Evolution of Host Countermeasure Traits against Symbiont Reproductive Manipulation

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Contents

Abstract

Vertically transmitted parasites are believed to have evolved to be less pathogenic. *Wolbachia* is an α-proteobacterial symbiont and cannot live outside a host, which is thought to be the reason for a host-*Wolbachia* coevolution toward benign parasitism, especially since the fitness of *Wolbachia* is traded against its host's fitness. However, some *Wolbachia* are known to induce cytoplasmic incompatibility (CI) in their hosts. Since *Wolbachia* invades the host population effectively by CI, high intensity of CI is favorable for the spread of *Wolbachia*, but weakened or eradicated CI has been reported. I examined whether the moderate CI intensity is caused by hosts in a coevolutionary interaction where hosts struggle to fight against *Wolbachia*'s reproductive manipulation. In Chapter 1, I investigated CI intensity of *Callosobruchus analis* infected with wCana1 and wCana2 strains of *Wolbachia* and discuss it in connection with host resource competition. In Chapter 2, I focus on the coevolutionary association between hosts and *Wolbachia* to investigate the reduction of the CI intensity. I examined whether female as well as male hosts once infected with *Wolbachia* but removed by antibiotics have the ability to lower the CI intensity. In Chapter 3, I focus on antagonisms that have evolved in response to the CI-phenotype. Since insect melanism has been reported to have a positive effect on pathogen resistance, I investigated whether the infection of CI-inducing *Wolbachia* causes fitness decline of melanic hosts in *C. analis*. In Chapter 4, I examined whether host oviposition site selection is altered by *Wolbachia* infection.

Key-words

Bean beetle, Benign parasitism, *Callosobruchus analis*, CI variation, Cytoplasmic incompatibility, Host-symbiont coevolution, Melanic mutant, Fitness decline, Oviposition, *Wolbachia*

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General Introduction

Coevolution is defined as reciprocal evolutionary change in interacting species (Futuyma and Slatkin 1983). Maternally inherited intracellular symbionts and hosts are one example of coevolution because they have the potential for speciation through reproductive isolation among populations (Thompson 1989). Interactions between symbionts and hosts range from mutually beneficial to antagonistic predator-prey interactions. Such diversity may be induced by the various ways in which populations of symbionts and hosts coadapted. In fact, rapid evolution of mutualism with antagonistic symbiont-host interactions has been reported under laboratory conditions (Jeon and Jeon 1976; Bouma and Lenski 1988). Here, rapid evolution is defined as genetic change occurring rapidly enough to have a measurable impact on simultaneous ecological change (Hairston et al. 2005).

In pathogen-host interactions, a theory called the trade-off hypothesis suggested by Anderson and May (1982) predicts that transmission modes (vertical or horizontal) are correlated with pathogen virulence (e.g., host life span) (for review, see Alizon et al. 2009). If virulence is strong, there is a cost in terms of reduced frequency of pathogen spread in a host population. Thus, Ewald (1987) suggested that vertically transmitted parasites should evolve benign parasitism. However, the endosymbiont *Wolbachia*, which is transmitted strictly vertically, is known to manipulate its hosts, mostly by inducing cytoplasmic incompatibility (CI), to maximize its own transmission (Laven 1951; Laven 1959).

Tuda et al. (2006) reported that in *Callosobruchus* bean beetle species, *Wolbachia*

infection was somewhat correlated with the beetles' use of dry beans. *Callosobruchus* species are distributed all over the world except in Antarctica (Borowiec 1987; Daglish et al. 1993). There are two types of bean beetles, the 'field' and 'storage' species (Southgate 1979). Field species eat beans that are not dried, while storage species are thought to have adapted to dry beans. According to Tuda et al. (2006), *Callosobruchus* beetles are well-adapted to using dry-bean resources, which is thought to be the reason that *Callosobruchus* beetles diverged as pest species that attack stored legumes and seeds eaten by humans. Primitive farming occurred between the 9th and 5th millennia B.C. in the Fertile Crescent and Aykroyd and Doughty (1982) infers that cultivation of legumes occurred nearly 8,000 years ago. Since the storage species of bean beetles are thought to have appeared at around the same time, the association between *Wolbachia* and *Callosobruchus* may have begun since then.

In this work, I investigate host adaptation in response to *Wolbachia*-inducing reproductive manipulation with *C. analis*. I chose this host because its association with *Wolbachia* is thought to be shorter than that of *Drosophila* or *Aedes* hosts, which are known as model species of *Wolbachia* research. Kondo et al. (2011) reported that only three out of 12 *Callosobruchus* species examined were infected with *Wolbachia*, suggesting that the *Wolbachia* infection occurred during the diversification of the *Callosobruchus* species. CI, a form of reproductive manipulation, can create isolating barriers, meaning that gene flow between two different strains of the same host species may be prevented by bi-directional CI, which can lead to host speciation. For reproductive isolating barriers to occur, CI must be complete, which is to say that there must be no surviving offspring in CI-occurring parental combinations. Thus, *Wolbachia* seems to be an exception to the evolution of benign parasitism in strictly vertically transmitted parasites discussed above. However, various intensities of CI have been reported to date, which is why I speculate that host evolution toward benign parasitism does, indeed, occur. The mechanism of CI has not been understood for over 40 years, but LePage et al. (2017) and Beckmann et al. (2017) identified CI-related sequences

recently. How they affect the CI intensity is still unknown.

In Chapter 1, I introduce the reproductive manipulation caused by *Wolbachia* in *C. analis* hosts. In Chapter 2, I investigate the factors of the various intensities of CI that is caused by the same strain of *Wolbachia*. Where *Wolbachia* employs specific host-reproductive manipulation, one might expect hosts to exhibit specific counter-adaptations such as avoiding CI partners. In Chapters 3 and 4, I examine whether it is possible for hosts to have countermeasure traits against CI-inducing *Wolbachia*. Then, by integrating all of the results obtained in this study, I discuss the possibility of coevolutionary association between hosts and *Wolbachia* that generates host counter-adaptations.

Chapter 1

Variation of intensity of cytoplasmic incompatibility in *Wolbachia*-infected *Callosobruchus analis*

Note: Since this chapter is in preparation for submission to a scientific journal, I include summary instead of full manuscript.

1.1 Summary

The genus *Wolbachia* is widely spread among arthropods, nematodes, and crustaceans. *Wolbachia* is known to manipulate host reproduction to invade the host population effectively by means of cytoplasmic incompatibility (CI). CI occurs when female hosts lack the same *Wolbachia* strain as their mates harbor, leading to the embryonic death of host progeny. High CI intensity is favorable for the spread of *Wolbachia*, but weakened or eradicated CI has been reported. The interaction between hosts and *Wolbachia* may

affect CI intensity. To examine this interaction, I focused on the resource competition type of hosts. There are two types of competition: contest and scramble. Contest-type hosts monopolize resources, and hence subordinates die out. Conversely, scramble-type hosts share resources, but they die out when their intake is insufficient for development. It has been reported that a *Wolbachia* infection negatively affects a host's fitness when the host exhibits contest-type resource competition. I examined the CI intensity of *Callosobruchus analis*, known as a contest-type bean beetle. *C. analis* has been reported as being infected with the wCana1 strain of *Wolbachia*. I found a second *Wolbachia* strain, wCana2, and investigated whether the host's competition type affects CI intensity. CI intensity was intermediate and differed between as well as within host lines in *C. analis* even when hosts had the same infection status. This CI variation was not observed in *C. chinensis*, which employs scramble-type competition.

Chapter 2

Taming of cytoplasmic incompatibility in *Wolbachia*-infected *Callosobruchus analis* (Coleoptera: Chrysomelidae)

Note: Since this chapter is in preparation for submission to a scientific journal, I include summary instead of full manuscript.

2.1 Summary

Theories on parasite virulence predict that vertically transmitted parasites with high virulence are excluded in the evolutionary interaction between hosts and parasites. *Wolbachia* is known to manipulate host reproduction to increase its vertical transmission. When uninfected females are crossed with infected male hosts, the

viability of their offspring decreases because of cytoplasmic incompatibility (CI) induced by *Wolbachia*. Although high CI intensity is favorable for spreading *Wolbachia*, the intensity of CI varies widely. In previous studies, low CI intensity was shown to have an association with the coevolutionary history of *Wolbachia* in its male hosts. In this study, I focused on the coevolutionary history between hosts and *Wolbachia* to investigate the reduction of CI intensity. I hypothesized that female and male hosts that had been infected with *Wolbachia* and subsequently treated with antibiotics to remove it should have the ability to lower CI intensity. I examined CI intensity by mating female and male hosts with a different host-*Wolbachia* associations. Females without a coevolutionary association were more sensitive to CI caused by infected males than females with an association. Males without a coevolutionary association caused higher CI intensity than males with an association. Reduced CI intensity with the presence of a coevolutionary association with *Wolbachia* was observed in both female and male hosts.

Chapter 3

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

3.1 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 bacteria detected by Hertig and Wolbach (1924), seems to strengthen parasitism. Yen and Barr (1973) found that cytoplasmic incompatibility (CI) was induced by *Wolbachia*. 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 and 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 the *Spodoptera* moth, melanic larvae exhibited lower mortality than did non-melanic ones when they were exposed to pathogens (Wilson et al. 2001). Melanic *Galleria* moths also have a higher tolerance to pathogen penetration than do non-melanic ones, though their melanism is accompanied by fitness costs (Dubovskiy et al. 2013). Infected *Anopheles* mosquitoes can delay internal pathogen proliferation through melanism (Yassine et al. 2012).

In *Callosobruchus* bean beetles, the ease with which the melanic-body-color mutation can be seen enables its isolation from wild type beetles. While recessive mutations have been reported in *C. chinensis* (Kashiwagi and Utida 1972), dominant, incomplete dominant and recessive mutations have been reported in *C. maculatus* (Breitenbecher 1921; Eady 1991; Mano and Toquenaga 2011). The melanic mutation in *C. analis* found in the current study was recessive. Since *Wolbachia* infection has been reported only in *C. analis*, *C. chinensis*, and *C. latealbus* (Kondo et al. 2011), the inheritance patterns of melanic gene(s) in *Callosobruchus* may not have an effect on *Wolbachia* infection.

However, Kashiwagi and Utida (1972) reported low fecundity and fertility among *C. chinensis* with the melanic-body mutation, concluding that the mutants could not survive in nature. Since I 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 since *C. chinensis* is infected with a CI-inducing strain of *Wolbachia* (Kondo et al. 2002), I suspected that the decline is caused by an association between *Wolbachia*'s parasitism and host mutation. To investigate this association, I checked 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*. Then I hypothesized that fitness decline in infected melanic hosts may offset the reduction of uninfected hosts caused by CI. I set up a mathematical model of the spread of the *Wolbachia* infection to examine whether *Wolbachia* can invade a host population with the existence of melanic mutants (see the supplementary information). *Wolbachia* could not invade the melanic host population if melanic female hosts exhibited complete sterility.

3.2 Materials and Methods

Source populations

C. analis is a pest of stored legumes (Haines 1989). Eggs are laid on the seed surface and hatched larvae grow inside a seed. Since larvae exhibit contest-type resource competition (Toquenaga and Fujii 1990; Mano et al. 2002), which is to say that 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*, at a market in 2005 in Colombo, Sri Lanka (S line). It is maintained on mung beans, *Vigna radiata*, under laboratory conditions (30°C, 60 to 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 fertilized females with the melanic mutation were found (see Figure 3.10 in the supplementary information). 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 in diameter and 5 mm in length). 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 in the relevant section of '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 were prepared (Braig et al. 1994; Grenier et al. 1998) 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 NaH_2PO_4, 8.1 mM Na_2HPO_4, 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 4731 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. I 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, living 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 one hour 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), and observed using a blue light trans-illuminator (Invitrogen, Thermo Fisher Scientific). In order to control for the failure of amplification with the primers, I 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 sec, 60°C for 30 sec and 72°C for 1 min.

Establishment of infected and uninfected melanic lines

I 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 3.1. 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 hours 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

number of 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 checked by counting days from the adult emergence to its death (13 to 77 replicates). Adults were given no food.

CI assay

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

Statistical analysis

I applied the Bayesian hierarchical model below with a joint-scaling method (JSM, Jinks and Mather 1982; Takano et al. 2001). JSM uses a design matrix (Table 3.1) 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:

$$fecundity \sim Poisson(\mu_i) \tag{3.1}$$

$$\mu_i = D_{fec,i}\ \beta^t_{fec} \tag{3.2}$$

where $D_{fec,i}$ represents the design matrix for crossing type i as represented in Table 3.1, β_{fec} is the vector $\beta_{fec} = [\beta_{icp}, \beta_A, \beta_D, \beta_M, \beta_I, \beta_{A\times D}, ...\beta_{A\times D\times I}, ...\beta_{A\times D\times I\times M}]$ of parameters for the effects of intercept (icp), additive (A), dominance (D), maternal (M), infection (I), and their interaction effects. μ is the mean fecundity of crossing type i. Priors were assigned to βs and σ: $Normal(0, \sigma^2)$ and $Uniform(0, 100)$ (Gelman and Hill 2006).

I applied the following model for hatch rates:

$$hatchedeggs \sim Binomial(p_i, t) \tag{3.3}$$

$$logit(p_i) = D_{h,i}\ \beta_h^t \tag{3.4}$$

where p_i is the hatching probability when crossing type i, t is the total number of eggs oviposited, $D_{h,i}$ represents a part of the design matrix for crossing type i, as in Table 3.1, 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. I assigned $Normal(0, \sigma^2)$ to each β and $Uniform(0, 100)$ to σ.

For longevity the following model was applied:

$$longevity \sim Normal(\mu_l, \sigma^2) \tag{3.5}$$

$$\mu_l = icp + \beta_G + \beta_I + \beta_S + \beta_{G\times I} + \beta_{G\times S} + \beta_{I\times S} \tag{3.6}$$

where βs represents host genotype (G), infection (I) and sex (S) effects and their interaction effects. μ_l is mean longevity and icp is the intercept. $Normal(0, \sigma^2)$ 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 and Yajima 2015) on R 3.3.3 (R Core Team 2017). 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 and Rubin 1992) for each parameter by comparing within-chain and between-chain variances. I also calculated R^2, the proportion of variance explained by the hierarchical model at the data level (Gelman and Hill 2006).

3.3 Results

Declines in host fecundity, fertility, and longevity were observed when mutant hosts were infected with the wCana2 strain of *Wolbachia*. Figure 3.2 shows the estimated mean fecundity ($R^2 = 0.83$). Both wild type and heterozygous parents showed few differences in fecundity between infected and uninfected groups. 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 eggs and 69 eggs, respectively, in infected and uninfected $b/b \times b/b$ (female $\times$ male) crosses, and six eggs and 69 eggs, respectively, in infected and uninfected $b/b \times B/b$ 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 3.3. Negative values of estimates indicate reduced host fecundity. The parameter for the intercept effect was distributed more positively than the others (95% CrI: 3.2 to 5.5). Effects of infection (I) and its interaction with additive ($A \times I$) were distributed positively (95% CrI: -0.2 to 2.1 for I, -0.1 to 2.1 for $A \times I$), but its interaction effect with maternal ($M \times I$) was distributed negatively (-4.5 to -0.1). Thus, the loss of fecundity in the infected b/b females was caused by the $M \times I$ effect.

Figure 3.4 shows the estimated host hatch rates ($R^2 = 0.97$). Reduced hatch rates were observed when parents were infected with *Wolbachia* compared to the hatch rates for uninfected parents. 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 $b/b \times b/b$ (female $\times$ male) crosses, and 0.4 and 0.8, respectively, in infected and uninfected $b/b \times B/b$ crosses. The distributions of parameters for host hatch rates are shown in Figure 3.5. The parameters for the I and $M \times I$ effects were distributed negatively (95% CrI: -1.1 to 0.1 for I and -3.8 to -1.4 for $M \times I$) while positive distributions were observed for the effects of intercept, $A \times I$ (0.2 to 1.4) and $D \times I$ (1.3 to 2.7). The hatch rate reduction observed

in infected compared to uninfected hosts was caused by the I effect. The $M \times I$ effect contributed negatively to hatch rates when female parents were infected b/b mutants. The M effect partly contributed to the hatch rate reduction (CrI: -3.4 to 0.8), indicating that the maternal b allele also causes reduction independently of host infection status.

Figure 3.6 shows the estimated mean longevity ($R^2 = 0.86$). Both infected and uninfected males of B/B and B/b had shorter longevity than females. The mutant b/b hosts had more greatly reduced longevity than the others, especially when hosts were *Wolbachia* infected. The mean longevity was four days in both infected b/b females and males. Estimated parameter distributions for host longevity are shown in Figure 3.7. Most of the parameters were distributed positively, but negative distributions were observed for the b/b effect (95% CrI: -7.6 to 1.1) and its interaction effects with female ($b/b \times female$: -6.7 to 1.3) and infection ($b/b \times I$: -9.3 to -1.1). Thus, host longevity was shortened by the effects of b/b, $b/b \times female$ and $b/b \times I$.

CI assays revealed that the wCana2 strain of *Wolbachia* induced almost complete CI (Figure 3.8, $R^2 = 0.99$). The mean hatch rate was 0 in CI-occurring groups. It is notable, however, that non-CI-occurring parental combinations (i.e., infected females $\times$ 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 3.9. Negatively distributed parameters of I (95% CrI: -8.3 to -1.9) and $D \times I$ (-3.1 to 0.1) effects indicate reduced hatch rates due to these effects. The interaction effect of $A \times I$ was distributed positively (95% CrI: -0.7 to 5.7).

3.4 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).

There are some studies that have previously reported that mutations of melanic body color, controlled by a single recessive autosomal gene, exhibit a fitness decline in other Coleoptera species. Ebony mutants in the flour beetle, *Tribolium confusum*, exhibit reduced fecundity compared to heterozygous and wild type adults (Park et al. 1945). Black mutants in the Colorado potato beetle, *Leptinotarsa decemlineata*, exhibit low egg viability (Boiteau 1985). In *C. chinensis*, declines in fecundity and fertility have also been reported (Kashiwagi and 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 (Figures 3.2 and 3.4: uninfected b/b females, Figure 3.6: uninfected b/b). Possibly, *Wolbachia* contributes to a worsening of the fitness decline caused by the b/b genotype in *C. analis*. Starr and 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 I did not examine oogenesis in *C. analis*, fecundity reduction in infected b/b females suggests that *Wolbachia* contributes to fecundity reduction. In this light, the results provide an example of the *Wolbachia* association with host oogenesis that contrasts with that reported by Starr and Cline (2002).

Since the wCana2 strain induced CI in hosts, I 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 the *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.

Because the sterility of infected *b*/*b* females is incomplete, meaning that it cannot be an antagonism against CI-inducing *Wolbachia* in *C. analis*, how the wCana1-infected hosts prevent the wCana2 invasion is unclear. 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 hosts during the coevolutionary history between hosts and *Wolbachia*; host infection experience has been reported to have an effect on the reduction of CI intensity (Poinsot and Merçot 2001). The wCana1-infected hosts may be eliminated by the wCana2-infected hosts in the future.

In conclusion, I found that *C. analis* body-color mutants exhibited a fitness decline in fecundity, fertility, and longevity when they 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 association between the coevolutionary history and the ability of *Wolbachia* to induce CI, further investigations are needed.

Table 3.1: Design matrix for joint scaling method

Status	Cross type	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
	Female × Male																
Uninfected	$b/b \times b/b$	1	1	0	1	0	0	0	1	0	0	0	0	0	0	0	0
	$b/b \times B/b$	1	1/2	1/2	1	0	1/4	0	1/2	0	1/2	0	0	1/4	0	0	0
	$B/b \times b/b$	1	1/2	1/2	1/2	0	1/4	0	1/4	0	1/4	0	0	1/8	0	0	0
	$B/b \times B/b$	1	0	1/2	1/2	0	0	0	0	0	1/4	0	0	0	0	0	0
	$B/B \times B/B$	1	-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Infected	$b/b \times b/b$	1	1	0	1	1	0	1	1	0	0	1	0	0	1	0	0
	$b/b \times B/b$	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
	$B/b \times b/b$	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
	$B/b \times B/b$	1	0	1/2	1/2	1	0	0	0	1/2	1/4	1/2	0	0	0	1/4	0
	$B/B \times B/B$	1	-1	0	0	1	0	-1	0	0	0	0	0	0	0	0	0

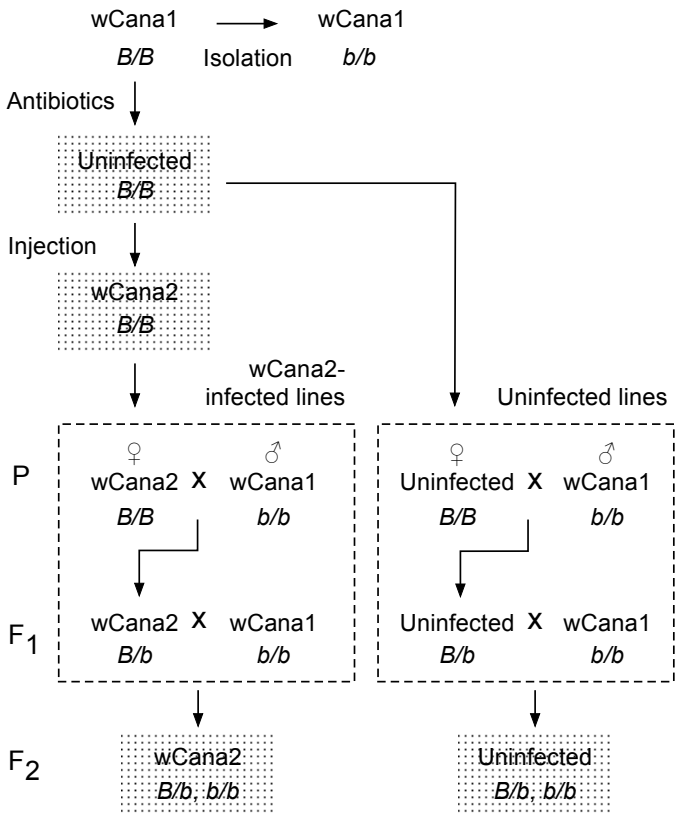

Figure 3.1: Experimental flow for the preparation of wCana2-infected (W+) and uninfected (W-) host lines with three genotypes. The recessive melanic mutation of body color is expressed as b/b. Wild type (B/B) and heterozygous (B/b) individuals exhibited normal body color. Wild type individuals of the 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 I used the six shaded host lines: W- (B/B, B/b, b/b) and W+ (B/B, B/b, b/b).

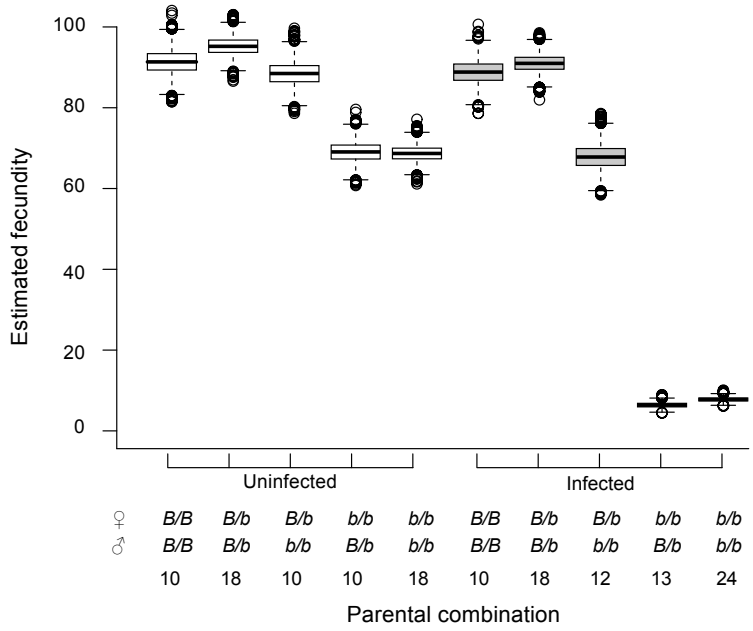

Figure 3.2: Estimated mean fecundity (total number of eggs produced) for the assigned parental combinations ($R^2 = 0.83$). Three genotypes represent wild type (B/B), heterozygous (B/b), and melanic mutant (b/b) parents. Shaded boxes indicate the *Wolbachia* infection and the others are infection-free. Numbers along the x-axis indicate replicates.

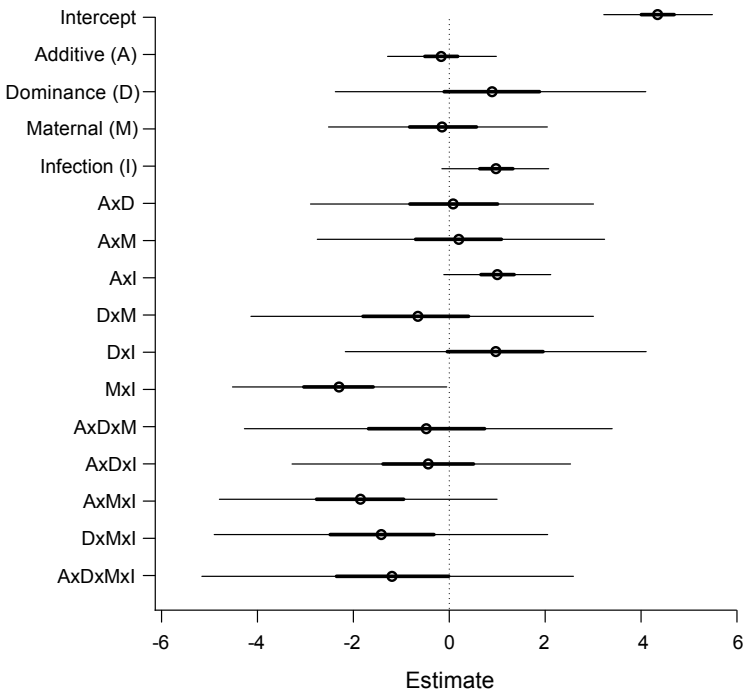

Figure 3.3: Estimated parameter distributions affecting host fecundity. The effects are listed along the y-axis. Circles represent means of the estimated posteriors. The 50% (thick line) and 95% (thin line) posterior credible intervals are shown. Negative values represent reduced fecundity.

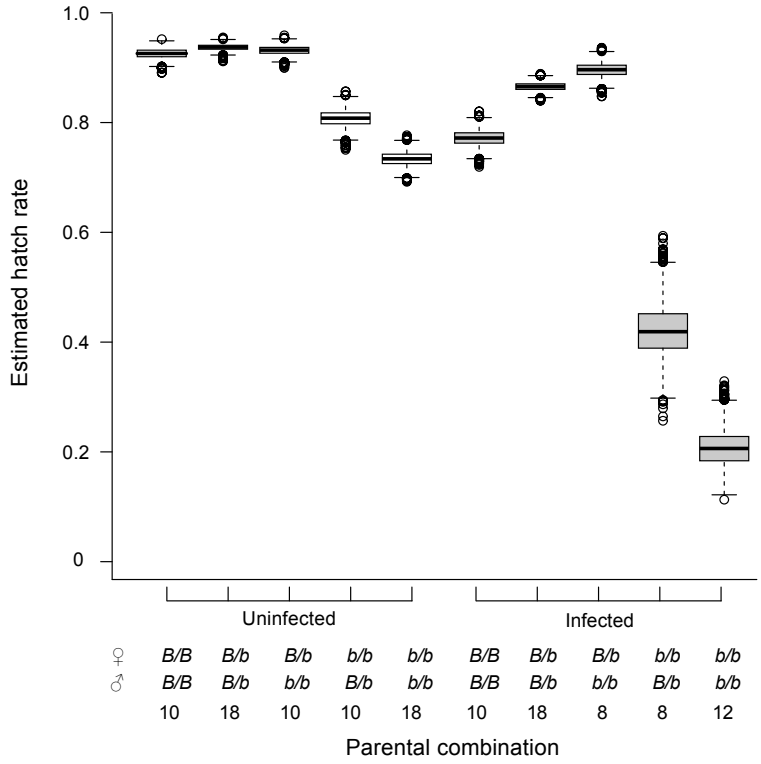

Figure 3.4: Estimated hatch rates obtained from melanic and normal colored parental combinations ($R^2 = 0.97$). Three genotypes represent wild type (B/B), heterozygous (B/b), and melanic mutant (b/b) parents. Shaded boxes indicate the *Wolbachia* infection and the others are infection-free. Numbers along the x-axis indicate replicates.

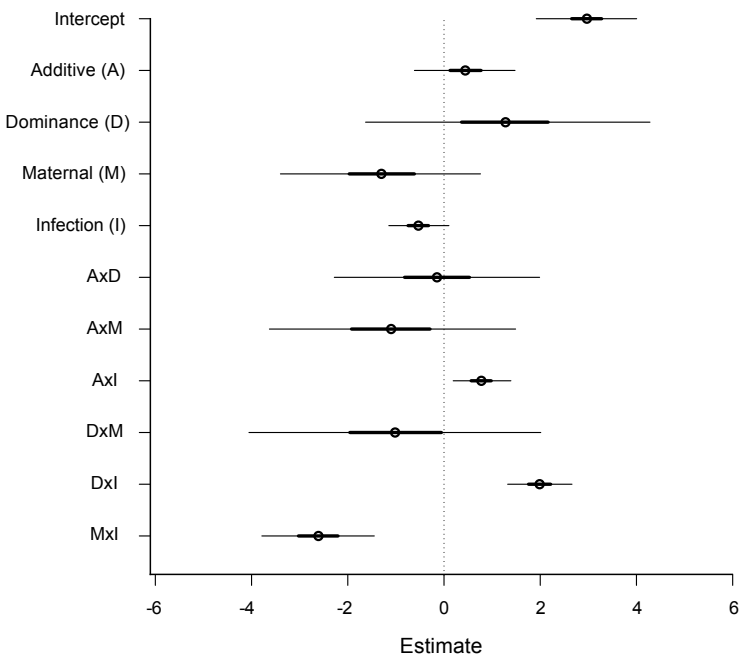

Figure 3.5: Estimated parameter distributions affecting host hatch rates. The effects are listed along the y-axis. Circles represent means of the estimated posteriors. The 50% (thick line) and 95% (thin line) posterior credible intervals are shown. Negative values represent reduced hatch rates.

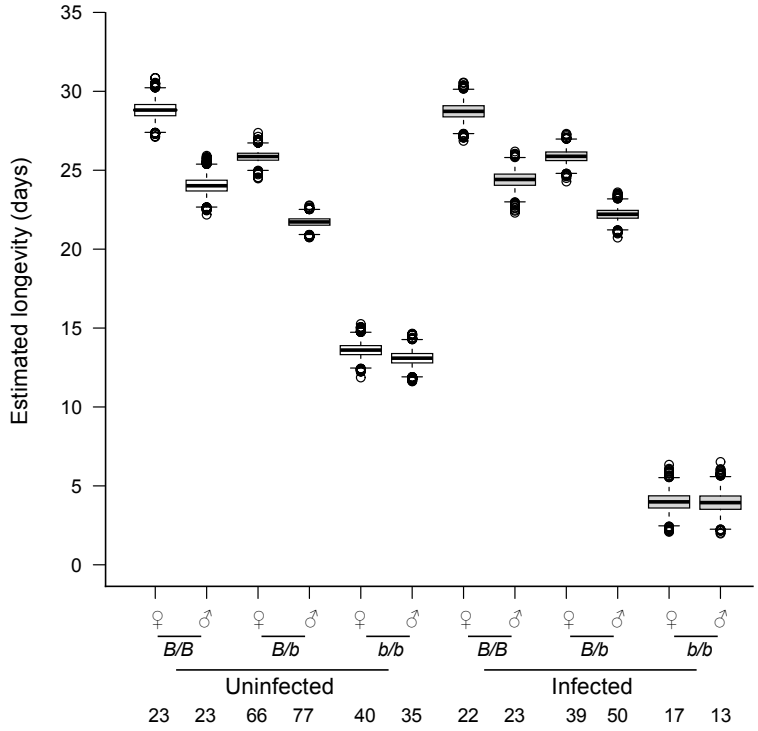

Figure 3.6: Estimated mean longevity of adult beetles ($R^2 = 0.86$). Three genotypes represent wild type (B/B), heterozygous (B/b), and melanic mutant (b/b) parents. Numbers along the x-axis indicate replicates.

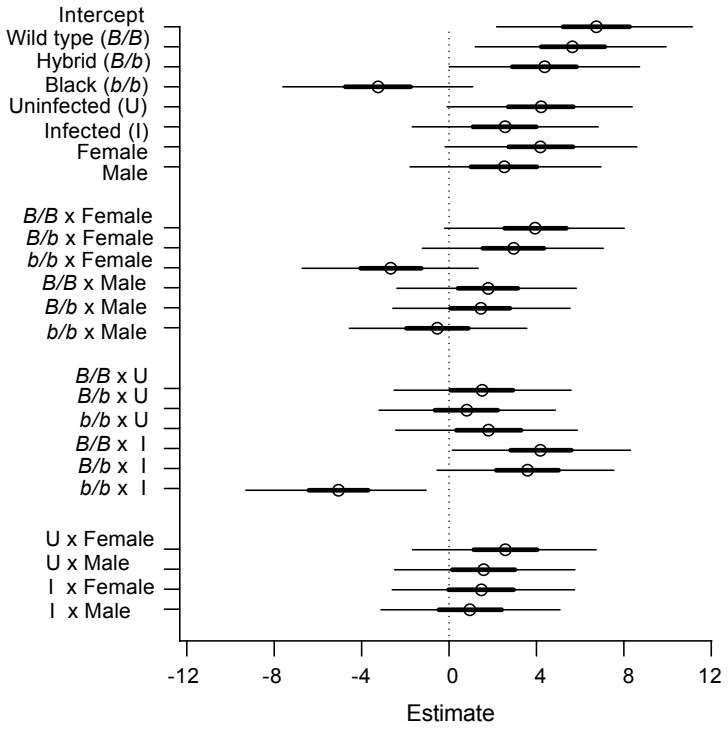

Figure 3.7: Estimated parameter distributions are shown for the effects of genotype, infection, and sex. Circles represent means of the estimated posteriors. The 50% (thick line) and 95% (thin line) posterior credible intervals are shown. Negative values represent reduced longevity.

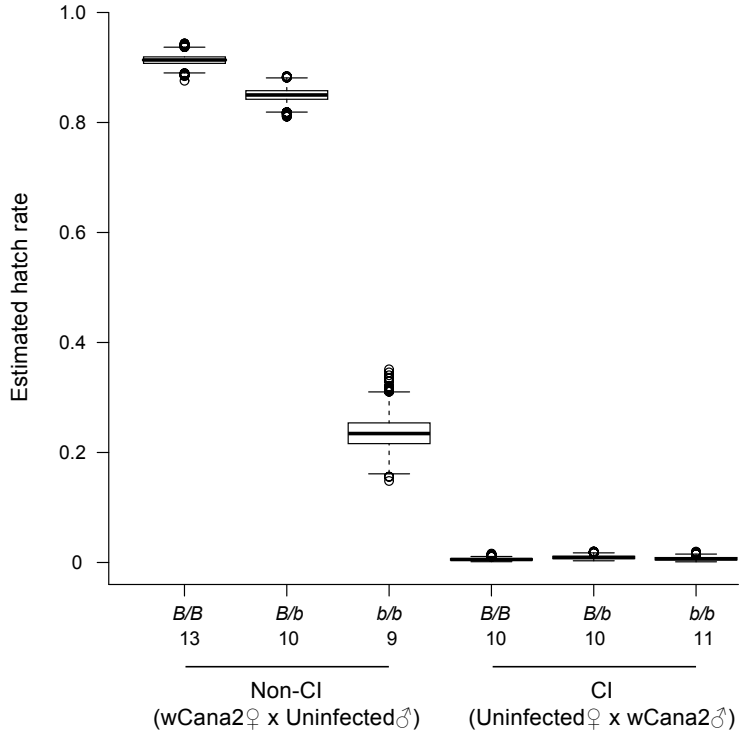

Figure 3.8: Estimated hatch rates obtained from CI and non-CI occurring parental combinations ($R^2 = 0.99$). Three genotypes represent wild type (B/B), heterozygous (B/b), and melanic mutant (b/b) parents. Parents of infected (W+) females and uninfected (W-) males are non-CI occurring combinations, while the opposite combinations have CI. Numbers along the x-axis indicate replicates.

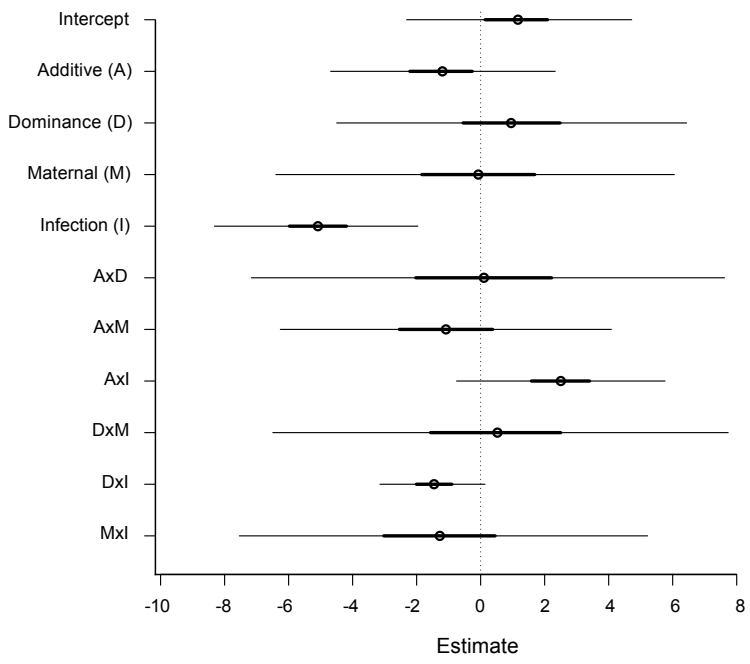

Figure 3.9: Estimated parameter distributions for host hatch rates. The effects are listed along the y-axis. Circles represent means of the estimated posteriors. The 50% (thick line) and 95% (thin line) posterior credible intervals are shown. Negative values represent reduced hatch rates.

Supplementary

Model for *Wolbachia* infection spread

CI enables the spread of *Wolbachia* by increasing the relative frequency of infected hosts. However, the sterility of infected b/b females reduces infected hosts. I modeled the infection dynamics considering both CI and incomplete b/b sterility based on the *Wolbachia* maintenance model proposed by Fine (1978). Figure 3.11 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. I 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. I also assumed that *Wolbachia* transmission failure was negligible but intensity of CI was incomplete ($s \neq 0$). I 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:

$$p_{t+1} = \frac{p_t(1-\beta q_t^2)}{w_t} \tag{3.7}$$

$$q_{t+1} = \frac{p_t(p_t(q-t-r_t)+q_t+r_t-\beta q_t^2(p_t q_t - p_t r_t + r_t + 1))}{2w_t} \tag{3.8}$$

$$r_{t+1} = \frac{(1-p_t)(p_t s(q_t+r_t)+2(1-p_t)r_t)}{2w_t} \tag{3.9}$$

$$w_t = 1 - p_t(1-p_t)(1-s) - \beta p_t q_t^2 \tag{3.10}$$

Results for infection dynamics

For infection dynamics, I considered difference in p between time t and $t+1$.

$$\Delta p = p_{t+1} - p_t = \frac{p_t(1-p_t)(p_t(1-s) - \beta q_t^2)}{w_t} \tag{3.11}$$

Figure 3.12 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 3.12 A) and $b = 0.5$ (Figure 3.12 B). If q is constant, the above recursion equation has three points of equilibrium: $p_1^* = 0$, $p_2^* = \beta q^2/(1-s)$, $p_3^* = 1$. 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, I considered Δq as well.

$$\Delta q = q_{t+1} - q_t \tag{3.12}$$

$$= \frac{\beta p_t(2-p)q_t^3 + \beta p_t(p_t r_t - r_t - 1)q_t^2 + (p_t - 1)(2p_t s - p_t + 2)q_t + p_t(1-p_t)r_t}{2(1 - p_t(1-p_t)(1-s) - \beta p_t q_t^2)} \tag{3.13}$$

Figure 3.13 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.

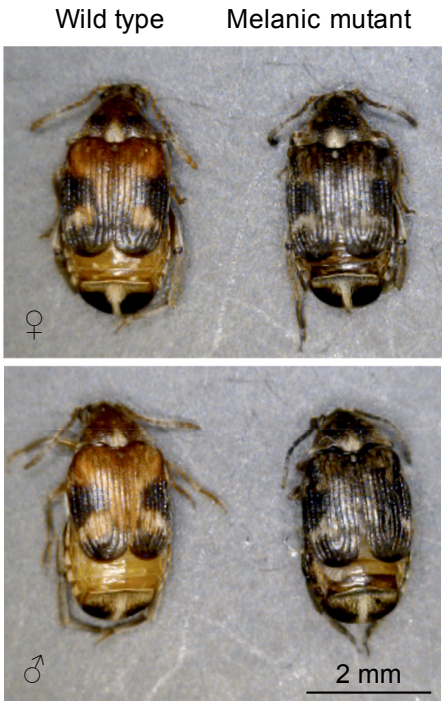

Figure 3.10: Females (above) and males (below) of normal and melanic colored *C. analis*.

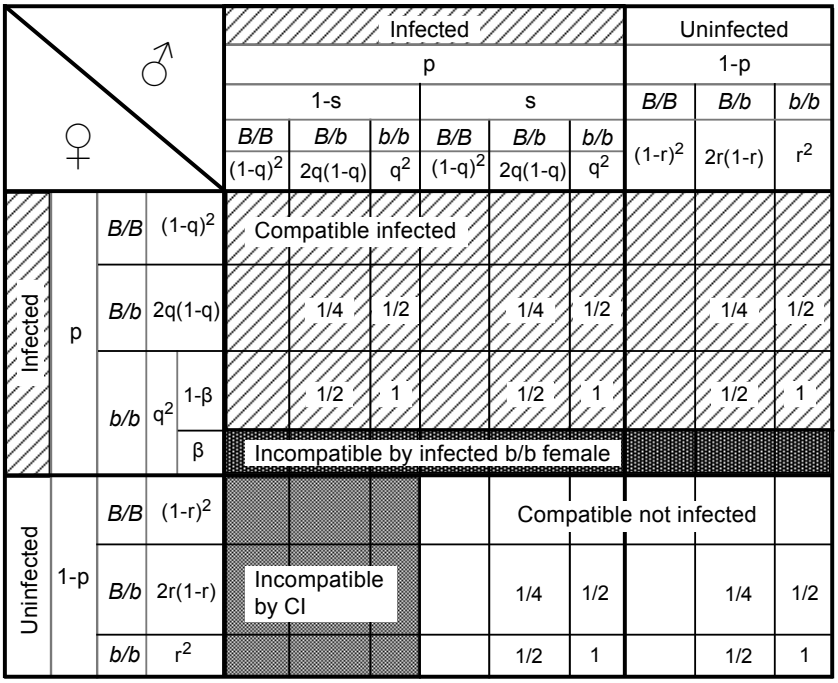

Figure 3.11: Infection status of zygotes. p is the proportion of infected hosts. q and r are frequency of allele b in infected and uninfected hosts, respectively. Each box represents the relative proportion of zygotes produced by random mating between host females and males. Numbers in the boxes represent 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 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.

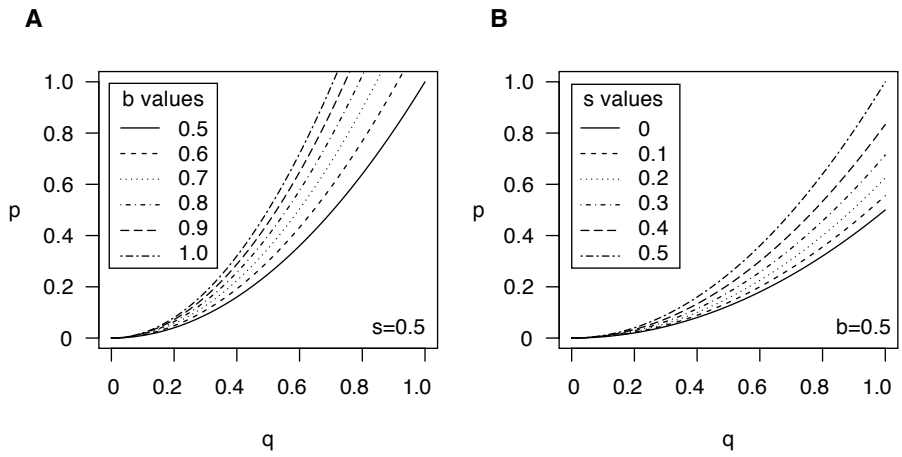

Figure 3.12: Parameter space of Δp, when $s = 0.5$ (A) and $b = 0.5$ (B). Combinations of (p, q) represented by the various dotted lines below satisfy the condition to stop the *Wolbachia* invasion ($\Delta p \leq 0$). p is the frequency of infected zygotes. q is the frequency of the b gene in the infected zygotes. s is the CI intensity (offspring survival rate).

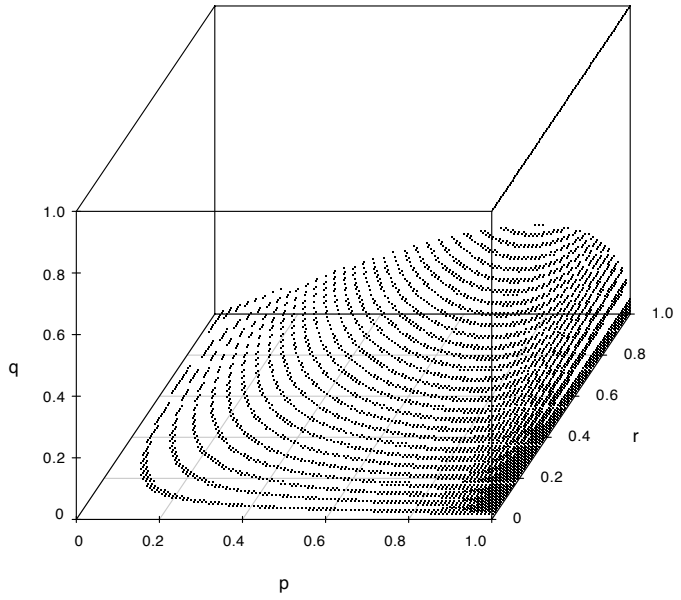

Figure 3.13: Parameter space of Δq ($b = 0.8, s = 0.5$). Combinations of (p, q, r) represented by the dotted lines below satisfy the condition $\Delta q \geq 0$.

Chapter 4

Wolbachia alters egg-laying behavior of bean beetle hosts

Note: Since this chapter is in preparation for submission to a scientific journal, I include summary instead of full manuscript.

4.1 Summary

Intracellular bacteria of the genus *Wolbachia* are widely spread possibly through the manipulation of host reproduction. Most commonly observed manipulation is cytoplasmic incompatibility (CI) leading to early embryonic death of host offspring when female parents lack the same *Wolbachia* strain that their mates harbor. CI enables the infected hosts to increase effectively in a host population, though some *Wolbachia* infections do not involve CI nor any other reproductive manipulations. While *Wolbachia* can alter host development, it can affect host behavior as well. Previous studies have been reported that female oviposition site selection is affected by the infection of *Wolbachia* in a few host species. Oviposition site selection affects the survivorship of

host offspring when larval resource competition is severe. Females of *Callosobruchus analis*, known as a bean beetle, lay their eggs on the surface of a seed. Since resources for larvae are limited, larvae compete with one another within a seed. Thus, females should disperse their eggs across seeds to increase the proportion of adult emergence when larval competition is contest. I hypothesized that *C. analis* females alter their egg-laying behavior as a countermeasure trait against CI that was induced by *Wolbachia*. I first examined whether *Wolbachia* in *C. analis* induces CI, then investigated whether *Wolbahcia* infection alters host egg-laying behavior. Two out of four host lines examined in this chapter were infected with CI-inducing *Wolbachia*. The host egg-laying behavior of *C. analis* was altered by CI-inducing *Wolbachia*. Since previous studies that examined the infection effect on the host behavior do not consider the influence of parasitism of *Wolbachia*, the results will provide new insight into the study of host behavior alteration by *Wolbachia*.

General Discussion

The objective of this work was to examine whether hosts can evolve countermeasure traits against CI-inducing *Wolbachia*. In Chapter 1, I investigated the CI intensity of *Wolbachia* in *C. analis* hosts. All examined CI parental combinations exhibited intermediate CI intensity. Interestingly, CI intensity varied among host strains, even when parents had the same infection status. In Chapter 2, I examined whether CI variation is caused by the *Wolbachia*-host association, which is to say whether coevolutionary history moderates CI intensity. Results revealed that female hosts that had once been infected with CI-inducing *Wolbachia*, but from whom the infection had been removed, exhibited a lower CI intensity than those that had never been infected. Male hosts naturally infected with CI-inducing *Wolbachia* exhibited lower CI intensity than those newly infected. Hosts can evolve to have the ability to moderate *Wolbachia*-induced CI; therefore, CI variation among *C. analis* hosts may be caused by differences in the duration of their respective coevolutionary histories with *Wolbachia*.

In Chapters 3 and 4, I analyzed whether hosts possess countermeasure traits to prevent invasion of CI-inducing *Wolbachia*. While melanin production is costly for insects (González-Santoyo and Córdoba-Aguilar 2012), melanism is correlated with increased fungal resistance (Wilson et al. 2001). By comparing life history traits of infected and uninfected melanic hosts, I revealed that melanism contributes to fitness reduction in hosts infected with CI-inducing *Wolbachia*. Then, I examined theoretically whether melanism can prevent the invasion of CI-inducing *Wolbachia*. I determined that although the number of infected hosts is reduced by the low viability of infected melanic

hosts, melanism cannot prevent infection. As counter-adaptations, some hosts are known to avoid mates that would lead to CI in their offspring and other hosts are unable to identify good oviposition sites when infected with *Wolbachia*, leading to the wastage of infected eggs. In *C. analis*, oviposition sites must be chosen so as to distribute eggs uniformly on multiple beans in order to be effective because only one adult can emerge from one bean with multiple eggs. However, hosts infected with a CI-inducing strain of *Wolbachia* tended to lay eggs more randomly than those infected with a CI-free strain. Moreover, the egg distribution became increasingly random when hosts were treated with antibiotics to remove CI-inducing *Woblachia*, which suggests that hosts with CI-inducing *Wolbachia* have no recourse other than to directly moderate CI in order to resist CI-inducing *Wolbachia*.

This study illustrates that *C. analis* hosts are not helpless against CI-inducing *Wolbachia*. They can render infected eggs unviable by distributing them randomly and they can moderate CI intensity through their coevolutionary history. The wCana2 strain of *Wolbachia* in *C. analis* may become non-parasitic in the future, as evidenced by the existence of a CI-free *Wolbachia* strain, wCana1. All laboratory host lines are infected with wCana1 despite its non-parasitic nature, raising the question of how the wCana1 strain spreads through *C. analis* host populations. One possible explanation is that wCana1 was once a CI-inducing strain of *Wolbachia* and hence spread, subsequently becoming CI-free through host moderation.

Wolbachia strains have 16 clades, and two of them (supergroups A and B) are thought to have diverged 58 to 67 million years ago (Werren 1997). Arthropod hosts are commonly infected with the A or B supergroups of *Wolbachia*. In *Drosophila simulans*, both A- and B-group strains of *Wolbachia* (A: wAu, wRi and wHa; B: wNo) have been reported, suggesting a longer coevolutionary association than that of the *C. analis* infected with wCana1 and wCana2 strains that belong to supergroup B. Considering that the moderation of CI by *C. analis* hosts may have developed in approximately 8,000 years, this study will have an impact on our understanding of host-symbiont

coevolution in terms of rapid evolution.

While the observed transmission mode in the present study was strictly vertical, examination of the *Wolbachia* phylogeny using several molecular markers has revealed the possibility of horizontal transfers of *Wolbachia* between lepidopteran host species (Ahmed et al. 2016; Li et al. 2016) and other orders (for review, see Werren 1997). While Ewald (1987) discussed a less parasitic evolution of vertically transmitted parasites, horizontal transmission, which is infrequent, may negatively affect on CI moderation. Thus, these two transmission modes may be antagonistic to one another in regard to CI moderation by hosts. Since it is unknown how CI-related *Wolbachia* genes identified by LePage et al. (2017) and Beckmann et al. (2017) affect CI moderation, further study is needed from a molecular viewpoint. In order for *Wolbachia* to contribute to host speciation, one might think that CI should be complete and should occur bidirectionally (Telschow et al. 2005); however, bidirectional CI is rarely reported. Therefore, if host genetic isolation would occur with unidirectional and incomplete CI, *Wolbachia* may be considered as speciation promoter in arthropod hosts.

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