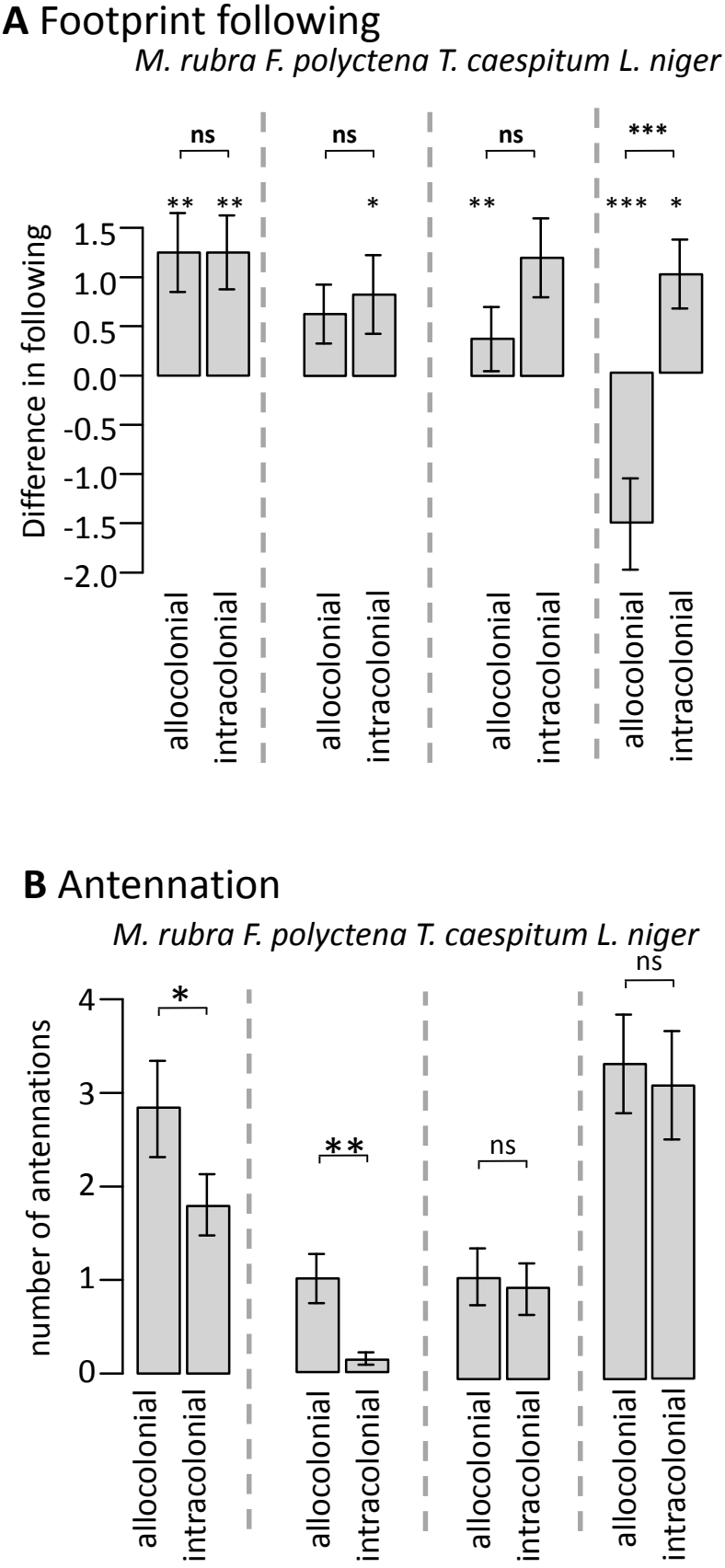

A Footprint following
M. rubra F. polyctena T. caespitum L. niger
Difference in following
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
ns
ns
ns
***
**
**
*
**
***
*
allocolonial
intracolonial
allocolonial
intracolonial
allocolonial
intracolonial
allocolonial
intracolonial
B Antennation
M. rubra F. polyctena T. caespitum L. niger
number of antennations
4
3
2
1
0
*
**
ns
ns
allocolonial
intracolonial
allocolonial
intracolonial
allocolonial
intracolonial
allocolonial
intracolonial

Fig. 3

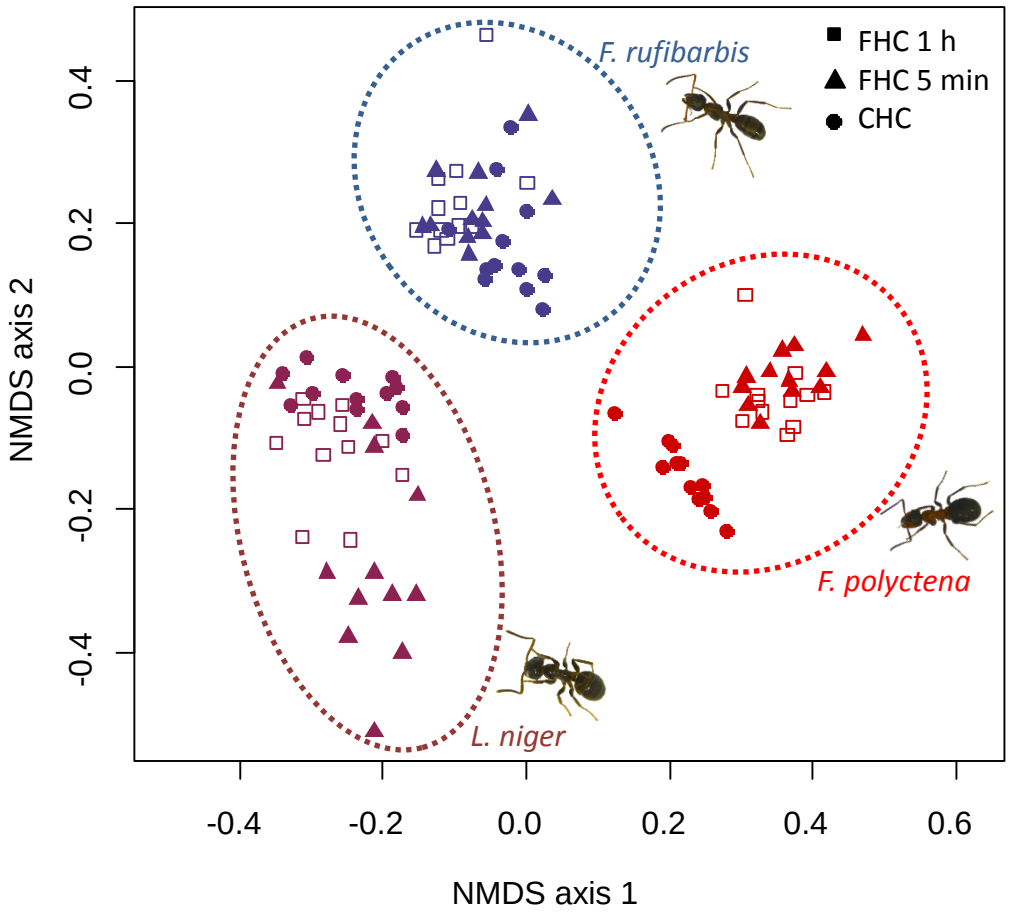
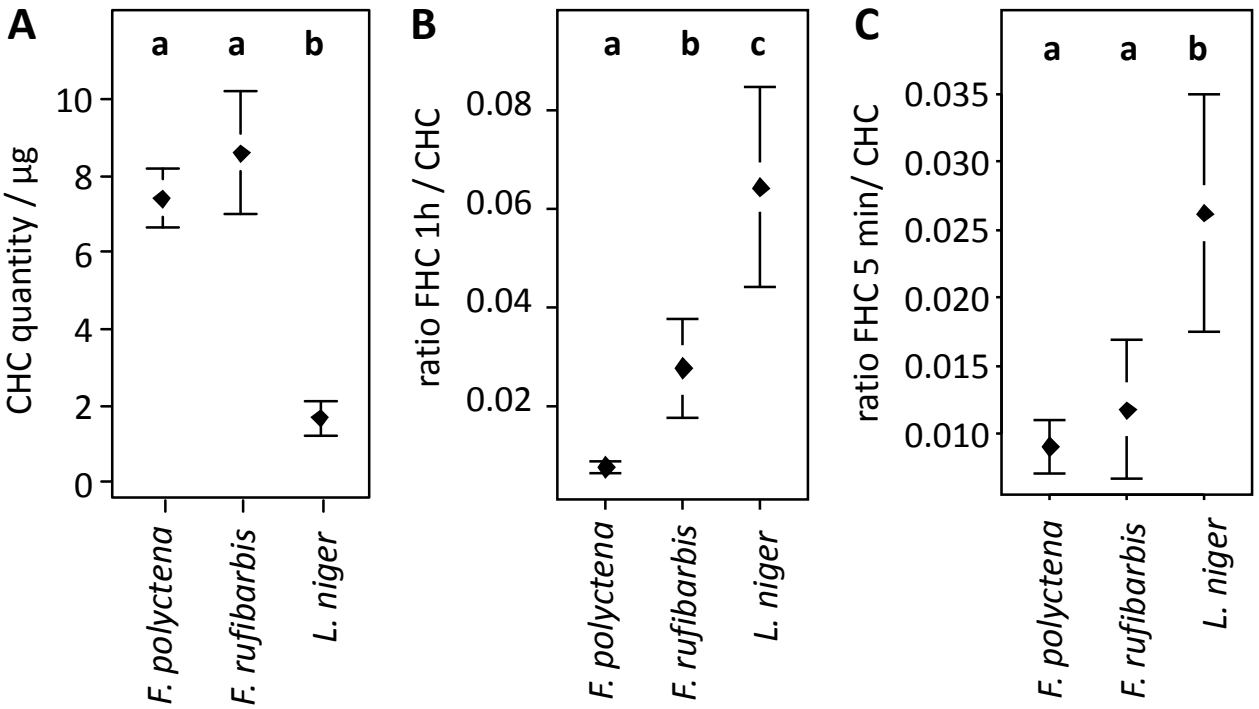

FHC 1 h
FHC 5 min
CHC
F. rufibarbis
F. polyctena
L. niger
NMDS axis 1
NMDS axis 2
-0.4
-0.2
0.0
0.2
0.4
0.6

Fig. 4


A
a
a
b
CHC quantity / µg
0
2
4
6
8
10
F. polyctena
F. rufibarbis
L. niger
B
a
b
c
ratio FHC 1h / CHC
0.02
0.04
0.06
0.08
F. polyctena
F. rufibarbis
L. niger
C
a
a
b
ratio FHC 5 min/ CHC
0.010
0.015
0.020
0.025
0.030
0.035
F. polyctena
F. rufibarbis
L. niger