



Coexistence of Two Male-Killers and Their Impact on the Development of Oriental Tea Tortrix *Homona magnanima*

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Abstract

Male-killing, the death of male offspring induced by maternally transmitted microbes, is classified as early, or late, male-killing. The primary advantage afforded by early male-killing, which typically occurs during embryogenesis, is the reallocation of resources to females, that would have otherwise been consumed by males. Meanwhile, the key advantage of late male-killing, which typically occurs during late larval development, is the maximized potential for horizontal transmission. To date, no studies have reported on the associated developmental and physiological effects of host coinfection with early and late male-killers, which may have a significant impact on the population dynamics of the male-killers. Here we used a lepidopteran tea pest *Homona magnanima* as a model, which is a unique system wherein an early male-killer (a *Spiroplasma* bacterium) and a late male-killer (an RNA virus) can coexist in nature. An artificially established matriline, coinfecting with both *Spiroplasma* and RNA virus, exhibited embryonic death (early male-killing) as seen in the host line singly infected with *Spiroplasma*. Moreover, the coinfecting line also exhibited developmental retardation and low pupal weight similar to the host line singly infected with the RNA virus. A series of field surveys revealed that *Spiroplasma*-RNA virus coinfection occurs in nature at a low frequency. Hence, although the two male-killers are capable of coexisting within the *H. magnanima* population independently, high associated fitness cost appears to limit the prevalence of male-killer coinfection in the field host population.

Keywords Male-killing · Endosymbiont · Coinfection · *Wolbachia* · *Spiroplasma* · RNA virus · *Homona magnanima*

Introduction

The symbiotic microbes of insects are often inherited from mothers to offspring via transovarian transmission and interact with the host in a mutualistic or parasitic manner [1–7]. Since

the survival of such intracellular microbes relies primarily on female hosts, they are considered to have developed unique adaptive strategies to manipulate the host [2]. Of particular focus in the present study is male-killing, which is a male-specific lethal phenomenon caused by a diverse array of intracellular bacteria [8, 9], microsporidia [10, 11], and certain RNA viruses [12–14]. Male-killing is classified by its timing of action; those typically occurring during embryogenesis, or early larval stages, are referred to as early male-killing, while those typically occurring during late larval stages are designated as late male-killing. The primary advantage associated with early male-killing is considered to be the reallocation of resources to female insects (transmitting sex) that were to have been consumed by males, while the advantage of late male-killing is considered to be the maximization of the male-killer's density in the host allowing for horizontal transmission [15].

One of the major unresolved questions in the study of male-killers is how they persist in host populations. Stable persistence of such microbes relies on both “microbe – microbe” interactions, as well as the “microbe – host” interactions as

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multiple intracellular microbes frequently coinfect the same insect, and their phenotypes are readily affected by the interactions between microbes within a host individual [16–21]. For example, the intracellular bacteria *Wolbachia* can affect other coinfecting symbionts. Specifically, they can affect the strength of cytoplasmic incompatibility (CI) induced by other coinfecting *Wolbachia* strains [16–18], or other intracellular bacteria [19–21], while also suppressing the replication of pathogenic viruses [22–24]. Hence, the positive effects associated with coinfection can facilitate the propagation of coinfecting hosts, while the negative effects can reduce, or even eliminate, coinfecting hosts. Thus, the complicated interaction between microbes within a host individual (host vs microbe vs microbe interactions) must be considered to accurately understand the population dynamics of coinfection.

Intriguingly, multiple male-killers have been defined for various insect species. For instance, in a ladybird beetle *Adalia bipunctata*, early male-killing can be caused by at least three different intracellular bacteria, i.e., *Wolbachia* [25], *Spiroplasma* [26], and *Rickettsia* [27]. Although some are distributed throughout the same region, theoretical models based on vertical transmission efficiency, cost of infection, and the level of fitness compensation suggest that two or more male-killers cannot stably coexist in a single population [28–30]. However, there is no empirical evidence demonstrating the interaction between several male-killers (i.e., early and late male-killers) in a population, and the phenotypic outcome of coinfection with multiple male-killers in a single individual.

A serious tea pest, *Homona magnanima* (Tortricidae, Lepidoptera), is infected with three different male-killers: *Wolbachia* (early and late male-killers) [31], *Spiroplasma* (early male-killer) [32], and an RNA virus (late male-killer) [12, 13, 33, 34]. The male-killing *Wolbachia* was only isolated from a Taiwanese population [31]; meanwhile, three non-male-killing *Wolbachia* strains (*wHm-a*, *wHm-b*, and *wHm-c*) prevail in Japanese populations [17]. Of the three Japanese *Wolbachia* strains, *wHm-c* has positive effects on female development and fecundity. Interestingly, an *H. magnanima* population in the tea field located at Shimada City, Shizuoka Prefecture, Japan, harbor the early male-killing *Spiroplasma*, and the late male-killing RNA virus, as well as three strains of non-male-killing *Wolbachia* [32], providing an ideal model to investigate the population dynamics of multiple male-killers as well as non-male-killing symbionts. Herein, we established a host line harboring both *Spiroplasma* and the viral male-killers, excluding *Wolbachia*. The effects of coinfection were then investigated and compared with singly infected host lines. Furthermore, the prevalence of *Spiroplasma*, RNA virus, and *Wolbachia* and their infection status (i.e., single, dual, or multiple infection) in the host population were surveyed seasonally for 4 years. We then discuss (1) the interactions of two male-killers within a host individual, (2) population dynamics of two male-killers, and (3) the possible

contribution of a non-male-killing *Wolbachia* to the persistence of the male-killers.

Methods

Insects

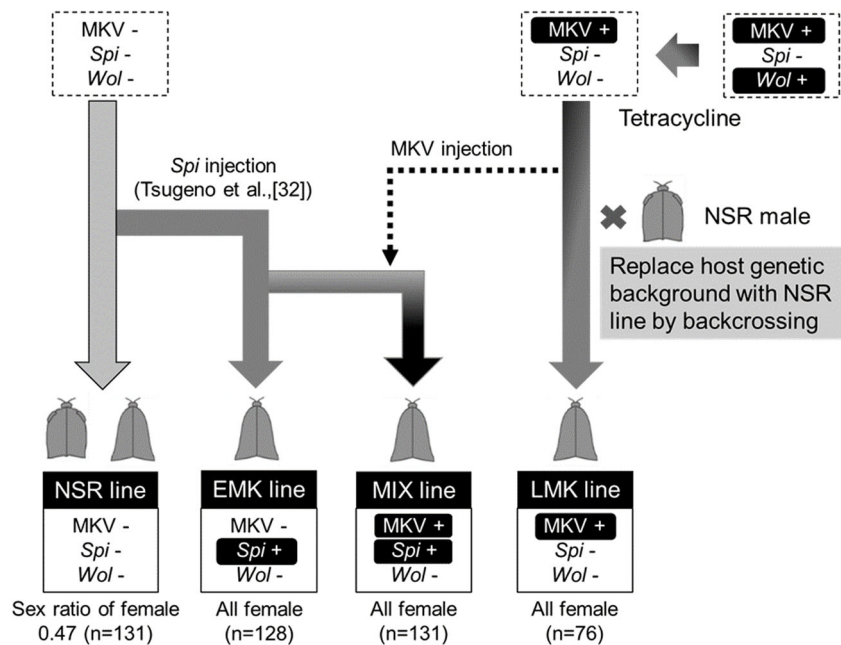
We maintained four *H. magnanima* lines with different infection patterns of intracellular bacteria and male-killing virus. The normal sex ratio (NSR) and the early male-killing (EMK) line of *H. magnanima* were established as previously reported [32]. The NSR line was free of intracellular bacteria, while the male-killing RNA virus and the EMK line were singly infected with male-killing *Spiroplasma*. The late male-killing (LMK) line was established from a maternal line derived from a single female collected in 2009 from a tea field of Shimada City, where Tsugeno et al. [32] surveyed. This line consisted of only females and was coinfecting with *Wolbachia* and the RNA virus. To establish a line harboring only male-killing RNA virus (LMK), *Wolbachia* was eliminated by treating the host larvae with tetracycline as described previously [17, 31] (Fig. 1). The host line coinfecting with *Spiroplasma* and the RNA virus (MIX) was established as described below.

The females from the four lines with different symbiont states (NSR, EMK, LMK, and MIX) were maintained in the laboratory for over 5 years by crossing with NSR males to homogenize host nuclear genetic backgrounds. Briefly, fifteen adult females of each line were mated with fifteen males of the NSR line in a plastic container (20 × 30 × 5 cm). Each egg mass was placed into a plastic box (20 × 15 × 5 cm) containing the artificial diet Silk Mate 2S (Nosan Co., Yokohama, Japan), at 25 °C under a 16L:8D photoperiod until adult eclosion [17, 31, 32].

Establishment of Host Line Coinfecting with *Spiroplasma* and RNA Virus (MIX)

A total weight of 1.0 g of abdomens collected from adult females of the LMK line was homogenized in 5 mL of homogenate buffer (0.1% ascorbic acid, 1 mM Tris-HCl [pH 8.0], 1 mM EDTA) on ice and filtered by single, double, and quadruple gauze layers in turn. The filtrate was then centrifuged twice at 1500×g for 20 min, and 10,000×g for 60 min at 4 °C. The supernatant was filtered using a 0.45-μm and 0.22-μm pore size filter (Merck Millipore, MA, USA). Next, 1.0 μL filtrate was injected into a newly molted fourth-instar larva of the EMK or NSR line using a micro syringe. The injected larvae were reared individually in a 15-mL plastic cup with a piece of INSECTA LF (Nosan Co.). Each female adult was allowed to mate randomly with two or three males of the NSR line in a plastic container (20 × 30 × 5 cm). The obtained egg masses were reared until adult emergence, and

Fig. 1 Procedures to establish host lines. Boxes at the bottom are the *H. magnanima* lines used in this study. Boxes drawn with dashed lines indicate their ancestors. Infection statuses (positive “+” or negative “-”) shown in the boxes indicate those of male-killing RNA virus (MKV), *Spiroplasma* (*Spi*), and *Wolbachia* (*Wol*). Shown in brackets are total number of individuals in sex ratio. The all-female host lines (EMK, MIX, and LMK lines) were backcrossed with NSR males for more than 60 generations (5 years)



microbial infection was assessed by PCR analysis. A matriline (MIX line) harboring both *Spiroplasma* and the RNA virus was maintained as described above.

Detection of *Wolbachia*, *Spiroplasma*, and the RNA Virus by PCR Analysis

The abdomens of *H. magnanima* were dissected into two pieces with forceps and each piece was transferred to a collection tube; one half was used for DNA extraction, and the other for RNA extraction. DNA was extracted as described previously [17, 31, 32]. The DNA was dissolved in TE buffer and stored at 4 °C. Total RNA was extracted using the ISOGEN kit (Nippon Gene Co., Ltd., Japan) following the manufacturers' protocol. The extracted RNA was treated with RNase-free recombinant DNase I (Takara Bio Inc., Shiga, Japan) and was reverse transcribed using AMV Reverse Transcriptase XL (Takara Bio Inc.) and Oligo dT 15mer primer.

PCR primers used to detect intracellular bacteria and male-killing virus are as follows: *wspF81* (5'-TGGT CCAATAAGTGATGAAGAAAC-3') and *wspR691* (5'-TGGAGTAGCGTTAATTTTT-3') to amplify *Wolbachia* *wsp* gene sequence (Zhou et al., [35]), *Haln1* (5'-GCTC AACCCTAACCGCC-3') and *SP-ITS-N2* (5'-GGTA CTCACGTCTTCATCG-3') to amplify *Spiroplasma* ribosomal ITS region (Hurst et al., [26] and Schulenburg et al. [36]), and *C3-F* (5'-AAGATGCAAGCC-3') and *C3-R* (5'-CCCTACCGCCTCACATC-3') to amplify the male-killing RNA virus MK1241 regions (Nakanishi et al., [13]). The reaction was conducted using Ex Taq DNA polymerase or Emerald Amp MAX master mix (Takara Bio Inc.) under the

following conditions: initial denaturation at 94 °C for 2 min; 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C (*Wolbachia*) and 55 °C (*Spiroplasma* and the RNA virus) for 30 s, and extension at 72 °C for 30 s; followed by final extension at 72 °C for 3 min.

Egg Hatchability, Development, Mortality, and Vertical Transmission Rate

Egg masses were randomly selected from ten females of each strain that were mated with ten males of the NSR line and were scanned using PIXUS MP970 (Canon Inc., Tokyo, Japan). The size of each egg mass was measured by an image analysis software ImageJ [37]. A linear regression line between egg mass size (x) and egg number (y) was constructed on the NSR line [17, 31]. We then estimated the number of eggs in a given egg mass based on the regression line. The number of hatchlings was counted for 4 days after the first hatchling appeared. To assess survivorship throughout embryogenesis, the hatchability (number of hatchlings/estimated number of eggs) was determined for each line. The survival rate during early to late embryogenesis was calculated by the number of pharate larvae (i.e., mature embryos forming black-colored head capsules) divided by the estimated number of eggs. The survival rate during late embryo to egg hatching was calculated by the number of hatchlings divided by the number of pharate larvae.

Hatchlings from the egg masses were individually reared in a plastic cup, as described above, until adult emergence. Development time, mortality at larval stage, and pupal weight were recorded. Mortality data for each larval instar was obtained using six egg masses of the LMK line, and five egg

masses each of the NSR, EMK, and MIX lines. To measure transmission rate of *Spiroplasma* and RNA virus to EMK, LMK, and MIX lines, five female insects and their ten daughters (50 in total) of each line were subjected to DNA and RNA extraction and microbial detection.

Field Collection of *H. magnanima*

H. magnanima progress through four generations per year in Japan: 1st generation in May–June, 2nd generation in July–August, 3rd generation in September–October, and 4th generation in November–March. To survey the infection dynamics of *Wolbachia*, *Spiroplasma*, and the RNA virus, individuals of *H. magnanima* were collected from a tea field located in Shimada City, Shizuoka Prefecture, Japan. Larvae collected from damaged tea leaves in 2012, 2013, and 2014 were reared individually in a plastic cup with an artificial diet of INSECTA LF (Nosan Co.) until adult emergence. Adults were collected from the same tea field using light trap or pheromone trap in 2019. All adults were stored in ethanol at $-80\text{ }^{\circ}\text{C}$ until microbial detection.

All adult individuals were subjected to *Wolbachia*, *Spiroplasma*, and late male-killing RNA virus detection. For adult samples collected in 2019, *Wolbachia*-infected samples were subjected to *wHm*-a, *wHm*-b, and *wHm*-c strain detection. Briefly, Emerald Amp MAX master mix (Takara Bio Inc.) and specific primer sets for *wsp* of *wHm*-a (F173: 5'-CCTATAAGAAAGACAATA-3' and R565: 5'-TTTGATCATTACACAGCGT-3'), *wHm*-b (F176 5'-GGTGCTAAAAGAAGACTGCGG-3' and R667: 5'-CCCCCTTGTCTTTGCTTGC-3'), and *wHm*-c (F188 5'-CATA TAAATCAGGTAAGGACAAC-3' and R603 5'-CACCAGCTTTTGCTTGATA-3') were used for PCR amplification under the following conditions: initial denaturation at $94\text{ }^{\circ}\text{C}$ for 3 min; 35 cycles of denaturation at $94\text{ }^{\circ}\text{C}$ for 30 s, annealing at $50\text{ }^{\circ}\text{C}$ for *wHm*-a, $55\text{ }^{\circ}\text{C}$ for *wHm*-b, and $60\text{ }^{\circ}\text{C}$ for *wHm*-c for 30 s, and extension at $72\text{ }^{\circ}\text{C}$ for 30 s; followed by final extension for 7 min at $72\text{ }^{\circ}\text{C}$ [17].

Statistical Analysis

Based on the sample size, data sets were tested for normality (for development time, pupal weight, mortality, survival traits through embryogenesis, and hatchability) using Shapiro-Wilk test on R 4.0.0 (<https://cran.r-project.org/>). Since all data was not normally distributed, data sets were analyzed by Steel Dwass nonparametric multiple comparison (JMP[®] 9.0.0 software; SAS Institute, Cary, NC).

Survival data for larval period was analyzed using Kaplan-Meier survival estimates and the Holm-Sidak test for multiple comparisons using survival package on R 4.0.0.

To compare the frequency of *Spiroplasma* and RNA virus coinfection to single infections by season, expected numbers

were calculated with frequencies of two male-killers and compared with the number of coinfecting individuals observed via Fisher's exact test on R 4.0.0. To determine (1) if the infection patterns of *Spiroplasma*, RNA virus, and *Wolbachia* correlate with each other, and (2) if *Wolbachia* genotype correlates with male-killer's infection, we fit the generalized linear mixed model (GLMM) using lme4 package [38] in R 4.0.0 assuming binomial error on the basis of the presence/absence of the respective endosymbionts within an individual *H. magnanima*. In the statistical modeling, we considered the infection status (i.e., *Spiroplasma* presence or absence) as a fixed factor and sampling season (month and year) as a random factor. Based on the GLMM, an analysis of variance (ANOVA) was conducted to assess and evaluate the significance of the individual model terms (effects of each explanatory variable).

Results

Establishment of MIX Line Coinfected with *Spiroplasma* and the RNA Virus

The MIX line harboring the male-killing *Spiroplasma* and RNA virus was successfully established via microinjection (Fig. 1). More specifically, *Wolbachia* was firstly eliminated from the ancestor of LMK line by tetracycline treatment, and a line harboring only the male-killing RNA virus was established (referred to as "LMK line" hereafter). A homogenate of the LMK female adults was then injected to fourth-instar larvae of "EMK line" (previously established by Tsugen et al., [32]), and to fourth-instar larvae of NSR line as control (Fig. S1). Among injected individuals (G_0 generation), no significant differences were observed in the survival rates between NSR females and EMK females ($p = 0.442$). Although 4/8 NSR males died after injection, the survival rates did not differ significantly from NSR females ($p = 0.074$; Fig. S2). Offspring (G_1 generation) were obtained from two G_0 EMK females that were all females (Brood S-3 consisting of 11 females and Brood S-10 consisting of 23 females; Fig. S1) and two G_0 NSR females were all females (Brood F-2 consisting of 14 females and Brood F-3 consisting of 14 females; Fig. S1). We also confirmed, by RT-PCR, that the offspring of injected females (S-10, F-2, and F3) were infected with the male-killing RNA virus (Fig. S1). A matriline successfully established from S-10 was referred to as "MIX line," which was confirmed to harbor both *Spiroplasma* and the RNA virus (Fig. S1). As shown in Fig. 1, the all-female lines (EMK, MIX, and LMK) were maintained by backcrossing with the NSR males for more than 5 years (ca. 60 generations) to homogenize their nuclear genetic background. At adult stage, the NSR line always consisted of males and females with a near 1:1 ratio (e.g., 69 males, 62 females in one brood),

while EMK, LMK, and MIX lines consisted of only females (Fig. 1).

In both the EMK and MIX lines, *Spiroplasma* transmission rates to the next generation were 100%; in each line, all 50 individuals were confirmed *Spiroplasma*-positive by PCR. In both the LMK and MIX lines, vertical transmission rates of the RNA virus were 100%; in each line, all 50 individuals were RNA virus-positive by RT-PCR.

Impact of Single or Dual Infection of Male-Killers on Host Survivorship

To assess embryonic lethality, survival rates of eggs were examined during early to late embryogenesis, and late embryogenesis to egg hatch, wherein the number of egg masses (y) was estimated by the regression line $y = 4.2828x + 6.8581$ with $R^2 = 0.9896$, $n = 16$, where x denotes the area of the egg mass [mm^2]. The survival rates during early to late embryogenesis (number of pharate larvae/estimated number of eggs) were significantly higher in NSR and LMK lines than in EMK and

MIX lines ($p < 0.01$; Fig. 2a). No significant difference was detected between NSR and LMK lines ($p = 0.442$) and between EMK and MIX lines ($p = 0.132$).

Survival rates in late embryogenesis to egg hatch (number of hatchlings/number of pharate larvae) did not differ significantly between NSR and LMK lines ($p = 0.741$), which were significantly higher than EMK and MIX lines (Fig. 2b), while that for the MIX line was significantly lower than the EMK line ($p < 0.05$).

Moreover, the hatchability (number of hatchlings/estimated number of eggs) was significantly higher in the NSR ($38.3 \pm 1.8\%$) and LMK lines ($41.0 \pm 3.2\%$) compared with EMK ($13.7 \pm 1.4\%$) or MIX line ($6.19 \pm 0.7\%$; Fig. 2c). The hatchability was also significantly higher in the EMK line than the MIX line ($p < 0.01$).

Lastly, the LMK line showed significantly lower survival rate (32.6%, $n = 184$) during larval to pupal stages than in the EMK (73.8%, $n = 145$), NSR (70.6%, $n = 163$), and MIX lines (65.1%, $n = 158$; $p < 0.01$). The survival rate of the MIX line did not differ significantly from that of the EMK line ($p = 0.29$) or NSR line ($p = 0.81$; Fig. 2d).

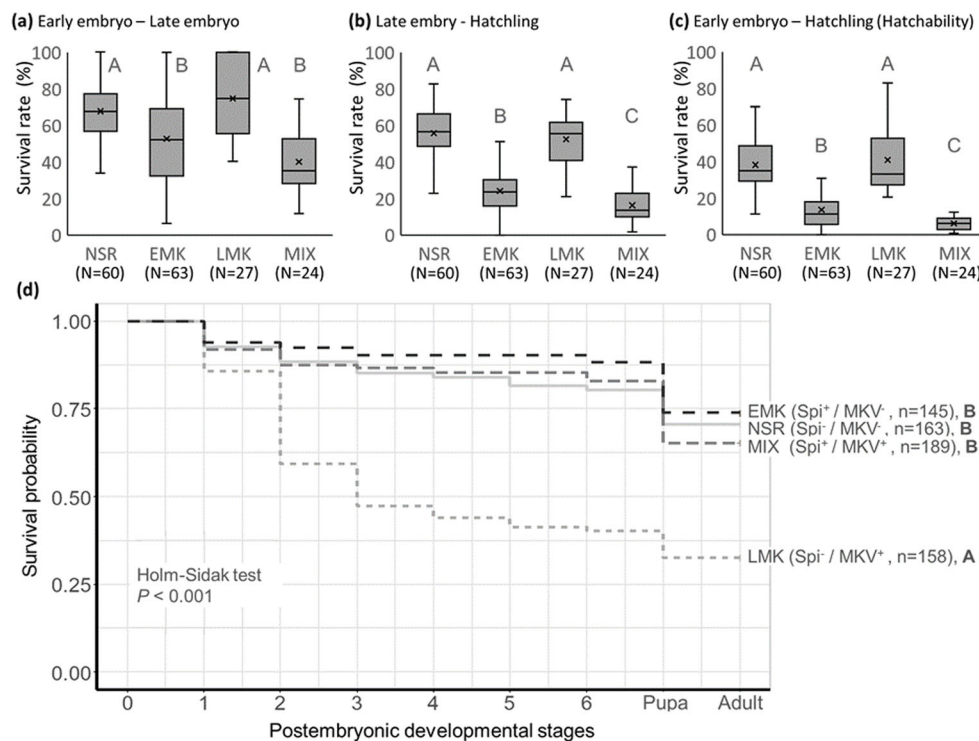


Fig. 2 Survivorship of *H. magnanima* lines during embryogenesis and postembryonic stages. Different letters indicate significant differences between the lines (Steel Dwass test, $p < 0.05$) in survival rate during early to late embryo (a), late embryo to hatchling (b), and total survival rate throughout embryogenesis: hatchability (early embryo to hatchling) (c). In each box-and-whisker plot (a–c), the center line and “X” within the box represent the median and the average value, respectively. The upper and lower boundaries of the box indicate upper quartile and lower

quartile, respectively. Sample size (number of subjected egg masses) in each data point is indicated in parentheses below the plots. Postembryonic survival (d) is shown with Kaplan-Meier survival curves and analyzed with log rank test adjusted by the Holm-Sidak method for multiple comparisons. Different letters indicate significant differences ($p < 0.05$). Numbers shown in parentheses on the right of infection statuses indicate sample size (hatchlings). Numbers below the x axis indicate larval instar. Spi, *Spiroplasma*; MKV, male-killing RNA virus; +, positive; –, negative

Impact of Single or Dual Male-Killer Infection on Host Physiology

To verify the fitness cost of the two male-killers besides the male-killing effect per se, we examined the larval development time and pupal weight, both of which should correlate with life cycle and fecundity [17]. Since all males in EMK, MIX, and LMK lines die before adult eclosion, female data was compared on these two factors. The female larval development time of LMK (31.6 ± 0.7 days) and MIX lines (30.0 ± 0.6 days) was significantly longer than that of NSR (24.3 ± 0.5 days) and EMK lines (24.5 ± 0.4 days; $p < 0.05$). No significant difference was detected between LMK and MIX lines (30.0 ± 0.6 days, $p = 0.175$), or between EMK and NSR lines ($p = 0.784$, Fig. 3a). The pupal weight of LMK (79.1 ± 1.7 mg) and MIX lines (82.8 ± 1.9 mg) was significantly lower than that of NSR (89.9 ± 2.0 mg) and EMK lines (88.8 ± 1.7 mg; $p < 0.01$); however, no significance was detected between LMK and MIX lines ($p = 0.50$, Fig. 3b). Taken together, lines harboring the RNA virus (LMK and MIX) showed negative growth and development phenotypes.

Prevalence of Intracellular Bacteria and Male-Killing Virus in the *H. magnanima* Field Population

In total, 1499 *H. magnanima* individuals (721 males and 778 females) collected in 2012, 2013 and 2014 (as larvae; Table S1), and 2019 (as adults) were examined at the adult stage for the presence of *Wolbachia*, *Spiroplasma*, and the RNA virus (Fig. 4; Table S2). Frequencies of *Spiroplasma*-positive individuals ranged from 1.8 to 21.7% in females (9.63% in total), and 0.0 to 10.8% in males (3.49% in total); frequency of the RNA virus-positive individuals ranged from 0.0 to 27.1% in females (13.72% in total), and 0.0 to 14.6% in

males (5.08% in total). Alternatively, frequencies of *Wolbachia*-positive individuals were very high, ranging from 94.4 to 100% in females and 89.8 to 100% in males (Table S2). Infection frequencies of *Wolbachia* among individuals positive for male-killers (either *Spiroplasma*, MKV, or both; 100% for both males and females) were not significantly different from those of individuals negative for male-killers (96.9%; Fisher's exact test, $p = 0.70$ for males and 98.6%; $p = 0.95$ for females). Frequencies of individuals infected with both *Spiroplasma* and the RNA virus (0.13% ($n = 1$) for males and 1.54% ($n = 12$) for females) were not significantly different from the assumption that the two male-killers distribute randomly in the population (expected number = 1.28 ($721 \times 3.49 \times 5.08/100$); Fisher's exact test, $p = 1.00$ for male and expected number = 10.27 ($778 \times 9.63 \times 13.72/100$); $p = 0.67$ for female), which does not support any positive or negative effects of coexistence of the two male-killers. Furthermore, the frequency of the male-killing RNA virus was not significantly affected by *Spiroplasma* in females (GLMM, $p = 0.5793$, Table S3), or males ($p = 0.8151$). Similarly, frequency of *Spiroplasma* was not significantly affected by the male-killing RNA virus in females ($p = 0.4108$), or males ($p = 0.8268$).

Infection frequencies of the *Wolbachia* strains (wHm-a, wHm-b, and wHm-c type) were 66.9%, 94.6%, and 95.3%, respectively, for females ($n = 148$), and 22.6%, 9.1%, and 96.3% for males ($n = 168$; Fig. 5). Remarkably, the triple infection dominated in females (ranged 41.7 to 77.1%) but not in males (ranged 0.0 to 7.9%). Alternatively, single infection of wHm-c type *Wolbachia* dominated in males (ranged 62.5 to 89.7%) but not in females (ranged 0.0 to 8.3%). Furthermore, *Spiroplasma* and RNA virus always coinfect the same host individual with wHm-c type *Wolbachia* (Fig. 5a–c). The frequency of male-killing RNA virus was significantly affected by wHm-c in females (GLMM, $p < 0.05$, Table S4) but not in

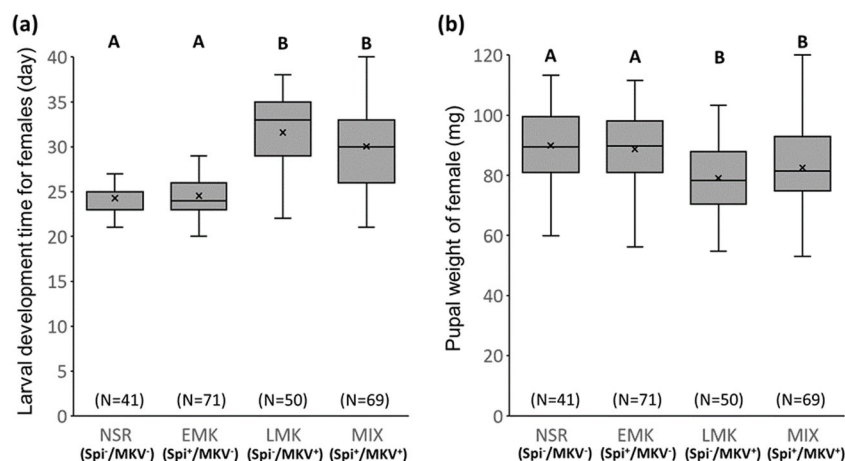
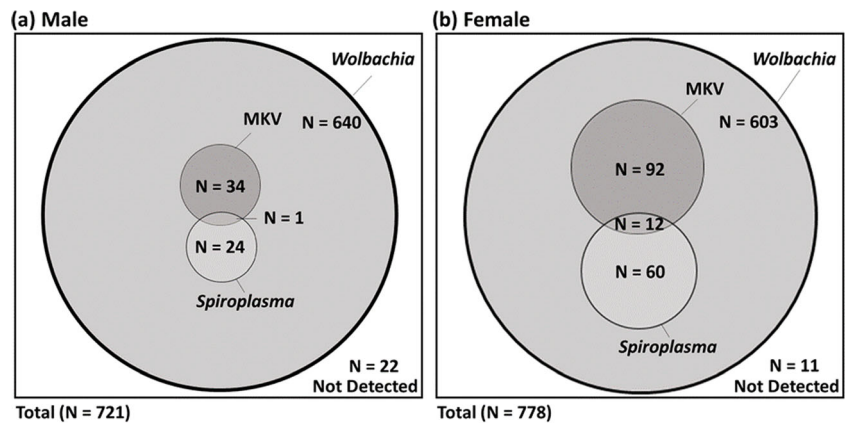


Fig. 3 Effects of infection status on host development. Larval development time (a) and pupal weight (b) were examined for females of each line. The horizontal line and “x” within the box represent the median and the average value, respectively. The upper and lower boundaries of the box indicate upper quartile and lower quartile,

respectively. N, number of individuals. Different letters indicate significant differences (Steel Dwass test, $p < 0.05$). Infection statuses (positive “+” or negative “-”) shown in the boxes indicate those of *Spiroplasma* (*Spi*) and male-killing RNA virus (MKV)

Fig. 4 Infection patterns of *Wolbachia*, *Spiroplasma*, and the male-killing RNA virus (MKV) in *H. magnanima*. Venn diagrams based on infection status of adults collected over 4 years at Shimada City, Sizuoka Pref. **a** Males. **b** Females. N indicates the number of individuals



males ($p = 0.32$). Similarly, frequency of *wHm-c* was significantly affected by the male-killing RNA virus in females ($p < 0.05$) but not in males ($p = 0.26$). Although frequency of *Spiroplasma* was significantly affected by *wHm-c* in females ($p < 0.01$), not males ($p = 1.00$), frequency of *wHm-c* was not affected by *Spiroplasma* both in females ($p = 0.10$) or males ($p = 0.48$). Hence, in terms of population prevalence, RNA virus and *Spiroplasma* do not influence one another; however, both are dependent on the presence of *Wolbachia*, particularly, *wHm-c*.

Discussion

Here we established an *H. magnanima* line doubly infected with the two male-killers, *Spiroplasma* and RNA virus, which

exhibited an early male-killing phenotype similar to the *Spiroplasma* singly infected line, however, showed developmental retardation and weight loss, pathogenic effects observed in the RNA virus singly infected line. Besides, both *Spiroplasma* and the RNA virus were vertically transmitted to all offspring of the coinfecting host line. A series of field surveys revealed that the two male-killers coinfecting the same host individuals at a low frequency, which did not support any positive or negative effects of coinfection.

Generally, the ecological reasons for the evolution of early male-killers are based on two types of detrimental conditions occurring in nature: inbreeding depression and competition between siblings for resources [28]. In Coccinellidae beetles, e.g., *A. bipunctata*, (i) eggs are laid in tight clutches, (ii) neonates consume unhatched eggs and are not efficient at foraging aphids, (iii) aphids are ephemeral and unpredictable, and

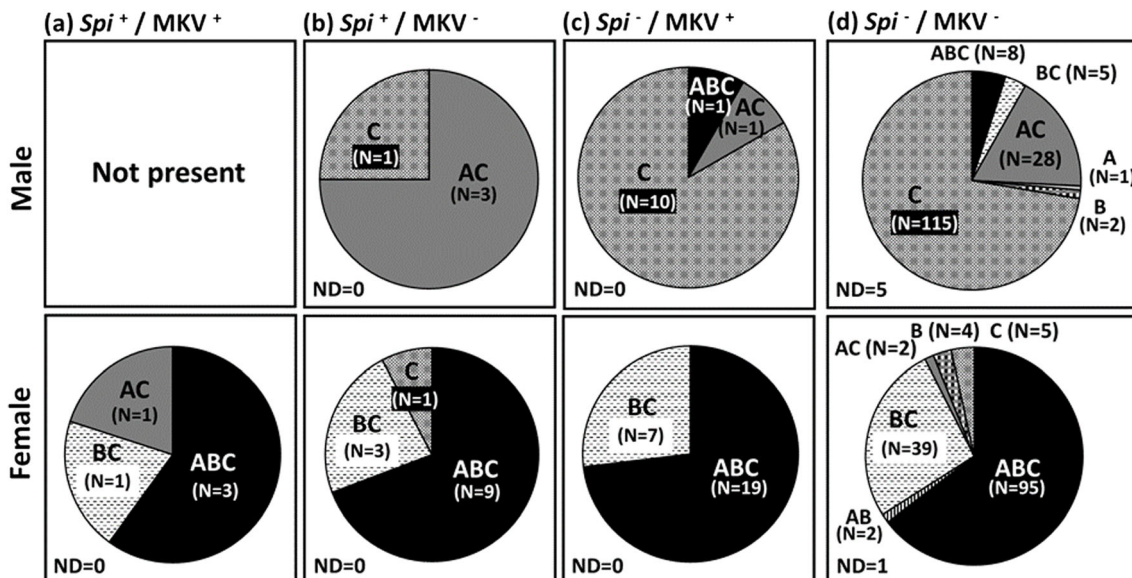


Fig. 5 Infection patterns of *Wolbachia* strains and the two male-killers in 2019. Infection status of the two male-killers in males (upper) and females (bottom) were classified as **a** *Spiroplasma*-positive and RNA virus-positive, **b** *Spiroplasma*-positive and RNA virus-negative, **c** *Spiroplasma*-negative and RNA virus-positive, and **d** *Spiroplasma*-negative and RNA virus-negative. Infection patterns of the three *Wolbachia* strains *wHm-a*,

wHm-b, and *wHm-c* are shown in pie charts. N indicates number of individuals. *Spi*, *Spiroplasma*; MKV, male-killing RNA virus; +, positive; -, negative; Not present, none of the individuals were positive for *Spiroplasma* or male-killing RNA virus; ND (not detected), *Wolbachia*-free individuals

(iv) mortality of neonates are very high due to starvation, facilitating the evolution of early male-killing, which can reduce risks of inbreeding and reallocate resources to females [15, 29]. Meanwhile, in *H. magnanima*, several lines have been maintained via sib-mating for over 20 years in our laboratory, suggesting the level of inbreeding depression is quite low. Although eggs are oviposited as egg masses, (i) *H. magnanima* neonates do not consume dead pharate males and immediately disperse, (ii) cannibalism does not occur, and (iii) herbivory larvae are surrounded by plenty of food (tea leaves), which does not experience seasonal fluctuations. Hence, resource reallocation via early male-killing does not appear advantageous in *H. magnanima*. Instead, the early male-killers (*Spiroplasma* [32], and Taiwanese *Wolbachia* strain wHm-t [31]), may reduce competition between larvae to ensure shelter. In fact, *H. magnanima* larvae frequently reuse nests made by previous generations in the tea field (personal observation by HA); hence, early male death may increase the chance for female larvae to find old nests, allowing them to evade natural enemies.

Alternatively, strategies of late male-killers maximize the chance of horizontal transmission [28, 39]. In *H. magnanima*, the late male-killing RNA virus was horizontally transmitted to a new host by microinjection. Therefore, it is possible that parasitoids act as vectors for horizontal transmission of symbionts (particularly the RNA virus), as have been demonstrated for parasitic wasps as vectors of *Wolbachia* in whiteflies [40], and ectoparasitic mites as vectors of male-killing *Spiroplasma* in *Drosophila* flies [41]. The RNA virus was transmitted horizontally via oral infection (personal observation by MNI), while oral intake of regurgitated digesta, feces, and male cadavers may explain how the late male-killing virus is maintained in the population. It is possible that male death in a nest triggers subsequent virus infection to new hosts in the contaminated shelter. Considering the fitness costs of the virus not only on males (late male-killing) but also females (weight loss and developmental retardation), persistence of the virus in various tea fields in Japan [12, 13, 32, 34] may be achieved by high efficiency of horizontal transmission of the virus.

Although numerous studies have investigated interactions between *Wolbachia* and viruses [22–24, 42], to our knowledge, no studies have reported on how *Spiroplasma* responds to subsequent viral infection. Successful establishment of the MIX line herein implies that primevally infecting *Spiroplasma* does not prevent subsequent infection with the RNA virus. Furthermore, MIX line did not show any obvious difference in the intensity and timing of male-killing compared with EMK line. Differences in tissue tropism, a low level of competition within the same niche, or large margin capacity of the two male-killers within host cells, may permit high vertical transmission efficiency, and independent expression of their own phenotypes. To verify these speculations, infection density and

localization patterns of the two male-killers in singly and coinfecting host lines must be investigated.

Coinfections with reproductive parasites can be stably maintained within host populations if multiple infections are associated with higher fitness than single infections [43, 44]. However, it has been predicted that multiple male-killers cannot coexist at equilibrium in a genetically and environmentally homogeneous population as the one with a higher transmission efficiency, lower cost, and greater benefit for female survival will eliminate the others [26, 28, 30]. In *H. magnanima*, the vertical transmission rate of *Spiroplasma* and the RNA virus in the coinfecting matriline seemed to be very high (none of 50 individuals lost the infection). In the coinfecting matriline, *Spiroplasma* has lower female fitness imposed by the RNA virus. Hence, the *Spiroplasma*-positive matrilines are considered to decrease the frequency upon secondary acquisition of the RNA virus. Meanwhile, upon acquisition of *Spiroplasma*, the RNA virus will lose horizontal transmission from the late-instar males although it may gain fitness compensation by early male-killing. Hence, the coinfecting individuals can be eliminated from the population [28–30, 44]. Therefore, the existence of coinfecting individuals in the field, albeit at a low frequency, may be explained by horizontal transmission of RNA virus to *Spiroplasma*-infected females, which may occur at a certain frequency in nature.

Natural existence of coinfecting individuals may also be explained by the effect of the accompanying *Wolbachia* as all individuals infected with the male-killers were also infected with *Wolbachia*. A previous study demonstrated that wHm-c did not cause CI but shortened the larval development time and increased pupal weight [17]. Although it is unknown precisely how *Wolbachia* strains (wHm-a, wHm-b, and wHm-c) interact with the male-killing RNA virus or *Spiroplasma*, the wHm-c strain, and possibly wHm-a as well, may mitigate the adverse effect of the RNA virus, thereby serving as a driver for the coexistence of the two male-killers. It is likely that *Wolbachia* firstly invaded the *H. magnanima* population, followed by the two male-killers independently; otherwise, the two male-killers would be expelled by the invasion of *Wolbachia* as a consequence of the wHm-b strain CI phenotype [17]. Cumulative, these results imply that complicated host-microbe-microbe interactions, including those between the two male-killers as well as between host and *Wolbachia*, could determine the ecological dynamics of microbes in *H. magnanima*.

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Authors' Contributions TT conducted characterization of host lines and conducted field surveys. HA conducted field surveys, data analyses, and preparation for the manuscript. TT and HA contributed equally to the present work. NA established the host lines and conducted field surveys. MN supported entire experiments and contributed to discussion. YK supported the works, arranged surveys, and contributed to entire discussions of this study. MNI managed experiments and preparations for the manuscript and contributed to discussions of this study. Corresponding authors HA and MNI have full access to all data and had responsibility for the decision to submit for publication.

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Data Availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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Consent to Participate Not applicable.

Consent for Publication Not applicable.

Code Availability Not applicable.

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