Chemical deterrent enables a socially parasitic ant to invade multiple hosts

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Social parasites are involved in a coevolutionary arms race, which drives increasing specialization resulting in a very narrow host range. The Formicoxenus ants are a small group of social parasites with a xenobiotic lifestyle. Formicoxenus quebecensis and Formicoxenus provancheri are highly specialized ants using chemical mimicry to blend into their respective Myrmica ant host colonies. However, Formicoxenus nitidulus is unique in being able to survive in over 11 different ant host species. We observed that when live or dead F. nitidulus adults are seized by their host they are immediately dropped undamaged, despite possessing a cuticular hydrocarbon profile that differs markedly from its host. Hexane extracts of the F. nitidulus cuticle made previously acceptable prey items unattractive to their Formica host, indicating a chemical deterrent effect. This is the first time that a social parasite has been shown to exploit the generalized deterrence strategy to avoid host aggression over long periods of time. This supports the idea that coevolved and generalist diseases or parasites require fundamentally different defence mechanisms. We suggest that F. nitidulus uses its cuticular chemistry, possible alkadienes, as a novel deterrent mechanism to allow it to switch hosts easily and so become a widespread and abundant social parasite.

Keywords: Formicoxenus nitidulus; chemical deterrent; alkadiene; cuticular hydrocarbons; coevolution

1. INTRODUCTION

All parasites are locked into a coevolutionary arms race with their host as their interests are diametrically opposed. Overcoming complex recognition systems has led to increasing specialization and narrow host ranges. This is not only exemplified by the bird cuckoos (Brooke & Davies 1988; Marchetti et al. 1998), but also occurs in insects that parasitize social insect colonies (Lenoir et al. 2001). In the vast majority of cases in social insects where two unrelated species inhabit the same colony, the cuticular hydrocarbon (CHC) profiles, which encode the recognition signals (Howard & Blomquist 2005), are similar, leading to the conclusion that ‘chemical mimicry is responsible for mutual tolerance’ (Lenoir et al. 1997). Integration into a host colony by a social parasite occurs by two different methods: ‘chemical camouflage’, where the parasite acquires the colony odour from the host species or the nest surroundings by allogrooming, or ‘chemical mimicry’, where the intrinsic biosynthesis of host CHCs by the parasite actively reproduces the host’s odour profile (Dettner & Liepert 1994). This may explain why most social parasites are exceedingly rare in comparison with their hosts (Elmes et al. 1999; Thomas et al. 2006).

The small genus of Formicoxenus ants are xenobionts rearing their brood within the host colony and stealing its resources such as food and space. The brood of xenobionts are reared in separate chambers where the host workers cannot physically enter due to their small size. The Formicoxenus ants represent one of the best examples of chemical mimicry through odour acquisition since Formicoxenus provancheri and Formicoxenus quebecensis are strongly attracted to their respective ant hosts, Myrmica incompleta and Myrmica alaskensis, and spend up to 45% of their time licking the host adults and larvae, earning them the name ‘shampoo ants’ (Errard et al. 1997). This behaviour allows them to directly acquire their hosts’ colony odour (Lenoir et al. 1997). This results in a strong host specificity since strong inter-species aggression between all ants inhibits social parasites moving between species (Holdoebler & Wilson 1990). However, in Europe, Formicoxenus nitidulus, the only Formicoxenus species, is unusual in that it has been recorded from nine mound-building species of Formica wood ants (Formica rufa, Formica polyctena, Formica pisarskii, Formica lugubris, Formica aquilonia, Formica pratensis, Formica truncorum, Formica uralensis and Formica exsecta; Holdoebler & Wilson 1990; Busch 2001) as well as Polyergus rufescens and Tetramorium caespitum (Wilson 1971). Despite strong colony recognition between the wood ant species, F. nitidulus colonies are well integrated into their host colonies and adults are able to switch freely between mound-building Formica species when they occur in the same area. Unlike the shampoo ants, F. nitidulus appears to have very little direct interaction with its hosts and is largely ignored (Stumper 1918), but when they are seized by a host worker they are dropped rapidly and left unharmed (Robinson 2005). Stumper (1949) concluded that Formicoxenus must have a special odour whose perception does not elicit any hostile response. The system employed by F. nitidulus to avoid being killed or
damaged by the host must not be error prone since although encounters appear rare, they do occur and since F. nitidulus is continuously exposed to the hosts during its lifetime, it must survive being attacked on many occasions. This is a fundamentally different problem faced by social insect parasites that invade and usurp host colonies.

We hypothesize that some characteristic of the F. nitidulus cuticular chemistry deters a fully aggressive response in the Formica host workers. We investigated the CHC chemistry of F. nitidulus and used laboratory and field bioassays to investigate the behaviour of Formica hosts towards F. nitidulus.

2. MATERIAL AND METHODS

(a) Sample collection

During August 2005, samples of adult F. nitidulus ants and their Formica hosts were collected from Ballater, Aberdeenshire (Formica lugubris), Ashness, Cumbria (Formica lugubris), Arnside, Lancashire (Formica rufa) and Sheffield, South Yorkshire (Formica lugubris). A total of 3 winged queens (gynes), 11 workers and 28 male F. nitidulus ants were collected over the four sites, along with 5 host Formica workers from each colony. During August 2006, we collected approximately 200 F. nitidulus adults (workers and males) from the Sheffield site, for use in the bioassays. In F. nitidulus, workers and males are very similar in appearance, cuticular chemistry and behaviour, so in the bioassays we did not discriminate between the sexes and refer to them collectively as adults. Furthermore, all bioassays used Formica lugubris workers from the Sheffield population, which is parasitized by F. nitidulus.

(b) Interaction bioassays

Direct interactions between Formica lugubris and F. nitidulus adults were rarely observed in the field. We therefore created an artificially high encounter rate in the laboratory by placing one Formica lugubris adult in a 50 mm diameter Petri dish containing 10 live F. nitidulus adults. If the Formica lugubris worker seized an F. nitidulus adult, picked it up and then immediately dropped it, then this was classified as ‘dropped’, whereas it was classified as ‘carried’ if it was picked up and carried off. As soon as a behavioural interaction was observed, the Formica lugubris worker was replaced with a new individual. The behaviour of 20 different Formica lugubris individuals were observed with each trial lasting between 1 and 5 min. To exclude the possibility of avoidance behaviour, we introduced 20 freshly killed F. nitidulus adults individually onto a 50 mm² piece of white paper placed near a busy foraging trail of a Formica lugubris. We then recorded the number of dead F. nitidulus that were either dropped or carried. We then tried to remove or at least reduce the amount of CHC from F. nitidulus adults by washing 10 dead F. nitidulus adults in HPLC-grade hexane for 10 min and recorded whether they were carried or dropped using the foraging trail bioassay just described.

Owing to the large number of foraging host workers, all bioassays were completed within a 5 to 10 min period and it is unlikely that the behaviour of the same host worker was recorded more than once.

(c) Formicoxenus extract bioassay

A CHC extract of F. nitidulus was prepared by placing 50 adults in 300 µl of HPLC-grade hexane for 10 min. Fifteen microlitres of extract were then applied via a Hamilton syringe to each of 20 fruitflies (Drosophila pseudoobscura) that had previously been washed in hexane for 10 min to remove their own CHC profile. The application of 15 µl of extract to each fruitfly is roughly equivalent to one ant, as the dry weight of an F. nitidulus (1–2×10⁻³ g) is two to three times lower than that of a fly. A further 20 washed flies were treated only with 15 µl of hexane and used as the control. The flies were offered to Formica lugubris workers using the same methodology as the dead F. nitidulus ants and their behaviour towards the two groups of flies was recorded as dropped or carried.

(d) Chemical analysis

Individual F. nitidulus or Formica lugubris/rufa ants were placed into vials containing 30 or 50 µl of hexane, respectively. After 10 min, the ants were removed, hexane evaporated and the vials sealed and stored at 5°C. Prior to analysis, 30 µl of hexane was added to the vial and the sample analysed on an HP 6890 GC (equipped with an HP-5MS 30 m column) connected to an HP 5973 MSD (mass spectrometer). The oven was programmed from 70 to 200°C at 40°C min⁻¹ and then from 200 to 320°C at 15°C min⁻¹ and held for 2 min at 320°C. CHCs were characterized by the use of standard MS databases, diagnostic ions and their Kovats indices. Double-bond positions were determined by DMS derivatization of a pooled extract of 10 F. nitidulus adults.

(e) Data analysis

The peak area for each compound was calculated for each ant and then normalized by calculating the percentage abundance of each compound in the sample, i.e. as a proportion of all compounds present. For each group (queens, workers or males) at each location, an average profile was calculated by taking the mean percentage for each compound across all the ants in that group. These data were then collated with the published data (Lenoir et al. 2003) in order to investigate the wider relationships between Formicoxenus and their hosts. To determine the level of similarity of the CHC profile of the three Formicoxenus spp. and their hosts, we used hierarchical cluster analysis (Euclidean distances, Ward’s method) to construct a single linkage dendrogram (SPSS v. 14) using all 77 CHCs described from this study and published data (Lenoir et al. 1997).

3. RESULTS

(a) Bioassays

Our field studies confirm previous observations (Robinson et al. 2005) that all adult F. nitidulus ants (queens, males and workers) are largely unmolested by their much larger hosts but if seized by a Formica lugubris or Formica rufa worker they are rapidly dropped unharmed. In both our laboratory and field bioassays, we found that in all encounters between Formica lugubris workers and F. nitidulus adults, living (20 out of 20 cases) or dead (20 out of 20 cases), resulted in F. nitidulus

| Table 1. Behaviour of Formica lugubris workers towards Drosophila fruitflies that had been treated with a hexane control or a hexane extract of the cuticular hydrocarbons of Formica nitidulus. |
|---|---|---|
| flies carried | flies dropped |
| hexane only | 17 | 3 |
| hexane+F. nitidulus CHC extract | 4 | 16 |
being immediately dropped. This indicates that *F. nitidulus* possesses a strong and effective deterrent that remains functional in the absence of other behaviours. We were able to transfer this effect to prey items. The application of *F. nitidulus* cuticle extract to fruitflies significantly decreased ($\chi^2$-test with Yates correction: $\chi^2 = 14.44$, $p = 0.001$, $n = 40$) their attractiveness to *F. lugubris* foragers. Only 15% of control flies were dropped compared with 80% of the *F. nitidulus* extract-treated flies (table 1). Furthermore, by washing *F. nitidulus* adults in hexane for only 10 min, we were able to partially remove the deterrent effect, since the number of *F. nitidulus* ants picked up and carried away increased significantly ($\chi^2$-test with Yates correction: $\chi^2 = 10.7$, $p = 0.001$, $n = 60$) from 0 out of 50 cases (untreated) to four out of 10 cases (washed).

(b) **Comparison of hydrocarbon profiles**

The CHC profile of *F. nitidulus* and their *Formica* hosts was strikingly different (figure 1; electronic supplementary material). The CHC profile of all adult *F. nitidulus* ants ($n = 42$) were similar irrespective of sex ($n = 2$), caste ($n = 2$), host ($n = 2$) or location ($n = 4$), and always contained a high proportion (16–43%) of alkanes ($C_{23}-C_{33}$), alkenes ($C_{25:1}-C_{33:1}$), alkadienes ($C_{29:2}-C_{33:2}$) and methyl-branched alkanes (e.g. $3MeC_{25}$). The compounds present are identified as alkanes ($C_{23}-C_{33}$), alkenes ($C_{25:1}-C_{33:1}$), alkadienes ($C_{29:2}-C_{33:2}$) and methyl-branched alkanes (e.g. $3MeC_{25}$). See the electronic supplementary material for proportions of each cuticular hydrocarbon (CHC). The $12MeC_{30}$ was not detected consistently in all *F. nitidulus* ants (see electronic supplementary material).

For *M. incompleta* and *M. alaskensis*, we found the CHC of *F. nitidulus* and their hosts was more strongly similar to that of their *Formica* hosts. In fact, the CHC profile of *F. provancheri* and *F. quebecensis* and their hosts *M. incompleta* and *M. alaskensis* (Lenoir et al. 1997) is more strongly dissimilar to the CHC of their *Formica* hosts (figure 2). The number of individual ants analysed is given in parentheses. W, worker; M, male; Q, queen.

Figure 1. Comparison of typical total ion chromatograms for a *F. lugubris* host worker and *F. nitidulus* worker from the same colony. The compounds present are identified as alkanes ($C_{23}-C_{33}$), alkenes ($C_{25:1}-C_{33:1}$), alkadienes ($C_{29:2}-C_{33:2}$) and methyl-branched alkanes (e.g. $3MeC_{25}$). See the electronic supplementary material for proportions of each cuticular hydrocarbon (CHC). The $12MeC_{30}$ was not detected consistently in all *F. nitidulus* ants (see electronic supplementary material).

Figure 2. Hierarchical cluster analysis (single linkage, Euclidean distances, Ward’s method) of the CHCs of *F. nitidulus* and their hosts *F. lugubris* and *F. rufa* (this study) from four locations in the UK combined with the data on *F. provancheri* and *F. quebecensis* and their hosts *M. incompleta* and *M. alaskensis* (Lenoir et al. 1997). The chemical dissimilarity between *F. nitidulus* and their *Formica* hosts became very clear when we included *F. provancheri* and *F. quebecensis* and their *Myrmica* hosts in the analysis. Our dendrogram (figure 2) shows that *F. provancheri* and *F. quebecensis* have an almost identical CHC profile to

Formica hosts to be strikingly different. This strongly suggests that chemical mimicry is not being used by F. nitidulus and explains the lack of chemical congruency found between F. nitidulus and Fo. rufa (Lenoir et al. 2001). The results of our bioassays confirm previous observations that when F. nitidulus is seized by a Fo. rufa (Robinson 2005) or Fo. lugubris (this study) worker, it is rapidly dropped unharmed. Our study shows that this deterrent effect is probably attributable to a chemical cue found on the cuticle, because dead F. nitidulus ants are dropped on contact rather than simply avoided. Furthermore, we were able to transfer the deterrent effect via a hexane CHC extract of F. nitidulus to make previously attractive prey (fruitflies) unattractive. This suggests that the flies were not simply ignored but had become distasteful. If flies or F. nitidulus ants had been ignored, there is a possible interpretation that F. nitidulus is chemically insignificant (Lenoir et al. 2001), but this was clearly not the case. In contrast to previous studies of social parasites, including that of the closely related species F. provancheri and F. quebecensis, we found the CHC profile of F. nitidulus is dominated by alkanes and alkenes (figure 1; electronic supplementary material). Alkenes are known to be used as recognition cues in insects (Lockey 1988; Akino et al. 2004; Howard & Blomquist 2005) and so we reiterate that it is highly unlikely that F. nitidulus are chemically insignificant to their hosts.

The presence of large amounts of normally rare alkadienes (Akino 2006) in the CHC profile of all F. nitidulus adults makes them good candidates for further investigation of their deterrent effect. Many social wasps (Polistes, Vespa and Stenogastra) also secrete substances that protect their colonies against attack from ants (Jeanne 1970; Martin 1991). These ant repellents are also composed of hydrocarbons (Sledge et al. 2000; Dani et al. 2003), although the specific hydrocarbons that act as deterrents have yet to be determined. Although we cannot completely rule out other compounds such as esters and fatty acids, which have also been suggested as acting as ant repellents (Henderson & Jeanne 1989), their absence from all F. nitidulus GC traces (figure 1) makes it unlikely.

We confirm that two very different strategies have evolved within the Formicoxenus genus, which enables them to survive within their respective host colonies (Lenoir et al. 2001). First, the socially parasitic shampoo ants, F. provancheri and F. quebecensis, closely mimic the chemical profiles of their respective ant hosts (M. incompleta and M. alaskensis), and so are obligately associated with their host species (Lenoir et al. 1997) and accordingly cannot survive within Formica colonies (Buschinger 1976). In contrast, the chemical deterrent used by F. nitidulus allows it to inhabit at least nine mound-building species of Formica wood ants as well as Polyergus rufescens and Tetramorium caespitum. F. nitidulus can also survive in laboratory-maintained Leptothorax acervorum nests (Buschinger 1976). This suggests that the natural restriction of F. nitidulus to mound-building Formica species may be a function of its ecology rather than its chemistry. However, L. acervorum, a species closely related to Formicoxenus, is unusual in possessing a CHC profile similar to F. nitidulus, i.e. one dominated by the same alkadienes and alkenes (Kaib et al. 1993), so chemical mimicry could also explain the mixed laboratory nests of L. acervorum and F. nitidulus. The evolution of a chemical deterrent in some Formicoxenus species may have occurred prior to their invasion of into Europe and Asia, since the Nearctic species, Formicoxenus diversipilus and Formicoxenus hirticornis, also inhabit the thatched mounds of several Formica spp. (Francoeur et al. 1985) and share a similar biology to F. nitidulus (Alpert & Akre 1973).

Although queens of social parasitic ants (D’Ettorre et al. 2000; Ruano et al. 2005) and bumble-bees (Zimma et al. 2003) use chemicals as a repellent or deterrent, these are short lasting propaganda substances produced by the Dufour’s or poison gland, which allow the parasite to invade and usurp the host colony or steal its brood (Lenoir et al. 2001). Therefore, the use of a cuticular ant deterrent by F. nitidulus, which allows it to survive within the host nests over long periods of time, is a novel strategy among social parasites. Such a strategy has clear advantages for exploiting a wide host range, but this is at odds with the apparent rarity of this strategy. Clearly, deterrence is a successful strategy for F. nitidulus, which is a widespread and abundant social parasite, with some host Formica mounds parasitized by over 100 F. nitidulus colonies (Buschinger et al. 1994). However, this is in stark contrast to the specialized chemical mimicry of F. quebecensis, which is regarded as a rare species, occurring in only a few locations (Buschinger et al. 1994). These findings lend weight to the idea that coevolved and generalist diseases or parasites require fundamentally different defence mechanisms (Boomsma & Franks 2006). The use of chemical deterrents to avoid predation is widespread among insects, but this is the first time it has been recorded in any of the predicted 10 000–20 000 species of insects that have evolved as social parasites of ants (Thomas et al. 2006). However, it is possible that other generalized xenobionts of ants use a similar mechanism, such as the Myrmecophila crickets (Holdodoler & Wilson 1990).

It is usually assumed that chemical mimicry is responsible for mutual tolerance in most social insect parasites (Lenoir et al. 1997, 2001), and when an ant species becomes an obligate parasite it seems to evolve quickly into a state of abject dependence on its host (Wilson 1971). However, our findings reveal another mechanism of social parasitism that allows the exploitation of multiple host species. We suspect that the use of cuticular-based deterrents by insect social parasites may be a more widespread strategy than previously thought.

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REFERENCES


NOTICE OF CORRECTION

The author names are now presented in the correct form.

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