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AN EVALUATION OF THE POSSIBLE ADAPTIVE FUNCTION OF FUNGAL BROOD COVERING BY ATTINE ANTS

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Fungus-growing ants (Myrmicinae: Attini) live in an obligate symbiotic relationship with a fungus that they rear for food, but they can also use the fungal mycelium to cover their brood. We surveyed colonies from 20 species of fungus-growing ants and show that brood-covering behavior occurs in most species, but to varying degrees, and appears to have evolved shortly after the origin of fungus farming, but was partly or entirely abandoned in some genera. To understand the evolution of the trait we used quantitative phylogenetic analyses to test whether brood-covering behavior covaries among attine ant clades and with two hygienic traits that reduce risk of disease: mycelial brood cover did not correlate with mutualistic bacteria that the ants culture on their cuticles for their antibiotics, but there was a negative relationship between metapleural gland grooming and mycelial cover. A broader comparative survey showed that the pupae of many ant species have protective cocoons but that those in the subfamily Myrmicinae do not. We therefore evaluated the previously proposed hypothesis that mycelial covering of attine ant brood evolved to provide cocoon-like protection for the brood.

KEY WORDS: **Formicidae, fungus-growing ants, mycelium, parasites, pupae, prophylactic behavior.**

Larvae of many holometabolous insects spin silk cocoons just prior to metamorphosis to the pupal stage (Chapman 1998), which provides multiple forms of protection during this vulnerable immobile stage. This protection may extend to larger enemies such as predators and macroparasites, but also to less conspicuous risk factors related to desiccation and parasitic microbes. In a phylogenetic survey across holometabolous insect taxa, Craig (1997) suggested that larval silk production has primarily protective functions, but also showed that the trait is absent in some clades.

Among Hymenoptera (bees, wasps, and ants), the ants are remarkably variable with respect to cocoon formation (Wheeler 1915). Although eusocial wasps have mostly retained silk cocoons that adhere to the inside of the brood cells (e.g., Chao and Hermann 1983; Ishay and Ganor 1990) and eusocial bees often spin silk to cap brood cells (e.g., Oertel 1930; see Michener 1977 for some exceptions), ant brood is normally piled within the nest, where the microclimate is either relatively well-controlled or where workers can move brood to deeper chambers when upper nest sections become too warm or dry. Constant monitoring of pupae by ant workers thus provides considerable protection against parasites (sensu Cremer et al. 2007) and desiccation. However, metamorphosing in well-buffered and well-defended eusocial nests may

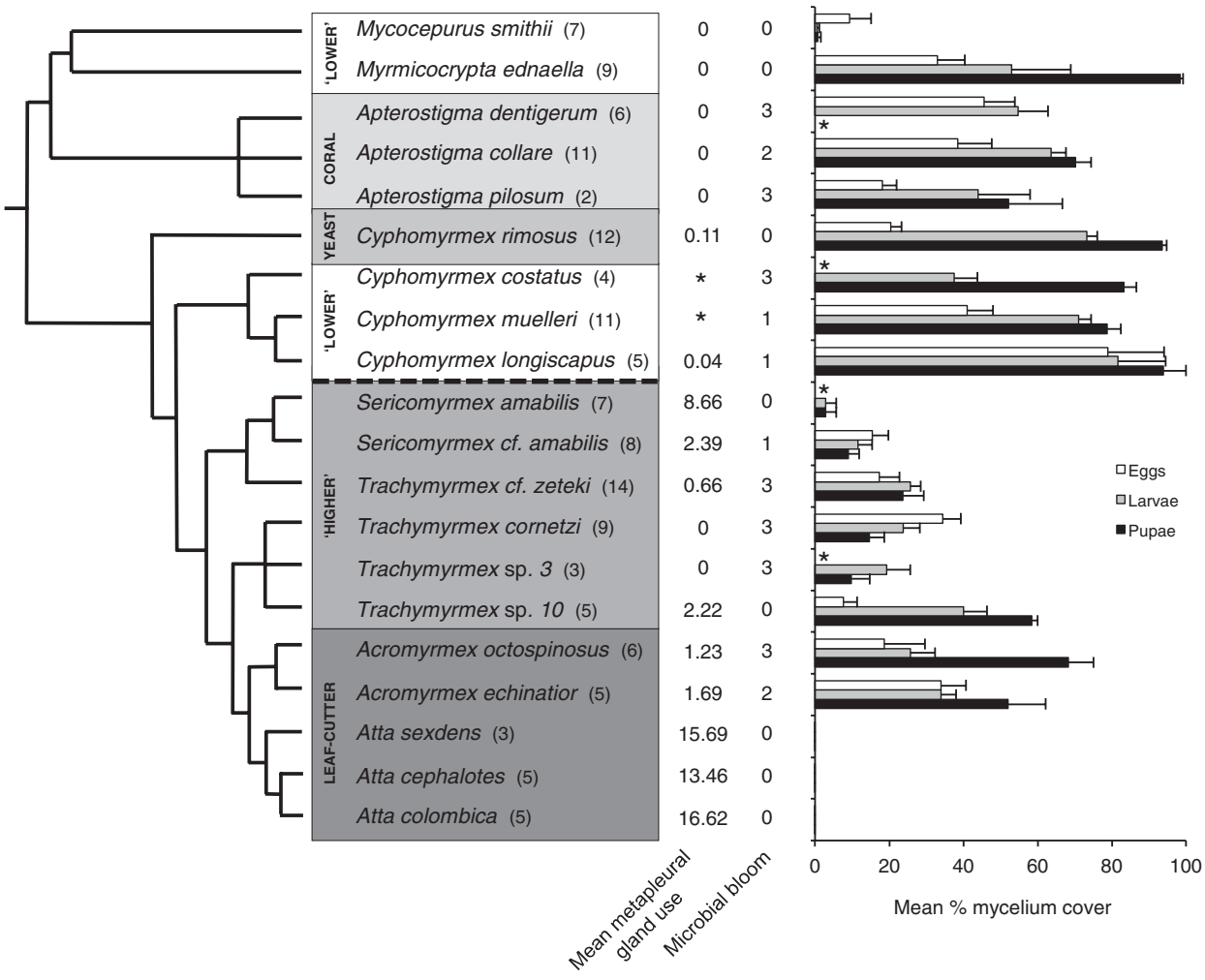

Figure 1. **Summary of information from attine species that were surveyed. Phylogeny after Villesen et al. (2002), Sumner et al. (2004), Schultz and Brady (2008), and Bacci et al. (2009). Agricultural systems are shown as shaded rectangles and follow the scheme in Schultz and Brady (2008), and the dashed black line marks the evolutionary transition between "lower" and "higher" attines. Numbers in parentheses after species names indicate the total number of colonies examined. Mean metapleural gland-grooming data after infection come from different colonies to the rest of the data. The presence of visible actinomycetes on the workers is indicated by the variables 0, 1, 2, and 3, where 0 is no workers from any colonies with visible bacteria, 1 is less than 50% of colonies had workers with visible bacteria, 2 is more than 50% of colonies had workers with visible bacteria, and 3 is bacteria visible on workers for all colonies examined. The bar chart shows the mean percentage mycelial cover on brood (eggs, larvae, and pupae) from all surveyed species. Stars indicate missing data due to low sample sizes (mycelial cover data) or data not available (metapleural gland grooming).**

simultaneously introduce a new trade-off that may select against the maintenance of cocoons. With external threats being reduced to low levels, cocoons may become a hindrance if they delay detection of infections that are readily curable if detected early. It may thus be more effective to have naked pupae that can be efficiently groomed and monitored for parasites.

The Attini are a monophyletic tribe with more than 230 described species, which have evolved an obligate dependency on cultivating fungi for food. Five different types of fungal cultivation are used, each of which represents an evolutionary transition (Schultz and Brady 2008), with the transition between the "lower" and "higher" attines being the most distinct (Fig. 1). At the base of the attine tree, the "paleo" and "lower" attine ants are generally small in body and colony size, lack worker caste polymorphism, and manure their gardens with dead organic matter. This contrasts with the "higher" attines (including the leaf-cutting ants), which rear specialized fungi, have larger colonies, increasing worker caste differentiation, and partly or completely herbivorous-foraging habits (Mueller 2002). Wheeler (1907) and others have noted that many attine ants cover naked brood with tufts of the symbiotic fungus, and that this behavior varies among taxa (summarized in Table 1, see also LaPolla et al. 2002 and Mueller 2002). In some species, such brood cover effectively creates partial to complete cocoon-like envelopes around the brood,

Table 1. Mycelial covering behavior from literature sources, combining both field and laboratory studies (Wheeler 1907; Weber 1945 and 1946 in Wheeler 1948; Schultz and Meier 1995; Adams et al. 2000; Dijkstra et al. 2005; Lopes et al. 2005; Camargo et al. 2006a,b). Taxonomic identifications are given at the genus level. Numbers refer to the number of species within a genus for which authors specifically mentioned the presence or absence of mycelial cover on particular developmental stages. Lack of data is indicated by -.

	Presence/Absence		
Genera	Eggs	Larvae	Pupae
Myrmicocrypta	-/-	1/-	-/1
Apterostigma	-/-	1/-	-/-
Cyphomyrmex	1/-	8/-	5/-
Sericomyrmex	-/-	2/2	1/-
Trachymyrmex	1[1]/-	2[1]/1	1[1]/1
Acromyrmex	1/-	6/1	2/-
Atta	-/3	1[2]/2	-/2

[1]Three of the four *Trachmyrmex* records come from to LaPolla et al. (2002) and represent very small amounts as the mycelial cover was reported to be "virtually absent" in eggs, larvae, and pupae of *T. arizonensis*. [2]According to Weber (1972), the mycelial cover of larvae "may be largely absent in older *Atta* broods," Weber (1945 in Wheeler 1948) elsewhere noted the "scanty" mycelial cover on *Atta* larvae.

but the mycelial cover adheres directly to the cuticular surface instead of having an airspace in between, as is found between a pupa and its silk cocoon. The function of this behavior is not resolved, although three untested hypotheses have been proposed: (1) protection against predators such as army ants or parasitism by wasps (LaPolla et al. 2002; Powell and Clark 2004; Fernández-Marín et al. 2006a; Pérez-Ortega et al. 2010); (2) protection against potentially adverse environmental factors (Mueller et al. 2010); and (3) protection from microbial parasites (Lopes et al. 2005; Mueller et al. 2010).

We focus our analyses on the third hypothesis, because there is ample evidence that the advent of attine fungus farming around 50 million years ago (Schultz and Brady 2008) has confronted these ants with multiple challenges from parasites that are absent in many other ants. First, the humid nest environment that needs to be maintained for optimal growth of the fungus garden also provides ideal conditions for the survival and growth of other, possibly parasitic, microorganisms. Second, a continuous dependence on fungus gardens likely meant that avoiding infections by moving brood around to safer nest chambers became less effective and impractical, given the many (up to thousands) of brood in mature nests. One solution for these novel nursing constraints may have been to increase brood grooming rates that could explain why metapleural glands are relatively large in attine ants, especially in the most derived genera (Hughes et al. 2008; see also Do Nascimento et al. 1996; Fernández-Marín et al. 2006b, 2009; Yek and Mueller 2011). A number of attine ants use metapleural gland secretions to target point sources of infection in the fungus garden and on leaf substrate, and these behaviors can be experimentally induced (Fernández-Marín et al. 2006b, 2009). In addition, attine ants have had to cope with a specialized ascomycete parasite, *Escovopsis*, that infects their fungal crops (Currie et al. 2003) and which can be suppressed by filamentous actinomycete bacteria that many of these ants culture on their cuticle (Currie et al. 2006). Some actinomycete bacteria may also inhibit the in vitro growth of other fungi, including entomopathogens (Sen et al. 2009), but whether this has a protective effect in vivo is unknown. Recent data have shown that the cuticle of attine ants can contain a mixed community of antibiotic-producing actinomycetes (*Pseudonocardia* and *Streptomyces*; Barke et al. 2010; Schoenian et al. 2011) as well as black yeast symbionts (Little and Currie 2008) and other bacterial strains (Mueller et al. 2008; Boomsma and Aanen 2009; Sen et al 2009; Cafaro et al. 2011), but it remains unknown whether such diversity is operational in the field. In this article, we continue to use the term "actinomycete cover" as most researchers cited above appear to agree that the microbial biomass consists largely of actinobacteria and that they are somehow beneficial for the prevention or control of parasites.

Our analyses explicitly consider the effects of actinomycete cover and metapleural gland grooming on the prevalence of fungal brood-covering behavior. First, we surveyed 20 species of fungus-growing ants (Myrmicinae: Attini) to map the incidence of mycelial cover on the brood, and we discuss the likelihood of our results being compatible with cocoon-like protection driven by parasite pressure or other threats. Second, we provide a comparative analysis of the evolutionary lability of cocoon use in ants (Formicidae), which confirmed earlier assessments that the ant subfamily Myrmicinae invariably lacks pupal cocoons, so that the ancestral attine ant must have had naked pupae.

Materials and Methods

ATTINE FIELD STUDIES

Nests from 20 species were excavated near Gamboa, Panama in May 2006, 2007, and 2010. We examined brood from a minimum of three colonies for each species, with the exception of *Apterostigma pilosum* for which only two colonies with reasonable numbers of brood could be obtained (see Fig. 1 for the full species list). Voucher specimens of these species are deposited in the Colección Nacional de Referencia, Museo de Invertebrados G. B. Fairchild, Universidad de Panamá. We used a dissecting microscope to examine a minimum of 11 eggs, 14 larvae, and 14 pupae per species for the presence of mycelial cover, which meant that we secondarily excluded three species (*Cyphomyrmex costatus*, *S. amabilis,* and *Trachymyrmex* sp. 3) with too low a

number of eggs, and one species (*Apt. dentigerum*) with too few pupae. Each brood item was carefully rotated with forceps so that all sides could be examined without removing any cover. Approximate percent cover was scored by eye for each brood item and observers practiced beforehand to ensure reliability of the estimates. For analyses, we used the mean percent cover for eggs, larvae, and pupae for each species, which was calculated from the mean values for each colony within a species. As a covariate, for each colony, we examined adult workers for the presence/absence of visible actonomycete bacteria on the cuticle. Per nest, approximately 25 adult workers of a similar cuticular color (no callow workers were used) were removed from the inside and the outside of the fungus garden, placed underneath a dissecting microscope, and examined all over their bodies for the presence of the whitish actinomycete bacteria. For polymorphic species (*Atta* and *Acromyrmex*), we examined 10 small, 10 medium, and five large workers. The observations were made from the same colonies as those used for observing mycelial coating of the brood, and therefore not recorded blindly.

METAPLEURAL GLAND GROOMING

We used data collected in 2004, 2009, and 2010 on the frequency of metapleural gland grooming after infection of subcolonies with dry conidia of the fungal parasite *Metarhizium anisopliae*. Three colonies per species were collected near Gamboa and Parque Nacional Soberania (Panama). We collected data for all species for which we assessed mycelial cover, except *C. costatus* and *C. muelleri*, which thus became missing values. For each colony we set up one subcolony for observation. We placed 0.3 g of fungus garden (1 g for leaf-cutting ants) into a sterile Petri dish (100-mm diameter) with three larvae. An area of 9 mm^2 of conidia (25 mm^2 for leaf-cutting ants) from a culture plate was placed in the center of the fungus garden. Twenty workers of a similar cuticular color (fully sclerotized) were added to the fungus garden. The subcolonies were placed under a dissecting microscope, allowed to acclimatize for 5 min, and every 10 min for the following 60 min (it has been shown for three *Atta* species that this behavior peaks during the first hour after fungal infection; Fernández-Marín et al. 2006b) we recorded the number of workers grooming the fungus garden. We also recorded the number of metapleural gland grooming events (Fernández-Marín et al. 2006b, 2009) for the whole 60-min period. For analyses, we used the average metapleural gland-grooming rate per worker. Data were collected over a few interspersed years, and so inevitably the observer had prior knowledge about one of the other variables for some species.

PHYLOGENETIC AND STATISTICAL ANALYSES

We mapped species-specific data onto the most recent attine phylogenies (Schultz and Brady 2008; Bacci et al. 2009). Some species in our sample were not included in these phylogenies, so we inferred their most likely phylogenetic placement. Thus, we placed *Apt. pilosum* near *Apt. dentigerum* and *Apt. collare* within the main "*pilosum* group," and set branch lengths as equal to the latter two species. Likewise, *Trachymyrmex* sp. 3 and *Trachymyrmex* sp. 10 became nested within the main *Trachymyrmex* clade (Hughes et al. 2008). Branch lengths were estimated from Schultz and Brady (2008; Figure 1) and unknown branch lengths were placed half way between the closest known nodes. For all the subsequent analyses, controlling for phylogeny, we assessed relationships (using linear correlation through the origin; Garland et al. 1992) between phylogenetically independent contrasts, generated using the PDAP version 1.14 module (Midford et al. 2008) of the programme Mesquite version 2.6 (Maddison and Maddison 2009). We also examined the results after reducing degrees of freedom to adjust conservatively for unresolved polytomies (Midford et al. 2008). However, we only present data based upon no adjustment, as this adjustment did not significantly affect our results.

First, we tested whether mycelial cover is related to the predictor variable "higher" versus "lower" attines (Fig. 1). Second, to test whether mycelial cover on eggs, larvae, and pupae is related to actinomycete presence on the cuticle of the workers, we used the following scale: 0 = no workers from any colonies with visible bacteria, 1 = less than 50% of colonies having workers with visible bacteria, 2 = more than 50% of colonies having workers with visible bacteria, and 3 = bacteria visible on workers for all colonies examined. The reason for testing this relationship is that recent work has suggested that cuticular microbes have a less-specific role than originally thought (Sen et al. 2009), so that they could potentially protect or harm the ant brood that would then predict a negative or positive relationship with brood cover. Third, we tested for a relationship between mycelial cover and metapleural gland grooming, the rationale being that if mycelial cover serves a defensive function for the brood against parasites, species with more mycelial cover may use their metapleural glands to a lesser degree.

PUPAL COCOON PRESENCE ACROSS ANT SUBFAMILIES

Through a literature search and from personal communication with taxonomic authorities we obtained data on the presence and absence of cocoons across ant subfamilies [using the AntWeb (2009) classification], and mapped these prevalences onto the ant phylogenies of Brady et al. (2006) and Moreau et al. (2006). Despite being counterintuitive, we coded cocoon presence as (0) and cocoon absence as (1), to remain consistent with the notation already used in ant taxonomy (e.g., Baroni Urbani 1992). This analysis allowed us to formally establish that cocoons are a labile trait across the ant phylogeny and that the attine ants had direct ancestors with naked pupae.

Table 2. Statistical results for the relationships between mycelial cover on eggs, larvae, and pupae and the transition from "lower" to "higher" attines, actinomycete presence/absence, and metapleural gland grooming frequency. Statistically significant results are in bold.

	"lower" to "higher" attine transition			Actinomycete presence/absence			Metapleural gland grooming frequency		
	t	df	*p*	*r*	df	*p*	*r*	df	*p*
Eggs	−1.14	15	0.277	0.385	15	0.127	−0.514	14	**0.042**
Larvae	−2.419	18	**0.026**	−0.073	18	0.760	−0.549	16	**0.018**
Pupae	−2.219	17	**0.040**	−0.0129	17	0.958	−0.480	15	0.051

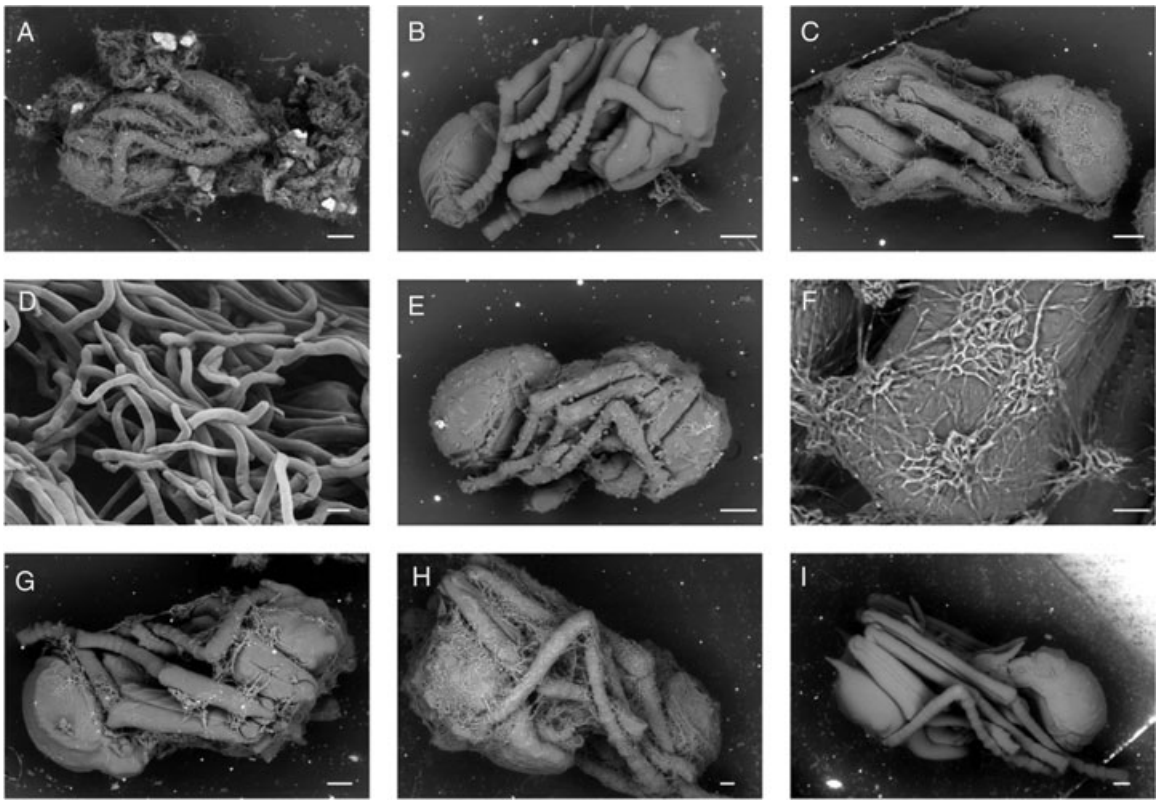

Figure 2. Scanning electron micrographs of attine worker pupae showing a diversity of mycelial cover. (A) *Myrmicocrypta ednaella* pupa with mycelial cover; (B) A naked *Mycocepurus smithii* pupa; (C) *Apterostigma collare* pupa; (D) Fungal hyphae of *Acromyrmex echinatior*; (E) *Cyphomyrmex rimosus* pupa; (F) Close-up of yeast covering *Cyphomyrmex rimosus* pupa; (G) *Trachymyrmex zeteki* with mycelial cover, and (H) *Acromyrmex echinatior* with mycelial cover; (I) A naked *Atta colombica* pupa. Scale bars indicate 200 μm, except for in Figures (D) and (F), which indicate 20 μm.

Results

COMPARATIVE DATA ON BROOD COVER IN ATTINE ANTS

Mycelial cover was found on eggs, larvae, and pupae for all genera that we studied, except for *Atta*, but the extent of the cover varied across species (Figs. 1, 2). Both phylogenetically basal and more derived species exhibited mycelial-covering behavior and it occurred in species practicing all types of fungal agriculture (Figs. 1, 2; Table 1). In all genera, however, the workers occasionally cleaned the brood, and partially or totally removed the mycelial cover (HFM, personal observation). The extent of the mycelial cover on larvae and pupae was significantly correlated with the transition from "lower" to "higher" attines, with the evolutionarily derived "higher" attines having less fungal covering (larvae $P = 0.026$; pupae $P = 0.040$; Table 2), but there was no significant difference for cover on eggs ($P > 0.20$; Table 2). The presence of actinomycetes on worker cuticles had no effect on mycelial cover in any brood stage ($P > 0.05$; Table 2), but species with workers that extensively groomed gardens covered their brood to a lesser

extent (eggs, $P = 0.042$; larvae, $P = 0.018$; pupae, $P = 0.051$; Table 2).

PUPAL COCOON PRESENCE ACROSS THE ANT SUBFAMILIES

The occurrence of cocoons is labile among ants (Table S1), irrespective of the details of a given phylogenetic hypothesis (Fig. S1A and B). We had no information on cocoons in Agroecomyrmecinae, Heteroponerinae, and Martialinae (described from a single worker; Rabeling et al. 2008), but among the 17 ant subfamilies for which we had information, six had cocoons present in all species, four subfamilies did not have cocoons in any species, and the remaining seven subfamilies were variable for this trait at different levels (genera within subfamily, species within genera, castes within species, and within castes; Fig. S1, Table S1). None of the 30 genera of the Myrmicinae (the subfamily that includes Attini) had observations on pupal cocoons in any species.

Discussion

Our analysis of cocoon use among extant ants confirmed that cocoon spinning is a labile trait (Wheeler 1915; Baroni Urbani et al. 1992; Shattuck 1992). Although most authors agree that having cocoons was ancestral and that cocoons have been secondarily lost multiple times (e.g., Wheeler 1915; Taylor 2007), the overall pattern of Figure S1 suggests that cocoon loss also happened in basal lineages (e.g., Leptanillinae). It is beyond the scope of this article to evaluate in detail the multiple selective forces that may induce loss of cocoons in some ant clades, but data from other taxa are illustrative of the possible adaptive value of cocoons. For example, in the braconid parasitoid wasp, *Cotesia glomerata*, cocoons protect pupae against desiccation (Tagawa 1996), and in eusocial wasps (Vespidae) cocoons have been shown to act as a thermostabilizer at both high (Plotkin et al. 2007) and low (Ishay and Ruttner 1971) temperatures. The multilayered structure of the silk cocoon in *Vespa orientalis* is such that it also acts as an efficient filter to remove very small particles such as bacteria from the surrounding air (Shabtai and Ishay 1998). Furthermore, at least in some taxa the silk may act as more than just a physical barrier to parasites, as two peptides in the silk cocoon of the wax moth, *Galleria mellonella*, have been shown to inhibit bacterial and fungal proteinases (Nirmala et al. 2001). To shed further light on this, it would be interesting to compare pairs of relatively closely related ant species with and without cocoons (e.g., *Amblypone pallipes* vs. *A. celata* or *Hypoponera opacior* vs. *H. monticola*), and evaluate the relative importance of ecological factors such as parasite pressure, nesting habitat, and food type.

More importantly for our present analysis, Figure S1 and Table S1 show that the subfamily Myrmicinae consistently lacks pupal cocoons, confirming earlier conclusions by Hölldobler and Wilson (1990) and others. Therefore, the ancestors of the attine ants had naked larvae and pupae, and shortly after the origin of fungus farming the garden symbionts were apparently coopted for being used as brood cover. There are long-standing anecdotal reports of mycelial brood covers in some taxa (e.g., Wheeler 1907), but comparisons between Table 1 and Figure 1 show that our compilation of data greatly expands our present knowledge of the phylogenetic distribution of fungal brood covering across the attine ants. Interestingly, the most consistent brood covering appeared to occur in the paleo- and lower attines, whereas average brood cover was significantly less on the higher attine larvae and pupae. This suggests that selection for brood covering became secondarily relaxed, possibly because the incorporation of live plant material in fungus gardens made ongoing inspection of brood for novel infections relatively more important. Another potential explanation could be that paleo- and lower attine ant larvae eat mycelium, whereas higher attine and leaf-cutting ants need to be fed with fungal gonglydia, so that fungal covers in the latter clades would have lost their function. However, this would leave unexplained the difference in pupal covering and the high frequency of brood covering in *Acromyrmex*, and it would also be inconsistent with (Weber's 1972) observation that attine larvae are actively fed by workers. Although several hypotheses for the adaptive significance of fungal brood covering have been proposed, the results of our comparative analysis lend strength to the idea that protection against parasites is the most general explanation for our observations, although alternative factors may be important in some cases.

PREDATORS, PARASITOIDS, AND ENVIRONMENTAL FLUCTUATIONS SEEM LESS LIKELY AS GENERAL EXPLANATIONS

The hypothesis that brood covering might be a form of chemical camouflage against predators such as army ants (LaPolla et al. 2002) seems less compelling as a general observation, and it is unclear whether the mycelial cover can reduce predation risk. For example, *Megalomyrmex* agropredators actively remove the mycelium cover of attine larvae before feeding them to their own larvae (Adams et al. 2000), and the army ant *Eciton hamatum* frequently preys on *Acromyrmex octospinosus* brood (Powell and Franks 2006), a species with substantial mycelial cover. Mycelial cover may act as a physical barrier against tiny parasitoid wasps. Observations that larvae of species from several attine genera with extensive mycelial covers are heavily parasitized by diaprinae wasps (Loiácono et al 2000; Fernández-Marín et al. 2006a; Pérez-Ortega et al. 2010) neither support nor refute this idea because we do not know if parasitism would be higher without the mycelial cover. These parasitoids are little studied, but they are relatively diverse and abundant in both *Trachymyrmex* and

Cyphomyrmex, which have a low and high extent of fungal brood covering, respectively. Finally, the alternative hypothesis of brood covering providing protection against fluctuations in temperature or humidity would imply that brood covering is positively correlated with seasonal variation in abiotic conditions, but there are no data to test this idea. In a north-temperate species, *Mycetosoritis clorindae,* which hibernates at relatively low temperatures, adults are also enveloped in mycelia (Mueller et al. 2010), but it is unknown if this provides temperature-related protection or protection against parasites. These observations do not negate the potential importance of predation, parasitism, or environmental variation in shaping the expression of fungal brood covering, but they highlight the fact that none of these hypotheses offer a convincing general explanation for brood covering in the wet or seasonal tropics, where attine ants evolved and where most extant species live.

BROOD COVERING, METAPLEURAL GLAND GROOMING RATE, AND ACTINOMYCETES

Although our data for metapleural gland grooming did not come from the same colonies as those for which we collected mycelial cover data, we found that attine ant species with a lower tendency to cover their brood had increased frequencies of metapleural gland grooming, supporting a trade-off between these behaviors at the species level. One could argue that this reflects the fact that severe parasite pressure would necessitate a response in both putative defensive behaviors, which may be a reasonable supposition if higher parasite pressure would somehow make more resources available for defense. However, given that the metapleural gland secretion is known to be costly in *Ac. octospinosus* (Poulsen et al. 2002) and that active planting of small mycelial fragments on each brood item by workers is time-consuming, it seems more likely that both types of defense are traded-off within a limited overall energy budget (van Noordwijk and de Jong 1986; Fernández-Marín et al. 2009). The logic of this trade-off hypothesis is exemplified by the fact that *Atta* never had mycelial brood cover, but has the highest frequency of metapleural gland grooming, whereas the reverse occurs in *Acromyrmex*. However, we did not examine mycelial cover in response to parasite infection, and the relationship between metapleural gland grooming and mycelial cover could be parasite dependent. There was no relationship between the presence of actinomycete bacteria on the cuticle of adult workers (Currie et al. 1999) and the degree of mycelial brood covering. This lack of relationship would be consistent with the actinomycetes primarily playing a role in fungus garden protection, rather than ant protection. As mentioned above, the microbial cover on the cuticle of attine ants is now known to often contain multiple microorganisms, so it would be premature to draw firmer conclusions about the interaction dynamics between this cuticular community and the ant-fungus symbiosis.

FUNGAL SYMBIONT PARASITE DEFENSES AND FURTHER PERSPECTIVES

Thus far we have argued that: (1) hypotheses unrelated to protection against parasites seem unlikely to be generally applicable and (2) indirect support for a parasite-defense function of fungal brood covering emanates from the negative correlation with an alternative parasite defense where a trade-off may be expected (metapleural gland grooming), and from the lack of correlation with an alternative defense function (actinomycete cover). There are also other observations that suggest that further work on parasite-defense functions of fungal brood cover in attine ants will be worthwhile. First, some studies have shown that the fungal garden symbiont has antimicrobial properties, as antibacterial lactol lepiochlorin was isolated from a fungus garden of the ant *C. costatus* (Hervey and Nair 1979; Nair and Hervey 1979) and several antifungal diketopiperazines were isolated from the fungus garden of *C. minutus* (Wang et al. 1999). In addition, metabolites produced in *A. colombica* fungus gardens inhibit the growth of some endophytic fungi (Van Bael et al. 2009), but it remains to be tested whether metabolites of the fungus gardens cultivated by species with mycelial brood cover (e.g., the leafcutting sister genus *Acromyrmex*) also inhibit endophytes or entomopathogenic fungi.

Freeland (1976) argued that parasites play a significant role in social evolution. Since then a substantial body of literature suggests that parasite pressure is perhaps the most persistent threat to eusocial insects (e.g., Hamilton 1987; Sherman et al. 1988; Schmid-Hempel 1998; Boomsma et al. 2005; Cremer et al. 2007), which strengthens our inference that further studies on population-level variation in attine ant fungal brood covering would be worthwhile in the context of defense against parasites. Focused experimental challenges with parasites could shed interesting light on the substantial variation in brood covering within species (cf. the error bars in Fig. 1) and on the possible adaptive benefits of brood covers of varying thickness (Fig. 2). Although fungal covering removes the energetic costs of silk spinning, additional studies might reveal intriguing costs of fungal brood covering (e.g., does the mycelium obtain resources from the brood?). Although silk cocoon presence is a binary trait, fungal brood covering is a continuous variable that would allow optimal compromises between the benefits of prophylactic protection against parasites, and the need for inspection to detect infections. In addition, using fungus rather than silk has also allowed the attine ants to extend protection to larvae and eggs, a flexibility that silk cocoons fail to provide.

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Supporting Information

The following supporting information is available for this article:

Table S1. Presence and absence of pupal cocoons across ant subfamilies (subfamilies after Brady et al. 2006; Moreau et al. 2006 have essentially the same subfamilies but without subdivison of the dorylomorph ants into five groups).
Figure S1. Presence and absence of pupal cocoons mapped onto ant subfamily phylogenies.

Supporting Information may be found in the online version of this article.