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Novel fungal disease in complex leaf-cutting ant societies

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Abstract. 1. The leaf-cutting ants practise an advanced system of mycophagy where they grow a fungus as a food source. As a consequence of parasite threats to their crops, they have evolved a system of morphological, behavioural, and chemical defences, particularly against fungal pathogens (mycopathogens).

2. Specific fungal diseases of the leaf-cutting ants themselves have not been described, possibly because broad spectrum anti-fungal defences against mycopathogens have reduced their susceptibility to entomopathogens.

3. Using morphological and molecular tools, the present study documents three rare infection events of *Acromyrmex* and *Atta* leaf-cutting ants by *Ophiocordyceps* fungi, a genus of entomopathogens that is normally highly specific in its host choice.

4. As leaf-cutting ants have been intensively studied, the absence of prior records of *Ophiocordyceps* suggests that these infections may be a novel event and that switching from one host to another is possible. To test the likelihood of this hypothesis, host switching was experimentally induced, and successfully achieved, among five distinct genera of ants, one of which was in a different sub-family than the leaf-cutter ants.

5. Given the substantial differences among the five host ants, the ability of *Ophiocordyceps* to shift between such distant hosts is remarkable; the results are discussed in the context of ant ecological immunology and fungal invasion strategies.

Key words. Ants, defence, ecological immunology, horizontal transmission, *Ophiocordyceps*, parasitism, plasticity, societies.

Introduction

Leaf-cutting ant societies represent one of the pinnacles of social living (Wilson, 1983, 1985). Their societies may comprise up to five million individuals that typically occupy huge subterranean nests, in which they practice agricultural mycophagy. The ancient practice of rearing fungus for food originated only once in the attine ants, around 50 million years ago (Schultz & Brady, 2008). The evolutionarily derived leaf-cutting ant genera *Atta* and *Acromyrmex*, rear their fungal symbionts on fresh leaf fragments that the ant workers cut and bring back to the nest. The evolutionarily less derived genera also grow fungus, but they use more varied material such as flowers, leaf litter, and dead insect parts as substrate. As with any intensive monoculture agricultural system, disease is a constant threat (Wolfe *et al.*, 2007). In order to protect their food fungus from being overrun by specialist and generalist pathogenic fungi (e.g. *Escovopsis* and *Trichoderma* respectively), the fungus-growing ants evolved a symbiotic partnership with antibiotic producing bacteria shortly after they adopted fungus-farming (Currie *et al.*, 1999 , 2006). This, in combination with effective behavioural defences (Little *et al.*, 2005), limits the emergence of agricultural disease inside the colony.

Vigorous general defences against parasitic fungi of food crops (i.e. mycopathogens) could indirectly enhance the exclusion of parasitic fungi of the ants themselves (i.e. entomopathogens). Leaf-cutting ants have the ability to recognise and avoid generalist

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insect pathogens such as *Metarhizium* (Jaccoud *et al.*, 1999), which is similar to their defence-style response when encountering spores capable of infecting their fungus gardens (Little *et al.*, 2005). Similar to almost all other ants, they have also paired exocrine metapleural glands that produce broad-spectrum antibiotics to protect themselves, their nestmates and even occasionally their fungus gardens against fungal spores and other disease agents (Fernández-Marín *et al.*, 2006). Finally, the very structure of a large social insect colony limits the spread of infectious agents, because of their organisational immunity (Cremer *et al.*, 2007; Naug & Smith, 2007). It is therefore possible that non-stressed long-lived ant colonies are to a large degree, devoid of pathogens and not conducive to their spread (Boomsma *et al.*, 2005).

The principal pathogenic fungi infecting ants (and other insects) belong to the genus *Cordyceps* (Evans, 1982; Sung *et al.*, 2007). There are at least 400 *Cordyceps* species and they infect a wide host range comprising nine orders of insects, one order of spiders and also other fungi. *Cordyceps* infections are highly virulent and kill their hosts as a developmental necessity to produce aerial structures from which spores are dispersed to infect new hosts. The fungal life-cycle is always direct, so that no other hosts are involved. Recently, a multi-gene study confirmed the long-standing suspicion of the genus being paraphylectic (Sung *et al.*, 2007). Three monophyletic genera were proposed and all ant-associated *Cordyceps* were placed in *Ophiocordyceps*. Therefore, this name will be used throughout the present paper.

During fieldwork, we found *Ophiocordyceps* parasitising leaf-cutting ants in Panama. As no such cases have been reported before, the present study set out to discover whether this finding represented a novel emergent disease in leaf-cutter societies. We sequenced some of the strains to assess their genetic similarity and we attempted to reproduce host-shifting events in the laboratory. The results are used to appraise aspects of ant–fungal parasite evolutionary biology in relation to insect host defences.

Materials and methods

Ophiocordyceps *in leaf-cutting ants*

Queens of *Acromyrmex octospinosus* (Q1) and *Atta colombica* (Q2) infected by *Ophiocordyceps* were found during fieldwork in Gamboa, Panama, in May 2005 and 2006, respectively. Q1 was collected when she already had an incipient nest, and Q2 was collected 1 day after her mating flight. Both ants died shortly after having been brought into the laboratory, and within 7 days after death, fungal stroma were observed growing from them (Fig. 1). Q1 was surface sterilised after death by placing it in 70% ethanol for a few seconds, before rinsing briefly in three changes of distilled water. The ant was then left in 1% NaClO for 1 min, blotted dry with sterile filter paper, and transferred to a Petri dish with damp filter paper and a wet cotton wool ball. It was left at ambient Panamanian air temperature (approx. 25 °C) and the characteristic aerial growth of multiple hyphae, i.e. the *Ophiocordyceps* stroma, was noted 7 days later. Q2 was not surface sterilised, and was discovered dead with a stroma growing out of her, 6 days after having been set up (alive) in a nest box containing moist soil. In 2007, a third infected queen, Q3, of *A. octospinosus* was discovered in a natural soil nest cavity with a stroma growing out of her. Finally, in 2006, a dead *Acromyrmex* sp. Worker (W1) was found with its mandibles clamped onto a tree in Vicosa, Minas Gerais, Brazil. It was not showing any external signs of infection, but when dissected it was found to contain fungal structures characteristic of *Ophiocordyceps* infection.

Determining the number of strains of Ophiocordyceps *in leaf-cutter ants*

DNA was extracted both from the fungi growing from the dead ants and from fungal cultures that were grown on agar

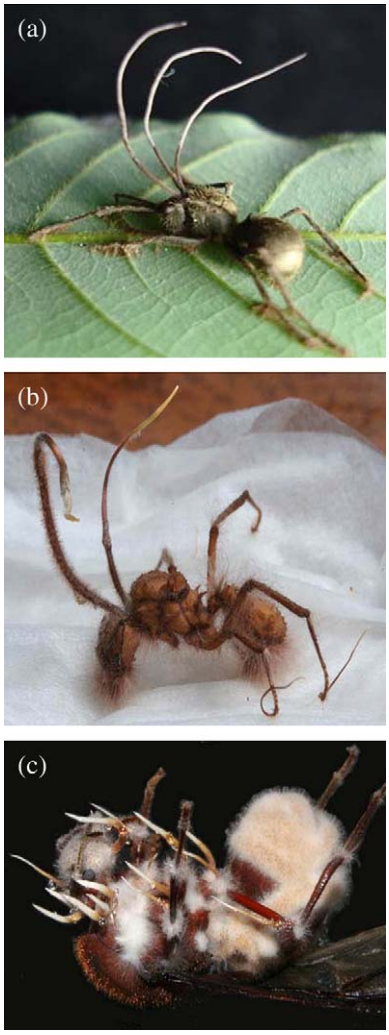

Fig. 1. Ants displaying natural infections by *Ophiocordyceps*. (a) *Polyrachis* spp. from Thailand with *Ophiocordyceps* (*Hirsutella*) *unilateralis* emerging from the head; (b) *Acromyrmex octospinosus* queen from Panama with two *Ophiocordyceps stilbelliformis* stroma erupting from between the head and pronotum; note also the fungus growing out of the ends of the legs; (c) *Atta colombica* also from Panama, displaying multiple stroma of *Ophiocordyceps* (*Hirsutella*) *subramanianii* and hyphal matting especially visible on the abdomen. Note that in each of the cases the fungus is in the asexual (anamorphic) state.

plates (culturing details below). Extractions were carried out with cetyltrimethylammonium bromide (CTAB) after grinding the tissue in liquid nitrogen. Fungal genes from the three infected queens were sequenced using the conserved primers EukF1 and EumycR2, and cycling conditions detailed in Van Borm and Boomsma (2002). DNA was also extracted from clumps of mycelium taken from agar plates. Fungal tissue from artificially infected ants (see below) was removed, had its DNA extracted and sequenced using the ITS region (primers ITS1, ITS4), because this gene is less conserved and thus more informative for grouping related fungi into species. The cycling conditions of White *et al.* (1990) were followed. All sequencing was direct and done without a cloning step. Alignments of the two obtained sequences was carried out using 'Blast2Sequences' available from GenBank. The strain infecting the *Acromyrmex* worker from Brazil could not be sequenced as export permits were not available.

Artificial host jumping

Ophiocordyceps cultures were initiated from Q1 and Q2 by transferring pieces of fungal tissue from the cadavers of the ants onto potato dextrose agar (PDA) plates that were subsequently kept upside down at 26 °C. After approximately 3–4 weeks, the fungus was producing infective asexual spores, which allowed us to perform two separate infection experiments.

Infection of Atta *and* Acromyrmex *workers*

The first experiment was to test if laboratory infections were possible and took place in Copenhagen, Denmark in June 2006. It included 10 workers of *A. colombica* from colony Ac20, and six workers of *A. octospinosus* from colony Ao169. Both colonies originated from Gamboa, Panama and were collected in 2004 and 2002, respectively. Workers were infected with spores originating from Q1 and Q2.

Infection of workers of five ant species

The second experiment was performed 4 months later and included five species of ants (colony identity codes are indicated in parentheses): *A. octospinosus* (Ao169), *A. colombica* (Ac20), two other species of fungus-growing ants – *Sericomyrmex amabilis large species* (Sam2005-1L, collected in 2006 from Gamboa, Panama) and *Apterostigma small species 1 'collare'* (Aco2006-9, collected in 2006 from Gamboa, Panama), and a European ant species – *Camponotus vagus* (Cv5, collected in 2006 near Imola, Italy). These ants were only infected with *Ophiocordyceps* that originated from Q1. To do this, 20 ants of each species were removed from their source colonies (one colony per species) and weighed to the nearest 0.1 mg. Taking the species possessing the extremes of weight, the ants varied by around 1.4 orders of magnitude (*Apterostigma* average = 0.6 mg; *C. vagus* average = 23.2 mg). Half of the ants were treated with *Ophiocordyceps* spores and half of the ants were left as controls. Treatment ants were chilled on ice to restrict their movement, and controls were sham treated. In total, six *Ophiocordyceps* culture plates were used for the infections, of which three had *Ophiocordyceps* in the Ariel Hyphae Conidia stage (AHC), and three had *Ophiocordyceps* in the Stilbelliformis Conidia stage (SC). As such multiple morphological spore types are commonly found in fungi, the asexual spores of both types were used.

While placed under a stereomicroscope, each ant had its pronotum rubbed five to six times against the spore-producing part of the *Ophiocordyceps* plate. Since the aim was not to test the differential infection ability of diverse spore types, but simply to expose worker ants to the range of spore types that were observed, a new untouched part of the *Ophiocordyceps* culture was used for each ant infection. Each ant was thus infected with only one 'type' of conidia, and each 'set' of five ants (a 'set' comprising one of each species of ants) was always infected from the same plate. Control ants were treated similarly, but did not have contact with the *Ophiocordyceps* plate. All ants were placed in individual tubes (40 mm high × 28 mm diameter) with a piece of damp cotton wool to maintain a high humidity. The ants were placed in an incubator at 26 °C and checked daily for 16 days for deaths and the growth of fungus from their joints. Ant survival was analysed using a Cox proportional hazards regression model in R (Development Core Team R, 2008) and ants that were still alive after 16 days were included in the model as censored cases. A model was fitted with day of death as the response variable, treatment and species as factors, and weight as a covariate.

To determine via molecular methods whether the infections which had been successful were due to the same fungus isolate that was applied, we took any fungal material that appeared morphologically similar to *Ophiocordyceps* and carefully removed it from the ant without touching the cuticle. This was then sequenced as described above. The fungus has a characteristic brown tint to its hyphae which made recognition relatively easy (Figs 1 and 2).

Results

Ophiocordyceps *is found in leaf-cutting ants and occurs in multiple strains*

The phenotype expressed by the fungus growing out of Q1 and Q2 was the asexual or anamorphic state, i.e. *Hirsutella*: the designation *Ophiocordyceps* refers to the sexual state. To convey this additional piece of information, we use the genus name *Ophiocordyceps* (*Hirsutella*) when information from both sexual and asexual stages is available. The asexual phenotypes could be further distinguished morphologically based upon the spores grown in agar culture (data available on request). Those from Q1 and Q3 (*A. octospinosus*) were *Hirsutella stilbelliformis* and those from Q2 (*A. colombica*) were *Hirsutella subramanianii* (Evans, 1985). The *Acromyrmex* worker discovered in the Mata Atlantica rainforest of Brazil, shows that workers can become naturally infected. In this case, no external stroma was seen but a dissection revealed the presence of a glistening white mass of hyphal bodies that are characteristic of *Ophiocordyceps* infection.

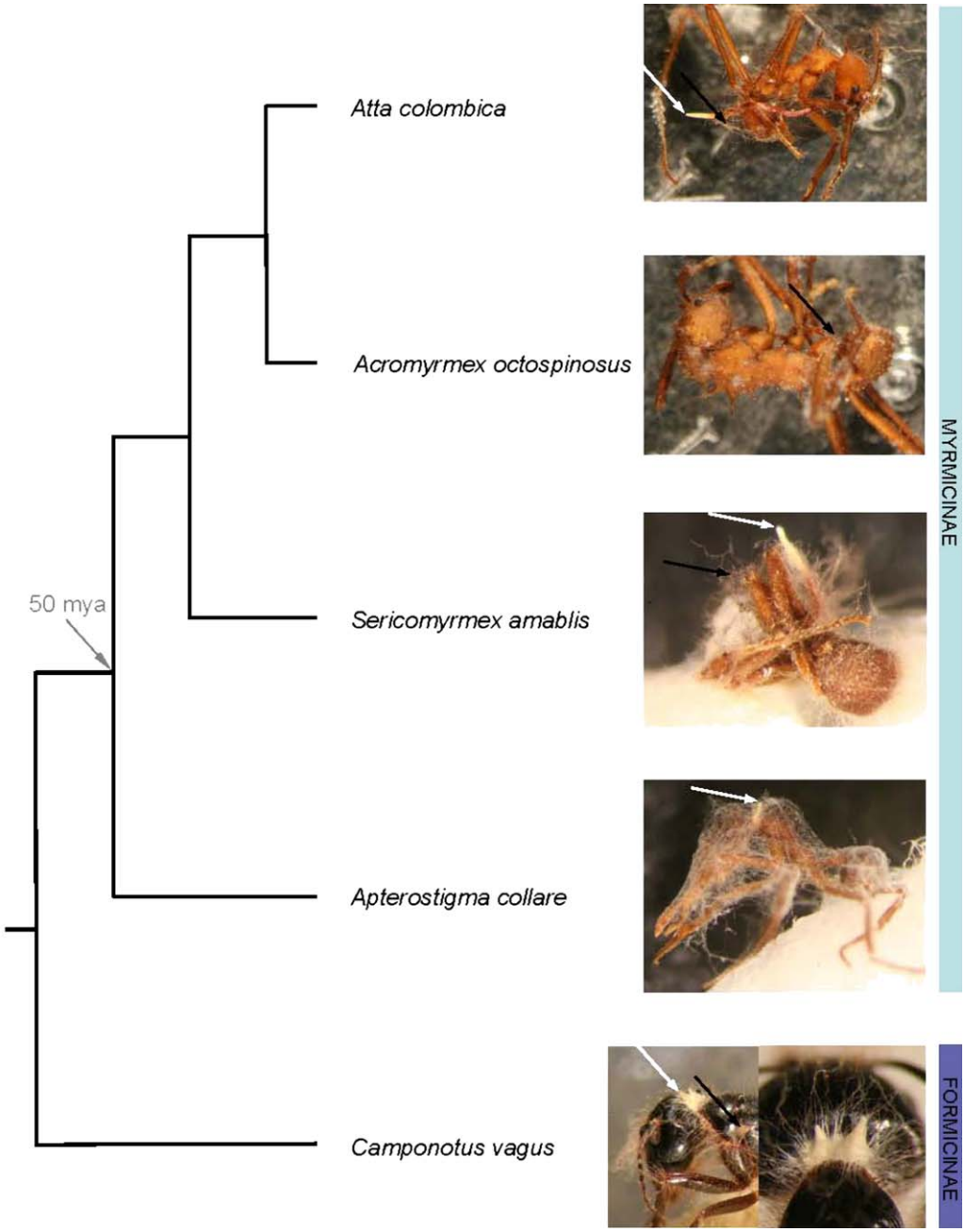

Fig. 2. Phylogenetic relationships between the experimentally infected ants. Simplified phylogenetic tree of the ant hosts that were experimentally infected with *Ophiocordyceps*. The date on the tree is an estimate of the origin of the attine tribe of the fungus-growing ants (Schultz & Brady, 2008). The upper four species belong to the subfamily Myrmicinae, whereas *Camponotus vagus* belongs to the sister subfamily, the Formicinae. White arrows indicate stroma, and black arrows indicate mycelia.

This was confirmed when the hyphal bodies, which were aseptically excised from the abdomen and transferred to nutrient agar, produced colonies in vitro with fruiting structures diagnostic for *Ophiocordyceps stilbelliformis* (Evans & Samson, 1982).

There was considerable difference in sequences between *Ophiocordyceps* infections found in the three queen ants (roughly 8–9% difference in base pair sequences, Table 1). These three strains were very different from a fungal strain (recorded as '*Cordyceps*-like') previously identified in *Acromyrmex* ants (Van Borm *et al.*, 2002). Our three sampled ant queens were infected by three different *Ophiocordyceps* strains.

Artificial host jumping

In the first infection experiment, we successfully infected 4 out of 10 *A. colombica* workers and 4 out of 6 *A. octospinosus*

workers, showing that laboratory-based infections of leaf-cutting ants is feasible and repeatable. Using the ITS1 primers, a 100% match was established between the *Ophiocordyceps* used to infect *A. colombica* and *A. octospinosus* workers, and the fungus growing from successfully infected *A. colombica* and *A. octospinosus* workers ($n = 1$ and 3 respectively).

In the second infection experiment, control ants lived significantly longer than ants that had been infected with *Ophiocordyceps* (Cox proportional hazards: $z = 2.50$, $P = 0.013$). Overall, there was a borderline significant effect of weight upon survival ($z = -1.96$, $P = 0.050$). Some species differed significantly in their survival in direct comparisons: *A. collare* ($z = -3.17$, $P = 0.0015$) and *C. vagus* ($z = -3.04$, $P = 0.0023$) lived significantly longer than *A. octospinosus*. None of the control ants developed what could be identified morphologically as an *Ophiocordyceps (Hirsutella)* stroma or hyphae. However, nine of the *Ophiocordyceps*-treated ants were successfully infected. Seven of these (three *A. colombica*, one *S. amabilis*, one *A. collare* and two *C. vagus*) had one or two *Hirsutella* stroma growing out between the head and thorax of their dead body, whereas two showed only hyphal growth (one *A. octospinosus* and one *C. vagus*) (Fig. 2). The ability of *Ophiocordyceps* to infect this broad range of hosts under artificial conditions is a novel finding. In particular the successful infection of a *Camponotus* ant which belongs to a different sub-family of ants from a different continent was surprising.

Discussion

The present study presented pathological and molecular evidence for a novel disease occurring in leaf-cutting ants. This disease, which is widespread among other ants in similar ecological conditions (Evans, 1982; Evans & Samson, 1982, 1984), has not been found previously in leaf-cutting ants, except for the four examples that have been described here. Given the high level of research on leaf-cutting ants and their symbiotic relationships with fungi (see e.g. Schultz *et al.*, 2005) and the Boomsma group's large research effort in Gamboa, Panama during the last 14 years, it would appear that *Ophiocordyceps* infections of leaf-cutting ants remain a rare event. Further work is needed to identify whether infection is in fact more common, but remains cryptic in many of the cases. If it were to be confirmed that infections are indeed rare, then understanding how infection is maintained in the population, and whether reservoirs of alternative host exist, would be interesting areas for further work. As the leaf-cutting ants are established model systems for the study of social evolution and symbiosis, *Ophiocordyceps* might also be a promising model to study infectious diseases in complex societies.

Queen ants were found infected after the nuptial flight and in one case, after a fungal (food) garden had been established. Queens are thought to be vulnerable to parasitic infections at the colony founding stage, due to multiple energetic demands that have to be met simultaneously (Baer *et al.*, 2006). In the early days of *Acromyrmex* colony founding, before the workers emerge, the queens must forage for leaves to nourish their fungal garden and at this stage they can presumably acquire infections. In addition, they could be exposed to infection whilst digging their nest. Only the latter would apply for *Atta* species where the queens do not forage, but rely entirely on their own body reserves to nourish the fungus garden.

The death position of ants may offer insight into the specificity of the host–parasite relationship. Ants infected with *Ophiocordyceps* are typically assumed to die in one of two ways (Evans, 1982; Evans & Samson, 1982, 1984). The first is to die away from the colony in the leaf-litter with a large fungal stroma arising from the body to discharge spores. The second also involves dying away from the colony, but makes the ant climb up the vegetation ('Summit disease') and eventually bite onto a surface such as the underside of a leaf, a tree trunk or a small branch before death. Both cases appear to be examples of adaptive parasite manipulation of host behaviour that benefits the fungus, because of more efficient spore dispersal. The abnormal behaviour of infected ant hosts is thus an extended phenotype of the fungus, *sensu stricto* Dawkins (1982). However, a recently discovered association between *Ophiocordyceps* (*Paraisaria*) and *Myrmica* ants in a temperate grassland ecosystem in southern England, has suggested that infected ants can die in less prominent places – hidden at soil level in grass swards or deposited in discrete middens (H. C. Evans *et al.*, in prep.). Of the two ants that were found to be infected in the field, the Brazilian *Acromyrmex* worker was clamped to a branch, but the Panamanian *A. octospinosus* queen was found within her excavated nest burrow (Henrik de Fine Licht, pers. comm.). The absence of an apparent behavioural change preceding death in the latter

Table 1. Pairwise comparisons between *Ophiocordyceps* fungi from different leaf-cutting ants displaying specific percentages similarity in base pairs (EukF1 and EumycR2 primer pair). '*Cordyceps*-like' is the strain discovered by Van Borm *et al.* (2002). AttaQ, queen (Q2) of *Atta colombica*; AcroQ, queen (Q1 and Q3) of *Acromyrmex octospinosus*; AcroW, worker of *A. octospinosus*. The accession numbers are: EU864317, EU864318, and EU864319.

			% base pair similarity				
	Place	Year	*Cordyceps*-like	AttaQ	AcroQs	AcroQ	AcroW
Cordyceps-like	Gamboa	2000		77.67	76.45	77.67	–
AttaQ (Q2)	Gamboa	2005	77.67		91.25	91.70	–
AcroQ (Q1)	Gamboa	2006	76.45	91.25		91.46	–
AcroQ (Q3)	Gamboa	2007	77.67	91.70	91.46		
AcroW	Brazil	2006					

case suggests that this association may be less specialised, although a larger sample of *Ophiocordyceps*-killed leaf-cutter ants will be necessary to support this assertion.

It may be that each of the three *Ophiocordyceps* strains that we discovered in Panama represents a novel infection, which would be in line with the absence of a specialised extended phenotype, but would also imply horizontal transmission from another host. A recent multi-gene phylogenetic reconstruction has determined that host jumping is a common feature, over long evolutionary time periods, in *Ophiocordyceps* and other members of the Order Hypocreales (Nikoh & Fukatsu, 2000; Øyvind *et al.*, 2005; Spatafora *et al.*, 2007; Sung *et al.*, 2007). Cases of host jumping inferred from phylogenies are often between different orders of hosts, which will hamper the search for reservoir populations in other hosts occurring in the same habitat. Our laboratory infections are the first example of experimentally induced artificial host jumping by *Ophiocordyceps* and indicates that host jumps in ants are possible both between genera within a tribe (*Atta*, *Acromyrmex*, *Sericomyrmex*, *Apterostigma)* and between sub-families within the family Formicidae.

After behavioural defence to avoid infection has failed, the insect cuticle is the decisive barrier to fungal invasion. The thickness of the cuticle, the tensile strength, and the amount of sclerotisation will determine whether the fungal infection structure, the appresoria, can pass through it (Hajek & Leger, 1994). From the perspective of an insect pathogenic fungus that needs to initiate infection, the host cuticle contains a wide variety of chemicals that may either inhibit germination or act as cues for germination (the latter involves adhesive chemicals that bind the infective spore to the cuticle prior to appresoria development). Thus, demonstrating an ability of *Ophiocordyceps* fungi to jump hosts, raises the question of which chemical and topographical features (Hajek & Leger, 1994) of ants are important, and whether they are normally different enough to prevent invasion on alternative hosts.

Once the cuticular barrier has been breached, the fungus must overcome the cellular and humoral immune defences that insects employ against parasites (Siva-Jothy *et al.*, 2005). There is currently little information on insect host immune defence against *Ophiocordyceps*. However, from the parasite perspective, there have been some studies examining the proteases and toxins produced by *Cordyceps*/*Hirsutella* that could be active against the insect host. For example, Mazet and Vey (1995) discovered that the toxin Hirsutellin A (HtA) is produced by *Hirsutella thompsonii*, and that injection of this protein into the waxmoth, *Galleria mellonella*, resulted in a high mortality rate. However, Maimala *et al.* (2002) also found that the HtA gene is present in many, but not all of the 162 *H. thompsonii* isolates that they studied, suggesting that HtA is not the only key factor for toxic activity. Another study by Kim *et al.* (2006) discovered that *Cordycepsin* produced by *Cordyceps militaris* had immuno-stimulating activity when it was tested on vertebrates. These recent examples indicate that our knowledge of the mechanisms of *Ophiocordyceps* infection is scant, and that highly interesting insights may be obtained from the further study of the interaction of this fungal pathogen with host immune systems and host brains, as far as it is able to manipulate host behaviour.

Clearly the newly discovered *Ophiocordyceps* strains that we studied were able to overcome the ant immune systems, although the stressful conditions under which the experiments were performed probably enhanced the vulnerability of the ant hosts. As we did not quantify the number of spores or adjust this according to ant size, we cannot say whether the ants were exposed to an ecologically relevant spore dose. However, even if the results would not be repeatable with lower doses, it would still indicate that rare natural host shifts are possible in unusual cases of heavy infection loads. It is interesting that the *Ophiocordyceps* stroma that we were able to induce experimentally, did grow out of the head region of the ant host, which is the specific phenotype seen in *Ophiocordyceps* infecting ants (Evans, 1982; Evans & Samson, 1982, 1984). This could indicate that the strains that we discovered were transmitted from other ants, as expressing this characteristic growth probably requires specific fungal responses to the host's anatomy.

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