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Opposite sex-specific effects of *Wolbachia* and interference with the sex determination of its host *Ostrinia scapulalis*

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In the adzuki bean borer, *Ostrinia scapulalis*, the sex ratio in most progenies is 1 : 1. Females from *Wolbachia*-infected matrilines, however, give rise to all-female broods when infected and to all-male broods when cured of the infection. These observations had been interpreted as *Wolbachia*-induced feminization of genetic males into functional females. Here, we show that the interpretation is incorrect. Females from both lines have a female karyotype with a WZ sex-chromosome constitution while males are ZZ. At the time of hatching from eggs, WZ and ZZ individuals are present at a 1 : 1 ratio in broods from uninfected, infected and cured females. In broods from *Wolbachia*-infected females, ZZ individuals die during larval development, whereas in those from cured females, WZ individuals die. Hence, development of ZZ individuals is impaired by *Wolbachia* but development of WZ females may require the presence of *Wolbachia* in infected matrilines. Sexual mosaics generated (i) by transfection of uninfected eggs and (ii) by tetracycline treatment of *Wolbachia*-infected mothers prior to oviposition were ZZ in all tissues, including typically female organs. We conclude that: (i) *Wolbachia* acts by manipulating the sex determination of its host; and (ii) although sexual mosaics can survive, development of a normal female is incompatible with a ZZ genotype.

Keywords: Lepidoptera; male killing; *Ostrinia scapulalis*; sex chromosomes; W chromatin; *Wolbachia*

1. INTRODUCTION

Maternally inherited female-biased sex ratios have been found repeatedly in arthropod species (reviewed by Hurst 1993). In insects, selective male death at early developmental stages is the most common mechanism: it involves infection by bacteria such as *Spiroplasma*, *Rickettsia*, *Wolbachia* and Flavobacteria (reviewed by Hurst *et al.* 1997). Bacteria of the genus *Wolbachia* are commonly found in arthropod species (Werren *et al.* 1995; Jeyaprakash & Hoy 2000; Werren & Windsor 2000) and many of them manipulate the reproductive systems of their hosts in a variety of ways that tend to increase their own transmission (reviewed by Stouthamer *et al.* 1999). Most of the *Wolbachia*-induced reproductive manipulations can be assigned to one of four basic categories: (i) cytoplasmic incompatibility, which is a reduction of the viability of offspring produced in crosses between infected males and uninfected females; (ii) thelytoky, the generation of females from unfertilized eggs in haplodiploid insects; (iii) feminization of genetic males; and (iv) selective male killing at early developmental stages.

In the Asian corn borer *Ostrinia furnacalis* and the adzuki bean borer *O. scapulalis* (Lepidoptera: Crambidae), *Wolbachia*-induced female-biased sex ratios have been reported (Kageyama *et al.* 1998, 2002, 2003*a*). In both species, elimination of the bacterium by treatment with tetracycline at the larval stage causes the production of exclusively male progenies by the cured females. This has been interpreted as *Wolbachia*-induced feminization in *O. furnacalis* (Kageyama *et al.* 2002). In lepidopterans, females have a ZW or ZO and males a ZZ sex-chromosome constitution. According to that interpretation, *Wolbachia*-infected females are feminized genetic males (ZZs), which, after mating with normal ZZ males, produce only ZZ progenies. ZZ individuals become females if their mothers are infected, but males if their mothers have been completely cured of the infection. Independently, feminization by *Wolbachia* was indicated by the regular appearance of sexual mosaics among the offspring of recently cured females in *O. scapulalis* (Kageyama *et al.* 2003*b*). Feminization of genetic males has also been reported for another lepidopteran species, the pierid butterfly *Eurema hecabe* (Hiroki *et al.* 2002).

In the present study, we show that *O. scapulalis* has a WZ–ZZ sex-chromosome system. In interphase nuclei, the W chromosome forms a conspicuous heterochromatin body, the sex chromatin. We used the sex-chromatin status as a diagnostic tool to identify genetic females and genetic males among larvae and adults of uninfected, *Wolbachia*-infected and cured *O. scapulalis*. This revealed a more complex situation, which resembles that predicted by the alternative interpretation presented by Kageyama *et al.* (2002) for the similarly infected *O. furnacalis*. Genetic males die in broods from infected mothers, whereas genetic females die in broods from cured mothers. In the *Wolbachia*-infected matriline, *Wolbachia* is indispensable for female survival. We generated sexual mosaics from

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genetically male eggs by transfection with *Wolbachia* and by incomplete curing of mothers prior to oviposition. This shows that *Wolbachia* acts by feminization of males. But, while partial feminization can be a viable condition, complete feminization of genetic males appears to be lethal.

2. MATERIAL AND METHODS

(a) *Insects*

The *Wolbachia*-infected matriline MD014 and the uninfected matriline MD068 were derived from single *O. scapulalis* females collected in the field near Matsudo, Chiba prefecture, Japan in 2000 (Kageyama *et al.* 2003*a*). The two matrilines were maintained for more than 10 generations in the Laboratory of Applied Entomology, University of Tokyo, by outcrossing with males of the uninfected stock. Larvae were fed with an artificial diet (Silkmate; Nihon Nosan, Yokohama, Japan). Insects were reared at 23 °C and under a 16 L : 8 D photoperiod.

(b) *Transfection*

Transfection was performed using a three-dimensional micromanipulator and a microinjector (Eppendorf, Hamburg, Germany). For microinjection, glass capillaries were made with an electrode puller and the tips bevelled to an oblique end with a diameter of *ca.* 10–12 μm. Freshly laid uninfected eggs (less than 1 h old) were injected with ooplasm procured from oocytes of *Wolbachia*-infected females.

(c) *Curing*

Cured females were reared from cured larvae of the infected matriline. The larvae had been fed continuously with a tetracycline-containing diet (0.06%, w/w) from hatching to pupation. Cured females give rise to all-male broods. To obtain sexual mosaics, adult females of the *Wolbachia*-infected matriline were mated with uninfected males. Two days later, males were removed and a 3% sucrose solution containing 0.24% w/v tetracycline hydrochloride was given to the females for 1 day. Only one female proved fertile after the treatment. Eggs were collected daily and reared separately.

(d) *PCR for* Wolbachia *detection*

To examine the infection status, PCR specific to *wsp*, a surface protein gene of *Wolbachia*, was conducted in 10 μl reaction volumes including 1 μl of DNA sample, using the *wsp* 81F (5′-TGGTCCAATAAGTGATGAAGAAAC-3′) and *wsp* 691R (5′-AAAAATTAAACGCTACTCCA-3′) primers designed by Braig *et al.* (1998).

(e) *Chromosome preparations*

Meiotic metaphase I preparations were obtained from larval testes and adult ovaries fixed in Carnoy's fluid (ethanol : chloroform : acetic acid, 6 : 3 : 1), and squashed in 60% acetic acid. After freezing on dry ice, the coverslips were removed with a scalpel and the slides were passed through 100% ethanol and air dried. The preparations were stained and mounted in lactic acetic orcein or stained with 4′,6-diamidino-2-phenylindole dihydrate (DAPI) and mounted in antifade (0.233 g of 1,4-diazobicyclo(2,2,2)octane, 1 ml of 0.2 M Tris–HCl at a pH of 8.0 and 9 ml of glycerol).

(f) *Sex chromatin*

From last-instar larvae, Malpighian tubules were dissected out, fixed in methanol : acetic acid (3 : 1) for *ca.* 1 min and transferred to slides. Hatching larvae were placed *in toto* in a drop of Ringer solution, opened with fine needles, fixed in methanol : acetic acid (3 : 1) for *ca.* 1 min and then minced in a drop of 60% acetic acid on a slide. The slides were placed on a heating plate at 70 °C. Rings of dissociated cells dried to the slide when the drop was moved around. The remaining liquid was discarded. The preparations were stained and mounted in lactic acetic orcein and examined by light microscopy.

(g) *Comparative genomic hybridization*

Diploid and low-ploidy nuclei were prepared from imaginal discs (wing discs) of last-instar larvae. Polyploid nuclei were prepared from larval midguts. Pieces of tissue were fixed in either Carnoy's fluid or methanol : acetic acid (3 : 1), minced in a drop of 60% acetic acid and fixed to the slide using a heating plate at 60 °C. Slides were checked with phase-contrast optics and stored in the freezer at −20 °C until needed. Whole genomic DNAs were isolated according to Kageyama *et al.* (2002); this was followed by RNase treatment with 0.02 mg ml^{-1} of RNaseA at 65 °C for 80 min. DNA from females was labelled by nick translation (Bionick Labeling system, Life Technologies, Karlsruhe, Germany) with FluorX-dCTP (green); DNA from males was labelled with Cy3-dCTP (red) (Amersham Life Science, Arlington Heights, IL, USA). Comparative genomic hybridization (CGH) was carried out according to Traut *et al.* (2001) with slight modifications. The probe cocktail for one slide (10 μl, 50% formamide, 10% dextran sulphate, 2 × saline-sodium citrate (SSC)) contained 400 ng of labelled DNA from females and 400 ng of labelled DNA from males but no unlabelled competitor DNA. Hybridization was for 3 days at 37 °C. A stringent wash at 62 °C in 0.1 × SSC and 1% Triton X-100 was followed by washes at room temperature, counterstaining in DAPI and mounting in antifade. Black-and-white images of the FluorX, Cy3 and DAPI signals were recorded separately with a cooled charge-coupled device (CCD) camera, which was mounted on a fluorescence microscope equipped with the Zeiss filter sets 02, 10 and 15.

(h) *Fluorescence measurements*

Fluorescence measurements of single bivalents were performed on digital micrographs of well-spread DAPI-stained metaphase I plates. The histogram function together with the lasso tool of Adobe Photoshop was used. Background fluorescence was subtracted and the fluorescence of single bivalents expressed as the fraction (as a percentage) of that of the full complement. Using the same tools, DAPI fluorescences of the sex chromatin and the bound female and male probes were measured and compared with the remaining chromatin, essentially according to Sahara *et al.* (2003).

3. RESULTS

(a) *Meiotic chromosome complements*

Mitotic chromosome numbers are high and mitoses difficult to obtain in Lepidoptera. Therefore, we investigated the meiotic complements of *O. scapulalis*. Metaphase I of male meiosis displayed 31 bivalents (figure 1*c*). Meiotic metaphase I of both infected and uninfected females had the same number of bivalents, $n = 31$ (figure 1*a*,*b*). Hence, the female sex-chromosome constitution is WZ, as normally encountered in Lepidoptera (and not ZO, which is found in a minority of species), while males are ZZ.

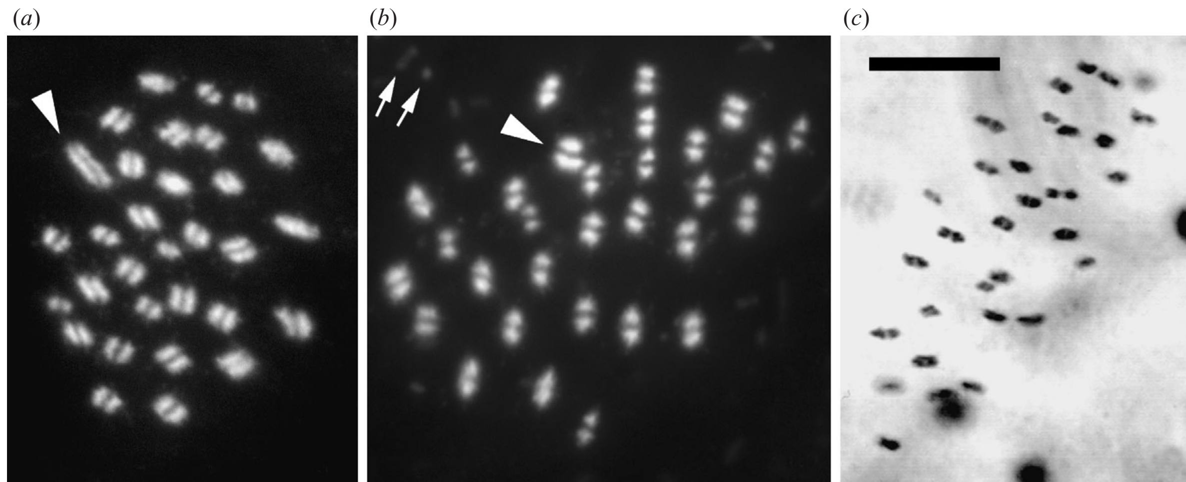

Figure 1. Meiotic metaphase I of: (*a*) an uninfected female; (*b*) a *Wolbachia*-infected female; and (*c*) an uninfected male. Arrowheads, presumed sex-chromosome bivalents; arrows, *Wolbachia* nucleoids. Scale bar, 20 µm; magnification of (*a*)–(*c*) is equal.

Table 1. WZ : ZZ ratios of larvae, inferred from sex-chromatin status.
(In the last instar, all WZ larvae were females and all ZZ larvae were males according to the gonad anlagen.)

	larvae at hatching stage			last-instar larvae
mother	not hatched WZ : ZZ	hatched WZ : ZZ	hatched plus not hatched WZ : ZZ	WZ : ZZ
uninfected	14 : 20	31 : 31	45 : 51	35 : 32
infected	12 : 30*	46 : 33	58 : 63	40 : 0**
cured	54 : 3**	16 : 72**	70 : 75	0 : 26**

* χ^2-test, deviation from the 1 : 1 ratio significant ($p < 0.01$); ** χ^2-test, deviation from the 1 : 1 ratio significant ($p < 0.001$).

In both types of female, one bivalent was conspicuous because of its larger size (figure 1*a*,*b*, arrowheads). Among the remaining bivalents, none was prominent enough to be recognized individually. Fluorescence measurement of the individual bivalents confirmed the presence of one large bivalent (6.2% of total fluorescence) and 30 smaller bivalents in a series of decreasing size (from 4.4% to 1.3% of total fluorescence). The large bivalent appeared to be slightly unequal and, hence, was considered the candidate sex-chromosome bivalent consisting of the Z and the W chromosomes. In DAPI-stained preparations, oocytes from infected females could be distinguished from those of uninfected females. Oocytes of infected females were crowded with small DAPI-positive patches, presumably the nucleoids of *Wolbachia* (figure 1*b*, arrows).

(b) *Sex chromatin*

Lepidopteran species with WZ–ZZ sex-chromosome systems normally display sex chromatin (also called W chromatin), a heterochromatin body derived from the W chromosome, in female interphase nuclei (Traut & Marec 1996). On inspection of female adults and female last-instar larvae, sex chromatin was present in polyploid interphase nuclei, e.g. of Malpighian tubule cells, as well as in diploid or low-ploidy nuclei of imaginal discs and ovaries. This was true for females from the uninfected and the infected lines. Sex chromatin was absent in all male adults and male last-instar larvae from uninfected and cured progenies (figure 2*a*–*f*). CGH with differently labelled genomic DNAs from females (FluorX labelled) and males (Cy3 labelled) highlighted the sex chromatin in the interphase nuclei of infected and uninfected females (figure 2*d*,*e*), while no such highlighted element was apparent in nuclei of males (figure 2*f*). DAPI fluorescence in the sex chromatin was stronger than in the remaining chromatin (figure 2*d*,*e*). This reflects the denser packaging of the DNA in the heterochromatic sex chromatin body than in euchromatin. Image analysis of the respective CGH signals showed that both female DNA probe and (to a lesser degree) male DNA probe bound to the sex chromatin overproportionally compared with the DNA concentration. Differences between the uninfected and the infected lines, however, were not significant (relative signal intensity (mean ± s.d.) is as follows: female probe/DAPI: 1.33 ± 0.13 and male probe/DAPI $= 1.17 \pm 0.16$ ($n = 13$, uninfected line); female probe/DAPI $= 1.31 \pm 0.11$ and male probe/DAPI $= 1.14 \pm 0.12$ ($n = 10$, infected line). The higher concentration of bound probe is thought to be the result of the faster hybridization kinetics of repetitive sequences and indicates an accumulation in the sex chromatin (W chromatin) of such repetitive sequences (Sahara *et al.* 2003).

(c) *Sex ratio and genetic sex in last-instar larvae*

We determined the phenotypic and genetic sex (WZ or ZZ sex-chromosome constitution) of last-instar larvae

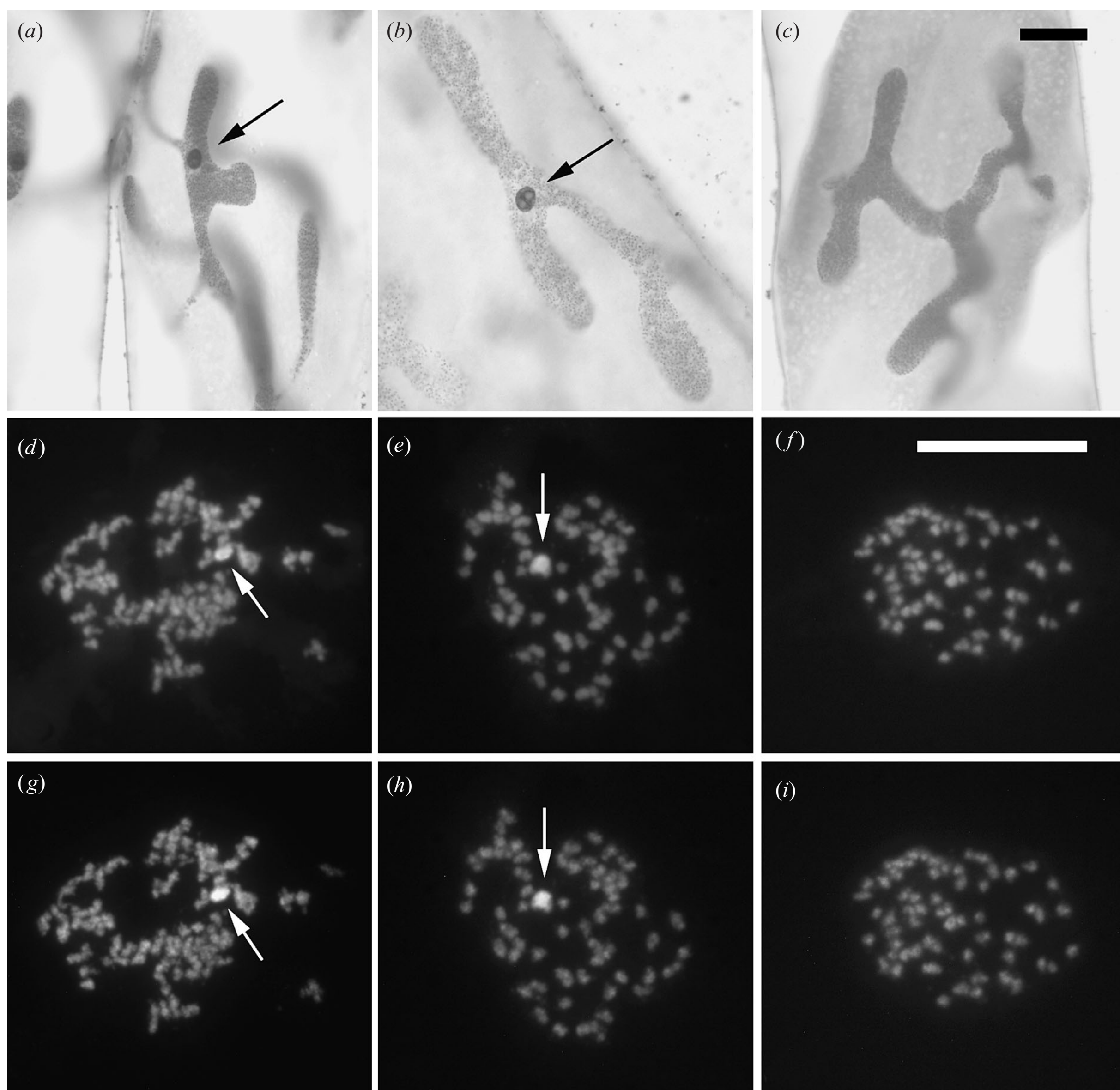

Figure 2. Sex chromatin in interphase nuclei of last-instar larvae. (*a–c*) Malpighian tubule cells with highly polyploid branched nuclei, stained with lactic acetic orcein, from: (*a*) a female from the uninfected line; (*b*) a female from the infected line; and (*c*) a male from the uninfected line. Magnification of (*a*)–(*c*) is equal. (*d–i*) CGH of prophase-like diploid nuclei from imaginal wing discs, images without contrast enhancement: (*d–f*) DAPI stained; and (*g–i*) signals of the female probe hybridized to the nuclei. Nucleus of (*d*,*g*) a female from the uninfected line; (*e*,*h*) a female from the infected line; and (*f*,*i*) a male from the uninfected line. Arrows, W chromatin bodies. Magnification of (*d*)–(*i*) is equal. Scale bars, 20 μm.

according to the presence of ovary or testis anlagen and the presence or absence of sex chromatin, respectively (table 1, last instar). In the uninfected line, sex chromatin was present in every female examined and absent from every male examined. Females, therefore, had a WZ genotype and males a ZZ genotype. In the infected strain, all individuals were female and had a WZ genotype. In the progenies produced by cured females, all larvae were ZZ males. Hence, all last-instar larvae investigated had developed according to their genetic sex. The results show that the all-female progenies in the infected line consist of genetic females and the all-male progenies consist of genetic males. No exception to the rule was detected in random inspections of the sex-chromatin status of adults in our lines (not shown).

(d) *Genetic sex ratio at hatching stage*

Larvae hatching from eggs were examined for the presence or absence of sex chromatin to identify genetically female (WZ) and male (ZZ) individuals (figure 3; table 1, hatching stage). In the uninfected line, the ratio of WZ to ZZ individuals among freshly hatched larvae was *ca.* 1 : 1. Some larvae, although apparently well developed, had not left the eggshell 2–3 days after hatching had started in that batch of eggs ('not hatched' in table 1). The WZ : ZZ ratio of these larvae did not significantly deviate from 1 : 1 either. In broods from infected mothers, the WZ : ZZ ratio was also *ca.* 1 : 1 when counts of 'hatched' and 'not hatched' larvae were combined. The WZ : ZZ ratio was biased towards ZZ, however, when 'not hatched' larvae were considered separately. ZZ larvae tended not to hatch

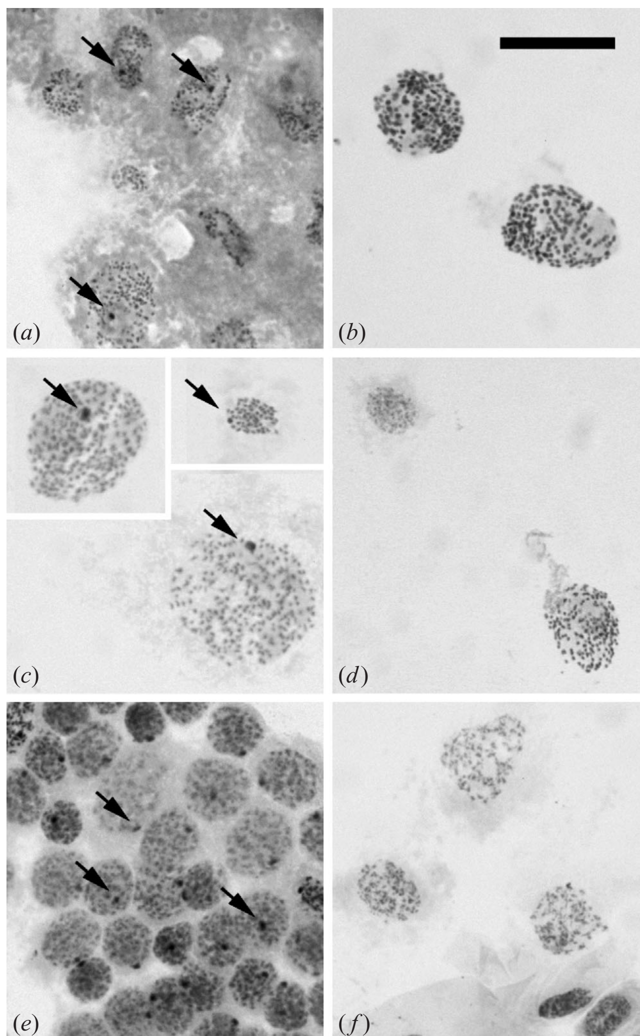

Figure 3. Sex-chromatin status of hatching larvae. Interphase nuclei of offspring from: (*a*,*b*) an uninfected female; (*c*,*d*) a *Wolbachia*-infected female; and (*e*,*f*) a cured female. (*a*,*c*,*e*) WZ larvae and (*b*,*d*,*f*) ZZ larvae. Spread nuclei stained with lactic acetic orcein. Arrows, sex-chromatin bodies. Magnification of (*a*)–(*f*) is equal. Scale bar, 20 μm.

in this line. But even 'hatched' ZZ larvae die before they reach the last instar (table 1, last column). In broods from cured females, the overall WZ : ZZ ratio in the 'hatched' and 'not hatched' larvae was again *ca.* 1 : 1. In these broods, WZ larvae tended not to hatch (table 1). The last column in table 1 shows that 'hatched' WZ larvae in these broods die before the last instar.

(e) *Sexual mosaics generated by transfection*

We injected 1227 freshly laid eggs of the uninfected line with ooplasm from the *Wolbachia*-infected line; 184 of these eggs hatched. A total of 100 moths developed from the injected eggs: 47 were females, two were sexual mosaics and 51 were males. The females were crossed with males from the uninfected line but no newly infected line could be established.

Sexual mosaics were recognized from the appearance of the wings: they had dark sectors typical of males and light sectors typical of females (figure 4*b*). External genitalia displayed a combination of female and male elements (figure 4*f*). When dissected, both mosaics displayed testes and a bursa copulatrix. W chromatin was absent from interphase nuclei of the testes, bursa copulatrix, midgut and Malpighian tubules (figure 4*i*). Hence, the sex-chromosome constitution of the two mosaics was ZZ in the male parts as well as in the indifferent and female parts of the mosaics.

Table 2. Fates of successive egg batches laid by a *Wolbachia*-infected female after tetracycline treatment.

day of oviposition (after treatment)	eggs laid	adults eclosed		
		females	mosaics	males
1	24	8	0	0
2	30	5	0	0
3	32	6	0	0
4	44	6	9	5
5	28	2	1	5
6	46	0	0	19
7	20	0	0	8
8	9	0	0	4
9	15	0	0	5

(f) *Sexual mosaics generated by curing*

Sexual mosaics appeared also among the offspring of females that were cured in the adult stage (Kageyama *et al.* 2003*b*). To investigate the genetic sex of the mosaics, we subjected adult females from the infected line to tetracycline treatment (see § 2c). The eggs laid on successive days were reared separately. 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, sexual mosaics or males. Eggs laid from the sixth to ninth days developed as males only (table 2). The successive appearance of females, mosaics and males suggests that eggs laid early after treatment began were still under the influence of the infection, eggs laid 4–5 days after treatment began were partly cured, and those laid from day six onwards were completely cured of the *Wolbachia* infection. This was confirmed by PCR: four out of six mosaics tested displayed the *Wolbachia*-specific amplification product (not shown).

The mosaics were recognized from the mosaic appearance of the wings (figure 4*c*). Morphological details of this type of mosaic are published elsewhere (Kageyama *et al.* 2003*b*). Six females, nine mosaics and two males were dissected, and midgut, Malpighian tubules, testes and bursa copulatrix were investigated to determine their sex-chromatin status. Phenotypic females were genetically female (WZ) and males were genetically male (ZZ). All mosaics were genetically male in all tissues (figure 4*j*).

4. DISCUSSION

(a) *WZ–ZZ sex-chromosome system*

The identical number of bivalents in female and male meioses and the presence of sex chromatin in the interphase nuclei of females show that *O. scapulalis* has a WZ–ZZ sex-chromosome system like the majority of

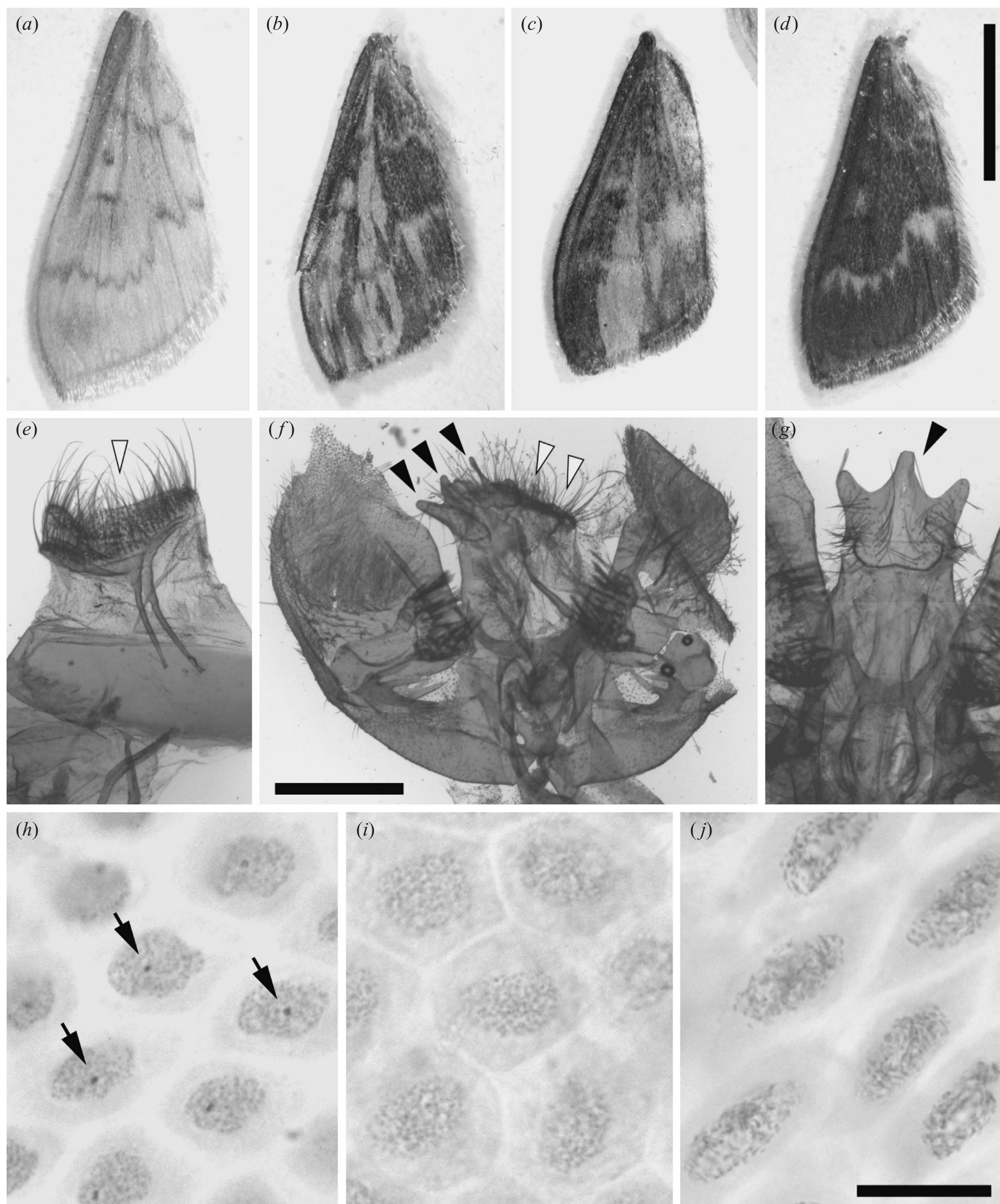

Figure 4. Morphological and cytogenetical features of sexual mosaics. Left forewing of: (*a*) an uninfected female; (*b*) a mosaic generated by transfection; (*c*) a mosaic generated by tetracycline treatment; and (*d*) an uninfected male. External genitalia of: (*e*) an uninfected female; (*f*) a sexual mosaic generated by transfection; and (*g*) an uninfected male. Interphase cells of the bursa copulatrix from: (*h*) an uninfected female; (*i*) a mosaic generated by transfection; and (*j*) a mosaic generated by tetracycline treatment. Open arrowheads, *ovipositor* and *ovipositor*-like structures; filled arrowheads, *uncus* and *uncus*-like structures; arrows, sex chromatin. Magnifications of (*a*)–(*d*), (*e*)–(*g*) and (*h*)–(*j*) are equal. Scale bars: (*a–d*) 5 mm; (*e–g*) 0.5 mm; and (*h–j*) 20 μm.

butterfly and moth species (reviewed by Traut & Marec 1996). CGH on sex chromatin (W chromatin) reveals a molecularly differentiated W chromosome that has accumulated repetitive sequences. Some of the repetitive sequences are presumably W-chromosome specific because the relative amount of female probe bound to the sex chromatin was higher than that of male probe. The presence of sex chromatin in interphase nuclei allowed us to diagnose the genetic sex, WZ or ZZ, of early stages of development as well as that of adults. The polyploid nuclei of most tissues from larvae and adults are rather convenient material for diagnosis because they usually contain only one sex-chromatin body albeit larger than that found in diploid nuclei.

Table 3. Sex-specific viability.

mother	genotype	sexual phenotype	viability
uninfected	WZ	female	viable
uninfected	ZZ	male	viable
uninfected, but eggs transfected	ZZ	mosaic	viable
infected	WZ[a]	female	viable
infected	ZZ	female?	inviable
cured	WZ[a]	?	inviable
cured	ZZ	male	viable
incompletely cured	ZZ	mosaic	viable

[a] A female genetic function temporarily or permanently lost (see § 4b).

(b) *Opposite sex-specific effects*

Wolbachia induces the generation of all-female broods in matrilines of the adzuki bean borer, *O. scapulalis* (Kageyama *et al.* 2003*a*). We show here that *Wolbachia* kills genetic males (ZZ individuals) during larval development while the *Wolbachia* infection is compatible with the development of genetic females (WZ individuals). This indicates that *Wolbachia* in *Ostrinia* is a male killer and does not induce ZZ individuals to become functional females as previously concluded (Kageyama *et al.* 2002). When females are cured of the *Wolbachia* infection by treatment with tetracycline at an early larval stage, they produce all-male broods. In this case, we see an inverse situation: genetic females (WZ individuals) die during larval development while males (ZZ individuals) survive (table 3). The respective adverse effects on development start early: they are already recognizable at the stage of hatching from the eggs. Also they cannot be reversed by curing in the larval stage even when tetracycline treatment starts immediately after hatching. Development of ZZ individuals is obviously incompatible with the *Wolbachia* infection, while development of WZ females is probably dependent on the presence of *Wolbachia* in the earliest stages of development. Females in this line may have lost a vital function that is now performed by the bacteria. In this regard, the *Wolbachia*-infected matrilines might be in the same category as the parasitic wasp *Asobara tabida*, in which removal of *Wolbachia* specifically inhibits oogenesis (Dedeine *et al.* 2001).

The loss of a vital function may be temporary, for the generation of the cured females only, or permanent, i.e. a genetic loss in the infected strain. If the former, we are dealing with a maternal effect: when we removed the *Wolbachia* parasites by administering tetracycline in the early larval stage, the effect was visible only in the next generation. In cytoplasmic incompatibility, which is the most commonly found phenotype in *Wolbachia*-infected insect species, obviously paternal effects are involved (Stouthamer *et al.* 1999). Our alternative hypothesis assumes a genetic difference between the infected matrilines and the uninfected matrilines that affects female survival (mutation hypothesis). The genomes of infected and uninfected matrilines are basically identical except for the W chromosome and cytoplasmic factors because infected females are mating with males from uninfected matrilines in every generation. Thus any permanent genetic difference is restricted to maternally inherited genes (W-linked or cytoplasmic factors). In the infected matrilines, a W-linked gene or cytoplasmic factor that is necessary for female survival or female sex determination may have lost its function by mutation while *Wolbachia* was performing that function.

Fujii *et al.* (2001) transfected the mealmoth, *Ephestia kuehniella*, with *Wolbachia* from the presently investigated matrilines of *O. scapulalis*. The results were interpreted as the induction of two different phenotypes by the same strain of *Wolbachia*: feminization in *O. scapulalis* and male killing in *E. kuehniella*. The results have to be reinterpreted in the light of our present results. *Wolbachia* kills males in both *O. scapulalis* and *E. kuehniella*, but, after curing, *E. kuehniella* returns to a 1 : 1 sex ratio while *O. scapulalis* produces all-male progeny.

(c) *The mechanism of sex-specific interference*

Incomplete curing of *Wolbachia* results in the development of sexual-mosaic individuals (Kageyama *et al.* 2003*b*). We show here that the mosaics are not genetic mosaics but genetically homogeneous male individuals. They have a ZZ genotype in female and male organs as well as in those that cannot be distinguished as female or male. The same type of sexual mosaic developed from experimentally transfected ZZ eggs of the uninfected line. Both types are partly feminized genetic males.

A rather similar type of sexual intergrade with mosaic wings and male and female organs has been bred from crosses between geographical races of the gypsy moth, *Lymantria dispar* (Goldschmidt 1934; Mosbacher 1973, 1975). As in *O. scapulalis*, they were of a homogeneous ZZ genotype (in reciprocal crosses, WZ intersexes arise, but they are mostly not mosaics). The sexual mosaics are thought to have arisen as a result of a strong feminizing maternal effect partly overriding the genetic ZZ male condition. When the feminizing effect was strong (in some crosses between geographical races), most of the ZZ individuals died before hatching (Mosbacher 1973), no different from what we saw in the *Wolbachia*-infected line. Sexual mosaics were also found in crosses between tetraploid females and diploid males in the psychid moth *Solenobia triquetrella* (Seiler 1965). The resulting triploid offspring with two Z chromosomes and three sets of autosomes developed into sexual intergrades owing to the intermediate ratio (2 : 3) between Z chromosomes and autosome sets, which is neither typically female (1 : 2) nor typically male (1 : 1).

The origin of the sexual mosaics renders it most probable that, in the infected line, *Wolbachia* also feminizes the ZZ individuals. We assume that development as a

complete female is incompatible with the ZZ genotype, not only in this but also in other cases of early male killing by *Wolbachia*, e.g. in *Hypolimnas bolina* (Clark *et al.* 1975; Dyson *et al.* 2002). In consequence, as in *Lymantria*, ZZ individuals die at an early stage of development. It is conceivable, though there is no evidence presently, that lethality of the WZ genotype in the offspring of cured mothers is caused by the inverse lethal condition: masculinization of the female genotype.

Lethality for the whole organism and viability when only parts of the organism are affected has been observed in the *Sex-lethal* (*Sxl*) mutant of *Drosophila melanogaster*. Activities of the gene product involve sex-specific regulation of downstream parts of the sex-determining cascade and sex-specific regulation of *msl-2*, which in turn controls dosage compensation of X chromosomal genes. Sex-specific lethality of *Sxl* mutants is thought to be caused by the faulty regulation of dosage compensation. Small segments of male tissue, however, generated by mitotic recombination in females, survive even with a female-type dosage compensation (Sánchez & Nöthiger 1983). Although the similarity to the mosaics in *O. scapulalis* is suggestive, dosage compensation is probably absent in Lepidoptera (Johnson & Turner 1979; Suzuki *et al.* 1998). Hence, we conclude that there must be other causes of incompatibility of sex reversal in *Ostrinia*, e.g. the requirement of a W chromosomal gene (or genes) for the viability of females and/or a male-killing activity of the W chromosome, which has been proposed for *E. kuehniella* (Marec *et al.* 2001).

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