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A short, high-temperature treatment of host larvae to analyze *Wolbachia*–host interactions in the moth *Ostrinia scapulalis*

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ABSTRACT

Maternally inherited endosymbiotic bacteria of the genus *Wolbachia* cause various reproductive alterations in their hosts. *Wolbachia* induces male-specific death during embryonic and larval stages in the moth *Ostrinia scapulalis*. To investigate how the density of *Wolbachia* affects their performance in the host, we attempted to reduce its density using a short, high-temperature treatment of the host at the larval stage. Individuals cured of infection as well as sexual mosaics, which harbor *Wolbachia*, were obtained by this method in the next generation. The sex of uninfected offspring was exclusively male, similar to that of the offspring of larvae treated with antibiotics. A strong correlation was found between *Wolbachia* density in female moths and the sex ratio of their progeny. These results suggest that a short, high-temperature treatment at the larval stage reduced the density of *Wolbachia* in the adult stage, and, hence, inhibited interference with the host's development in the next generation. Since the direct effects of the heat treatment on *Wolbachia* were transient, this method may be useful for specifying the critical time for interference by *Wolbachia* in host development.

1. Introduction

Endosymbiotic bacteria of the genus *Wolbachia* are transmitted exclusively from females to their progeny, and cause various reproductive alterations such as cytoplasmic incompatibility, male killing, feminization, and the induction of parthenogenesis (Werren et al., 2008). In the adzuki bean borer *Ostrinia scapulalis* and its congener *Ostrinia furnacalis* (Lepidoptera: Crambidae), female moths infected with *Wolbachia* were found to produce female-only progeny (Kageyama et al., 1998, 2003a). Since the elimination of *Wolbachia* from infected strains by an antibiotic (tetracycline) did not restore the sex ratio of their progeny to 1:1, but rather gave rise to male-only progeny, the mode of action of *Wolbachia* was initially considered to be due to the feminization of genetic males (Kageyama et al., 2002, 2003a). However, subsequent studies showed that female-only progeny were produced as a result of male-specific death, whereas male-only progeny were produced as a result of female-specific death during embryonic and larval development (Kageyama and Traut, 2004). Female-specific lethality in disinfected insects was attributed to the deterioration of the sex determination system on the W chromosome in the *Wolbachia*-infected strain of *O. scapulalis* (Sugimoto and Ishikawa, 2012).

The density of *Wolbachia* affects their transmission rate and level of interference in host reproduction in various insect species (reviewed in Jaenike, 2009). In *O. scapulalis*, the treatment of infected female adults (moths) with antibiotics occasionally gives rise to sexually mosaic (gynandromorph) progeny that express both male and female morphological features (Kageyama et al., 2003b), suggesting that a reduction in the density of *Wolbachia* in female moths influenced the actions of *Wolbachia* in their progeny. A heat treatment of *Wolbachia*-infected female moths also gave rise to a few sexually mosaic progeny (Sakamoto et al., 2008). However, unlike antibiotic treatments, heat treatments did not result in male-only progeny, indicating that it was not as effective at removing *Wolbachia*. In the present study, we established a new heat treatment protocol that efficiently cured *Wolbachia* infections. Using this method, we found that the density of maternal *Wolbachia* correlated with the sex ratio of their progeny, suggesting that a reduction in *Wolbachia* density decreased transmission to the next generation.

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2. Materials and methods

2.1. Insects

Adult females of *O. scapulalis* were collected at Matsudo, Chiba Prefecture, Japan in 2008 and 2009, and then individually allowed to lay eggs in the laboratory. One *Wolbachia*-infected matriline and four uninfected matrilines were used in the present study. The infection of females with *Wolbachia* was diagnosed by polymerase chain reaction (PCR) using a primer pair specific to the *Wolbachia surface protein* gene (wsp in Table S1; Zhou et al., 1998). The *actin* gene, amplified with the primers actin-F and actin-R in Table S1, was used as the reference. PCR conditions were 98 °C for 2 min followed by 35 cycles of 98 °C for 15 s, 55 °C for 15 s, and 68 °C for 1 min, with a final extension at 72 °C for 10 min. The offspring from single females were separately reared on a commercial diet (Silkmate™ 2 M, Nosan Corp) at 23 °C under a 16L: 8D photoperiod, and were maintained as matrilines (Sugimoto and Ishikawa, 2012).

2.2. Heat treatment

We first explored the most severe conditions, in terms of a combination of temperature and period, under which some larvae survived and subsequently developed into adults. The heat treatment was performed as follows: ten *Wolbachia*-infected 5th instar larvae were placed on a small piece of artificial diet in each of four plastic cups (10 cm in diameter, 4.5 cm in depth). These cups were placed in an incubator maintained at 53 ± 3 °C, and the cups were then removed after 40, 50, 70, and 100 min. The heat treatment of *Wolbachia*-infected and uninfected larvae at 63 ± 3 °C for 20, 30, 40, 55, and 100 min was performed in a similar manner. Control larvae were not subjected to any heat treatment (0 min). Treated larvae were subsequently reared under normal conditions at 23 ± 1 °C, and the number of individuals that successfully developed into adults was counted to calculate the survival rate. The female moths that emerged were allowed to mate with uninfected males in a screen cage (20 cm × 20 cm × 20 cm) for two days. Mated females were individually transferred into plastic cups, and maintained at 23 ± 1 °C under a 16L: 8D photoperiod to allow for oviposition. Eggs were collected and offspring were reared by brood. The pupae were sexed by the morphology of the midleg and terminal abdominal segments under a stereomicroscope, and then maintained separately in plastic cups with a piece of moistened filter paper.

Subsequently, another set of heat treatment experiments at 63 °C for 20–30 min were carried out to investigate the relationship between *Wolbachia* density in female moths and sex ratio of their progeny. The experimental procedures were basically the same as described in the above section, but the survival rate was not recorded. In total, fifteen mated female moths were obtained from the treatment of >50 *Wolbachia*-infected larvae, and 5 mated female moths were obtained from the treatment of 36 uninfected larvae. After allowing these mated females to lay eggs for two days, they were subjected to DNA extraction for quantification of *Wolbachia*. The eggs were reared by brood until adulthood. Probit analysis of the relationship between *Wolbachia* density in female moths and the sex ratio of their progeny was performed using software R ver. 2.15.2. (R Development Core Team, 2014).

2.3. Antibiotic treatment to induce sexual mosaics

Ten *Wolbachia*-infected adult females and >10 uninfected males were housed in a mesh cage for mating. Two days later, after removing males, a 3% sucrose solution containing 0.24% tetracycline hydrochloride was provided for mated females for one day (Kageyama et al., 2003b). Thereafter, females were separately housed in plastic cups provided with water and allowed to lay eggs for a few days. The eggs were reared by brood. Male and female moths were distinguished by sexually dimorphic characters such as morphology of the genitalia and internal reproductive organs, coloration of the wings, and thickness of the midtibia (Kageyama et al., 2003b).

2.4. Antibiotic treatment to eliminate Wolbachia

The neonates of a *Wolbachia*-infected matriline were fed an artificial diet containing tetracycline hydrochloride (0.06%, w/w) throughout the entire larval stage. Only female moths that are free of *Wolbachia* were obtained in the treated generation (Kageyama et al., 2003b). When these females were mated with uninfected males, all give rise to all-male progeny (Kageyama et al., 2003b; Kageyama and Traut, 2004).

2.5. Determination of the genetic sex of mosaics

The genetic sex of sexually mosaic individuals produced by heat treatment was determined by the presence (female) or absence (male) of sex chromatin in interphase nuclei in the cells of Malpighian tubules as described previously (Sugimoto and Ishikawa, 2012).

2.6. Quantitative PCR analysis

The density of *Wolbachia* was estimated by quantitative PCR (qPCR) using genomic DNA as a template. Female moths were allowed to mate and lay eggs before sampling their genomic DNA. Genomic DNA was extracted from a 2–3-mm section of the terminal abdomen (mostly occupied by ovaries) using the Blood & Tissue Genomic DNA Extraction Miniprep System (Viogene-Biotek, Taipei, Taiwan). qPCR was performed as previously described (Sugimoto and Tsuchida, 2015) using an Applied Biosystems 7300 Realtime PCR System (Applied Biosystems, Foster City, CA) and Thunderbird SYBR qPCR mix (Toyobo) with a primer pair designed to amplify a fragment of the *Wolbachia surface protein* (*wsp*) gene (*wsp*-Q-F and wsp-Q-R in Table S1). The *Ribosomal protein subunit 3* (*RPS3*) gene (DDBJ/EMBL/GenBank accession number AB775139), which was amplified with the primers RPS3-Q-F and RPS3-Q-R (Table S1; Sugimoto and Tsuchida, 2015), was used as an internal standard. Three technical replicates were made for each sample in the qPCR assay. PCR conditions were 98 °C for 2 min followed by 40 cycles of 98 °C for 10 s, 60 °C for 15 s, and 68 °C for 30 s. The quantity of the *wsp* gene was normalized to that of *RPS3*.

3. Results

3.1. Heat treatment conditions for curing infection

We exposed the final (5th) instar larvae of a *Wolbachia*-infected matriline to a high temperature for a short period (Fig. 1). When the larvae were treated at 53 °C for different periods of time ($n = 10$ for each), some survived the treatment for up to 50 min and eventually developed into adult moths. When the temperature was raised to 63 °C, some larvae survived a 40-min treatment and developed into adults, whereas no larvae developed into adults when the treatment was extended to 55 min (Fig. 1). The changes in the survival rate of uninfected larvae along with the extension of treatment at 63 °C were similar to those of *Wolbachia*-infected