

# Negative Fitness Consequences and Transmission Dynamics of a Heritable Fungal Symbiont of a Parasitic Wasp<sup>∇</sup>

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**Heritable bacterial symbionts are widespread in insects and can have many important effects on host ecology and fitness. Fungal symbionts are also important in shaping their hosts' behavior, interactions, and evolution, but they have been largely overlooked. Experimental tests to determine the relevance of fungal symbionts to their insect hosts are currently extremely rare, and to our knowledge, there have been no such tests for strictly predacious insects. We investigated the fitness consequences for a parasitic wasp (*Comperia merceti*) of an inherited fungal symbiont in the Saccharomycotina (Ascomycota) that was long presumed to be a mutualist. In comparisons of wasp lines with and without this symbiont, we found no evidence of mutualism. Instead, there were significant fitness costs to the wasps in the presence of the yeast; infected wasps attacked fewer hosts and had longer development times. We also examined the relative competitive abilities of the larval progeny of infected and uninfected mothers, as well as horizontal transmission of the fungal symbiont among larval wasps that shared a single host cockroach egg case. We found no difference in larval competitive ability when larvae whose infection status differed shared a single host. We did find high rates of horizontal transmission of the fungus, and we suggest that this transmission is likely responsible for the maintenance of this infection in wasp populations.**

The majority of heritable bacterial symbionts associated with insects either provide nutritional benefits for hosts that feed on nutrient-poor diets, such as blood (e.g., *Wigglesworthia* sp. [1]) or sap (e.g., *Buchnera* spp. [33]), or manipulate the hosts' reproduction to benefit their own transmission (e.g., *Wolbachia* spp. [38] or *Cardinium* sp. [40]). Thanks in part to these examples, research efforts have become more diverse, leading to the discovery of additional benefits, such as heat tolerance (29) and protection from parasitism (26).

Despite growing interest in the cryptic roles of insect associates, fungal symbionts have largely been overlooked, and their prevalence, ecological importance, and evolutionary implications for hosts are still poorly understood. Yet we have reason to suspect that fungal symbionts may be as diverse and functionally important as bacteria in insects. Buchner's (5) foundational work on arthropod-microbe symbioses included many fungi, and anecdotal reports of such symbioses are scattered throughout the literature (e.g., fire ants [3]; stingless bees [28]; earwigs, scale insects, flies, andrenid bees, and ants [39]; and leafhoppers [30]). Recent surveys of insects for fungi have resulted in an astonishing diversity, including fungi in beetles (35), a cockroach and five other neuropteran families (24), sap-feeding beetles, and flies and bees (15), and it has been suggested that the majority of unicellular fungal diversity may be in insects (35). It is often suggested that such associations are mutualistic, with the fungus presumably providing enzymes, essential amino acids, vitamins, or sterols (37) and the insect vectoring and providing a habitat for the fungus. Fitness

consequences of these associations have been assessed in only a few cases, including associations in planthoppers (31), anobiid beetles (23, 32), and scolytid beetles (2). In most instances the significance of the relationship is not clear, especially in the many cases where the fungi are not obligate associates.

In 1985, LeBeck (18) reported a unicellular fungal symbiont in *Comperia merceti* (Compere) (Hymenoptera: Encyrtidae), a gregarious endoparasitoid wasp that specializes on the egg cases of brown-banded cockroaches [*Supella longipalpa* (Serville) (Blattaria: Blattellidae)]. The fungus is found throughout the hemocoel in juvenile wasps, in adult males, and in the venom gland of adult females (18). In addition, the fungus is vertically transmitted from mother to offspring via the external surface of wasp eggs during oviposition into cockroach egg cases. Vertical transmission via the egg surface is a common method in other fungal symbiont systems (e.g., planthoppers [19]; lacewings [10]; and wood wasps, anobiid beetles, and cerambycid beetles [5]). LeBeck (18) characterized the fungus as a *Candida* sp. and suggested that it might alter the nutritional value of the host cockroach egg case for the benefit of the developing wasp larvae. However, this claim has never been tested. Further, the predacious diet of immature parasitic wasps would make them unusual candidates for nutritional symbionts; parasitic wasps consume other insects and do not ordinarily require the complementary nutrients that many fungal and bacterial symbionts provide to insects with unbalanced diets. To our knowledge, our study is the first to specifically test the role of an inherited fungus in an insect with a strictly predacious diet.

*C. merceti* wasps house a single known fungal symbiont belonging to the Ascomycota (Saccharomycotina) and no detectable bacterial symbionts (9). Further, these wasps do not become infected with any of their host cockroaches' symbionts (9). In *in vitro* trials of the *C. merceti* wasp fungus with other

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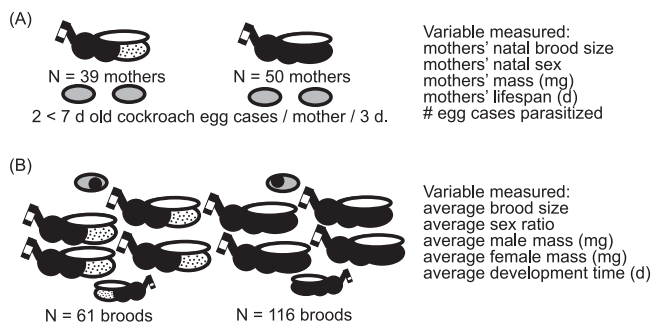


FIG. 1. Diagram of the experiment to determine the effects of the fungal endosymbiont on wasp fitness. Female wasps are larger than males and have white-banded antennae pointing toward the left (males are pointing toward the right). Infected wasps are indicated by stippled abdomens, and uninfected wasps are indicated by filled abdomens. (A) Experimental setup and variables measured for the mothers. (B) Experimental setup and variables measured for the offspring, d, days.

microbes there was no evidence of inhibition or any type of interaction (C. M. Gibson, unpublished). The current research tests the hypothesis that the wasps' fungal symbiont is a mutualist and explores alternative means by which this fungus could be maintained in wasp populations.

#### MATERIALS AND METHODS

A brown-banded cockroach culture was started using several hundred feral individuals collected in the Marley Building of the University of Arizona between 2003 and 2006. The colony was maintained on autoclaved dog food (Science Diet, Topeka, KS) and sterile water. A *C. merceti* wasp culture was initiated from parasitized brown-banded cockroach egg cases sent in 2003 from a feral population at the University of Massachusetts (R. VanDriesche) and was maintained continuously on brown-banded cockroach egg cases (larvae) and on honey and sterile water (adults).

During the course of a multigeneration experiment in which hundreds of individual wasps were isolated and maintained separately on hosts, three lines of wasps were identified as fungus-free using microscopy and PCR tests (the methods used are described in reference 8). Calculations from this multigeneration experiment suggest that the vertical transmission rates are normally >90%.

The progeny of the uninfected wasps were isolated and used to initiate an uninfected *C. merceti* culture. Subsequent experiments were conducted 17 to 20 generations after the infection was lost. Periodic PCR tests (with 15 individuals) and dissections ( $n = 150$ ) verified the infection status of wasps from each culture.

**Effects of the fungal endosymbiont on wasp fitness.** Parasitized cockroach egg cases were collected from both infected and uninfected culture stocks and kept individually in 13-ml glass vials with cotton stoppers until adults emerged. Emerging wasps of both sexes were given honey and water ad libitum and held for 1 day. Females were removed from each of the vials, weighed with a Cahn C-31 microbalance to the nearest 1  $\mu$ g, and placed into plastic boxes (125 mm by 82 mm by 22 mm) with mesh windows (diameter, 65 mm) individually. Five microliters of honey was placed on the mesh window every 3 days. These mothers' natal brood sizes, natal sex ratios, and life spans were recorded. Over the duration of these mothers' lives, two weighed, <7-day-old cockroach egg cases were provided every 3 days, and equivalent host resources were allotted to each treatment (Fig. 1A). After exposure to wasps, egg cases were placed individually into 35-mm petri dishes and kept at 27°C with 65% relative humidity and with a daily cycle consisting of 16 h of light and 8 h of darkness in an environmental chamber until wasps or cockroaches emerged or until the egg case desiccated. The brood sizes, sex ratios, and development times for the wasp progeny that emerged from egg cases were recorded, and the masses of male and female offspring were determined (Fig. 1B).

Two multivariate analyses of variance were conducted for the data for the mothers and the data for the offspring; all statistical analyses were conducted using JMP statistical discovery software (version 5.0.1; SAS Institute, Cary, NC). There were no differences in the identity responses, and therefore individual Student's *t* tests were conducted independently for each normally distributed

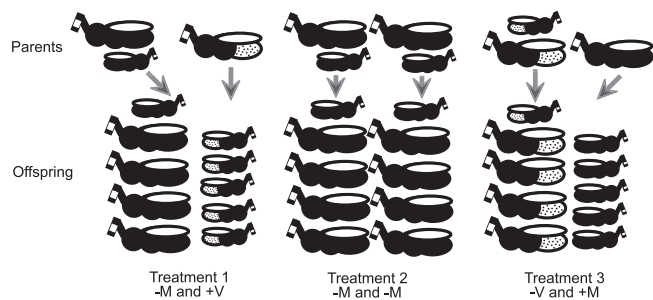


FIG. 2. Diagram of the experiment to determine fungal transmission and intraspecific competition. Female wasps are larger than males and have white-banded antennae pointing toward the left (males are pointing toward the right). Infected wasps are indicated by stippled abdomens, and uninfected wasps are indicated by filled abdomens. The predicted offspring sex ratios and infection states in the absence of horizontal transmission are indicated. See Fig. 4 for experimental results. -M, uninfected mated mother; +M, infected mated mother; -V, uninfected virgin mother; +V, infected virgin mother.

variable. The life span of mothers was transformed with a  $\log x + 1$  transformation. The Wilcoxon rank sum nonparametric test was used to analyze the number of egg cases parasitized, the offspring sex ratio data, and the offspring development time data, as these data were not normally distributed and no transformation was sufficient to normalize them.

Life table analyses for both the infected and uninfected groups of *C. merceti* wasps were conducted using Populus (version 5.3; D. N. Alstad, 2003 [http://www.cbs.umn.edu/populus/]) and empirical values obtained in this study.

**Fungal transmission and intraspecific competition among larvae.** We examined the relative competitive abilities of offspring from infected and uninfected mothers and also examined the potential for the fungus to be transmitted horizontally among larvae within individual cockroach egg cases.

*C. merceti*, like other Hymenoptera, is haplodiploid; unmated females lay unfertilized, haploid eggs that mature into sons, while mated females lay both haploid eggs (sons) and fertilized, diploid eggs (daughters). For the offspring of mated *C. merceti* females the sex ratio is relatively invariant (20% males), in accordance with local mate competition theory for inbreeding gregarious broods (11). In this experiment, the mating status of the ovipositing mother wasps was used to determine which offspring came from an infected mother and which offspring came from an uninfected mother, both of which were allowed to oviposit on a single cockroach egg case.

In treatment 1, a mated, uninfected female wasp (capable of producing male and female offspring) shared an egg case with a virgin, infected wasp (capable of producing only male offspring). In this scenario, daughters from the mated, uninfected mother should have remained infection free in the absence of horizontal transmission. In treatment 2, two mated, uninfected females shared a host. This treatment established that each mated female still produced 20% male offspring when she shared a cockroach egg case with a second female. In treatment 3, a mated, infected female wasp (capable of producing both male and female offspring) shared an egg case with a virgin, uninfected wasp (capable of producing only male offspring). In this treatment, no more than one or two of the male offspring in an emerging adult brood should have been infected in the absence of horizontal transmission. Treatment 3 was designed for comparison with treatment 1 to explore whether there might be a competitive advantage to infection. For example, one would expect a more male-biased sex ratio in treatment 3 than in treatment 1 if housing the fungal symbiont has a cost for developing infected female larvae (Fig. 2 shows the experimental setup).

To obtain female wasps with the appropriate mating and infection status, egg cases from both the infected and uninfected culture stocks were dissected, and the sexes of individual wasps were determined in the pharate pupal stage. Female *C. merceti* wasps have three or more white antennal segments, while males have a maximum of two white antennal segments (16). Pupae were placed on sterile filter paper in 35-mm plastic petri dishes lined with 1% water agar. Emerging adult females that were to be mated were placed in 0.6-ml Eppendorf tubes with honey and three or four males with the same infection status for 24 h. Before the experiment, cockroach egg cases were weighed and assigned to treatments such that equal host resources were apportioned to each treatment. The two females used for each replicate were then placed on a single egg case inside a plastic box

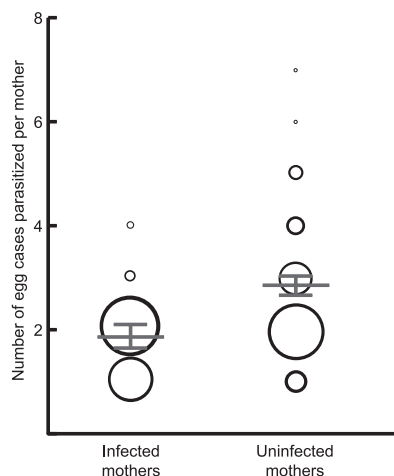


FIG. 3. Numbers of egg cases parasitized in the two infection groups in the fitness experiment. The sample size for infected mothers 39, with 61 of the 276 egg cases parasitized, and that for uninfected mothers was 50, with 116 the 342 egg cases parasitized. The sizes of the circles are proportional to the number of replicates with that particular value. The long and short horizontal bars indicate means and standard errors, respectively.

with a mesh window as described above. After 7 days, egg cases were examined for oviposition stalks and then kept at 27°C with 65% relative humidity and with a daily cycle consisting of 16 h of light and 8 h of darkness in an environmental chamber until wasp progeny emerged. Following emergence, replicates of the experimental treatments in which there were too few males (<15%) were excluded from the analysis because of the likelihood that the virgin mother had not oviposited. Conversely, replicates in which there were male-only broods were also excluded, as this indicated either that the mated female had not oviposited or that she was unmated.

Horizontal transmission was calculated for treatment 1 by counting the number of female progeny (from the mated, uninfected mother), determining the number of male progeny for the uninfected brood that had a sex ratio that was 20% or just less than 20% (more conservative), and finally determining the numbers of the female and male offspring that were infected. For treatment 3 (with uninfected virgin mothers), the number of uninfected sons was estimated by determining the number of males after the sons and daughters of the infected mother were accounted for. Horizontal transmission to these sons was then tallied. Although some of the progeny from the infected mother may have lost the infection through incomplete vertical transmission (as discussed above), this would affect only the calculation for the uninfected sons and therefore underestimate the rate of horizontal transmission in treatment 3. (Males that were infection free upon dissection would have been counted as offspring of the uninfected mother, despite being sons of the infected mother.) Sex ratios in the horizontal transmission experiment were compared using a Student's *t* test with JMP (SAS Institute, Cary, NC).

## RESULTS

**Effects of the fungal endosymbiont on wasp fitness.** We observed no significant differences between infected and uninfected mothers with respect to the mother's natal brood size, natal sex ratio, mass, or life span. However, on average, uninfected mothers oviposited into one more egg case ( $2.83 \pm 0.19$ ) than infected mothers ( $1.85 \pm 0.21$ ) ( $Z = -3.79$ ;  $P = 0.0001$ , Wilcoxon rank sum test) (Fig. 3).

We observed no significant differences between the progeny of infected mothers and the progeny of uninfected mothers with respect to average sex ratio, average mass of the female offspring, average mass of the male offspring, or average brood size. The greatest difference was the difference in development

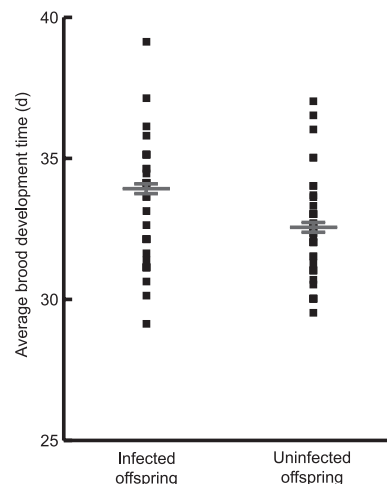


FIG. 4. Average brood development times for the two infection groups in the fitness experiment. The sample size for infected offspring was 475 individuals from 61 parasitized egg cases, and that for uninfected offspring was 1,032 individuals from 116 parasitized egg cases. The long and short horizontal bars indicate means and standard errors, respectively. d, days.

time; on average, uninfected offspring developed 1.8 days faster than infected offspring ( $Z = 3.46$ ;  $P = 0.0005$ , Wilcoxon rank sum test) (Fig. 4). For uninfected wasps, a development time of 32 days was used in the life table calculation, along with 0.46 egg cases parasitized per day, a sex ratio of 0.17 males, and, therefore, 7.65 females per brood. For infected wasps, a life span of 34 days was used in the life table calculation, along with 0.30 egg cases parasitized per day, a sex ratio of 0.19 males, and, therefore, 6.60 females per brood. Juvenile mortality (egg to pupal stages) was the same for infected and uninfected wasps,  $\sim 7\%$ . The differences translated to instantaneous growth rates of 0.17 for uninfected wasps (as calculated using Populus [see above]) and 0.13 for infected wasps and resulted in doubling times of 8.3 and 10.9 days, respectively.

**Fungal transmission and intraspecific competition among larvae.** We also found that the fungal endosymbiont, although previously documented to be vertically transmitted, was transmitted horizontally among developing wasps with extremely high frequency (Fig. 5). In treatments 1 and 3, 94.5% and 98.9%, respectively, of the progeny of uninfected individuals were found to be infected. If uninfected larvae were better competitors than infected larvae in the same egg case (as one might imagine from the results of the fitness experiment), then it is possible that there would be a sex ratio bias toward males in treatment 3 compared to treatment 1. The sex ratios for the two experimental treatments (treatment 1, 55.2% males; treatment 3, 64.5% males) were not significantly different ( $t_{1,17} = -0.75$ ;  $P = 0.46$ , two-tailed test). This result suggests that the mated mothers used in the two treatments contributed equally to the progeny produced, despite the difference in their infection status. Therefore, there appears to be no competitive difference between the progeny of uninfected wasps and the progeny of infected wasps when they share a single host egg case.

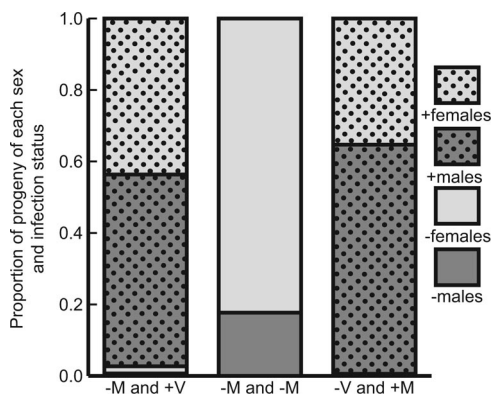


FIG. 5. Proportion of progeny with each infection type in the horizontal transmission experiment. Light gray indicates females, and dark gray indicates males. Stippling indicates yeast-infected individuals. The first column shows the results for treatment 1 with an uninfected mated mother ( $-M$ ) and an infected virgin mother ( $+V$ ) (eight replicates and a total of 145 progeny assessed). The second column shows the results for treatment 2 with two uninfected mated mothers (seven replicates and a total of 130 progeny assessed). The third column shows the results for treatment 3 with an uninfected virgin mother ( $-V$ ) and an infected mated mother ( $+M$ ) (10 replicates and a total of 169 progeny assessed). The sex ratios for treatments 1 and 3 are not significantly different ( $t_{1,17} = -0.75$ ;  $P = 0.46$ , two-tailed test).

## DISCUSSION

The fungal symbiont of *C. merceti* was previously thought to be transmitted solely vertically and to be mutualistic with its hosts (17). Under controlled laboratory conditions, however, we found that the fungal symbiont provided no clear fitness benefit to wasps in either the larval or adult stage. Instead, we documented that there are clear fitness costs; infected wasps attack fewer hosts (Fig. 3) and have longer development times (Fig. 4). These fitness costs translate into a difference in doubling time of 2.6 days; the uninfected wasps develop 1.8 days faster than the infected wasps. Moreover, we found that there is no difference in competitive ability between the infected and uninfected larvae.

Given the frequency of vertical transmission of the fungus (>90%), it is initially surprising that it provides no apparent benefit (17). Although vertical transmission could be either intentional (mutualistic) or orchestrated (parasitic) behavior designed to package fungal cells along with offspring, the fungal symbiont colonizes *C. merceti*'s venom gland, which normally secretes fluid during oviposition (27). Therefore, a significant behavioral change would not be required for inclusion of the symbiont along with wasp eggs. Symbionts that are transmitted strictly vertically must provide fitness benefits to their hosts or manipulate host reproduction to spread in host populations (6). In the *C. merceti* system there is no obvious fitness benefit to housing the fungus or evidence of symbiont-mediated sex ratio distortion. However, our demonstration that the rates of horizontal transmission are extremely high (Fig. 5) provides a compelling mechanism by which a detrimental fungal symbiont could be maintained in wasp populations. Although wasps can discriminate between unparasitized and previously parasitized egg cases in choice arenas (36), superparasitism (deposition of eggs by multiple females into a single host) is common in *C. merceti* (12) where broods of

emerging adults must compete for patchily distributed hosts (4). Interestingly, one might predict barriers to invasion by this fungus when the frequency of infection is low, the relative ratio of wasps to cockroach hosts is low, and the cost of infection is not balanced by opportunities for horizontal transmission.

Horizontal transmission could also occur via mating (e.g., in cactophilic yeasts transmitted by *Drosophila* males [34] and paternal transmission of some aphid bacterial symbionts [22]). We tested for sexual horizontal transmission in a pilot experiment with 23 uninfected *C. merceti* wasp females enclosed with infected males for 24 h after emergence. Experimental mothers were not assessed directly, but none of the 77 offspring assessed were infected (*C. M. Gibson*, unpublished data). This experiment suggests that it is horizontal transmission within the egg case and not sexual transmission that is key for the spread of this fungus in wasp populations.

Despite the fact that there are fitness costs to infection and frequent horizontal transmission, it may be premature to conclude that the wasp's symbiont is a parasite. Under different ecological conditions (e.g., host cockroach nutritional stress), infection may provide a benefit. This is the case for some documented types of conditional mutualism, including thrips infected with *Erwinia* sp. (8), black flies infected with trichomyces fungi (20), and eumenine wasps infected with mites (25). However, our data clearly rule out the possibility that the fungus that we studied is an obligate nutritional mutualist. The wasps survive and reproduce readily in the absence of the symbiont, and further, the masses of the uninfected wasp progeny were not significantly different from those of the infected wasp progeny in the fitness experiment. The vigor of the uninfected wasps suggests that larvae do not need to consume the fungus directly like other insect associates of fungi do (37). Some of the close relatives of the wasp symbiont are also insect associates (9). However, the phenotypes of these fungi in their beetle hosts are unknown (14). The fungus most closely related to *C. merceti*'s symbiont is a fungal symbiont of the host cockroaches (9). The cockroach fungal symbiont is not fixed in its hosts, and there is no evidence that it infects the wasps (9). It is possible that historically the fungus colonized the wasps from the cockroaches and is currently a mutualist of the cockroaches. Molecular data for a fast-evolving gene (e.g., internal transcribed spacer region) would be useful for testing this hypothesis.

Uncellular fungi have also been found in other parasitic wasps. Keilin and Tate (13) documented that yeasts occur in the gut of a braconid wasp, *Dapsilarthra apii*. Middeldorf and Ruthmann (21) noted vertically transmitted yeasts inside the eggs of an ichneumonid wasp, *Pimpla turionellae*, and Cooper (7) observed vertically transmitted "tubular bodies" in the parasitic wood wasp *Orrusus sayi*. It is often speculated that the fungi in these parasitoid associations are injected maternal factors for suppression of the wasps' hosts (27); however, the precise nature of these relationships has never been tested.

Our data do not support a nutritional role or any type of mutualistic role for the *C. merceti*-associated yeast. Instead, this fungal symbiont provides no apparent benefit; rather, it imposes fitness costs on its wasp host. We anticipate that future explorations of this and other insect-fungus symbioses will reveal the nature and diversity of roles that eukaryotic microbes play in their hosts' ecologies.



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