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Fitness consequences of nest infiltration by the mutualist-exploiter *Megalomyrmex adamsae*

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Abstract. 1. Fungus-growing ants are obligate mutualists. Their nutrient-rich fungus garden provides a valuable food store that sustains the ant hosts, but can also attract social parasites.

2. The 'guest ant' *Megalomyrmex adamsae* Longino parasitises the fungus-growing *Trachymyrmex zeteki* Weber queen just after nest founding. The parasitic queen infiltrates the incipient nest, builds a cavity in the fungal garden, and lays eggs that develop into workers and reproductive males and females.

3. This study compared young parasitised and non-parasitised laboratory colonies by measuring garden growth and biomass, and the number of host workers and reproductives. Host queen survival and parasite colony growth were also monitored.

4. Parasitised *Trachymyrmex* colonies had reduced host worker and alate numbers, as well as lower garden biomass, compared with non-parasitised control colonies, confirming that *M. adamsae* is a xenobiotic social parasite. Host queen survival was not significantly different between parasitised and control colonies.

5. This is the first study that experimentally infects host colonies with a xenobiotic social parasite to measure fitness cost to the host. The natural history of *M. adamsae* and the fungus-growing ant mutualism are evaluated in the context of three general predictions of (Bronstein, *Ecology Letters*, **4**, 277–287, 2001a) regarding the cost of mutualism exploiters.

Key words. Attini, fitness cost, fungus-growing ant, host fitness, *Megalomyrmex*, social parasite, Solenopsidini, *Trachymyrmex*.

Introduction

In the tangled web of species associations, symbiotic interactions can range from mutualistic to parasitic. Mutualists benefit one another, whereas parasites obtain resources at the expense of their host. Parasites often take advantage of species interactions and attack mutualistic traits, exploiting the resources exchanged between partners (Letourneau, 1990; Yu, 2001; Bronstein, 2001b; Little, 2010; Palmer *et al*., 2010). These complex relations are a fundamental innovation in evolution, shaping diversity and stabilising species networks (Stanton, 2003; Moran, 2007). Understanding how species co-evolve and shape these networks is one of the major challenges in evolutionary biology (Thompson, 2006).

There is a growing number of examples of multilateral symbioses with participants from all domains of life, making the study of species interactions increasingly complex (e.g. yucca – yucca moth mutualism and the corresponding florivores, dual bacterial symbiosis of sharpshooters, fungus-growing ant – microbe symbiosis) (Althoff *et al*., 2005; Wu *et al*., 2006; Little, 2010; Mueller, 2012). Establishing the effects on fitness in such multilateral interactions is crucial to fully understand these complex systems (Palmer *et al*., 2010). This knowledge is requisite to explain how such mutualisms persist, when services or rewards exchanged

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between partners are stolen or disrupted by obligate mutualist exploiters.

Social parasitism, defined as one social organism parasitising another social organism (Hölldobler & Wilson, 1990; Buschinger, 2009), is common in social insects. The social parasite exploits colony resources by infiltrating or attacking the host colony and either cohabiting with or evicting the host. Established colonies of social insects face parasitic infection collectively; nestmates protect each other and their queen, the key individual responsible for colony reproduction (Schmid-Hempel, 1998; Cremer *et al*., 2007). These homeostatic fortress conditions within the nest are thought to provide resistance to highly virulent pathogens (Hughes *et al*., 2008). However, when a single queen is in the nest-founding stage, she remains vulnerable to nest infiltration and usurpation (Lorenzi *et al*., 1992; Lorenzi & Thompson, 2011). Social parasites can influence the host colony by altering host worker behaviour (Allies *et al*., 1986), by reducing colony resources (i.e. food and workers) (Foitzik & Herbers, 2001) or by killing current or future queens (Wheeler, 1910; Lorenzi *et al*., 1992; Lorenzi & Thompson, 2011).

The newly described social parasite *Megalomyrmex adamsae* Longino (Tribe: Solenopsidini) (Longino, 2010) parasitises two *Trachymyrmex* host species in Panama, *T. zeteki s.s.* Weber (Weber, 1940) and the larger, behaviourally distinct *T.* cf. *zeteki* (Tribe: Attini) (T. R. Schultz, pers. comm.). These 'guest ants' (xenobionts) infiltrate incipient colonies and nest in the fungus garden of their host, where they care for their own brood in isolation. *Megalomyrmex adamsae* is a perennial social parasite which integrates seamlessly into *T. zeteki* colonies, following a mating flight that appears to be synchronised with (or slightly after) the mating flight of the host (April to May in Panamá). Individual and multiple *M. adamsae* queens can be found in young *T. zeteki* and *T.* cf. *zeteki* colonies with no or few host workers. Larger parasitised *T. zeteki* colonies can also be found, with approximately 100 parasite workers and hundreds of host workers. Parasitised and non-parasitised laboratory colonies can reach a similar size and can live, respectively, for over 2 or 5 years (R. M. M. Adams, pers. obs.; Wheeler, 1925; Weber, 1972). Currently, there is no evidence that the *M. adamsae* parasites provide any benefit to their hosts. Instead, *M. adamsae* colonies seem to forage exclusively within the host nest, consuming host brood and the cultivated fungal garden.

This study examines the impact of *M. adamsae* on the mutualistic network involving the fungus-growing ant *T. zeteki*. Specifically, the hypothesis that *M. adamsae* is a xenobiotic social parasite was tested, by measuring direct and indirect fitness effects on the ant–fungus mutualism. By quantifying changes in worker number, reproductive output, garden growth and host queen survival, the fitness costs to both mutualistic partners are established and discussed in the broader context of species interactions. This is the first study to empirically determine the fitness costs that xenobiotic social parasitism imposes on a mutualistic network.

Materials and methods

Megalomyrmex social parasites and their fungus-growing ant hosts

The genus *Megalomyrmex* comprises 38 described species (Brandão, 1990, 2003; Longino, 2010). At least 10 *Megalomyrmex* species are thought to be associated with fungus-growing ant hosts, whereas the rest are free-living predators (R. M. M. Adams, unpublished; Brandão, 1990). Within the *silvestrii* species group, there is a gradation of parasitic behaviour that includes lestobiotic parasitism, also described as raiding 'thief ants' (e.g. *M. mondabora, M. mondaboroides*, and *M. silvestrii*), agro-predatory associations, where the parasite usurps the nest and fungus garden (e.g. *M. wettereri*), and, the most derived, xenobiotic parasitism, or cohabiting 'guest ants', as described above (e.g. *M. wettereri*, *M. symmetochus*, and *M. adamsae*) (Brandão, 1990, 2003; Hölldobler & Wilson, 1990; Adams *et al*., 2000a; Adams & Longino, 2007; Longino, 2010). All of the aforementioned species appear to be obligate associates of attine ants, except for *M. silvestrii*, which is a facultative parasite that has been found both free-living and associated with fungus-growing ant hosts (Brandão, 1990). *Megalomyrmex* parasites produce workers and have been observed caring for and feeding their own brood, unlike most inquiline social parasites (Buschinger, 2009). Although they often commingle with their fungus-growing ant hosts, the range of tasks that *Megalomyrmex* workers perform is still unknown.

In contrast, the fungus-growing ants, or attines, are among the textbook examples of coevolution and mutualism (Fremann & Herron, 2003; but see Mueller, 2012) and have been widely studied for decades (Wheeler, 1907; Weber, 1958; Mehdiabadi & Schultz, 2009; Suen *et al*., 2011). Comprising over 230 described species, the attines maintain a ≈50-million-year-old mutualistic association with their fungus garden. Vertically transmitted by future queens (i.e. gynes), the garden is dispersed and cultivated. The ant associates provide nutritional substrate and protection from diseases that curb their cultivar's growth (Weber, 1972; Currie & Stuart, 2001). In turn, the ants rely on the fungus garden as a primary food source (Weber, 1972; Quinlan & Cherrett, 1979). The cultivar lineages fall into two distantly related families, Agaricaceae (formerly Lepiotaceae) and Pterulaceae, in the phylum Basidiomycota (Schultz & Brady, 2008). In addition, the gardens contain a diverse assemblage of associates that either attack the cultivar (e.g. *Escovopsis* and *Trichoderma*) (Currie & Stuart, 2001; Gerardo *et al*., 2004, 2006; Pagnocca *et al*., 2008; Mehdiabadi & Schultz, 2009; Rodrigues *et al*., 2009, 2011) or interact synergistically to protect it (Rodrigues *et al*., 2009). Furthermore, the ants accumulate bacterial growth on their integument (Currie *et al*., 2006) which are thought to help combat *Escovopsis* (Currie *et al*., 1999; Caldera *et al*., 2009) and/or entomopathogens (Mattoso *et al*., 2012).

Most attine species are inconspicuous and maintain relatively small nests, when compared with the leaf-cutting genera, *Atta* and *Acromyrmex*. Ant species in the *Trachymyrmex*

clade (sister to the leaf-cutting clade) are semi-claustral nest founders, where the queen supplements her stored fat reserves by foraging for vegetable material to feed the garden, which provides a nutrient-rich diet for herself and her worker force (Weber, 1972; Fernández-Marín *et al*., 2004). The queen grows her cultivar from a minuscule piece of garden that she transported from her natal nest. It is during this critical period in the life cycle of an ant colony that mortality is highest (Hölldobler & Wilson, 1990) and queen immunity is compromised (Baer *et al*., 2006), particularly for fungus-growing ants, where mortality can be 73.3–97.5% in the first year of life (Autuori, 1950; Vieira-Neto & Vasconcelos, 2010).

Colony collection and maintenance

Colonies used in the study were collected between 21 July and 18 August 2005 (two other colonies were collected in 1999, see *Gyne castration* below) in the forests adjacent to Pipeline Road, Soberanía National Park, Republic of Panamá. The colonies were found in primary and older secondary rainforest, along creek embankments and on steep slopes nearby. *Trachymyrmex zeteki* colonies are easily located by their characteristic auricle-shaped nest entrance (Fernández-Marín *et al*., 2004), although parasitised and non-parasitised colonies are indistinguishable before excavation. First-year incipient colonies, established after mating flights during the early rainy season in 2005 (approx. May or June), were collected in small five-dram vials, then transferred to small 60 × 15 mm diameter Petri dishes, lined with a ring of moistened cotton to maintain high humidity.

Of the 250 first-year *T. zeteki* colonies inspected in the field, 15 (6%) contained *M. adamsae* queens. Of these, four (26.7%) did not contain a host queen, possibly because the *Trachymyrmex* queen was foraging at the time of collection or had died, and two (13.3%) had two parasite queens each. Sixty-five colonies (15 parasitised and 50 non-parasitised) of the 250 inspected, each containing 0–10 host workers, were brought to the laboratory at the University of Texas at Austin. Voucher specimens of *M. adamsae*, *T. zeteki*, and *T*. cf. *zeteki* have been deposited at the National Museum of Natural History, Smithsonian Institution, Washington, DC.

Colonies were transferred into permanent nest-boxes in early September 2005 at the University of Texas, allowing for more controlled conditions. The square plastic nest-boxes (7.5 × 2 cm) had moist plaster bottoms, connected to a dry foraging and refuse chamber of the same size. Within the nest chamber, the fungal garden was grown in the bottom portion of a Petri dish (60 mm diameter, 15 mm height), which was removed for biweekly weighing. When the garden filled the dish, a new nest-box with a Petri dish was attached and a small amount of cultivar was moved to the dish, stimulating occupation of this second nest chamber by the ants. The ants were given a mix of UV-sterilised pecan catkins, organic oats, and organic polenta, and allowed to feed their fungus gardens *ad libitum*. Unused substrate was removed from the foraging chamber every 2 weeks. Discarded garden was removed regularly to discourage fungal contaminant growth in the nest chamber. Waste pellet piles were allowed to accumulate in the nest or refuse chamber but were removed every 2 weeks, as their function is still uncertain (Little *et al*., 2003). Colonies were kept in the dark, except during data collection and general maintenance, and at room temperature (approximately 20–23 °C).

Experimental design

In August 2005, 34 colonies were randomly chosen from 50 non-parasitised colonies. The 15 parasitised and 34 non-parasitised colonies were divided into 3 experimental treatments. The 'Complement Control' (CC) treatment group consisted of presumably naïve parasite-free colonies ($n = 17$). In the '*Megalomyrmex* Minus' (M−) treatment colonies, the parasite queen(s) was removed, leaving the host colony unparasitised. This resulted in $n = 11$ M− colonies, as four *T. zeteki* queens were missing at the time of collection and two colonies contained two parasite queens. Finally, in the 'New Host' (M+) treatment group, social parasite queens, removed from their original host colonies, were introduced into naïve colonies that had been collected parasite free ($n = 17$). Parasite queens were first exposed to a mixture of the original and new host fungus garden in a new Petri dish, then host workers (one to three at a time) were added and, lastly, the host queen was placed in the Petri dish. Eleven replicates consisting of all three types and six consisting of M+ and CC were standardised for worker number and garden biomass. Colonies were rotated together in replicates on the lab-bench every 2 weeks.

In all treatments, original gardens and brood were removed and replaced with a new fungal strain. The feeding of the fungus garden by the ants was carefully monitored and used as an indicator of the acceptance of the new strain. Four different fungal strains were used (29 September 2005) across replicates but were controlled for within each replicate. By 26 October 2005, two fungal strains seemed to be preferred, therefore most gardens were changed to the RMMA050105-29 strain, whereas a few replicates remained on RMMA041228-05. Subsequently, all gardens were consistently fed and maintained by the ants, and colonies were determined stable. To control for the fitness effect of the cultivated fungus across the experiment, the fungal strain with the highest acceptance and growth rate (fungal strain RMMA050105-29) was given to all colonies by 9 January 2006. By this date, host colonies from six separate replicates had become non-viable. The host queen was either found dead, or covered in an unidentified fungus (Figure S1, Table S1), in which case the colony was immediately quarantined and removed from the experiment. Queens infected with fungus stopped feeding their garden and it either became depleted or died. In an effort to keep the block experiment design, three new colonies, two parasitised and one control, were added to the experiment in December 2005. One of the new *M. adamsae* queens was found in the same population as the others but in a *T*. cf. *zeteki* colony. By March 2006, five additional *T. zeteki* queens had died, three CC, one M−, and one M+, as well as one parasite queen. The remaining colonies were determined stable on

20 March 2006, when they were all feeding their cultivar and had not experienced garden death. As a result of the high mortality, CC and M− were combined into a control category. Treatments are referred to hereafter as 'Control' (CC and M−) and 'Parasitised' (M+) unless otherwise indicated.

Data collection

Data collected between 20 March and 2 November 2006, hereby referred to as the 'experimental period', were used in the analyses. The experimental period began approximately 200 days after the colonies were moved to their permanent nest-boxes and 70 days after all colonies received the same fungal strain. Data were collected approximately every 2 weeks over 228 days.

The weights of the garden, brood, and ants were recorded together, as their separation would risk the health of the brood and fungus garden (Explorer Ohaus E0RR80, SD ± 0.1–0.5 mg). A single worker weighs 2.1 mg on average (R. M. M. Adams, pers. obs.). This was multiplied by the number of ants counted for that data collection, then subtracted from the total garden weight, to derive what is referred to hereafter as the 'garden biomass'. The effect of the brood biomass was assumed negligible. Measurements were taken blindly, where the person reporting the weights did not know which treatment colony was being weighed. The number of living ant workers in a colony was quantified, by counting workers visible amongst the fungal garden three times in close succession, then averaging them. These counts became less accurate as the garden grew larger, because ants located within the garden were less visible. Therefore, the worker counts in larger colonies may be an underestimate of the actual ants present, until November, when all ants were counted. Because too few host colonies produced reproductives by November 2006, data were collected again approximately 7 months later (May 2007, nearly 2 years after colony collection), to capture a second reproductive bout. In nature, fungus-growing ants typically mate at the start of the rainy season (May in Panamá), when the ground is moist and easier to dig (Weber, 1972; Fernández-Marín *et al*., 2004), thus the production of reproductives is expected at this time of the year. All colonies were maintained in the same way as in the first year during the extended 7-month period, except that information on garden biomass, garden replacement, and worker number was not gathered. Because gardens were torn apart in November 2006 to count all of the ants, some gardens died and were replaced.

The progression of parasite colony growth was opportunistically monitored. As in *Megalomyrmex wettereri* (Adams *et al*., 2000a), queens of *M. adamsae* build a fungal cavity in the host garden, where they remain hidden most of the time with their brood and reproductives. This sequestration made it difficult to monitor parasite brood and reproductive output accurately, without opening the cavity and destroying its structure. For this reason, the parasite colony growth was estimated every 2 weeks and colonies were censused whenever it could be done with minimal disturbance. Once an individual was discovered, they were assumed alive until a body was collected in the refuse pile.

Statistical analyses

Survival analyses of data from 36 experimental colonies, viable on 20 March 2006, were conducted in R version 2.14.0 (R Development Core Team, 2011) (Table S1). Eleven colonies, five CC, four M−, and two M+, were excluded as a result of mortality before this date. The final data set consisted of $n = 12$ CC, $n = 7$ M−, and $n = 17$ M+ colonies (Table S1). Host queen survival was analysed with a Cox proportional hazards regression model with Type I censoring, using the survival package of Therneau & Lumley (2012). Treatment (Parasitised vs. Control) and parasite exposure (M+ and M− vs. CC) were factors for the fitted model, whereas the response variable was the data collection day for the period when a queen was found dead or covered in an unknown fungus, collectively referred to as 'queen death' hereafter. The 19 host queens that were alive after 228 days were right censored. The proportional hazards assumptions of the Cox model were verified using Shoenfeld residuals ($P = 0.44$ for treatment, $P = 0.091$ for parasite exposure) (Grambsch & Therneau, 1994). A Fisher's exact test was used to determine if parasitised host queens were more likely to exhibit fungal growth on their exoskeleton.

For all fitness analyses, additional colonies were excluded because of complex experimental histories (i.e. failed parasite queen introduction), *Megalomyrmex* queen death or *Trachymyrmex* queen death or fungal infection. The final data set comprised nine Control and five Parasitised colonies (Table S1).

In order to assess direct fitness, Fisher's exact test was used to compare the number of Control and Parasitised colonies that produced alates during the two reproductive bouts in 2006 and 2007. Fungus garden growth and worker number were used to assess fitness indirectly.

The garden growth rates were lower at the beginning and end of the experiment, with accelerated growth in between, so a logistic growth model was fitted to the data and model parameters were compared between the Control and Parasitised groups. Host worker production was evaluated as the change in live workers, as well as in the total number of workers produced (live and dead), between the start and end dates of the experimental period. Exact two-sample permutation tests, implemented by the coin package (Hothorn *et al*., 2008), as well as a custom R script, were used for analysing these salient life-history traits, as this method is more appropriate for data with departures from parametric and classic non-parametric assumptions (i.e. non-normal distribution, asymmetrical data, and small sample size) (Adams & Anthony, 1996; Peres-Neto & Olden, 2001; LaFleur & Greevy, 2009). It has been shown that permutation tests for equality of means on unbalanced data (i.e. with unequal sample sizes) that come from distributions that are not identical, can have inflated Type I error rates (Huang *et al*., 2006). Therefore, it is important to note that the permutation test assumption of exchangeability under the null hypothesis holds for this data set, as a result of the experimental design of the standardised initial garden biomass and worker number for incipient colonies derived from the same population. One-sided tests were performed, owing to

the *a priori* decision to test for the cost of parasitism (i.e. the parasite was expected to have negative impact on host fitness, based on previous knowledge).

Results

Host queen mortality

Mortality of the host and parasite queens was a limitation to this study. Before the experimental period began, 25% of the host queens had died. During the experimental period, 47% of the queens died. Cox proportional regression analysis determined there was no significant difference in *Trachymyrmex* queen mortality between the Parasitised and Control treatments ($P = 0.45$), or based on parasite exposure ($P = 0.068$).

Trachymyrmex queens that died were no more likely to be found with fungal growth on their exoskeleton in the Controls (three out of eight) versus Parasitised colonies (six out of nine) ($n = 17$; $P = 0.35$). The putative ant pathogens were identified, using morphology and ITS sequencing, by A. Rodrigues as *Fusarium solani* (plant pathogen) (Zaccardelli *et al*., 2008), *Simplicillium lanosoniveum* (mycoparasite) (Ward *et al*., 2011), *Purpureocillium lilacinum* (ubiquitous fungus isolated from soil, insects and air and used as a biocontrol against nematodes) (Luangsa-ard *et al*., 2011), and *Cladosporium cladosporioides* (commonly isolated from the fungal pellet of *Atta laevigata* gynes) (Pagnocca *et al*., 2008) (Figure S1).

Garden growth and worker number

Total workers produced over the lifetime of the colonies were significantly different between the Control ($n = 11$) and Parasitised ($n = 5$) groups ($Z = 2.2483$, $P = 0.0065$), as was the number of live host workers ($Z = 1.787$, $P = 0.019$). The difference is illustrated in the biweekly averages of the produced workers (Fig. 1a). Garden biomass was asymptotically significantly different under the logistic growth model ($P = 0.0020$), whereas the growth curve inflection point and growth rate coefficient were not ($P = 0.22$ and $P = 0.054$, respectively) (Fig. 1b).

Host reproductives

Six colonies produced males between April and July 2006 but four were removed from the experiment as a result of the *Trachymyrmex* queen death (M-2, CC7, CC15) or *Megalomyrmex* queen death (M + 11) (Table S1). By the end of May 2007, nearly 2 years after colony collection and the start of the rainy season in Panamá, four additional colonies produced males. Over the two reproductive bouts (2006 and 2007), six Control colonies ($n = 8$) and none of the Parasitised colonies ($n = 5$) produced male reproductives ($P = 0.021$) (Fig. 2). No gynes were produced by any of the colonies.

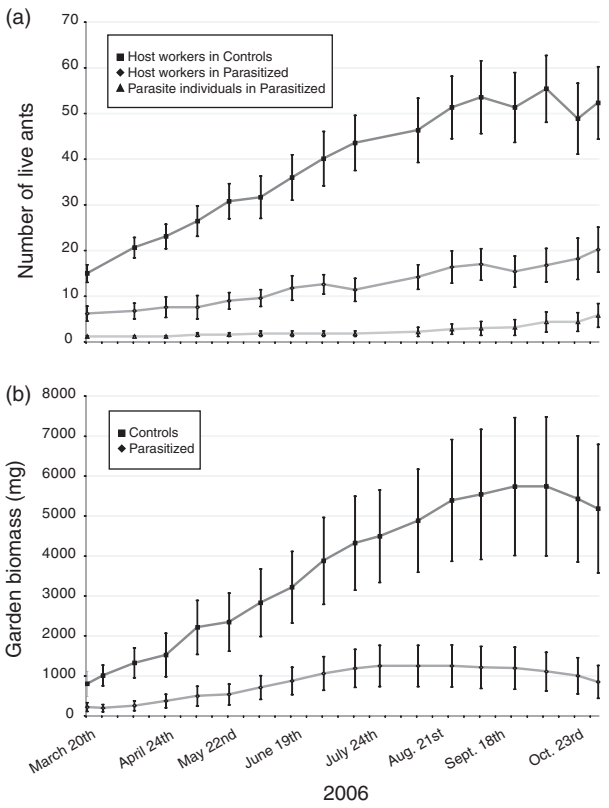

Fig. 1. (a) Average live workers in parasitised and control nests. Worker counts were estimated in larger colonies with the exception of the exact count at the end. Square markers represent host workers in control colonies ($n = 11$). Diamond markers represent host workers in parasitised colonies ($n = 5$). Triangle markers represent an estimate of the number of parasite individuals including the queen, workers, and males in the parasitised colonies ($n = 5$). (b) Average garden biomass (mg). Square markers represent control colonies and diamonds represent parasitised colonies. Both (a) and (b) data were recorded approximately every 2 weeks. Error bars indicate standard errors.

Parasite colony growth

Parasite colony growth was opportunistically monitored, because the parasites build nest cavities within the fungus garden, where the brood, queen and some workers reside. However, based on the final count and the number of dead, it was clear that the parasite colonies remained small (Fig. 1a). Two colonies never produced workers. Four colonies produced a single male alate each and one colony produced a female alate, although all were asynchronous with the expected host alate production. By November 2006, 3 of the 5 *Megalomyrmex* colonies produced 3–14 workers. Parasite males were generally short-lived and rarely seen, until their corpses appeared in the refuse area. The colony with the highest number of parasite workers was in the host nest with the largest garden biomass (2289 mg), whereas the colonies that did not produce workers were in the host nests with the lowest garden biomass (55 and 81 mg, respectively) and host worker number (7 and 6, respectively). As a result of the low

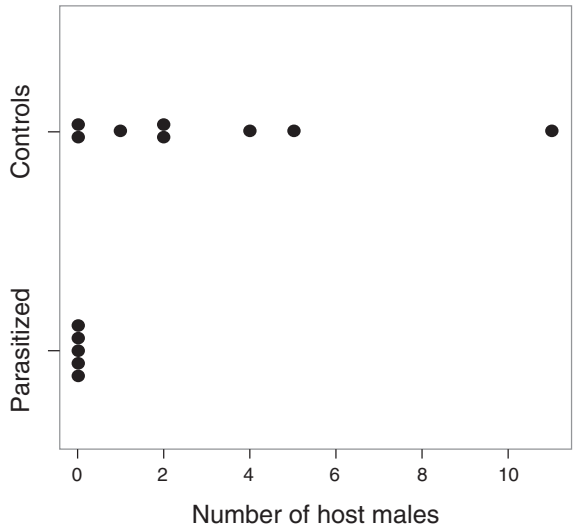

Fig. 2. Total reproductives produced in controls ($n = 8$) and Parasitised ($n = 5$) colonies after two reproductive bouts in 2006 and 2007.

sample size, the impact of the number of parasite workers on the fitness parameters of the host colonies could not be statistically evaluated.

Host–parasite interactions

To ensure the highest rate of successful introductions, parasite queens were gradually introduced to their new host colonies, starting with the host workers. Surprisingly, the parasite queens were not attacked by the host workers. However, there was obvious aggression between the host queen and the parasite queen. Successful introductions were those in which aggressive behaviour eventually ceased between 40 and 160 min after the first contact with the host queen and the two queens coexisted peacefully thereafter. Two introductions failed because the host queen was killed by the parasite.

During the experiment, two surprising behaviours were observed. First, host workers groomed the parasite queens for mostly brief intervals, but as long as 30 min. The gaster of the parasite was groomed most often but the head and thorax was also targeted (Figure S2). A separate study, using the same colonies, has shown that total nestmate grooming is three times more frequent in parasitised colonies ($n = 14$) than in control colonies ($n = 14$), with 40% of the interactions involving host workers grooming the *Megalomyrmex* queen (Tseng & Adams, 2006). Second, on two occasions host workers were observed placing small fragments of fungus garden on the thorax of the parasite queen (Figure S3), much like they plant fungus onto their larvae and pupae (Lopes *et al*., 2005; Camargo *et al*., 2006).

Gyne castration

Two parasitised *T. zeteki* colonies, not included in the experiment and collected in 1999, were kept in the lab for over 2 years (RMMA990929-06 and RMMA990928-04). When these colonies reached the size of approximately 75–200 workers, they produced both male and female reproductives. Remarkably, *M. adamsae* workers were observed chewing the wings off host female reproductives (Fig. 3). A few workers would begin the attack but then one would slowly chew either at the base or in the middle of the wing, until both wings were fully or partially removed. All host females present were attacked (2–6 females per nest), whereas host males were left alone (approximately 4–10 males per nest).

Fig. 3. Parasite worker wing-clipping and thus castrating a reproductive female in a mature host colony. A parasite worker mutilates the wings of a host female, resulting in stubby, non-functional wings (insert).

Discussion

Parasitised and non-parasitised fungus-growing ant colonies were examined by quantifying fungus garden growth and colony size change, to test the hypothesis that parasitised colonies suffer fitness costs in the first 2 years of colony life. *Megalomyrmex adamsae* parasites inflict costs to both partners in the ant-fungus mutualism, by reducing colony resources, altering host worker behaviour, and preventing production of host reproductives.

The fungus-growing ant mutualism is an obligate association and the two main partners are reproductively bound, reciprocal cooperators. Vertical transmission, where young queens disperse with the fungus, ensures continuation of the association across generations (i.e. high partner fidelity). Cooperative reciprocity and partner fidelity are two criteria that have been proposed to allow a mutualism to persist in spite of a parasitic attack (Yu, 2001). Even under high partner fidelity, third parties such as parasites may influence selection pressures important to mutualist evolution, affecting co-evolutionary dynamics between partners but may not necessarily destroy the mutualistic association.

Reduction in garden biomass

Garden biomass was lower in parasitised colonies, suggesting that either the host and parasite ants consume a large portion of the garden, and/or the host workers are less efficient at caring for and feeding their garden in the presence of a parasite. Recent work on invertebrate grazers of free-living basidiomycete fungi shows that different grazing species can have a varied and costly impact on the growth of the mycelium (Crowther *et al*., 2011). It is therefore possible that parasite grazing results in different growth patterns than host grazing. Furthermore, the parasite manipulates the garden to construct a cavity and the mycelium for this construction does not receive substrate directly for growth, unlike typical parts of an attine garden.

The lower number of host workers found in parasitised colonies could lead to less substrate added to the fungus garden, reducing overall fungal biomass. In addition, the unusual host worker behaviour (i.e. parasite grooming and fungal planting) could reduce foraging efficiency. The differences in growth between parasitised and control colonies under the logistic growth model seem to support these hypotheses. Future work measuring worker efficiency and fungus growing patterns in both parasitised and non-parasitised colonies would help elucidate the mechanisms underlying the observed reduction in garden biomass.

Alate production in host colonies

Reproductive output of the host colony may be mediated by the host or the parasite (Forbes, 1993). The host colony may curtail reproductive effort in response to a smaller garden or to a low number of workers. In *Trachymyrmex septentrionalis*, reproductive production is a linear function of the amount of standing worker biomass (Seal & Tschinkel, 2008), thus a growing colony may first invest in building a work force, then produce reproductives once it reaches an optimal size (Oster & Wilson, 1978). A parasite can therefore affect reproductive output of a host indirectly, by impeding the growth of the host colony (i.e. worker number and garden biomass). Alternatively, the parasite might directly control the number of reproductives eclosing via differential consumption of worker versus reproductive brood. Although the cause could not be determined in this study, the observed zero reproductive output of parasitised host colonies suggests that both mechanisms may be at play in this system.

Host–parasite balance

This study suggests there is a delicate balance of resource exploitation strategies used by the parasite. *Megalomyrmex adamsae* colonies remained small but most produced alates. Parasitised *T. zeteki* colonies also remained small and similar to young non-parasitised colonies with low garden biomass and few workers. They stayed in an ergonomic stage rather than entering a reproductive stage, as is seen in other parasitised organisms (Burns *et al*., 2005). Regardless of social parasite exposure, *T. zeteki* colonies had high mortality in the first 2 years of life. Over the lifetime of the colony, mortality of parasitised colonies is likely higher than non-parasitised colonies, because they remain smaller longer. Parasitised colonies therefore are less likely to benefit from the homeostatic fortress conditions of large colonies (Hughes *et al*., 2008), remaining vulnerable to parasites and predators. However, it is notable that even in the presence of the putative entomopathogenic fungal parasites observed attacking the host queens (Figure S1), parasitised colonies were not more likely to die, suggesting a prudent exploitation strategy by the parasite in the first 2 years of colony life.

Attack of two partners

The results suggest that, in young host colonies, parasites have an equal negative impact on both partners in the ant–fungus mutualism. For the ants, either male or female alate production would provide a fitness gain, but the garden gains fitness only through dispersal by female alates. Neither female nor male reproductives were produced in young parasitised colonies and only males were produced in unparasitised control colonies. The lack of female alate production prevents the ants from delivering the 'service' of fungal dispersal to its fungal partner, although they still provide food, shelter, and garden maintenance (e.g. weeding).

The parasite impact is likely different in young versus older host colonies. Because *M. adamsae* and *T. zeteki* cohabit for multiple years, there may be selection for characteristics in the parasite to prolong the association and limit the damage inflicted on the host (Holmes, 1983). The discovery of larger parasitised host colonies in the field suggests that the fungus garden can be maintained and become large enough to support the hosts and parasites for years. Larger parasitised laboratory colonies collected in 1999 produced both male and female alates. The males were unharmed but the female alates were mutilated by the parasites and their wings removed (Fig. 3). Wing removal is, essentially, castration because these females would normally mate during flight and disperse. By removing the wings, the parasite forces female alates to forfeit personal reproduction, remain in the nest, and perform worker tasks (R. M. M. Adams, pers. obs.). By behaving as a worker, the castrated female presumably improves host survivorship and garden growth, which also benefits the parasite. This suggests that males could disperse from older, larger parasitised colonies, propagating host ant genes and providing a fitness benefit. On the other hand, female alates could not disperse with the fungus garden, thus the fitness of the fungus garden is zero, just as it is in young colonies. Interestingly, host female castration also occurs in the *Sericomyrmex amabilis*/*Megalomyrmex symmetochus* system, where *M. symmetochus* is another xenobioic 'guest ant' parasite that is closely related to *M. adamsae* (R. M. M. Adams, pers. obs.).

Costliness of mutualism exploiters

Bronstein (2001a) provides a general discussion of evolutionary implications of mutualism exploiters and gives a list

of three predictions regarding the cost of exploitation in mutualisms.

First, Bronstein (2001a) predicts that, if the mutualism exploiter prevents one mutualist from receiving a benefit from the other mutualistic partner and this benefit is tied to the success of the first partner, then the cost of mutualism–exploitation will increase as the benefit decreases. The extreme case would be if a mutualist avoids its partner because it is being exploited (e.g. nest and garden abandonment in *M. wettereri* hosts). It is likely that there is a low-level cost to parasite-disrupted behaviour in the *T. zeteki* hosts. If the hosts are grooming the parasite, rather than foraging or tending the garden, then the fungus is likely to suffer. Parasitised colonies were shown to remain smaller and, therefore, likely to be more susceptible to pathogens than non-parasitised colonies, which could have detrimental impacts on both mutualists.

Second, Bronstein (2001a) predicts that the cost of exploitation increases with the 'value' of the exploited commodity, such as the garden or workers in this study. The value of a commodity increases with the host's investment level and when the resource is difficult to replace. The most severe case is when the exploiter kills one of the mutualists. The entire garden is difficult to replace (however, see Adams *et al*., 2000b), but if it is only fed upon, the fungal biomass could regenerate. The larvae are also replaceable, when consumed by the parasites. However, if reproductive brood is consumed, there is a direct fitness cost to the ants and, in the case of female brood, to the garden as well. Moreover, parasitised colonies delay production of alates and, when gynes are produced, they are castrated by the parasites. This results in the prevention of garden and ant dispersal and has a high cost to the ant-fungus mutualism.

Third, ecological context plays a key role in the costs and benefits of exploitation (Bronstein, 2001a; Althoff *et al*., 2005). If there are other selection pressures, such as predation or resource limitations, mutualism exploitation is predicted to be even more detrimental. Naturally, this cost increases when the exploiters are abundant and is negligible when they are rare. The rareness of *M. adamsae* parasites, 6% in incipient colonies and less than 1% in older colonies (i.e. >1 year), and the flexible host choice (i.e., *T. zeteki* and *T.* cf. *zeteki*), suggests that the impact the parasites have on the ant–fungus mutualism may be limited. With low parasitism rates, the cost of losing dispersal services of the fungus by the ants in a few colonies in the population is likely insignificant, when considering the stability of the mutualism (Bronstein, 2001a). If co-evolutionary alteration is occurring, then there would be a cyclical host defence response to parasitism, and host switching to the least defended species by the parasites is expected (Nuismer & Thompson, 2006), further diminishing the selection pressure exerted by the parasites on the host symbiont system.

This study provides conclusive empirical evidence that *M. adamsae* is exploiting the 50-million-year-old fungus–ant mutualism. The ecological environment influences the mutualism and the role of additional predators or pathogens will further elucidate the complexities of this species network. The impact of brood and fungus consumption by the parasite at the colony level would be an interesting focus for future work. Finally, quantifying lifetime host reproductive fitness costs is the next logical step in understanding the ecological and evolutionary significance of these remarkable social parasites and their impact on the stability of the fungus-growing ant mutualism.

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Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: 10.1111/j.1365-2311.2012.01384.x

Figure S1. Live *Trachymyrmex zeteki* queens with fungal growth on their exoskeleton. (a) A queen with the most common fungal growth morphology. Photo taken by Alex Wild. (b and c) Another queen attacked with two unique but unidentified fungal pathogens. The yellow growth on the head was likely a species in the order Hypocreales.

Figure S2. Video of a host worker grooming the *M. adamsae* parasite queen.

Figure S3. Picture of a host worker covering the *M. adamsae* parasite queen with fungal fragments.

Table S1. Colony information, experimental history, and analyses.

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