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Research Article

Sporadic Infection of *Wolbachia* in a Recently Established Population of *Formica fusca*

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This study examines the distribution and invasion dynamics of *Wolbachia* in a recently established *Formica fusca* population. Preliminary data revealed the intermittent infection of *Wolbachia* across colonies, providing the opportunity to test for ecological factors affecting the acquisition and spread of the parasite. Only 35% of colonies are infected in this population. Both infected and noninfected nests have similar dispersion patterns that approximate a random distribution, suggesting that transmission of *Wolbachia* between adjacent colonies is not common. There is no difference in the infection rate between workers and brood, indicating that workers are not actively eliminating the infection. Our results show no significant association between *Wolbachia* infection and nest size; however, infected colonies tend to be larger than noninfected colonies. Finally, *Wolbachia* infection was not associated with queen number. Overall, our results suggest no large fitness differences between infected and noninfected colonies, although small fitness effects cannot be ruled out for this population.

1. Introduction

Wolbachia is common endosymbiotic bacteria of arthropods, crustaceans, mites, and nematodes that induces a variety of effects on their hosts to promote their own spread within the host population [1–6]. It is estimated that *Wolbachia* is present in 20 to 75% of all arthropods [2, 7] including more than 90 species of ants [2, 8–14]. Within ant species, high levels of multiple *Wolbachia* infection are documented, including up to 4 strains of *Wolbachia* in single individuals [10, 13].

Wolbachia transmission normally occurs through vertical maternal transmission [5, 14, 15]. The parasite has been shown to increase transmission via manipulation of reproduction and the sex ratio of the host using a number of different mechanisms (reviewed in [16–19]). *Wolbachia* infection can benefit host females through positive fitness effects or via cytoplasmic incompatibility (CI) [4]. CI prevents infected males from successfully mating with a noninfected female or with a female infected with a different strain of *Wolbachia* [19]. Other mechanisms by which *Wolbachia* can bias host sex ratio in favor of infected females include male killing, parthenogenesis, and feminization [19]. In social insects, worker control of sex allocation requires *Wolbachia*-mediated manipulation of worker's behavior to result in a favorable sex ratio [20]. To date, studies of sex ratio in ants have provided little evidence for *Wolbachia*-induced manipulations of sex ratio in ants [14, 15, 20].

Wolbachia can also spread via horizontal transmission of the parasite between species [13, 14, 21–23]. Occasional horizontal transmission has been documented and occurs most frequently between related species [5, 24]. In addition, the presence of multiple *Wolbachia* strains within a species shows evidence for horizontal gene transfer between host species or recombination events among *Wolbachia* strains [3, 10]. Less is known about the infection dynamics of *Wolbachia* within a single host species. Across several ant species, *Wolbachia* infection prevalence appears near fixation within some populations [5, 10, 14]. However, other populations vary in the prevalence of infection across colonies. Even within infected colonies, not all workers harbor the infection [14, 22], suggesting that *Wolbachia* is not transferred readily between workers. There is also evidence that infection rates of workers are

lower than infection rates of worker brood [14] suggesting a loss of infection with age.

We studied the distribution and infection dynamics of the *Wolbachia* parasite in a recently established *Formica fusca* population. *F. fusca* is a pioneering species and rapidly colonizes open environments prior to competition from other species [25, 26]. Within a single population, this species can establish both monogynous and polygynous colonies [25, 27]. A preliminary study suggested that *Wolbachia* is present in this population, but that only a subset of colonies is infected, contrasting previous findings of near fixation prevalence rates in related ants species [5, 14, 15, 20]. We surveyed the population to determine the prevalence of *Wolbachia* infection across colonies and to test whether infection is associated with nest size, a proxy for colony size [28], nest location, production of sexuals, and queen number.

2. Methods

2.1. Study Population. The isolated study population of *Formica fusca* inhabits a disturbed meadow of grasses and goldenrod that borders a temperate conifer forest in Hamilton, New York (N 42° 48.134 W 075° 30.343). While the exact colonization date is not known, estimates of the appearance of nest mounds at the site range from 10 to 15 years ago. Other ant species present at or near the site include *Formica species*, *Leptothorax longispinosus, Tapinoma sessile*, *Camponotus americanus*, *Lasius species*, *Myrmica punctiventris*, and *Monomorium minimum*. *Formica fusca* ants were the most common ants found at the site. We did not check for the presence of *Wolbachia* in other ant species.

2.2. Nest Characteristics. All nests within the study site were mapped with GPS coordinates using Google Earth and ArcView (Figure 1). The Clark-Evans nearest neighbor method was used to infer dispersion of *Wolbachia* across colonies [29], with $R = 1$ indicating random dispersion and $R = 0$ indicating clumped dispersion. In order to test whether R was significantly different from 1, a critical value, c, was calculated according to Clark and Evans [29] using a t-distribution. Significant differences between infected and noninfected colonies were tested by comparing R-values using the F distribution.

The size of the nest mound was measured in two directions across the nest entrance; the longest diameter and the one perpendicular to the longest. Measurement extended to the edge of the raised mound. The area of the nest mound was calculated as the area of an ellipse with the two perpendicular measures halved as radii. The average nest mound area was calculated, and mean nest mound area of infected and noninfected nests was compared with a two-tailed t-test. Nests were designated as either "small," nests smaller than the mean nest area, or "large," nests larger than the mean. A Fisher's Exact Test was used to determine association between infection and nest size. Across infected colonies, the proportion of infected individuals was compared to nest area using Kendall's coefficient of rank correlation. Nest size was used as a proxy for colony size following the association described in Tuzzolino [28].

2.3. Sample Collection. Workers, worker brood, and reproductive brood were collected from all 35 colonies within the boundaries of the sampling site from late June to early August, 2011, during the period when reproductives are most abundant (unpublished data, [28]). Samples were collected from nests in both shady and sunny locations during late morning hours. Temperature during collection averaged between 24 and 29°C. Nests were watered with approximately 10 liters of water in the afternoon preceding collection to facilitate the sampling of reproductives [28]. During collection, small areas were probed with trowels to determine location of brood chambers and workers and brood were aspirated into vials with minimum disturbance to the nest. When no brood chambers were found, shovels were used to extract more dirt from the surface to collect workers. The duration of collection was limited to 20 minutes. A Fisher's Exact Test was used to compare the number of reproductives obtained during this sampling period in infected and noninfected colonies.

Worker and brood samples were immediately frozen at −20°C. DNA was extracted from all samples using 100 uL of a 10% Chelex solution (Bio-Rad), and samples were boiled for 15 min and spun for 1 min at 13,000 rpm. The supernatant from worker samples was placed directly into a PCR reaction; the supernatant from brood samples was diluted 1 : 10 with water.

We sampled 20 colonies for presence of *Wolbachia*; for one of these colonies, microsatellite data was not available, resulting in a sample size of 19 colonies for comparisons of infection with queen number.

2.4. Population Survey of Wolbachia. All samples were amplified with 18S primers (18SF1 and 18SR1; [30]) to confirm that the DNA extractions were successful. Each sample was then amplified twice with *Wolbachia* specific primers (wsp 81F and wsp 691R; [31]) to confirm presence or absence of *Wolbachia* infection. Detection rate of *Wolbachia* infection was estimated at greater than 99%. For all reactions, samples were run in 25 uL of the following reaction mixture: 1X of 10X PCR buffer, 0.2 mM each of dNTPs, 1.5 mM $MgCl_2$, 0.5 uM each of primer, 1 unit of Taq polymerase (5 U/uL), and 1 uL DNA. Samples were run on a Bio-Rad DNAengine PTC thermocycler with the following protocol: 94°C for 5 min, 47 cycles of 94°C for 30 s, 55°C for 45 s, 72°C for 1:30 min, hold at 72°C for 7 min. Amplified products were run on 1.5% agarose gels and analyzed as presence/absence of a 610 bp band. Samples with no band after two runs were designated as noninfected samples.

2.5. Microsatellite Analysis. We genotyped 20 workers per colony from a total of 34 colonies at 5 microsatellite loci: FE42 [32], FL12, FL29, [33], FY7, FY15 [34]. PCR amplifications were performed in a 25 μL final volume containing 1 X of 10X PCR buffer, 0.2 mM each of dNTPs, 1.5 mM $MgCl_2$, 0.5 uM each of primer, 1 unit of Taq polymerase (5 U/uL), and 1 uL DNA. Samples were run on a Bio-Rad DNAengine

Figure 1: Map of the study site showing all colonies in the population. Infected colonies: red circles; noninfected colonies: blue circles; untested colonies: yellow circles.

PTC thermocycler with the following protocol: 94°C for 5 min, 27 cycles of 94°C for 30 s, 48/55°C for 30 s, 72°C for 45 s, hold at 72°C for 3 min. Amplified fragments were analyzed on an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) and sized using GeneMapper 4.1 and 400ROX size standard from Applied Biosystems. All allele calls were manually verified.

Effective queen number was estimated from pairwise worker-worker relatedness values between colonies using Relatedness 4.2 according to the equation outlined in Krieger and Keller [35].

3. Results

In this population, 35% (7/20) of colonies were infected with Wolbachia. In infected colonies, the average proportions of individuals that were infected per colony (±SD) were workers, 0.62 ± 0.31; worker brood, 0.83 ± 0.32; reproductive brood, 1.00. In infected colonies, there was no difference between the proportions of infected workers per colony versus the proportions of infected worker brood per colony (t = 0.82, P = 0.44). Noninfected colonies were no more likely to have reproductives than infected colonies (P = 0.53). Only one of the seven infected colonies and three out of 13 noninfected colonies produced sexuals, so it was difficult to test associations between infection and colony sex ratio.

The ratio (R) of average distance to nearest neighbor to the expected distance based on density was 1.08 and 0.83, respectively, for noninfected and infected colonies, suggesting that both noninfected and infected nests occur in a random distribution. The R-value for infected colonies was not significantly different from one (R = 0.83, t = 0.43, P = 0.85). The distribution of infected nests was not significantly different from noninfected nests (F = 0.24, P = 0.70).

The average nest mound size was 2203 cm^2. Prevalence of infection is not associated with large nest size when comparing small and large nests (P = 0.12). Mean nest size of infected colonies was nearly double the size of noninfected colonies, but this difference was not significant (infected = 3101 cm^2; noninfected = 1632 cm^2; t = 1.16, P = 0.26). Among infected colonies, nest area was not related to the proportion of infected individuals within a colony (τ = 0.41, P = 0.249).

The population has a high level of genetic diversity with the number of alleles per locus ranging from 6 to 14 and expected heterozygosities for each locus ranging from $H_e = 0.44$ to 0.83. There is significant genetic structure between nests in the population ($F_{ST} = 0.20 \pm 0.13$), and there is no evidence for isolation by distance across nests ($y = 9E^{-06}x + 0.28$; $R^2 = 0.014$), suggesting that dispersal occurs primarily via mating flights and not by budding of queens and workers to adjacent nest sites (unpublished data).

Of the 19 colonies for which both *Wolbachia* infection and queen number were tested, 40% of infected colonies were monogynous and only 9% of noninfected colonies were monogynous, but this difference was not significant ($P = 0.30$). The average queen number in infected colonies was 2.37 ± 1.06 and was not significantly different from the average queen number in noninfected colonies, 2.17 ± 0.86 ($t = 0.46$, $P = 0.65$).

4. Discussion

The results from this study reveal a snapshot of early *Wolbachia* infection in a recently established *Formica fusca* population. The recent introduction of this population offers the unique opportunity to test for ecological correlates of *Wolbachia* infection and spread. Infected nests in the population were broadly scattered throughout the study site, and the probability of infection was not predicted by closest neighbors, indicating little or no transmission of *Wolbachia* between colonies. In one area, three infected nests are closely clumped together, but these nests are likely satellite nests of the same colony due to their close proximity. This result contrasts previous studies of other *Formica* species where *Wolbachia* seems to infect a high proportion of colonies within populations [5, 10, 14]. In addition, a survey of 32 species of *Formica* found multiple strains of *Wolbachia* infection in all species and sharing of parasite haplotypes across distant host mtDNA haplotypes, suggesting historical horizontal transmission between species [5].

One possible explanation for these differences is that many *Formica* species tend to form long-term, stable populations and these species may have enhanced opportunities for horizontal transmission. *Formica fusca* is an ephemeral species that invades relatively open spaces and establishes colonies that later may be outcompeted by more aggressive species [25]. These short-lived populations may not persist long enough to permit extensive horizontal transmission. Alternatively, the difference in prevalence rate among populations may be due to the fact that a population that spreads from an initial infected foundress or group of infected queens may also have complete transmission of infection to all nests via vertical transmission. For example, the fact that three closely spaced nests are infected likely resulted from vertical transmission of *Wolbachia* preceding the split into satellite colonies. Our results suggest that this newly established population was founded by multiple introductions of *Formica fusca*, some of which were infected with *Wolbachia* and others that were not. If *Wolbachia* is transmitted between colonies in this host population, it has not had time to spread. Determining whether the current infections across this population represent the same *Wolbachia* strain or represent introductions of separate strains of the parasite will help disentangle the history of *Wolbachia* infection in this population. An additional possibility is that newly established colonies in this population were infected with *Wolbachia* via horizontal transmission of neighboring ant species. A parallel study of *Wolbachia* infection in other ant species within the population would provide the data necessary to test this hypothesis.

Although no significant association between nest size and infection prevalence was found, 9 out of 12 "small" nests do not harbor infections and mean nest size of infected nests is nearly double that of noninfected nests. The trends suggest that nest size may be positively associated with the prevalence of *Wolbachia* given a larger sample size. Such a finding would contradict the argument, as proposed and rejected by Wenseleers et al. [14], that *Wolbachia* may have deleterious effects on the colony via reducing worker biomass. Alternatively, because nest size is correlated generally with colony age, older colonies may have been established from foundress queens that emigrated from an infected population and the newly established, smaller colonies represent a recent introduction from a noninfected population. Multiple introductions and/or recent acquisition of the *Wolbachia* parasite might also explain the low prevalence rate of *Wolbachia* in this population (34%), which contrasts previous findings of near fixation of the parasite [5, 10, 14]. Interestingly, in other species, *Wolbachia* infections are not common across introduced populations [11, 36]. In Argentine ants and fire ants, selective pressures of colonization may impede the ability of an infected population to successfully colonize [11, 36]. This effect is not seen in this *Formica* population.

Within colonies, the infection rate can vary between workers, brood, and reproductives. In our study population, 100% of reproductives, 87% of worker brood were infected and 63% of workers were infected. This pattern resembles that seen in *Formica truncorum* species (95% in sex M, 94% sex F, 87% worker brood, and 45% workers) [14], but the differences in infection rate in this study were not significant. Thus, our results provide little support to the hypothesis that adult workers may be able to rid themselves of infection. There is no strong selective pressure inhibiting the loss of infection in workers because workers produce only males and are essentially an evolutionary dead-end for this parasite.

Overall, the results of this study show no large deleterious fitness effects of infection on colony size or longevity; infection does not appear to decrease longevity of the colony, at least within the time scale of this population expansion. *Wolbachia* infection could also cause a decrease in the number of reproductives produced, limiting the reproductive success of colonies. In this population, few colonies produce sexuals, but the production of sexuals does not appear to be linked to the presence of infection as both infected and noninfected colonies produce sexuals. It is important to note that the infected colony that produced sexuals produced only 4 males, compared to the greater than 16 reproductives produced in noninfected colonies. This result does support the finding of reduced biomass of sexual brood in infected *Formica truncorum* colonies [14]. Differences in sex ratios of infected

colonies could also result from infections [14, 15, 20, 37], but the small numbers of reproductives produced in this population make it difficult to test sex ratio predictions.

Finally, *Wolbachia* infection is not correlated with queen number in this population of *Formica fusca*. In native populations of fire ants, monogyne colonies harbor a higher frequency of *Wolbachia* infection than polygyne colonies. The difference in prevalence rate may be due to a reproductive advantage to monogyne colonies because these queens are less likely to produce diploid males when founding new colonies [11]. This pattern was not seen in our study or in studies of related species of *Formica ants* [8, 20]. Both studies show no difference in the infection rate of monogyne versus polygyne colonies. Thus, *Wolbachia* infection does not appear to be associated with queen number in *Formica* ants and is not likely to affect the genetic diversity of *Formica fusca* colonies.

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