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

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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, intraspecific cultivar switches did not significantly affect ant 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)]*

Recent evolutionary theory has regarded mutualisms as reciprocal exploitations in which each partner receives net benefits from the other (Axelrod and Hamilton, 1981; Futuyma and Slatkin, 1983; Herre et al., 1999; Leigh and Rowell, 1995; Sachs et al., 2004; Thompson, 1982). This view of mutualisms emphasizes the importance of identifying and measuring reproductive costs as well as benefits, for it is net fitness that is likely to shape such interactions (Bronstein, 1994, 2001; Connor, 1995).

The attine ant-fungus symbiosis is one mutualism in which the benefits and costs to each party can be readily identified and measured. Fungus-growing ants in the tribe Attini obligately depend on fungus as their major food source (Hölldobler and Wilson, 1990; Weber, 1972) and in exchange provide the fungus with nourishment in the form of vegetative substrate as well as protection from garden pathogens (Currie et al., 1999; Martin, 1987; Mueller 2002; Mueller and Gerardo, 2002; Mueller et al., 2005). Here, with the aid of a new method, we are able to rigorously identify and measure the benefits and costs of this association for the first time and to explore some of the evolutionary interests of the cultivated fungi.

First, we focus on the benefits of the association between the ant host and its native, coevolved fungus. This is especially relevant because the long-standing assumption that fungi are only transmitted vertically (i.e., from mother to daughter colonies; Chapela et al., 1994) has recently been shown to be incorrect (Green et al., 2002; Mueller et al., 1998, 2001). Rather, cultivars are occasionally transmitted horizontally between ant species, and at least some cultivars appear to be

closely related to free-living fungal populations (Bot et al., 2001; Green et al., 2002; Mueller et al., 1998). These modes of horizontal cultivar transmission cause the genetic lineages of ant and fungus to become more loosely coupled to each other in an evolutionary sense, creating novel types of ant-cultivar conflict (Mueller, 2002).

Second, we examine the costs of the mutualistic association. An intriguing aspect of the attine ant-fungus mutualism is its possible role in conflict over the sex ratio, that is, the ratio of male to female reproductives produced by a social insect colony. In social Hymenoptera, including those lacking heterospecific symbionts, conflict over the sex ratio exists between the mother queen and her daughters (the workers) because of differences in their genetic relatedness to the reproductives inherent in the haplodiploid mechanism of sex determination (Trivers and Hare, 1976). In the simplest case of one singly mated queen, the queen should prefer a 1:1 sex ratio (male:female) because she is equally related to her sons and daughters. Workers, however, should prefer a 1:3 sex ratio as they are more closely related to their sisters ($r = .75$) than to their brothers ($r = .25$). When there is more than one queen or when queens are multiply mated, predicting and measuring sex-ratio conflict in social insect colonies become more complex (Bourke and Franks, 1995; Crozier and Pamilo, 1996; Mehdiabadi et al., 2003).

The presence of heterospecific symbionts in a social insect colony, such as fungus-growing ants, causes further complications for studying conflict over the sex ratio. In such cases, three parties may have conflicting interests: the mutualistic fungus, the workers, and the queen. Besides the conflict of interests between queen and workers, the fungus itself should prefer the production of only female reproductives as only virgin queens disperse the fungus (they carry small pieces of the natal fungus to start their own garden; Huber, 1905a,b; Ihering, 1898; Mueller et al., 2001). From the evolutionary viewpoint of the fungus, then, males are a waste of colony resources. Indeed, it seems possible for the fungus to manipulate the ant sex ratio because brood are covered with and are

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 ant-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 cutivar 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 [$(CS_{end} - CS_{start})/CS_{start}$], (2) relative changes in garden biomass (GB) between the start and the end of

the experiment [(ln GB_{end} − ln GB_{start})/210 experimental days], and (3) the number of reproductives produced by colonies.

All data except for data on reproductives were analyzed with a univariate mixed model ANOVA (PROC MIXED) using SAS v.8 (SAS Institute, 2000). The fixed effect in each analysis was treatment, and the random effect was colony (each of the four colonies per replicate standardized according to worker number and garden biomass). Differences among pairs of treatments were determined using Tukey honestly significant difference (HSD) post hoc tests. Differences among treatments in number of reproductives produced were analyzed with a general linear model with Poisson errors in SAS (SAS Institute, 2000).

Survivorship of reproductives on a novel fungus

This experiment tested whether survivorship of male and female reproductives 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 reproductives on their native fungus (controls). We also reared reproductives of *C. muelleri* on the cultivar of *C. longiscapus* (switch treatment). We used a paired design: half of the reproductives 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 reproductives and garden biomass: each colony fragment contained two male reproductives and two female reproductives, 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 reproductives and cultivar
The reproductives 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 auto-clave-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 reproductives 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* reproductives from a given colony that were randomly assigned to either the control or the experimental treatment within a replicate).

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 reproductives 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 reproductives 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 reproductives 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 reproductives). 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: $F_{3,42} = 6.39$, $p = .0011$; relative growth rate of cultivar: $F_{3,42} = 4.00$, $p = .0136$; Figures 1 and 2). In addition, *C. muelleri* colonies reared on their native, coevolved cultivar produced significantly more reproductives compared to those reared on the novel, non-coevolved cultivar ($\chi_3^2 = 34.28$, $p \leq .0001$; Figure 3). Clearly, both the ants and the introduced fungus show reduced fitness due to associating with a novel, non-coevolved partner.

Survivorship of reproductives 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
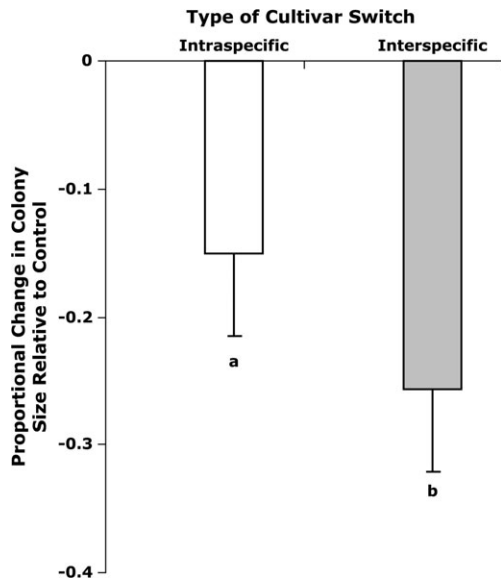
Figure 1
Percentage change (+SE) in *Cyphomyrmex muelleri* colony size relative to controls (cultivated original native fungus) for intraspecific cultivar switches (cultivated native fungus but taken from another *C. muelleri* colony) and for interspecific cultivar switches (cultivated novel, non-coevolved 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: $t = 0.0076$, $p = .0365$.

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: $t_{113} = 1.44$, $p = .32$).
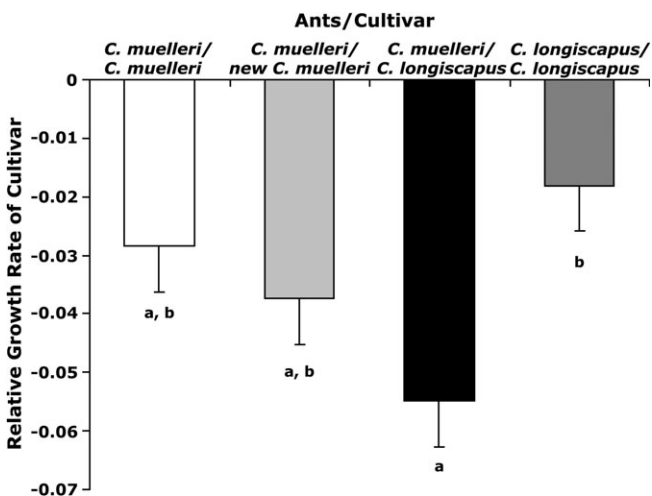

Figure 2
Relative growth rate of cultivar ([ln GB$_{end}$ − ln GB$_{start}$]/210 experimental days; +SE.) for four treatments from the experiment on Cooperation Between Ants and Coevolved Fungus: (1) *Cyphomyrmex muelleri* ants on original *C. muelleri* fungus, (2) *C. muelleri* ants on a different *C. 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.
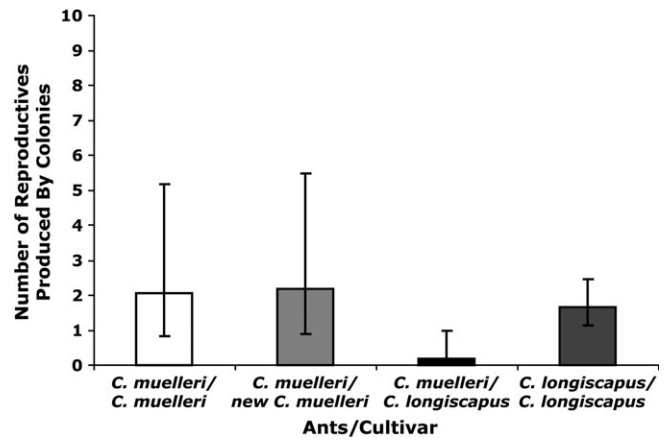

Figure 3
Number of *Cyphomyrmex muelleri* reproductives produced (±Wald 95% confidence limits) for four treatments from the experiment on Cooperation Between Ants and Coevolved Fungus.

Sex-ratio conflict—ants versus fungus

Our results show that neither colony size nor garden biomass was altered by within-species cultivar switches (colony size: $F_{3,13} = 2.00$, $p = .1642$; garden biomass: $F_{3,13} = 0.89$, $p = .4713$; Table 1). The ant sex ratio also did not differ among treatments ($F_{3,9} = 1.55$, $p = .2686$; Table 1; Figure 4); obviously, fungus from a nest with an extreme sex ratio in either direction did not seem to affect—at least during the course of this study—the sex ratio of the nest into which the fungus is introduced. In addition, the sex ratio did not show a strong comparison with the prior sex ratio of the ants, that is, the ant sex ratio before cultivar switches took place. Nevertheless, the proportion of males surviving to the end of the experiment differed among treatments ($F_{3,6} = 17.89$, $p = .0021$). Males had higher survivorship when reared by ants from nests with highly female-biased sex ratios compared to when they were reared by ants from nests with highly male-biased sex ratios (Figure 5).

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

Table 1

Summary data for sex-ratio experiment

Ants from[a]	Fungus from[a]	Number of colonies	Change in colony size[b]	Change in garden biomass[c]	Total number of reproductives[d]	Sex ratio[e]
Female-biased nest	Male-biased nest	8	−0.4399 (±0.2982)	−0.0131 (±0.008)	11.8 (7.0)	0.5313 (0.1451)
Male-biased nest	Female-biased nest	8	−0.3444 (±0.2982)	−0.0122 (±0.008)	33.3 (14.6)	0.9400 (0.1343)
Male-biased nest	Male-biased nest	3	0.6945 (±0.4556)	0.0040 (±0.0125)	86.3 (45.7)	0.8430 (0.2052)
Female-biased nest	Female-biased nest	5	−0.2189 (±0.3628)	−0.0213 (±0.010)	7.4 (6.2)	0.6667 (0.2052)

[a] Nest from which the ants or fungus were taken from: female-biased nest = nests taken from field with highly female-biased sex ratios ($\geq$75% female); male-biased nest = nests taken from field with highly male-biased sex ratios ($\geq$75% male).

[b] Number of workers = the proportional change in colony size at the beginning and at the end of the experiment [$(CS_{end} - CS_{start})/CS_{start}$]; SEs in parentheses.

[c] GB = the relative growth rate from the start to the end of the experiment [$(\ln GB_{end} - \ln GB_{start})/294$ experimental days (g g^{-1} day^{-1})]; SEs in parentheses.

[d] Average number of male and female reproductives produced by colonies; SEs in parentheses.

[e] (Number of males)/(number of males + number of females); SEs in parentheses.

produced. In contrast, intraspecific switches did not significantly alter these fitness measures relative to controls (Figures 1–3).

Despite compelling evidence for mutualism between co-adapted 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.
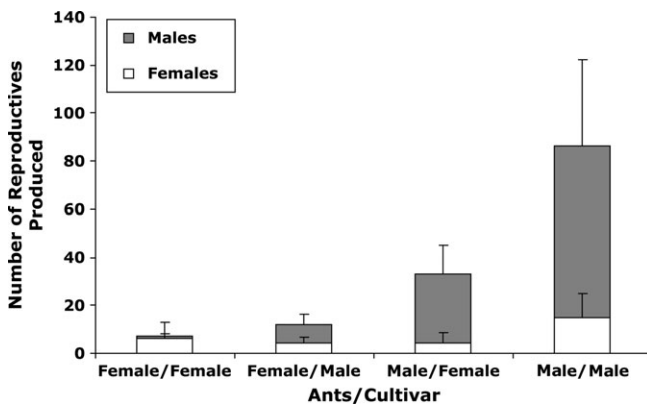

Figure 4
Average absolute numbers of male and female reproductives (+SE) for the experiment on Sex-Ratio Conflict—Ants Versus Fungus: male = cultivar or ants that came from a nest with highly male-biased sex ratios, female = cultivar or ants that came from a nest with highly female-biased sex ratios.
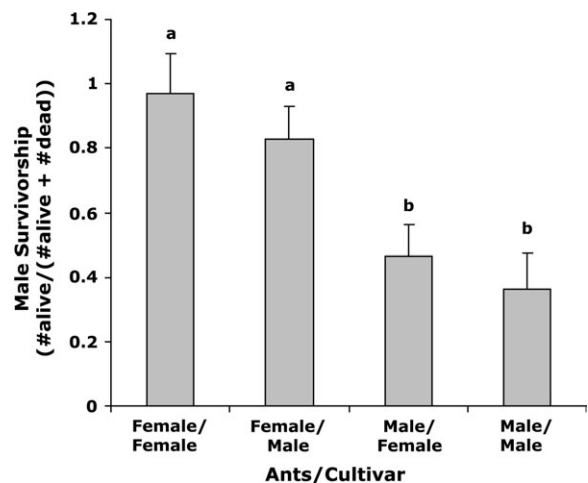

Figure 5
Male survivorship (+SE) for the experiment on Sex-Ratio Conflict—Ants Versus Fungus: male = cultivar or ants that came from a nest with highly male-biased sex ratios, female = cultivar or ants that came from a nest with highly female-biased sex ratios. Different letters represent statistically 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: $t = 1.28$, $p = .6083$.

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