chapter thirteen

Wolbachia-induced sex reversal in Lepidoptera

Satoko Narita and Daisuke Kageyama

Contents

Introduction .................................................................................................................................................. 296
Essence of the sex-determining mechanism in insects ........................................................................... 296
Non-genetic factors affecting sex determination or sex differentiation .................................................. 296
Endosymbiotic bacteria affecting sex determination or reproduction of arthropod hosts ...................... 298
Wolbachia-induced feminization in the butterfly E. haebe ................................................................ 299
Infection status of Wolbachia in E. haebe ............................................................................................... 299
Infection status of Wolbachia is associated with female-biased sex ratios ......................................... 299
Feminization as the underlying mechanism of the female-biased sex ratios ........................................ 300
Constant presence of wHecl and frequent loss of wHecFem: implications for the population ecology of E. haebe ........................................................................................................................................ 300
High and stable density of wHecl vs. low and fluctuating density of wHecFem ..................................... 300
Feminizing Wolbachia continuously act on E. haebe during larval development for maintenance of female phenotypes .................................................................................................................. 302
Key players of feminization in E. haebe .................................................................................................. 304
Wolbachia-induced feminizing effect and male killing in Ostrinia species moths .................................... 305
Infection status of Wolbachia in Ostrinia species ................................................................................... 305
Female-biased sex ratios in Wolbachia-infected matrilines .................................................................... 307
Appearance of all-male progeny from mothers treated with antibiotics during larval development: possible feminization of genetic males as the underlying mechanism of the female-biased sex ratios ................................................................. 307
Cytological observations reveal that feminization is not the underlying mechanism of the female-biased sex ratios ........................................................................................................................................ 308
Male killing when Wolbachia is present .................................................................................................. 308
Female killing when Wolbachia has been eliminated ............................................................................ 309
Antibiotic treatment of adult females leads to the production of progeny with intersexual phenotypes ........................................................................................................................................ 310
Integrated explanation of the mechanism underlying male killing ....................................................... 312
Mechanistic bases of male killing and feminization .............................................................................. 312
Does Wolbachia interfere with sex-determining genes? ....................................................................... 312
Does Wolbachia interfere with dosage compensation? ......................................................................... 313
Wolbachia genotype or host genotype: which is responsible for the reproductive phenotype? .......... 315

295
Evolutionary implications of male killing and feminization ........................................ 315
Acknowledgments ........................................................................................................... 316
References ....................................................................................................................... 316

Introduction

It is generally assumed that insect sexes are genetically determined. In some insect species, however, sexes can be partially or completely reversed by nongenetic factors such as temperature. Notably, endosymbiotic microorganisms can affect the reproduction of their arthropod hosts in various ways, such as feminization or male killing. In two groups of lepidopteran insects (i.e., moths of the genus Ostrinia and the butterfly Euromia lecabe), it has recently been discovered that sex reversal from male to female can be caused by endosymbiotic bacteria of the genus Wolbachia. In this chapter, we briefly review the general mechanism of sex determination in insects and then describe the Wolbachia-induced sex reversal found in these two groups of lepidopteran insects. We discuss the mechanistic bases and evolutionary implications of these phenomena and attempt to integrate our knowledge of male killing and feminization, which have been recognized as distinct phenomena caused by endosymbionts.

Essence of the sex-determining mechanism in insects

Sexes are genetically determined in the majority of insects. For example, dipteran insects like the fruitfly Drosophila melanogaster have a male-heterogametic sex chromosome constitution, in which XX zygotes become females and XY zygotes develop into males. Lepidopteran insects like the silkworm Bombyx mori have a female-heterogametic chromosomal constitution, in which ZZ zygotes become males and ZW zygotes develop into females. Hymenopteran insects like the honeybee Apis mellifera have a haplodiploid sex-determination system, in which fertilized eggs (2n) become females and unfertilized eggs (n) develop into males (Bull, 1983; Werren and Beukeboom, 1998). The molecular mechanisms underlying sex determination and sex differentiation in the model insect D. melanogaster are well understood. Each cell determines its sex independently at a very early embryonic stage, and once determined, the sex of each cell is maintained during later development through a gene expression cascade consisting of Sex lethal, transformer, doublesex and other genes, in which sex-specific mRNA splicing plays an important role (Schütz and Nöthiger, 2000). Sex determination at a very early embryonic stage in a cell-autonomous manner is believed to be widespread among insects, on the basis that sexually mosaic individuals often occur in a wide variety of insects (Laugé, 1985). Although the molecular mechanisms of sex determination are very poorly understood in species other than D. melanogaster, all the sex-determining mechanisms in insects are proposed to be variations of a single model consisting of a master regulator gene (like Sex lethal in D. melanogaster) at the top of the cascade and the highly conserved doublesex gene at the bottom of the cascade (Figure 13.1) (Nöthiger and Steinmann-Zwicky, 1985; Bownes, 1992; Hoy, 2003). However, evidence for this general model is scarce at the present time.

Nongenetic factors affecting sex determination or sex differentiation

As stated above, sexes are basically determined by genetic factors in insects. However, in some insect taxa, nongenetic factors, such as temperature, hormonal substances, and endosymbiotic microorganisms, are believed to affect sex determination or sex differentiation.
A proposed general model of sex determination in insects. This model assumes that the actions of both maternal genes and zygotic genes affect the expression of a master regulator gene, which corresponds to Sex lethal in *D. melanogaster*. The expression of the master regulator gene activates or suppresses the expression of subsequent genes (downstream genes). At the end of the hierarchical gene expression cascade, a highly conserved *doublesex*-like gene is subjected to alternative RNA splicing and produces the male-specific protein DSX\(^m\) or female-specific protein DSX\(^f\). The sex-specific DSX proteins activate and suppress a series of sex-specific differentiation genes, leading to either the female phenotype or male phenotype. (Modified from Bownes, 1992).

**Figure 13.1**

During development or even after maturation (Bull, 1983; Werren and Beukeboom, 1998; De Loof and Huybrechts, 1998). A switch from maleness to femaleness was reported in mosquito species of the genus *Aedes* after exposure to high temperatures (Brust, 1966, 1968; Brust and Horsfall, 1965; Horsfall et al., 1964; Horsfall and Anderson, 1961, 1965; Craig, 1965). On the other hand, a switch from femaleness to maleness after exposure to high temperatures was reported in the bagworm *Solenobia triquetrella* (Seiler, 1935) and stick insect *Carausius morosus* (Bergereard, 1958, 1961).

In the firefly *Lampyris noctiluca*, transplantation of larval male gonads into female larvae resulted in masculinization of female individuals (Naisse, 1966a, 1966b). Therefore, it has long been assumed that, unlike the majority of insects, an androgenic hormone secreted from the gonads of male larvae induces male differentiation in fireflies (De Loof and Huybrechts, 1998). Recently, the experiments carried out by Naisse in 1966 were reexamined by Maas and Dorn (2005). When larval male gonads were transplanted into female larvae,
masculinization of ovaries was never observed, and the sex of the recipient was always in accordance with the sex of its own gonads. It was therefore concluded that an androgenic hormone is not circulating in *L. noctiluca* larvae and that sex differentiation is probably regulated in the same manner as in other insect species (Maas and Dorn, 2005). At present, nothing is known about the causal agent of the masculinization of *L. noctiluca* females observed by Naissé.

De Loof and Huybrechts (1998) proposed the possible presence of a sex hormone in the tussock moth *Orgyia postica* on the basis that males exhibited higher ecdysteroid titers than females (Gu et al., 1992). At present, however, a direct causal link between ecdysteroid and sex differentiation has not been proven.

In insects, some maternally inherited microorganisms can drastically affect the sex determination (e.g., via feminization), and these effects are described in the next section.

**Endosymbiotic bacteria affecting sex determination or reproduction of arthropod hosts**

The reproductive systems of arthropod hosts are often manipulated by endosymbiotic bacteria such as *Spiroplasma*, *Rickettsia*, *Wolbachia*, *Arsenophonus*, and *Cardinium* (O’Neill et al., 1997; Bourtzis and Miller, 2003, 2006). Among these, *Wolbachia* are particularly focused upon due to their high prevalence (approximately 30% of insect species are infected) and the various types of reproductive manipulations they induce.

The most common type of *Wolbachia*-induced reproductive manipulation is cytoplasmic incompatibility. Cytoplasmic incompatibility results in embryonic mortality after matings between insects with differing *Wolbachia* infection statuses (Bourtzis et al., 2003), and can be either unidirectional or bidirectional. Unidirectional cytoplasmic incompatibility is typically expressed when an infected male mates with an uninfected female. The reciprocal mating is fully compatible, as are matings between infected individuals. Bidirectional cytoplasmic incompatibility usually occurs in matings between infected individuals harboring different strains of *Wolbachia* (Bourtzis and Miller, 2003). The underlying mechanism of cytoplasmic incompatibility is basically considered to be a modification-rescue system. In other words, a *Wolbachia* strain in males modifies the sperm in order to kill the offspring during embryogenesis. If the same *Wolbachia* strain is also possessed by females, the offspring will be rescued by removal of the modification (Poinsot et al., 2003, Bourtzis and Miller, 2003).

*Wolbachia* also induces various types of sex-ratio distortion, such as male killing, whereby male individuals (i.e., the nontransmitting sex) are selectively killed (Bourtzis and Miller, 2003), thelytokous parthenogenesis, whereby females reproduce without fertilization (O’Neill et al., 1997), and feminization, whereby genetic males are transformed into functional females (O’Neill et al., 1997; Hiroki et al., 2002; Negri et al., 2006). Feminization is likely to occur in a relatively small number of species. At present, naturally occurring feminization has only been reported in the butterfly *E. hecate* (Hiroki et al., 2002, 2004; Narita et al., 2007a) and a leafhopper, *Zyginaidae pullula* (Negri et al., 2006). In *E. hecate*, genetic males are completely transformed into functional females, whereas in *Z. pullula*, genetic males are incompletely feminized and exhibit deformed morphologies. Outside insects, *Wolbachia*-induced feminization is known to occur in crustacean species, such as woodlice, and has been extensively examined in *Aradillidium vulgare* (for a review, see Rigaud, 1997).
Wolbachia-induced feminization in the butterfly *E. hecabe*

**Infection status of Wolbachia in *E. hecabe***

In Japanese populations of the butterfly *E. hecabe* (Lepidoptera: Pieridae), two distinct *Wolbachia* strains have been identified (Hiroki et al., 2004; Narita et al., 2007a) (Figure 13.2). One strain, designated *w*HeC1 (corresponding to *w*HeC12 or *w*HeC1 in Hiroki et al., 2004), is prevalent throughout Japanese populations, except northern populations. It exhibits infection frequencies of almost 100% and causes cytoplasmic incompatibility (Narita et al., 2006; Hiroki et al., 2005). The other strain, designated *w*HeCFem (corresponding to *w*HeCFem2 in Hiroki et al., 2004), has been detected in individuals collected in Okinawajima, one of the subtropical southwestern islands of Japan (Okinawa Prefecture), and Tanegashima, one of the temperate islands of Japan (Kagoshima Prefecture). In Okinawajima, approximately 20% of individuals are doubly infected with *w*HeC1 and *w*HeCFem, whereas 80% are singly infected with *w*HeC1 (n = 24; summarized data of Hiroki et al., 2002 and 2004). In Tanegashima, approximately 90% of individuals are doubly infected with *w*HeC1 and *w*HeCFem (n = 23; Narita et al., unpublished).

**Infection status of Wolbachia is associated with female-biased sex ratios**

Female butterflies collected in Okinawajima and Tanegashima were individually allowed to oviposit and their offspring were reared until adult emergence. The progeny produced by females doubly infected with *w*HeC1 and *w*HeCFem consisted of all or nearly all females, whereas the progeny produced by females singly infected with *w*HeC1 consisted of males and females at sex ratios of nearly 1:1 (Figure 13.2).
Feminization as the underlying mechanism of the female-biased sex ratios

In many lepidopteran species, including *E. hecabe*, the sex chromosome constitution is female-heterogametic (i.e., WZ females and ZZ males), and the W chromosome is cytologically observable as a condensed sex chromatin body in interphase nuclei (Traut and Marec, 1996). Cytological observations of Malpighian tubule cells and bursa copulatrix cells revealed that sex chromatin bodies were present in females of normal 1:1 sex-ratio broods, but were not observed in females of female-biased broods (Figure 13.3) (Hiroki et al., 2002; Narita et al., 2007a). These results strongly suggest that the female-biased sex ratios were caused by feminization of genetic males (ZZ). These feminized genetic males are able to copulate with normal males and produce subsequent generations that are all females.

Like many other endosymbiotic bacteria, *Wolbachia* are susceptible to tetracycline, a bacteriostatic antibiotic that inhibits bacterial growth by interfering with protein synthesis. When a tetracycline-containing honey solution was fed to adult females of female-biased broods prior to oviposition, they exclusively produced male progeny. The antibiotic treatment did not influence the 1:1 sex ratios of normal broods (Hiroki et al., 2002). Therefore, the results for antibiotic treatment (i.e., all-male production) also strongly support the notion that feminization of genetic males is the underlying mechanism of *Wolbachia*-induced female-biased sex ratios in *E. hecabe*.

Constant presence of wHecCI and frequent loss of wHecFem: implications for the population ecology of *E. hecabe*

The vertical transmission rates of wHecCI and wHecFem in singly infected and doubly infected matrilines were examined. The transmission rates of wHecCI were nearly 100% in both singly infected and doubly infected matrilines. The transmission rate of wHecFem was significantly lower than that of wHecCI, because approximately 20% of offspring failed to inherit wHecFem (Figure 13.4).

wHecCI causes cytoplasmic incompatibility with 100% intensity in *E. hecabe* (Hiroki et al., 2002, 2004). Previous studies on infection frequencies among field populations and molecular phylogeography revealed that wHecCI has spread rapidly from the southwest to the northeast of mainland Japan (Hiroki et al., 2005; Narita et al., 2006). The high transmission fidelity and high cytoplasmic incompatibility intensity of wHecCI clearly support the biogeographical data.

Although lower than wHecCI, wHecFem still has a transmission rate as high as 80%. Because nearly 100% of the offspring of doubly infected mothers are feminized, wHecFem has the potential to spread in host populations if at least 50% of the offspring inherit wHecFem. Considering the observed wHecFem infection frequency of 80%, it is reasonable to assume that wHecFem can spread and be maintained in *E. hecabe* populations. In future studies, it will be of great interest to examine the population dynamics of *E. hecabe* in populations where individuals with the two different infection types coexist.

High and stable density of wHecCI vs. low and fluctuating density of wHecFem

Although the two *Wolbachia* strains coinfect the same host insect, the cytoplasmic incompatibility-inducing strain wHecCI consistently exhibited 10²- to 10³-fold higher infection densities (10⁻³-10⁻⁶ copies per mitochondrial COI copy) than the feminizing strain wHecFem (10⁻¹-10⁻⁷ copies per mitochondrial COI copy) (Figure 13.5). In a previous study, the wHecCI densities were consistently high and stable and the wHecFem densities were
constantly low and fluctuating irrespective of the adult ages and tissues (Narita et al., 2007b). The high and stable densities of \( w \)HecCI may be considered as an adaptive strategy to maximize the efficiency of its vertical transmission, while the imperfect vertical transmission of \( w \)HecFem may be attributable to its low and fluctuating densities. The different infection densities between \( w \)HecCI and \( w \)HecFem may be relevant to their reproductive
Figure 13.4  Percentages of offspring infected with each *Wolbachia* strain in the ovary. Left: Offspring of mothers doubly infected with *w*HecCl and *w*HecFem. Right: Offspring of mothers singly infected with *w*HecCl. Gray: Individuals positive for both *w*HecCl and *w*HecFem. White: Individuals positive for *w*HecCl alone. The sample size is given on each bar.

Figure 13.5 Densities of *Wolbachia* strains *w*HecCl and *w*HecFem in the ovaries of adult females examined at day 4 after adult emergence. (a): Densities of *w*HecCl in the offspring of mothers singly infected with *w*HecCl (left). Densities of *w*HecCl (middle) and *w*HecFem (right) in the offspring of mothers doubly infected with *w*HecCl and *w*HecFem. Each circle represents an individual. (b): Relationship between *w*HecCl and *w*HecFem densities within single individuals. Each dot represents an individual.

phenotypes (cytoplasmic incompatibility vs. feminization) or their different levels of adaptation to the host insect (widespread *w*HecCl vs. infrequent *w*HecFem).

_Feminizing Wolbachia continuously act on E. hecabe during larval development for maintenance of female phenotypes_

How and when do the *Wolbachia* endosymbionts feminize genetically male butterflies? To answer these questions, larvae were fed a tetracycline-containing diet from different
developmental stages until pupation. When the adults emerged, most of them had wings with abnormal morphologies (e.g., curled, folded, or asymmetric) and were unable to fly. Strikingly, their wing morphologies were sexually intermediate (Figure 13.6). The expression of intersexual phenotypes in wing morphologies was strong in butterflies treated from the first instar stage, moderate in butterflies treated from third instar stage, and weak in butterflies treated from the fourth instar stage (Table 13.1). The reproductive organs and genitalia also exhibited these tendencies (Figure 13.7; Figure 13.8), because they exhibited sexually intermediate traits according to the timing and duration of the tetracycline treatment. These results strongly suggest that the sexually intermediate traits were caused by attenuated feminization due to suppression of the function of Wolbachia by the antibiotic treatment. Continuous infection with the feminizing Wolbachia during the period from the first to third instar stages appears to be required for complete expression of female phenotypes under the male genotype.

On the basis of the well-understood molecular mechanisms underlying sex determination in D. melanogaster (Schütt and Nöthiger, 2000) and the universal occurrence of sexual mosaicism in diverse insects (Laugé, 1985), it has been proposed that sex determination in insects generally occurs at an early embryonic stage in a cell-autonomous manner. Wolbachia-induced parthenogenesis makes unfertilized eggs develop into female embryos (Arakaki et al., 2001; Hagimori et al., 2006; Stouthamer, 1997), whereas Wolbachia-induced cytoplasmic incompatibility results in arrested embryogenesis in incompatible crosses (Bourtzis and Miller, 2003; O’Neill et al., 1997), and Wolbachia-induced male killing causes
male-specific embryonic mortality (Bourtzis and Miller, 2003; Hurst and Jiggins, 2000; O’Neill et al., 1997). From these circumstantial lines of evidence, it appears natural to assume that Wolbachia-induced feminization should involve the transformation of genetic males into phenotypic females at an early embryonic stage. In this context, the discovery that the feminizing Wolbachia act continuously on the larvae of E. hecabe for the consummation of female phenotypes is quite unexpected and may provide some novel insights into the mechanisms underlying symbiont-induced reversal of insect sex.

**Key players of feminization in E. hecabe**

The strong association of Wolbachia infection statuses with feminization phenotypes, i.e., matrilinea doubly infected with ωHecCI and ωHecFem exhibit feminization, whereas matrilinea singly infected with ωHecCI do not, may lead us to naïvely assume that ωHecFem is the only causal agent of feminization. However, a more complex situation is implied by several observations as described below. ωHecCI exhibited a high and stable density and was constantly present (transmission efficiency of 100%) irrespective of the presence or absence of ωHecFem. In contrast, ωHecFem exhibited an extremely low and fluctuating density and was frequently lost (transmission efficiency of 80%) (Narita et al., 2007b). Notably, offspring that spontaneously failed to inherit ωHecFem were completely feminized (Narita et al., 2007b).

These observations may indicate that ωHecFem does not have a feminizing effect by itself, and that factors other than ωHecFem may act directly or cooperatively during feminization of genetically male butterflies. The host nuclear background and/or ωHecCI can be suggested as candidates for the other factors. Because antibiotic treatment of larvae affected the sexual phenotype of feminized butterflies (Narita et al., 2007a), the Wolbachia strain ωHecCI rather than the host nuclear background could play an important role in

---

**Table 13.1** Sexually Intermediate Phenotypes in Wing Morphology of Antibiotic-Treated E. hecabe

<table>
<thead>
<tr>
<th>Treated Stage</th>
<th>No. of Individuals with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Feminine Color, Sex Brand</td>
</tr>
<tr>
<td>From 1st instar until pupation</td>
<td>0</td>
</tr>
<tr>
<td>From 2nd instar until pupation</td>
<td>0</td>
</tr>
<tr>
<td>From 3rd instar until pupation</td>
<td>11</td>
</tr>
<tr>
<td>From 4th instar until pupation</td>
<td>3</td>
</tr>
<tr>
<td>No treatment</td>
<td>168</td>
</tr>
</tbody>
</table>

*Note: Feminine color, soft yellow typical of normal females; masculine color, bright yellow typical of normal males; 0, absence of sex brand; ?, sex brand not examined or unrecognized; 2, sex brand present in both forewings. The phenotypes presented range from most feminine to most masculine (left to right).*

Figure 13.7 (Color figure follows p 238.) Reproductive organs of *E. hebbe* adults that emerged after larval antibiotic treatment: (a) Two deformed testes coexisting with a mature ovary obtained after antibiotic treatment from the first to fourth instar stages of an insect line doubly infected with *wHeCIC* and *wHeCFem*. (b) and (c): Two deformed testes obtained after antibiotic treatment from the first to fourth instar stages of an insect line doubly infected with *wHeCIC* and *wHeCFem*. (d): A deformed testis obtained after antibiotic treatment from the first to fourth instar stages of an insect line doubly infected with *wHeCIC* and *wHeCFem*. (e): A normal testis from a nontreated insect line singly infected with *wHeCIC*. (f): A normal ovary from a nontreated insect line singly infected with *wHeCIC*. Note that a pair of testes are often fused into one testis in lepidopteran adult insects. Arrows indicate testes. Bar, 1 mm. (Adapted from Narita S, Kageyama D, Nomura M, and Fukatsu T. (2007a). *Appl. Environ. Microbiol.* 73: 4332-4341. With permission.)

feminization (Figure 13.9). To directly confirm this idea, the sexual phenotypes of the offspring of individuals singly infected with *wHeCFem* would need to be examined. However, due to the absence of individuals singly infected with *wHeCFem* in nature (Figure 13.2) and the difficulty in selectively eliminating *wHeCIC* in the laboratory, this issue remains to be elucidated.

Wolbachia-induced feminizing effect and male killing in *Ostrinia* species moths

*Infection status of Wolbachia in Ostrinia species*

*Wolbachia* infection has been reported in four species in the *Ostrinia furnacalis* species complex (Lepidoptera: Crambidae), namely *O. furnacalis*, *Ostrinia scapulalis*, *Ostrinia orientalis*, and *Ostrinia zagulaeaei* (Kageyama et al., 2004). Based on detailed analyses of their biological and genetic traits, it was recently proposed that *O. scapulalis* and *O. orientalis* are mor-
Genitalia preparations of *E. lecaeb* adults that emerged after larval antibiotic treatment. (a) and (b): Sexually intermediate genitalia obtained after antibiotic treatment from the first to fourth instar stages of an insect line doubly infected with *vhecCl* and *vhecFem*. (c): Male genitalia from a nontreated insect line singly infected with *vhecCl*. (d): Female genitalia from a nontreated insect line singly infected with *vhecCl*. Blue arrowheads indicate male traits (*bicuspid apex of volva*), and pink arrowheads indicate female traits (*papilla analis*). Bar, 1 mm. (Adapted from Narita, S., Kageyama, D., Nomura, M., and Fukatsu, T. (2007a). *Appl. Environ. Microbiol.* 73: 4332–4341. With permission.)

Phenotypical variants and form a single species designated *O. scapulalis* (Frolov et al., 2007). Although *O. furnacalis* and *O. scapulalis* are closely related, they are clearly distinct species that are commonly found in Japan. *O. furnacalis* mainly feeds on maize, whereas *O. scapulalis* feeds on legumes and a wide range of plants. Among *O. furnacalis* and *O. scapulalis*, nearly 5% of wild-caught females are infected with Wolbachia (Kageyama et al., 1998, 2002, 2003a) (Figure 13.10). In each of the two Wolbachia genes, i.e., *wsp* (555 bp) and *ftsZ* (1023 bp), DNA fragment sequences were found to be identical among different individuals within species and among different species, suggesting that they are infected with a single strain of Wolbachia. The Wolbachia-induced reproductive manipulations have been relatively well examined in *O. furnacalis* and *O. scapulalis* and were found to be substantially the same. Thus, the Wolbachia-induced reproductive manipulations in *Ostrinia* are hereafter described for these two species.
Figure 13.9 What determines the sex of *E. hecate*? (a): Mothers singly infected with αHeCt produce αHeCt-infected offspring with 1:1 sex ratios. (b): Mothers doubly infected with αHeCt and αHeFem produce offspring consisting of all or nearly all females in the normal condition (left and middle). Approximately 80% of the offspring are doubly infected (left), while 20% of the offspring spontaneously lose αHeFem (middle). When offspring are treated with an antibiotic (tetracycline) during larval development, they develop as intersexes (right). These results suggest that not only αHeFem but also αHeCt may play important roles in feminizing genetic males of *E. hecate*.

**Female-biased sex ratios in Wolbachia-infected matriline**

Adult females collected in six geographic locations across central and northern parts of the Honshu mainland of Japan (Figure 13.10) were individually allowed to oviposit and their offspring were reared until adult emergence. The progeny produced by Wolbachia-infected females consisted of all or nearly all females, whereas most of the progeny produced by uninfected females consisted of males and females with sex ratios of nearly 1:1. Wolbachia-infected matriline were maintained by crossing with normal males and consistently produced all or nearly all females for more than 20 generations.

**Appearance of all-male progeny from mothers treated with antibiotics during larval development: possible feminization of genetic males as the underlying mechanism of the female-biased sex ratios**

To investigate the effects of Wolbachia, Wolbachia-infected larvae were fed an antibiotic (tetracycline)-containing diet until pupation and found to develop into healthy female adults free from Wolbachia infection. Strikingly, however, the progeny produced from these cured females only consisted of males at the adult stage. These results are reminiscent of Wolbachia-induced feminization in *E. hecate* (Hiroki et al., 2002; Narita et al., 2007a), and Kageyama et al. (1998 and 2002) erroneously concluded that feminization of genetic males was the underlying mechanism of the female-biased sex ratios in *Ostrinia*. However, the cytological examinations described in the next section clearly exclude the possibility of feminization as the underlying mechanism of the female-biased sex ratios.
Cytological observations reveal that feminization is not the underlying mechanism of the female-biased sex ratios

Cytological observations of sex chromatin bodies (condensed W chromosome in highly polyploid interphase nuclei) in Malpighian tubule cells and bursa copulatrix cells are often useful for clarifying the sex chromosome constitution (WZ in females; ZZ in males) (Traut and Marec, 1996). In *Ostrinia*, all the *Wolbachia*-infected mothers that produced female-biased progeny and their daughters had the WZ karyotype. All the uninfected mothers that produced progeny with 1:1 sex ratios also had the WZ karyotype (Kageyama and Traut, 2004). These results indicate that the sex ratio distortion found in this species is not due to feminization.

Male killing when *Wolbachia* is present

Larvae at the hatching stage were examined for the presence or absence of sex chromatin bodies to identify their genetic sexes (WZ or ZZ). Some larvae, which apparently developed well but did not leave the eggshell, were regarded as unhatched. In broods from *Wolbachia*-infected mothers, the WZ:ZZ ratios of unhatched larvae were significantly biased toward
Table 13.2 WZ:ZZ Ratios of Larvae, Inferred from Sex-Chromatin Status

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected</td>
<td>14:20</td>
<td>31:31</td>
<td>45:51</td>
<td>35:32</td>
</tr>
<tr>
<td>Infected</td>
<td>12:30*</td>
<td>46:33</td>
<td>58:63</td>
<td>40:0**</td>
</tr>
<tr>
<td>Cured</td>
<td>54:3”</td>
<td>16:72”</td>
<td>70:75</td>
<td>0:26”</td>
</tr>
</tbody>
</table>

* In the last instar, all WZ larvae were females and all ZZ larvae were males according to the gonad anlagen.
** X2-test, deviation from the 1:1 ratio significant (P < 0.01).


ZZ (i.e., genetic males). The WZ:ZZ ratios of the survivors increased with larval development and finally reached 1:0 (i.e., genetic females only) at the last instar stage (Kageyama and Traut, 2004; Sakamoto et al., 2007) (Table 13.2). These results clearly indicate that Wolbachia kills genetic males (ZZ individuals) during larval development and that Wolbachia infection is compatible with the development of genetic females (WZ individuals). These findings indicate that Wolbachia in Ostrinia is a male killer and does not feminize ZZ individuals into functional females.

Female killing when Wolbachia has been eliminated

In contrast, the WZ:ZZ ratios of unhatched larvae in broods from cured mothers were significantly biased toward WZ (i.e., genetic females) (Kageyama and Traut, 2004; Sakamoto et al., 2007). The WZ:ZZ ratios of the survivors decreased with larval development and finally reached 0:1 (i.e., genetic males only) at the last instar stage (Kageyama and Traut, 2004; Sakamoto et al., 2007) (Table 13.2). These results clearly indicate that, when mothers are cured of Wolbachia infection by antibiotic treatment during larval stages, genetically female offspring (WZ individuals) die during larval development while male offspring (ZZ individuals) survive.

There are two hypotheses that can account for the female-killing mechanism, which we refer to as the compensation hypothesis and the modification-rescue hypothesis (Figure 13.11). The compensation hypothesis assumes host genetic differences in maternally inherited factors (i.e., cytoplasmic elements or W-linked genes) between infected and uninfected matrilines. Due to this difference, infected matrilines lack some essential genetic factors necessary for the early development of females, but Wolbachia does compensate for these factors. In other words, this hypothesis assumes a historical coevolution between the bacteria and the hosts. In contrast, the modification-rescue hypothesis does not assume host genetic differences between infected and uninfected matrilines. Wolbachia are assumed to modify Ostrinia maternally in order to kill the daughters. Furthermore, the transmitted Wolbachia are assumed to rescue the modified daughters. In other words, daughters of infected mothers are rescued and can survive if Wolbachia have been successfully transmitted. Otherwise, they are killed by the effect of the modification.

These two hypotheses are mutually exclusive and testable by transfection of Wolbachia. If female-biased matrilines are established by transfecting Wolbachia from infected matrilines into uninfected matrilines, all we need to do is eliminate the Wolbachia infection from
Figure 13.11 Two hypotheses can account for the female-killing mechanism when Wolbachia have been removed from mothers. (a): The compensation hypothesis assumes that infected matrilines lack some matrilineal genetic factors (i.e., W-linked or cytoplasmic factors) that are necessary for the survival of daughters, although the autosomal genes of infected and uninfected matrilines are homogeneous. This hypothesis also assumes that Wolbachia compensate for the deficiency of the matrilineal trait of infected matrilines. (b): Instead of assuming genetic differences, the modification-rescue hypothesis assumes that Wolbachia modify mothers in order to selectively kill the daughters (maternal imprinting). This hypothesis also assumes that inherited Wolbachia (i.e., those successfully transmitted to daughters) rescue the daughters from being killed.

The newly established matrilines and examine the sex ratios of their offspring. If the sex ratios are 1:1, the compensation hypothesis would be correct. In contrast, if the sex ratios are male-biased, the modification-rescue hypothesis would be correct.

Antibiotic treatment of adult females leads to the production of progeny with intersexual phenotypes

When mothers were fed an antibiotic-containing sucrose solution during the adult stage prior to oviposition, a considerable number of offspring with intersexual phenotypes appeared (Figure 13.12) (Kageyama et al., 2003b; Kageyama and Traut, 2004). Eggs laid during the first to third days after tetracycline treatment developed as females only. Eggs laid on the fourth and fifth days developed as females, intersexes, or males. Eggs laid from the sixth to ninth days developed as males only (Table 13.3). The successive appearance of females, intersexes, and males suggests that eggs laid early after treatment onset were still under the influence of the Wolbachia infection, whereas eggs laid 4–5 days after treatment onset were partly cured of the Wolbachia infection, and those laid from day 6 onwards were completely cured. Cytological observations of Malpighian tubules, testes, and bursa copulatrix cells revealed that phenotypic females were genetically female (WZ), phenotypic males were genetically male (ZZ), and all intersexual individuals were genetically male (ZZ) in all tissues. Strikingly, the bursa copulatrix, which is a female-specific organ, had the male genotype (ZZ). In other words, this organ had the female phenotype under
Figure 13.12 Morphological and cytological features of intersexual individuals. (a): Left forewing of an uninfected female. (b): An intersexual individual generated by transfection. (c): An intersexual individual generated by tetracycline treatment. (d): An uninfected male. (e)–(g): External genitalia of an uninfected female (e), an intersexual individual generated by transfection (f) and an uninfected male (g). (h)–(k): Interphase cells of the bursa copulatrix from an uninfected female (h), an intersexual individual generated by transfection (i), and an intersexual individual generated by tetracycline treatment (j). *Ovipositor* and *ovipositor*-like structures; *uncus* and *uncus*-like structures; sex chromatin bodies. The magnifications of (a)–(d), (e)–(g), and (h)–(j) are equal. Scale bars: 5 mm (a–d); 0.5 mm (e–g); 20 mm (h–j). (Adapted from Kageyama, D. and Traut, W. (2004). Proc. R. Soc. Lond. B. 271: 251–258.)
Table 13.3 Fates of Successive Egg Batches Laid by a Wolbachia-Infected Female after Tetracycline Treatment

<table>
<thead>
<tr>
<th>Day of Oviposition (after Treatment)</th>
<th>Eggs Laid</th>
<th>Females</th>
<th>Adults Eclosed</th>
<th>Intersexes</th>
<th>Males</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>24</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>32</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>44</td>
<td>6</td>
<td>9</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>28</td>
<td>2</td>
<td>1</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>46</td>
<td>0</td>
<td>0</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>


the male genotype (i.e., feminization). Intersexual individuals were also generated in O. scapulalis after transfecting Wolbachia from an infected matriline to an uninfected matriline (Kageyama and Traut, 2004). These intersexual individuals were not genetic mosaics but genetically homogeneous male individuals. In this sense, the fundamental mechanism of intersexual development in Ostrinia species is likely to be the same as that in E. hecabe.

Integrated explanation of the mechanism underlying male killing

As shown above, Wolbachia cause male killing in Ostrinia. It is obvious from the generation of intersexual individuals with the male genotype that Wolbachia have a feminizing effect on genetic males. How can we reconcile these seemingly distinct phenomena of male killing and feminization? We consider that, in Ostrinia, the fully expressed feminizing effect of Wolbachia is lethal to genetic males, whereas the weakly expressed feminizing effect can be nonlethal to genetic males. We therefore propose that the intersexual individuals obtained after antibiotic treatment of mothers could have survived because they were only partially feminized (Figure 13.13).

Mechanistic bases of male killing and feminization

Does Wolbachia interfere with sex-determining genes?

Both Wolbachia in Ostrinia and Wolbachia in E. hecabe have feminizing effects on their genetic male hosts. Thus, it is natural to assume that Wolbachia manipulates genes among the sex-determining gene cascades of their hosts. Unlike D. melanogaster (Baker et al., 1987; Schütt and Nöthiger, 2000), the sex-determining mechanisms are not well understood in lepidopteran insects, except for the fact that doublesex (dsx) gene expression is sex-specifically spliced in the silkworm B. mori (Ohbayashi et al., 2001; Suzuki et al., 2003, 2005, 2008). By investigating the splicing patterns of the dsx gene expression in normal males, normal females, feminized individuals, and intersexual individuals of E. hecabe and
### Genotype vs. Phenotype

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>(ZZ)</td>
<td>completely feminized</td>
</tr>
<tr>
<td></td>
<td>inviable</td>
</tr>
<tr>
<td></td>
<td>partially feminized</td>
</tr>
<tr>
<td></td>
<td>viable</td>
</tr>
<tr>
<td></td>
<td>(intersex)</td>
</tr>
</tbody>
</table>

**Figure 13.13** Proposed mechanism of male killing in *Ostrinia*. Upper: Under complete influence of *Wolbachia*, genetic males are completely feminized into phenotypic females but are inviable due to some forms of incompatibility between the genotypic sex and the phenotypic sex. Middle: Under incomplete influence of *Wolbachia* (i.e., by incomplete curing of *Wolbachia* infection from mothers or transfection of *Wolbachia* into uninfected eggs), genetic males are partially feminized and develop into intersexes, at least some of which are viable. Bottom: In the absence of *Wolbachia*, genetic males develop into phenotypic males.

*Ostrinia*, we may be able to clarify whether the target of *Wolbachia* is upstream or downstream of *d sx* within their sex-determining gene cascades (Figure 13.14). Future elucidation of the whole sex-determining mechanism in *B. mori* will provide strong reference information when investigating the effects of *Wolbachia* on the sex-determining mechanisms in *Ostrinia* and *E. hecabe*.

**Does Wolbachia interfere with dosage compensation?**

Organisms with male heterogametic (XX in females and XY in males) or female heterogametic (WZ in females and ZZ in males) sex-determining systems have different numbers of X chromosomes (or Z chromosomes) between males and females. To equalize the titers of X-linked (or Z-linked) gene expression between males and females, many organisms adopt two alternative processes: overexpression of X-linked genes in males or underexpression of X-linked genes in females (Marín et al., 2000; Parkhurst and Meneely, 1994; Charlesworth, 1996). These mechanisms are collectively referred to as dosage compensation. It is known that *Drosophila*, *Caenorhabditis elegans*, and mammals undertake dosage compensation. In the lepidopteran insect *B. mori*, however, dosage compensation is absent, because Z-linked gene expression is twofold higher in males than in females (Suzuki et al., 1998, 1999). Therefore, in *B. mori*, males and females function normally despite the unequal amounts of Z-linked gene products between males and females.

In *D. melanogaster*, the endosymbiotic bacteria *Spiroplasma* are known to cause male killing (Williamson and Poulson, 1979). A male-specific protein complex (dosage compensation
Figure 13.14 Hypothetical molecular mechanisms for sex determination in *E. hecabe* and *Ostrinia*. (a): In uninfected matrilines, morphological sexual dimorphism is generated by proper expression of sex-determining and sex-differentiation genes according to their genetic sexes. (b): In feminized matrilines, the expression of one of the sex-determining or sex-differentiation genes is switched from the male-type to the female-type at a particular point. In the downstream of this point, the gene expressions are consistently of the female type. Consequently, phenotypically female adults are generated under the male genotype in *E. hecabe*, whereas genetic males die during larval development in *Ostrinia*. (c): Intersexual individuals generated in *E. hecabe* and *Ostrinia* are purely genetic males, but are comprised of mosaics of phenotypically female and male tissues.

complex: DCC), which is necessary for dosage compensation, was found to be required for the expression of Spiroplasmap-induced male killing (Veneti et al., 2005).

The death of *Ostrinia* genetic males is considered to be due to their intolerance of the feminizing effect of *Wolbachia* (Kageyama and Traut, 2004). This may imply that genetic males (ZZ individuals) cannot survive as phenotypic females due to the adverse effects of the excessive expression of Z-linked genes. Therefore, why do feminized genetic males of *E. hecabe* survive and function normally? To answer or validate this question, we need to
examine the Z-linked gene expression levels among normal females, normal males, and feminized individuals of E. hecabe and Ostrinia.

Wolbachia genotype or host genotype: which is responsible for the reproductive phenotype?

Wolbachia exhibit various types of reproductive manipulations in their hosts. Which genotype is responsible for the types of reproductive manipulations, the Wolbachia genotype or the host genotype? Fujii et al. (2001) transferred Wolbachia from O. scapulalis into the moth *Ephestia kuehniella*, which was previously cured of a naturally occurring Wolbachia infection. A newly established Wolbachia-infected matriline of E. kuehniella exhibited male killing (i.e., female-biased sex ratios, egg hatch rates of nearly 50%, and 1:1 sex ratios following tetracycline treatment). Because Wolbachia was assumed to cause feminization in Ostrinia at that time, this result was considered to indicate that the types of reproductive manipulations, i.e., feminization and male killing, are attributable to host genetic differences between Ostrinia and Ephestia. Because naturally occurring Wolbachia in Ostrinia actually cause male killing instead of feminization, this result does not support the assumption that host genetic differences determine the types of reproductive manipulation.

There is a convincing case in which the host genetic background is responsible for the types of reproductive manipulations. The almond moth *Cadra cautella* is doubly infected with two Wolbachia strains, vCauA and vCauB, and expresses strong cytoplasmic incompatibility. Tetracycline treatment generated a C. cautella strain singly infected with vCauA and this strain was found to express strong cytoplasmic incompatibility by itself (Sasaki et al., 2005). Wolbachia were artificially transferred from C. cautella into E. kuehniella and an E. kuehniella strain singly infected with vCauA was generated. All-female production, egg hatch rates of 50%, and 1:1 sex ratios following tetracycline treatment showed that the vCauA strain expressed male killing in the E. kuehniella host (Sasaki et al., 2002). In other words, vCauA caused cytoplasmic incompatibility in C. cautella and male killing in E. kuehniella.

In the flour beetle *Tribolium confusum*, cytoplasmic incompatibility is caused by a naturally occurring Wolbachia strain, vCon (Fialho and Stevens, 1997). It is interesting that, in the closely related species *Tribolium madidens*, a naturally occurring Wolbachia strain indistinguishable from vCon by DNA sequencing of the *wsp* and *ftsZ* genes causes male killing (Fialho and Stevens, 2000). Although it is unclear whether the Wolbachia genome or the host genome is responsible for determining the type of reproductive manipulations in Tribolium, these insects may represent an ideal system for investigating the mechanisms of Wolbachia-induced reproductive manipulations.

Although not technically easy, reciprocal transfection of Wolbachia strains between various insects that exhibit different reproductive manipulations, such as Ostrinia and E. hecabe, may greatly contribute to clarifying the important issue of whether the types of reproductive manipulations are determined by the Wolbachia genotype or the host genotype, or both genotypes. Clarification of this issue will lead to future understanding of the mechanisms of Wolbachia-induced reproductive manipulations.

Evolutionary implications of male killing and feminization

To date, male killing has been reported in various species of insects, including fruitflies, mosquitoes, butterflies, moths, ladybird beetles, and parasitic wasps. Furthermore, the
causal agents of the male killing belong to taxonomically diverse microorganisms, such as bacteria belonging to Wolbachia, Ricketttsia, Arsenophonus, Spiroplasma, and Flavobacterium and unicellular prokaryotes belonging to Microsporidia (Hurst and Majerus, 1993; Hurst and Jiggins, 2000; Hurst et al., 2003). Therefore, male killing is considered to be a trait that is easy to evolve (Hurst et al., 2003). On the other hand, endosymbiont-induced complete feminization has only been reported in E. hecabe among insects. Even among all arthropods, microbe-induced feminization has only been found in a few species, such as woodlice and shrimps (Rigaud, 1997; Dunn et al., 1993). Despite its rare occurrence, feminization is a more advantageous strategy for maternally transmitted endosymbionts than male killing, because all the offspring of infected mothers can transmit the infection to subsequent generations in the case of feminization, whereas only half the offspring can transmit the infection to subsequent generations in the case of male killing.

The male killing observed in Ostrinia is considered to be death of genetic males due to the feminizing effect of Wolbachia. Some endosymbionts may have a feminizing effect on genetic male hosts and this effect can often be lethal. In E. hecabe, this feminizing effect may somehow be nonlethal, such that genetic males completely revert to functional females. Evolutionary transitions in either the host (E. hecabe) or the endosymbiont (Wolbachia) can account for the nonlethal complete feminizing effect on genetic males of E. hecabe, i.e., E. hecabe may have evolved a trait in genetic males such that they are not killed by feminization or Wolbachia may have evolved a trait not to kill genetic males while feminizing them.

Overall, it is undoubtedly the case that male killing and feminization are both outcomes of the close associations between endosymbionts and the sex-determining systems of their hosts. By untangling such complex interactions between endosymbionts and their hosts, we may be able to reveal unknown aspects of sex determination or sex differentiation in insects.

Acknowledgments

We thank Drs. Masashi Nomura, Takema Fukatsu, Sugihiko Hoshizaki, Yukio Ishikawa and Walther Traut for valuable advice during the course of this study. We thank Dr. Hiroaki Noda for helpful comments on an early version of the manuscript. SN was supported by a Japan Society for the Promotion of Science (JSPS) fellowship for Young Scientists. DK was supported by a Grant-in-Aid for Young Scientists (No. 19780046) from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT).

References

Chapter Thirteen: Wolbachia-induced sex reversal in Lepidoptera


**Color Figure 13.2** Different Wolbachia infection types and their phenotypes in *E. hecabe*. (a): In most populations, butterflies singly infected with *wHeCIC* exhibit cytoplasmic incompatibility. (b): In populations from Okinawajima and Tanegashima, butterflies doubly infected with *wHeCIC* and *wHeFCeM* exhibit femization. (c): Butterflies singly infected with *wHeFCeM* have never been found in natural populations or in the laboratory. Right: A female adult of *E. hecabe* in the natural condition. (Photo provided by Dr. Masashi Nomura, Chiba University.)

**Color Figure 13.6** *E. hecabe* adults that emerged after larval antibiotic treatment. (a) and (b): Emerged adult insects with deformed wings obtained after antibiotic treatment from the third to fourth instar stages of an insect line doubly infected with *wHeCIC* and *wHeFCeM*. (c): Adult insect that failed to escape from the pupal case obtained after antibiotic treatment from the first to fourth instar stages of an insect line doubly infected with *wHeCIC* and *wHeFCeM*. (d) and (e): Normal adult females, pale in ground color and without sex brands, representing a nontreated insect line singly infected with *wHeCIC*. (f) and (g): Normal adult males, bright in ground color and with sex brands (arrows), representing a nontreated insect line singly infected with *wHeCIC*. Bars, 10 mm. (Adapted from Narita, S., Kageyama, D., Nomura, M., and Fukatsu, T. (2007a). *Appl. Environ. Microbiol.* 73: 4332–4341. With permission.)
Color Figure 13.7  Reproductive organs of *E. heliothis* adults that emerged after larval antibiotic treatment. (a): Two deformed testes coexisting with a mature ovary obtained after antibiotic treatment from the first to fourth instar stages of an insect line doubly infected with *α*HecCl and *α*HecFem. (b) and (c): Two deformed testes obtained after antibiotic treatment from the first to fourth instar stages of an insect line doubly infected with *α*HecCl and *α*HecFem. (d): A deformed testis obtained after antibiotic treatment from the first to fourth instar stages of an insect line doubly infected with *α*HecCl and *α*HecFem. (e): A normal testis from a nontreated insect line singly infected with *α*HecCl. (f): A normal ovary from a nontreated insect line singly infected with *α*HecCl. Note that a pair of testes are often fused into one testis in lepidopteran adult insects. Arrows indicate testes. Bar, 1 mm. (Adapted from Narita, S., Kageyama, D., Nomura, M., and Fukatsu, T. (2007a). *Appl. Environ. Microbiol.* 73: 4332–4341. With permission.)
Color Figure 13.8  Genitalia preparations of E. hirci adults that emerged after larval antibiotic treatment. (a) and (b): Sexually intermediate genitalia obtained after antibiotic treatment from the first to fourth instar stages of an insect line doubly infected with αHecCl and αHecFem. (c): Male genitalia from a nontreated insect line singly infected with αHecCl. (d): Female genitalia from a nontreated insect line singly infected with αHecCl. Blue arrowheads indicate male traits (bicuspied apex of valves), and pink arrowheads indicate female traits (papilla analis). Bar, 1 mm. (Adapted from Narita, S., Kageyama, D., Nomura, M., and Fukatsu, T. (2007a). Appl. Environ. Microbiol. 73: 4332–4341. With permission.)