Cooperation, conflict, and coevolution in the attine ant-fungus symbiosis

Natasha J. Mehdiabadi, Benjamin Hughes, and Ulrich G. Mueller

Section of Integrative Biology, School of Biological Sciences, University of Texas at Austin, Austin, TX 78712, USA

Fungus-growing ants in the tribe Attini represent a classic example of a mutualism. These ants obligately depend on fungus as their major food source, while the fungus receives both vegetative substrate (nourishment) from the ants and protection from pathogens. Here, we try to identify both benefits and costs of the association by using cultivar switch experiments. We assessed the benefits to each mutualistic partner by replacing the native fungus (cultivar) used by the primitive attine ant species *Cyphomyrmex muelleri* with a novel cultivar, that of the closely related ant species *Cyphomyrmex longiscapus*. We show that interspecific cultivar switches caused a significant decline in worker number, garden biomass, and the number of reproductives produced by colonies. In contrast, these effects were not seen in intraspecific switches. We also examined possible costs of the mutualistic association. We estimated colony sex ratios for *C. longiscapus* to determine whether cultivars can bias reproductive allocation toward females; such bias may evolve because only female reproductives can disperse the fungus, and males are therefore of no value to the fungus. However, interspecific cultivar switches did not significantly affect sex ratios. Cultivar switch experiments represent a new tool for studying cooperation, conflict, and coevolution between mutualistic partners in the attine ant-fungus symbiosis.

Key words: Attini, benefits, costs, *Cyphomyrmex longiscapus*, *Cyphomyrmex muelleri*, Formicidae, fungus-growing ants, Hymenoptera, mutualism, sex allocation, sex ratios. [Behav Ecol 17:291–296 (2006)]
embedded in the fungus (Weber, 1972; Mehdiabadi NJ, personal observation). The goal of this study was to explore whether sex-ratio conflict occurs between the ants and the cultivar in a primitive attine ant-fungus system, as well as to identify and measure aspects of anti-fungus cooperation in this symbiosis.

For this study, we used the primitive attines Cyphomyrmex longiscapus and Cyphomyrmex muelleri (Mueller and Wcislo, 1998; Schultz et al., 2002). Population genetic analyses using allozyme and microsatellite markers showed that the previously recognized C. longiscapus sensu lato actually comprises two cryptic species (C. longiscapus sensu stricto and C. muelleri; Schultz et al., 2002). These two species are sympatric in Panama, and reproductive isolation between them is indicated by the absence of heterozygotes at differentially fixed microsatellite and allozyme loci (Schultz et al., 2002). Each of these cryptic species cultivates only one type of fungus, and these two cultivars are distantly related (Mueller et al., 1998). Nevertheless, horizontal transmission is common for members of this genus (Green et al., 2002; Mueller et al., 1998). C. muelleri and C. longiscapus are useful for our studies because C. muelleri accepts the cultivar of C. longiscapus (Mueller et al., 2004). However, C. longiscapus will not readily accept the cultivar of C. muelleri, preventing reciprocal switches (Himler A and Mueller UG, in preparation). Nevertheless, the ability to remove a native, coevolved cultivar and replace it with a novel, non-coevolved cultivar allows us to examine aspects of cooperation and conflict in this mutualism.

Using intraspecific (i.e., within species) and interspecific (i.e., between species) cultivar switch experiments, we asked the following questions:

**What aspects of cooperation derive from the mutualistic association?**

The prediction is that colonies that switch to a novel fungus, which occurs occasionally under horizontal transmission of cultivars between colonies (Green et al., 2002; Mueller et al., 1998), will have lower fitness than those colonies that cultivate their native fungus. We measured cooperation by estimating fitness of both the fungus and the ants in switched nests and controls. A correlate of fitness for the fungus is garden biomass. We assume that the larger the biomass of a garden, the greater the chance of fungal propagation. Such propagation is a benefit not only to the fungus but also to the ants as a larger garden biomass means more food for the ants. In addition, we estimated ant colony fitness by measuring worker population size as well as the production of reproductives. A larger colony size and a greater number of reproductives increase the fitness for the colony (as well as for the fungus, unless the colony sex ratio becomes male biased).

**What forms of conflict are involved in this mutualism?**

We focused on the potential for conflict over the sex ratio between the ants and the fungus. Sex allocation varies from one colony to another for both C. muelleri and C. longiscapus (Mueller, 2002). Such differences might be explained by the differential ability of fungal cultivars to manipulate the ant sex ratio to become more female biased. Specifically, we examined the potential for fungal manipulation of the ant sex ratio by measuring numerical sex ratios after eclosion of reproductive brood, even though such manipulation can occur at earlier developmental stages. Nevertheless, adult reproductives can remain in nests for long periods of time until nuptial flights, representing an energy cost to the colony (Mueller UG, personal observation). As noted above, these parties should have conflicting sex-ratio interests, with the fungus predicted to prefer a sex ratio exclusively or highly biased toward females and the queens and workers predicted to prefer a less female-biased sex ratio (i.e., production of proportionally more males), against the interests of the fungus.

**METHODS**

**Cooperation between ants and coevolved fungus**

Here, we examined the possibility of coevolution between C. muelleri and its host fungus by having the ants cultivate a novel, non-coevolved fungus.

**Collection and construction of colonies**

We used 60 queenright colonies (i.e., colonies with a queen present) of C. muelleri \((n = 45)\) and C. longiscapus \((n = 15)\) collected in the Republic of Panama in the spring and fall of 2001. C. muelleri colonies were maintained in the laboratory on either (1) their original host cultivar, (2) the same type of cultivar from a different conspecific colony, (3) or a novel cultivar from the closely related species C. longiscapus. In addition, C. longiscapus colonies were reared on their native fungus. All four treatments were replicated 15 times for a total of 60 colonies. Using a block design, we standardized worker number and garden biomass within each of the 15 replicates. Colony size ranged from 6 to 42 workers (average colony size of a field-collected C. muelleri colony is 44 ± 28 SD workers; range 6–109; \(n = 106\) [Schultz et al., 2002]), and garden biomass ranged from 80.5 to 1943.1 mg across all replicates. For example, all four treatments from replicate one had six workers and a garden biomass of 80.5 mg, all four treatments from replicate fifteen had 42 workers and a garden biomass of 1943.1 mg, and so on. Before the experiment began, we removed brood (eggs, all larval instars, and pupae) and reproductives from all colonies by carefully searching through the entire garden under a microscope. Newly emerged workers or reproductives produced within the first 4 weeks of the experiment were not counted in case some brood was missed during the removal process. Nevertheless, only a few colonies produced new workers within this time period.

**Maintenance of colonies**

Each colony was housed in a square plastic nest-box (side, 7.5 cm; height, 3.0 cm), which was connected by transparent Tygon tubing (diam: 5/16 inches) to another box of identical size where the ants foraged and discarded dead ants and refuse (i.e., second chamber). Each week we moistened the plaster on the bottom of the nest-boxes and placed UV-sterilized organic oats, ad libitum, in the second chambers for use as garden substrate. We reared colonies under an approximate 8:16 h light:dark cycle at room temperature.

**Data collection and analyses**

For all colonies, we measured ant mortality weekly (i.e., counted corpses in the second chamber) and garden biomass monthly during the entire experiment, which lasted 210 days. To minimize disturbance to colonies, we kept all ants on their cultivars during garden biomass measurements. Nevertheless, ant biomass was negligible relative to fungal biomass (Mehdiabadi NJ, unpublished data). In addition to collecting data on ant mortality and garden biomass, we counted the number of live and dead reproductives produced by each colony at the end of the experiment.

We examined the effects of interspecific cultivar switches on (1) the relative change in colony size (CS, number of workers) between the beginning and the end of the experiment for each colony \([(C_{S_{end}} - C_{S_{start}})/C_{S_{start}}]\), (2) relative changes in garden biomass (GB) between the start and the end of...
Sex-ratio conflict—ants versus fungus

Using within-species cultivar switches, this experiment tested whether the ants or the cultivar controlled the numerical sex ratios of ants. To do this, we switched cultivars between nests having very different sex ratios to determine if the cultivars could affect the sex ratios of colonies in which they were placed.

Collection and setup of colonies

We used 24 queenright colonies of *C. longiscapus* collected in the Republic of Panama in December 2001. This experiment incorporated three treatments, each with eight replicates. We took ants from nests collected in the wild that had highly male-biased sex ratios (≥75%) and reared them on cultivars from nests that had highly female-biased sex ratios (≥75%) and vice versa (ranges of numbers of male and female reproducives per nest: 3–15). In addition, we included controls in which we placed both the ants and cultivars from nests with highly male-biased sex ratios together (*n* = 3) and did the same for those from nests with highly female-biased sex ratios (*n* = 5). Using a block design, we standardized worker number and garden biomass within each of the eight replicates. Colony size ranged from 1 to 40 workers, and garden biomass ranged from 29.2 to 624.0 mg across the replicates. We removed brood and reproducives from all colonies before starting this experiment. We reared colonies as we did for the first experiment, except we placed autoclave-sterilized polenta, as needed, in the second chamber for use as garden substrate.

Data collection and analyses

For all colonies, we measured ant mortality every 2 weeks, garden biomass every 6 weeks, and production of reproducives every month for 6 months. To minimize disturbance to colonies, we again kept all ants on their cultivars during garden biomass measurements as well as during measurement of sex ratios.

We analyzed data in the same way as we did for the interspecific switch experiment. In addition, we examined the effects of within-species cultivar switches on colony sex ratio as estimated by (number of males)/(number of males + number of female reproducives). The fixed effect was treatment (control and switch treatments), and the random effect was colony (each of the three colonies per replicate standardized according to worker number and garden biomass). We used Tukey HSD post hoc tests to distinguish among all pairs of treatments.

RESULTS

Cooperation between ants and coevolved fungus

To examine the benefits and costs of this mutualism, we replaced the native, coevolved cultivar of the ant *C. muelleri* with a novel, non-coevolved cultivar, that of the ant *C. longiscapus*. Both colony size and the relative growth rate of the cultivar decreased over time for groups with the switched fungus in comparison to controls (colony size: *F* = 6.39, *p* = .0011; relative growth rate of cultivar: *F* = 4.00, *p* = .0136; Figures 1 and 2). In addition, *C. muelleri* colonies reared on their native, coevolved cultivar produced significantly more reproducives compared to those reared on the novel, non-coevolved cultivar (*χ^2^ = 34.28, *p* ≤ .0001; Figure 3). Clearly, both the ants and the introduced fungus show reduced fitness due to associating with a novel, non-coevolved partner.

Survivorship of reproducives on a novel fungus

This experiment shows that, in contrast to the results of colony biomass, there was no effect of treatment on either male

---

**Survivorship of reproducives on a novel fungus**

This experiment tested whether survivorship of male and female reproducives changed when they were switched to a novel cultivar in the absence of any potential sex-ratio influences from the queen and workers (i.e., in reproductive-only colony fragments with the queen and workers removed).

**Collection and setup**

We used 22 colonies of *C. muelleri* (*n* = 11) and *C. longiscapus* (*n* = 11) collected in the Republic of Panama in the spring and fall of 2001. From colonies of both species, we reared only male and female reproducives on their native fungus (controls). We also reared reproducives of *C. muelleri* on the cultivar of *C. longiscapus* (switch treatment). We used a paired design: half of the reproducives from a given *C. muelleri* colony constituted the control and the other half constituted the switch treatment for each of the 11 replicates. For all replicates, we standardized the number of reproducives and garden biomass: each colony fragment contained two male reproducives and two female reproducives, as well as a small piece of fungal garden (about 50 mg). We performed this experiment over 80 days: from February 2003–May 2003. We used a double-blind protocol to control for potential bias.

**Maintenance of reproducives and cultivar**

The reproducives and cultivar were placed in small plaster-bottom petri dishes (diam: 5 cm). We moistened the plaster on the bottom of the dishes and provided the ants with autoclave-sterilized polenta, *ad libitum*, for garden substrate. Dishes were regularly inspected for fungal contaminants and waste products (e.g., dead fungal fragments, dead ants, and unused polenta). We reared colony fragments in the dark during the entire experiment except during data collection.

**Date collection and analyses**

For all colony fragments, we collected data on male and female reproductive survivorship (day of death) three to five times per week (every 1–2 days). We also qualitatively noted the general health and activity of ants and cultivars. The experiment was terminated on day 80, at which time all remaining reproducives were recorded as having lived to at least that day. Life span (e.g., longevity of the first male to die, second male to die, and so on) was tabulated for each treatment (two controls and one switch treatment). We compared male and female reproductive survivorship (number of days until death) in the two treatments using a univariate mixed model ANOVA (PROC MIXED) in SAS v.8 (SAS Institute, 2000). The fixed effects were treatment (two controls and the switch treatment) and sex (male and female). The random effect was colony (the eight *C. muelleri* reproducives from a given colony that were randomly assigned to either the control or the experimental treatment within a replicate).
or female reproductive survivorship ($F_{2,113} = 1.21, p = .3034$). The only significant result was that females lived longer than males ($F_{1,113} = 142.25, p < .0001$; $C. muelleri$ reproductive survivorship did not change when male and female reproductives were reared on the cultivar of $C. longiscapus$ (Tukey HSD post hoc test: $t_{113} = 1.44, p = .32$).

**DISCUSSION**

The two main results of this experiment are (1) the ants and fungi appear to be coadapted, at least for the two ant-fungus associates studied, and (2) the fungi seem unable to bias colony sex ratios in a way that would enhance the fitness of the fungus.

Two factors may explain why $C. muelleri$ colonies raised on their host cultivar had higher fitness than those raised on the novel cultivar. First, the host (native) fungus might be nutritionally superior to less preferred cultivars. This itself could result from simple acclimation but, if present, is more likely to reflect adaptations in the ant and/or fungus. Second, the ants may not be behaviorally or genetically adapted to meet the cultivation requirements of the novel cultivar (e.g., ants may not carry the appropriate antibiotic-producing bacterium to kill garden parasites specialized on a novel cultivar).

Our results adduce at least preliminary evidence for species-specific coevolution, in which each species in a partnership does better with its own partner than with the partners of a related species. This is the first demonstration, to our knowledge, that coexisting attine ants and fungus also suffer fitness losses when presented with new partners. Switching fungi between species for $C. muelleri$ resulted in a decrease in colony size, garden biomass, and the number of reproductives.

**Figure 1**
Percentage change (+ SE) in $Cyphomyrmex muelleri$ colony size relative to controls (cultivated original native fungus) for intraspecific cultivar switches (cultivated native fungus but taken from another $C. muelleri$ colony) and for interspecific cultivar switches (cultivated novel, non-coevolved fungus) from the experiment on Cooperation Between Ants and Coevolved Fungus. Tukey HSD post hoc tests—control versus intraspecific switch: $t = 1.62, p = .3805$; control versus interspecific switch: $t = 0.0076, p = .9365$.

**Figure 2**
Relative growth rate of cultivar ([ln GBend − ln GBstart]/210 experimental days; + SE) for four treatments from the experiment on Cooperation Between Ants and Coevolved Fungus: (1) $Cyphomyrmex muelleri$ ants on original $C. muelleri$ fungus, (2) $C. muelleri$ ants on a different $C. muelleri$ fungus, (3) $C. muelleri$ ants on $Cyphomyrmex longiscapus$ fungus, and (4) $C. longiscapus$ ants on $C. longiscapus$ fungus. Different letters represent statistically significant differences.

**Figure 3**
Number of $Cyphomyrmex muelleri$ reproductives produced (± Wald 95% confidence limits) for four treatments from the experiment on Cooperation Between Ants and Coevolved Fungus.
produced. In contrast, intraspecific switches did not significantly alter these fitness measures relative to controls (Figures 1–3).

Despite compelling evidence for mutualism between coadapted ant and fungus, the possibility still exists that they could be in conflict over the optimal colony sex ratio. Our data seem to suggest that if such sex-ratio conflict exists between these ants and the fungus, it is resolved in favor of the ants (Table 1). The failure of such cultivar switches to affect sex ratios suggests that the fungus lacks sufficient control over the colony sex ratio, even though it would seem in the interest of the fungus to produce more female reproductives (Mueller, 2002). The lack of a fungal effect could result from several possible factors: (1) the cultivar might be unable to manipulate the ant sex ratio, either because it is physiologically impossible to do so or the fungus simply lacks the requisite genetic variation; (2) the time frame of our experiment was insufficient to reveal such an effect; (3) the so-called "lower" attine fungi are too recently domesticated within the symbiosis and thus did not have time to counter the sex-ratio-biasing mechanisms of the ants; (4) the fungi are transferred between ant species too frequently such that there is not enough time when associated with a single species to evolve effective sex ratio–biasing mechanisms against the sex-ratio interests of a new ant host (Mueller et al., 1998); (5) behavioral control of the sex ratio by the ants is for some reason more effective than the presumably chemical control by the fungi; and (6) expression of ant-fungus conflict is selected against because it reduces the efficiency of the overall symbiosis. Fungal manipulation of the ant sex ratio deserves further exploration, especially in more derived attine species (e.g., the leaf-cutting ants) thought to have longer evolutionary associations with their cultivars (Mueller et al., 1998). The ease of manipulating and substituting mutualistic partners in the attine ant-fungus symbiosis makes them ideal for such studies. Cultivar switch experiments may emerge as a major new research tool for unraveling the evolutionary dynamics of conflict and cooperation between mutualistic partners in the attine ant-fungus symbiosis.

Figure 5
Male survivorship (+SE) for the experiment on Sex-Ratio Conflict—Ants Versus Fungus: male = cultivar or ants that came from a nest with highly male-biased sex ratios, female = cultivar or ants that came from a nest with highly female-biased sex ratios. Different letters represent statistically significant differences. Tukey HSD post hoc tests (ants/cultivar): female/female versus female/male: $t = 1.33, p = .5785$; female/female versus male/female: $t = 5.19, p = .0081$; female/female versus male/male: $t = 5.02, p = .0095$; female/male versus male/female: $t = 5.13, p = .0022$; female/male versus male/male: $t = 5.24, p = .0019$; and male/female versus male/male: $t = 1.28, p = .2083$.
We thank Jerry Coyne for insightful discussions, Saara DeWalt for statistical help, Abigail Green for help with rearing colonies, Anna Himler for sharing unpublished data, and Jerry Coyne, Michiel Dijkstra, Mark Elgar, and an anonymous reviewer for comments on the manuscript. We thank the Autoridad Nacional del Ambiente of the Republic of Panama and the Smithsonian Tropical Research Institute for collecting and research permits. This work was supported by a National Science Foundation CAREER Award DEB-9985879 to U.G.M. and a Carl Gottfried Hartman Graduate Research Fellowship and a Dorothea Bennett Memorial Graduate Research Fellowship from the University of Texas at Austin to N.J.M.

REFERENCES


