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Melanic mutation causes a fitness decline in bean beetles infected by *Wolbachia*

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Abstract

Wolbachia cannot live outside a host, which is thought to be the reason for host-*Wolbachia* coevolution toward benign parasitism, especially because the fitness of *Wolbachia* is traded against its host's fitness. Insect melanism has been reported to have a positive effect on pathogen resistance, but melanic mutants of *Callosobruchus analis* (Fabricius) and *Callosobruchus chinensis* (L.) (Coleoptera: Chrysomelidae) are infected with *Wolbachia*. *Callosobruchus chinensis* is infected with CI-inducing *Wolbachia* and melanic mutants exhibit fitness decline. Interestingly, this decline is not observed in *C. analis* melanic mutants that are infected with CI-free *Wolbachia*. Our research question is whether the infection of CI-inducing *Wolbachia* causes fitness decline of melanic hosts in *C. analis*. We examined fecundity, fertility, and longevity of *C. analis* melanic mutants and compared them between uninfected and infected hosts with CI-inducing *Wolbachia*. Infected melanic mutants of *C. analis* exhibited fitness decline leading to reduced hatch rates even when parental combinations were compatible. *Wolbachia* can invade a host population by causing CI in order to decrease the fraction of uninfected hosts, but melanic mutant hosts decrease the number of infected hosts through fitness decline. Nevertheless, the melanism in hosts is not able to stop *Wolbachia* invasion in *C. analis.*

Introduction

Symbiosis, first defined by de Bary (1879), broadly includes all interactions that have mutualistic, commensal, and parasitic effects on the host. Since symbionts with a vertical transmission mode share the same fate as their hosts, they should evolve to be harmless to the hosts (Ewald, 1987). However, vertically transmitted *Wolbachia*, an intracellular genus of bacteria detected by Hertig & Wolbach (1924), seems to strengthen parasitism. Yen & Barr (1973) found that cytoplasmic incompatibility (CI) is one of the reproductive manipulations that *Wolbachia* can induce. CI leads to early embryonic death in host offspring when female parents lack the same *Wolbachia* strain harbored by their mates (Werren et al., 2008). CI-inducing *Wolbachia* can invade a host population effectively because the relative infection frequency increases with CI (Turelli & Hoffmann, 1991; Turelli, 1994).

One antagonism against CI-inducing *Wolbachia* is reduced maternal transmission, which could indirectly contribute to reduced CI intensity (Turelli, 1994). Others are assortative aggregation of host eggs and pre-copulatory mate choice where hosts avoid choosing mates with which CI would occur (Vala et al., 2004; Jaenike et al., 2006). However, the evolution of these antagonisms is debatable because the number of hosts that exhibit such behaviors is very low, and they would be eliminated through drift in a population (Sahoo, 2016).

Previous studies have reported that melanism had a positive effect on pathogen resistance (Wilson et al., 2001; Yassine et al., 2012; Dubovskiy et al., 2013). In *Spodoptera exempta* (Walker) (Lepidoptera: Noctuidae), melanic larvae exhibited lower mortality than non-melanic ones when they were exposed to pathogens (Wilson et al., 2001). Melanic *Galleria mellonella* (Linnaeus) (Lepidoptera: Pyralidae) also have a higher tolerance to pathogen penetration than non-melanic ones, though their melanism is accompanied by fitness costs (Dubovskiy et al., 2013). *Infected Anopheles gambiae* (Giles) (Diptera: Culicidae) can delay internal pathogen proliferation through melanism (Yassine et al., 2012).

In *Callosobruchus* spp. bean beetles, the ease with which the melanic-body-color mutation can be seen enables its isolation from wild type beetles. Recessive mutations have been reported in *Callosobruchus chinensis* (L.) (Kashiwagi & Utida, 1972), but dominant, incomplete dominant and recessive mutations in *Callosobruchus maculatus* (Fabricius) (Breitenbecher, 1921; Eady, 1991; Mano & Toquenaga, 2011). The melanic mutation in *Callosobruchus analis* (Fabricius) found in the current study was recessive. As *Wolbachia* infection has been reported in *C. analis*, *C. chinensis*, and *Callosobruchus latealbus* (Pic)

(Kondo et al., 2011), recessiveness of melanic gene(s) in *Callosobruchus* may not have an effect on *Wolbachia* infection. However, Kashiwagi & Utida (1972) reported low fecundity and fertility among *C. chinensis* with the melanic-body mutation, concluding that the mutants could not survive in nature. As we do not observe such decline in melanic mutants of *C. analis* infected with the wCana1 (accession no. AB545608) strain of *Wolbachia*, which is CI-free, and as *C. chinensis* is infected with a CI-inducing strain of *Wolbachia* (Kondo et al., 2002), we suspected that the decline is caused by an association between *Wolbachia*'s parasitism and host mutation. To investigate this association, we determined host fitness traits such as fecundity, longevity, and hatchability in *C. analis* mutants with melanic bodies, and compared those traits between uninfected and infected mutant hosts with the CI-inducing wCana2 (accession no. LC090027) strain of *Wolbachia*. We hypothesized that fitness decline in infected melanic hosts may offset the reduction of uninfected hosts caused by CI. We built a mathematical model of the spread of the *Wolbachia* infection to examine whether *Wolbachia* can invade a host population with melanic mutants (see the supplementary information).

Materials and methods

Source populations

Callosobruchus analis is a pest of stored legumes (Haines, 1989). Eggs are laid on the seed surface and hatched larvae grow inside a seed. As larvae exhibit contest-type resource competition (Toquenaga & Fujii, 1990; Mano et al., 2002), i.e. only one adult can emerge from a seed with multiple eggs, the population size of *C. analis* is small. A population of *C. analis* was derived from infested brown rice beans, *Vigna umbellata* (Thunb.) Ohwi & H. Ohashi (Fabaceae), at a market in 2005 in Colombo, Sri Lanka (S line). It is maintained on mung beans, *Vigna radiata* (L.) R. Wilczek, under laboratory conditions (30 °C, 60-70% r.h., L24:D0) with about 180 adults per generation. The S line is naturally infected with a CI-free wCana1 (accession no. AB545608) strain of *Wolbachia* (Kageyama et al., 2010; Kondo et al., 2011). At least 30 generations after the establishment of the S line, two mated females with the melanic mutation were found (see Figure S1). All their offspring exhibited normal body color. Two females of the normal-colored offspring were crossed with two mutant males that were newly found. Their offspring exhibited both melanic and normal body colors; one normal-colored female produced 29 mutants out of 62 offspring and the other produced 28 mutants out of 50 offspring. This indicates that the mutation was controlled by a single

recessive autosomal gene, establishing the mutant line as the melanic S (*b/b*) line. Wild type (*B/B*) and heterozygous (*B/b*) individuals exhibited normal body color.

Antibiotic treatment

Wolbachia was removed from the S line using a tetracycline (TC) treatment. Larvae from the S line were fed with artificial beans containing 0.03% (wt/wt) TC. The artificial beans were made by mixing and kneading mung bean powder and TC, and pressing them to the bean size with a pollen press (6 mm diameter, 5 mm long). Artificial seed coats were added by dipping the artificial beans in collodion. Inseminated females from the S line were maintained and allowed to oviposit on the artificial beans. Emerged females were used to establish iso-female lines and were checked for infection using molecular identification of the wCana1 strain of *Wolbachia* with specific primers for the *wsp* gene as noted below ('Molecular identification of *Wolbachia*'). Uninfected females used to establish the non-infected (W-) line with *B/b* and *b/b* had been maintained in the laboratory for at least 21 generations.

Microinjection of *Wolbachia*

Wolbachia pellets (Braig et al., 1994; Grenier et al., 1998) were prepared by obtaining singly infected hosts with a wCana2 (accession no. LC090027) strain of *Wolbachia* as donors. The donor line singly infected with the wCana2 strain was established previously by injecting *Wolbachia* pellets, including both wCana1 and wCana2 strains from the Indonesian population of *C. analis*, into the uninfected S line. One injected host, which exhibited a single-strain wCana2 infection, was used to establish an iso-female S line. For the preparation of *Wolbachia* pellets, the whole body of the donor pupa was put into a 1.5-ml microtube and homogenized in 200 µl of 30% sucrose in PBS (1.9 mM NaH2PO4, 8.1 mM Na2HPO4, 175 mM NaCl, pH 7.4). The homogenate was centrifuged at 1 509 ***g*** for 2 min to remove cellular debris. The supernatant was again centrifuged to remove any remaining debris. The supernatant then was centrifuged at 4 731 ***g*** for 5 min to pellet the *Wolbachia*. The resulting pellet was suspended in 5 µl of PBS buffer by gently pipetting the solution on ice. *Wolbachia* pellet suspension (1 µl) was microinjected directly in the ventral region between the thorax and abdomen of each uninfected pupa using glass needles with manually cut tips. After injections, emerged virgin females were mated with untreated males. We established an infected iso-female line (W+) by isolating a generation-one (G1) female that was infected. The single infection with the wCana2 strain of *Wolbachia* was checked using molecular identification with specific primers for the *wsp* gene as noted in the following section.

Molecular identification of *Wolbachia*

Infection status was confirmed before crossing experiments. For DNA extraction, live insects were first preserved in acetone (99.5%). One hind leg for each sample was put into a 0.2-µl plastic tube with 100 µl lysis buffer (1 mM EDTA, 10 mM Tris-HCl, 50 mM NaCl) and proteinase K, incubated at 55 °C for 1 h and 99 °C for 10 min, and preserved at 4 °C. The supernatant was used as the template in subsequent PCR reactions. PCR detection of *Wolbachia* from total DNA of *C. analis* was conducted using GoTaq hot-start green master mix (Promega) with specific primers for the *wsp* gene. A 361-bp fragment of the *wsp* gene in a wCana1 strain was amplified using Cana1F [5'-GCCTGCAGTACAATGGTGAA-3'] and Cana1R [5'-TGATCCTTAACTGCGTCAGC-3'] under a temperature profile of 95 °C for 10 min followed by 30 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min. The last extension step lasted 5 min at 72 °C. A 333-bp fragment of the *wsp* gene in a wCana2 strain was also amplified using Cana2F [5'-GTTCGTTTGCAATATAATGGTGA-3'] and Cana2R [5'-GCTTACATACGCTGCACCAA-3'] under the same temperature profile. The PCR products were electrophoresed in TAE-agarose gels, stained with SYBR-safe DNA gel stain (Invitrogen, Carlsbad, CA, USA), and observed using a blue light transilluminator (Thermo Fisher Scientific, Inc., Waltham, MA, USA). In order to control for the failure of amplification with the primers, we tested whether the samples scored as negative for primers would result in positive amplification of the host mitochondrial cytochrome oxidase subunit I gene (*COI*) by amplifying a 608-bp fragment of the *COI* gene using CanaCOIF [5'-TCCTTTTATTACTTTCTCTACCCGTTT-3'] and CanaCOIR [5'-TTCCTGTAAATAAGGGGAATCA-3'] under a temperature profile of 95 °C for 10 min followed by 30 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min.

Establishment of infected and uninfected melanic lines

We used uninfected (W-) and wCana2-infected (W+) lines for all three genotypes (*B/b*, *B/B*, and *b/b*) produced as shown in the procedure illustrated in Figure 1. To obtain heterozygous (*B/b*) offspring, wild type (*B/B*) females of W- and W+ lines were crossed with melanic males from the S (*b/b*) line whose wCana1 was CI-free with three replicates each. All first-generation (F1) offspring exhibited a normal body color. Heterozygous F1 females from each replicate were crossed again with the melanic males from the S (*b/b*) line with three replicates. Second generation (F2) offspring included both *B/b* and *b/b* genotypes. The F2 offspring and *B/B* individuals from the W- and W+ lines were used to examine host fitness traits.

Host fitness traits

Fecundity and hatch rates were examined by conducting four reciprocal crossings among *B/b* and *b/b* individuals and one crossing between *B/B* females and males. Fecundity was checked by depositing virgin adults onto beans within 24 h of emergence. Virgin males were individually mated with virgin females. Each of 10 to 24 pairs was set in a 10-cm-diameter dish filled with about 60 mung beans. Males and females were allowed to mate and oviposit until they died. Fecundity was assessed by counting the eggs that a female laid. Hatch rates were checked by counting hatched and unhatched eggs. Longevity of infected and uninfected S lines of all three genotypes was determined by counting days from the adult emergence to its death (13-77 replicates) in absence of food.

Cytoplasmic incompatibility assay

To examine whether the wCana2 strain of *Wolbachia* induced CI or hatch rate reduction, we investigated hatch rates of incompatible (i.e., uninfected female × infected male) parental combinations and of reciprocal combinations for three genotypes (10-15 replicates). Hatch rates were determined using the same procedure noted above. We excluded the crosses with no oviposition.

Statistical analysis

We applied the Bayesian hierarchical model below with a joint scaling method (JSM; Mather & Jinks, 1982; Takano et al., 2001). JSM uses a design matrix (Table 1) to assign additive and dominance effects of a gene (Wolf et al., 2000), and other effects such as maternal and infection effects. For fecundity the following model was applied:

$$\text{Fecundity} \sim \text{Poisson}\left(\mu_i\right)$$

$$M_i = D_{fec,i}\,\beta_{fec}^{t},$$

where $D_{fec,i}$ represents the design matrix for crossing type i as represented in Table 1, β_{fec} is the vector $\beta_{fec} = [\beta_{icp}, \beta_A, \beta_D, \beta_M, \beta_I, \beta_{A\times D}, \ldots \beta_{A\times D\times I}, \ldots \beta_{A\times D\times I\times M}]$ of parameters for the effects of intercept (icp), additive (A), dominance (D), maternal (M), infection (I), and their interaction effects. μ_i is the mean fecundity of crossing type i. Priors were assigned to βs and σ: Normal$(0,\sigma^2)$ and Uniform(0,100) (Gelman & Hill, 2006).

We applied the following model for hatch rates;

$$\text{hatched eggs} \sim \text{Binomial}(p_i, t)$$

$$\text{logit}(p_i) = D_{h,i}\,\beta_h^t,$$

where p_i is the hatching probability when crossing type i, t is the total number of eggs deposited, $D_{h,i}$ represents a part of the design matrix for crossing type i, as in Table 1, and β_h is the vector $\beta_h = [\beta_{icp}, \beta_A, \beta_D, \beta_M, \beta_I, \beta_{A\times D}, ...]$ of parameters for the effects as explained above. We assigned Normal(0, σ^2) to each β and Uniform(0, 100) to σ.

For longevity the following model was applied:

$$\text{longevity} \sim \text{Normal}(\mu_l, \sigma^2)$$

$$\mu_l = \text{intercept} + \beta_G + \beta_I + \beta_s + \beta_{G\times I} + \beta_{G\times S} + \beta_{I\times S},$$

where βs represents host genotype (G), infection (I), and sex (S) effects and their interaction effects. μ_l is mean longevity. Normal(0, σ^2) and Uniform(0,100) were assigned to β and σ, respectively.

Samplings from posterior distributions of the parameters using Markov Chain Monte Carlo (MCMC) methods were performed using the R2jags package (Su & Yajima, 2015) on R v.3.3.1 (R Core Team, 2016). The posterior samples were obtained by running 10 000 iterations (the first 5 000 iterations were discarded as a burn-in) for each of four independent MCMC chains. The convergence of MCMC calculations was confirmed by evaluating the results of Gelman and Rubin's convergence diagnostic (Gelman & Rubin, 1992) for each parameter by comparing within-chain and between-chain variances. We also calculated R^2, the proportion of variance explained by the hierarchical model at the data level (Gelman & Hill, 2006).

Results

Declines in host fecundity, fertility, and longevity were observed when mutant hosts were infected with the wCana2 strain of *Wolbachia*. Both wild type and heterozygous parents showed few differences in fecundity between infected and uninfected groups (Figure 2). However, reduced fecundity was observed in infected hosts compared to uninfected hosts when *b/b* parents were included. In particular, infected *b/b* females produced fewer eggs than the others. The mean fecundity was eight and 69 eggs, respectively, in infected and uninfected *b/b* × *b/b* (female × male) crosses, and six and 69 eggs, respectively, in infected and uninfected *b/b* × *B/b* crosses. A slight reduction in fecundity was also observed in uninfected *b/b* mutants compared to the other uninfected groups. Estimated parameters for the examined effects of fecundity are shown in Figure 3. Negative values of estimates

indicate reduced host fecundity. The parameter for the intercept effect was more positive than the others (95% CrI: 3.2–5.5). Effects of infection (I) and its interaction with additive (A×I) were distributed positively (95% CrI: -0.2–2.1 for I, -0.1–2.1 for A×I), but its interaction effect with maternal (M×I) was distributed negatively (-4.5– -0.1). Thus, the loss of fecundity in the infected *b/b* females was caused by the M×I effect.

Reduced hatch rates were observed when parents were infected with *Wolbachia* compared to the hatch rates of uninfected parents (Figure 4). Mutant *b/b* females exhibited lower hatch rates than the others when they were infected with the wCana2 strain of *Wolbachia*. The mean hatch rate was 0.2 and 0.7, respectively, in infected and uninfected *b/b* × *b/b* (female × male) crosses, and 0.4 and 0.8, respectively, in infected and uninfected *b/b* × *B/b* crosses. The distributions of parameters for host hatch rates are shown in Figure 5. The parameters for the I and M×I effects were distributed negatively (95% CrI: -1.1–0.1 for I, -3.8– -1.4 for M×I) whereas positive distributions were observed for the effects of intercept, A×I (0.2–1.4) and D×I (1.3–2.7). The hatch rate reduction observed in infected compared to uninfected hosts was caused by the I effect. The M×I effect contributed negatively to hatch rates when female parents were infected *b/b* mutants. The M effect partly contributed to the hatch rate reduction (95% CrI: -3.4–0.8), indicating that the maternal *b* allele also causes reduction independently of host infection status.

Both infected and uninfected males of *B/B* and *B/b* had shorter longevity than females (Figure 6). The mutant *b/b* hosts had more reduced longevity than the others, especially when hosts were *Wolbachia* infected. The mean longevity was 4 days in both infected *b/b* females and males. Estimated parameter distributions for host longevity are shown in Figure 7. Most of the parameters were distributed positively, but negative distributions were observed for the *b/b* effect (95% CrI: -7.6–1.1) and its interaction effects with female (*b/b*×female: -6.7–1.3) and infection (*b/b*×I: -9.3– -1.1). Thus, host longevity was shortened by the effects of *b/b*, *b/b*×female, and *b/b*×I.

Cytoplasmic incompatibility assays revealed that the wCana2 strain of *Wolbachia* induced almost complete CI ($R^2 = 0.99$; Figure 8) with a mean hatch rate of 0. Note, however, that non-CI-occurring parental combinations (i.e., infected females × uninfected males) also exhibited hatch rate reduction (mean hatch rate: 0.2) when host genotypes were *b/b*. This is consistent with the results described above that infected *b/b* females had reduced hatch rates. The parameter estimation for the effects of CI is shown in Figure 9. Negatively distributed parameters of I (95% CrI: -8.3– -1.9) and D×I (-3.1–0.1) effects indicate reduced hatch rates due to these effects. The interaction effect of A×I was distributed positively (95% CrI: -0.7–

5.7).

Discussion

The combination of CI-inducing *Wolbachia* and host mutation of body color affected host fitness traits negatively. When infected with the wCana2 strain of *Wolbachia*, *b/b* mutants had reduced fecundity, fertility, and longevity. The fitness decline indicates that the *b/b* mutant hosts infected with wCana2 cannot survive in nature. The fitness decline observed in the infected *b/b* mutant hosts was caused by the interaction of hosts and *Wolbachia* because the decline was accompanied by the combination of host mutation and the infection of CI-inducing *Wolbachia*. CI assay revealed that the reduction in hatch rates occurred not only in the incompatible parental combinations but also in compatible combinations when hosts had *b/b* genotypes. Although the number of infected hosts was reduced by the infected *b/b* females, the model for *Wolbachia* infection spread suggested that the host *b/b* mutation could not stop the *Wolbachia* invasion (see the supplementary information).

Some studies have reported that mutations of melanic body color, controlled by a single recessive autosomal gene, are associated with a fitness decline in other Coleoptera species. Ebony mutants in the flour beetle, *Tribolium confusum* Jaquelin Du Val, exhibit reduced fecundity compared to heterozygous and wild type adults (Park et al., 1945). Black mutants in the Colorado potato beetle, *Leptinotarsa decemlineata* (Say), exhibit low egg viability (Boiteau, 1985). In *C. chinensis*, declines in fecundity and fertility have also been reported (Kashiwagi & Utida, 1972). The fitness decline is accompanied by a mutation of melanic body color independently of CI-inducing *Wolbachia* infection because even uninfected mutants in *C. analis* had a slight decline in fitness traits (cf. uninfected *b/b* females in Figures 2 and 4; uninfected *b/b* in Figure 6:). Possibly, *Wolbachia* contributes to a worsening of the fitness decline caused by the *b/b* genotype in *C. analis*. Starr & Cline (2002) reported that host fitness was modified by *Wolbachia* infection. They found that removing *Wolbachia* induced an oogenesis deficit in *Drosophila* hosts and concluded that *Wolbachia* remedied the deficit. Although we did not examine oogenesis in *C. analis*, fecundity reduction in infected *b/b* females suggests that *Wolbachia* contributes to fecundity reduction. In this light, our results provide an example of the *Wolbachia* association with host oogenesis that contrasts with that reported by Starr & Cline (2002).

As the wCana2 strain induced CI in hosts, we emphasize that the reduction in examined host traits was caused by CI-inducing *Wolbachia*. The *b/b* mutants in *C. analis*

were first discovered in hosts originally infected with the wCana1 strain of *Wolbachia*. As the wCana1 strain was CI-free, the host fitness decline had an association with CI induced by *wCana2* in *C. analis*. The relative percentage of the *b* allele can become high in a small population due to random drift. However, although the population size of *C. analis* in nature is thought to be small, the frequency of the *b* allele seems to be low: all of our *C. analis* lines obtained in nature exhibited an initially normal body color. Even if *b/b* mutants are fixed in a host population, incomplete sterility of infected *b/b* females produces a fraction of infected zygotes and the sterility reduces the fraction of infected *b/b* zygotes. Because of the reduction of infected *b/b* zygotes, *Wolbachia* can easily overcome the invasion threshold in the following host generation, leading to the fixation of *Wolbachia*. If the sterility of infected *b/b* females was complete and the *b/b* mutation was fixed in a host population by random drift (i.e., all hosts showed the *b/b* genotype) *Wolbachia* would not be able to invade the host population because infected *b/b* females would not produce viable infected offspring.

How the wCana1-infected hosts prevent the wCana2 invasion is unclear, because the sterility of infected *b/b* females is incomplete, meaning that it cannot be an antagonism against CI-inducing *Wolbachia* in *C. analis.* One possible explanation is that the wCana1 strain was once CI-inducing *Wolbachia*, as is the wCana2 strain, and that CI was moderated after the wCana1 fixation by the host during the coevolutionary history between host and *Wolbachia*. Indeed, host infection experience has been reported to have an effect on the reduction of CI intensity (Poinsot & Merçot, 2001). The wCana1-infected hosts may be eliminated by the wCana2-infected hosts in the future.

In conclusion, we found that *C. analis* body-color mutants exhibited a fitness decline in fecundity, fertility, and longevity when hosts were infected with a CI-inducing *Wolbachia* strain. The decline caused by infected *b/b* hosts cannot stop a *Wolbachia* invasion unless the sterility of infected *b/b* females is complete and the melanic mutants are fixed in a host population. If the CI-free wCana1 strain was caused by CI moderation during the coevolutionary history, the wCana2 strain may also become CI-free. To confirm the role of coevolutionary history in the ability of *Wolbachia* to induce CI, further investigations are needed.

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Figure captions

Figure 1 Experimental flow for the preparation of wCana2-infected (W+) and uninfected (W-) host lines with three genotypes. The recessive melanic mutation of body color is expressed as *b/b*. Wild type (*B/B*) and heterozygous (*B/b*) individuals exhibited normal body color. Wild type individuals of the W-line were established using antibiotic treatment for the wild type S line. Some of these were injected with the wCana2 strain of *Wolbachia* to establish the wild type W+ line. These *B/B* females were crossed with the mutant *b/b* males from the S line to obtain *B/b* offspring. The heterozygous first generation (F1) females were crossed again with the *b/b* males and the second generation (F2) *B/b* and *b/b* offspring were obtained. For experiments we used the six shaded host lines: W- (*B/B*, *B/b*, *b/b*) and W+ (*B/B*, *B/b*, *b/b*).

Figure 2 Estimated fecundity (total no. eggs produced) is shown for the assigned parental

combinations ($R^2 = 0.83$). Three genotypes represent wild type (*B/B*), heterozygous (*B/b*), and melanic mutant (*b/b*) parents. Grey boxes indicate the *Wolbachia* infection and the others are infection-free. The thick lines represent the median value and the limits of the box represent inter-quartile range. The whiskers outside the box extend to the highest and lowest values within 1.5 times the inter-quartile range. Points outside the whiskers are outliers. Numbers along the x-axis indicate replicates.

Figure 3 Estimated parameter distributions for the effects listed along the y-axis. Circles represent means of the estimated posteriors. The 50% (thick line) and 95% (thin line) posterior credible intervals are shown. Negative values represent reduced fecundity.

Figure 4 Estimated hatch rates of offspring from the assigned parental combinations ($R^2 = 0.97$). Three genotypes represent wild type (*B/B*), heterozygous (*B/b*), and melanic mutant (*b/b*) parents. Grey boxes indicate the *Wolbachia* infection and the others are infection-free. The thick lines represent the median value and the limits of the box represent inter-quartile range. The whiskers outside the box extend to the highest and lowest values within 1.5 times the inter-quartile range. Points outside the whiskers are outliers. Numbers along the x-axis indicate replicates.

Figure 5 Estimated parameter distributions for the effects listed along the y-axis. Circles represent means of the estimated posteriors. The 50% (thick line) and 95% (thin line) posterior credible intervals are shown. Negative values represent reduced hatch rates.

Figure 6 Estimated longevity of adult beetles ($R^2 = 0.86$). Three genotypes represent wild type (*B/B*), heterozygous (*B/b*), and melanic mutant (*b/b*) parents. The thick lines represent the median value and the limits of the box represent inter-quartile range. The whiskers outside the box extend to the highest and lowest values within 1.5 times the inter-quartile range. Points outside the whiskers are outliers. Numbers along the x-axis indicate replicates.

Figure 7 Estimated parameter distributions are shown for the effects of genotype, infection, and sex. Circles represent means of the estimated posteriors. The 50% (thick line) and 95% (thin line) posterior credible intervals are shown. Negative values represent reduced longevity.

Figure 8 Estimated hatch rates in offspring from parents with assigned genotypes ($R^2 = 0.99$).

Three genotypes represent wild type (*B/B*), heterozygous (*B/b*), and melanic mutant (*b/b*) parents. Parents of infected (W+) females and uninfected (W-) males are non-CI occurring combinations, whereas the opposite combinations have CI. The thick lines represent the median value and the limits of the box represent inter-quartile range. The whiskers outside the box extend to the highest and lowest values within 1.5 times the inter-quartile range. Points outside the whiskers are outliers. Numbers along the x-axis indicate replicates.

Figure 9 Estimated parameter distributions for the effects listed along the y-axis. Circles represent means of the estimated posteriors. The 50% (thick line) and 95% (thin line) posterior credible intervals are shown. Negative values represent reduced hatch rates.

Supporting Information

Additional Supporting Information may be found in the online version of this article.

Model for *Wolbachia* infection spread

Results for infection dynamics

Figure S1 Females (above) and males (below) of normal and melanic colored *Callosobruchus analis*.

Figure S2 Infection status of zygotes. p is the proportion of infected hosts, q and r are frequencies of allele *b* in infected and uninfected hosts, respectively. Each box represents the relative proportion of zygotes produced by random mating between host females and males. Numbers in the boxes represent the *b/b* proportion among zygotes. The status of female parents is represented along the vertical axis and that of the males along the horizontal axis. s and β are cytoplasmic incompatibility (CI) intensity and sterility level of *b/b* females, respectively. Zygotes fail to develop with the presence of CI and with *b/b* females. *B/B*, wild type; *B/b*, heterozygotes; *b/b*, melanic mutant.

Figure S3 Parameter space of Δp when (A) $s = 0.5$ and (B) $b = 0.5$. Combinations of (p,q) below the various dotted lines satisfy the condition to stop the *Wolbachia* invasion ($\Delta p \leq 0$). p is the frequency of infected zygotes, q is the frequency of the *b* gene in the infected zygotes, and s is the cytoplasmic incompatibility intensity (offspring survival rate).

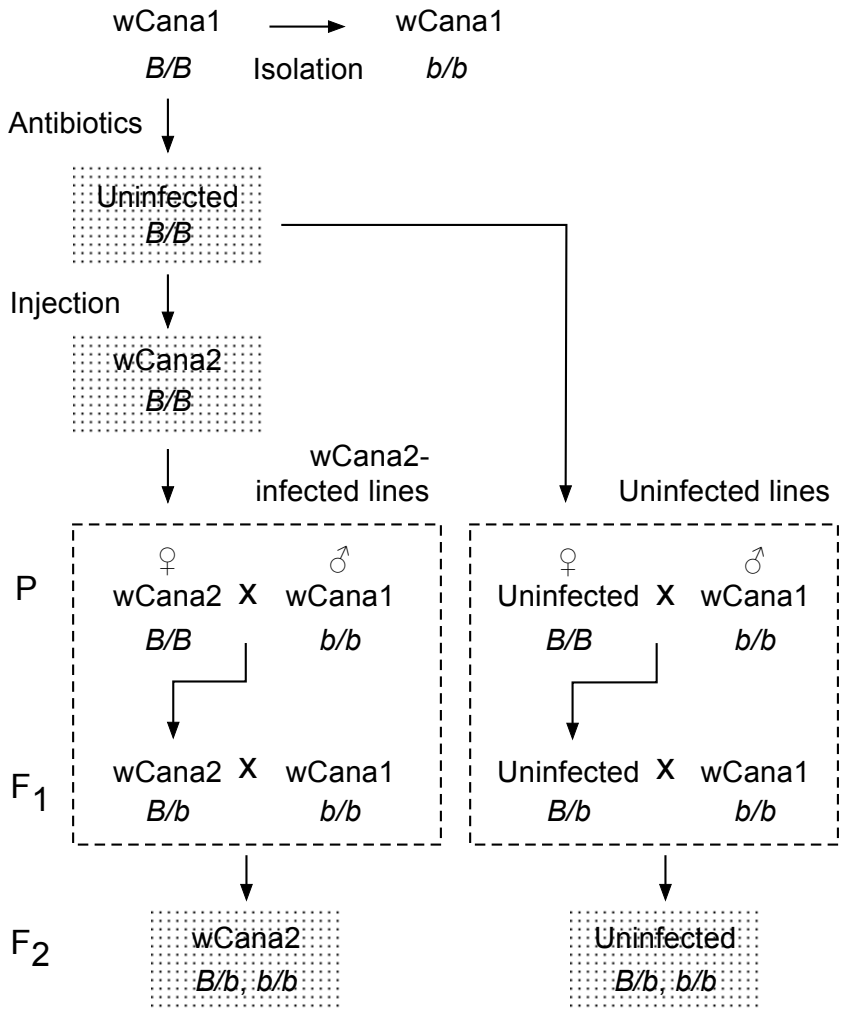
Figure S4 Parameter space of Δq ($b = 0.8$, $s = 0.5$). Combinations of (p,q,r) below dotted lines satisfy the condition $\Delta q \geq 0$.

Table 1 Design matrix for joint scaling method

Status	Cross type (female × male)	Intercept	A	D	M	I	A×D	A×I	A×M	D×I	D×M	I×M	A×D×I	A×D×M	A×I×M	D×I×M	A×D×I×M
Uninfected	*b/b* × *b/b*	1	1	0	1	0	0	0	1	0	0	0	0	0	0	0	0
	b/b × *B/b*	1	1/2	1/2	1	0	1/4	0	1/2	0	1/2	0	0	1/4	0	0	0
	B/b × *b/b*	1	1/2	1/2	1/2	0	1/4	0	1/4	0	1/4	0	0	1/8	0	0	0
	B/b × *B/b*	1	0	1/2	1/2	0	0	0	0	0	1/4	0	0	0	0	0	0
	B/B × *B/B*	1	-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Infected	*b/b* × *b/b*	1	1	0	1	1	0	1	1	0	0	1	0	0	1	0	0
	b/b × *B/b*	1	1/2	1/2	1	1	1/4	1/2	1/2	1/2	1/2	1	1/4	1/4	1/2	1/2	1/4
	B/b × *b/b*	1	1/2	1/2	1/2	1	1/4	1/2	1/4	1/2	1/4	1/2	1/4	1/8	1/4	1/4	1/8
	B/b × *B/b*	1	0	1/2	1/2	1	0	0	0	1/2	1/4	1/2	0	0	0	1/4	0
	B/B × *B/B*	1	-1	0	0	1	0	-1	0	0	0	0	0	0	0	0	0

Effects: A, additive; D, dominance; M, maternal; I, infection.

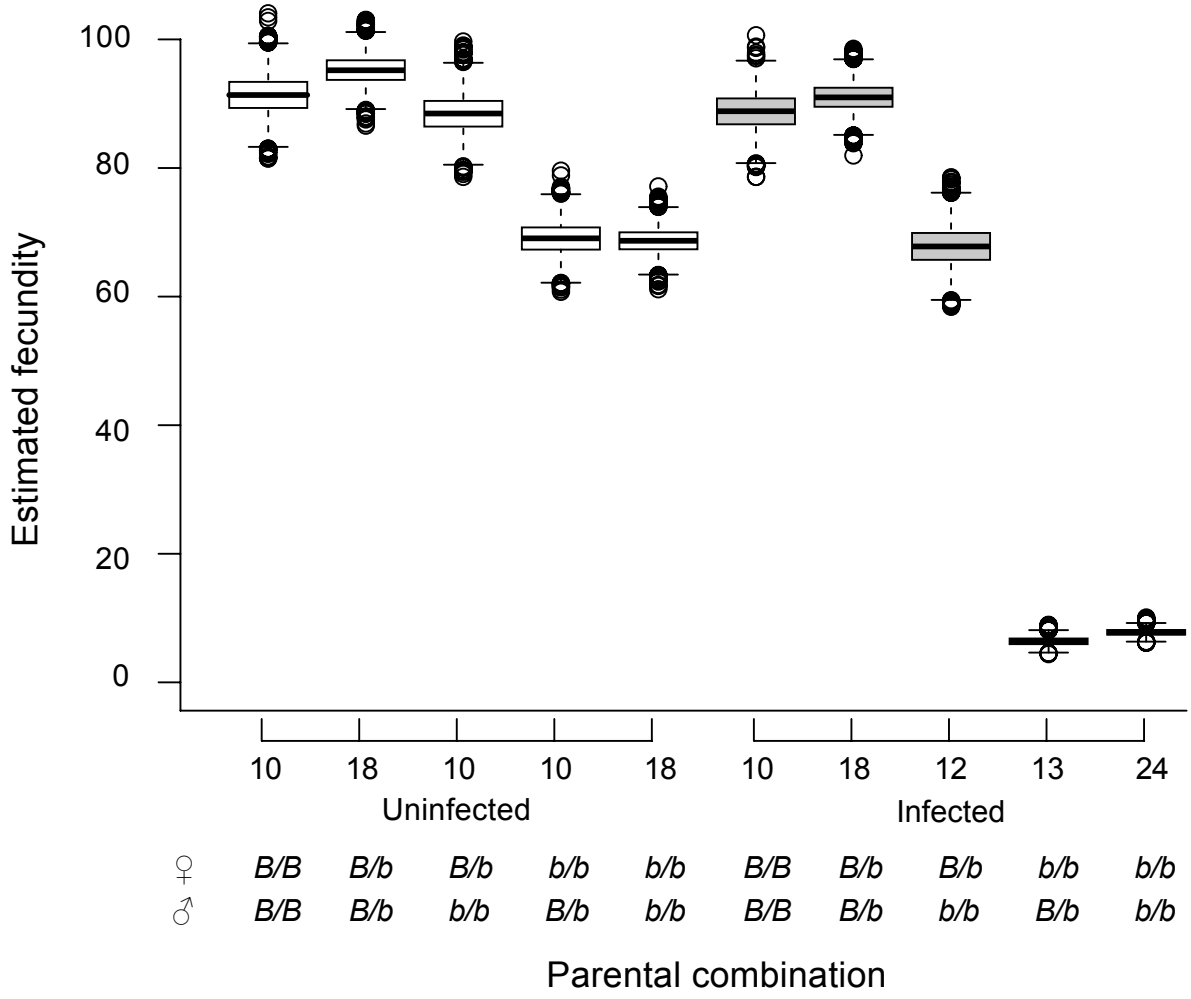
1 Figure 1


wCana1
B/B
Isolation
wCana1
b/b
Antibiotics
Uninfected
B/B
Injection
wCana2
B/B
wCana2-
infected lines
Uninfected lines
P
♀
♂
wCana2 X wCana1
B/B b/b
Uninfected X wCana1
B/B b/b
F1
wCana2 X wCana1
B/b b/b
Uninfected X wCana1
B/b b/b
F2
wCana2
B/b, b/b
Uninfected
B/b, b/b

2

3

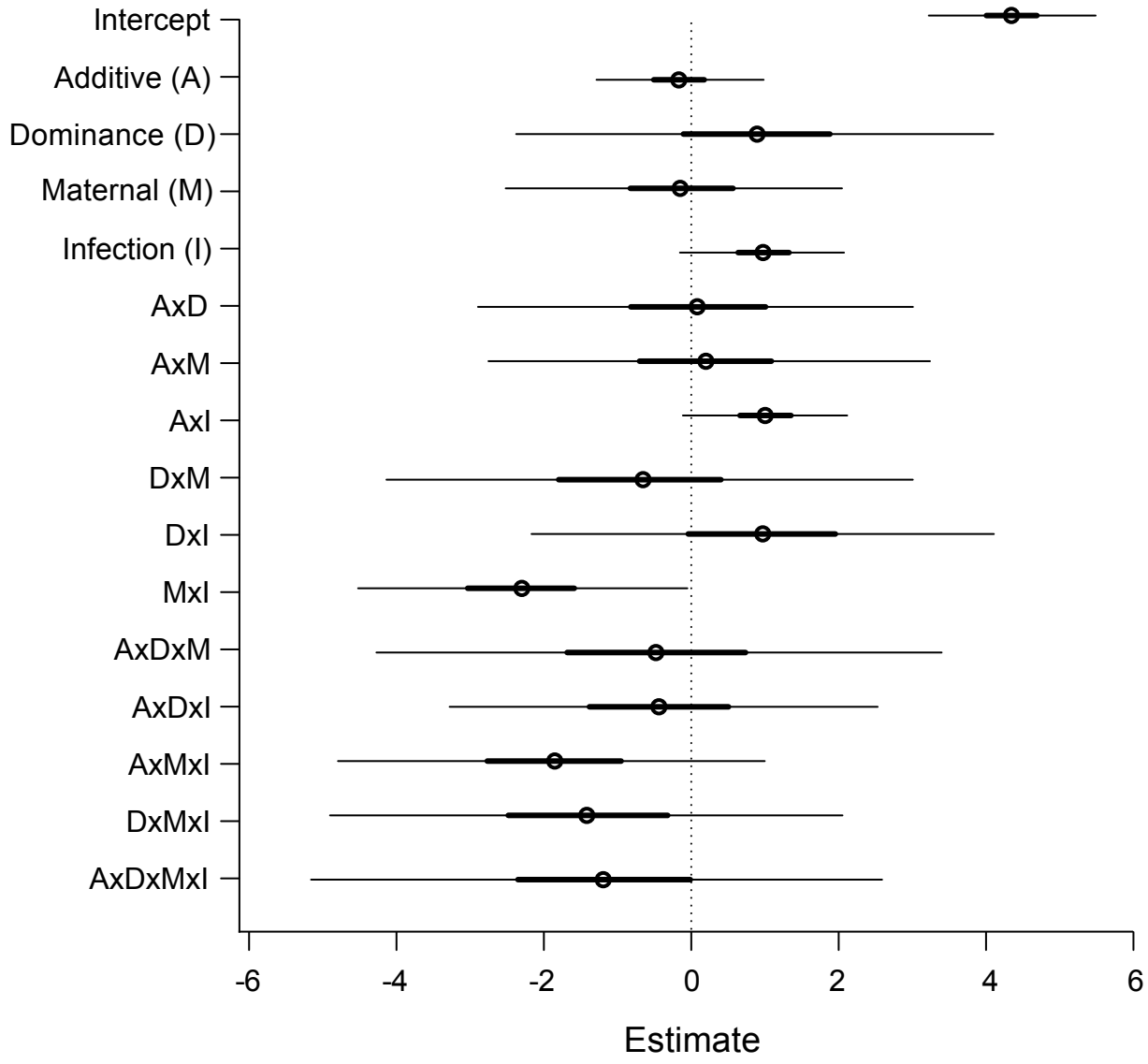
1 Figure 2


Estimated fecundity
100
80
60
40
20
0
10 18 10 10 18 10 18 12 13 24
Uninfected
Infected
♀ B/B B/b B/b b/b b/b B/B B/b B/b b/b b/b
♂ B/B B/b b/b B/b b/b B/B B/b b/b B/b b/b
Parental combination

2

3

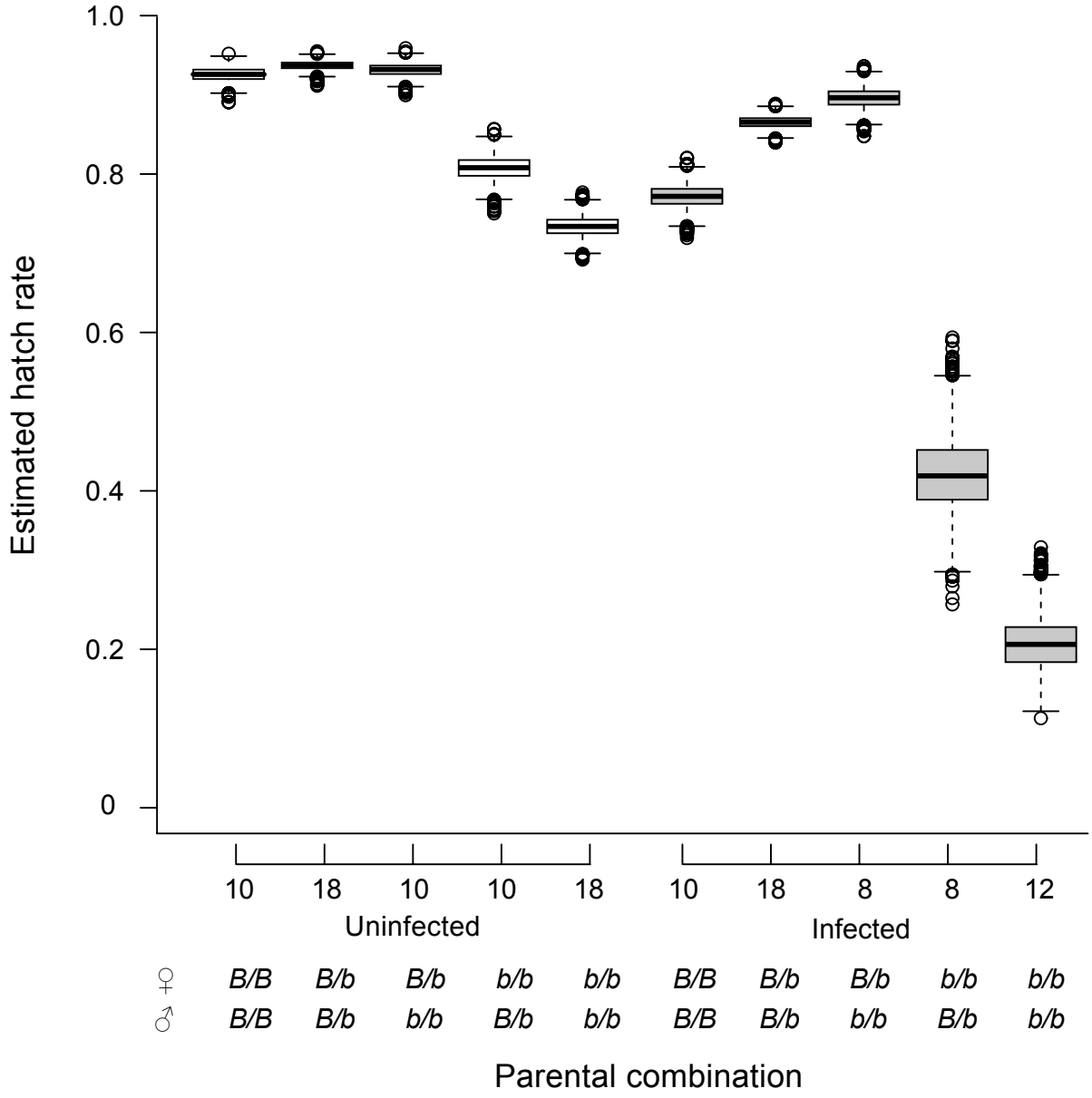
1 Figure 3


Intercept
Additive (A)
Dominance (D)
Maternal (M)
Infection (I)
AxD
AxM
AxI
DxM
DxI
MxI
AxDxM
AxDxI
AxMxI
DxMxI
AxDxMxI
-6
-4
-2
0
2
4
6
Estimate

2

3

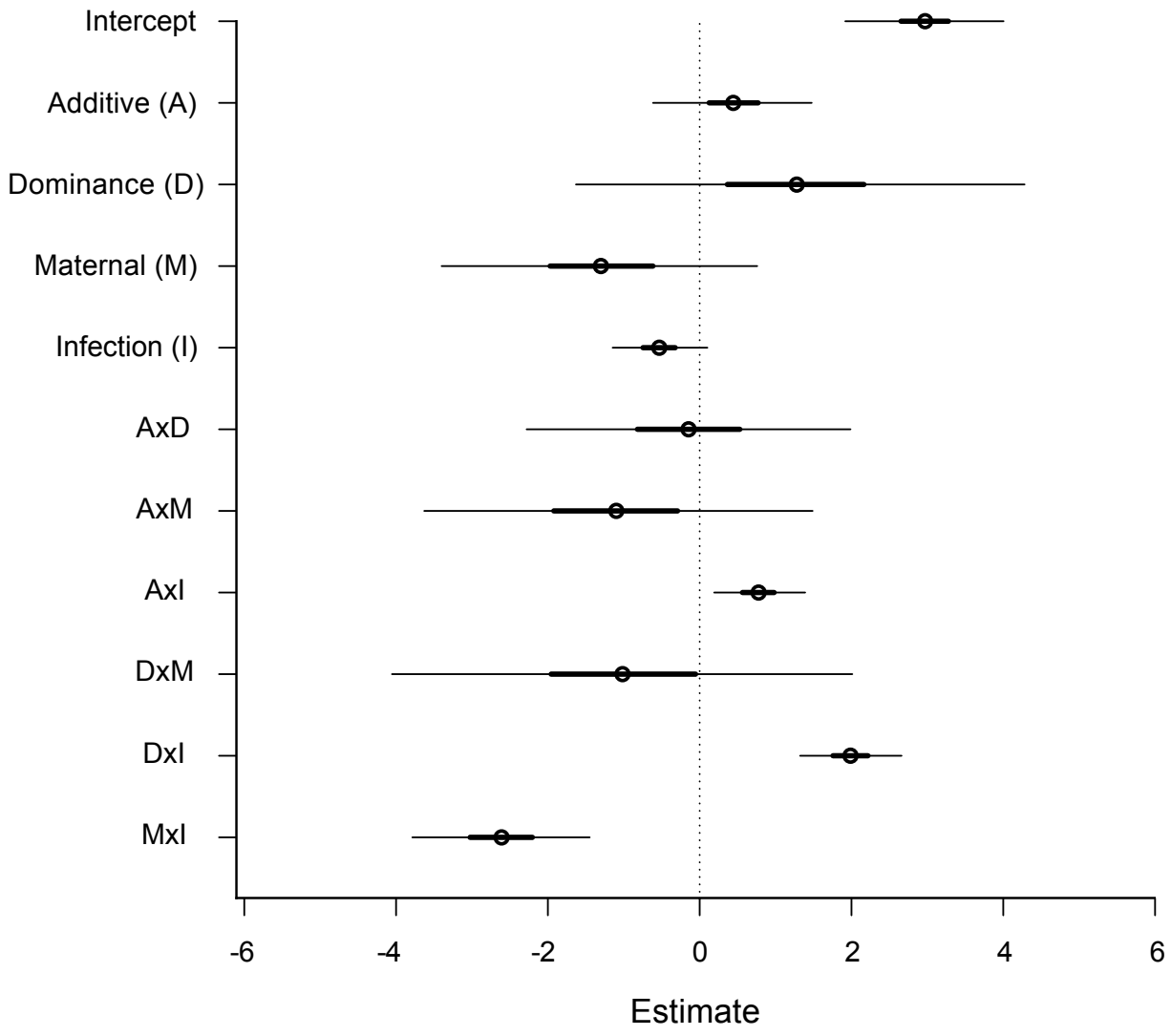
1 Figure 4


1.0
0.8
0.6
0.4
0.2
0
Estimated hatch rate
10 18 10 10 18 10 18 8 8 12
Uninfected
Infected
♀ B/B B/b B/b b/b b/b B/B B/b B/b b/b b/b
♂ B/B B/b b/b B/b b/b B/B B/b b/b B/b b/b
Parental combination

2

3

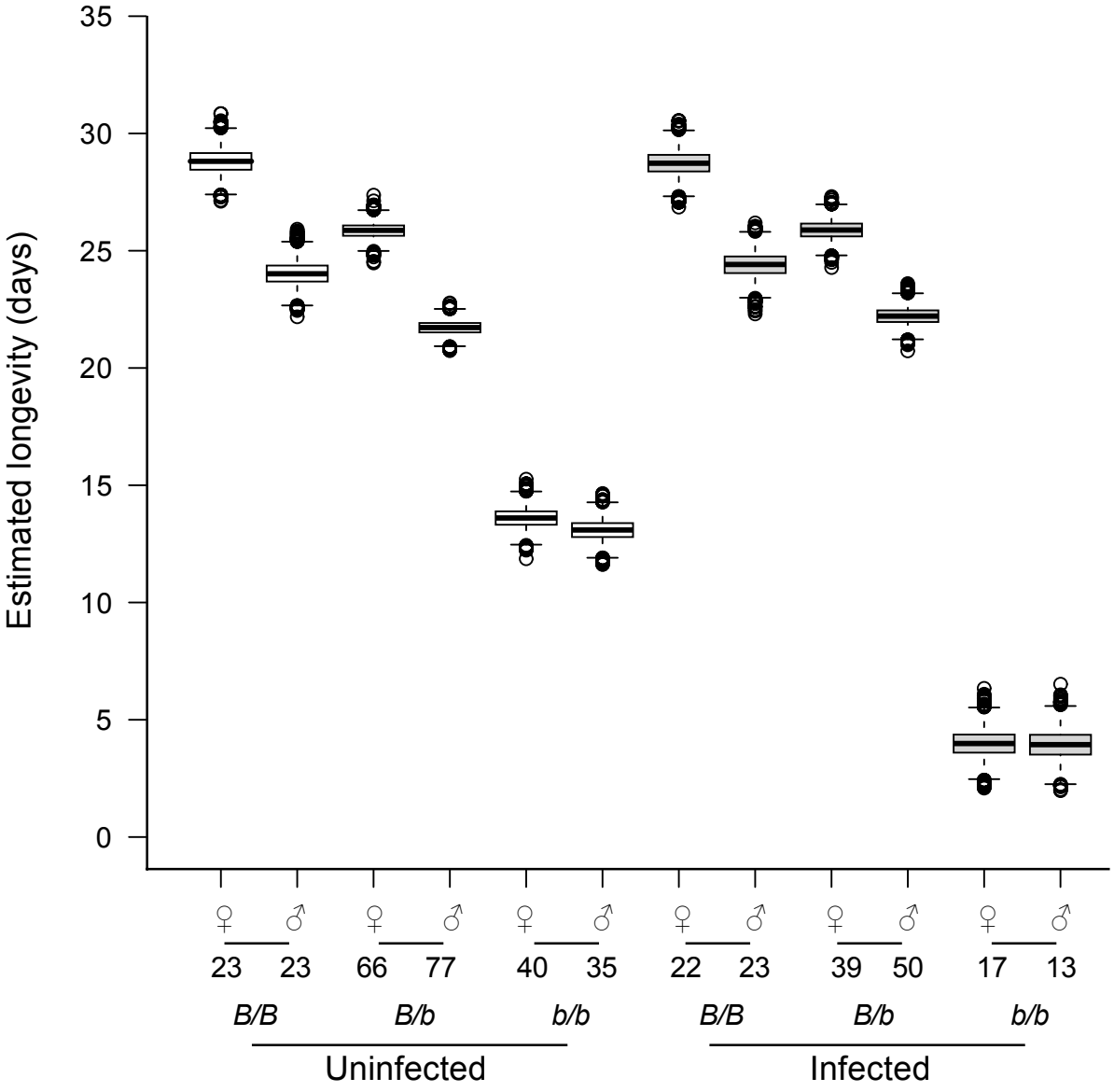
1 Figure 5


Intercept
Additive (A)
Dominance (D)
Maternal (M)
Infection (I)
AxD
AxM
AxI
DxM
DxI
MxI
-6
-4
-2
0
2
4
6
Estimate

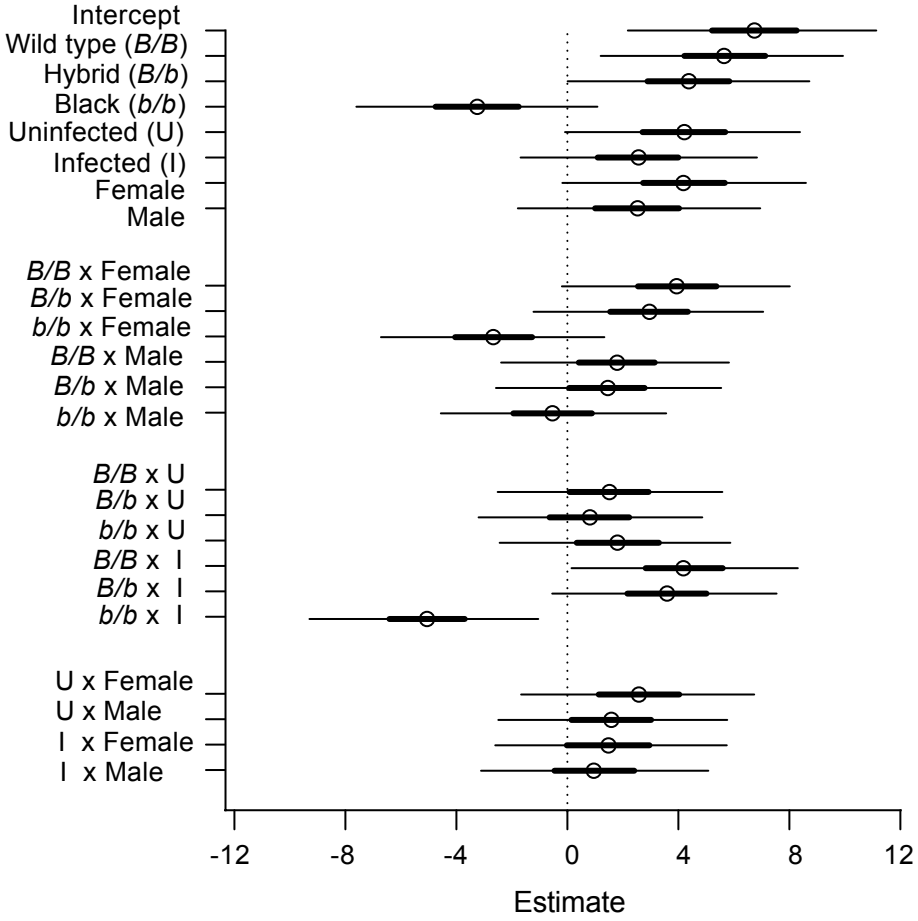
2

3

Figure 6


Estimated longevity (days)
35
30
25
20
15
10
5
0
♀ ♂ ♀ ♂ ♀ ♂ ♀ ♂ ♀ ♂ ♀ ♂
23 23 66 77 40 35 22 23 39 50 17 13
B/B B/b b/b B/B B/b b/b
Uninfected
Infected

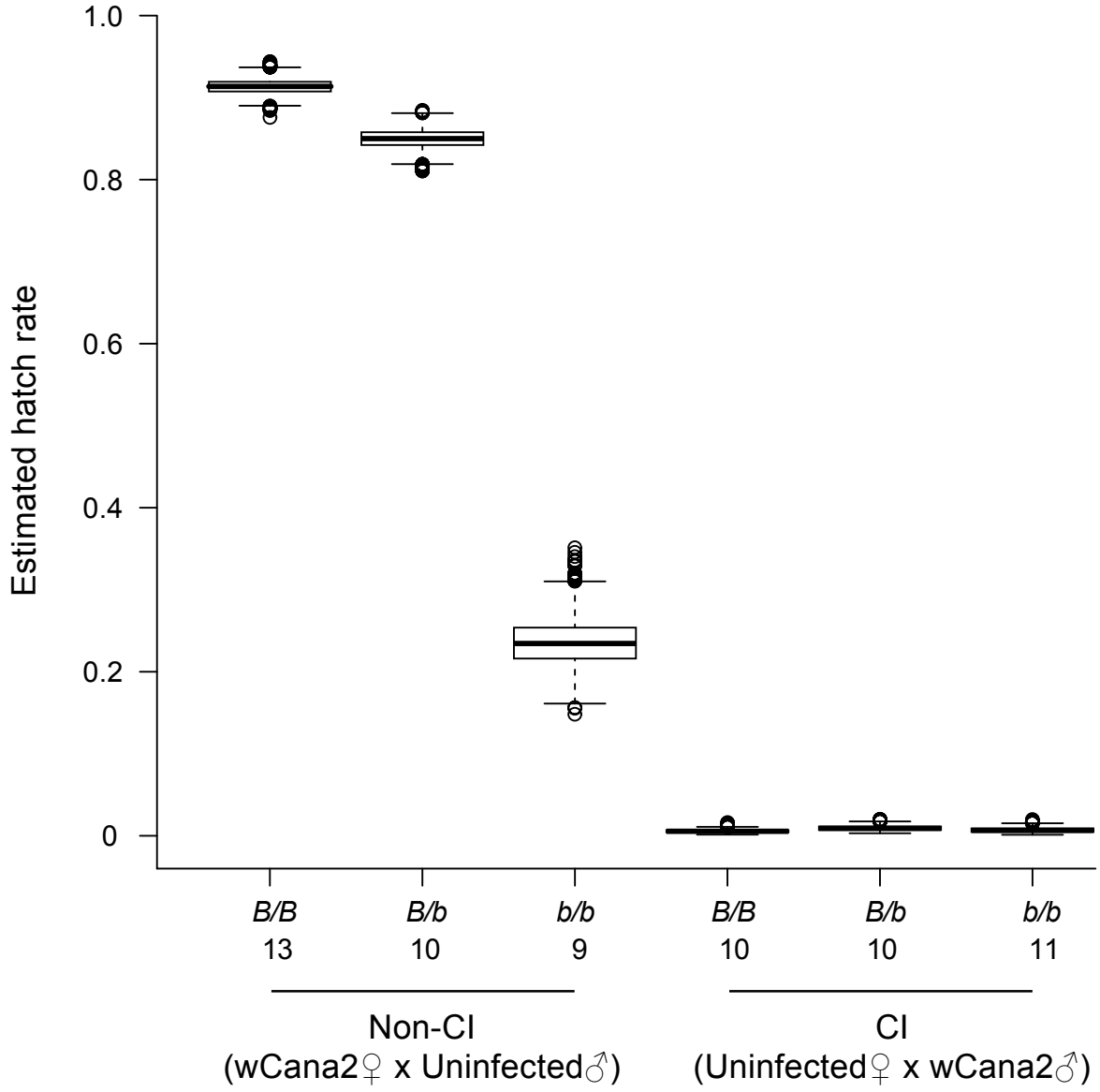
1 Figure 7


Intercept
Wild type (B/B)
Hybrid (B/b)
Black (b/b)
Uninfected (U)
Infected (I)
Female
Male
B/B x Female
B/b x Female
b/b x Female
B/B x Male
B/b x Male
b/b x Male
B/B x U
B/b x U
b/b x U
B/B x I
B/b x I
b/b x I
U x Female
U x Male
I x Female
I x Male
-12
-8
-4
0
4
8
12
Estimate

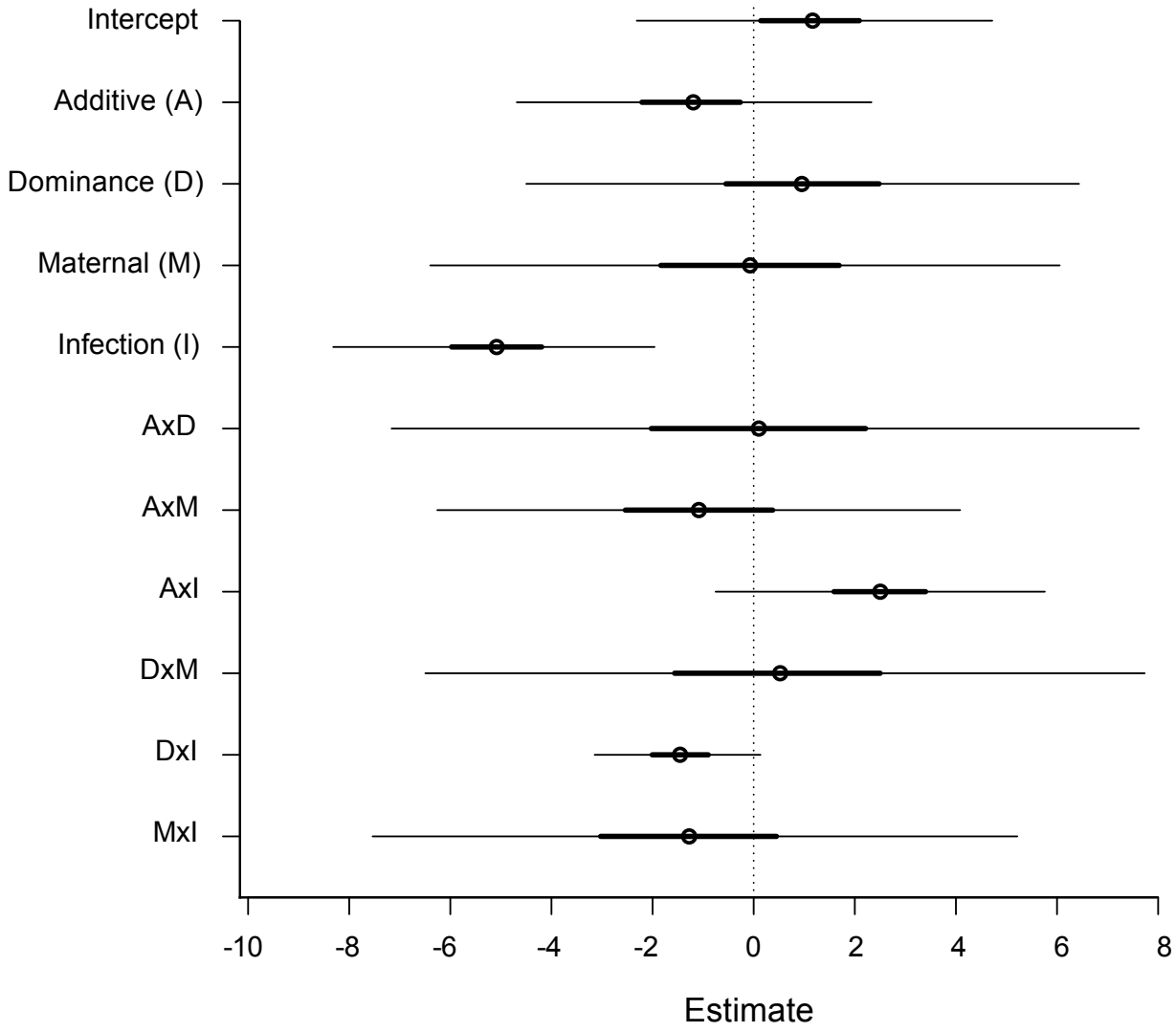
2

3

Figure 8


1.0
0.8
0.6
0.4
0.2
0
Estimated hatch rate
B/B
13
B/b
10
b/b
9
B/B
10
B/b
10
b/b
11
Non-CI
(wCana2♀ x Uninfected♂)
CI
(Uninfected♀ x wCana2♂)

1 Figure 9


Intercept
Additive (A)
Dominance (D)
Maternal (M)
Infection (I)
AxD
AxM
AxI
DxM
DxI
MxI
-10
-8
-6
-4
-2
0
2
4
6
8
Estimate

2

3

Supporting information

Model for *Wolbachia* infection spread

Cytoplasmic incompatibility enables the spread of *Wolbachia* by increasing the relative frequency of infected hosts. However, the sterility of infected *b/b* females reduces infected hosts. We modeled the infection dynamics considering both CI and incomplete *b/b* sterility based on the *Wolbachia* maintenance model proposed by Fine (1978). Figure S2 shows the schematic diagram of the relative proportions of compatible infected, compatible uninfected, and incompatible zygotes produced by a randomly mating population. In the figure, the numeric values inside the boxes represent proportions of *b/b* zygotes. We assumed that the proportion of *Wolbachia*-infected hosts was p_t, and that the gene frequency of the recessive melanic mutant at time t in infected and uninfected hosts was q_t and r_t, respectively. We also assumed that *Wolbachia* transmission failure was negligible but intensity of CI was incomplete ($s \neq 0$). We introduced mortality (β) of *Wolbachia*-infected zygotes that were reproduced by *b/b* females. The frequency of infected zygotes (p_t), and the *b* frequency of infected (q_t) and uninfected (r_t) zygotes can be expressed as follows:

$$p_{t+1} = \frac{p_t\left(1-\beta\, q_t^2\right)}{w_t},$$

$$q_{t+1} = \frac{p_t\left(p_t(q_t-r_t)+q_t+r_t-\beta\, q_t^2(p_t q_t-p_t r_t+r_t+1)\right)}{2w_t},$$

$$r_{t+1} = \frac{(1-p_t)(p_t s(q_t+r_t)+2(1-p_t)r_t)}{2w_t}, \text{ and}$$

$$w_t = 1 - p_t(1-p_t)(1-s) - \beta p_t q_t^2 .$$

Results for infection dynamics

For infection dynamics, we considered difference in p between time t and t+1:

$$\Delta p = p_{t+1} - p_t = \frac{p_t(1-p_t)\left(p_t(1-s)-\beta\, q_t^2\right)}{w_t}.$$

Figure S3 shows the combinations of p_t and q_t that satisfy the condition of $p_t < \beta q_t^2/(1-s)$ where *Wolbachia* invasion is stopped by *b/b* hosts when s = 0.5 (Figure S3A) and b = 0.5 (Figure S3B). If q_t is constant, the above recursion equation has three points of equilibrium: $p_1^* = 0$, $p_2^* = \beta q^2/(1-s)$, and $p_3^* = 1$. Equilibrial points p_1^* and p_3^* are stable, but p_2^* is unstable. For a successful invasion, the initial frequency of *Wolbachia* needed to overcome the invasion threshold, p_2^*. However, as noted above, q_t is not constant. Thus, we considered Δq as well:

$$\Delta q = q_{t+1} - q_t = \frac{\beta\, p_t(2-p)q_t^3 + \beta\, p_t(p_t r_t - r_t - 1)q_t^2 + (p_t - 1)(2p_t s - p_t + 2)q_t + p_t(1-p_t)r_t}{2\left(1 - p_t(1-p_t)(1-s) - \beta\, p_t q_t^2\right)}.$$

Figure S4 shows the combinations of p_t, q_t, and r_t satisfying the condition of $\Delta q \geq 0$. The condition $p_t < \beta q_t^2/(1 - s)$ is easily violated after a couple of time steps because q_t^2 soon diminishes to zero. Then $\Delta p = 0$ has two equilibrium points (0 and 1), meaning that *b/b* hosts cannot stop the *Wolbachia* invasion.

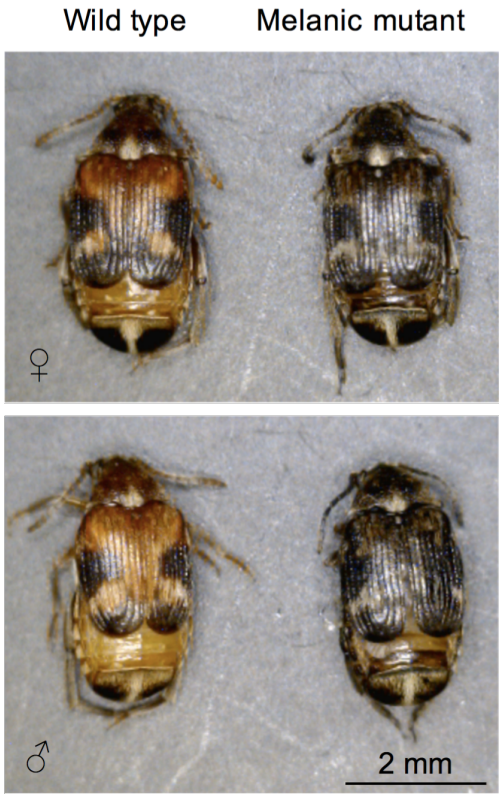


Figure S1 Females (above) and males (below) of normal and melanic colored *Callosobruchus analis*.

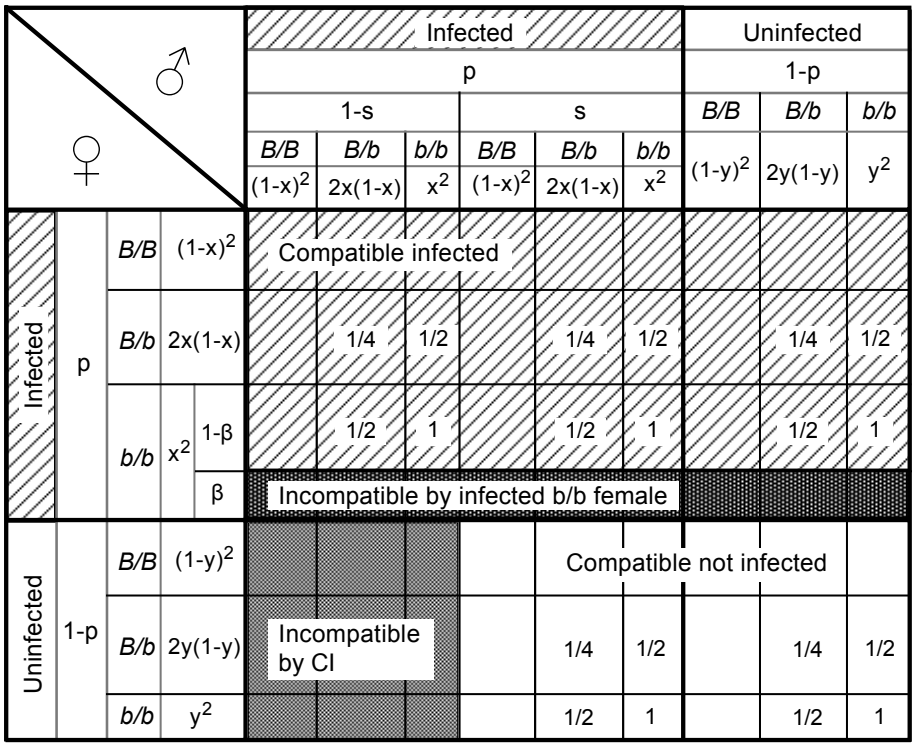


Figure S2 Infection status of zygotes. p is the proportion of infected hosts, q and r are frequencies of allele *b* in infected and uninfected hosts, respectively. Each box represents the relative proportion of zygotes produced by random mating between host females and males. Numbers in the boxes represent the *b/b* proportion among zygotes. The status of female parents is represented along the vertical axis and that of the males along the horizontal axis. s and β are cytoplasmic incompatibility (CI) intensity and sterility level of *b/b* females, respectively. Zygotes fail to develop with the presence of CI and with *b/b* females. *B/B*, wild type; *B/b*, heterozygotes; *b/b*, melanic mutant.

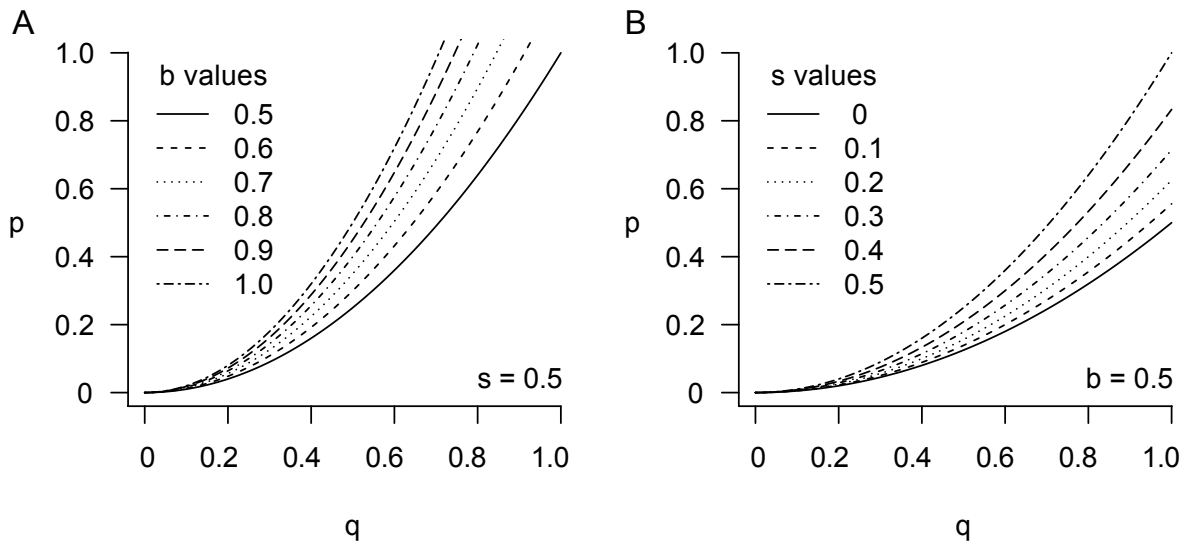


Figure S3 Parameter space of Δp when (A) s = 0.5 and (B) b = 0.5. Combinations of (p,q) below the various dotted lines satisfy the condition to stop the *Wolbachia* invasion ($\Delta p \leq 0$). p is the frequency of infected zygotes, q is the frequency of the *b* gene in the infected zygotes, and s is the cytoplasmic incompatibility intensity (offspring survival rate).

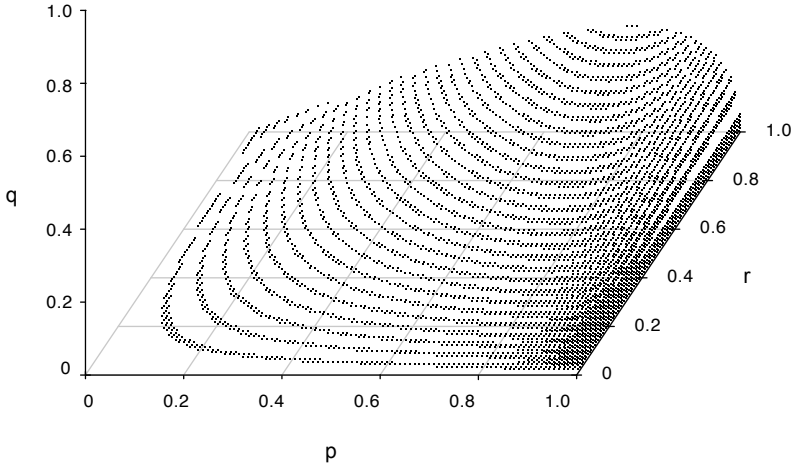


Figure S4 Parameter space of Δq (b = 0.8, s = 0.5). Combinations of (p,q,r) below dotted lines satisfy the condition $\Delta q \geq 0$.