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Frequent intertrophic transmission of *Wolbachia* by parasitism but not predation

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eLife Assessment

Using experiments in the white fly, this manuscript provides evidence that the bacterial symbiont Wolbachia can be transmitted from parasitoid wasps to their insect hosts. Characterizing the transfer of Wolbachia between insect species is a **valuable** attempt to explain the widespread of this intracellular bacterium. This paper is **incomplete** as it does not furnish sufficient data to support several of its claims for which additional methods and data are necessary.

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Abstract

Wolbachia is one of the most pervasive symbionts, estimated to infect ~50% of arthropod species. It is primarily transmitted vertically, inducing a variety of fascinating reproductive manipulations to promote its spread within host populations. However, incongruences between host and *Wolbachia* phylogenies indicate substantial horizontal transmissions, the mechanisms of which are largely unexplored. By systematically analyzing *Wolbachia* surface protein (*wsp*) sequences on NCBI, we found that parasitism, not predation, is the primary route of intertrophic *Wolbachia* transmission. This conclusion held after accounting sampling bias. One example of frequent *Wolbachia* transfers is between egg parasitoid wasps, *Trichogramma*, and their lepidopteran hosts. Moreover, both bioinformatics and experimental results showed that *Wolbachia* from the parasitoid wasp *Encarsia formosa* can be transmitted to its whitefly host *Bemisia tabaci*, through unsuccessful parasitism. Once *En. formosa Wolbachia* is transferred to whiteflies, it can be vertically transmitted within whiteflies and induce fitness costs. To our knowledge, this is the first compelling evidence that *Wolbachia* can be transmitted from parasitoid wasps to their hosts, revealing the bidirectional nature of *Wolbachia* transfers between parasitoids and their hosts. Overall, our findings enrich the current understanding of the horizontal transmission of *Wolbachia* and shed new light on its ecology and evolution.

Introduction

Symbiosis with microbes, ranging from parasitism to mutualism, is prevalent in both plants and animals (1, 2). The ubiquity of microbial symbionts is likely attributed to their profound impact on host biology, including host survival, development, immunity, reproduction, and even behavior (2, 3). The transmission mode of symbionts is a key factor in shaping the ecology and evolution of both symbionts and their hosts (4–6). In addition to vertical transmission, incongruence between symbionts and host phylogenies indicates a large number of horizontal symbiont transfers across species (1). These events are important, as they allow symbionts to expand their host range and enable hosts to acquire new symbionts and alter their fitness.

Wolbachia (Rickettsiales, Alphaproteobacteria) is intracellular gram-negative bacteria and one of the most famous endosymbionts that infests ~50% arthropod and several filarial nematode species (7–9). On the one hand, *Wolbachia* can induce a range of fascinating phenotypes, including a variety of reproductive manipulations, provision of nutrients, and alteration of host behavior, thus facilitating its spread among populations (7). On the other hand, although *Wolbachia* is primarily maternally transmitted, there are widespread and frequent horizontal transfers across hosts (10, 11). Together, these characteristics make *Wolbachia* likely the most common microbe on Earth in terms of the number of species it infects (7, 8).

Wolbachia has also received much attention for its applications in controlling pests and vector-borne diseases (8). Specific strains of *Wolbachia* are artificially transfected into target pests and subsequently released into the field to either suppress pest populations or replace populations to depress the spread of vector-borne diseases (12–15). Understanding how *Wolbachia* spreads horizontally is critical in assessing its successful application and potential risks (16). This is because the released *Wolbachia* may leak into natural pest populations, frustrating population suppression strategies based on the cytoplasmic incompatibility (CI) of *Wolbachia*. It may also spread to nontarget organisms, potentially disrupting their population dynamics. Therefore, the lack of a thorough understanding of *Wolbachia* transmission and its consequences could hinder its broader application (16).

Despite the extensive interest in the horizontal transmission of *Wolbachia*, our understanding of this subject remains incomplete (17). Similar to other symbionts, *Wolbachia* host shifts may occur through three main routes: parasitism, predation, and shared plant or other food sources (17). However, it is important to note that these are not the only routes through which transmission may occur, and the specific contributions of each to the overall process of host shift are not yet fully understood. Multiple surveys report a significant similarity in *Wolbachia* sequences between parasitoid wasps and their respective hosts, suggesting that parasitism may serve as a primary route for *Wolbachia*'s host shift (18–23). However, there is still a lack of systematic statistical analyses to support this hypothesis. For the intertrophic transmission of *Wolbachia* between parasitoid wasps and their hosts, experimental evidence has shown that parasitoid wasps can acquire hosts' *Wolbachia* and vertically transmit them for several generations (24). However, it remains uncertain whether *Wolbachia* can be transferred from parasitoid wasps to their hosts. Some have argued that the transfer of *Wolbachia* between parasitoid wasps and their hosts is unidirectional, from host to wasp, as all parasitoid wasps emerge from their hosts, but parasitized hosts are eventually killed or castrated if not killed immediately (19, 25, 26). However, it is common in nature for hosts to survive parasitoid attacks (27–29). For example, whiteflies can survive after attacks of *Eretmocerus* parasitoids (27). These parasitoids can serve as phoretic vectors for the transmission of *Wolbachia* among whitefly populations, with contamination of *Wolbachia* on their mouthparts and ovipositors during the probing process (27).

In this study, our objective was to elucidate the role of parasitism and other potential routes in *Wolbachia* horizontal transmission and to investigate whether *Wolbachia* can be transferred from parasitoids to their hosts. We conducted a systematic survey of *Wolbachia* surface protein (*wsp*) sequences from the NCBI database and executed experiments using the whitefly *Bemisia tabaci* and its parasitoid wasp *Encarsia formosa*. Our study illuminates the crucial role of parasitism in *Wolbachia* intertrophic transmission, demonstrates the bidirectional nature of *Wolbachia* transfer between parasitoids and their hosts, and thus expands the current understanding of *Wolbachia* horizontal transmission.

Results

Wolbachia is frequently transmitted between parasitoid wasps and their hosts

To investigate potential horizontal transmission of *Wolbachia*, we retrieved 4685 *wsp* sequences from the NCBI database, and species interaction relationships were extracted from the GloBI database (for details, see Methods and Materials). Out of these 4685 sequences, 4253 could be assigned to 1377 species. We constructed a phylogenetic tree of *wsp* sequences (**Fig. 1a**) and extracted the minimum genetic distances of *wsp* between every species pair. Based on the relationships between species, we defined the species pairs into categories “Parasitism”, “Plant-sharing”, “Predation” and “Others”. Among these species pairs, 16.5% (53 out of 321) in “Parasitism” pairs had the minimum interspecific *wsp* distances less than 0.01 (i.e., > 99% identity). This proportion is significantly greater than the 6.3% (145 out of 2294) in “Plant-sharing” pairs, the 1.1% (13 out of 1146) in “Predation” pairs, and the 1.5% (14120 out of 943315) in “Others” pairs (χ^2 test; all comparisons: $p < 1e^{-5}$). Consistently, the minimum interspecific *wsp* distances in “Parasitism” relationships were significantly shorter than those in “Plant-sharing”, “Predation”, and “Other” relationships (**Fig. 1b**; Mann-Whitney U test (MWUT); all comparisons: $p <$ 1e-12). The minimum *wsp* distances in “Plant-sharing” were also significantly smaller than those in “Others” (**Fig. 1b**; MWUT; $W = 730215564$, $p < 2.2e^{-16}$). However, the minimum *wsp* distances showed no significant difference between “Predation” and “Others” (**Fig. 1b**; MWUT; $W =$ 699285646, $p = 0.096$).

To test whether these effects were merely due to sampling bias, we further obtained divergent times from TimeTree for 95.2% (901,615 out of 947,376) of the species pairs. As expected, the minimum interspecific *wsp* distance increased as the divergent time increased (**Fig. 1c**; Spearman’s correlation; $\rho = 0.14$, $p < 2.2e^{-16}$). Considering the impact of divergent time, linear regression analyses were conducted with the *wsp* distance as the dependent variable and divergent time as the independent variable. We found that both “Parasitism” and “Plant-sharing” had significant effects (both tests: $p < 2e^{-16}$). The estimated effect of “Parasitism” (−0.28) was more profound than that of “Plant-sharing” (−0.13) ($p = 3.9e^{-16}$). Similar results were observed even after considering phylogenetic correction (Supplementary Note1). Given that we cannot obtain divergence times for all species pairs, especially for those closely related, we also classified the species pairs according to their last common ancestor. We divided all species pairs into six categories based on whether the two species belonged to the same genus, family, order, class, phylum, or kingdom. Similar results were observed (Supplementary Note 2). To further rule out potential influences of sampling bias, subsampling analyses were conducted using three methods: 1) shuffling species relationships, 2) subsampling by controlling divergent time, and 3) subsampling by controlling the last common ancestor (for details, see Methods and Materials). For all three methods, both “Parasitism” and “Plant-sharing”, but not “Predation”, exhibited significantly shorter minimum interspecific distances of *wsp* than randomly generated controls (Fig. S1; both “Parasitism” and “Plant-sharing”: $p < 0.001$; “Predation”: $p > 0.05$). These results confirmed that parasitism and plant-sharing promote interspecific transfers of *Wolbachia*, rather than being due to sampling bias.

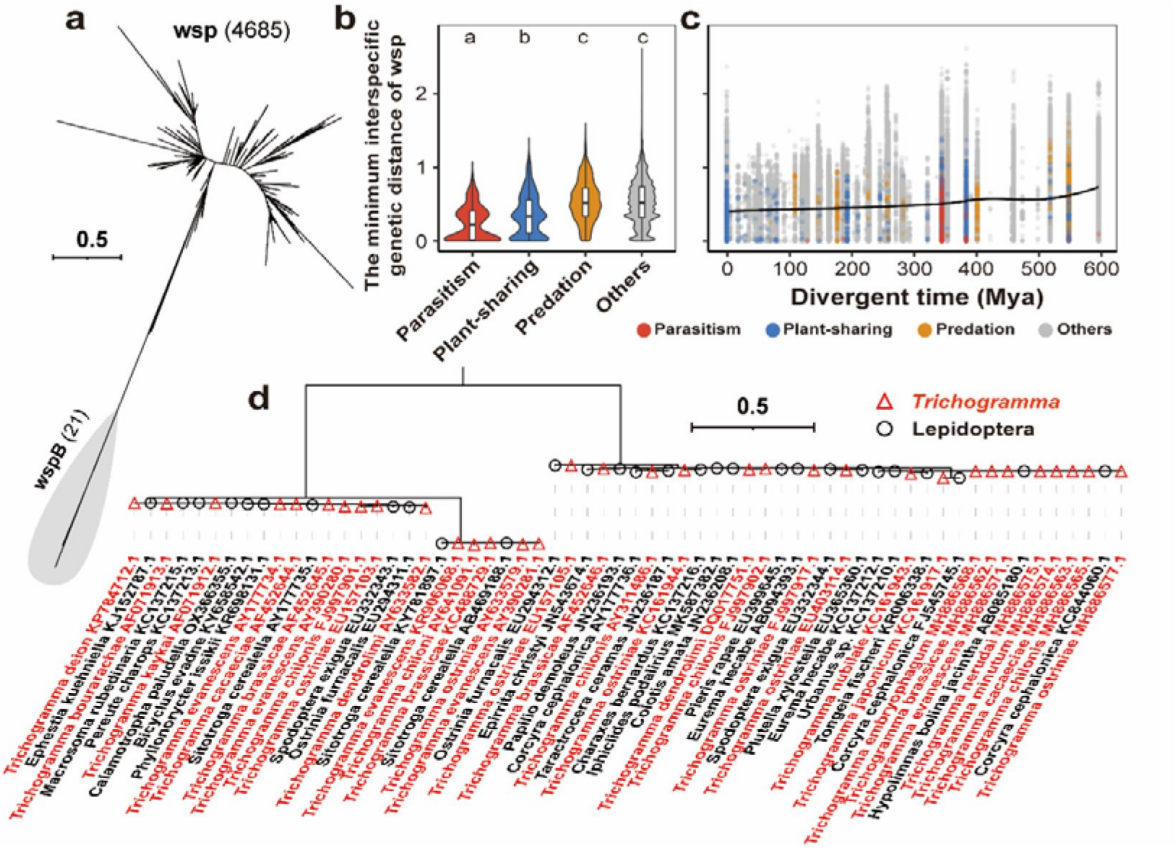

Fig. 1.

Parasitism facilitates interspecific horizontal transfer of *Wolbachia*.

(a) Phylogeny of *Wolbachia* surface protein (*wsp*) genes retrieved from NCBI. **(b)** Effect of parasitism, plant-sharing, and predation on the genetic distance of *wsp* between species. **(c)** Relationship between species divergence time and the genetic distance of *wsp* between species. **(d)** Phylogeny of representative *wsp* sequences from *Trichogramma* wasps and lepidopterans. Color red and black represent *wsp* sequences from *Trichogramma* wasp and lepidopterans, respectively.

An example of frequent *Wolbachia* transfers between parasitoids and their hosts is observed in *Trichogramma* wasps and their lepidopteran hosts (**Fig. 1d**). *Trichogramma* is a genus of generalist egg parasitoids, targeting mainly lepidopterans such as moths and butterflies (30). **Fig. 1d** displays representative *wsp* sequences from *Trichogramma* and lepidopterans, illustrating potential interspecific transitions of *Wolbachia*. In the analyzed dataset, 16 out of 23 (69.6%) *Trichogramma* species exhibited the minimum interspecific *wsp* distance of less than 0.01 with at least one lepidopteran species. Similarly, 79 out of 254 (31.1%) surveyed lepidopteran species displayed a minimum interspecific *wsp* distance of less than 0.01 with at least one *Trichogramma* species. These results suggest frequent *Wolbachia* transitions between *Trichogramma* wasps and lepidopterans.

Collectively, these findings support that *Wolbachia* are frequently transmitted between parasitoid wasps and their hosts.

Wsp phylogeny suggests transfer directions of *Wolbachia* between the whitefly *B. tabaci* and its parasitoid wasps

However, the interspecific identities of *wsp* between parasitoids and their hosts typically offer no clues about the direction of *Wolbachia* transmission. We noticed frequent *Wolbachia* transfers between the whitefly *B. tabaci* and its parasitoid wasps (**Fig. 2a**). Notably, one of the juvenile parasitoids of *B. tabaci* is *En. formosa*. *Wolbachia* induces parthenogenesis and has evolved into an obligate symbiont in *En. formosa*, exhibiting a 100% infection rate (31, 32). This system presents a unique opportunity to infer the directions of *Wolbachia* transmission between parasitoids and their hosts.

One clade of the *wsp* phylogeny contained 99 sequences from *B. tabaci*, 14 sequences from *Encarsia* and *Eretmocerus* parasitoid wasps of *B. tabaci*, and 8 other sequences (**Fig. 2a**). Based on ancestral state reconstruction, the ancestral state of the Order for these *wsp* sequences was Hemiptera with a high likelihood (100.00%), while all other orders were negligible (<0.001%). This overwhelming probability strongly suggests that the transmission direction of *Wolbachia* in this clade is predominantly from *B. tabaci* to its parasitoid wasps. Notably, in another clade of *wsp*, nine *En. formosa* and five *B. tabaci wsp* sequences are clustered together, along with one *wsp* sequence detected in cotton and 12 sequences from other parasitoid wasps of *B. tabaci*, such as *En. inaron*, *En. lutea*, *En. bimaculata*, *Er. mundus*, and *Aimtus hesperidum* (**Fig. 2a**). The ancestral state of the Order for these *wsp* sequences was estimated to be Hymenoptera with a near certain likelihood of 100.00%. Given that *Wolbachia* is obligate with a 100% infection rate in *En. formosa*, it is reasonable to infer that the transmission direction of *Wolbachia* in this clade was from *En. formosa* to *B. tabaci*.

Wolbachia can be transmitted from the parasitoid wasp *En. formosa* to its whitefly host in cage experiments

Bemisia tabaci is a species complex of at least 40 cryptic species (33). Infection rates of *Wolbachia* reported across multiple populations within these cryptic species exhibit dramatic fluctuations, ranging from 0% to 100% (33–36). To investigate whether *Wolbachia* can be transmitted from parasitoid wasps to their whitefly hosts, we first established a *Wolbachia*-free iso-female line of *B. tabaci* Q biotype. Subsequently, we conducted outdoor cage experiments using the *Wolbachia*-free *B. tabaci* and its parasitoid *En. formosa*, which was 100% infected with *Wolbachia* (**Fig. 2b**). Notably, each cage setup was replicated three times to ensure experimental rigor. After introducing *En. formosa*, the *Wolbachia* infection rate in whiteflies increased from zero to 4.17% after 20 days and reached 10.83% after 100 days (**Fig. 2c**; ANOVA; $F_{5,17}$=11.44, p < 0.001). Correspondingly, the female ratio of whiteflies decreased from 69.17% to 60.08% (**Fig. 2d**; ANOVA; $F_{5,17}$=3.14, p = 0.048). In contrast, the whitefly population with no exposure to wasps maintained a zero infection rate of *Wolbachia* (**Fig. 2c**), and the female ratio showed no

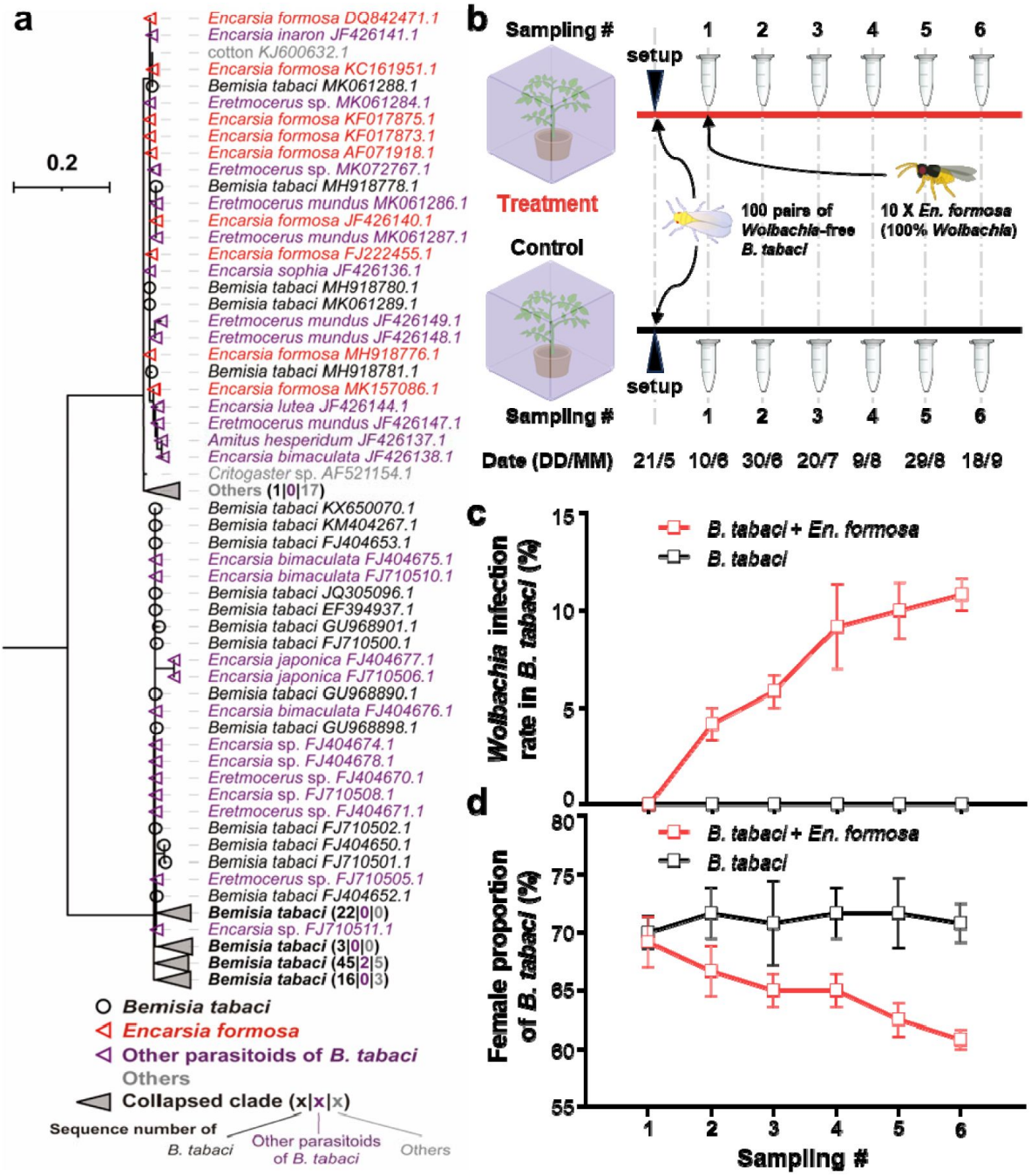

Fig. 2.

Transfer of *Wolbachia* from the parasitoid wasp, *Encarsia formosa*, to its host, *Bemisia tabaci*.

(a) Phylogenetic analysis of the *wsp* gene from *B. tabaci* and its parasitoid wasps. The colors black, red, purple, and gray represent *wsp* sequences from *B. tabaci*, *En. formosa*, other parasitoids of *B. tabaci*, and other species, respectively. **(b)** Scheme of the experimental design for studying the transmission of *Wolbachia* from *En. formosa* to *B. tabac*. **(c–d)** Effects of *En. formosa* on **(c)** the infection rate of *Wolbachia* and **(d)** the proportion of females in *B. tabaci* populations. Data are presented as the means ± standard errors (SE) (n = 3).

significant changes (**Fig. 2d**; ANOVA; $F_{5,17} = 0.076$, $p = 0.99$). Additionally, the Sanger sequencing results showed that *wsp* sequences from both whitefly and *En. formosa* were identical (Fig. S2). These results indicate that *Wolbachia* from *En. formosa* can be rapidly transmitted to its whitefly host.

Parasitism failure transmits *Wolbachia* from the parasitoid wasp *En. formosa* to its whitefly host

We hypothesized that *Wolbachia* was transmitted from parasitoid wasps to their hosts through unsuccessful parasitism. To test this hypothesis, we performed parasitism experiments on wasp individuals and applied irradiation treatment to wasps to reduce their parasitism success rate. After 60 Gy irradiation, the fecundity of *En. formosa* in 12 h significantly decreased (**Fig. 3a**; Student's t test; $t = 12.91$, $p < 0.001$), and the parasitism success rate on whiteflies drastically declined from 78.8% to 9.9% (**Fig. 3b**; Student's t test; $t = 13.09$, $p < 0.001$). Correspondingly, the *Wolbachia* infection rate increased from 7.5% to 78.3% in whiteflies survived from parasitism (**Fig. 3c**; Student's t test; $t = 6.61$, $p < 0.001$), despite a decrease in the *Wolbachia* titer in *En. formosa* post irradiation (Fig. S3). More details can be found for irradiation treatments at different dosages (Supplementary Note 3). Fluorescence in situ hybridization (FISH) assays further showed that *Wolbachia* was injected into the host nymphs along with *En. formosa* eggs (**Fig. 3** d-f and S4). These results indicate that parasitism failure can transfer *Wolbachia* from the parasitoid wasps *En. formosa* to their whitefly hosts.

Vertical transmission and fitness cost of *Wolbachia* in whiteflies after horizontal transfer from *En. formosa*

Next, we investigated the vertical transmission and fitness cost of *Wolbachia* in its new host, *B. tabaci*, following its horizontal transfer from *En. formosa*. The vertical transmission rate varied from 22.2% to 33.3% across generations in whiteflies, with a slight but statistically insignificant increase from G_1 to G_5 (**Fig. 4b**; ANOVA; $F_{4,40} = 2.09$, $p = 0.10$). Moreover, *Wolbachia* was also detected in the G_3 whiteflies, specifically in the nymph's bacteriocytes, the abdomen of male adults, and the ovaries of female adults (**Fig. 4** and S5). MLST typing confirmed that the *Wolbachia* strain introduced into the whiteflies was identical to the strain from *En. formosa* (Fig. S6). We then examined the impact of the introduced *Wolbachia* on the fitness of whiteflies. Compared to uninfected females, *Wolbachia*-infected females showed decreased fecundity (**Fig. 5a**; Student's t test; $t = 8.51$, $p < 0.001$). Moreover, offspring from infected mothers exhibited a diminished egg hatching rate (**Fig. 5b**; Student's t test; $t = 8.33$, $p < 0.001$), a lower survival rate among nymphs (**Fig. 5c**; Student's t test; $t = 13.54$, $p < 0.001$), and a decreased ratio of females in adults (**Fig. 5d**; Student's t test; $t = 12.29$, $p < 0.001$), although there was no significant difference in the development time from egg to adult (**Fig. 5e**; Student's t test; $t = 1.51$, $p < 0.001$). We also investigated the effects of paternal infection with *Wolbachia*. Regardless of whether the female was infected with *Wolbachia*, the infection status in males showed no significant influence on their fecundity, the hatching rate of their offspring's eggs, the survival rate of their nymph offspring, or the female ratio of their adult offspring (**Table 1**; Student's t test; all comparisons: $p > 0.05$).

Collectively, these results indicate that *Wolbachia* from *En. formosa*, when shifted into the new host *B. tabaci*, exhibits a low rate of vertical transmission and a substantial fitness cost, without apparent reproductive manipulation phenotypes.

Discussion

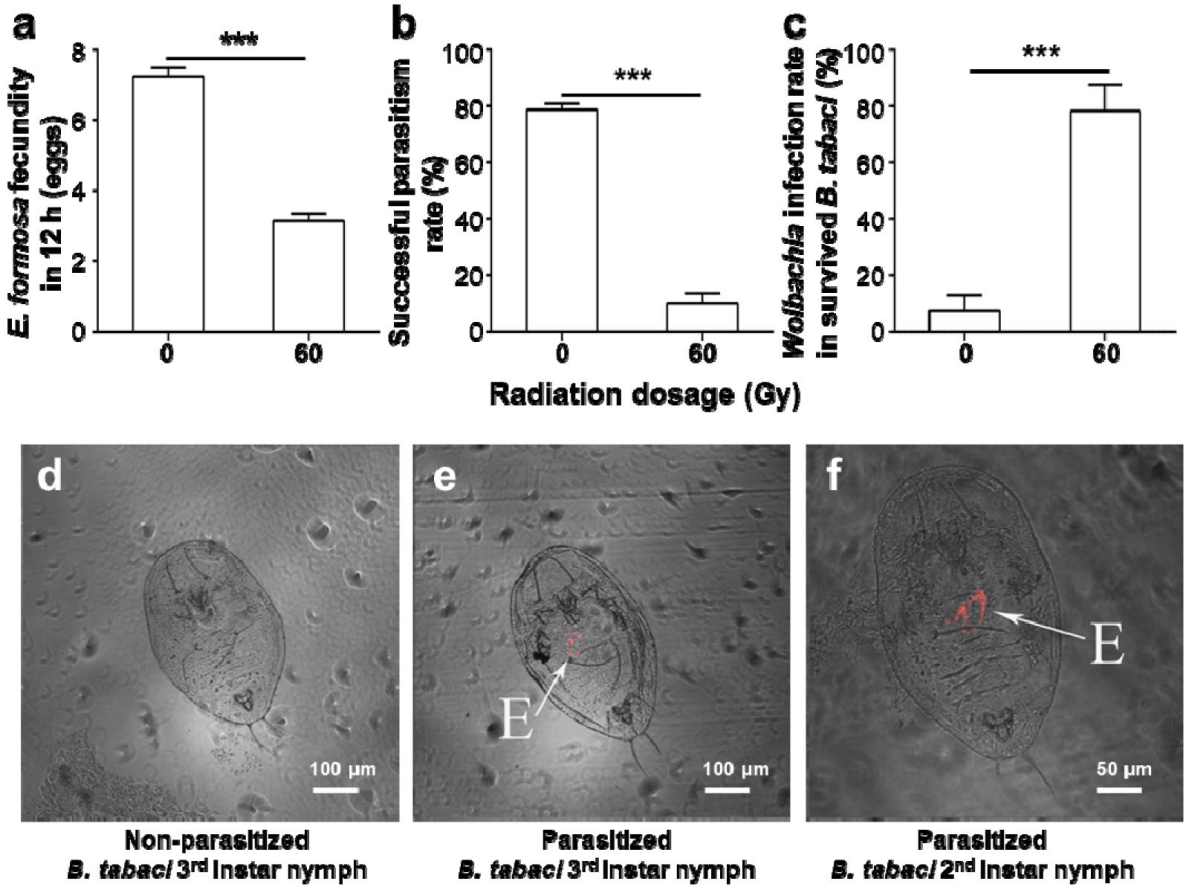

Fig. 3.

Parasitism failure mediates *Wolbachia* transfer from *En. formosa* to *B. tabaci*.

(a–c) Effects of 60 Gy radiation on **(a)** the fecundity of *En. formosa* over a 12-hour period, **(b)** the rate of successful parasitism, and **(c)** the *Wolbachia* infection rate in surviving whiteflies. Data are presented as the means + SEs (n = 20). ***: $p < 0.001$. **(d–f)** Fluorescence in situ hybridization (FISH) visualization of *Wolbachia* in nonparasitized and parasitized 3rd instar nymphs, as well as parasitized 2nd instar nymphs of *B. tabaci*. The images present a combination of bright field and fluorescence. E: injected eggs from *En. formosa*.

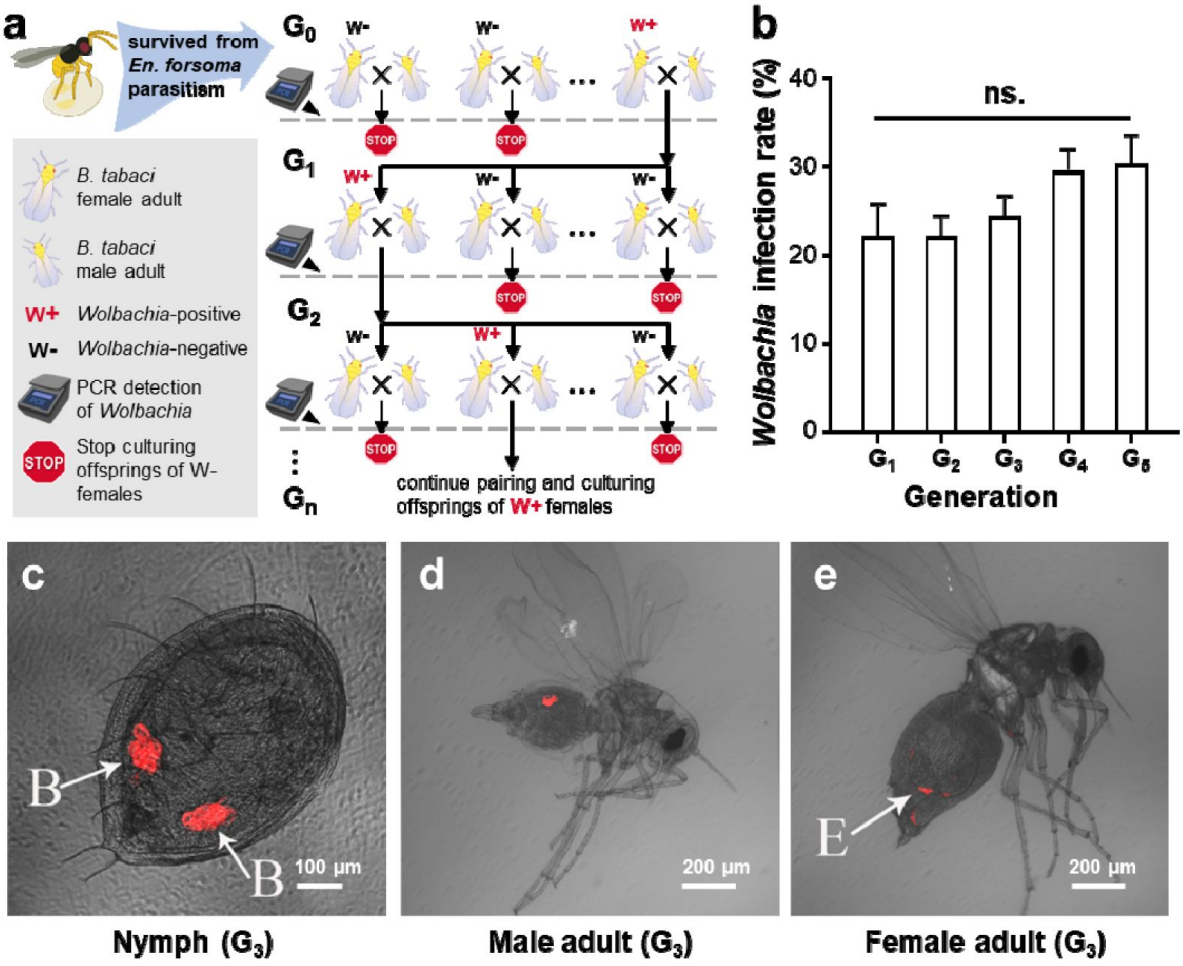

Fig. 4.

Vertical transmission of *Wolbachia* from *En. formosa* in *B. tabaci*.

(a) Scheme of the study design for *Wolbachia* vertical transmission in *B. tabaci*. Adult whiteflies that survived from *En. formosa* parasitism were denoted as G_0. After pairing and oviposition, the infection status of *Wolbachia* in the female parent was examined. Only the offsprings from *Wolbachia*-infected female whiteflies were maintained. **(b)** The vertical transmission rate of *Wolbachia* across five generations in *B. tabaci*. Data are presented as the means + SEs (n = 9). ns.: no significant differences. **(c–e)** FISH visualization of *Wolbachia* in G_3 *B. tabaci* **(c)** nymph, **(d)** male adult, and **(e)** female adult. The images present a combination of bright field and fluorescence. B: bacteriocyte; E: eggs in the ovary of a female whitefly.

Fig. 5.

Fitness costs in *B. tabaci* induced by *Wolbachia* from *En. formosa*.

(a–e) The effects of *En. formosa Wolbachia* on *B. tabaci* **(a)** female fecundity, **(b)** egg hatching rate, **(c)** immature survival rate, **(d)** female proportion, and **(e)** developmental time. Data are presented as the means + SEs (n = 60). ***: $p < 0.001$; ns.: no significant differences.

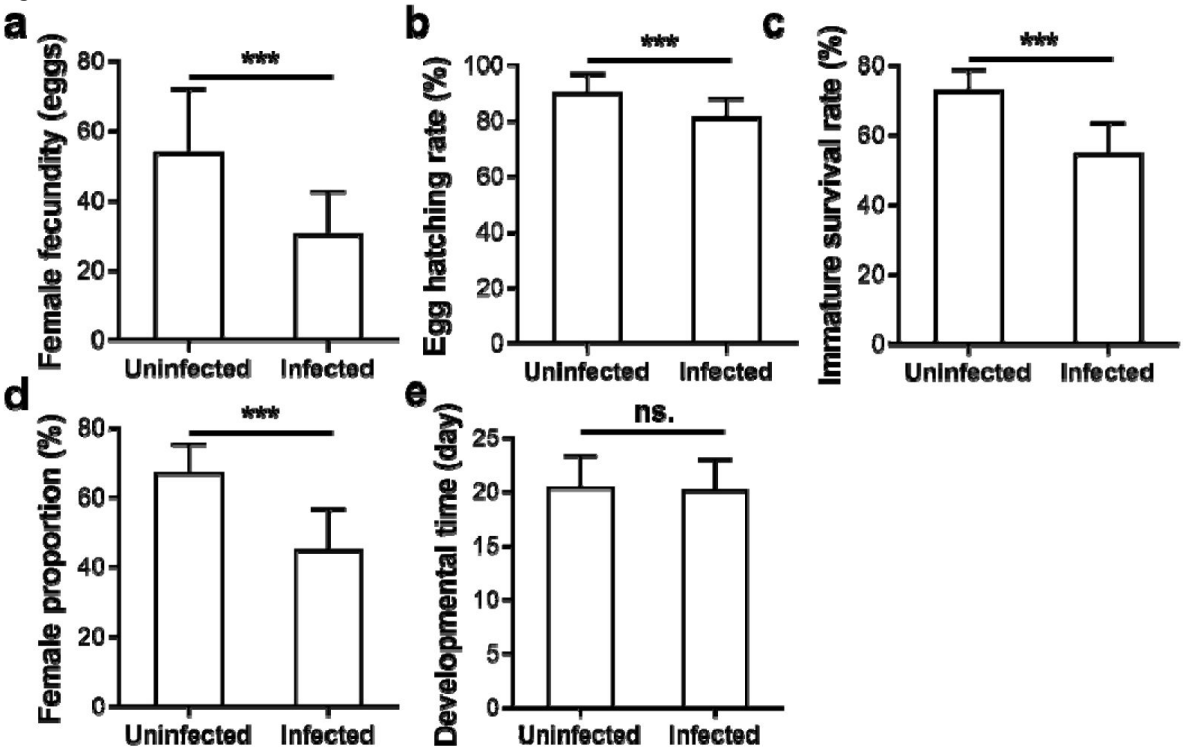

Table 1.

Fitness in *Bemisia tabaci* of different cross combinations

Cross	n	Fecundity (eggs)	Egg hatching rate (%)	Immature survival rate (%)	Female proportion (%)
♀- × ♂-	20	54.0 ± 19.5 a	91.61 ± 2.99 a	73.31 ± 5.29 a	65.81 ± 8.19 a
♀- × ♂+	20	51.7 ± 20.5 a	90.88 ± 4.99 a	72.85 ± 6.33 a	66.43 ± 7.43 a
♀+ × ♂-	20	34.0 ± 12.3 b	82.19 ± 4.54 b	53.66 ± 6.58 b	42.57 ± 9.10 b
♀+ × ♂+	20	28.8 ± 11.1 b	82.16 ± 5.36 b	55.35 ± 7.21 b	48.03 ± 11.99 b

Data are presented as the means ± standard errors (SE) (n = 20). ♀: female adult; ♂: male adult; +: *Wolbachia* infected; -: *Wolbachia* uninfected.

The effect of parasitism on *Wolbachia* horizontal transmission

As with previous studies, we utilized the sequence similarities of *wsp* to infer potential horizontal transfers of *Wolbachia*. Here, we systematically investigated all *wsp* sequences in the NCBI database, enabling us to examine the effect of potential factors such as parasitism, plant-sharing, and predation. Our findings clearly indicate that parasitism exhibits shorter interspecific *wsp* distances than plant-sharing and predation. For example, we found that some *wsp* sequences from multiple *Trichogramma* species and their various lepidopteran hosts are almost identical. A previous study has found common horizontal transmission between butterflies and moths, and speculated that parasitoids might be one of routes that contribute to the horizontal transmission (37). Our results suggest potential horizontal transmission between *Trichogramma* wasps and their lepidopteran hosts, and *Trichogramma*, which is a kind of egg parasitoids of many lepidopterans, may act as vectors facilitating the transfer of *Wolbachia* among lepidopteran hosts.

Moreover, the fact that herbivores sharing plants have identical *wsp* sequences does not necessarily imply plant-mediated horizontal transfer of *Wolbachia*. This is because species that share the same plants often have recent divergent times (**Fig. 1b**), which increases the potential for hybridization and also sharing common parasitoid wasps. Therefore, the plant-sharing category overestimates the extent of plant-mediated *Wolbachia* horizontal transmission, further supporting the notion that parasitism is the primary route of *Wolbachia* horizontal transmission.

Directions of *Wolbachia* transmission between parasitoids and their hosts

However, investigations based on *Wolbachia* sequence similarity have significant limitations. First, the PCR detection of *wsp* does not necessarily indicate horizontal transfer of *Wolbachia* (26). Instead, it could merely represent contamination that arises during predation or parasitism. The contamination can be *Wolbachia*-infected tissues or even just fragmented *Wolbachia* DNA, which could be found on the surface, within the gut, or inside the body cavity (as in the case of parasitized hosts). Experimental confirmations of *Wolbachia* horizontal transfer remain relatively rare, with only a limited number of documented cases (24, 27, 38, 39). Additionally, some experiments have found no evidence of horizontal transmission of *Wolbachia* (40–42). Second, determining the direction of transfer is challenging based solely on the identity of *Wolbachia* strains. In certain exceptional cases, inferences of transfer direction might be drawn from the prevalence of *Wolbachia* infection in the two species (17). For example, *Wolbachia* is obligate in *En. formosa* and exhibits a 100% infection rate across all populations (32). This unique instance of horizontal *Wolbachia* transfer between *En. formosa* and its whitefly hosts provides compelling evidence, suggesting that *Wolbachia* is likely transmitted from the parasitoid wasp to its host, rather than the reverse.

To verify *Wolbachia* transmission from parasitoid wasps to their hosts, we conducted outdoor cage experiments and indoor tests using *Wolbachia*-free whitefly *B. tabaci* and its parasitoid *En. formosa*. *Wolbachia* was detected by nested PCR in whitefly adults (G_0) that survived parasitism. *Encarsia formosa* parasitizes *B. tabaci* nymphs, which undergo a pseudo-pupal stage to reach adulthood (31, 43). In our experiments, PCR detection of *Wolbachia* typically occurred 3~7 days post-parasitism, minimizing risks of contamination from parasitism. *Wolbachia* was also detected by PCR in subsequent generations (G1–G5) of whiteflies and induced notable fitness costs. PCR sequencing confirmed that the *Wolbachia* strain in the *B. tabaci* matched that of *En. formosa*. Furthermore, FISH assays revealed a tissue-specific distribution of *Wolbachia* in both nymphs and adults of the whiteflies, matching previously reported patterns (44). Collectively, these findings provide compelling evidence that *Wolbachia* from *En. formosa* can be horizontally transmitted to *B. tabaci*, beyond mere DNA contamination.

Although previous research has demonstrated that parasitoids can acquire *Wolbachia* from their hosts, the reverse direction of transmission, from parasitoid to host, has been largely overlooked and lacks supportive experimental evidence. One possible reason for this oversight is that all parasitoids emerge from their hosts, but hosts are eventually killed by the parasitism of parasitoid wasps (19, 25, 26). However, parasitoid wasps' success in parasitizing their hosts does not always reach 100% (28). Some hosts can manage to survive after parasitism. Various factors can influence the outcome of parasitoid-host interactions, including environmental conditions, the species and genotype of both wasps and hosts, the host's age, and the presence of symbiotic bacteria within the host (29, 45, 46). Moreover, we used radiation to reduce the parasitism success rate, which notably enhanced the transfer of *Wolbachia* from *En. formosa* to its whitefly host.

Potential intertrophic transmission network of *Wolbachia*

A previous study reported that parasitoid wasps can act as vectors to transmit *Wolbachia*, without the necessity of being infected themselves (27). Through the probing actions of the *Eretmocerus* parasitoid, *Wolbachia* can be transmitted among whitefly hosts (27). This is often referred to as the 'dirty needle' model. Conversely, hosts can also serve as vectors for *Wolbachia* transmission among parasitoid wasps. *Wolbachia* can be transferred from infected to noninfected *Trichogramma* wasps through superparasitism (38, 47). However, the *Wolbachia* transmission of these two modes is restricted within the same trophic level. In contrast, the transmission of *Wolbachia* between parasitoid wasps and hosts can cross trophic levels. Our findings, when combined with existing knowledge, suggest that the intertrophic transmission of *Wolbachia* is bidirectional between parasitoid wasps and their hosts. This greatly enhances our understanding of the horizontal transmission of *Wolbachia*.

Interestingly, we found on NCBI that a strain of *Wolbachia* detected in the cotton plant was identical to the *Wolbachia* from *En. formosa*, based on the *wsp* sequence and MSLT typing (**Fig. 2** and S6). This *Wolbachia* strain was probably transmitted to the cotton plant from the feeding of whiteflies (39). Given that *En. formosa* is 100% infected with its obligate *Wolbachia* strain, a possible transmission route could be from *En. formosa* to whiteflies via parasitism and then to the cotton plant through the feeding of whiteflies. This finding indirectly supports our conclusion that *Wolbachia* can be transmitted from parasitoid wasps to their hosts. This suggests that once the *Wolbachia* of parasitoids is transmitted to herbivorous hosts, it may further spread to host plants. The reverse transmission route can also be possible. It is likely that *Wolbachia*'s widespread and complex horizontal transmission network is established through such bidirectional transmissions across multiple trophic levels, e.g., "plant-herbivore-parasitoid-hyperparasitoid". Further investigations are needed to test these hypotheses.

Wolbachia establishment after host transfers

Moreover, the physical transfer of *Wolbachia* often represents merely the first step of its establishment in a new host (17). Several subsequent steps are required for *Wolbachia* to establish itself within new species, e.g., entry into germ cells, vertical transmission, and mechanisms that promote its spread within the population (17). First, we found that *Wolbachia* from *En. formosa* was enriched in the ovaries of whiteflies and vertically transmitted after entering *B. tabaci*. However, the vertical transmission efficiency is low, ranging from 22.2% to 33.3%. We also noted an absence of reproductive manipulations by the newly introduced *Wolbachia* in whiteflies. The reduced female ratio after infection does not support the induction of parthenogenesis, feminization or male-killing by *Wolbachia*. We neither observed cytoplasmic incompatibility, where the mating of infected males and uninfected females resulted in reduced offspring hatching.

In contrast, the introduced *Wolbachia* from *En. formosa* reduced whiteflies' egg laying and hatching, larval survival, and female proportion, demonstrating significant fitness costs in the new host. This is likely due to *Wolbachia*'s coevolution with its host in *En. formosa*, which may have led to the loss of its ability to colonize new host species. Given the low vertical transmission rate, high fitness cost, and lack of clear reproductive manipulations, it is reasonable to predict that the spread of *Wolbachia* in its new host population will be limited. Finally, these factors, together with the frequency of *Wolbachia* introductions by parasitoids and its spread via parasitoid or plant vectors, shape the dynamics and equilibrium of *Wolbachia*. These dynamics could shift with the emergence of reproductive manipulation or other beneficial phenotypes that promote *Wolbachia* spread, probably through gene mutation, recombination or horizontal gene transfer within *Wolbachia* (2). There are still many questions waiting to be further studied in these steps of *Wolbachia* host shifts.

Conclusions

By investigating *wsp* sequences from the NCBI database, we found frequent intertrophic transmission of *Wolbachia* by parasitism but not predation. Combining bioinformatics and experimental approaches, we demonstrated that *Wolbachia* can be transmitted from the parasitoid wasp *En. formosa* to the host *B. tabaci*. To our knowledge, this is the first compelling evidence that *Wolbachia* can be transmitted from parasitoid wasps to their hosts, thus revealing the bidirectional nature of *Wolbachia* transfers between parasitoids and their hosts. These findings enrich our knowledge of the *Wolbachia* transmission network and have significant implications for understanding the ecology of *Wolbachia*, as well as for evaluating the release of *Wolbachia* in pest control.

Materials and Methods

Wsp sequence retrieval and phylogenetic analyses

TBLASTN was conducted against the NCBI "nr/nt" database using the *wsp* protein AAS14719.1 from *Wolbachia* of *Drosophila melanogaster* (July 2023). Default settings were used with a maximum target sequence of 5000 and the organism limitation of *Wolbachia* (taxid:953). The sequences were filtered to remove those shorter than 300 bp or containing premature stop codons. *Wsp* sequences were translated into proteins, aligned using MAFFT v7.475 (48), and reverse translated into codons using PAL2NAL v14 (49). The phylogenetic trees were constructed using IQ-Tree v2.2.0 (50), with the best model selected by the built-in ModelFinder (51). The phylogenetic trees were visualized using iTOL v6 (52).

Statistics for genetic distances of *wsp*

Genetic distances of *wsp* sequences were extracted from the *wsp* phylogeny using a custom Python script. Species interaction relationships were extracted from the GloBI database (August 2023) (53). Parasitic associations were extracted using interaction types of "parasiteOf" or "parasitoidOf", excluding social parasitism to focus on direct biological parasitism. Predation associations were extracted using interaction types of "preysOn" or "eats". Given the relatively broad dietary range of predators, a genus-to-genus expansion was adopted for the predation relationships. Herbivorous interactions were extracted using the interaction types "hasHost" or "eats", specifically targeting taxa within the kingdom Plantae. Extracted relationships were manually curated to verify the accuracy. Divergent times between species were extracted from TimeTree v5 via its application programming interface (API) (September 2023) (54). To test the effects of special features of sampled species pairs, three subsampling methods were employed using a custom Python script. For each method, 1000 replicates were randomly generated. For species pair shuffling, in every replication, the pairs of species were randomly rearranged to create new combinations. To control divergent time, pairs of species were randomly sampled from

the background to match the divergent times in the tested category (i.e., parasitism, plant-sharing or predation). The sampling background pools were all species pairs excluding the specific tested category. A similar process was applied to control the last common ancestor. Linear model analyses, statistical tests, and data visualization were executed using R v4.3.1.

Phylogenetic correction

We treated the minimum interspecific *wsp* distance between two species as a trait for each species pair (*i*, *j*). For any two pairs of species (*i*, *j*) and (*k*, *l*), we postulate that the covariance between their traits is given by: $Cov[Y_{ij},Y_{kl}]=\sigma^2\cdot(T_{ik}+T_{jl})$,where T_{ik}denotes the evolutionary time from the root to the most recent common ancestor (MRCA) of species *i* and *k*, and *Tjl* represents the evolutionary time from the root to the MRCA of species *j* and *l*. This formulation allows us to estimate the covariance structure of the traits, which is then integrated into our linear model analysis to adjust for phylogenetic non-independence. To manage computational constraints, we employed a random sampling strategy, selecting 1,000 species pairs from the "Others" category. We analyzed the relationship between the minimum interspecific *wsp* distance and group categories using a Generalized Least Squares model, accounting for the covariance structure. The model was implemented using the "gls" function in R package "nlme" v3.1.164.

Ancestral state reconstruction

The taxonomic order of species from which wsp sequences originated was regarded as a discrete character trait. Ancestral state reconstruction was performed across the *wsp* phylogeny using the "ace" function in R package "phytools" v2.1.1. Three models were applied: Equal-Rates (ER), Symmetry (SYM), and All-Rates-Different (ARD). Among these three models, ARD was chosen as the best-fitting model, based on its lowest Information Criterion (AIC) value.

Insect rearing

Both whiteflies *B. tabaci* and the parasitoid wasps *En. formosa* were maintained in nylon cages at 25±1°C, 70±5% RH, and a L:D photoperiod of 16:8 h. *Bemisia tabaci* was originally collected from the campus of Nanjing Agricultural University in 2012 and determined to be the Q biotype. An isofemale line of *B. tabaci*, which is naturally *Wolbachia*-free and has not been treated with antibiotics, was established. *En. formosa* was initially acquired from Beijing Ecoman Biotechnology Co., Ltd. in 2013 and was maintained on *B. tabaci* on tomato plants.

PCR and Sanger sequencing

Bemisia tabaci or *En. formosa* were initially washed using ethanol and ultrapure water three times each. DNA was extracted from individuals using the STE method (55). To avoid false-negative results, a nest-PCR targeting the *wsp* gene was employed for detecting *Wolbachia* in whiteflies (Fig. S7) as previously described (55). PCRs were performed using DreamTaq Green PCR Master Mix (Thermo Scientific) according to the manufacturer's protocol. To confirm whether the *Wolbachia* present in whiteflies and their parasitoid wasps belonged to the same strain, PCR and Sanger sequencing were performed on *Wolbachia* genes, namely, *wsp*, *gatB*, *coxA*, *hcpA*, *ftsZ*, and *fbpA*, for MSLT typing (56). The primers used for *wsp* nest-PCR and MSLT typing are listed in Table S1.

FISH detection of *Wolbachia*

The location of *Wolbachia* in whiteflies was determined using fluorescence in situ hybridization as previously reported (32). To enhance the detection signal, two 5' rhodamine-labeled probes, W1: 5'-AATCCGGCCGARCCG ACCC-3' and W2: 5'-CTTCTGTGAGTACCGTCATTATC-3', were used to target *Wolbachia* 16S rRNA (57). Stained samples were examined under a Zeiss LSM 700 confocal microscope (Carl Zeiss, Germany). The specificity of the method was confirmed using *Wolbachia*-free whiteflies as negative controls.

Outdoor cage experiments for *Wolbachia* transmission

To assess the potential transmission of *Wolbachia* from *En. formosa* to *B. tabaci*, six cages (80 cm x 80 cm x 80 cm, enclosed with a 120 mesh nylon screen) were established on the Nanjing Agricultural University campus. Initially, six 20-cm tall tomato plants were placed in each cage, with two additional plants introduced at 30-day intervals. Each cage was populated with 100 female and 100 male *Wolbachia*-free whiteflies. After a 20-day period, ten *En. formosa* wasps were introduced into each of three randomly selected cages, following the random sampling of 40 adult whiteflies from each cage. Subsequently, an additional 40 adult whiteflies were sampled from each cage at 20-day intervals. These sampled whiteflies were sexed based on their morphologies and screened for *Wolbachia* infection using *wsp* nest PCR.

Wolbachia transmission from irradiated *En. formosa* to *B. tabaci*

To explore the possibility of *Wolbachia* transmission from *En. formosa* to *B. tabaci* due to unsuccessful parasitism, we subjected *En. formosa* wasps to radiation at the Nanjing Aerospace Irradiation Center. Wasps that had emerged within 24 h were exposed to Co^{60} radiation at doses of 60, 80 and 100 Gy (dose rate of 2 Gy/min). A single irradiated wasp was subsequently introduced into a Petri dish, which contained a tomato leaf infested with *Wolbachia*-free 3^{rd} or 4^{th} instar whitefly nymphs, and wet cotton was used to wrap the end of the leaf petiole to keep the leaf fresh. The parasitic behaviors of *En. formosa* were monitored under a Nikon SMZ800 stereomicroscope (Nikon Instrument Inc., Tokyo, Japan). Wasps were removed after 12 h of parasitism. Only whitefly nymphs that had been punctured by wasps were kept until the emergence of either parasitoid wasps or whiteflies. The newly emerged whiteflies were then collected for *Wolbachia* detection using *wsp* nested PCR. In each replication, five irradiated *En. formosa* were randomly selected for parasitization, with four replications performed in total. Nonirradiated wasps were used as controls.

Wolbachia transmission across generations in whiteflies

The parasitized and subsequently emerged whiteflies were denoted as the initial generation (G_0). To investigate the transmission rate of *Wolbachia* across whitefly generations, a pair of female and male newly emerged whitefly adults (G_0) were randomly chosen and introduced into a Petri dish containing a tomato leaf. After 5 d of oviposition, the whitefly adults (G_0) were removed and sampled for *Wolbachia* detection using *wsp* nested PCR. Only the eggs laid by *Wolbachia*-infected females were allowed to develop into adults. In a similar manner, upon the emergence of G_1 adults, whiteflies were paired and placed into individual Petri dishes to produce the G_2 generation and then sampled for *Wolbachia* detection. This procedure was repeated from the G_0 to G_5 generations. A parallel control experiment was conducted using *Wolbachia*-free whiteflies. Nine replications were conducted for each generation.

Effects of *Wolbachia* on whitefly fitness

Given the inability to obtain a whitefly strain with 100% *Wolbachia* infection, we selected individuals from the infected whitefly population, which initially acquired *Wolbachia* through parasitism of irradiated *En. formosa* and subsequently maintained for over five generations. Whitefly nymphs were individually isolated into PCR tubes at their 4^{th} instar stage. After emergence, female and male whitefly adults were paired and allowed to oviposit for five days within a Petri dish containing a tomato leaf.

These adults were then removed for *Wolbachia* detection via PCR. For the *Wolbachia*-infected female adults, we recorded several fitness parameters. These included the total number of eggs laid, the number of first instar nymphs, the developmental time from egg to eclosion, and the number of eclosed male and female offspring. This procedure was replicated with 60 pairs, and whiteflies from the original *Wolbachia*-free population were used as controls. Additionally, we

examined the effects of various paternal and maternal infection status combinations on whitefly fitness. A similar procedure was employed, with the exception that all whitefly adults were sourced from the infected population, and their infection status was determined through *wsp* nested PCR.

Statistics of the experimental data

Statistical analysis of experimental data was conducted using SPSS version 18.0 (SPSS Inc., Chicago, USA). The proportions were arcsine-square root transformed. Two independent samples were compared using Student's T test. One-way ANOVA was performed for analysis of multiple samples. Means were then compared using Honest Student's Tukey test. Data were visualized using GraphPad Prism 6 software (GraphPad Software, San Diego, USA).

Data and code availability

The alignment files, phylogenetic trees, and custom scripts can be accessed on FigShare (*https://doi.org/10.6084/m9.figshare.24718119*).

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Additional information

Author contributions

Y.X.L., X.Y.H., and Z.C.Y. conceptualized and designed the research; Z.C.Y. carried out the bioinformatics analysis; L.D.Q., H.L.J., and X.X.W. conducted the experiments; Y.X.L. and Z.C.Y. interpreted the results; Z.C.Y. and L.D.Q. prepared the visualization; Z.C.Y. wrote the initial manuscript; Y.X.L. and X.Y.H revised the manuscript.

Additional files

Supplementary information

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Reviewer #2 (Public review):

The resubmitted version of the paper by Yan et al. titled "Frequent intertrophic transmission of Wolbachia by parasitism but not predation" contains all the major flaws I found in the original submission. As far as I could see, the authors did not address my original concerns.

In short:

(1) A control of Portiera MUST be included in the FISH experiments, if the claim that Wolbachia is not only transferred from a parasitoid to the whitefly, but finds its way to the bacteriocytes. This is especially true for the Q, a biotype for which the pattern of Wolbachia distribution has been documented as scattered in naturally infected populations. The very strong signal in the whitefly bacteriocytes implies Portiera.

(2) In my original review I wrote: "The authors fail to discuss, or even acknowledge, a number of published studies that specifically show no horizontal transmission such as the one claimed to be detected in the study presented." In return the authors wrote in their rebuttal letter: "We have made corresponding modifications to the discussion section (Lines 256-271in the revised manuscript) and have discussed the published studies that report no evidence of horizontal transmission (Lines 260-263 in the revised manuscript)." However, the stated lines are concerned with a different subject. In addition, in their letter the authors write "Additionally, some experiments have found no evidence of horizontal transmission of Wolbachia (39- 42) (Lines 260-263 in the revised manuscript)." Beside the fact that the line numbers are wrong, the papers cited are entirely irrelevant as they do not discuss parasitoids.

(3) My original comment on the origin of sequences used for the phylogenetical analysis still stands. It is hard to claim a data-based search, when most of the data originate in the authors lab. The explanation of the confusion with the Qi et al. (2019) paper should at least be mentioned in the M&M. Apologies if it has been included and I missed it.

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Author response:

The following is the authors' response to the original reviews.

> ***Public Reviews:***
>
> ***Reviewer #1 (Public Review):***
>
> *Summary and Strengths:*
>
> *The ability of* Wolbachia *to be transmitted horizontally during parasitoid wasp infections is supported by phylogenetic data here and elsewhere. Experimental analyses have*

> *shown evidence of wasp-to-wasp transmission during coinfection (eg Huigins et al), host to wasp transmission (eg Heath et al), and mechanical ('dirty needle') transmission from host to host (Ahmed et al). To my knowledge this manuscript provides the first experimental evidence of wasp to host transmission. Given the strong phylogenetic pattern of host-parasitoid* Wolbachia *sharing, this may be of general importance in explaining the distribution of* Wolbachia *across arthropods. This is of interest as* Wolbachia *is extremely common in the natural world and influences many aspects of host biology.*
>
> *Weaknesses:*
>
> *The first observation of the manuscript is that the* Wolbachia *strains in hosts are more closely related to those in their parasitoids. This has been reported on multiple occasions before, dating back to the late 1990s. The introduction cites five such papers (the observation is made in other studies too that could be cited) but then dismisses them by stating "However, without quantitative tests, this observation could simply reflect a bias in research focus." As these studies include carefully collected datasets that were analysed appropriately, I felt this claim of novelty was rather strong. It is unclear why downloading every sequence in GenBank avoids any perceived biases, when presumably the authors are reanalysing the data in these papers.*

Thank you for bringing this to our attention. In this study, we downloaded all *wsp* sequences from GenBank and conducted a systematic analysis. We acknowledge that there could still be a bias in research focus, but a systematic analysis, compared to a limited dataset, may reduce this bias. We agree with the reviewer's point, and we have revised this statement to make it more accurate. Now the new sentence reads: "However, there is still a lack of systematic statistical analyses to support this hypothesis." (Lines 69–70 in the revised manuscript)

> *I do not doubt the observation that host-parasitoid pairs tend to share related* Wolbachia*, as it is corroborated by other studies, the effect size is large, and the case study of whitefly is clearcut. It is also novel to do this analysis on such a large dataset. However, the statistical analysis used is incorrect as the observations are pseudo-replicated due to phylogenetic non-independence. When analysing comparative data like this it is essential to correct for the confounding effects of related species tending to be similar due to common ancestry. In this case, it is well-known that this is an issue as it is a repeated observation that related hosts are infected by related* Wolbachia*. However, the authors treat every pairwise combination of species (nearly a million pairs) as an independent observation. Addressing this issue is made more complex because there are both the host and symbiont trees to consider. The additional analysis in lines 123-124 (including shuffling species pairs) does not explicitly address this issue.*

We agree with your point about the non-independence of data due to phylogenetic relationships. In the analysis of species traits, a conventional phylogenetic correction assumes that traits follow a Brownian motion model (Felsenstein, 1985). The variance of the trait values for a species i is given by:

$Var[Yi]=\sigma 2Ti$,

Where Ti represents the time from the root to the tip for species i. Consequently, the covariance between traits of species i and j is:

$Cov[Yij,Yj]=\sigma^2 Tii$,

where Tij is the time from the root to the most recent common ancestor (MRCA) of species i and j. Linear model analysis incorporates the covariance matrix to correct for the effects of

non-independence. Mathematically, this method is equivalent to the independent contrasts approach (Felsenstein, 1985).

In our analysis, we treat the minimum interspecific *wsp* distance between two species as a trait for the species pair (*i*, *j*). Similarly, for any two pairs of species (*i*, *j*) and (*k*, *l*), we postulate that the covariance between their traits is given by:

$Cov[Y_{ij},Y_{kl}]=\sigma 2\cdot(T_{ik}+T_{jl})$,

where *Tik* denotes the time from the root to the MRCA of species *i* and *k*, and *Tjl* represents the time from the root to the MRCA of species *j* and *l*. This covariance matrix is then incorporated into our linear model analysis to account for the effects of phylogenetic non-independence.

However, when extending trait analysis to pairs of species, the computational demands increase substantially. For instance, with a dataset of 1,377 species, forming all possible pairs yields 947,376 unique species combinations. Consequently, constructing a covariance matrix for these pairs would necessitate storing 897,521,285,376 entries, a requirement that far exceeds the memory capabilities of standard computing systems.

To address this, we randomly sampled 1,000 pairs from the total of 947,376 species pairs within the 'Others' category, thereby reducing the computational load without compromising the representativeness of our analysis. Ultimately, even after accounting for phylogenetic correction using covariance, the effect of parasitism remains highly significant ($p < 0.0001$).

We have added a “Phylogenetic correction” section to Materials and Methods (Lines 392–405 in the revised manuscript). The corresponding results are described on lines 120–121 and in supplementary Note 1. The data and scripts for this analysis are available at https://doi.org/10.6084/m9.figshare.24718119.

REFERENCE

Felsenstein J, 1985. Phylogenies and the comparative method. The American Naturalist, 125(1), 1-15.

> *The sharing of* Wolbachia *between whitefly and their parasitoids is very striking, although this has been reported before (eg the authors recently published a paper entitled "Diversity and Phylogenetic Analyses Reveal Horizontal Transmission of Endosymbionts Between Whiteflies and Their Parasitoids"). In Lines 154-164 it is suggested that from the tree the direction of transfer between host and parasitoid can be inferred from the data. This is not obvious to me given the poor resolution of the tree due to low sequence divergence. There are established statistical approaches to test the direction of trait changes on a tree that could have been used (a common approach is to use the software BEAST).*

We thank the reviewer for this constructive feedback on our interpretation of *Wolbachia* transfer between whiteflies and their parasitoids. Inspired by the reviewer's comments, we have now incorporated a trait-based approach, using the taxonomic order of the source species of the *wsp* gene as a discrete trait for ancestral state reconstruction on the *wsp* tree. The estimated ancestral trait state for one clade, which clusters *wsp* sequences from whiteflies and parasitoids, is Hymenoptera, suggesting that within this clade, the direction of *Wolbachia* transfer may have been from parasitoids to hosts. Conversely, in another clade characterized by the ancestral trait state of Hemiptera, the inferred direction of transfer appears to be from hosts to parasitoids. We have added a “Ancestral state reconstruction” section to Materials and Methods (Lines 406–412 in the revised manuscript). The corresponding results are described on lines 159–163 and 167–168. The data and script for this analysis is available at https://doi.org/10.6084/m9.figshare.24718119.

> ***Reviewer #2 (Public Review):***
>
> *The paper by Yan et al. aims to provide evidence for horizontal transmission of the intracellular bacterial symbiont* Wolbachia *from parasitoid wasps to their whitefly hosts. In my opinion, the paper in its current form consists of major flaws.*
>
> *Weaknesses:*
>
> *The dogma in the field is that although horizontal transmission events of* Wolbachia *occur, in most systems they are so rare that the chances of observing them in the lab are very slim.*
>
> *For the idea of bacteria moving from a parasitoid to its host, the authors have rightfully cited the paper by Hughes, et al. (2001), which presents the main arguments against the possibility of documenting such transmissions. Thus, if the authors want to provide data that contradict the large volume of evidence showing the opposite, they should present a very strong case.*
>
> *In my opinion, the paper fails to provide such concrete evidence. Moreover, it seems the work presented does not meet the basic scientific standards.*

We are grateful for your critical perspective on our work. Nonetheless, we are confident in the credibility of our findings regarding the horizontal transmission of *Wolbachia* from *En. formosa* to *B. tabaci*. Our study has documented this phenomenon through phylogenetic tree analyses, and we have further substantiated our observations with rigorous experiments in both cages and petri dishes. The horizontal transfer of *Wolbachia* was confirmed via PCR, with the *wsp* sequences in *B. tabaci* showing complete concordance with those in *En. formosa*. Additionally, we utilized FISH, vertical transmission experiments, and phenotypic assays to demonstrate that the transferred *Wolbachia* could be vertically transmitted and induce significant fitness cost in *B. tabaci*. All experiments were conducted with strict negative controls and a sufficient number of replicates to ensure reliability, thereby meeting basic scientific standards. The collective evidence we present points to a definitive case of *Wolbachia* transmission from the parasitoid *En. formosa* to the whitefly *B. tabaci*.

> *My main reservations are:*
>
> *- I think the distribution pattern of bacteria stained by the probes in the FISH pictures presented in Figure 4 looks very much like* Portiera*, the primary symbiont found in the bacterium of all whitefly species. In order to make a strong case, the authors need to include* Portiera *probes along with the* Wolbachia *ones.*

We thank you for your critical evaluation regarding the specificity of FISH in our study. We assure the reliability of our FISH results based on several reasons.

(1) We implemented rigorous negative controls which exhibited no detectable signal, thereby affirming the specificity of our hybridization. (2) The central region of the whitefly nymphs is a typical oviposition site for *En. formosa*. Post-parasitism, we observed FISH signals around the introduced parasitoid eggs, distinct from bacteriocyte cells which are rich in endosymbionts including *Portiera* (Fig 3e-f). This observation supports the high specificity of our FISH method. (3) In the G3 whiteflies, we detected the presence of *Wolbachia* in bacteriocytes in nymphs and at the posterior end of eggs in adult females (Fig. 4). This distribution pattern aligns with previously reported localizations of *Wolbachia* in *B. tabaci* (Shi et al., 2016; Skaljac et al., 2013). Furthermore, the distribution of *Wolbachia* in the whiteflies does indeed exhibit some overlap with that of *Portiera* (Skaljac et al., 2013; Bing et al., 2014). 4) The primers used in our FISH assays have been widely cited (Heddi et al., 1999)

and validated in studies on *B. tabaci* and other systems (Guo et al., 2018; Hegde et al., 2024; Krafsur et al., 2020; Rasgon et al., 2006; Uribe-Alvarez et al., 2019; Zhao et al., 2013).

Taking all these points into consideration, we stand by the reliability of our FISH results.

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Zhao DX, Zhang XF, Chen DS, Zhang YK, Hong XY, 2013. *Wolbachia*-host interactions: Host mating patterns affect *Wolbachia* density dynamics. PLoS One, 8: e66373.

> *- If I understand the methods correctly, the phylogeny presented in Figure 2a is supposed to be based on a wide search for* Wolbachia wsp *gene done on the NCBI dataset (p. 348). However, when I checked the origin of some of the sequences used in the tree to show the similarity of* Wolbachia *between* Bemisia tabaci *and its parasitoids, I found that most of them were deposited by the authors themselves in the course of the current study (I could not find this mentioned in the text), or originated in a couple of papers that in my opinion should not have been published to begin with.*

We appreciate your meticulous examination of the sources for our sequence data. All the sequences included in our phylogenetic analysis were indeed downloaded from the NCBI database as of July 2023. The sequences used to illustrate the similarity of *Wolbachia* between *B. tabaci* and its parasitoids include those from our previously published study (Qi et al., 2019), which were sequenced from field samples. Additionally, some sequences were also obtained from other laboratories (Ahmed et al., 2009; Baldo et al., 2006; Van Meer et al., 1999). We acknowledge that in our prior research (Qi et al., 2019), the sequences were directly

submitted to NCBI and, regrettably, we did not update the corresponding publication information after the article were published. It is not uncommon for sequences on NCBI, with some never being followed by a published paper (e.g., FJ710487- FJ710511 and JF426137-JF426149), or not having their associated publication details updated post-publication (for instance, sequences MH918776-MH918794 from Qi et al., 2019, and KF017873-KF017878 from Fattah-Hosseini et al., 2018). We recognize that this practice can lead to confusion and apologize for the oversight in our work.

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Qi LD, Sun JT, Hong XY, Li YX, 2019. Diversity and phylogenetic analyses reveal horizontal transmission of endosymbionts between whiteflies and their parasitoids. J Econ Entomol, 112(2):894-905.

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> *- The authors fail to discuss or even acknowledge a number of published studies that specifically show no horizontal transmission, such as the one claimed to be detected in the study presented.*

Thank you for bringing this to our attention. We have made corresponding modifications to the discussion section (Lines 256–271 in the revised manuscript) and have discussed the published studies that report no evidence of horizontal transmission (Lines 260–263 in the revised manuscript). The added sentences read: “Experimental confirmations of *Wolbachia* horizontal transfer remain relatively rare, with only a limited number of documented cases (24, 27, 37, 38). Additionally, some experiments have found no evidence of horizontal transmission of *Wolbachia* (39-42).” (Lines 260–263 in the revised manuscript)

> ***Reviewer #3 (Public Review):***
>
> *This is a very ordinary research paper. The horizontal of endosymbionts, including* Wolbachia, Rickettsia *etc. has been reported in detail in the last 10 years, and parasitoid vectored as well as plant vectored horizontal transmission is the mainstream of research. For example, Ahmed et al. 2013 PLoS One, 2015 PLoS Pathogens, Chiel et al. 2014 Enviromental Entomology, Ahmed et al. 2016 BMC Evolution Biology, Qi et al. 2019 JEE, Liu et al. 2023 Frontiers in Cellular and Infection Microbiology, all of these reported the parasitoid vectored horizontal transmission of endosymbiont. While Caspi-Fluger et al. 2012 Proc Roy Soc B, Chrostek et al. 2017 Frontiers in Microbiology, Li et al. 2017 ISME Journal, Li et al. 2017 FEMS, Shi et al. 2024 mBio, all of these reported the plant vectored horizontal transmission of endosymbiont. For the effects of endosymbiont on the biology of the host, Ahmed et al. 2015 PLoS Pathogens explained the effects in detail.*

Thank you for the insightful comments and for highlighting the relevant literature in the field of horizontal transmission of endosymbionts, including *Wolbachia* and *Rickettsia*. After

careful consideration of the studies mentioned in the commences, we believe that our work presents significant novel contributions to the field. 1) Regarding the parasitoid-mediated horizontal transmission of *Wolbachia*, most of the cited articles, such as Ahmed et al. 2013 in PLoS One and Ahmed et al. 2016 in BMC Evolutionary Biology, propose hypotheses but do not provide definitive evidence. The transmission of *Wolbachia* within the whitefly cryptic species complex (Ahmed et al. 2013) or between moths and butterflies (Ahmed et al. 2016) could be mediated by parasitoids, plants, or other unknown pathways. 2) Chiel et al. 2014 in Environmental Entomology reported "no evidence for horizontal transmission of *Wolbachia* between and within trophic levels" in their study system. 3) The literature you mentioned about *Rickettsia*, rather than *Wolbachia*, indirectly reflects the relative scarcity of evidence for *Wolbachia* horizontal transmission. For example, the evidence for plant-mediated transmission of *Wolbachia* remains isolated, with Li et al. 2017 in the ISME Journal being one of the few reports supporting this mode of transmission. 4) While the effects of endosymbionts on their hosts are not the central focus of our study, the effects of transgenerational *Wolbachia* on whiteflies are primarily demonstrated to confirm the infection of *Wolbachia* into whiteflies. Furthermore, the effects we report of *Wolbachia* on whiteflies are notably different from those reported by Ahmed et al. 2015 in PLoS Pathogens, likely due to different whitefly species and *Wolbachia* strains. 6) More importantly, our study reveals a mechanism of parasitoid-mediated horizontal transmission of *Wolbachia* that is distinct from the mechanical transmission suggested by Ahmed et al. 2015 in PLoS Pathogens. Their study implies transmission primarily through dirty needle, without *Wolbachia* infection of the parasitoid, suggesting host-to-host transmission at the same trophic level, where parasitoids serve as phoretic vectors. In contrast, our findings demonstrate transmission from parasitoids to hosts through unsuccessful parasitism, which represents cross-trophic level transmission. To our knowledge, this is the first experimental evidence that *Wolbachia* can be transmitted from parasitoids to hosts. We believe these clarifications and the novel insights provided by our research contribute valuable knowledge to the field.

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Liu Y, He ZQ, Wen Q, Peng J, Zhou YT, Mandour N, McKenzie CL, Ahmed MZ, Qiu BL, 2023. Parasitoid-mediated horizontal transmission of *Rickettsia* between whiteflies. Front Cell Infect Microbiol, 12:1077494.

Qi LD, Sun JT, Hong XY, Li YX, 2019. Diversity and phylogenetic analyses reveal horizontal transmission of endosymbionts between whiteflies and their parasitoids. J Econ Entomol, 112(2):894-905.

Shi PQ, Wang L, Chen XY, Wang K, Wu QJ, Turlings TCJ, Zhang PJ, Qiu BL, 2024. *Rickettsia* transmission from whitefly to plants benefits herbivore insects but is detrimental to fungal and viral pathogens. mBio, 15(3):e0244823.

> *Weaknesses:*
>
> *In the current study, the authors downloaded the MLST or* wsp *genes from a public database and analyzed the data using other methods, and I think the authors may not be familiar with the research progress in the field of insect symbiont transmission, and the current stage of this manuscript lacking sufficient novelty.*

We appreciate your critical perspective on our study. However, we respectfully disagree with the viewpoint that our manuscript lacks sufficient novelty.

> ***Recommendations for the authors:***
>
> ***Reviewer #1 (Recommendations For The Authors):***
>
> *The data and scripts from the experimental section of the paper are not made publicly available. This would be good practice. It may well be a requirement for this journal too, but I have not read the journal policy on this matter.*

Thank you for the kind reminder, we have uploaded the data and scripts to the public database at https://doi.org/10.6084/m9.figshare.24718119.

> *• Line 16 should read 'intertrophic' not 'intertropical'.*

Corrected.

> *• Line 50 should not say 'the most infectious' as this is an incorrect use of the word 'infectious'. Maybe 'common'? Should also add something like 'likely' here.*

Corrected. The new sentence reads “Together, these characteristics make *Wolbachia* likely the most common microbe on Earth in terms of the number of species it infects (7, 8).” (Lines 47–49 in the revised manuscript).

> *• Line 54 These references are all about mosquito disease vectors, not pests. More generally, in this paragraph, the research interest in* Wolbachia *relates overwhelmingly to blocking arbovirus transmission and not controlling pest populations.*

To enhance consistency with our statements, we have revised the supporting references as follows:

X. Zheng et al., "Combined incompatible and sterile insect techniques eliminate mosquitoes," Nature 572, 56-61 (2019).

A. A. Hoffmann et al., "*Wolbachia* establishment in *Aedes* populations to suppress dengue transmission," Nature 476, 454-457 (2011).

J. T. Gong, T. P. Li, M. K. Wang, X. Y. Hong, "Prospects of *Wolbachia* in agricultural Pest Control," Current Opinion in Insect Science 57, 101039 (2023).J. T. Gong et al., "Stable integration of plant-virus-inhibiting *Wolbachia* into planthoppers for rice protection," Current Biology 30, 4837-4845.e4835 (2020).

Regarding the content of the articles:

Zheng et al. (2019) detail the successful suppression of wild mosquito populations through the release of male mosquitoes artificially infected with *Wolbachia*.

Gong et al. (2020) present the potential of releasing *Wolbachia*-infected brown planthoppers to inhibit plant viruses and control pest populations.

Gong et al. (2023) provide a comprehensive review on the application and future of *Wolbachia* in managing agricultural pests.

> *• Line 60-61. This sentence seems poorly supported by theory or data. I suggest it is deleted. Why should CI cause extinction, and why would it have a major effect on genetic diversity beyond mtDNA?*

We have deleted the statements about extinction or genetic diversity. Now the sentence reads “It may also spread to nontarget organisms, potentially disrupting their population dynamics.” (Lines 57–58 in the revised manuscript)

> *• Line 66. Reword to make clear these routes are not an exhaustive list.*

We have reworded these sentences. The new sentences now read “Similar to other symbionts, *Wolbachia* host shifts may occur through three main routes: parasitism, predation, and shared plant or other food sources (*17*). However, it is important to note that these are not the only routes through which transmission may occur, and the specific contributions of each to the overall process of host shift are not yet fully understood.” (Lines 62–66 in the revised manuscript).

> *• Line 77-79. This could do with mentioning studies of parasitoid-to-host transmission like Ahmedd et al given that it is common knowledge that insects commonly survive parasitoid attacks.*

We have added sentences acknowledging the common occurrence of insects surviving parasitoid attacks and referenced and described the Ahmed et al. 2015 study. The added sentences read:

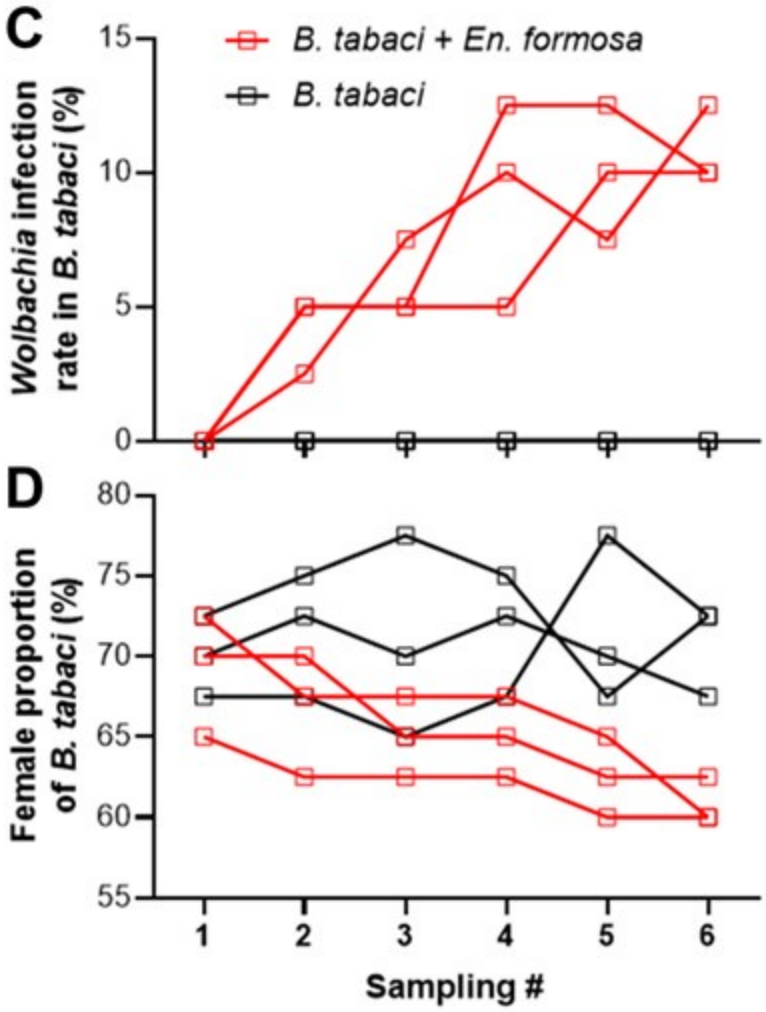
“However, it is common in nature for hosts to survive parasitoid attacks (27-29). For example, whiteflies can survive after attacks of *Eretmocerus* parasitoids (27). These parasitoids can act as phoretic vectors, facilitating the spread of *Wolbachia* within whitefly populations through the contamination of their mouthparts and ovipositors with *Wolbachia* during the probing process (27).” (Lines 77–82 in the revised manuscript).

> *• Line 173. Mention that there are three replicates of each cage. In Figures 2C and D, it is better to show each replicate as a separate line to see how consistent they are.*

In accordance with the reviewer's suggestion, we have included a statement highlighting the replication of our experiments: “Notably, each cage setup was replicated three times to ensure experimental rigor.” (Lines 179–180 in the revised manuscript).

Regarding Figures 2C and D, we have revised the figures to display each replicate as a separate line, as suggested. However, we have encountered a visual clutter that may detract from the clarity of the figures. Additionally, in Figure C, the three black lines, all representing zero values, do not allow for the distinction of individual trends. Therefore, we recommend retaining the original figure format. In accordance with eLife's data policy, we have also provided the source data for all figures, ensuring that readers can access to the detailed data, thus balancing the need for visual simplicity with the provision of comprehensive data.

Author response image 1.



> • *The GloBI database is central to the phylogenetic analysis and it would be helpful to have a few words in the results stating where this information comes from.*

The revised sentence now reads: “To investigate potential horizontal transmission of *Wolbachia*, we retrieved 4685 *wsp* sequences from the NCBI database, and species interaction

relationships were extracted from the GloBI database (for details, see Methods and Materials).” (Lines 94–96 in the revised manuscript).

> ***Reviewer #3 (Recommendations For The Authors):***
>
> *To improve the quality of this manuscript, I have some questions and suggestions.*
>
> *Introduction:*
>
> *Line 41-42, I don't agree with this statement, as mentioned above, the ways of insect symbiont transmission have been studied in the last 10 years.*

According to the reviewer’s suggestion, we have deleted this statement.

> *Line 75-76, Again, the statement is not correct, many studies have clearly revealed and confirmed that* Wolbachia *CAN be transferred from parasitoid to their insect hosts including whitefly* Bemisia tabaci*.*

Thank you for your insightful comments. After careful consideration of the studies you have mentioned above, none of these articles provided definitive evidence supporting the transfer of *Wolbachia* from parasitoids to their insect hosts. A closely related study is Ahmed et al. (2015) in PLoS Pathogens. This article demonstrates that parasitoid wasps can act as phoretic vectors mediating the transmission of *Wolbachia* between whiteflies. However, *Wolbachia* did not infect the parasitoid wasps themselves. Therefore, this study does not provide evidence for intertrophic transmission of *Wolbachia* from parasitoids to their hosts. To avoid confusion, we have cited the Ahmed et al. (2015) reference following this statement and described its findings accordingly. (Lines 88-92 in revised manuscript).

> *Results:*
>
> *Line 133-134, Ahmed et al. 2016 BMC Evolution Biology, clearly revealed and confirmed the "common horizontal transmission of* Wolbachia *between butterflies and moths".*

We thank you for guiding us to the relevant study. Ahmed et al. 2016 BMC Evolution Biology suggested common horizontal transmission of *Wolbachia* between butterflies and moths and proposed that this horizontal transmission might be caused by parasitoid wasps. Here, we present the potential *Wolbachia* transfer between *Trichogramma* and their lepidopteran hosts (Lines 135–136 in revised manuscript). Integrating the results from Ahmed et al. 2016, our result also suggests that *Trichogramma* wasps may be the vectors for horizontal transmission of *Wolbachia* among lepidopteran hosts. We have discussed this point in the discussion section and cited Ahmed et al. 2016 BMC Evolution Biology (Lines 239–246 in revised manuscript).

> *Line 176-177, as we know* Wolbachia *in* Encarsia formosa *is a strain of parthenogenesis, why did it reduce the female ratio of whitefly progeny after it was transmitted to whitefly* B. tabaci*, it needs a convincing explanation.*

Wolbachia induces parthenogenesis in *En. formosa*. However, we observed that *Wolbachia* from *En. formosa* failed to induce parthenogenesis in *B. tabaci*, possibly due to the requirement for host gene compatibility. Additionally, we noted a reduced female ratio in *B. tabaci* infected with *En. formosa Wolbachia*. We speculate that this might result from the burden imposed by *En. formosa Wolbachia* on the new host, potentially reducing fertilization success rates and indirectly leading to a decrease in the female ratio. Similarly, we observed a decline in female fecundity, egg hatching rate, and immature survival rate in *B. tabaci*

infected with *En. formosa Wolbachia*. We speculate that this might result from the burden imposed by *En. formosaWolbachia* on the new host, potentially reducing fertilization success rates and indirectly leading to a decrease in the female ratio. Similarly, we observed a decline in female fecundity, egg hatching rate, and immature survival rate in *B. tabaci* infected with *En. formosaWolbachia*. The mechanisms underlying these fitness costs remain unclear and warrant further in-depth research.

> *Line 189-190, do the authors have convincing evidence that the 60Gy irradiation only has effects on the reproduction of* En. formosa*, but does not have any negative effects on the activity of* Wolbachia*? I think there may be.*

We observed that after irradiation, the titer of *Wolbachia* within *En. formosa* significantly decreased (Fig S3). We agree that the irradiation may cause other negative effects on *Wolbachia* which is worth of close investigation. However, even with a significant reduction in *Wolbachia* titer, irradiation increased the infection rate of *Wolbachia* in surviving *B. tabaci* after wasp attacks (Fig 3C). We speculate that this may be due to irradiation of *En. formosa* increasing the rate of parasitic failure. While the full extent of the effects of irradiation on *Wolbachia* is not yet clear in our experiments, it does not alter our conclusion that *Wolbachia* can be transmitted from *En. formosa* to whitefly hosts through failed parasitism.

> *Discussion:*
>
> *Line 289-290, I don't understand, why the authors think from parasitoid* Eretmocerus *to whitefly, and from* Trichogramma *to moth, are the same trophic level, they are indeed two different trophic levels.*

Thank you for your feedback. We have conducted a thorough search but were unable to locate the specific statement you are referring to. If there has been any ambiguity in our manuscript that has led to confusion, we sincerely apologize for any misunderstanding it may have caused. We agree with your perspective and have always considered the parasitoid *Eretmocerus* and whitefly, as well as *Trichogramma* and moth, to be at different trophic levels. However, in the context of specific references, such as Ahmed et al. 2015 in PLoS Pathogens, we believe that *Wolbachia* is transmitted within the same trophic level without infecting the parasitoid *Eretmocerus*, merely serving as a phoretic vector to facilitate the spread of *Wolbachia* among whitefly hosts. Similarly, in the case of Huigens et al. 2000 in Nature, *Wolbachia* uses lepidopteran hosts as vectors to promote its transmission among *Trichogramma* without the need to infect the lepidopteran hosts themselves.

> *Materials and Methods*
>
> *Line 348, what is tblastn?*

We have corrected tblastn to TBLASTN. We are grateful to the reviewer for pointing this out. Here, we utilized TBLASTN instead of BLASTN, to avoid missing the rapidly evolving *wsp* sequences. Because alignment at the protein level is generally more sensitive than at the nucleotide level. TBLASTN is a bioinformatics tool within the BLAST (Basic Local Alignment Search Tool) suite used for comparing a protein query sequence against a nucleotide database. Specifically, TBLASTN aligns a given protein sequence with nucleotide sequences in a database by translating the nucleotide sequences into all possible protein sequences (considering different reading frames) and comparing them to the query protein sequence.

> *Line 383, how was the* Wolbachia*-free line of* B. tabaci *established, by antibiotics? If so, how do we ensure the antibiotic does not have any negative to other symbionts in whitefly* B. tabaci*?*

The *Wolbachia*-free line of *B. tabaci* was collected from field, without the treatment of antibiotics. We have made revisions in the Materials and Methods section to clarify this, stating, "An iso-female line of *B. tabaci*, which is naturally *Wolbachia*-free and has not been treated with antibiotics, was established." (Lines 417–418 in the revised manuscript)

> *Line 419-421 as I mentioned before, the irradiation may have negative effects on* Wolbachia *too, so change the biology of both* Encarsia *and whitefly host.*

We observed that after irradiation, the titer of *Wolbachia* within *En. formosa* significantly decreased (Fig S3). However, even with a significant reduction in *Wolbachia* titer, irradiation increased the infection rate of *Wolbachia* in surviving *B. tabaci* after wasp attacks (Fig 3C). We speculate that this may be due to irradiation of *En. formosa* increasing the rate of parasitic failure. While the full extent of the effects of irradiation on *Wolbachia* is not yet clear in our experiments, it does not alter our conclusion that *Wolbachia* can be transmitted from *En. formosa* to whitefly hosts through failed parasitism.

> *Line 452-453, From egg to eclosion, it needs about 21 days to understand suitable temperature and other conditions, during this period, the egg and nymphs can not move, so how to keep the cut-leaf fresh enough in a Petri dish for 21 days?*

We apologize for not clearly describing the materials and methods. By using wet cotton to wrap the end of petiole of the leaf, we can keep the leaves fresh for up to a month. We have included this detail in the materials and methods to enhance the reproducibility of the experiment. “A single irradiated wasp was subsequently introduced into a Petri dish, which contained a tomato leaf infested with *Wolbachia*-free third or fourth instar whitefly nymphs, and wet cotton was used to wrap the end of the leaf petiole to keep the leaf fresh.” (Lines 455–458 in the revised manuscript)

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