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# Regulation of *Wolbachia* Density in the Mediterranean Flour Moth, *Ephestia kuehniella*, and the Almond Moth, *Cadra cautella*

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**ABSTRACT**—The Mediterranean flour moth, *Ephestia kuehniella*, is infected with A-group *Wolbachia* (*w*Kue), and the almond moth, *Cadra cautella*, is doubly infected with A- and B-group *Wolbachia,* which are designated as *w*CauA and *w*CauB, respectively. In both insects, the *Wolbachia* populations increased greatly during embryonic and larval stages. The *Wolbachia* population doubled every 3.6 days on average in *E. kuehniella* larvae, whereas those of *w*CauA and *w*CauB doubled every 2.1 days in *C. cautella* larvae. The populations of *w*CauA and *w*CauB that had been transferred into the *E. kuehniella* background increased at similar rates to that of *w*Kue in the natural host *E. kuehniella*, suggesting that the host genetic background influences *Wolbachia* proliferation. To examine whether the populations of the two *Wolbachia* variants in double infection is regulated collectively or independently, we measured the infection load in the ovaries of three transfected *E. kuehniella* lines in different infection states: single infection with *w*CauA, single infection with *w*CauB, and double infection. The density of each *Wolbachia* variant did not differ significantly between the singly and doubly transfected hosts, suggesting independent regulation.

**Key words:** *Wolbachia*, *Ephestia kuehniella*, *Cadra cautella*, infection density, real time quantitative PCR

## INTRODUCTION

*Wolbachia* is a group of *Rickettsia*-like bacteria that are present in a wide range of arthropods and filarial nematodes. *Wolbachia* resides intracellularly and is inherited maternally by transovarial transmission. In arthropod hosts, the infection can cause various reproductive alterations, such as cytoplasmic incompatibility (CI), thelytokous parthenogenesis, male killing and feminization (reviewed in Werren, 1997; Bourtzis and Braig, 1999; Stouthamer *et al*., 1999). These reproductive alterations enhance the spread of *Wolbachia* in the host population by increasing the number of infected females either directly (feminization and parthenogenesis) or indirectly by increasing the relative fitness of infected females against uninfected counterparts (male killing and CI).

Since *Wolbachia* is inherited vertically from generation to generation and establishes a long term association with its host, its proliferation in a host is expected to be controlled so that the density is maintained high enough to ensure transovarial transmission while being low enough not to cause pathology. The discovery of a virulent *Wolbachia* variant in *Drosophila melanogaster* may imply the importance of such regulation of *Wolbachia* proliferation. This variant, named *popcorn*, proliferates to abnormally high density especially in nervous tissue, and reduces the host lifespan (Min and Benzer, 1997).

The density of *Wolbachia* has often been measured in conjunction with the intensity of CI. Many studies have suggested a positive correlation between the infection load and CI strength (*e.g.*, Breeuwer and Werren 1993; Bressac and Rousset, 1993; Turelli and Hoffmann, 1995; Noda *et al*., 2001). It has also been shown that *Wolbachia* density varies depending on both bacterial genotype and host species. For example, two *Wolbachia* variants, *w*Ri and *w*Ha, differ widely in density although they are carried by the same host species, *Drosophila simulans* (Bourtzis *et al*., 1996). The influence of host genetic background on the infection load was revealed by artificial transfers of *Wolbachia* in *Drosophila*, in which the same *Wolbachia* variant was maintained at higher densities in *D. simulans* than in *D. melanogaster* (Boyle *et al*., 1993; Poinsot *et al*., 1998).

The density of *Wolbachia* is determined by the original infection in host egg and subsequent bacterial proliferation. To examine whether *Wolbachia* proliferation is determined by the bacterial genotype or is regulated by the host, it is of use to analyze the infection dynamics in various host-*Wol-*

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*bachia* combinations in which either the host or bacterial effect can be extracted. The involvement of *Wolbachia* genotype in the determination of proliferation patterns has recently been demonstrated in the adzuki bean beetle, *Callosobruchus chinensis*, and in *D. simulans*. In *C. chinensis* three *Wolbachia* variants harbored by the same multiply infected host exhibited different population growth patterns (Ijichi *et al*., 2002). In *D. simulans*, the *popcorn* variant transferred from *D. melanogaster* and *w*Ri showed different infection dynamics (McGraw *et al*., 2002). On the other hand, a comparison of *popcorn* infection between *D. melanogaster* and *D. simulans* revealed that host species affects the bacterial proliferation rate (McGraw *et al*., 2002). Thus it appears that *Wolbachia* proliferation is controlled by a complex interaction of bacterium and host.

*E. kuehniella* is infected with A-group *Wolbachia* (*w*Kue) designated by Werren *et al*. (1995), and expresses a partial CI (Sasaki and Ishikawa, 1999). *C. cautella* is doubly infected with A- and B-group *Wolbachia* variants, which are referred to as *w*CauA and *w*CauB, respectively, and expresses complete CI (Sasaki and Ishikawa, 1999). We recently performed a transfer of *Wolbachia* from *C. cautella* to *E. kuehniella*, and generated three transfected lines, namely singly infected with *w*CauA, singly infected with *w*CauB, and doubly infected (Sasaki *et al*., 2002). These doubly infected hosts and transfected lines provide an ideal system for studying the extent to which the control of *Wolbachia* proliferation is influenced by bacterial genotype and host species.

In the present study, to further understand the basis for the regulation of *Wolbachia* proliferation, we investigated the growth pattern of the *Wolbachia* population in two naturally infected hosts and the transfected *E. kuehniella* carrying *w*CauA and *w*CauB. We also measured the infection load in the doubly transfected and singly transfected *E. kuehniella* lines, in order to determine whether the densities of the two *Wolbachia* variants in double infection are regulated collectively or independently.

## MATERIALS AND METHODS

### Insects

*Wolbachia*-infected *E. kuehniella* and *C. cautella* were originally collected in Tsuchiura, Japan. Transfer of *Wolbachia* from *C. cautella* to *E. kuehniella* was performed by embryonic microinjection as described by Sasaki and Ishikawa (2000). Although naturally infected *C. cautella* and *E. kuehniella* expressed CI, *w*CauA induced male killing at host embryonic stage when transferred into *E. kuehniella*. The *E. kuehniella* line transfected with only *w*CauB expressed partial CI (Sasaki *et al*., 2002). The transfected lines had been reared for more than 10 generations before the present study was performed.

The insects were maintained on a diet consisting of wheat bran, water, glycerol and dried yeast (100 : 5 : 10 : 5 w/w) under a 16 hr : 8 hr light : dark cycle at 25°C. To minimize the possible effects of rearing density on the infection load of *Wolbachia* (Sinkins *et al*., 1995; Hoffmann *et al*., 1998), the larvae were reared under uncrowded conditions (approximately 1 insect per 1 g of diet).

### DNA extraction

DNA was extracted from the whole tissues of insects or ovaries collected by dissecting adult females. Each sample was homogenized in 500 μl of a buffer (10 mM Tris-HCl, 150 mM NaCl, 10 mM EDTA, 0.1% SDS, pH=7.5) containing proteinase K at 0.1 mg/ml, and incubated at 55°C for 3 hr. The solution was extracted with phenol saturated with TE (10 mM Tris-HCl, 1 mM EDTA, pH=7.5), and then extracted with chloroform. DNA was precipitated by adding 500 μl of ethanol to 250 μl of the aqueous phase and dissolved in 500 μl of TE. When the samples were prepared from the eggs or one-day-old larvae, 20 μg of glycogen was added to the aqueous phase prior to ethanol precipitation, and DNA was finally dissolved in 200 μl TE.

### Real time quantitative (RTQ)-PCR

To estimate *Wolbachia* densities, the copy number of the *groEL* gene was quantified by RTQ-PCR in a Light Cycler (Roche Diagnostics). The amplification reaction was monitored using a set of fluorescent probes specific for the PCR product.

In the measurement of *w*Kue and *w*CauA, a 264 bp fragment

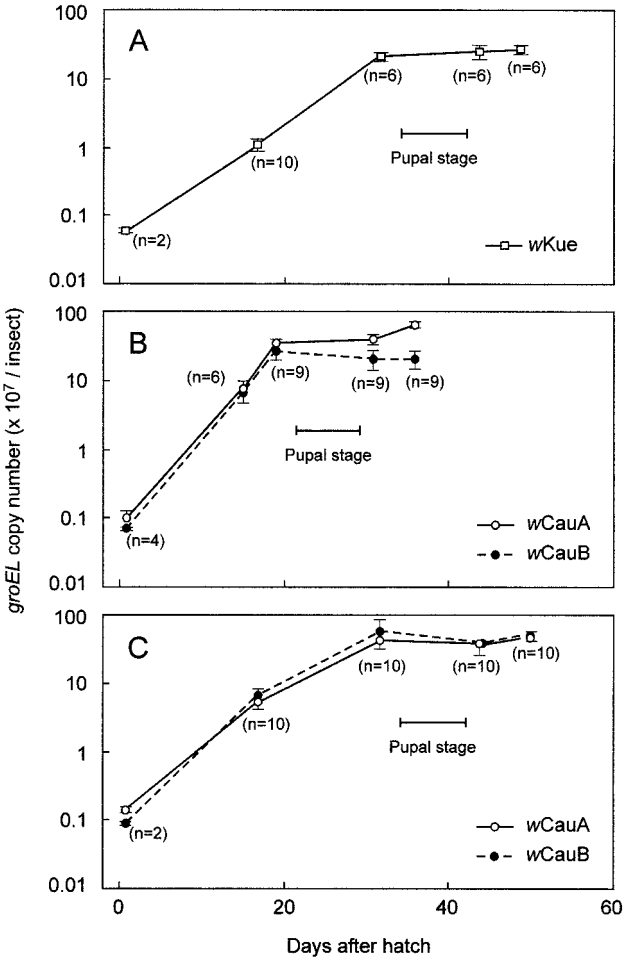


**Fig. 1.** The changes in the *Wolbachia* population during the development of naturally infected *E. kuehniella* (A), *C. cautella* (B) and transfected *E. kuehniella* that carries *w*CauA and *w*CauB (C). Values in parentheses indicate the number of samples tested. Each DNA sample was prepared from a single individual except that each sample of one-day-old larva (younger than 24 hr) was prepared from five individuals. The mean and standard deviation are shown in terms of *groEL* copies per insect.

of the *groEL* gene was amplified by using the primers groEL415AF [5'-CAA TGT CTC GTA CAA TTT CTC-3' (positions 395–415)] and groEL641AR [5'-GAT TAT CAA GCT CCA CGA-3' (positions 658–641)]. The PCR product was detected with the probes groELA1 [5'-AAA GAG TTA GAA GTT GAG CTG ACT ACT GG-3'-FITC (positions 553–581)] and groELA2 [LC-Red 640-5'-ATG CAA TTT GAT CGC GGT TAT CTC TCT CCG-3' (positions 583-612)]. These primers and probes were designed based on the region where the sequences are identical between *w*Kue and *w*CauA. The *groEL* gene of *w*CauB was amplified with the primers groEL415BF [5'-CAA TGT CTC GCA CAA TTT CTT-3' (positions 395–415)] and groEL641BR [5'-GAT CAT CAA GCT CCA CGC-3' (positions 658–641)], and the product was detected with the probes groELB1 [5'-GAA GTT GAA CTT ACA ACT GGT ATG CAG TT-3'-FITC (positions 562–590)] and groELB2 [LC-Red 640-5'-GAC CGT GGT TAT CTC TCT CCA TAC-3' (positions 592–615)].

Standard solutions were prepared from the PCR products amplified from *w*CauA and *w*CauB using the primers groEL49F [5'-GTT GCA AGA AGC CTT TCG TG-3' (positions 29-49)] and groEL827R [5'-CCA AAA CCT GGA GCT TTT ACT G-3' (positions 848-827)]. The PCR products were electrophoresed on 1.0% agarose gel, extracted from the gel using QIAquick Gel Extraction Kit (Qiagen), and quantified based on the optic absorbance at 260 nm. Ten-fold dilutions from 10 pg/μl to 1 fg/μl were used to draw standard curves.

## RESULTS

### Infection dynamics of *Wolbachia* in *E. kuehniella* and *C. cautella*

The changes of *Wolbachia* titer during host development were monitored in *E. kuehniella* and *C. cautella* by a quantitative PCR. Females were subjected to the measurements, except for one-day-old larvae in which the females and males could not be distinguished.

Fig. 1 shows the temporal infection dynamics of the *Wolbachia* population in terms of *groEL* gene copy number. In naturally infected *E. kuehniella*, the population of *w*Kue increased from $5.7\times10^5$ to $2.2\times10^8$ during larval development, and thereafter remained at an almost constant level. At the larval stage, the *w*Kue population doubled every 3.6 days ($R^2$=0.97) on average (Fig. 1A). The proliferation rates of the two *Wolbachia* variants in *C. cautella* larvae were similar to each other: the populations of *w*CauA and *w*CauB doubled every 2.1 days ($R^2$=0.97) and 2.1 days ($R^2$=0.98), respectively (Fig. 1B). After pupal ecdysis, the population of *w*CauA increased, especially at adult stage, while that of *w*CauB did not.

The changes of *Wolbachia* density in eggs are shown in Table 1. The duration of embryonic development of *E. kuehniella* and *C. cautella* was about 6 days and 4 days at 25°C, respectively. The density of *w*Kue doubled in 3.6 days ($R^2$=0.96), whereas that of *w*CauA and *w*CauB doubled in 2.5 days ($R^2$=0.54) and 1.6 days ($R^2$=0.85), respectively. Thus, the growth rate of the *Wolbachia* population during the host embryonic development was higher in *C. cautella* than in *E. kuehniella* as during the larval development.

The differential proliferation rates of *Wolbachia* observed in the two naturally infected hosts can be accounted for by the differences in both the bacterium and host species. In order to examine the extent to which the proliferation rate is influenced by the bacterial genotype and host genetic background, the infection dynamics in a transfected *E. kuehniella* line that carried *w*CauA and *w*CauB

**Table 1.** Changes of *Wolbachia* density during embryonic development of *E. kuehniella* and *C. cautella*

| Host insect | *Wolbachia* variant | *groEL* copies ($\times 10^5$) / embryo (mean ± S.D.) | | No. of samples |
|---|---|---|---|---|
| | | Early embryo* | Late embryo** | tested |
| *E. kuehniella* | *w*Kue | 3.35 ± 0.65 | 9.07 ± 0.51 | 2 |
| *C. cautella* | *w*CauA | 5.01 ± 2.83 | 10.53 ± 3.12 | 3 |
| | *w*CauB | 1.88 ± 0.71 | 6.62 ± 1.14 | 3 |

* Samples were prepared from embryos younger than 12 hr old.
** Samples of *E. kuehniella* and *C. cautella* were prepared from 120–132 hr old and 72–84 hr old embryos, respectively. DNA was extracted from 5 embryos for each sample.

**Table 2.** Infection density of *Wolbachia* in the ovary of *E. kuehniella* and *C. cautella*

| Host insect | *Wolbachia* variant | *groEL* copies ($\times 10^7$) / ovary | *groEL* copies (x $10^7$) / mg ovary |
|---|---|---|---|
| *E. kuehniella* | *w*Kue | 5.75 ± 1.43 | 1.01 ± 0.24[a] |
| *C. cautella* | *w*CauA | 13.34 ± 4.00 | 3.11 ± 0.51[b] |
| | *w*CauB | 7.63 ± 2.59 | 1.78 ± 0.36[c] |
| *E. kuehniella* | *w*CauA | 14.21 ± 3.46 | 2.17 ± 0.41[d] |
| *E. kuehniella* | *w*CauA | 13.33 ± 3.52 | 2.20 ± 0.50[d] |
| | *w*CauB | 8.87 ± 3.54 | 1.48 ± 0.55[e] |
| *E. kuehniella* | *w*CauB | 9.59 ± 1.83 | 1.49 ± 0.27[e] |

Ovaries were collected from females 60–72 hr after adult emergence.
The mean ± S.D. (n=15) is given for each *Wolbachia* variant.
The values indicated by different letters were significantly different by Student's *t*-test ($P$<0.05).

was investigated (Fig. 1C). In the transfected larvae, the *Wolbachia* populations increased more slowly than those in *C. cautella*, and the growth rates were similar to that of *w*Kue in *E. kuehniella*: the populations of *w*CauA and *w*CauB doubled every 4.2 days ($R^2$=0.95) and 3.9 days ($R^2$=0.90), respectively. The proliferation rates in eggs were not examined because *w*CauA causes male killing in *E. kuehniella* embryos.

### *Wolbachia* density in host ovary

In Table 2, are given the *Wolbachia* densities in the ovaries of the two naturally infected hosts and three transfected *E. kuehniella* lines in different infection states: single infection with *w*CauA, single infection with *w*CauB, and double infection. The density of *w*CauA was significantly higher than that of *w*CauB in *C. cautella* ovary. A difference in infection load of *w*CauA and *w*CauB was also observed in comparisons of the two variants in the doubly transfected *E. kuehniella* line and also between the two singly transfected lines. For each of *w*CauA and *w*CauB, the infection load did not differ when compared between the doubly and singly transfected *E. kuehniella* lines.

## DISCUSSION

In this study, we estimated the population growth rate of different *Wolbachia* variants in the same host and that of the same *Wolbachia* in different hosts. Using this approach we can determine the relative importance of bacterial genotype and host genetic background in the determination of the proliferation rates. Comparisons of different *Wolbachia* variants in the same host did not show a remarkable difference in the rate of proliferation during the host larval development. The populations of *w*CauA and *w*CauB infecting the same host increased at similar rates in either *C. cautella* or *E. kuehniella* (Fig. 1, B and C). In addition, the proliferation rate of both *w*CauA and *w*CauB in the *E. kuehniella* background was similar to that of *w*Kue (Fig. 1, A and C). In contrast, host species had an obvious influence on *Wolbachia* proliferation: the populations of *w*CauA and *w*CauB increased more rapidly in *C. cautella* than in *E. kuehniella* (Fig. 1, B and C). Thus, our data suggested that host genetic background plays a major role in the determination of the *Wolbachia* proliferation rate, though it should be noted that *Wolbachia* genotype has also been shown to affect the proliferation rate in *C. chinensis* (Ijichi *et al*., 2002) and *D. simulans* (McGraw *et al*., 2002).

Interestingly, the densities of *w*CauA, *w*CauB and *w*Kue were comparable at the late larval stage regardless of the host species they infected, which is because the duration of larval development of *E. kuehniella* is longer than that of *C. cautella* while the *Wolbachia* proliferation rate is lower in *E. kuehniella* than in *C. cautella*. This may imply that the host growth rate influences *Wolbachia* proliferation, though the mechanism for this is unclear. The duration of larval development of *C. cautella* and *E. kuehniella* can be controlled by rearing conditions such as temperature and humidity. The relationship between *Wolbachia* proliferation and the growth rate of the host could be addressed by examining *Wolbachia* density in hosts maintained under different conditions.

After pupal ecdysis, the population of *Wolbachia* did not change much. It stayed at almost constant levels except for *w*CauA in *C. cautella* adult. The proliferation and death of *Wolbachia* may be in equilibrium in the pupae and adults. Many larval cells undergo apoptosis following metamorphosis in pupae, and the fatbody tissue in adults is degraded as the ovary develops. A portion of the *Wolbachia* population may be lost in tissues that are degraded as host development proceeds. The increase of *w*CauA population in *C. cautella* adult could be explained by its high infection density in the ovarian tissue (Table 2), considering that the ovaries develop greatly after adult emergence and amount to approximately half of the whole tissues in weight.

The density of *w*CauA was higher than that of *w*CauB in the ovary of *C. cautella* and *E. kuehniella* (Table 2) and in *C. cautella* eggs (Table 1). *w*CauA might enter the germ line cells more efficiently or proliferate more quickly in ovarian tissue than *w*CauB. It is possible that the two *Wolbachia* variants differ in tissue tropism. Indeed, a remarkable difference in tissue tropism has been described among the three *Wolbachia* variants in *C. chinensis* (Ijichi *et al*., 2002) and between *w*Ri and *popcorn* in *D. simulans* (McGraw *et al*., 2002).

In doubly infected hosts, two forms of density regulation can be assumed. One is that the total number of the two *Wolbachia* variants is regulated, which may occur if the two variants compete with each other for some limited resources in the same host cell. The other is that the presence of one *Wolbachia* variant does not affect the proliferation of the other variant, and the density of each variant is regulated independently. Our data on infection load in the three transfected *E. kuehniella* lines supported the latter possibility because the density of each of *w*CauA and *w*CauB did not differ between the singly and doubly infected lines. Since the host genetic background affects the growth rate of the *Wolbachia* population, it is conceivable that there may be a host factor that limits the bacterial proliferation. The proliferation of *w*CauA and *w*CauB may be limited by different host factors. It is also possible that *w*CauA and *w*CauB are spatially segregated and do not affect each other. Histological observations are needed to understand the dynamics of double infection further.

In the present study, quantitative PCR was performed with *groEL* as the target gene. The *groEL* gene is known to be present in a single copy per genome in the *Rickettsiaceae* group (Segal and Ron, 1996) to which *Wolbachia* belongs (O'Neill *et al*., 1992). Masui *et al*. (1997) analyzed the intergenic, less conserved, sequences between the *groES* and *groEL* genes in various *Wolbachia* variants, and reported that only one sequence was found from each *Wolbachia* variant, suggesting that *Wolbachia* also has one *groE* operon per genome. However, we should emphasize

that gene copy number does not always reflect the number of microbial cells. *Buchnera*, the intracellular symbiont of aphids, has more than 100 copies of a genome in a single bacterium (Komaki and Ishikawa, 1999). The copy number of the genome of *Wolbachia* has not been examined. In addition, quantitative PCR counts gene copies derived from dead cells as well as those from living cells. Therefore, the *groEL* copy number shown in this paper should be considered as an index of relative density rather than the number of viable *Wolbachia* cells.

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