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ARTICLE

Distinct effects of three *Wolbachia* strains on fitness and immune traits in *Homona magnanima*

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The endosymbiotic bacterium *Wolbachia* occasionally increases host fitness or manipulates host reproductions to enhance vertical transmission. Multiple *Wolbachia* strains can coinfect the same host individual, which alters the density as well as phenotypes of the bacteria. However, the effects of *Wolbachia* coinfection on host fitness remain largely unknown. Here, we examined the effects of three phylogenetically distinct *Wolbachia* strains, *w*Hm-a, *w*Hm-b, and *w*Hm-c, on host fitness by comparing non-infected, singly infected, and triply infected *Homona magnanima* lines within a fixed genetic background. By examining the effects of *Wolbachia* on host longevity, survivorship, and reproduction, we demonstrated that single infection with either *w*Hm-b or *w*Hm-c reduced host reproduction, but the triple infection led to the highest intrinsic growth rate. Susceptibility to the natural pathogens such as viruses and fungi was not different among the lines regardless of *Wolbachia* infection status. Cellular and humoral immunities were not affected by *Wolbachia* in females, whereas phenoloxidase activity was suppressed in males of all *Wolbachia*-infected lines, implying that it was a result of the mother's curse hypothesis or a strategy of *Wolbachia* to increase their horizontal transmission efficiency. Although how the host's genetic diversity affects the *Wolbachia* fitness effects is yet unknown, our findings indicated that the effects of *Wolbachia* are deeply influenced by infection status and that *Wolbachia* could change symbiotic strategy depending on host sex and transmission route.

Heredity (2023) 130:22–29; https://doi.org/10.1038/s41437-022-00574-6

INTRODUCTION

Wolbachia, an Alphaproteobacteria, is the most widespread endosymbiotic bacterium in arthropods and reside in 40–60% insect species (Duron et al. 2008; Werren et al. 2008; Zug and Hammerstein 2012). Since it localises in the host cytoplasm and is mostly transmitted vertically from the mother to offspring, *Wolbachia* is considered to have developed strategies to compensate host fitness and to manipulate host reproduction (Werren et al. 2008).

Wolbachia confers fitness advantages to its host by establishing mutualistic relationship. In *Bemisia tabaci*, *Wolbachia* shortens host developmental duration and alters larval survival (Xue et al. 2012). In the case of *Drosophia suzukii*, *Wolbachia* infection increases the number of eggs in the female hosts (Mazzetto et al. 2015). These characteristics are related to the intrinsic growth rate (*r*), a population dynamics measure, of hosts (Birch 1948). Furthermore, *Wolbachia* enhances host resistance to natural pathogens; in the case of *D. melanogaster*, *Wolbachia* helps induce resistance to RNA viruses (Teixeira et al. 2008). Certain *Wolbachia* strains reduce susceptibility to RNA viruses in dipteran insect hosts (Moreira et al. 2009; Stevanovic et al. 2015). *Wolbachia* also enhances humoral immune responses, such as phenoloxidase (PO) activity, and stimulates the expression of genes related to early immunity in dipteran insects (Thomas et al. 2011; Rancès et al. 2012).

Conversely, association with *Wolbachia* can sometimes incur costs for hosts. Some *Wolbachia* strains negatively affect host fitness by shortening adult longevity and reducing female host fecundity (Hoffmann et al. 1990; Fleury et al. 2000). However, it is considered that parasitic *Wolbachia* has evolved to form more mutualistic associations with their hosts (Weeks et al. 2007). Other costly traits are reproductive manipulations, such as male killing (a son killing phenomenon) and cytoplasmic incompatibility (CI), whereby a cross between males infected with *Wolbachia* and females uninfected or infected with different *Wolbachia* strain produces no or few offspring (Werren et al. 2008). Such reproductive manipulations enhance the prevalence of *Wolbachia* in host populations but could be costly, especially for male hosts, resulting in the development of resistance against the male-killing phenotype of *Wolbachia* (Hornett et al. 2006).

Furthermore, *Wolbachia* interacts with other *Wolbachia* strains or microbes within a single host individual, which affects the intensity of reproductive manipulations as well as population dynamics of *Wolbachia*, resulting in the evolution of host–symbiont interactions (Ikeda et al. 2003; Dobson et al. 2004; Kondo et al. 2005; Dean 2006; Mouton et al. 2006; Narita et al. 2007; Watanabe et al. 2011; Lu et al. 2012). Therefore, the host–bacteria and bacteria-bacteria interactions generate new outcomes and are important for the biology of *Wolbachia*.

Homona magnanima (Tortricidae, Lepidoptera), a serious tea pest, harbours three *Wolbachia* strains, *w*Hm-a, *w*Hm-b, and *w*Hm-c, that influence host fitness and reproductions: *w*Hm-a does not induce any reproductive phenotype, *w*Hm-b causes CI, and *w*Hm-c shortens the larval development time and increases pupal weight in female hosts (Arai et al. 2019; Arai et al. 2020; Takamatsu et al. 2021). Theoretically, multiple *Wolbachia* infections can be

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Received: 3 August 2022 Revised: 26 October 2022 Accepted: 28 October 2022
Published online: 9 November 2022

maintained in a host population if the coinfected hosts have more fitness advantages than the singly infected or uninfected hosts (Frank 1998, Vautrin and Vavre 2009). In this study, we hypothesise that the *Wolbachia* triple infection is more advantageous to its host *H. magnanima* than a single infection or a non-infection. To test this hypothesis, we compared multiple life history parameters of isogenic *H. magnanima* lines with different *Wolbachia* infection status and determined the advantages and strategies of triple infection of *Wolbachia* for its successful transmission in *H. magnanima*.

MATERIALS AND METHODS

Insects

Five lines of *H. magnanima*, namely W^{abc}, W^{a}, W^{b}, W^{c}, and W^{-}, established in the laboratory as described by Arai et al. (2019), were used. The W^{abc} line, harbouring *w*Hm-a, *w*Hm-b, and *w*Hm-c strains, was collected from Akiruno city, Tokyo, Japan, in 1999. The W^{a}, W^{b}, and W^{c} lines, established from the W^{abc} line via antibiotic treatments, are singly infected with *w*Hm-a, *w*Hm-b, and *w*Hm-c, respectively. The W^{-} line, free of any *Wolbachia* strains, was generated by subjecting the W^{abc} line to antibiotic treatment. We used the W^{abc} line from 116–240 generations W^{a} from 60–84 generations, W^{b} and W^{c} from 24–48 generations, W^{-} from 96–120 generations for the experiments. All the larvae hatched from an egg mass were mass-reared as previously described in Arai et al. (2019), under laboratory conditions (16: 8 h light/dark cycle, 25 °C, and 60% relative humidity). We used males of the W^{-} line to mate with females of each host line to homogenise their genetic backgrounds. The rearing and mating treatments were followed in each generation.

Fitness traits of *H. magnanima*

To investigate the longevity and fecundity of female adults, egg hatchability, and egg development time, a virgin female within 24 h after emergence and two males of the same host line were mated in a 120 mL plastic cup for oviposition. The mating sets comprised 50, 46, 40, 35 and 35 cups for the W^{-}, W^{a}, W^{b}, W^{c} and W^{abc} lines, respectively. The areas of egg masses collected every day were determined, and the estimated total number of eggs per female oviposition was calculated from the total egg mass area, as described previously (Arai et al. 2019). Egg development time was defined as the number of days from oviposition to hatching. After hatching, pharate larvae in each egg mass were counted. The embryonic developmental ratio of eggs per female was calculated as the total number of pharate larvae divided by the estimated total number of eggs. The hatchability of pharate larvae was calculated as the number of hatched larvae divided by the total number of pharate larvae. The number of hatched larvae was determined as the difference between the total number of pharate larvae and unhatched pharate larvae. Survival of the female adults was recorded every day. After the death of female adults, they were dissected and spermatophore presence was checked to confirm mating. Non-mating females were excluded from the oviposition data. The longevity of the female adult was defined as the number of days from emergence till death. To determine the longevity of male adults, males within 24 h after emergence were individually reared in a plastic cup. Male adult longevity was defined as the number of days from emergence till death.

The pupation rates were recorded using 105 individuals (35 × 3 replicates) of each host line. The neonates hatched within 24 h were individually reared on an artificial diet INSECTA LF (Nosan Co., Ltd., Yokohama, Japan). The pupal duration and emergence rate were calculated using another 105 individuals (35 × 3 replicates) from each host line. The final instar larvae were individually reared using INSECTA LF until their eclosion. Pupal duration was recorded as the number of days from pupation to eclosion; the eclosion rate was calculated as total number of adults divided by the number of pupae.

The net reproduction rate (R_0), the mean generation time (T), and the intrinsic growth rate (r) in each host line were calculated as follows (Birch 1948).

$$T = \frac{\sum x l_x m_x}{\sum l_x m_x}$$

$$R_0 = \sum l_x m_x$$

$$r = \frac{\ln R_0}{T}$$

Table 1. Life history parameters of the five *Homona magnanima* lines, W^{-}, W^{a}, W^{b}, W^{c} and W^{abc}.

Line	Total number of eggs per female[d]	The total number of pharate larvae per female[d]	Embryonic developmental ratio per female (%)[d]	Hatchability per female (%)[d]	Pupation rate[d] (%)	Emergence rate (%)		Egg development time (days)[d]	Pupal duration (days)[d]		Adult longevity (days)[d]	
						Female[e]	Male[d]		Female	male	Female	Male
W^{-}	522.3 ± 216.3 (40)[a]	396.5 ± 272.4 (40)[ab]	62.8 ± 23.4 (40)[a]	84.6 ± 2.1 (39)[a]	91.4 ± 2.9[a]	86.7 ± 5.3[a]	79.1 ± 5.3[a]	7.0 ± 0.0 (89)	6.6 ± 0.1 (91)[a]	7.0 ± 0.1 (83)[a]	8.5 ± 0.3 (50)[a]	7.0 ± 0.6 (6)
W^{a}	592.6 ± 313.5 (36)[a]	338.1 ± 210.5 (36)[ab]	55.1 ± 29.7 (36)[a]	82.8 ± 2.4 (32)[a]	92.4 ± 2.5[a]	89.1 ± 6.0[a]	83.3 ± 2.3[a]	7.0 ± 0.0 (82)	7.0 ± 0.1 (84)[b]	7.5 ± 0.1 (77)[b]	8.1 ± 0.4 (46)[a]	6.6 ± 0.8 (10)
W^{b}	498.0 ± 246.2 (32)[a]	248.8 ± 163.3 (32)[b]	50.3 ± 24.3 (32)[a]	71.3 ± 3.4 (28)[b]	91.4 ± 0.0[a]	88.4 ± 4.9[a]	87.5 ± 0.9[a]	7.0 ± 0.0 (53)	7.0 ± 0.1 (91)[b]	7.6 ± 0.1 (91)[bc]	8.9 ± 0.4 (40)[a]	5.8 ± 0.6 (12)
W^{c}	455.0 ± 207.6 (29)[b]	251.3 ± 165.5 (29)[b]	52.1 ± 27.7 (29)[a]	84.1 ± 2.9 (26)[a]	94.3 ± 0.0[a]	86.4 ± 3.4[a]	82.8 ± 4.3[a]	7.0 ± 0.0 (16)	6.9 ± 0.1 (89)[b]	7.5 ± 0.1 (83)[b]	8.1 ± 0.4 (35)[a]	4.6 ± 0.6 (5)
W^{abc}	690.0 ± 343.2 (31)[a]	392.0 ± 126.1 (31)[a]	62.9 ± 19.8 (31)[a]	84.8 ± 1.0 (31)[a]	89.5 ± 4.8[a]	87.5 ± 0.9[a]	76.3 ± 8.1[a]	7.0 ± 0.0 (66)	6.9 ± 0.1 (91)[b]	7.9 ± 0.1 (68)[c]	9.1 ± 0.1 (35)[a]	7.3 ± 1.3 (4)

Different letters indicate significant differences between the lines ($p < 0.05$). Sample sizes are given in brackets. Data are presented as mean ± SE.
[d]Data were analysed using Steel-Dwass test.
[e]Data were analysed using Tukey-Kramer test.

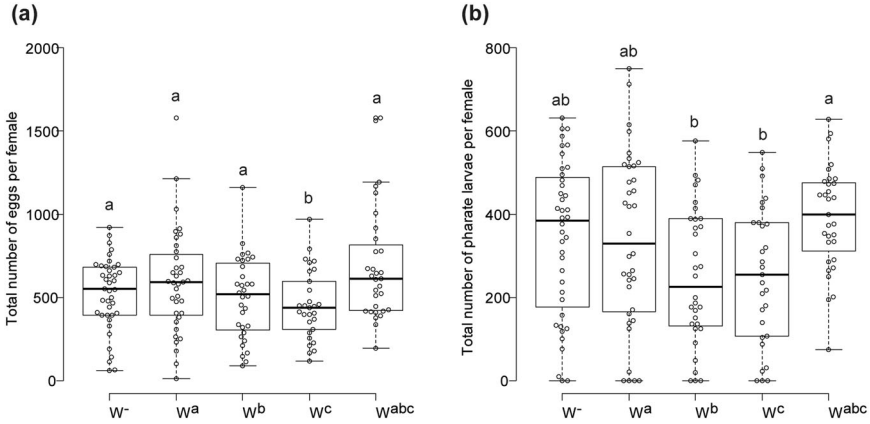

Fig. 1 Female fecundity of adults of the five *H. magnanima* lines. a Number of eggs per female and **b** the number of pharate larvae per female. Different letters indicate significant differences between lines ($p < 0.05$). In each box-and-whisker plot, the centre line indicates the median. The upper and lower boundaries of the box indicate the upper quartile and lower quartile, respectively. The points on plots indicate each sample.

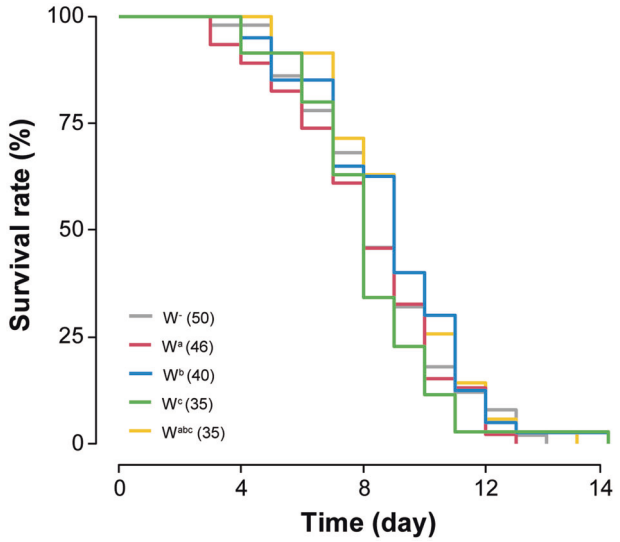

Fig. 2 Kaplan-Meier curves of *Homona magnanima* females after emergence.

Table 2. Fitness parameters of the five *Homona magnanima* lines, W^-, W^a, W^b, W^c and W^{abc}.

Line	Mean generation time (T)	Net reproduction rate (R_0)	Intrinsic growth rate (r)
W^-	38.7 ± 0.1^a	113.08 ± 8.38^{ac}	0.122 ± 0.002^a
W^a	39.5 ± 0.1^b	108.67 ± 10.25^{abc}	0.119 ± 0.002^a
W^b	40.6 ± 0.1^c	70.66 ± 7.74^b	0.105 ± 0.003^b
W^c	36.5 ± 0.1^d	83.98 ± 9.71^{ab}	0.122 ± 0.003^{ac}
W^{abc}	36.6 ± 0.1^d	131.32 ± 7.33^c	0.133 ± 0.002^c

Different letters indicate significant differences among the lines (Steel-Dwass test, $p < 0.05$). Data are presented as mean ± SE and estimated using Jackknife method.

where l_x is the survival rate of the female adult at x day old, and m_x is the mean oviposition number (the number of pharate larvae) per day. The sex ratio is assumed to be 1:1 (Arai et al. 2019). The value of x and l_x were defined as follows. Mean larval development times in each host line were cited from Arai et al. (2019).

x = (mean egg development time) + (mean larval development time) + (mean pupal duration) + (days after emergence).

l_x = 0.5 (sex ratio) × (mean hatchability) × (mean pupation rate) × (mean eclosion rate) × (survival rate of the female adult at x days old).

To perform statistical analysis, Jackknife estimate of r (r_j) was calculated as follows (Meyer et al. 1986):

$$r_j = n \times r_{all} - (n-1) \times r_i$$

where r_{all} is the intrinsic growth rate calculated from the mean oviposition number per day of all females ($m_{x,\ all}$), r_i is the intrinsic growth rate calculated from the mean oviposition number per day of all females, excluding the i number individual ($m_{x,\ i}$), and n is the number of all females. Jackknife estimates of R_0 and T were also calculated using a similar equation as used for the calculation of r_j (Maia et al. 2000).

Susceptibility of *H. magnanima* against viruses and fungi

The double-stranded DNA viruses *Adoxophyes honmai* entomopoxvirus (AHEV, Poxviridae) and *Homona magnanima* granulovirus (HomaGV, Baculoviridae) were isolated from diseased larvae collected from tea fields in Mizuho (Tokyo) and Kagoshima Prefecture Tea Experimental Station (Kagoshima), respectively. Occlusion bodies (OBs) of AHEV and HomaGV were purified as described previously (Takatsuka et al. 2010; Tsuruta et al. 2018). The concentration of AHEV OBs was determined via phase-contrast microscopy, using a Thoma hemocytometer, and that of Homa GV OBs was determined via Transmission electron microscopy using Latex beads (0.3 µm in diameter, Sigma Aldrich, St. Louis, MO). The purified OB suspensions of AHEV and HomaGV were adjusted to 2.0×10^7 OBs mL^{-1} and $2.0 \times 10^{6.5}$ OBs mL^{-1}, respectively, using sterilised distilled water. Neonates (within 24 h of hatching, three replicates with 5–35 individuals) were allowed to feed on droplets of a viral suspension containing 10% sucrose and 5% red dye, with 1.0×10^7 OBs mL^{-1} for AHEV and $1.0 \times 10^{6.5}$ OBs mL^{-1} for HomoaGV, as described previously (Ishii et al. 2003). The droplets without viral OBs served as controls. Larvae that ingested the suspension were individually reared on an artificial diet and observed every day until they pupated or died. The dead insects were checked for symptoms of lethality by viruses.

The insect pathogenic fungus *Beauveria bassiana* was isolated from BotaniGard wettable powder (Arysta Lifescience Corporation, Tokyo, Japan). BotaniGard wettable powder was diluted ten times with sterilised distilled water, plated on to the Sabouraud agar medium, and incubated under a 16 h: 8 h light/dark cycle at 25 °C until conidia formation. Fungal conidia were scraped and suspended in sterilised 0.02% Tween 80, followed by centrifugation at 3000 × g for 10 min. Pellets were suspended in 10 mL sterilised 0.02% Tween 80. The concentration of conidia was adjusted to 2.0×10^9 mL^{-1}. Fourth instar larvae (within 24 h of moulting, three replicates with 9–34 individuals) were soaked in the conidial suspension for 10 s, and sterilised 0.02% Tween 80 served as the control. After air-drying, the larvae were individually reared on an artificial diet and

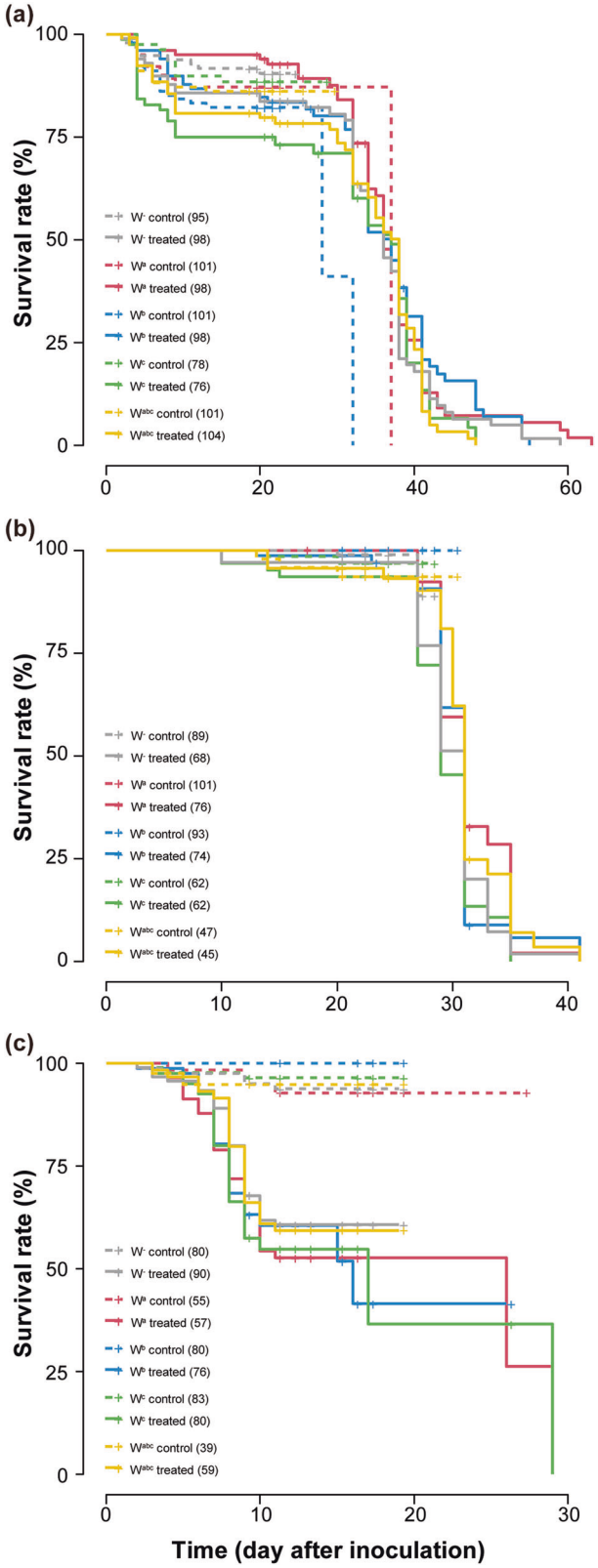

Fig. 3 Susceptibility of *Homona magnanima* in each line against natural pathogens. Kaplan-Meier curves of the larvae after each treatment in the inoculation tests of **a** AHEV and **b** HomaGV, and the fourth instar larvae for days post-inoculation of **c** *B. bassiana*.

observed every day for mortality until they pupated or died under conidial infestation.

Immune traits of *H. magnanima*

Haemolymph was withdrawn from the fifth instar larvae from each host line after 3 days of moulting using a thin needle. To evaluate total haemocyte count (THC), the haemolymph of each larva was immediately diluted five times with 1 × PBS (pH 7.0), and haemocyte number per μL of diluted haemolymph was estimated by counting haemocyte with a light microscope using a Thoma haemocytometer. The THC (cells μL^{-1}) of undiluted haemolymph was estimated as haemocyte number per μL of diluted haemolymph multiplied by the dilution rate.

To evaluate PO activity, the haemolymph of each larva was diluted ten times with 0.1 M potassium phosphate buffer (pH 6.0) and centrifuged at 9000 × *g* and 4 °C for 1 min. Then, 50 μL of the supernatant was dispensed into two wells of 96-well plate, and either 2 μL of ethanol or 5% phenylthiourea (PTU) in ethanol was added to the wells. Following incubation at room temperature (15–25 °C) for 15 min, 48 μL of 0.02 M L-dopa solution in 0.1 M potassium phosphate buffer was added to each well as the substrate. After incubation for 10 min at 30 °C, the absorbance at 490 nm was measured every 5 min for 2 h. PO activity was defined as the difference in absorbance between the two wells containing either ethanol or ethanol with PTU. The absorbance variation during the first 5 min was used for analysis because all samples showed a linear change for ~40 min.

Total PO activity was evaluated using the modified method of Bailey and Zuk (2008). After centrifuging the diluted haemolymph in PBS (pH 7.0), 50 μL each of the supernatants was dispensed into two wells of a 96-well plate containing 7 μL α- chymotrypsin solution (1.3 mg/mL DW, Sigma Aldrich C7762, St. Louis, Missouri, USA). After either ethanol or a 5% PTU and 0.02 M L-dopa solution in PBS was added, the absorbance was measured as mentioned above. Absorbance variation during the first 5 min was used for analysis because all samples showed a linear change by about 5 min. This experiment was done only for males because we could not get enough female larvae.

To examine the induced antibacterial activity of larval haemolymph, we conducted a growth inhibition assay. We injected 1 μL of a suspension of *Escherichia coli* (gram-negative) or *Micrococcus luteus* (gram-positive) (1.0×10^8 cells mL^{-1} in PBS) into the haemocoel of the fifth instar larvae from each host line (2 days after moulting), using a hand micro-applicator (Kiya Kogyo Seisakusho, Tokyo, Japan) fitted with a 50 μL micro-syringe (Ito Co., Shizuoka, Japan). After 24 h of individual rearing, haemolymph was collected on ice and centrifuged at 9000 × *g* and 4 °C for 1 min. Then, 5 μL of the supernatant was pipetted onto a 5 mm diameter filter paper, air-dried, and inoculated in to LB soft agar medium containing the same bacteria as mentioned above. After 24 h of incubation at 37 °C for *E. coli* or 30 °C for *M. luteus*, the area of growth inhibition zone was measured using Image J (https://imagej.nih.gov/ij/).

Statistical analysis

The survival data of *H. magnanima* larvae against viruses (AHEV and HomaGV) and fungi (*B. bassiana*), and adult longevity were analysed using the Kaplan-Meier survival estimates and pairwise log-rank tests between the curves, with *p*-values adjusted by Bonferroni using the "survival" package in R ver. 4.0.2 (R Core Team 2021). All data sets obtained in this study were tested for normality using Shapiro-Wilk test, and the data showing normal distributions (i.e., female emergence rate, female THC, and growth inhibition data) were analysed using a parametric Tukey-Kramer test. Other data sets (total number of eggs and pharate larvae per female, embryonic developmental ratio, hatchability, pupation rate, male emergence rate, pupal duration, adult longevity, *T*, R_0, *r*, male THC, PO activity and total PO activity) were analysed using a non-parametric Steel-Dwass test. The Shapiro-Wilk, Tukey-Kramer, and Steel-Dwass tests were performed using JMP9 (SAS Institute Inc., Cary, NC, USA).

RESULTS

Triple infection of *Wolbachia* resulted in high fitness advantages for *H. magnanima*

The W^{abc} line showed higher number of eggs per female individual than the W^c line ($p = 0.042$, Table 1, Fig. 1a) and a higher number of pharate larvae than the W^b and W^c lines

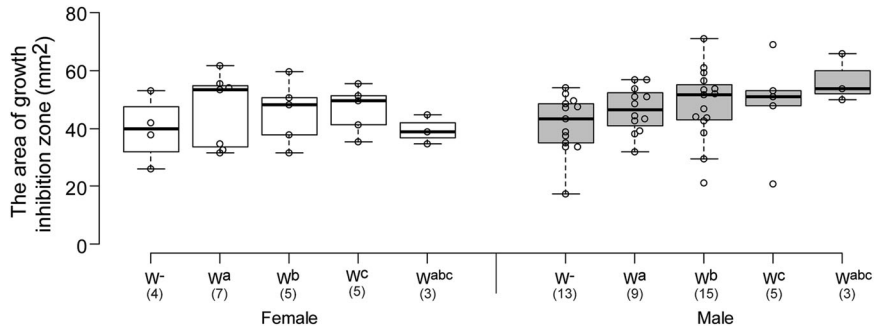

Fig. 4 Effects of *Wolbachia* infections on the induced antibacterial activity of host larvae. The area of growth inhibition zone of *E. coli* was measured using host larval haemolymph after 24 h of *E. coli* injection. The centre line and the upper and lower boundaries of the box indicate median, upper quartile, and lower quartile, respectively. The points on plots indicate each sample. The sample size is indicated in parentheses below host lines.

($p < 0.05$, Fig. 1b). Hatchability was lower in the W^b line than in other lines ($p < 0.05$, Table 1). Pupal duration was shorter in females of the W^- line than in those of the other lines and was longer in the males of the W^{abc} line than those of the W^-, W^a, and W^c lines ($p < 0.05$, Table 1). No significant difference was observed in the embryonic developmental ratio, egg development time, pupation rate, emergence rate, and female adult longevity between the host lines (Log-rank test, $p > 0.05$, Table 1, Fig. 2). Notably, no analysis was carried out on the data for adult male longevity because we could only observe very low number of males.

The intrinsic growth rate (r) was higher in the W^{abc} line than that in the singly infected or non-infected *H. magnanima* lines and the lowest in the W^b line harbouring only CI-inducing *w*Hm-b ($p < 0.01$, Table 2). For parameters relating to the r value, the net reproduction rate (R_0) was higher in the W^{abc} line than in the W^b and W^c lines ($p < 0.01$). Mean generation time (T) was shorter in the W^{abc} line than in the other lines ($p < 0.01$), except for the W^c line ($p > 0.05$), and longest in the W^b lines. All statistical data are shown in Tables S1 and S2.

Wolbachia did not protect hosts from natural pathogens but altered male immunity

Regardless of *Wolbachia* infection status, we observed no difference in larval susceptibility to AHEV, HomaGV, and *B. bassiana* among host lines (Log-rank test, $p > 0.05$, Fig. 3). Regarding immunity-related factors, the THC (cellular immunity) and induced antibacterial activity to *E. coli* of larval haemolymph (humoral immune response) were not different among host lines and sexes ($p > 0.05$, Fig. 4). Larval haemolymph of both sexes from each line ($n = 3$) did not inhibit the growth of *M. luteus*. No difference was observed in PO activity (humoral immune responses) among the female host lines ($p > 0.05$, Fig. 5a), while male larvae of all host lines harbouring *Wolbachia* showed lower PO activities than those in the W^- line (Fig. 5b). In contrast, there was no difference in total PO activity in the haemolymph of male hosts among the lines, regardless of *Wolbachia* infection ($p > 0.05$, Fig. 5c).

DISCUSSION

In the present study, we demonstrated that females of the triply infected *H. magnanima* showed higher intrinsic growth rates (r) than other non-infected or singly-infected host lines. Although *Wolbachia* infection did not protect the hosts from natural pathogen attack, it specifically altered the PO activity in males but not in females. These findings suggest that triple *Wolbachia* infection is more advantageous for *H. magnanima* females than for other singly or non-infected females (Table 3, Fig. S1).

We previously demonstrated that *w*Hm-b induced CI in the *H. magnanima* collected from Tokyo (Arai et al. 2019). The CI phenotype enabled rapid spreads of CI-inducing *Wolbachia* in its host population (Turelli and Hoffmann 1991; Ballad et al. 1996). Takamatsu et al. (2021) reported a high *w*Hm-b infection frequency (approximately over 90%) in Shizuoka population, wherein triple infection was more frequent than *w*Hm-b single infection. In this study, we found that *w*Hm-b single infection reduced female fitness parameters, such as egg number and hatchability, resulting in reduced net reproduction rate (R_0) and intrinsic growth rate (r). This result suggests that *w*Hm-b single infection is not preferable for the host. Even if a *Wolbachia* strain affects its host negatively, other mutualistic and parasitic microbes would compensate the infection costs and increase the overall fitness advantage (Gómez-Valero et al. 2004, Frank 1998, Engelstädter et al. 2004), which contributes to the persistence of microbes in host populations (Hoffmann et al. 1990; Vautrin and Fabrice 2009). In *H. magnanima*, CI intensity was lower in the triply infected line than in the *w*Hm-b singly infected line, corresponding to *w*Hm-b density (Arai et al. 2019). We speculate that the lowered *w*Hm-b density resulted in reduced fitness costs in the triply infected line. The hosts with *w*Hm-c triple and single infection exhibited fitness advantages in insect growth, while only *w*Hm-c single infection reduced the number of eggs laid per female host. Host fecundity is a crucial factor for *Wolbachia* transmission, but several studies have reported that *Wolbachia* infection reduces egg number (Hoffmann et al. 1990; Fleury et al. 2000). Generally, pupal weight and fecundity are positively correlated in lepidopteran insects such as *Cydia pomonella* (Tortricidae) (Deseo 1971; Danthanarayana 1975; Hough and Pimentel 1978). However, *w*Hm-c infection increased larval growth and pupal weight (Arai et al. 2019) and reduced the number of eggs, probably by negatively affecting oocyte maturation or oviposition behaviour in female hosts. Similar to that of *w*Hm-b, the lowered *w*Hm-c density (Arai et al. 2019) may reduce the negative effects in the triply infected line. Otherwise, the presence of *w*Hm-a may recover the negative effects in the triply infected line. Although the mechanism by which triply infected *Wolbachia* led to high intrinsic growth in their hosts is unknown, *Wolbachia* interactions may contribute to recover the negative effects observed in singly infected host lines. Future studies using double-infected lines would provide more information on how *Wolbachia* facilitates intrinsic growth rate.

We also identified the difference in *H. magnanima* immunity caused between the sexes by *Wolbachia*. Notably, the *Wolbachia*-infected *H. magnanima* males showed reduced PO activities (Fig. 5b). As total PO activity, all quantities of inactive precursors of PO (pro-PO) activated by α-chymotrypsin, did not differ regardless of the presence or absence of *Wolbachia*, reduced quantity of pro-PO activity may not be the cause of reduced PO activity in males.

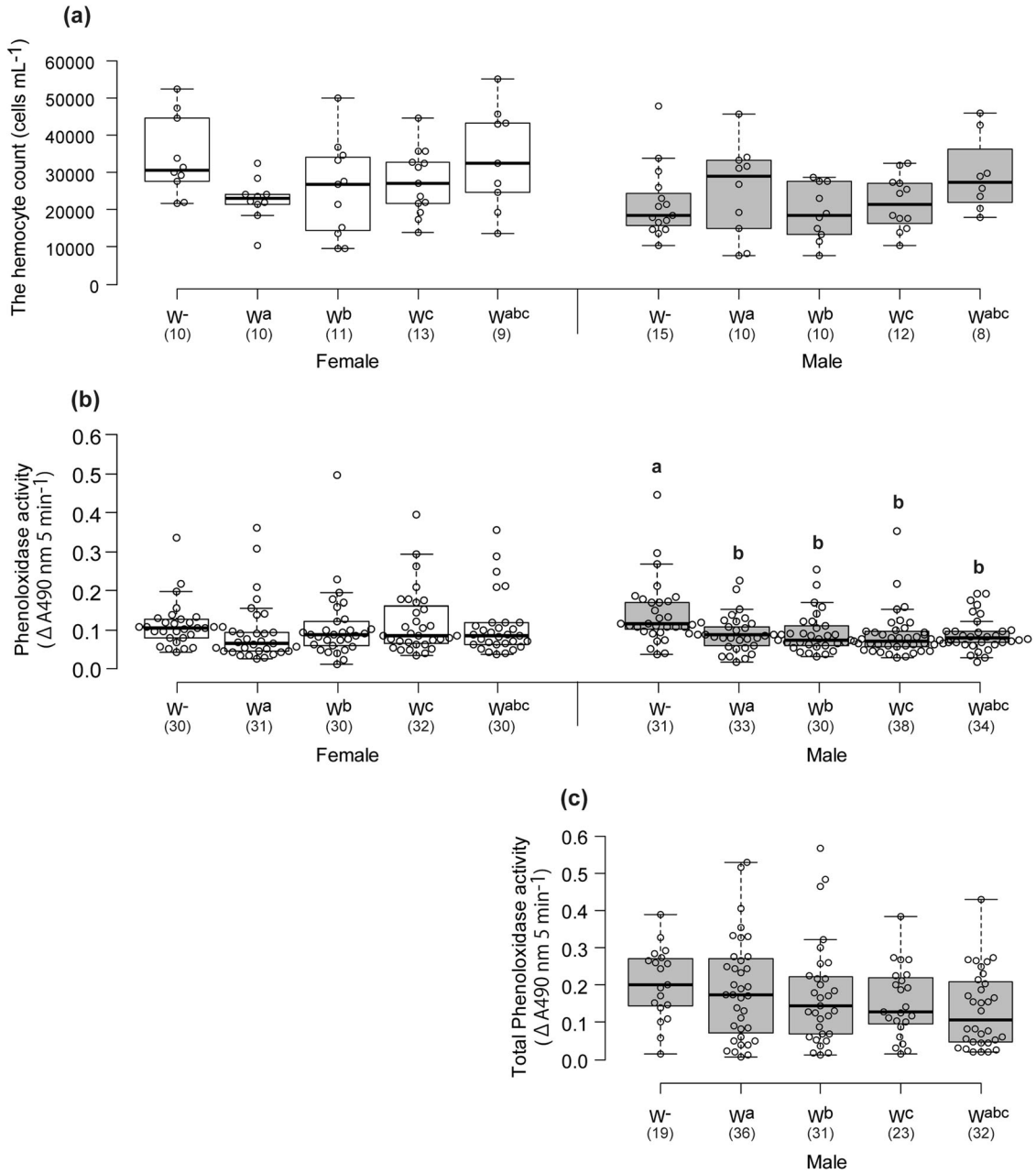

Fig. 5 Effects of *Wolbachia* infections on the host immunity. a Total haemocyte count and **b** phenoloxidase activities in female and male larval haemolymph, **c** total phenoloxidase activity in male larval haemolymph. Different letters indicate significant differences between lines (Steel-Dwass test, $p < 0.05$). The centre line and the upper and lower boundaries of the box indicate median, upper quartile, and lower quartile, respectively. The points on plots indicate each sample. The sample size is indicated in parentheses below host lines.

Wolbachia presence probably suppressed the pro-PO activation pathway, wherein pro-PO is cleaved by a proteolytic enzyme to form PO. *Wolbachia* sometimes increases host fitness by enhancing resistance to pathogens (Moreia et al. 2009; Stevanovic et al. 2015). Contrastingly, *Wolbachia* sometimes suppresses host immunity; the CI-inducing *w*Dil strain suppresses host immunity by reducing phagocytosis and PO activities in males the terrestrial isopods *Porcellio dilatatus* (Pigeault et al. 2014). *Wolbachia* is transmitted mostly vertically from female hosts to offspring but not from male hosts; therefore, suppressed immunities in male hosts is not adaptive. This can be a result of natural selection explained by the mother's curse hypothesis: inheritance through cytoplasm. If the elements inherited through cytoplasm (such as mitochondria) have mutation with severe effects for males but only mild effects for females, the mutation will undergo natural selection in females. The mutations increase to relatively a high frequency in a population because males do not transmit the mutation and male-specific phenotypes have no fitness consequences for the elements inherited through cytoplasm (Frank and Hurst 1996). Therefore, the inheritance through cytoplasm can result in sexually antagonistic traits. Since *Wolbachia* is also inherited through the cytoplasm of the female host, suppressed PO activity in only male hosts in *H. magnanima* would be a trait that has no fitness consequences for *Wolbachia* and does not support natural selection. Another possible interpretation is that the adaptation of *Wolbachia* for horizontal transmission from male hosts. In the manner of vertical transmission, infection in male host is a dead-end for *Wolbachia*. Accordingly, *Wolbachia* is

Table 3. Effects of *Wolbachia* strains on *Homona magnanima* lines.

Host lines (infection status)	Reproductive manipulation[d]	Fitness traits		Immune traits	
		Female	Male	Female	Male
W^a line (*w*Hm-a single)	No distinct effects	No distinct effects	No distinct effects	No distinct effects	Suppressed PO activity (Negative)
W^b line (*w*Hm-b single)	Cytoplasmic incompatibility (Higher intensity than W^{abc} line)	Low egg number (Negative) Low hatchability (Negative) Low intrinsic growth rate (Negative)	No distinct effects	No distinct effects	Suppressed PO activity (Negative)
W^c line (*w*Hm-c single)	No distinct effects	Low egg number (Negative) Increased pupal weight (Positive)[d] Shortened developmental duration (Positive)[d]	No distinct effects	No distinct effects	Suppressed PO activity (Negative)
W^{abc} line (triple infection)	Cytoplasmic incompatibility (Lower intensity than W^b line)	Shortened developmental duration (Positive)[d] Increased pupal weight (Positive)[d] Highest intrinsic growth rate (Positive)	No distinct effects	No distinct effects	Suppressed PO activity (Negative)

The *Wolbachia* interactions or triple infection play a mutualistic role in females.
The *Wolbachia* infection plays a potentially parasitic role in males.
[d]Arai et al. 2019.

thought to have evolved reproductive manipulations, such as male-killing, feminisation, and CI (Werren et al. 2008). But for the reproductive manipulations, *Wolbachia* would have developed horizontal transmission from males to other insects. It is unclear whether the horizontal transmission of *Wolbachia* occurs in *H. magnanima*, but several studies have suggested that parasitoid wasps harbour identical *Wolbachia* strains in their hosts and can transmit *Wolbachia* to a new insect from the original host (Vavre et al. 1999; Ahmed et al. 2015). The pro-PO activation in insect host is triggered by recognising the peptidoglycan layer of the cell wall of gram-negative bacteria (Takehana et al. 2002). Although a gram-negative *Wolbachia* does not have a proper cell wall, some studies have suggested the presence of peptidoglycan in *Wolbachia* (Vollmer et al. 2013; Voronin et al. 2014; Zug and Hammerstein 2015), which possibly triggers the pro-PO activation pathway. Despite of the presence of peptidoglycan, *Wolbachia* may prevent pro-PO activation in *H. magnanima* males with unknown manners and avoid elimination by the host immunity system, which could enhance its horizontal transmission from male host to other insects.

In conclusion, we demonstrated that triple infection of *Wolbachia* is more advantageous than single or non-infection in *H. magnanima*, which confirmed the prediction by Frank (1998). Our results imply that the *Wolbachia* interactions or triple infection plays a mutualistic role in females but a potentially parasitic role in males to maximise the transmission efficiency. The various strategies utilised by *Wolbachia* depending on the sex of the host and transmission route were revealed using isogeneic host lines. However, we have to take in to account the possibility that the genetic diversity of hosts as well as *Wolbachia* within or between populations can diverge their interaction (Hornett et al. 2006, Fry et al. 2004; Dean 2006; Capobianco et al. 2018). The complex *H. magnanima-Wolbachia* as well as *Wolbachia-Wolbachia* interactions may contribute to the maintenance of *Wolbachia* coinfections in nature. Further investigations on traits in other *H. magnanima* populations with *w*Hm-a, *w*Hm-b, and *w*Hm-c will need to understand the effects of the host's genetic diversity on the interactions with *Wolbachia*.

Data archiving

https://doi.org/10.6084/m9.figshare.20422587

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ACKNOWLEDGEMENTS

We thank Dr. T. Hiraoka (Tokyo University of Agriculture and Technology, Tokyo, Japan) for teaching the method to evaluate PO activity and Dr. S. Okuno (Arysta Lifescience Corporation, Tokyo, Japan) for providing the BotaniGard wettable powder. We also thank anonymous reviewers for their valuable suggestions.

AUTHOR CONTRIBUTIONS

MU conducted experiments, data analysis, and prepared the manuscript. HA contributed to the discussion and revision of the manuscript. KM conducted data analysis. MN contributed to discussion. MNI supervised all experiments, prepared the manuscript, and contributed to the discussion.

COMPETING INTERESTS

The authors declare that they have no conflict of interest.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41437-022-00574-6.

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