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Feminizing *Wolbachia* in an insect, *Ostrinia furnacalis* (Lepidoptera: Crambidae)

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Wolbachia, which forms a group of maternally inherited bacteria in arthropods, often cause reproduction alterations in their hosts, such as cytoplasmic incompatibility, parthenogenesis, male-killing, hybrid breakdown and feminization. To date, *Wolbachia*-induced feminization has been reported only in isopods. Here we report that a *Wolbachia* strain feminizes an insect host, *Ostrinia furnacalis*. Among 79 wild females of *O. furnacalis* examined, *Wolbachia* infection was detected in 13 females. Twelve of the 13 infected females produced all-female progenies, and this trait was maternally inherited. Tetracycline treatment of thelygenic matrilines resulted in the production of all-male progenies. The present findings indicate that the *Wolbachia* infection induces feminization of genetic males in *O. furnacalis*. Differences in the *Wolbachia*-induced feminization in *O. furnacalis* and that in isopods are discussed along with the differences in sex determination mechanisms between insects and isopods. Phylogenetic analysis of the *wsp* gene sequence of *Wolbachia* suggests independent evolutionary origins for the *Wolbachia*-induced feminizations in *O. furnacalis* and in isopods. Our findings over 5 years suggest that the infection has been maintained at a low prevalence in the *O. furnacalis* population.
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Introduction

Much attention has been increasingly paid to selfish genetic elements such as *Wolbachia* (eg, Werren *et al*, 1988; O'Neill *et al*, 1997). *Wolbachia* are a group of cytoplasmically transmitted bacteria prevailing widely among arthropods (eg Stouthamer *et al*, 1999). *Wolbachia* are primarily of interest because they can cause alterations to the reproduction of their hosts; male-killing (eg, Hurst *et al*, 1999), thelytokous parthenogenesis (eg, Stouthamer *et al*, 1993), feminization of genetic males (eg, Rousset *et al*, 1992), hybrid breakdown (Vala *et al*, 2000) and cytoplasmic incompatibility (eg, O'Neill *et al*, 1992). *Wolbachia* may have promoted rapid speciation in arthropods (Bordenstein *et al*, 2001).

The Asian corn borer, *Ostrinia furnacalis* (Lepidoptera: Crambidae) is a major pest of *Zea mays* in eastern and southeastern Asia. Miyahara (1984) first reported the occurrence of female-biased sex ratio (thelygeny) in Japanese populations of *O. furnacalis*, but its mechanism and causal agent were not identified. Kageyama *et al* (1998) found that thelygeny was inherited in a matriline of *O. furnacalis* and that feminization of genetic males was caused by a bacterial infection. However, the causal bacteria of the feminization in *O. furnacalis* remained unidentified.

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In the present study, we reveal that the feminization of genetic males in *O. furnacalis* is caused by infection with a *Wolbachia* strain, and estimate the phylogenetic position of the *Wolbachia* strain based on the *wsp* gene sequence. This is the first study to report the occurrence of a feminizing *Wolbachia* in insects.

Materials and methods

Insects

We collected female adults of *O. furnacalis* from Matsudo (Chiba pref., Japan) in the summers of 1996–2000. Some of the findings of the present study, for insects collected in 1996, has previously been published by Kageyama *et al* (1998).

The captured females were individually allowed to oviposit in the laboratory. Most of the collected females laid fertile eggs within a few days. Larvae were reared by broods on an artificial diet (Silkmate 2M, Nihon-Nosan, Yokohama, Japan). At the pupal stage, insects were sexed based on the abdominal tip morphology. A piece of cotton soaked with 3% sucrose was provided for adult moths. Insects were reared under the conditions of 23°C and 15L/9D. Twenty females and 20 males were put in a mating cage, and 2 days later, females were separately allowed to oviposit. After oviposition, ovaries of females were dissected and stored in STE buffer (O'Neill *et al*, 1992) at –20°C until extraction of DNA.

Maternal inheritance of female-biased sex ratio

When a wild female produced a thelygenic family ($P < 0.01$, chi-squared test), daughters were used to found a

matriline. Matrilines were maintained by crossing females with males from normal lines. A normal line was a pool of matrilines with the parental sex ratio not significantly distorted from 1:1.

Tetracycline treatment

Tetracycline hydrochloride was mixed into the larval diet at 0.06% wet weight and fed to the larvae from the neonate stage.

The tetracycline treatment was used to check whether the thelygeny was due to bacteria-induced feminization. Lepidopteran insects have been suggested to have either ZW/ZZ or ZO/ZZ sex chromosomes (Traut and Marec, 1996), and this was also assumed for *O. furnacalis*. If the production of all-male offspring occurs after antibiotic elimination of the feminizer, it indicates feminization of a genetic male, since the genotype of their mother should be ZZ to produce ZZ eggs exclusively. As another possibility, if a male-killing bacterium is causing the thelygeny, a 1:1 sex ratio should result from the tetracycline treatment.

PCR assay of *Wolbachia* infection

One of the pair of ovaries in a female adult was ground in 100 μl of STE buffer (O'Neill *et al*, 1992) with 2 μl of proteinase K (20 mg/ml), 1 μl of 2-mercaptoethanol and 10 μl of 10% SDS. The homogenate was incubated at 37°C for at least 30 min and at 95°C for 5 min. The lysate was extracted with phenol-chloroform and chloroform once each, and DNA was precipitated with ethanol. The DNA pellet was dissolved in 50 μl of TE buffer.

Polymerase chain reactions (PCR) specific to the *Wolbachia* 16S rDNA gene were conducted in 10 μl reaction volumes including 1 μl of DNA samples following O'Neill *et al*. (1992).

Sequencing and phylogenetic analysis

A PCR specific to the *wsp* gene of *Wolbachia* was performed (Zhou *et al*, 1998). The PCR products were purified using the GENE CLEAN III kit (Bio101, La Jolla, CA, USA), and both DNA strands were directly sequenced using the ABI 377 DNA sequencer with the BigDye terminator cycle sequencing kit (Perkin Elmer).

The *wsp* sequence of *Wolbachia* infecting *O. furnacalis* was initially aligned to 29 *wsp* sequences of other strains of B-group *Wolbachia* and two outgroup sequences from A-group *Wolbachia* (the *Wolbachia* strains from *Drosophila melanogaster* and *D. sechellia*) using the program package CLUSTAL W ver1.5 (Thompson *et al*, 1994), and the alignment was manually modified based on the inferred amino acid sequences using the sequence alignment editor BioEdit (Hall, 1999). The third hypervariable region (positions 518–565) was excluded from the analyses (Zhou *et al*, 1998). The molecular phylogenetic tree was estimated by the maximum likelihood method using the program package PAUP* (Swofford, 1996). We employed the general time reversible (GTR) model with discrete-gamma approximation (four rate categories). All model parameters were estimated from the data using a starting tree topology that had previously been inferred with unweighted maximum parsimony. These parameter estimates and the starting tree topology were then employed for a heuristic search based on tree bisection and reconnection (TBRs). Bootstrap analysis was done with 100 replications.

Results

Wolbachia infection and female-biased sex ratio

Three of 13 females collected in the field in 1996 were thelygenic (Figure 1). We found *Wolbachia* infection in two thelygenic females (M9 and M11, Table 1), but we did not examine infection in the other 11 wild females (Kageyama *et al*, 1998).

Wolbachia infection was found in 11 of 79 females collected in the field during 1997–2000 (Figure 1). Throughout the 4 years, the frequencies of infected females were rather low (0–43%). Ten of the 11 infected females produced all-female offspring (strong thelygeny), while no uninfected females produced the strongly thelygenic offspring (Table 1, Figure 1). One infected female (MD771)

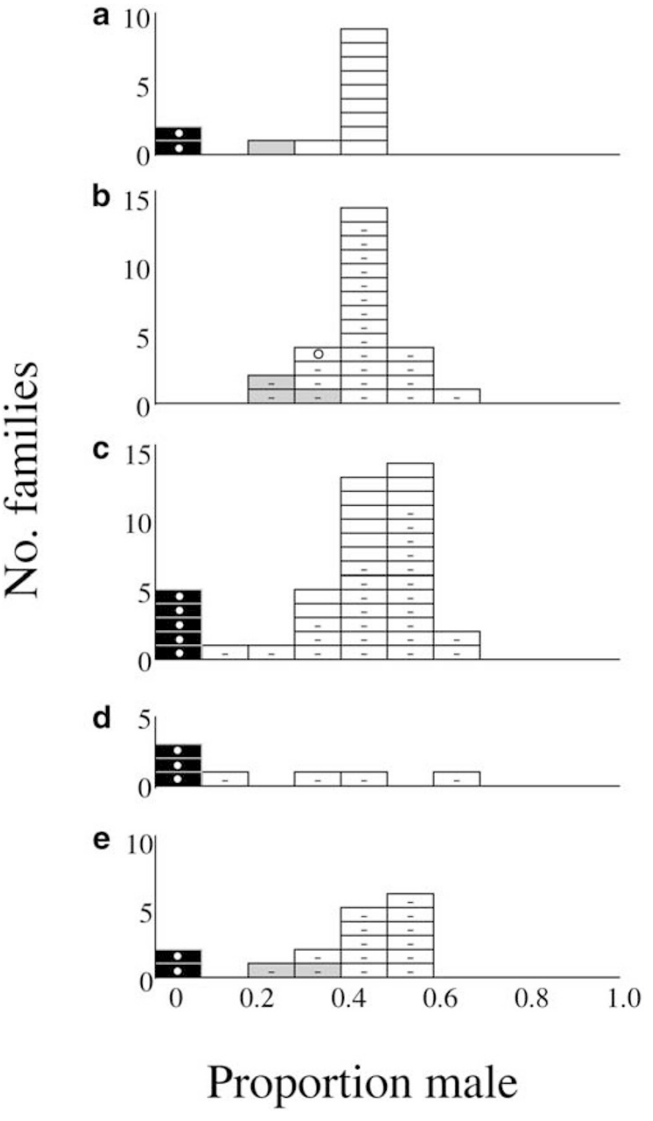

Figure 1 Distribution of sex ratio in progenies produced by wild females of *Ostrinia furnacalis* collected at Matsudo in (**a**) 1996, (**b**) 1997, (**c**) 1998, (**d**) 1999 and (**e**) 2000. Boxes with circles and minus signs indicate *Wolbachia*-infected and uninfected broods, respectively. Broods without these signs were not examined for *Wolbachia* infection. Black and grey boxes indicate strong and weak SR broods respectively. Two broods with size less than 10 in (**c**) and (**d**) each were excluded from the figure.

Table 1 *Wolbachia* infection and sex ratios of thelygenic matrilines (strongly and weakly) of *Ostrinia furnacalis* with egg hatch rates in parental families

Line	Wolbachia *infection*	*Parental*		*Egg hatch rate*	F_1		F_2		F_3	
		Female	*Male*		*Female*	*Male*	*Female*	*Male*	*Female*	*Male*
(**a**) *Strongly thelygenic matrilines*										
M9*	+	89	0	ne	184	27	15	0		
							9	0		
					22	7	81	0		
M11*	+	29	0	ne	56	0	37	8		
MD804	+	38	0	0.97 (123)	43	0	65	0	43	0
									55	0
									36	0
									65	0
MD807	+	32	0	0.96 (97)	35	0				
					34	0				
MD825	+	31	0	0.81 (392)	43	0				
MD826	+	25	0	ne	20	0				
					31	0				
MD846	+	42	0	0.95 (186)	45	0	45	0	10	1
MD903	+	48	0	ne	51	0				
MD910	+	58	0	ne	82	3	3	10		
							2	2		
MD920	+	28	0	ne	44	0				
					43	0				
MD030	+	14	0	ne	48	0	43	0	26	0
					51	0	29	0	25	0
									32	0
									21	0
									18	0
									33	0
MD049	+	47	0	ne	30	0	43	0	20	0
					56	0	49	0	12	0
									7	0
									9	0
(**b**) *Weakly thelygenic matrilines*										
M13*	–	22	6	ne						
MD743	–	94	48	ne						
MD745	–	39	11	ne	13	8				
MD747	–	44	16	ne						
MD003	–	59	29	1.00 (204)	16	12	34	33		
							3	11		
MD0037	–	31	10	ne						

Wild females were checked for *Wolbachia* infection by PCR. Egg hatch rates are given with total numbers of eggs in parentheses. A large number of eggs were used to check their hatchability, and not necessarily all of the hatched larvae were inoculated on the diet to avoid overcrowding. ne, not examined. *Sex ratio data on lines M9, M11 and M13 are from Kageyama *et al*, 1998.

produced a brood with a sex ratio not strongly distorted (22 females and 10 males, $P = 0.03$ by chi-squared test). Six wild females that were not infected produced families with significantly female-biased sex ratios ($P < 0.01$ by chi-squared test), but these families included males at more than 20% (defined as weak thelygeny, Table 1, Figure 1) in contrast to the strong thelygeny seen in the *Wolbachia*-infected lines. In addition, two wild uninfected females produced families that appeared to be biased to female (10 females and two males, $P < 0.05$; 13 females and three males, $P < 0.05$; Figure 1).

The strong thelygeny was maternally inherited for all of the seven matrilines examined albeit with four exceptional cases (Table 1). Two F_2 broods (three females and 10 males, and two females and two males) in matriline MD910 were not biased towards females. One F_1 brood (22 females and seven males) in matriline M9 and the F_2 brood (37 females and eight males) in matriline M11 showed rather weak thelygeny, although they are significantly female-biased ($P < 0.01$ by chi-squared test). On the other hand, the weak thelygeny disappeared in subsequent generations for both of the two matrilines examined (Table 1). We could not obtain offspring from the MD771 female.

Thus, *Wolbachia* infection was strongly associated with the all-female production in *O. furnacalis* females.

The mechanism of strong thelygeny

The average egg hatch rate for four families of strong thelygeny was 0.923 (Table 1), indicating that the *Wolbachia*-associated thelygeny was not due to early male-killing. All the larvae fed with tetracycline developed into females in all of seven matrilines of strong thelygeny, but these female adults produced all-male progenies (Table 2). The PCR assay confirmed that *Wolbachia* was eliminated from ovaries of tetracycline-treated females. These findings strongly suggest that the *Wolbachia* infection caused the strong thelygeny through feminization of

Table 2 Sex ratios of broods produced from tetracycline-treated strongly thelygenic females

Line	*Replicate*	*Female*	*Male*
M11*	a	0	24
	b	0	59
	c	0	58
	d	0	24
	e	0	50
	f	0	8
MD804		0	91
MD826		0	70
MD846		0	46
MD920		0	51
MD030		0	29
MD049	a	0	51
	b	0	49

*Data on line M11 are from Kageyama *et al*, 1998.

genetic males in *O. furnacalis*. Namely, *Wolbachia* feminizes individuals carrying ZZ sex chromosomes (male genome), and such feminized individuals solely produce ZZ eggs that develop into male adults in the absence of the *Wolbachia*.

If *Wolbachia*-infected genetic females (ZW*, an asterisk indicates *Wolbachia* infection) were included among the strongly thelygenic females in addition to the feminized genetic males (ZZ*), they would have produced broods with normal sex ratio after the tetracycline treatment. In the present study, however, no such female was found (Table 2). The present finding can be explained if we assume that the infection of *Wolbachia* is old; Since ZZ* females produce solely ZZ* females while ZW* females produce ZZ* and ZW* females, the proportion of ZW* females will decrease sharply, and eventually almost all of the *Wolbachia*-bearing females will be ZZ*, provided that the fitness of ZZ* and ZW* females is similar.

Molecular phylogenetic affiliation of the *Wolbachia* strain in *O. furnacalis*

Nucleotide sequences of a portion (555 bp) of the *wsp* gene of *Wolbachia* in the 13 infected *O. furnacalis* females were determined. All the 13 sequences were identical (DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB056664), suggesting infection with an identical strain of *Wolbachia*.

Molecular phylogenetic analyses have revealed the occurrence of two major clades (A and B) in *Wolbachia* strains infected with arthropods (Werren *et al*, 1995; van Meer *et al*, 1999; see also Vandekerckhove *et al*, 1999). The present analysis of *wsp* sequences showed that the *Wolbachia* strain in *O. furnacalis* is a member of the B group (Figure 2). Within the B group, the *Wolbachia* in *O. furnacalis* did not have a sister relationship to the feminizing *Wolbachia* strains in isopods, *Armadillidium vulgare*, *Oniscus asellus* and *Porcellio scaber*.

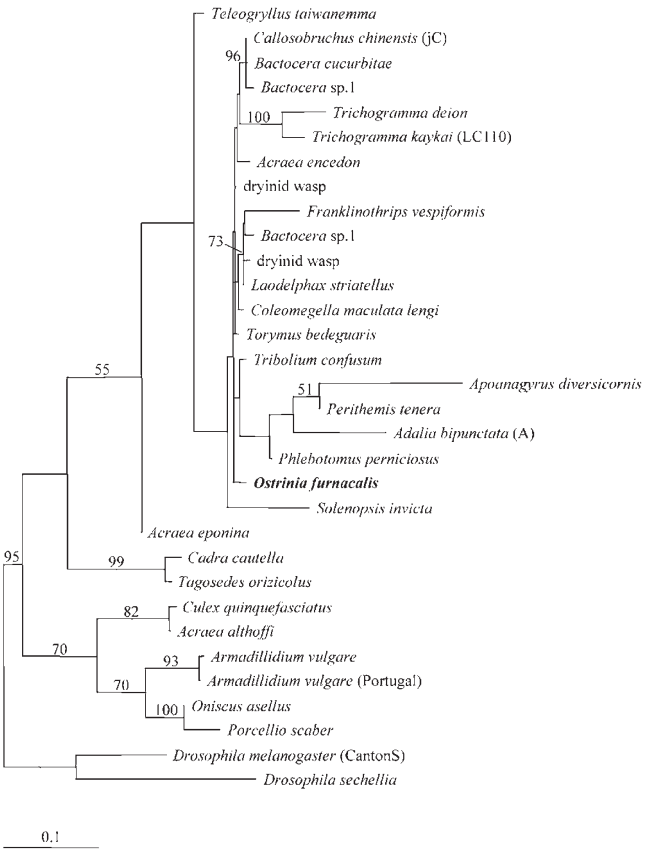

Figure 2 Phylogenetic tree of B-group *Wolbachia* based on *wsp* gene sequence data with two outgroups of A-group *Wolbachia* from *Drosophila sechellia* and *D. melanogaster*. *Wolbachia* strains are given as their host species names. The tree was constructed by the maximum likelihood methods using PAUP* (Swofford, 1996). The tree has log likelihood of –4136.75. The results of 100 bootstrap replicates are shown above the branches. GenBank accession numbers of included published sequences are as follows: AB035514, AB038326, AB039284, AB045314, AB046720, AF020060, AF020065, AF020073, AF020076, AF020080, AF020083, AF020084, AF020085, AF071915, AF071916, AF071917, AF071927, AF217720, AF217725, AF237884, AF243436, AF295346, AF295347, AF295348, AJ130714, AJ130716, AJ269474, AJ269475, AJ269476, AJ271194, AJ271197.

Discussion

In lepidopteran insects, thelygenies due to early (embryonic) male-killing have been known, some of which are caused by *Wolbachia* infection (Hurst *et al*, 1999; Jiggins *et al*, 2000, and references therein). In *O. furnacalis*, however, two lines of evidence indicated that *Wolbachia* infection causes feminization of genetic males. First, *Wolbachia* infection was strongly correlated to the strong thelygeny: this trait was found in 12 of 13 wild *Wolbachia*-infected females, but not in 68 uninfected females. Second, the result of antibiotic elimination of *Wolbachia* infection strongly suggested that feminization of genetic males underlay the strong thelygeny, although an alternative interpretation of the result is possible, as discussed below. This is the first study to report the occurrence of *Wolbachia*-induced feminization in insects, which has been known only from several isopod species (Rigaud *et al*, 1997).

It is possible to interpret the all-male production after antibiotic elimination of *Wolbachia* in a more complicated way; First, *Wolbachia*-infected females of *O. furnacalis* are assumed to be not viable without infection. This 'obligatory infection' hypothesis, in combination with a further hypothesis that *Wolbachia* causes male-killing during the larval stage (late male-killing), can explain the strong thelygeny in *O. furnacalis*. However, although a case of obligatory infection was recently reported in a parasitic

wasp (Dedeine *et al*, 2001), *Wolbachia* have never been demonstrated to be obligatory in any other host species. Moreover, late male-killing due to *Wolbachia* infection has not been found in any animal to date. Taken together, it is very unlikely that the *Wolbachia*-induced strong thelygeny in *O. furnacalis* is caused by 'obligatory infection and late male-killing'.

Among the 13 *Wolbachia*-infected females of *O. furnacalis*, one female (MD771) produced non-thelygenic progeny (22 females and 10 males, $P > 0.01$). Three explanations are possible for this exceptional case. First, the MD771 female may have harboured a *Wolbachia* strain different from the other 12 infections, although it was not distinguishable in terms of the *wsp* sequence. Secondly, the MD771 female may have failed to transmit the infection to some of the eggs. Lastly, the density of *Wolbachia* in MD771 females may have been low, leading to failure in feminizing some of the progeny. In addition, the latter two explanations might be relevant to the two non-thelygenic broods in matriline MD910 and the two weakly thelygenic broods in matrilines M9 and M11 (Table 1).

Wolbachia-induced feminization is known in isopods such as *Armadillidium vulgare* (Rigaud *et al*, 1997 for a review). In comparison, two phenotypic differences were found between the feminizations in *A. vulgare* and *O. furnacalis*. First, *Wolbachia*-infected lines of *A. vulgare* occasionally produce intersexes, which has not been found in *O. furnacalis*. Second, young females of *Wolbachia*-infected *A. vulgare*, when reared at 30°C for eliminating the infection, progressively acquired the male phenotype within the treated generation. In *O. furnacalis*, however, tetracycline did not change the sex of individuals in the treated generation, and all-male offspring was produced in the subsequent generation.

These distinct phenotypic characteristics of feminization are most likely to be relevant to differences in sex determination and/or differentiation processes between *A. vulgare* and *O. furnacalis*. In *A. vulgare*, a 'male gene' has been suggested to control development of the androgenic gland that produces androgenic hormone. The androgenic hormone triggers the male differentiation after the fourth moult. *Wolbachia* may affect the activity of the male gene (Rigaud *et al*, 1997).

In insects, the sex determination process has been well elucidated only in *D. melanogaster* (for a recent review, Schütt and Nöthiger, 2000). The sex in *Drosophila* is determined at the embryonic stage, and is not affected by diffusing substances such as sex hormones in the later developmental stages. This mechanism is also supported for insects other than Diptera (eg, Hoy, 1994; but see also De Loof and Huybrechts, 1998). In congruence with this widely accepted notion, tetracycline-treated larvae of infected *O. furnacalis* developed into female adults, suggesting that the feminizing action of *Wolbachia* operates at the embryonic stage. The target(s) of the feminizing action may be some molecule(s) that play a role in sex determination during the embryonic stage of *O. furnacalis*, although the molecular mechanism of sex determination in Lepidoptera has not been elucidated. The silkworm *Bombyx mori* possesses a homolog of *dsx*, which is one of the *Drosophila* sex determination genes (Ohbayashi *et al*, 2001). The *dsx* homolog in *B. mori*, as well as *dsx* in *Drosophila*, is subjected to sex-specific splicing.

On the maximum likelihood tree of *Wolbachia* strains based on *wsp* sequences, the feminizing *Wolbachia* in *Ostrinia* does not have a sister relationship with that in *A. vulgare*, while the *Wolbachia* strains in isopods were monophyletic. This suggests that the evolutionary origins of feminization were independent in *A. vulgare* and *O. furnacalis*, provided that there has been no recombination between *Wolbachia* strains (but see Werren and Bartos, 2001).

Feminization mediated by a cytoplasmic parasite such as *Wolbachia* can increase the frequency of the parasite in the host population (O'Neill *et al*, 1997). However, our observation of *Wolbachia* infection in the Matsudo population of *O. furnacalis* during 5 years (Figure 1) did not show that the frequency of infected moths were increasing, but rather strongly suggested that the infection was maintained at a low prevalence. In isopods, the presence of nuclear gene(s) that resist to the vertical transmission or feminizing activity of *Wolbachia* has been argued (Rigaud and Juchault, 1992; Juchault *et al*, 1994). A similar nuclear resistance gene may explain the low prevalence of *Wolbachia* infection in *O. furnacalis*. However, such a resistance factor in *O. furnacalis*, if present, appears not to be functioning, at least under the laboratory conditions, because the rates of vertical transmission of *Wolbachia* were high and *Wolbachia* infection induced strong, not weak, thelgeny in *O. furnacalis* (Table 1, Figure 1). As another possibility, sexual selection against the feminized genetic males may be preventing the spread of *Wolbachia* infection in the *O. furnacalis* population, as has been argued in the case of *A. vulgare* (Moreau *et al*, 2001); however, this hypothesis must be critically tested through future studies.

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