A natural population of the butterfly Eurema hecabe with Wolbachia-induced female-biased sex ratio not by feminization

Satoko Narita, Masashi Nomura, and Daisuke Kageyama

Abstract: In butterflies, the adult sex ratio observed in the field is usually male-biased, although the sex ratio of their progeny is 1:1. This is due to the higher motility and larger behavioral range of males than females. As expected, the sex ratio of Eurema hecabe butterflies collected at 6 localities throughout Japan was male-biased. However, in Tsukuba, located in the central part of Japan, the sex ratio was found to be biased toward females. Their progeny reared in the laboratory also exhibited a female-biased sex ratio. A single strain of Wolbachia is considered to be the cause of the sex-ratio distortion, because antibiotic treatment reversed the sex ratio to 1:1, and only a single nucleotide sequence of wsp, a highly variable Wolbachia gene, was detected by molecular analysis. Cytogenetic analysis excluded the possibility of feminization as the underlying mechanism. In addition, when the wild-caught females that had already mated in nature were treated with antibiotics before oviposition, egg-hatch rates were extremely low, suggesting that the same Wolbachia strain also caused cytoplasmic incompatibility. Our findings suggest the possibility that a single strain of Wolbachia induces 2 distinct reproductive manipulations in the same host.

Key words: butterfly, cytoplasmic incompatibility, Eurema hecabe, male killing, sex-ratio distortion, Wolbachia.

Introduction

In dioecious species, it is recognized that a 1:1 sex ratio has an evolutionary advantage (Fisher 1930, 1958). There are approximately equal numbers of males and females in most animal species. However, some species or populations show greatly distorted sex ratios, either because of their genotype or the endosymbionts they harbor (Bull 1983; Werren 1987; Terry et al. 1992; Hurst et al. 2003; Hurst 1991; Dunn and Smith 2001; Terry et al. 2004). These female-biased sex ratios are considered to be a selfish strategy adopted by endosymbionts (Werren and O’Neill 1997; Werren and Beukeboom 1998). The genetically determined female-biased sex ratios found in haplodiploid species, such as parasitic wasps and mites, are considered to be effective in reproduction because of their particular life history (Dyer and Swift 1979; Hamilton 1967; Herre 1985).

However, a female-biased sex ratio can be caused by maternally transmitted endosymbionts, such as bacteria in genera Spiroplasma, Rickettsia, Wolbachia, and Arsenophonus, and unicellular eukaryotic microsporidian parasites (O’Neill et al. 1992; Hurst et al. 2003; Hurst 1991; Dunn and Smith 2001; Terry et al. 2004). These female-biased sex ratios are considered to be a selfish strategy adopted by endosymbionts (Werren and O’Neill 1997).

Wolbachia are common in arthropod species (>30%) and induce various reproductive manipulations in their hosts. Some examples are cytoplasmic incompatibility (CI), which results in the sterility of uninfected females; male killing, which results in the selective death of male progeny; the
feminization of genetic males; and the induction of parthenogenesis. All these manipulations increase the proportion of infected females in a population (Werren and O’Neill 1997; Werren 1997; Stouthamer et al. 1999).

Wolbachia infections have also been reported in the common yellow butterfly *Eurema hecabe* (Lepidoptera: Pieridae). *Eurema hecabe* is widely distributed globally. In Japan, *E. hecabe* is ubiquitous, both on the mainland and on the southwestern islands. Butterflies infected with CI-inducing *Wolbachia* are found in high frequencies on the mainland (Hiroki et al. 2004, 2005). Moreover, some of the females (~20%) in Okinawajima, a southwestern island of Japan, were found to be feminized by *Wolbachia*. These females were infected with 2 distinct strains of *Wolbachia*, 1 of which was indistinguishable from the CI-inducing *Wolbachia* strain (Hiroki et al. 2002, 2004).

In many insect species, the field sex ratio assessed by walking census is usually male-biased, although the sex ratio of progeny is 1:1. This is due to the higher motility and larger behavioral range of males than of females. In this study, however, we found an extraordinarily female-biased sex ratio in *E. hecabe* collected in Tsukuba, in central Japan. To reveal the underlying mechanism and causative agent of this sex-ratio discrepancy, we performed rearing and crossing, cytogenetic analyses, antibiotic treatment, and detection and identification of the endosymbionts.

**Materials and methods**

**Field sampling**

In 2005 and 2006, the population sex ratio of *E. hecabe* was assessed by walking census at 7 geographic locations in Japan: Morioka, Zao, Tsukuba, Matsudo, Kawazu, Tsushima, and Ishigaki Island (Fig. 1, Table 1). For sexing, we used the characteristic “sex brand” that appears only on a male’s forewing underside. Sex ratios (proportions of females) were compared between local populations.

**Rearing**

Four female butterflies collected at the foot of Mt. Tsukuba in August 2006 were individually allowed to oviposit on leaves of *Albizia julibrissin* in plastic cups. Their progeny were kept individually in plastic cases and reared on an artificial diet containing cuttings of *A. julibrissin* (Kato and Sakakura 1994). The sex ratio of each brood was determined at adult emergence. Butterflies were reared at 26 °C under a 16 h light : 8 h dark photoperiod. Individual rearing allowed us to obtain precise data on sex ratio, egg-hatch rate, survival rate during larval stages, and emergence rate.

**Antibiotic treatment of adults**

To examine whether sex-ratio distortion was caused by endosymbiotic bacteria, 5 female adults (mothers of broods E, F, G, H, and I) collected at Tsukuba were treated with antibiotics. These adults were fed with 10% sucrose solution containing tetracycline hydrochloride (1 mg/mL) for 7 to 10 days, and were subsequently allowed to oviposit. Sex ratios of their progeny were examined.

**Antibiotic treatment of larvae**

To examine whether the sex-ratio distortion was caused by endosymbiotic bacteria, a portion of the F₁ progeny from 2 females (broods A and B) were continuously fed a tetracycline-containing diet (tetracycline hydrochloride: 0.7% w/w) from the second instar stage until pupation. Emerged female adults were crossed with other F₁ males (broods A and E) and allowed to oviposit. Sex ratios of their progeny were examined.

**Cytogenetic sexing**

In many species of Lepidoptera, including *E. hecabe*, sex-chromosome constitution is female-heterogametic (ZZ in male, WZ in female) and the W chromosome is conspicuous as a condensed sex-chromatin body (W chromatin) in an interphase nucleus (Traut and Marec 1996). In this study, Malpighian tubules were dissected from adult females, fixed in a mixture of methanol – acetic acid (3:1) for ~1 min, and stained and mounted in lactic acetic orcein. Preparations were examined under light microscopy, as described by Kageyama and Traut (2004). Genetic sexes were determined on the basis of the presence/absence of W chromatin.

**Detection and identification of endosymbiotic bacteria**

After oviposition, ovaries or thoracic muscles were dissected from female adults and stored at ~20 °C until DNA extraction. DNA was extracted using the DNeasy Tissue Kit (QIAGEN).

To examine endosymbiotic bacteria in 2 female butterflies (mothers of broods A and B) that produced a female-biased progeny, PCRs were conducted using a pair of primers, fD1 (5'-CCGATTCGTGACCAACAGATTTGATCCTGGCTCAG-3') and rP2 (5'-CCCGGGATCCACTTAGCTCTACATTGACTCCTTGTAAGCTTACGGCTAGTACCT-3'), that amplify nearly the full length of 16S ribosomal (r)DNA of most bacteria (Weisburg et al. 1991). The PCR products were ligated to the T-easy vector (Promega) and transformed into *Escherichia coli* JM109 competent cells. Inserted plasmids were cloned and extracted from the transformants and subsequently subjected to DNA sequencing with ABI PRISM® BigDye™ terminator chemistry and an ABI PRISM® 3100 capillary sequencer (Applied Biosystems).

PCR detection of a *Wolbachia* infection was performed with primers 5'-TTTGCAGCCTGTATGGTTAATCC-3' and 5'-GAATAGGTATGATTTTCACTGTAATCC-3' for the 16S rRNA gene (O’Neill et al. 1992), primers groEfl (5'-TGATTAGATAGTAAAGAGCTCTGGCTCGT-3') and groErl (5'-CCATTGCAAGAATTACTGCA-3') for the groE gene (Masui et al. 1997), and primers wsp81F (5'-TGGTCCAATAAGTGACAC-3') and wsp81R (5'-AAAATTTAAGCGTACTCCA-3') for the wsp gene (Zhou et al. 1998). PCR detection of *Spiroplasma* infection was performed with primers Spoul-F (5'-GGTTAACATTGCTGC-3') and Spoul-R (5'-CCTGTCTCAATGATGACCT-3'), which amplify a partial sequence of 16S rDNA (Montenegro et al. 2005).

To characterize *Wolbachia* strains, a 0.6 kb segment of the wsp gene was amplified with primers wsp81F and wsp81R, and PCR products were cloned and subjected to DNA sequencing.

Infection with 2 distinct strains of *Wolbachia* was discriminated with specific PCR detection, targeting the wsp gene with primers wsp81F and wHecFem1 (5'-AC-
which amplify the 232 bp DNA fragment of the CI-inducing Wolbachia strain, and primers wHec-Fem2 (5'-TTACTCAACAATTGGCCTAAAGAT-3') and wsp691R, which amplify the 398 bp DNA fragment of the Wolbachia strain that occurs only in feminized individuals. PCRs were conducted under the following temperature profile: 35 cycles at 95 °C for 1 min, 58 °C for 1.5 min, and 72 °C for 1.5 min, followed by 7 min at 72 °C.

**Results**

Sex ratio in the field was biased toward females at Tsukuba

The sex ratios of 7 local populations in Japan were measured by walking census and compared (Fig. 1, Table 1). The population sex ratio at Tsukuba was, on average, 70.5% female (n = 61). This contrasts with sex ratios measured in the other 6 populations, where the female fraction was 20.0%,

Table 1. *Wolbachia* infection status and population sex ratio of *Eurema hecabe* assessed by walking census at 7 geographic locations in Japan.

<table>
<thead>
<tr>
<th>Location</th>
<th>Date of sampling</th>
<th>Females</th>
<th>Males</th>
<th>Female ratio</th>
<th>Infection frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tsukuba, Ibaraki Prefecture</td>
<td>Aug. 19, 2006</td>
<td>13</td>
<td>11</td>
<td>0.54</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Aug. 23, 2006</td>
<td>30</td>
<td>7</td>
<td>0.81</td>
<td>100</td>
</tr>
<tr>
<td>Morioka, Iwate Prefecture</td>
<td>Sept. 3, 2006</td>
<td>4</td>
<td>15</td>
<td>0.21</td>
<td>100</td>
</tr>
<tr>
<td>Zao, Miyagi Prefecture</td>
<td>Sept. 3, 2006</td>
<td>8</td>
<td>12</td>
<td>0.40</td>
<td>100</td>
</tr>
<tr>
<td>Matsudo, Chiba Prefecture</td>
<td>Sept. 23, 2006</td>
<td>0</td>
<td>8</td>
<td>0.00</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Oct. 1, 2006</td>
<td>2</td>
<td>25</td>
<td>0.07</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Oct. 13, 2006</td>
<td>1</td>
<td>13</td>
<td>0.07</td>
<td>100</td>
</tr>
<tr>
<td>Kawazu, Shizuoka Prefecture</td>
<td>Sept. 29, 2006</td>
<td>1</td>
<td>9</td>
<td>0.10</td>
<td>100</td>
</tr>
<tr>
<td>Tsushima Island, Nagasaki Prefecture</td>
<td>Sept. 20, 2005</td>
<td>12</td>
<td>45</td>
<td>0.21</td>
<td>0</td>
</tr>
<tr>
<td>Ishigaki Island, Okinawa Prefecture</td>
<td>Mar. 20, 2006</td>
<td>6</td>
<td>9</td>
<td>0.40</td>
<td>100</td>
</tr>
</tbody>
</table>
on average (0%–40%). In butterfly population sampling, the population sex ratio measured in the field by walking census is male-biased because of differences in behavioral patterns between males and females. Therefore, the population sex ratio at Tsukuba is remarkably biased toward females.

To examine the endosymbiotic bacteria *Wolbachia*, diagnostic PCR was performed on all collected butterflies (*n* = 231). All butterflies were infected with *Wolbachia* except for the Tsushima Island population (Table 1).

### Progeny of females from Tsukuba exhibited a female-biased sex ratio

The sex ratio of *E. hecabe* butterflies collected at Tsukuba was found to be biased toward females. To reveal the underlying cause of this sex-ratio distortion in the field, 4 female butterflies (mothers of broods A, B, C, and D) collected at Tsukuba were individually allowed to oviposit, and their progeny were reared individually until the adult stage. The sex ratios of all 4 broods were female-biased (69%–89%). Statistical significance from the 1:1 ratio was detected in broods A, B, and C (*P* < 0.05, χ² test) (Table 2).

### The female-biased sex ratio is not associated with feminization

A previous report demonstrated that the feminization of genetic males is the mechanism of the strongly female-biased broods produced by some butterflies collected at Okinawajima, a southwestern island of Japan (Hiroki et al. 2002). We therefore suspected the occurrence of feminization in the Tsukuba population.

If feminization does indeed occur, all or some of the mothers/daughters should be genetically male, i.e., lacking the W chromatin. W chromatin was present in all 9 wild-caught females (mothers of broods A–I), indicating that the females were genetically female. Likewise, W chromatin was present in all females (*n* = 24) of female-biased brood D (Table 2). These results clearly indicate that the female-biased sex ratio is not associated with feminization.

### Antibiotics cured abnormal sex ratio

To reveal whether an endosymbiotic bacterium was the cause of the sex-ratio distortion, the sex ratio in progeny of antibiotic-treated females was examined. A clear effect of antibiotic treatment on the sex ratio was found. In brood E, which was produced by a female that was treated during the adult stage, the sex ratio was not significantly different from 1:1 (Table 2; *P* > 0.05, χ² test). Furthermore, 6 F2 broods produced by females (3 from brood A and 3 from brood B) treated during the larval stages also showed a normal sex ratio (Table 2; *P* > 0.05, χ² test). These results strongly suggest that the sex-ratio distortion was caused by endosymbiotic bacteria.

We subsequently compared egg-hatch rates and survival rates between progenies of antibiotic-treated and nontreated females. The mean egg-hatch rate of the nontreated progeny was 0.58, while that of the treated progeny was 0.67. The mean survival rate of the nontreated progeny was 0.36, while that of the treated progeny was 0.40. Although no statistical significance was detected (*P* > 0.05, χ² test), the treated progeny showed, on average, higher values than the nontreated progeny for egg-hatch and survival rates (Tables 2 and 3).

### A single strain of *Wolbachia* was detected and characterized

To examine the composition of bacteria in 2 female butterflies (mothers of broods A and B) that produced a female-biased progeny, bacterial 16S rDNA was cloned and sequenced. Thirty-four of 36 clones from the mother of brood A and 39 of 41 clones from the mother of brood B were identified as *Wolbachia*. Four of the 77 clones were identified as either *Serratia* sp. or *E. coli*. These were regarded as contamination.

Next, to characterize the *Wolbachia* strains detected in the mothers of broods A and B, a ~600 bp segment of the *wsp* gene was amplified, cloned, and subjected to DNA sequencing. The sequences of 84 of 84 clones from the mother of brood A and 40 of 40 clones from the mother of brood B

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**Table 2. Sex ratio and survival rate of F1 and F2 progenies derived from butterflies of the Tsukuba population.**

<table>
<thead>
<tr>
<th>Generation (tetracycline treatment)</th>
<th>Brood (mother × father)</th>
<th>No. of eggs (hatch rate)</th>
<th>1st</th>
<th>2nd</th>
<th>3rd</th>
<th>4th</th>
<th>Last</th>
<th>No. of pupae</th>
<th>No. of adults (adult/eggs)</th>
<th>No. of adults, female: male</th>
<th>Female ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1 (nontreated)</td>
<td>A</td>
<td>213 (0.37)</td>
<td>79</td>
<td>66</td>
<td>63</td>
<td>60</td>
<td>59</td>
<td>59</td>
<td>56 (0.26)</td>
<td>40 (0.16)</td>
<td>0.71</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>143 (0.56)</td>
<td>80</td>
<td>40</td>
<td>38</td>
<td>36</td>
<td>35</td>
<td>35</td>
<td>35 (0.24)</td>
<td>31 (0.14)</td>
<td>0.89</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>113 (0.72)</td>
<td>82</td>
<td>56</td>
<td>38</td>
<td>36</td>
<td>35</td>
<td>35</td>
<td>35 (0.31)</td>
<td>28 (0.14)</td>
<td>0.80</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>55 (0.75)</td>
<td>41</td>
<td>38</td>
<td>36</td>
<td>35</td>
<td>35</td>
<td>35</td>
<td>35 (0.63)</td>
<td>24 (0.11)</td>
<td>0.69</td>
</tr>
<tr>
<td>F1 (adult females treated)</td>
<td>E</td>
<td>73 (0.54)</td>
<td>40</td>
<td>39</td>
<td>36</td>
<td>31</td>
<td>31</td>
<td>31</td>
<td>29 (0.39)</td>
<td>15 (0.14)</td>
<td>0.52</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>31 (0.00)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0 (0.00)</td>
<td>0 (0.00)</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>25 (0.00)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0 (0.00)</td>
<td>0 (0.00)</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>28 (0.04)</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0 (0.00)</td>
<td>0 (0.00)</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>I</td>
<td>20 (0.00)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0 (0.00)</td>
<td>0 (0.00)</td>
<td>—</td>
</tr>
<tr>
<td>F2 (larvae treated)</td>
<td>a (A×E)</td>
<td>63 (0.69)</td>
<td>44</td>
<td>38</td>
<td>37</td>
<td>37</td>
<td>37</td>
<td>37</td>
<td>37 (0.58)</td>
<td>20 (0.14)</td>
<td>0.54</td>
</tr>
<tr>
<td></td>
<td>b (A×E)</td>
<td>123 (0.84)</td>
<td>103</td>
<td>88</td>
<td>84</td>
<td>81</td>
<td>81</td>
<td>81</td>
<td>80 (0.65)</td>
<td>41 (0.28)</td>
<td>0.51</td>
</tr>
<tr>
<td></td>
<td>c (A×E)</td>
<td>134 (0.50)</td>
<td>67</td>
<td>27</td>
<td>24</td>
<td>23</td>
<td>23</td>
<td>23</td>
<td>21 (0.16)</td>
<td>13 (0.08)</td>
<td>0.62</td>
</tr>
<tr>
<td></td>
<td>d (B×A)</td>
<td>77 (0.57)</td>
<td>44</td>
<td>11</td>
<td>11</td>
<td>11</td>
<td>11</td>
<td>11</td>
<td>11 (0.14)</td>
<td>3 (0.08)</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td>e (B×A)</td>
<td>69 (0.74)</td>
<td>51</td>
<td>28</td>
<td>27</td>
<td>27</td>
<td>27</td>
<td>27</td>
<td>26 (0.38)</td>
<td>12 (0.25)</td>
<td>0.46</td>
</tr>
<tr>
<td></td>
<td>f (B×E)</td>
<td>15 (0.67)</td>
<td>10</td>
<td>9</td>
<td>8</td>
<td>8</td>
<td>7</td>
<td>7</td>
<td>7 (0.46)</td>
<td>2 (0.54)</td>
<td>0.29</td>
</tr>
</tbody>
</table>

*Significantly deviated from 1:1 according to Fisher’s exact probability test: *P* < 0.05.

*Significantly deviated from 1:1 according to Fisher’s exact probability test: *P* < 0.001.
were identical to those of the CI-inducing *Wolbachia* strain (Hiroki et al. 2002, 2004). The nucleotide sequences of the *wsp* gene of *Wolbachia* from broods A and B were deposited in the DDBJ/EMBL/GenBank databases (accession Nos. AB285477 and AB285478, respectively).

In the Okinawajima population, some females were found to be feminized genetic males. Such females were infected with a unique strain of *Wolbachia* in addition to the CI-inducing *Wolbachia* strain (Hiroki et al. 2002, 2004). Therefore, we diagnosed all wild-caught females (mothers of broods A–I) by strain-specific PCR and found that all the females were positive for the *wsp* fragment specific to the CI-inducing *Wolbachia* strain, but none were positive for the *wsp* fragment that occurred only in feminized individuals. In addition, all wild-caught females were diagnosed with *Spiroplasma*-specific PCR; *Spiroplasma* is known to cause male killing in butterflies, fruit flies, and ladybird beetles. But none (broods A–I) were positive.

### The *Wolbachia* strain is likely to cause cytoplasmic incompatibility

Five wild-caught females were fed with antibiotic-containing sucrose solution. Egg-hatch rates of broods F to I were extremely low (1/104), except for brood E. The presence of spermatozoa in all 5 females indicated that they had already copulated in nature. Thus, the low hatching rates of eggs can be considered to be due to CI induced by *Wolbachia*. These females were infected with a CI-inducing *Wolbachia* strain and mated in the field with males that were presumably infected with the same strain of *Wolbachia*. Antibiotic treatment resulted in a drastic decrease in *Wolbachia* density in eggs that would have resulted in failure to unlock the modification made in sperm (i.e., CI).

### Discussion

#### Female-biased sex ratio in the Tsukuba population

In butterflies, the field sex ratio examined by walking censuses is usually male-biased, although the real population sex ratio in most species is 1:1. This is due to differences in behavior between males and females in the field; because males actively search for female mates, they display a much higher motility and larger behavioral range than females.

In *E. hecabe*, the observed sex ratios in 6 populations (except for Tsukuba) were consistently male-biased (proportion of females was 20%, on average). This can be interpreted as a real population sex ratio of approximately 1:1. However, the female-biased sex ratio observed in the Tsukuba population (proportion of females was 70%, on average) suggests that the population sex ratio is far from 1:1.

The sex ratio of the progeny of females collected at Tsukuba was also female-biased (69%–89% female). This sex ratio seems to coincide with the field sex ratio examined by walking census. In some populations of *Acraea* and *Hypolimnas* butterflies, the population sex ratio is extremely female-biased because of the prevalence of male-killing *Wolbachia*. In such populations, females are assumed to have changed their behavior to increase the chance of mating with extremely rare males (Jiggins et al. 2000a, 2000b; Dyson and Hurst 2004). It is possible that, in the case of *E. hecabe* in the Tsukuba population, females had difficulty finding males and behaved more actively.

#### Wolbachia as a causative agent of sex-ratio distortion

In insects, female-biased sex-ratio distortion is often caused by endosymbiotic bacteria, such as *Wolbachia* or *Spiroplasma* (Hurst et al. 2000). Restoration to a 1:1 sex ratio after antibiotic treatment indicated that the causative agent of the sex-ratio distortion is a bacterium or bacteria. PCR amplification and sequencing of the bacterial gene segment demonstrated that the causative agent of the sex-ratio distortion is likely to be a single strain of *Wolbachia*. This *Wolbachia* strain was indistinguishable from the one that causes CI in *E. hecabe* and occurs in most parts of mainland Japan, which includes the Tsukuba area.

This particular sequence of *wsp* is one of the most common among the diverse *Wolbachia* *wsp* sequence variations. Most of the *Wolbachia* strains with this sequence are likely to cause CI in various insect species, including *E. hecabe*, but some cause male killing in butterflies such as *Acraea encedon* and *Hypolimnas bolina* (Jiggins et al. 2000b; Dyson and Hurst 2004). Because the type of reproductive manipulation caused by a given *Wolbachia* strain can depend on the genetic background of the host (Sasaki et al. 2002, 2005), the sex-ratio distortion of *E. hecabe* observed in the Tsukuba population might be associated with differences in the genetic background of butterflies between Tsukuba and other populations.

Alternatively, the *Wolbachia* genome might be responsible for differences in the type of reproductive manipulations between Tsukuba and other populations. The types or combinations of bacteriophages harbored by *Wolbachia* (Fujii et al. 2004) might be responsible for this difference. Furthermore, we cannot rule out the possibility that other bacteria that could not be detected in this study because of low densities are responsible for the sex-ratio distortion.

#### Mechanism of sex-ratio distortion

*Wolbachia* has been strongly linked to the cause of the sex-ratio distortion in the Tsukuba population. How, then, does *Wolbachia* distort the host sex ratio toward the female? The mechanisms of *Wolbachia*-induced female-biased sex ratio previously known are thelytokous parthenogenesis, feminization, and male killing (Hurst 1993). Other potential

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**Table 3. Mean and standard deviation of egg-hatch rate, survival rate, and sex ratio.**

<table>
<thead>
<tr>
<th>Replicates</th>
<th>Egg-hatch rate</th>
<th>Survivorship of 1st-inst larva (2nd instar/1st instar)</th>
<th>Larval survivorship (pupae/hatched larvae)</th>
<th>Total survivorship (adults/eggs)</th>
<th>Female ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nontreated</td>
<td>4</td>
<td>0.58±0.18</td>
<td>0.74±0.19</td>
<td>0.58±0.19</td>
<td>0.36±0.18</td>
</tr>
<tr>
<td>Tetracycline-treated</td>
<td>6</td>
<td>0.67±0.12</td>
<td>0.64±0.28</td>
<td>0.58±0.24</td>
<td>0.40±0.21</td>
</tr>
</tbody>
</table>

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mechanisms, such as meiotic drive, might also lead to a female-biased sex ratio (Lyttle 1991, 1993; Jiggins et al. 1999).

Thelytokous parthenogenesis is a phenomenon in which females produce exclusively female offspring without fertilization; it is known only in haplodiploid insect groups, such as Hymenoptera and Thysanoptera (Stouthamer 1997; Arakaki et al. 2001). The diploidloid system of E. hecabe has been confirmed by cytogenetic analysis (unpublished data). Moreover, male-derived spermatophores were present in female adults of E. hecabe collected at Tsukuba, indicating that they experienced copulation in nature. Thus, the possibility that parthenogenesis induction is the cause of the sex-ratio distortion is extremely low.

Feminization is a phenomenon in which inherently genetic males are phenotypically changed into females; it is known only in the woodlice Armadillidium and the butterfly E. hecabe (Rigaud 1997; Rigaud et al. 1997; Hiroki et al. 2002, 2004). Both of these species belong to female-heterogametic groups (WZ in females and ZZ in males). In the presence of feminizing Wolbachia, ZZ zygotes develop as phenotypically female. In this study, all females that produced female-biased progeny and their daughters had the WZ karyotype, indicating that the sex-ratio distortion found in the Tsukuba population was not due to feminization.

Male killing is a phenomenon in which male progeny are selectively killed. Male killing occurs widely in insects, including Lepidoptera, and in many cases the causative agents are Wolbachia and Spiroplasma (Hurst and Jiggins 2000; Hurst et al. 2003). If male killing is the mechanism, the sex ratio should revert to 1:1 and egg-hatch rates and (or) survival rates should increase after antibiotic treatment. In E. hecabe in the Tsukuba population, the sex ratio reverted to 1:1 after antibiotic treatment. Egg-hatch rates and survival rates increased slightly but were not statistically significant. To detect the increase in egg-hatch rates and (or) survival rates, if present, we obviously need a larger sample size. A slight increase in egg-hatch rates and (or) survival rates might sufficiently explain the observed weak sex-ratio bias in adults. We consider, at present, that the possibility of male killing still remains.

Meiotic drive is a phenomenon in which certain gametes with driving alleles are preferentially transmitted to subsequent generations (Lyttle 1991, 1993; Kusano et al. 2003). The sex ratio would be distorted when the driving alleles are sex linked. Although microbe-associated meiotic drive is not documented, the possibility that Wolbachia-induced meiotic drive is the mechanism of the female-biased sex ratio in the Tsukuba population of E. hecabe still remains, together with other unknown mechanisms.

A single strain of Wolbachia induces both CI and female-biased sex-ratio distortion

It seems likely that the Wolbachia strain disturbing the sex ratio in the Tsukuba population can also cause CI in its host. CI, the most common type of host manipulation caused by Wolbachia (Bourtzis et al. 2003), typically occurs when infected males mate with uninfected females; crosses of other combinations are compatible (Werren 1997). The hatching rate of eggs produced by such incompatible crosses is low. It is assumed that Wolbachia modifies sperm and that the modification is rescued when females are infected but is not rescued when females are uninfected (Bourtzis et al. 2003).

In this study, most of the eggs laid by wild-caught females that were treated with antibiotics did not hatch, despite the presence of spermatophore(s) in every female. Antibiotic treatment might have inactivated the action of Wolbachia, and thus, the modification in sperm failed to be rescued, resulting in CI.

It follows that the single strain of Wolbachia found from the Tsukuba population seems to have the ability to cause both CI and female-biased sex-ratio distortion. Two reports have described a single Wolbachia strain that induce different types of reproductive manipulation in the host. In Drosophila bifasciata, male killing is induced by a naturally occurring Wolbachia strain. After high-temperature treatment, small numbers of male progeny appeared. These males were found to be infected with the Wolbachia strain, and exhibited weak CI when crossed with uninfected females (Hurst et al. 2000). Furthermore, a transfection experiment demonstrated that a Wolbachia strain that induces complete CI in a natural host, Cadra cautella, caused male killing in a new host, Ephesia kuehniella (Sasaki et al. 2002, 2005).

Our study suggests that a single Wolbachia strain can induce strong CI and weak sex-ratio distortion (likely to be male killing). Thus far, different reproductive manipulations caused by identical Wolbachia strains have been reported only in a combination of CI and male killing. The above observations suggest that a common mechanism for CI and male killing is more than just coincidence.

Conclusion

This study strongly suggests that the female-biased sex ratio of a butterfly, E. hecabe, collected at Tsukuba is the result of brood sex-ratio bias caused by a Wolbachia infection. In addition, this Wolbachia strain was also able to cause strong CI. We believe that the E. hecabe–Wolbachia relationship is so flexible that it can result in CI, feminization, and other sex-ratio biases, depending on the symbiont or host genotype. This endosymbiotic system will be a good model system for the integrative understanding of the various reproductive manipulations caused by Wolbachia.

Acknowledgements

We would like to thank Dr. A. Mochizuki, Dr. H. Tanaka, and Ms. C. Suzuki for samples of E. hecabe; Ms. Y. Koizumi and Ms. Y. Sato for technical assistance; and Dr. H. Noda for encouragement. This study was financially supported in part by a Japan Society for the Promotion of Science (JSPS) Fellowship for Young Scientists to S.N.

References


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