Evolutionary Biology

Prophage-encoded *Hm-oscar* gene recapitulates *Wolbachia*-induced male killing in the tea tortrix moth *Homona magnanima*

Reviewed Preprint
v2 • February 3, 2025
Revised by authors

Reviewed Preprint
v1 • September 6, 2024

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eLife Assessment

This **valuable** study implicates a specific Wolbachia gene in driving the male-killing phenotype in a moth: This is a contribution to a growing body of literature from the authors in which they authors have nicely teased apart the loci responsible for male killing across diverse insects. The conclusions are supported by **solid** evidence.

https://doi.org/10.7554/eLife.101101.2.sa4

Abstract

Wolbachia are maternally transmitted bacterial symbionts that are ubiquitous among arthropods. They can hijack host reproduction in various ways, including male killing (MK), where the sons of infected mothers are killed during development. The recent discovery of MK-associated *Wolbachia* genes, i.e., *oscar* in *Ostrinia* moths and *wmk* in *Drosophila* flies, stimulates our interest in the diversity and commonality of MK mechanisms, which remain largely unclear. We recently discovered that a *Wolbachia* symbiont of the moth *Homona magnanima* carries an MK-associated prophage region encoding homologs of *oscar* (*Hm-oscar*) and *wmk* (*wmk*-1–4). Here, we investigated the effects of these genes in the native host. Upon transient overexpression, *Hm-oscar*, but not *wmk*, induced male lethality in *H. magnanima*, in contrast to our observations in *Drosophila*, where the *wmk* homologs, but not *Hm-oscar*, killed the males. *Hm-oscar* disrupted sex determination in male embryos by inducing a female-type *doublesex* splicing and impaired dosage compensation, recapitulating the *Wolbachia* phenotype. Cell-based transfection assays confirmed that *Hm-oscar* suppressed the function of *masculinizer*, the primary male sex determinant involved in lepidopteran dosage compensation. Our study highlights the conserved roles of *oscar* homologs in *Wolbachia*-induced lepidopteran MK and argues that *Wolbachia* have evolved multiple MK mechanisms in insects.

Introduction

Arthropods commonly carry microbial symbionts that are passed from mother to offspring (Hurst & Frost, 2015; Werren et al., 2008; Hurst, 2017). The maternally transmitted bacteria, belonging to the genus *Wolbachia* (Alphaproteobacteria), are estimated to be present in at least 40% of all insect species, making them one of the most widespread endosymbiont genera on the planet (Werren et al, 2008; Zug & Hammerstein, 2012). *Wolbachia* have achieved evolutionary success by manipulating host reproduction through various means that enhance endosymbiont transmission (Werren et al., 2008). Such manipulation of the host's reproduction includes cytoplasmic incompatibility (CI), parthenogenesis, feminization, and male killing (MK), each of which affects the biological features, distribution, and evolution of the host. Among these strategies, MK directly skews the sex ratio of the host population toward females by killing male offspring of infected mothers during development. The lack of symbiont transmission through male hosts often leads to the evolution of MK. *Wolbachia* have been shown to induce MK in a wide range of insect taxa (e.g., dipterans, lepidopterans, and coleopterans) and other arthropods (Hurst et al., 1999; Kageyama & Traut, 2004; Hurst et al., 2000). Furthermore, various bacteria, viruses, and microsporidia induce MK phenotypes (Kageyama et al., 2012; Fujita et al., 2021; Kageyama et al., 2023; Nagamine et al., 2023), and recent studies have postulated that these microbes have evolved their MK ability independently (Harumoto & Lemaitre, 2018; Kageyama et al., 2023; Nagamine et al., 2023; Arai et al., 2023a).

The evolution and molecular mechanisms underlying *Wolbachia*-induced MK have attracted considerable attention for decades. It has been hypothesized that the mechanisms underlying MK are associated with sex determination cascades in insects (Hornett et al., 2022). Indeed, some MK-inducing *Wolbachia* strains have been shown to directly interact with the sex determination system in lepidopteran insects (Sugimoto and Ishikawa, 2012; Sugimoto et al., 2015; Fukui et al., 2015; Arai et al., 2023a). The Lepidopteran sex determination system consists of multiple transcriptional regulators, some of which exhibit sex-linked expression and/or splicing isoforms. Lepidoptera generally have a female heterogametic sex chromosome system (e.g., WZ/ZZ) and employ dosage compensation, which equalizes the sex-linked (Z-chromosome-linked) gene dose between males and females (Kiuchi et al., 2014). Dosage compensation is regulated by the *masculinizer* gene (*masc*), which is critical for male development. *masc* also controls the downstream master regulator of sex determination and differentiation, *doublesex* (*dsx*), which exhibits sex-dependent splicing isoforms (*dsxF* in females and *dsxM* in males) (Kiuchi et al., 2014). In *Ostrinia* and *Homona* male moths, MK-inducing *Wolbachia* strains impair dosage compensation by disrupting the expression of *masc*. Furthermore, they disrupt sex determination in male moths by inducing the "female" isoform of *dsx* (*dsxF*), leading to a "mismatch" between genetic sex (male: ZZ sex chromosome constitution) and phenotypic sex (female: based on *dsxF*) and consequently to male death (Arai et al., 2023a; Fukui et al., 2015; Sugimoto et al., 2015; Sugimoto & Ishikawa, 2012).

More recently, a protein produced in *Wolbachia wFur* strain named Oscar (Osugoroshi protein containing CifB C-terminus-like domain and many Ankyrin Repeats; Osugoroshi translates to male killing in Japanese) was shown to recapitulate the *Wolbachia*-induced MK phenotype in the native host *Ostrinia furnacalis* (Katsuma et al., 2022). The Oscar protein interacts with and degrades Masc protein, leading to the failure of dosage compensation and the production of female-type *dsx* isoforms in *Ostrinia* male moths (Fukui et al., 2024; Katsuma et al., 2022). Although *oscar* homologs have been identified in several MK-inducing *Wolbachia* strains in Lepidoptera, some MK *Wolbachia* strains in Diptera (*Drosophila*) and Lepidoptera do not carry this gene (Arai et al., 2023b; Arai et al., 2024a). Furthermore, *oscar* homologs are evolutionarily dynamic, with highly variable sequences and structures (classified as type I to type III *oscar* based on sequence homology) (Arai et al., 2024a), making it difficult to assess their functional relevance. In addition

to *oscar*, the helix-turn-helix domain-containing putative transcriptional regulator *wmk* is widely conserved among *Wolbachia* strains and induces various toxicities in *Drosophila* flies (i.e., no effect, weak male lethality [30% of males die], strong male lethality [90%–100% of males die], and death of all males and females) (Perlmutter et al., 2019; Perlmutter et al., 2020; Perlmutter et al., 2021; Arai et al., 2023b). Although the mechanisms underlying the *wmk*-induced toxicities and their connection to sex determination systems remain unclear, these findings suggest that *Wolbachia* strains carry multiple factors that cause male lethality. However, the diversity and commonality of these functions in insects remain largely unknown, partly due to the technical challenges in validating gene functions in non-model insects.

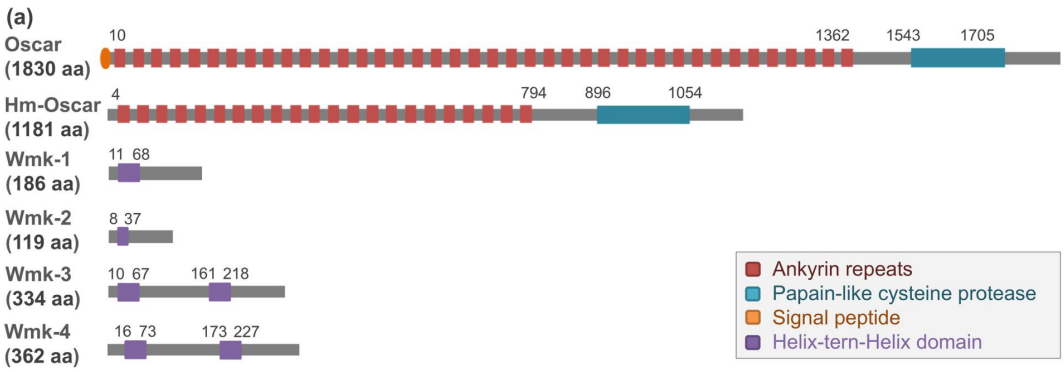
We recently discovered a prophage region responsible for a *Wolbachia*-induced MK by comparing closely related non-MK *Wolbachia* (*w*Hm-c) and MK *Wolbachia* (*w*Hm-t) in the tea tortrix moth *Homona magnanima* (Tortricidae) (Arai et al., 2023b; Arai et al., 2024b). The MK-associated prophage element encodes potential MK causative genes–four *wmk* homologs (*wmk*-1 to *wmk*-4) as well as an *oscar* homolog (*Hm-oscar*, encoding 1181 aa protein), which differs significantly in sequence length and structure from the *w*Fur-encoded *oscar* (encoding 1830 aa protein) (**Fig. 1a**). When these MK candidate genes are transgenically overexpressed in *Drosophila*, *wmk-1* and *wmk*-3 have a lethality of almost 100%, while *wmk-2*, *wmk-4*, and *Hm-oscar* induce no lethal effects when singly overexpressed. Furthermore, co-expression of the adjacent *wmk-3* and *wmk-4* has been shown to induce the death of 90% of male flies and restores female survival, suggesting their combined action (Arai et al., 2023b). However, transgenic expression of genes from a moth-derived male killer in flies does not necessarily replicate the native host–bacteria interaction, and the mechanistic links between prophage-encoded *Wolbachia* genes and MK in the native host *Homona* remain unclear.

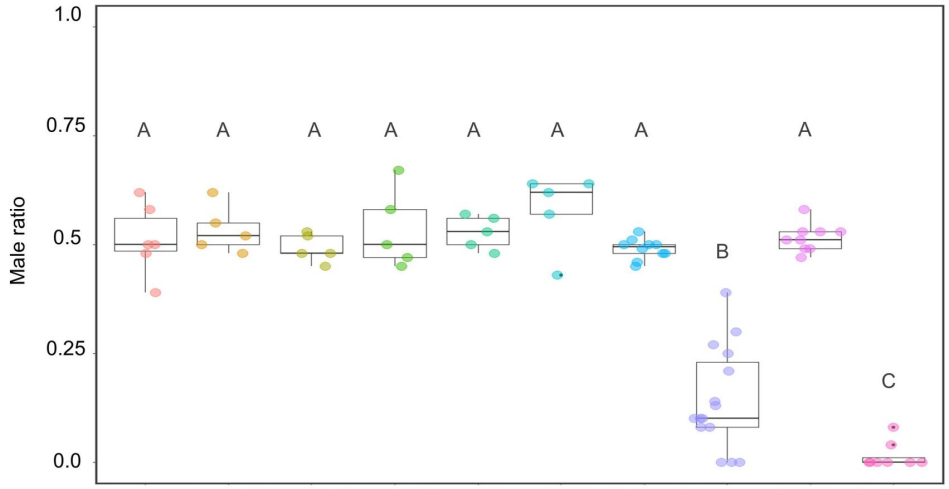
In this study, we showed that the prophage-encoded *Hm-oscar* recapitulates *Wolbachia*-induced MK in *H. magnanima*. Furthermore, we clarified the mechanistic links to host sex-determination cascades both *in vivo* and *in vitro* and discussed the underlying mechanisms of MK in Lepidoptera, arguing for the diverse evolutionary origin of *Wolbachia*-induced MK.

Results

Hm-oscar induces female-biased sex ratios

To achieve the transient overexpression of *Hm-oscar* and the four *wmk* genes (*wmk*-1, *wmk*-2, *wmk*-3, and *wmk*-4), constructed mRNA (cRNA) was injected into *Wolbachia*-free *H. magnanima* embryos. Subsequently, the adult moths that emerged from the cRNA-injected embryos were sexed based on their external morphology. When *Hm-oscar* was overexpressed, the sex ratio of adults was strongly female-biased (42 males and 218 females in total, 14.4% ± 11.1% male ratio in 15 replicates), which was in sharp contrast with the ratios observed in the *GFP*-injected (126 females and 123 males in total, 49.1% ± 2.08% male ratio in 10 replicates) and non-injected (NSR) (201 females and 215 males in total, 51.4% ± 3.08% male ratio in 9 replicates) groups (P = 0.001 and 0.002, respectively, Steel–Dwass test, **Fig. 1b**). Compared with the *GFP*-injected group, the sex ratio was not biased by the overexpression of *wmk*-1 (51.1% ± 7.27% male ratio in 6 replicates, P = 0.999), *wmk*-2 (53.2% ± 4.77% male ratio in 6 replicates, P = 0.810), *wmk*-3 (53.3% ± 7.91% male ratio in 5 replicates, P = 0.999), and *wmk*-4 (52.9% ± 3.47% male ratio in 5 replicates, P = 0.773). Although the dual expression of *wmk*-3 and *wmk*-4 induces strong male lethality in *Drosophila* (Arai et al., 2023b), here, it did not skew the sex ratio of *Homona* compared with the ratio detected in the *GFP*-injected group (57.9% ± 7.67% male ratio in 5 replicates, P = 0.704). Similarly, the dual expression of the tandemly arrayed *wmk*-1 and *wmk*-2 did not bias the sex ratio (49.4% ± 2.82% male ratio in 5 replicates) compared with the value in the *GFP*-injected group (P = 1.000). The sex ratio data of all replicates are available on the supplementary data file.





Gene	*wmk-1*	*wmk-2*	*wmk-1-2*	*wmk-3*	*wmk-4*	*wmk-3-4*	*GFP*	*Hm-oscar*	NSR	W^{T12}
Replicates	6	5	5	5	5	5	10	15	9	9
Male ratio	0.51	0.52	0.50	0.51	0.53	0.56	0.49	0.16	0.52	0.01
Female	47	62	72	84	50	72	126	218	201	213
Male	48	67	73	86	56	90	123	42	215	2
Binominal test (P =)	0.5000	0.3624	0.5000	0.4694	0.3137	0.0907	0.4496	0.0000	0.2172	0.0000

Figure 1

Transient expressions of the *Hm-oscar* gene resulted in female-biased sex ratios

(a) Structures of *w*Fur-encoded Oscar and *w*Hm-t-encoded Hm-Oscar and four Wmk proteins. (b) Male ratio of adult progeny obtained from cRNA-injected groups (*wmk*-1, *wmk*-2, T2A-brigged *wmk*-1 and *wmk*-2, *wmk*-3, *wmk*-4, T2A-brigged *wmk*-3 and *wmk*-4, GFP, and *Hm-oscar*; n=5–15 independent replicates using different egg masses), the non-injected NSR line, and the MK *w*Hm-t-positive W^{T12} line. The total numbers of adult females and males are shown at the bottom. Different letters indicate significant differences (Steel–Dwass test, $P < 0.05$). The dot plots show all data points individually.

Males are killed mainly at the embryonic stage

The sex of unhatched embryos and hatched larvae (neonates) was determined by karyotyping for W chromatin, with the presence and absence of this substance indicating females and males, respectively. MK *Wolbachia w*Hm-t kills *Homona* males during embryogenesis, resulting in a female-biased sex ratio in hatched larvae (neonates) and a male-biased sex ratio in unhatched embryos (Arai et al., 2023b; Arai et al., 2020). In line with this finding, the sex ratios of hatched larvae (34 females and 6 males) and unhatched embryos (27 females and 47 males) of the MK *w*Hm-t infected line (W^{T12}) were also female- and male-biased, respectively (P = 0.013 and 0, respectively). In the *Hm-oscar*-injected group, the sex ratio of the hatched larvae (neonates) was strongly female-biased (21 females and 3 males, P = 0.0001 in the binomial test) (**Fig. 2a**). In contrast, the unhatched mature embryos showed a male-biased ratio (18 females and 38 males, P = 0.005), suggesting that male mortality primarily occurred during the embryonic stage, reflecting the *w*Hm-t-induced MK phenotype. In the non-injected group (NSR), the sex ratios of hatched larvae (28 females and 28 males) and unhatched embryos (23 females and 25 males) were not biased (P = 0.553 and 0.443, respectively).

Female-type sex determination in male embryos that are destined to die

Splicing patterns of the downstream sex determinant *dsx* were assessed in each of the *H. magnanima* embryos that were *Hm-oscar*-expressed, *GFP*-expressed, *w*Hm-t-infected, or non-expressed (i.e., non-injected, NSR). In *GFP*-expressed and NSR embryos, females and males (which were identified by the presence and absence of W chromatin, respectively) exhibited a female- and male-type splicing variant of *dsx*, respectively. However, both *w*Hm-t-infected and *Hm-oscar*-expressed embryos induced female-type *dsx* splicing regardless of the presence or absence of W chromatin (**Fig. 2b**). Notably, male embryos expressing *Hm-oscar* also exhibited weak male-type *dsx* splicing in addition to the female-type splicing, resembling the previously observed pattern in male embryos infected with low-titer *w*Hm-t (Arai et al., 2023a).

Hm-oscar impairs dosage compensation in male embryos

To confirm the effects of *Hm-oscar* on dosage compensation, we quantified the expression of Z-linked genes using RNA-seq and qPCR. Unlike other lepidopteran species, the family Tortricidae, including *H. magnanima*, generally possess a large Z chromosome that is homologous to *B. mori* chromosomes 1 (Z) and 15 (autosome). RNA-seq analysis revealed that, in *Hm-oscar*-injected embryos, Z-linked genes (homologs on the *B. mori* chromosomes 1 and 15) were more expressed in males than in females (**Fig. 3a**), which was not observed in the *GFP*-injected group (**Fig. 3b**). Similarly, as previously reported by Arai et al. (2023a), high levels of Z-linked gene expression were also observed in *w*Hm-t-infected males, but not in NSR males (**Fig. 3c,d**). Statistical data are available on the supplementary data file. The high (i.e., doubled) Z-linked gene expression in both *Hm-oscar*-expressed and *w*Hm-t-infected males was further confirmed by quantification of the Z-linked *Hmtpi* gene (**Fig. 3e**).

Hm-oscar suppresses the masculinizing functions of lepidopteran *masc* genes

To confirm whether *Hm-oscar* suppresses the functions of the primary male sex determinant *masc*, their interactions were assessed by transfection using BmN-4 cells, which are derived from *B. mori* ovaries (Grace, 1967) and exhibit a female-type default sex determination (Kiuchi et al., 2014). Previous studies have shown that transfection of BmN-4 cells with *masc* cDNA from various lepidopteran insects, such as *O. furnacalis*, induces the expression of $Bmdsx^{M}$ and $BmIMP^{M}$ (Fukui et al., 2015; Katsuma et al., 2019; Katsuma et al., 2022). Since $BmIMP^{M}$

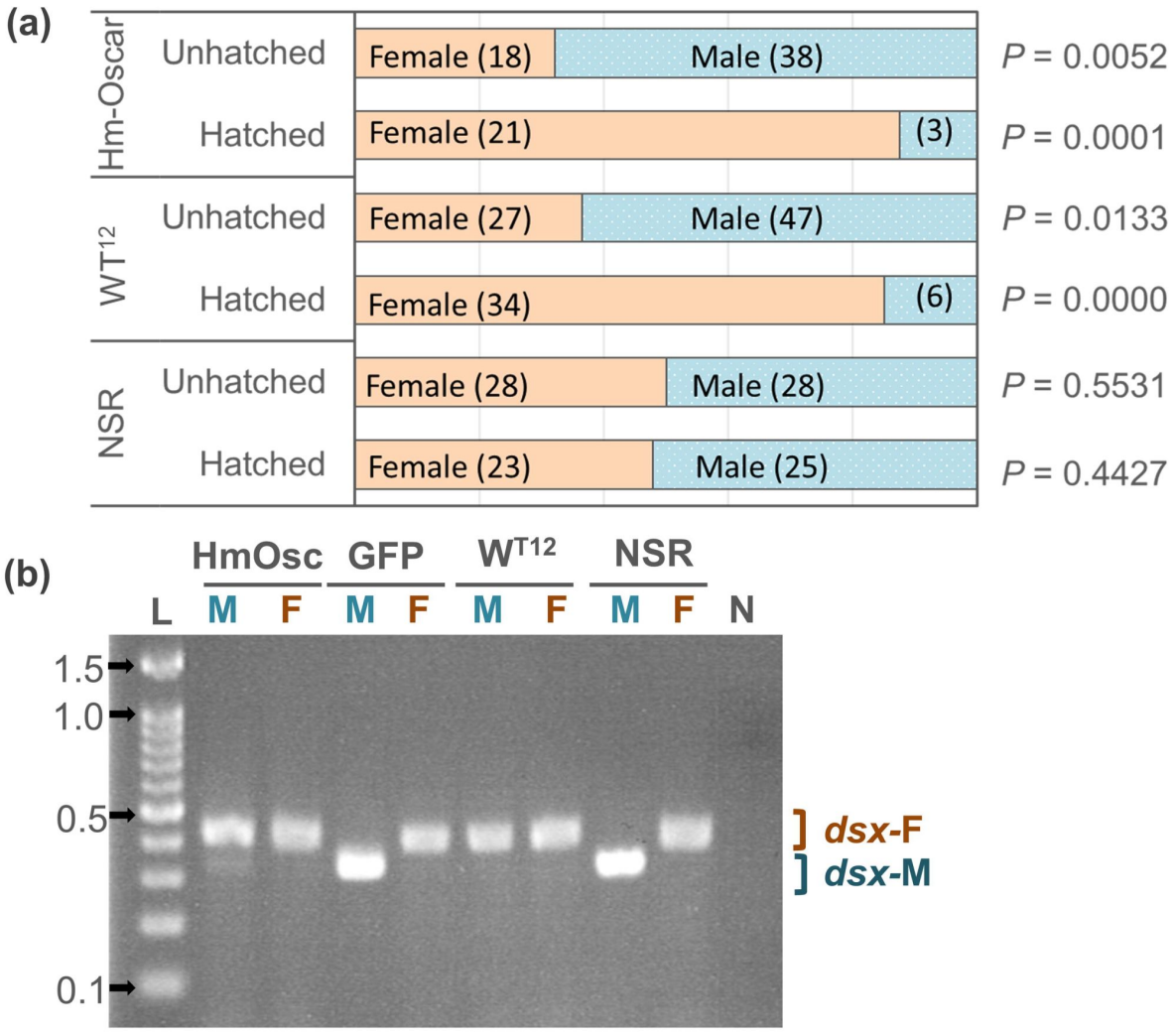
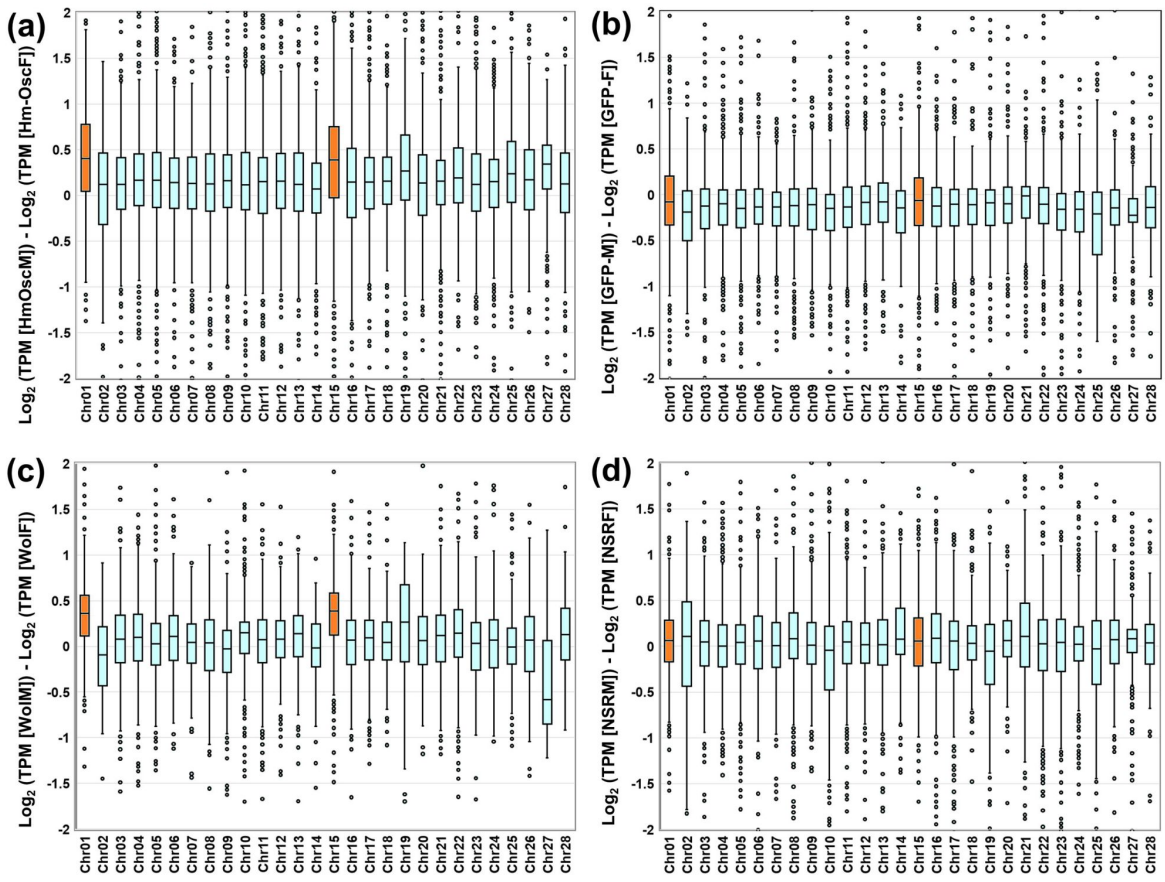
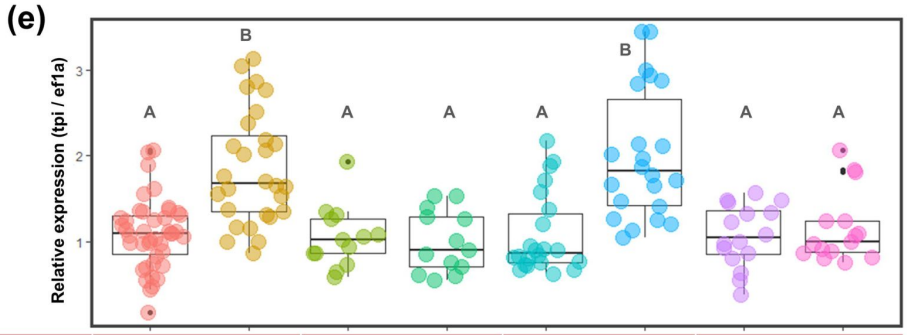


Figure 2

Hm-Oscar induced lethality in male embryos with the female-type sex determination

(a) Sex ratio of the hatched larvae and unhatched embryos in the *Hm-oscar*-expressed, *w*Hm-t-infected, and non-infected/expressed groups. Females and males were discriminated based on the presence or absence of W chromatin. The number of individuals is indicated in brackets. (b) Splicing patterns of the downstream sex-determining gene *dsx* of *H. magnanima* embryos (5 days post oviposition). Abbreviations: HmOsc, *Hm-oscar* injected group; GFP, GFP-injected group; W^{T12}, *w*Hm-t-infected line; NSR, non-infected/injected line. M and F indicate W chromatin-negative (ZZ: male genotype) and W chromatin-positive (ZW: female genotype) mature embryos, respectively. *dsx-F* and *dsx-M* represent female and male-specific splicing variants, respectively. L: 100 bp DNA ladder (ExcelBand 100 bp DNA Ladder, SMOBIO Technology, Inc., Hsinchu, Taiwan). 0.1, 0.5, 1.0, and 1.5 kb markers are indicated with arrows. N: negative control (water).





Line	*Hm-oscar*		*GFP*		W^{T12}		*NSR*	
Sex	Female	Male	Female	Male	Female	Male	Female	Male
Replicates	42	28	13	13	22	22	16	15

Figure 3

Hm-oscar-overexpressed male embryos showed higher levels of Z-linked gene expression

(a-d) Normalized expression levels (TPM) and chromosomal distributions of transcripts in *H. magnanima* embryos. RNA-seq data of embryos (108 hpo) were used to make the following comparisons: *Hm-oscar*-injected males versus *Hm-oscar* injected females (a), GFP-injected males versus GFP-injected females (b), W^{T12} males versus W^{T12} females (c), and NSR males versus NSR females (d). The chromosome number for each *H. magnanima* transcript-derived contig was assigned based on *Bombyx mori* gene models. The X axis represents the chromosome number of *B. mori* (shown as chr01 to chr28), and chr01 and chr15 (highlighted in orange) correspond to the Z chromosome of *H. magnanima* (Arai et al., 2023a). (e) Relative expression of a Z-linked *tpi* gene in *H. magnanima* groups. Statistical significance is highlighted with different alphabets.

regulates the splicing of *Bmdsx*^{M} and is exclusively expressed in males, we selected *BmIMP*^{M} as a quantitative marker of masculinization, as described in Katsuma et al. (2022). In contrast with the control groups, which were transfected with the non-inserted pIZ/V5-His vector and showed this default sex determination, the cells transfected with the *Hmmasc*-inserted pIZ/V5-His vector exhibited the male-type sex determination, as indicated by the increased expression of the male-specific splicing variant of the sex-determining gene *BmImp*^{M}, although this increase was not statistically significant (**Fig. 4a**). Furthermore, the masculinizing function of *Hmmasc* was suppressed by co-transfecting the *Hmmasc/Hm-oscar*-inserted pIZ/V5-His vectors, as manifested by the low expression levels of *BmImp*^{M}. *w*Fur-encoded Oscar, which differs from Hm-Oscar in structure and amino acid sequence (**Fig. 4b**), also suppressed *Hmmasc*, the function of this gene was less active than that of *Hm-oscar* (**Fig. 4a**). *Hm-oscar* also suppressed the masculinizing function of *masc* genes derived from various lepidopteran insects (i.e., *O. furnacalis, Spodoptera frugiperda, Bombyx mori, Trichoplusia ni,* and *Papilio machaon*), which are highly divergent in their amino acid sequences (**Fig. 4c,d**). These results suggest that *Hm-oscar* has a broad spectrum of activity in Lepidoptera.

Discussion

In this study, we showed that the phage-encoded *Hm-oscar*, but not *wmk*, induced male lethality and a female-biased sex ratio in *H. magnanima*. Furthermore, the overexpressed *Hm-oscar* impaired male sex determination in *Homona,* recapitulating the *w*Hm-t-induced phenotypes. Cell-based assays confirmed that *Hm-oscar* suppressed the masculinizing functions of *masc*. These results strongly suggested that *Hm-oscar* underlies the *w*Hm-t-induced MK function in *H. magnanima*. In light of our results and previous findings in *Homona*, *Ostrinia,* and *Bombyx* (Arai et al., 2023a; Katsuma et al., 2022; Kiuchi et al., 2014), we hypothesize the following molecular mechanisms for *w*Hm-t-induced MK: The Hm-Oscar protein targets and inhibits the function of the HmMasc protein, thereby resulting in female sex determination and disrupting dosage compensation, which ultimately leads to male death. Although the full mechanisms remain unclear, unbalanced Z-linked gene expression mediated by improper dosage compensation—resulting in Z-linked gene expression levels twice as high as those in normal males—appear to be lethal for lepidopteran males (Kiuchi et al., 2014; Fukui et al., 2015; Visser et al., 2021).

The *Wolbachia*-encoded *oscar* homologs identified so far show diversity in sequence length and are classified as type I, II, and III based on their amino acid sequence homologies. Type I and II Oscar proteins, derived from MK-inducing *Wolbachia*, contain many ankyrin repeats and a proteinase domain (Arai et al., 2024a). Although long ankyrin repeat sequences are postulated to be critical for the function of *w*Fur-encoded Oscar protein (1830 aa, type I) (Katsuma et al., 2022), our study revealed that the functions of Hm-Oscar protein (1181 aa, type II), which encodes fewer ankyrin repeats, were comparable with those of Oscar protein carried by *w*Fur. Interestingly, type II *oscar* gene is also present in the feminizing *Wolbachia w*Fem in *Eurema* butterflies (Arai et al., 2024a). *oscar* homologs, which inhibit the masculinizing function and induce female sex determination, may have a conserved function in *Wolbachia*-induced MK and feminization in Lepidoptera.

In contrast to the results of this study, we have previously demonstrated that the phage-encoded *wmk*, but not *Hm-oscar*, induces male lethality in *D. melanogaster* (Arai et al., 2023b). Although the means of expression are different (i.e., transient in *Homona* and transgenic in *Drosophila*), this finding highlighted the differences in the mode/range of action of *Wolbachia* genes between insect species. It has been hypothesized that microbes induce MK in insects by targeting molecular mechanisms involved in sex determination and differentiation (Hornett et al., 2022). Sex determination systems in insects are diverse; for example, Lepidoptera (including *H. magnanima*) and Diptera (including *D. melanogaster*) do not share any known sex-determining genes other than *dsx* (Suzuki, 2018). The different outcomes in *Homona* and *Drosophila* are probably due to

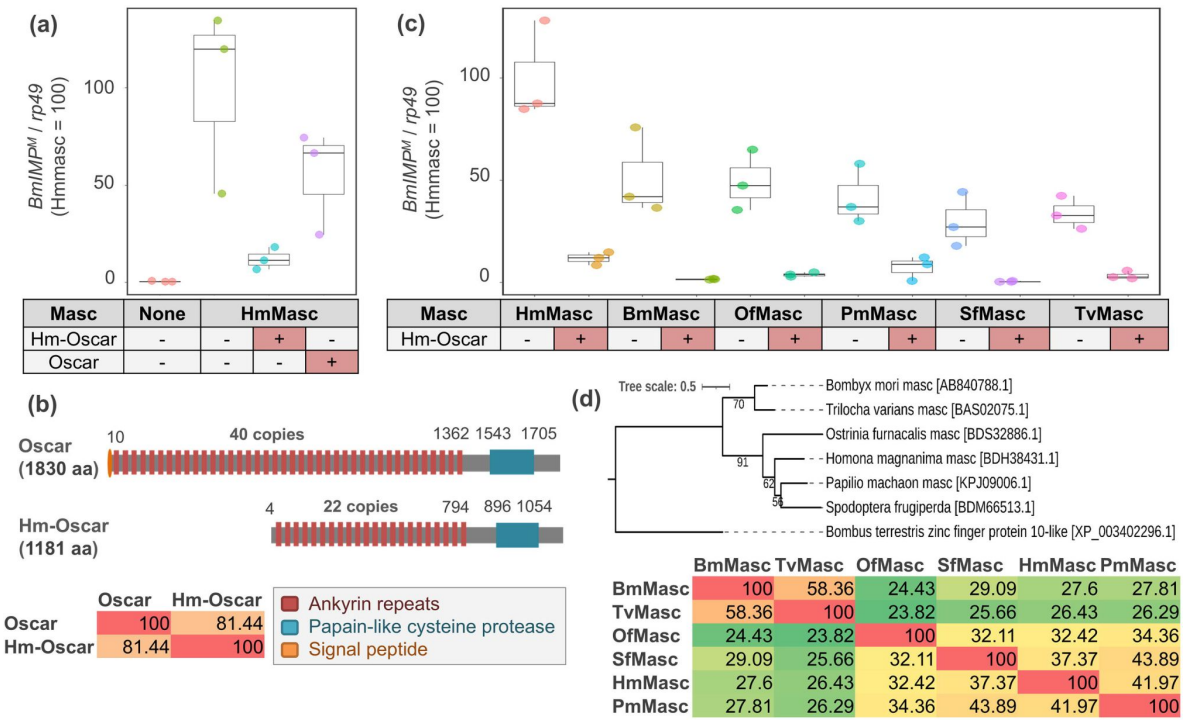


Figure 4

Hm-oscar suppressed the masculinizing function of lepidopteran Masc

(a) Relative expression levels of the male-specific *BmImp*^{M} variant in transfected BmN-4 cells. The relative expression of *BmImp*^{M} in the control (non-inserted pIZ/V5) and *Hm-oscar*/*oscar* co-transfected groups was normalized by setting the mean in the *Hmmasc* single transfected group as 100. (b) Protein structures and homologies of Hm-Oscar and Oscar proteins. (c) Relative expression levels of the *BmImp*^{M} in *Hm-oscar/masc* transfected BmN-4 cells. The relative expression of *BmImp*^{M} in each condition was normalized by setting the mean in the *Hmmasc* single transfected group as 100. The dot plots show all data points individually. Each experimental condition was replicated three times. Abbreviations: Hm, *Homona magnanima*; Bm, *Bombyx mori*; Of, *Ostrinia furnacalis;* Pm, *Papilio machaon;* Sf, *Spodoptera frugiperda*; and Tv, *Trilocha varians.* (d) Phylogeny and homologies of the Masc protein sequences used in this study.

their different sex determination systems. Because *oscar* interacts with and suppresses *masc*, *Hm-oscar* could induce mortality in *Homona* males that possess *masc*, but not in *Drosophila* males that lack it. Considering that *oscar* homologs are not present in known MK *Wolbachia* strains in dipteran insects (Arai et al., 2024c; Katsuma et al., 2022), the mechanisms (i.e., causative genes) of *Wolbachia*-induced MK probably differ between insect taxa (e.g., between Lepidoptera and Diptera). While the mechanisms underlying *wmk*-induced lethality remain unclear, the distinct effects associated with this gene between *Homona* and *Drosophila* may also reflect their genetic backgrounds (e.g., presence/absence of host factor(s) that interact with *wmk*). In addition, Katsuma et al. (2022) reported that the *wmk* homologs encoded by *w*Fur did not affect the masculinizing function of *masc in vitro*, indicating that *wmk* likely targets factors other than *masc*. Our results strongly suggested that *Wolbachia* strains have acquired multiple MK genes through evolution.

Wolbachia-induced phenotypes are known to be influenced by the genetic backgrounds of hosts (Hornett et al., 2006; Sasaki et al., 2002; Veneti et al., 2012). Our study showed that the *w*Hm-t-encoded *Hm-oscar* suppresses the function of *masc* in *H. magnanima* more efficiently than the *w*Fur-encoded *oscar*, suggesting that this *Wolbachia* factor has undergone evolutionary tuning to adapt to its natural host. However, the mere presence of *oscar* and *wmk* homologs does not ensure MK expression. For example, *w*CauA, bearing type I *oscar*, did not induce MK in *Ephestia* (*Cadra*) *cautella* collected around 2000, although it did induce MK when transferred to the closely related host *Ephestia kuehniella* (Sasaki et al., 2002). An MK phenotype, presumably induced by *w*CauA, was also recorded in *E. cautella* around the 1970s (Takahashi & Kuwahara, 1970). These findings suggest a suppressor evolution against *w*CauA-induced MK in *E. cautella*. In addition, *Wolbachia* typically encode a number of *wmk* homologs in their genomes, but their effects differ among insects and are not apparent in some species such as *Homona,* possibly due to the diversity in sex determination system or the evolution of suppressors. Nonetheless, given the high copy number of *wmk* homologs in *Wolbachia* genomes, they may still contribute to *Wolbachia* biology as transcriptional regulators and potential virulence factors. Virulence genes often undergo duplication and substitution under strong selective pressure (Hill et al., 2022; Jones & Dangl, 2006). An intense evolutionary arms race between *Wolbachia* and their hosts could have increased and diversified the MK-associated *Wolbachia* genes. Conversely, natural selection favors the rescue of males by suppressing the *Wolbachia*-induced reproductive manipulations (Hornett et al., 2006; Hornett et al., 2014), which may involve changes in the sex determination system because *Wolbachia* strains frequently hijack host reproduction systems. In this context, *Wolbachia* and other MK-inducing microbes may be a hidden driver for the diversification of complex insect sex determination systems.

In this study, we clarified the conserved roles of the *Wolbachia*-encoded *oscar* homologs in Lepidoptera and demonstrated that *Wolbachia* have evolved distinct MK mechanisms (through causative genes) in insect taxa. The diversification of phenotype/virulence-associated genes and the rampant horizontal transmission of phages carrying virulence genes between *Wolbachia* strains may have contributed to the outstanding success of this bacterial genus. In addition to *oscar* and *wmk, Wolbachia* may retain other uncharacterized genes that induce male lethality, and further studies on diverse *Wolbachia*–host systems are highly warranted. Our findings provide insights into the molecular mechanisms and evolutionary relationships between endosymbionts and their hosts, which may also contribute to the design of pest management strategies.

Materials and methods

Experimental model and subject details

Homona magnanima

A laboratory-maintained *H. magnanima* line with a normal sex ratio (NSR) that was negative for *Wolbachia* and other endosymbionts, was used in our experiments. This line was initially collected in Tokyo, Japan, in 1999 and has been maintained inbred for over 250 generations in the laboratory. Larvae were reared using the artificial SilkMate 2S diet (Nosan Co., Yokohama, Japan) at 25°C under a long photoperiod (16L:8D) (Arai et al., 2019).

The laboratory-maintained all female *H. magnanima* W^{T12} line, which was initially collected in Taiwan (Tea Research Extension Station, Taoyuan city) in 2015 (Arai et al., 2020), was also used in this study. This line was maintained for over 50 generations by crossing it with the males of the NSR line.

BmN-4 cell line

BmN-4 cells (provided by Chisa Yasunaga-Aoki, Kyushu University, and maintained in our laboratory) were cultured at 26°C in IPL-41 Insect Medium (Applichem, Darmstadt, Germany) supplemented with 10% fetal bovine serum.

Transient expression of MK-associated phage genes

(i) mRNA synthesis

Codon-optimized *wmk* genes (*wmk-1* to *wmk-4*), conjugated *wmk* pairs using a T2A peptide (i.e., *wmk*-1-T2A-*wmk*-2 and *wmk*-3-T2A-*wmk*-4), and *Hm-oscar* genes synthesized by Arai et al. (2023b) were used for mRNA synthesis. These synthetic genes were ligated into the plasmid pIZ/V5-His (Invitrogen, MA, USA) using the NEBuilder® HiFi DNA Assembly kit (New England Biolabs, MA, USA) following the manufacturer's protocol. The inserts of the vector (i.e., *wmk*-1, *wmk*-2, *wmk*-3, *wmk*-4, *Hm-oscar*, *wmk*-1-T2A-*wmk*-2, *wmk*-3-T2A-*wmk*-4, and *GFP* as a control gene) were amplified using KOD-one (TOYOBO, Osaka, Japan) with the primer set containing the T7 promoter described in Fukui et al. (2015) (i.e., pIZ-F-T7: 5'-TAATACGACTCACTATAGGGAGACAGTTGAACAGCATCTGTTC-3' and pIZ-R: 5'-GACAATACAAACTAAGATTTAGTCAG-3') under the following PCR conditions: 20 cycles at 98°C for 10 s, 62°C for 5 s, and 68°C for 15 s. The amplicons were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany), and 100–200 ng DNA was used for mRNA synthesis. The capped mRNA (cRNA) with poly(A) tail was synthesized using the mMESSAGE mMACHINE® T7 Ultra kit (Invitrogen, MA, USA) following the manufacturer's protocol with some modifications. In brief, the assembled transcription reaction (10 µL of T7 2X NTP/ARCA, 2 µL of 10X T7 reaction buffer, 2 µL of T7 enzyme mix, and 6 µL of linear DNA template diluted with nuclease-free water) was incubated at 37°C for at least 10 h to maximize RNA yields. After poly(A) tailing, cRNA was purified using ISOGEN II (Nippongene, Tokyo, Japan) and dissolved in up to 10 µL of nuclease-free water to achieve an RNA concentration of approximately 1500–2000 ng/µL. The synthesized cRNA was preserved at −80°C until further use.

(ii) Preparation of *H. magnanima* eggs

A total of 15 males and 10 females of *H. magnanima* were mated in a plastic box (30 cm × 20 cm × 5 cm) for 3–4 days. The collection of egg masses began the day after oviposition was confirmed. In brief, newly oviposited egg masses were collected at 30-min intervals during the dark period using red light (which the moths cannot perceive). Females started to oviposit eggs at least 4 h into the dark period and, within less than 30 min post oviposition (mpo), the egg masses were collected. The collection lasted until the start of the light period. The egg masses were then subjected to microinjection assays.

(iii) Inoculum preparation, microinjection, and maintenance of the injected embryos

A glass needle for microinjection was prepared using glass capillary GD-1 (Narishige, Tokyo, Japan) with a PC-10 puller (Narishige). The glass capillaries were pulled at two temperatures (first stage: 75°C, second stage: 65°C) using two heavy weights and one light weight. The movement position during the second heating stage was set to 3 mm (range 1–10 mm).

The cRNA solution was diluted in a buffer (100 mM potassium acetate, 2 mM magnesium acetate, and 30 mM HEPES-KOH; at pH 7.4) containing 0.2% (W/V) Brilliant Blue FCF (Wako, Osaka, Japan) to obtain an RNA concentration of 1000 ng/μL. Approximately 1–4 μL of dye-containing mRNA solution was aspirated into the glass needles (capillaries), and the edge of each needle was ground using Micro Grinder EG-402 (Narishige) at an angle of 20 degrees for 2 s.

The fresh egg masses of *H. magnanima* (collected at less than 30 mpo as described above) were put on a double-sided sticky tape (15 mm × 5 m T4612, Nitoms, Tokyo, Japan) and placed on glass slides. Under a Nikon SMZ1270 microscope (Nicon, Tokyo, Japan), the RNA solution (30–100 fL) was injected into individual eggs using an microinjector IM-400 (Narishige) (balance pressure set at 0.010–0.030; injection pressure set at 0.050-0.100) and a QP-3JOY-2R electric micromanipulator (MicroSupport, Shizuoka, Shizuoka).

The injected egg masses were maintained in a 90-mm plastic Petri dish fitted with a slightly wet filter paper. As high humidity interfered with the development of the embryos, the injected eggs (egg mass) were first maintained at a relative humidity of 30%–50% (0–3 days post injection) and then at a higher humidity until hatching (4–6 dpo, 60% relative humidity). The hatched larvae were reared separately with 1/2 ounces of SilkMate 2S (Nosan Co.) until eclosion. The adult *H. magnanima* moths that emerged from the cRNA-injected embryos were sexed based on their external morphology.

Sexing of embryos/neonates and RNA extraction

To verify the effect of the transient expression of *Hm-oscar* on sex determination in *H. magnanima*, *Hm-oscar*/*GFP*-expressed, non-injected, and *w*Hm-t-infected mature embryos showing black head capsule (1 day before hatching, 5–6 days post oviposition, dpo) were dissected on glass slides using forceps, as described in Arai et al. (2022). Their Malpighian tubules were fixed with methanol/acetic acid (50% v/v) and stained with lactic acetic orcein for W chromatin observations. The remaining tissues not used for sexing were stored in ISOGEN II (Nippon Gene) at −80°C until subsequent extraction. In total, 12 males or females (confirmed based on the presence or absence of W chromatin) were pooled and homogenized in ISOGEN II to extract RNA as described in Arai et al. (2023a). In brief, 0.4 times the volume of UltraPure distilled water (Invitrogen) was added to the ISOGEN II homogenates, which were centrifuged at 12000 × *g* for 15 min at 4°C to pellet proteins and DNAs. The resulting supernatant was mixed with the same volume of isopropanol to precipitate RNAs; then, the resulting solutions were transferred to EconoSpin columns (Epoch Life Science) and centrifuged at 17,900 × *g* and 4°C for 2 min. The RNAs captured in the column were washed twice with 80% ethanol and eluted in 20 μL of UltraPure distilled water (Invitrogen).

Hmdsx detection

Sex-specific *dsx* splicing variants of *H. magnanima* were detected as described in Arai et al. (2023a). In brief, total RNA (100–300 ng) extracted from sex-determined mature embryos was reverse-transcribed using PrimeScript™ II Reverse Transcriptase (TaKaRa, Shiga, Japan) at 30°C for 10 min, 45°C for 60 min, and 70°C for 15 min. Then, cDNA was amplified using KOD-FX Neo (Toyobo Co., Ltd.) with the following two primers: Hmdsx_long3F (5'-TGCCTAAAGTGAAAACGCCGAGGAGCC-3') and Hmdsx_Mrev (5'-TGGAGGTCTCTTTTCATCCGG-3'). The

PCR conditions used were as follows: 94°C for 2 min, followed by 45 cycles of 98°C for 10 s, 66°C for 30 s, and 68°C for 30 s. The amplicons were subjected to electrophoresis on 2.0% agarose Tris-borate-EDTA buffer (89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA) gels.

Quantification of Z chromosome-linked genes

The effects on dosage compensation were assessed by measuring differences in gene expression, as described in Fukui et al. (2015) and Arai et al. (2023a). A total of 1.0 µg of the total RNA extracted from *Hm-oscar* or *GFP*-overexpressed mature embryos (108 hpo, approximately 40-50 sex determined embryos) was used to prepare mRNA-seq libraries via the NEBNext Poly (A) mRNA Magnetic Isolation Module (New England Biolabs) and the NEBNext Ultra II RNA Library Prep kit for Illumina (New England Biolabs) following the manufacturer's protocol. The adaptor sequences and low-quality reads (Qscore <20) were removed from the generated sequence data [150 bp paired-end (PE150)] using Trimmomatic (Bolger et al., 2014). The trimmed reads were mapped to the previously assembled transcriptome database for *H. magnanima* (Arai et al., 2023a) using Kallisto (Bray et al., 2016) to generate the normalized read count data (transcripts per million, TPM). While we did not generate new RNA-seq data of the *w*Hm-t-infected and uninfected groups, the expression data included in Arai et al. (2023a) were re-analyzed along with the newly obtained RNA-seq data above. The binary logarithms of the TPM differences between males and females belonging to each *H. magnanima* group (i.e., *Hm-oscar*/*GFP*-expressed) were calculated to assess the fold-changes in gene expression levels between sexes. As described in Arai et al. (2023a), the transcriptome data of *H. magnanima* were annotated using the *B. mori* gene sets obtained from KAIKOBASE (*https://kaikobase.dna.affrc.go.jp*). The binary logarithms of the TPM differences between males and females in the *B. mori* chromosomes 1 to 28 were plotted.

To confirm the effects observed in the RNA-seq-based quantification of the Z chromosome genes, expression level of the Z-linked *Hmtpi* gene was quantified by reverse transcription-qPCR as described in Arai et al. (2023a). Briefly, total RNA (100–300 ng) extracted from pooled male or female mature embryos (108 hpo) was reverse transcribed using PrimeScript II reverse transcriptase (TaKaRa) as described above. The cDNA was used to quantify relative gene expression levels, with normalization to the control gene elongation factor 1a (*ef1a*). qPCR was performed using primer sets for *Hmtpi* (HmTpi_F180702_212: 5'-GCTGCGAGTGGGTGATTTTG-3'; HmTpi_R180702_326: 5'-GCGATCACTTTCAGACCCGA-3') and *Hmef1a* (Hmef1a_F_val1_85: 5'-TTTCCAGGGTGGTTGAGCA-3'; Hmef1a_R_val1_193: 5'-CCGTTAAGGAGCTGCGTCG-3'), and KOD SYBR qPCR Mix (Toyobo, Osaka, Japan) in a LightCycler 96 system (Roche, Basel, Switzerland). The qPCR reaction was made with 5 µl KOD SYBR (TOYOBO), 0.4 µL forward primer [10 pmol/µl], 0.4 µl reverse primer [10 pmol/µl], and 3.2 µl water. The qPCR was performed under the following conditions: 180 s at 95°C, 40 cycles of 8 s at 98°C, 10 s at 60°C, and 10 s at 68°C, followed by heating to 90°C for melting curve analysis. Mean cycle threshold (C_T) values of samples were calculated for at least 13 replicates, and both ΔC_T (C_T Z gene – C_T ef1a) and $\Delta\Delta C_T$ (ΔC_Tmale – ΔC_Tfem) values were calculated.

Transfection assays and quantification of *BmIMP*^{M}

The coding sequence of *Hmmasc* was amplified from cDNA derived from the RNA extracted from male embryos of the NSR line using KOD-one (TOYOBO) with the following primer set: HmMasc_CDS_HindIIIF: 5'-GCAAAGCTTCAACATGATCTCTCGCCAACCACAATCAACATCA-3' and HmMasc_CDS_BamHI R: 5'-GCAGGATCCCAACCTACTGATAAGGAGGGAAGTAAGGCTGCTG-3'. The following PCR conditions were applied: 20 cycles of 98°C for 10 s, 62°C for 5 s, and 68°C for 15 s. The amplicon was purified with the QIAquick PCR purification kit (Qiagen) and cloned into the pIZ/V5-His vector using the HindIII and BamHI enzymes (New England Biolabs). The codon-optimized *oscar* gene of *w*Fur (Katsuma et al. 2022) was also cloned into the pIZ/V5-His vector using the KpnI and NotI enzymes (TaKaRa).

To verify the masculinizing function of *Hmmasc*, BmN-4 cells (4×10^5 cells per dish, diameter 35 mm) were transfected with 1 μg of each plasmid DNA (pIZ/V5-His having *Hmmasc*) using FuGENE HD (Promega, WI, USA), as described in Katsuma et al. (2022). To clarify whether *Hm-oscar* suppressed the function of *Hmmasc*, 1 μg of plasmid DNA (pIZ/V5-His bearing either *Hmmasc* or *Hm-oscar* and non-inserted vector as negative control) was transfected to the BmN-4 cells using FuGENE HD (Promega). Three days after transfection, the cells were collected and subjected to RNA extraction via TRI REAGENT® (Molecular Research Center Inc., USA) and cDNA construction with AMV transcriptase (TaKaRa). The degree of masculinization in the BmN-4 cells (default female-type sex determination) was verified by quantifying the expression levels of $BmImp^M$ (Suzuki et al., 2010), which is involved in male-specific sex determination cascades, using primers BmIMP_F: 5'-ATGCGGGAAGAAGGTTTTATG-3' and BmIMP_R: 5'-TCATCCCGCCTCAGACGATTG-3', as described in Fukui et al. (2015). *BmRp49* gene was also amplified as a control gene using primers rp49_F: 5'-CCCAACATTGGTTACGGTTC-3' and rp49_R: 5'-GCTCTTTCCACGATCAGCTT-3'. Further interactions between *Hm-oscar* and *masc* genes derived from lepidopteran insects [i.e., *Trilocha varians masc* (*Tvmasc*), *Spodoptera frugiperda masc* (*Sfmasc*), *B. mori masc* (*Bmmasc*), *O. furnacalis masc (Ofmasc),* and *Papilio machaon* masc (*Pmmasc*)] (Katsuma et al., 2022 b) were assessed using the same procedures. The expression levels of $BmImp^M$ were analyzed as described in Katsuma et al. (2022). In brief, the relative expression of $BmImp^M$ ($C_T\,BmImp^M$ / $C_T\,BmRp49$) was calculated for each experimental group. Then, relative expression of $BmImp^M$ of each sample was normalized by setting the mean in the *Hmmasc* singly transfected group as 100. The normalized relative expression of $BmImp^M$ was visualized using R v4.0 with ggplot2 (*https://ggplot2.tidyverse.org/*).

Quantification and statistical analysis

The number of surviving *H. magnanima* injected with either *Hm-oscar*, *wmk*s, or *GFP* in each replicate were counted at the adult stage. The male ratios (number of adult males/numbers of all adults) under all conditions were compared using the Steel– Dwass test in R v4.0. The sex ratio bias was also assessed based on the total numbers of (i) male and female adults and (ii) male and female embryos under each condition using the binomial test in R v4.0.

Gene expression levels between chromosomes (RNA-seq), a Z-linked *Hmtpi* gene expression in *H. magnanima* (RT-qPCR), and normalized $BmIMP^M$ expression in BmN-4 cells under all conditions (RT-qPCR) were compared using the Steel– Dwass test in R v4.0.

A phylogenetic tree of Masc proteins used in this study was constructed based on maximum likelihood with bootstrap re-sampling of 1,000 replicates using MEGA7 (Kumar et al., 2016).

Acknowledgements

We thank Greg Hurst (Institute of Infection, Veterinary and Ecological Sciences, University of Liverpool, Liverpool, UK) and Takeshi Suzuki (Graduate School of Bio-Applications and Systems Engineering, Tokyo University of Agriculture and Technology, Tokyo, Japan) for their kind advice.

We acknowledge support from the Japan Society for the Promotion of Science (JSPS) Research Fellowships for Young Scientists [Grant Number 19J13123 and 21J00895 to H. Arai], JSPS Grant-in-Aid for Scientific Research [Grant Number 22K14902 to H. Arai, 23H02229 and 24H02293 to D. Kageyama, 22H00366 and 24H02289 to S. Katsuma], JSPS Fund for the Promotion of Joint International Research (Fostering Joint International Research (B)) [Grant Number 21KK0105 to H. Arai and MN. Inoue].

Additional information

Author contributions

H. Arai conducted gene functional validation assays, transcriptome assays, and data analysis; designed experiments; wrote the original manuscript; and revised the manuscript. S. Katsuma conducted transfection assays, quantified the gene expressions in the cells and presented the entire discussion. N. Matsuda-Imai constructed the pIZ/V5 vector having *oscar* gene derived from *Ostrinia furnacalis*. S-R. Lin revised the manuscript and contributed to the discussion. MN Inoue revised the manuscript and contributed to the discussion. D. Kageyama organized the project and revised the manuscript. Lastly, H. Arai and D. Kageyama took responsibility for the decision to submit the manuscript for publication and managed the experiments and discussion.

Inclusion and diversity

We support inclusive, diverse, and equitable conduct of research.

Benefit-Sharing

The *w*Hm-t-infected *H. magnanima* was collected from Tea Research and Extension Station (Taoyuan City, Taiwan), and imported with permission from the Ministry of Agriculture, Forestry and Fisheries (No. 27 - Yokohama Shokubou 891 and No. 297 - Yokohama Shokubou 1326). All collaborators are presented as co-authors, and the results have been shared with the provider communities. Moreover, our group is committed to international scientific partnerships as well as institutional capacity building. The authors declare no competing interests.

Data and code availability

High-throughput sequencing data are available under accession numbers DRA018708 and PRJDB18169 (BioProject). All data generated during this study are included in the manuscript and a supporting excel file. Further information and requests for resources and reagents are accessible from the lead contact, Hiroshi Arai (dazai39papilio@gmail.com/HArai@liverpool.ac.uk).

Additional files

Suppremental data files

References

Arai H, Anbutsu H, Nishikawa Y, Kogawa M, Ishii K, Hosokawa M, Lin SR, Ueda M, Nakai M, Kunimi Y, Harumoto T, Kageyama D, Takeyama H, Inoue MN 2023b) **Combined actions of bacteriophage-encoded genes in Wolbachia-induced male lethality** *iScience* **26** https://doi.org/10.1016/j.isci.2023.106842

Arai H, Herran B, Sugimoto TN, Miyata M, Sasaki T, Kageyama D 2024a) **Cell-based assays and comparative genomics revealed the conserved and hidden effects of Wolbachia on insect sex determination** *PNAS Nexus* **3** https://doi.org/10.1093/pnasnexus/pgae348

Arai H, Hirano T, Akizuki N, Abe A, Nakai M, Kunimi Y, Inoue MN 2019) **Multiple infection and reproductive manipulations of Wolbachia in Homona magnanima (Lepidoptera: Tortricidae)** *Microbial Ecology* **77**:257–266 https://doi.org/10.1007/s00248-018-1210-4

Arai H, Ishitsubo Y, Nakai M, Inoue MN 2022) **Mass-rearing and molecular studies in Tortricidae pest insects** *JoVE (Journal of Visualized Experiments)* **181** https://doi.org/10.3791/63737

Arai H, Lin SR, Nakai M, Kunimi Y, Inoue MN 2020) **Closely related male-killing and nonmale-killing Wolbachia strains in the oriental tea tortrix Homona magnanima** *Microbial Ecology* **79**:1011–1020 https://doi.org/10.1007/s00248-019-01469-6

Arai H, Takamatsu T, Lin SR, Mizutani T, Omatsu T, Katayama Y, Nakai M, Kunimi Y, Inoue MN 2023a) **Diverse molecular mechanisms underlying microbe-inducing male killing in the moth Homona magnanima** *Applied and Environmental Microbiology* **89**:e02095–22 https://doi.org/10.1128/aem.02095-22

Arai H, Ueda M, Hirano T, Akizuki N, Lin SR, Hanh DK, Widada J, Rohman MS, Nakai M, Kunimi Y, Vang LV, Wijonarko A, Inoue MN. 2024b) **Conserved infections and reproductive phenotypes of Wolbachia symbionts in Asian tortrix moths** *Environmental Microbiology Reports* **16** https://doi.org/10.1111/1758-2229.13219

Arai H, Watada M, Kageyama D 2024c) **Two male-killing Wolbachia from Drosophila birauraia that are closely related but distinct in genome structure** *Royal Society Open Science* **11** https://doi.org/10.1098/rsos.231502

Bolger AM, Lohse M, Usadel B 2014) **Trimmomatic: A flexible trimmer for illumina sequence data** *Bioinformatics* **30**:2114–2120 https://doi.org/10.1093/bioinformatics/btu170

Bray NL, Pimentel H, Melsted P, Pachter L 2016) **Near-optimal probabilistic RNA-seq quantification** *Nature Biotechnology* **34**:525–527 https://doi.org/10.1038/nbt.3519

Fujita R, Inoue MN, Takamatsu T, Arai H, Nishino M, Abe N, Itokawa K, Nakai M, Urayama S, Chiba Y, Amoa-Bosompem M, Kunimi Y 2021) **Late male-killing viruses in Homona magnanima identified as Osugoroshi viruses, novel members of Partitiviridae** *Frontiers in Microbiology* **11** https://doi.org/10.3389/fmicb.2020.620623

Fukui T, Kawamoto M, Shoji K, Kiuchi T, Sugano S, Shimada T, Suzuki Y, Katsuma S 2015) **The endosymbiotic bacterium Wolbachia selectively kills male hosts by targeting the**

masculinizing gene *PLOS Pathogens* **11** https://doi.org/10.1371/journal.ppat.1005048

Fukui T, Kiuchi T, Tomihara K, Muro T, Matsuda-Imai N, Katsuma S 2024) **Expression of the Wolbachia male-killing factor Oscar impairs dosage compensation in lepidopteran embryos** *FEBS Letters* **598**:331–337 https://doi.org/10.1002/1873-3468.14777

Grace TDC 1967) **Establishment of a line of cells from the silkworm Bombyx mori** *Nature* **216**:613–613 https://doi.org/10.1038/216613a0

Harumoto T, Lemaitre B 2018) **Male-killing toxin in a bacterial symbiont of Drosophila** *Nature* **557**:252–255 https://doi.org/10.1038/s41586-018-0086-2

Hill T, Unckless RL, Perlmutter JI 2022) **. positive selection and horizontal gene transfer in the genome of a male-killing Wolbachia** *Molecular Biology and Evolution* **39** https://doi.org/10.1093/molbev/msab303

Hornett EA, Charlat S, Duplouy AMR, Davies N, Roderick GK, Wedell N, Hurst GDD 2006) **Evolution of male-killer suppression in a natural population** *PLOS Biology* **4** https://doi.org/10.1371/journal.pbio.0040283

Hornett EA, Kageyama D, Hurst GDD 2022) **Sex determination systems as the interface between male-killing bacteria and their hosts** *Proceedings of the Royal Society B: Biological Sciences* **289** https://doi.org/10.1098/rspb.2021.2781

Hornett EA, Moran B, Reynolds LA, Charlat S, Tazzyman S, Wedell N, Jiggins CD, Hurst GDD 2014) **The evolution of sex ratio distorter suppression affects a 25 cM genomic region in the butterfly Hypolimnas bolina** *PLOS Genetics* **10** https://doi.org/10.1371/journal.pgen.1004822

Hurst GDD 2017) **Extended genomes: Symbiosis and evolution** *Interface Focus* **7** https://doi.org/10.1098/rsfs.2017.0001

Hurst GDD, Frost CL 2015) **Reproductive parasitism: maternally inherited symbionts in a biparental world** *Cold Spring Harbor Perspectives in Biology* **7** https://doi.org/10.1101/cshperspect.a017699

Hurst GDD, Johnson AP, Schulenburg JHG. v d, Fuyama Y. 2000) **Male-killing Wolbachia in Drosophila: A temperature-sensitive trait with a threshold bacterial density** *Genetics* **156**:699–709 https://doi.org/10.1093/genetics/156.2.699

Hurst GDD, von der Schulenburg JHG, Majerus TMO, Bertrand D, Zakharov IA, Baungaard J, Völkl W, Stouthamer R, Majerus MEN 1999) **Invasion of one insect species, Adalia bipunctata, by two different male-killing bacteria** *Insect Molecular Biology* **8**:133–139 https://doi.org/10.1046/j.13652583.1999.810133.x

Jones JDG, Dangl JL 2006) **The plant immune system** *Nature* **444**:323–329 https://doi.org/10.1038/nature05286

Kageyama D, Harumoto T, Nagamine K, Fujiwara A, Sugimoto TN, Jouraku A, Tamura M, Katoh TK, Watada M 2023) **A male-killing gene encoded by a symbiotic virus of Drosophila** *Nature Communications* **14** https://doi.org/10.1038/s41467-023-37145-0

Kageyama D, Narita S, Watanabe M 2012) **Insect sex determination manipulated by their endosymbionts: incidences, mechanisms and implications** *Insects* **3** https://doi.org/10.3390

/insects3010161

Kageyama D, Traut W 2004) **Opposite sex–specific effects of Wolbachia and interference with the sex determination of its host Ostrinia scapulalis** *Proceedings of the Royal Society of London. Series B: Biological Sciences* **271**:251–258 https://doi.org/10.1098/rspb.2003.2604

Katsuma S, Hirota K, Matsuda-Imai N, Fukui T, Muro T, Nishino K, Kosako H, Shoji K, Takanashi H, Fujii T, Arimura S, Kiuchi T 2022) **A Wolbachia factor for male killing in lepidopteran insects** *Nature Communications* **13** https://doi.org/10.1038/s41467-022-34488-y

Katsuma S, Shoji K, Sugano Y, Suzuki Y, Kiuchi T 2019) **Masc-induced dosage compensation in silkworm cultured cells** *FEBS Open Bio* **9**:1573–1579 https://doi.org/10.1002/2211-5463.12698

Kiuchi T, Koga H, Kawamoto M, Shoji K, Sakai H, Arai Y, Ishihara G, Kawaoka S, Sugano S, Shimada T, Suzuki Y, Suzuki MG, Katsuma S 2014) **A single female-specific piRNA is the primary determiner of sex in the silkworm** *Nature* **509**:633–636 https://doi.org/10.1038/nature13315

Kumar S, Stecher G, Tamura K 2016) **MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets** *Molecular Biology and Evolution* **33**:1870–1874 https://doi.org/10.1093/molbev/msw054

Nagamine K, Kanno Y, Sahara K, Fujimoto T, Yoshido A, Ishikawa Y, Terao M, Kageyama D, Shintani Y 2023) **Male-killing virus in a noctuid moth Spodoptera litura** *Proceedings of the National Academy of Sciences* **120** https://doi.org/10.1073/pnas.2312124120

Perlmutter JI, Bordenstein SR, Unckless RL, LePage DP, Metcalf JA, Hill T, Martinez J, Jiggins FM, Bordenstein SR 2019) **The phage gene wmk is a candidate for male killing by a bacterial endosymbiont** *PLOS Pathogens* **15** https://doi.org/10.1371/journal.ppat.1007936

Perlmutter JI, Meyers JE, Bordenstein SR 2020) **Transgenic testing does not support a role for additional candidate genes in Wolbachia male killing or cytoplasmic incompatibility** *mSystems* **5** https://doi.org/10.1128/msystems.00658-19

Perlmutter JI, Meyers JE, Bordenstein SR 2021) **A single synonymous nucleotide change impacts the male-killing phenotype of prophage WO gene wmk** *eLife* **10** https://doi.org/10.7554/eLife.67686

Sasaki T, Kubo T, Ishikawa H 2002) **Interspecific transfer of Wolbachia between two Lepidopteran insects expressing cytoplasmic incompatibility: A Wolbachia variant naturally infecting Cadra cautella causes male killing in Ephestia kuehniella** *Genetics* **162**:1313–1319 https://doi.org/10.1093/genetics/162.3.1313

Sugimoto TN, Ishikawa Y 2012) **A male-killing Wolbachia carries a feminizing factor and is associated with degradation of the sex-determining system of its host** *Biology Letters* **8**:412–415 https://doi.org/10.1098/rsbl.2011.1114

Sugimoto TN, Kayukawa T, Shinoda T, Ishikawa Y, Tsuchida T 2015) **Misdirection of dosage compensation underlies bidirectional sex-specific death in Wolbachia- infected Ostrinia scapulalis** *Insect Biochemistry and Molecular Biology* **66**:72–76 https://doi.org/10.1016/j.ibmb.2015.10.001

Suzuki MG, Kobayashi K., Kitano T., Iwao Y., Kondo M. 2018) **Sex Determination cascade in insects: a great treasure house of alternative splicing** *Reproductive and Developmental*

Strategies: The Continuity of Life Japan: Springer :267–288 https://doi.org/10.1007/978-4-431-56609-0_13

Suzuki MG, Imanishi S, Dohmae N, Asanuma M, Matsumoto S 2010) **Identification of a male-specific RNA binding protein that regulates sex-specific splicing of Bmdsx by increasing RNA binding activity of BmPSI** *Molecular and Cellular Biology* **30**:5776–5786 https://doi.org/10.1128/MCB.00444-10

Takahashi F, Kuwahara Y 1970) **Studies on sex pheromones of Pyralididae. III. The inheritance of the abnormal sex ratio condition in a strain of the almond moth** *Cadra cautella Walker (Phycitinae). Botyu-Kagaku = Scientific Insect Control* **35**:11–21 https://www.cabidigitallibrary.org/doi/full/10.5555/19710503687

Veneti Z, Zabalou S, Papafotiou G, Paraskevopoulos C, Pattas S, Livadaras I, Markakis G, Herren JK, Jaenike J, Bourtzis K 2012) **Loss of reproductive parasitism following transfer of male-killing Wolbachia to Drosophila melanogaster and Drosophila simulans** *Heredity* **109**:306–312 https://doi.org/10.1038/hdy.2012.43

Visser S, Voleníková A, Nguyen P, Verhulst EC, Marec F 2021) **A conserved role of the duplicated Masculinizer gene in sex determination of the Mediterranean flour moth, Ephestia kuehniella** *PLOS Genetics* **17** https://doi.org/10.1371/journal.pgen.1009420

Werren JH, Baldo L, Clark ME 2008) **Wolbachia: Master manipulators of invertebrate biology** *Nature Reviews Microbiology* **6**:741–751 https://doi.org/10.1038/nrmicro1969

Zug R, Hammerstein P 2012) **Still a host of hosts for Wolbachia: analysis of recent data suggests that 40% of terrestrial arthropod species are infected** *PLOS One* **7** https://doi.org/10.1371/journal.pone.0038544

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Reviewer #1 (Public review):

Summary:

Insects and their relatives are commonly infected with microbes that are transmitted from mothers to their offspring. A number of these microbes have independently evolved the ability to kill the sons of infected females very early in their development; this male killing strategy has evolved because males are transmission dead-ends for the microbe. A major question in the field has been to identify the genes that cause male killing and to understand how they work. This has been especially challenging because most male-killing microbes cannot be genetically manipulated. This study focuses on a male-killing bacterium called Wolbachia. Different Wolbachia strains kill male embryos in beetles, flies, moths, and other arthropods. This is remarkable because how sex is determined differs widely in these hosts. Two Wolbachia genes have been previously implicated in male-killing by Wolbachia: oscar (in moth male-killing) and wmk (in fly male-killing). The genomes of some male-killing Wolbachia contain both of these genes, so it is a challenge to disentangle the two.

This paper provides strong evidence that oscar is responsible for male-killing in moths. Here, the authors study a strain of Wolbachia that kills males in a pest of tea, Homona magnanima. Overexpressing oscar, but not wmk, kills male moth embryos. This is because oscar interferes with masculinizer, the master gene that controls sex determination in moths and butterflies. Interfering with the masculinizer gene in this way leads the (male) embryo down a path of female development, which causes problems in regulating the expression of genes that are found on the sex chromosomes.

Strengths:

The authors use a broad number of approaches to implicate oscar, and to dissect its mechanism of male lethality. These approaches include: a) overexpressing oscar (and wmk) by injecting RNA into moth eggs, b) determining the sex of embryos by staining female sex chromosomes, c) determining the consequences of oscar expression by assaying sex-specific splice variants of doublesex, a key sex determination gene, and by quantifying gene expression and dosage of sex chromosomes, using RNASeq, and d) expressing oscar along with masculinizer from various moth and butterfly species, in a silkmoth cell line. This extends recently published studies implicating oscar in male-killing by Wolbachia in Ostrinia corn borer moths, although the Homona and Ostrinia oscar proteins are quite divergent. Combined with other studies, there is now broad support for oscar as the male-killing gene in moths and butterflies (i.e. order Lepidoptera). So an outstanding question is to understand the role of wmk. Is it the master male-killing gene in insects other than Lepidoptera and if so, how does it operate?

Weaknesses:

I found the transfection assays of oscar and masculinizer in the silkworm cell line (Figure 4) to be difficult to follow. There are also places in the text where more explanation would be helpful for non-experts.

https://doi.org/10.7554/eLife.101101.2.sa3

Reviewer #2 (Public review):

Summary:

Wolbachia are maternally transmitted bacteria that can manipulate host reproduction in various ways. Some Wolbachia induce male killing (MK), where the sons of infected mothers are killed during development. Several MK-associated genes have been identified in Homona magnanima, including Hm-oscar and wmk-1-4, but the mechanistic links between these Wolbachia genes and MK in the native host are still unclear.

In this manuscript, Arai et al. show that Hm-oscar is the gene responsible for Wolbachia-induced MK in Homona magnanima. They provide evidence that Hm-Oscar functions through interactions with the sex determination system. They also found that Hm-Oscar disrupts sex determination in male embryos by inducing female-type dsx splicing and impairing dosage compensation. Additionally, Hm-Oscar suppresses the function of Masc. The manuscript is well-written and presents intriguing findings. The results support their conclusions regarding the diversity and commonality of MK mechanisms, contributing to our understanding of the mechanisms and evolutionary aspects of Wolbachia-induced MK.

Comments on revisions:

The authors have already addressed the reviewer's concerns.

https://doi.org/10.7554/eLife.101101.2.sa2

Reviewer #3 (Public review):

Summary:

Overall, this is a clearly written manuscript with nice hypothesis testing in a non-model organism that addresses the mechanism of Wolbachia-mediated male killing. The authors aim to determine how five previously identified male-killing genes (encoded in the prophage

region of the wHm Wolbachia strain) impact the native host, Homona magnanima moths. This work builds on the authors' previous studies in which
(1) they tested the impact of these same wHm genes via heterologous expression in Drosophila melanogaster
(2) also examined the activity of other male-killing genes (e.g., from the wFur Wolbachia strain in its native host: Ostrinia furnacalis moths).

Advances here include identifying which wHm gene most strongly recapitulates the male-killing phenotype in the native host (rather than in Drosophila), and the finding that the Hm-Oscar protein has the potential for male-killing in a diverse set of lepidopterans, as inferred by the cell-culture assays.

Strengths:

Strengths of the manuscript include the reverse genetics approaches to dissect the impact of specific male-killing loci, and use of a "masculinization" assay in Lepidopteran cell lines to determine the impact of interactions between specific masc and oscar homologs.

Weaknesses:

It is clear from Figure 1 that the combinations of wmk homologs do not cause male killing on their own here. While I largely agree with the author's conclusions that oscar is the primary MK factor in this system, I don't think we can yet rule out that wmk(s) may work synergistically or interactively with oscar in vivo. This might be worth a small note in the discussion. (eg at line 294 'indicating that wmk likely targets factors other than masc." - this could be downstream of the impacts of oscar; perhaps dependent on oscar-mediated impacts on masc first).

Regarding the perceived male-bias in Figure 2a: I think readers might be interpreting "unhatched" as "total before hatching". You could eliminate ambiguity by perhaps splitting the bars into male and female, and then within a bar, coloring by hatched versus unhatched. But this is a minor point, and I think the updated text helps clarify this.

The new Figure 4b looks to be largely redundant with the oscar information in Figure 1a.

Updated statistical comparisons for the RNA-seq analysis are helpful. However these analyses are based on single libraries (albeit each a pool of many individuals), so this is still a weaker aspect of the manuscript.

The new information on masc similarity is useful (Fig 4d) - if the authors could please include a heatmap legend for the colors, that would be helpful. Also, please avoid green and red in the same figure when key for interpretation.

Figure 1A "helix-turn-helix" is misspelled. ("tern").

https://doi.org/10.7554/eLife.101101.2.sa1

Author response:

The following is the authors' response to the original reviews.

> ***Public Reviews:***
>
> ***Reviewer #1 (Public review):***
>
> *Summary:*

> *Insects and their relatives are commonly infected with microbes that are transmitted from mothers to their offspring. A number of these microbes have independently evolved the ability to kill the sons of infected females very early in their development; this male killing strategy has evolved because males are transmission dead-ends for the microbe. A major question in the field has been to identify the genes that cause male killing and to understand how they work. This has been especially challenging because most male-killing microbes cannot be genetically manipulated. This study focuses on a male-killing bacterium called Wolbachia. Different Wolbachia strains kill male embryos in beetles, flies, moths, and other arthropods. This is remarkable because how sex is determined differs widely in these hosts. Two Wolbachia genes have been previously implicated in male-killing by Wolbachia: oscar (in moth male-killing) and wmk (in fly male-killing). The genomes of some male-killing Wolbachia contain both of these genes, so it is a challenge to disentangle the two.*
>
> *This paper provides strong evidence that oscar is responsible for male-killing in moths. Here, the authors study a strain of Wolbachia that kills males in a pest of tea, Homona magnanima. Overexpressing oscar, but not wmk, kills male moth embryos. This is because oscar interferes with masculinizer, the master gene that controls sex determination in moths and butterflies. Interfering with the masculinizer gene in this way leads the (male) embryo down a path of female development, which causes problems in regulating the expression of genes that are found on the sex chromosomes.*

We would like to thank you for evaluating our manuscript.

> *Strengths:*
>
> *The authors use a broad number of approaches to implicate oscar, and to dissect its mechanism of male lethality. These approaches include:*
>
> *(1) Overexpressing oscar (and wmk) by injecting RNA into moth eggs.*
>
> *(2) Determining the sex of embryos by staining female sex chromosomes.*
>
> *(3) Determining the consequences of oscar expression by assaying sex-specific splice variants of doublesex, a key sex determination gene, and by quantifying gene expression and dosage of sex chromosomes, using RNASeq.*
>
> *(4) Expressing oscar along with masculinizer from various moth and butterfly species, in a silkmoth cell line.*
>
> *This extends recently published studies implicating oscar in male-killing by Wolbachia in Ostrinia corn borer moths, although the Homona and Ostrinia oscar proteins are quite divergent. Combined with other studies, there is now broad support for oscar as the male-killing gene in moths and butterflies (i.e. order Lepidoptera). So an outstanding question is to understand the role of wmk. Is it the master male-killing gene in insects other than Lepidoptera and if so, how does it operate?*

Thank you for your comments. *Wolbachia* strains often carry *wmk* genes, but as observed in this study, the homologs in *Homona* showed no apparent MK ability. These showed strong male lethality in *D. melanogaster*, but it is still unclear whether the genes are the master male-killing gene in Diptera. It is also possible that the genes show toxicities in other lepidopteran insects as well as in other insect taxa. Further functional validation assays in different insects are warranted to clarify whether *wmk* shows toxicity in different insect taxa. We have also discussed the functions of *wmk* in the Discussion section (lines 301-306).

> *Weaknesses:*

> *I found the transfection assays of oscar and masculinizer in the silkworm cell line (Figure 4) to be difficult to follow. There are also places in the text where more explanation would be helpful for non-experts (see recommendations).*

Thank you for your suggestion. We have thoroughly revised the manuscript to address all the questions, comments and suggestions you raised in “recommendations”. In particular, we have revised the section on the transfection assays of Oscar and Masc in Bm-N4 cells (result section “Hm-oscar suppresses the masculinizing functions of lepidopteran masc genes” starts on line 214 and Fig. 4; materials and methods section ”Transfection assays and quantification of *BmIMP*^{M}”, starts on line 483). We have also provided more detailed explanations for non-experts in some contexts (in response to your recommendation). We believe that the resulting revisions have significantly improved the quality and comprehensiveness of our manuscript.

> ***Reviewer #2 (Public review):***
>
> *Summary:*
>
> *Wolbachia are maternally transmitted bacteria that can manipulate host reproduction in various ways. Some Wolbachia induce male killing (MK), where the sons of infected mothers are killed during development. Several MK-associated genes have been identified in Homona magnanima, including Hm-oscar and wmk-1-4, but the mechanistic links between these Wolbachia genes and MK in the native host are still unclear.*
>
> *In this manuscript, Arai et al. show that Hm-oscar is the gene responsible for Wolbachia-induced MK in Homona magnanima. They provide evidence that Hm-Oscar functions through interactions with the sex determination system. They also found that Hm-Oscar disrupts sex determination in male embryos by inducing female-type dsx splicing and impairing dosage compensation. Additionally, Hm-Oscar suppresses the function of Masc. The manuscript is well-written and presents intriguing findings. The results support their conclusions regarding the diversity and commonality of MK mechanisms, contributing to our understanding of the mechanisms and evolutionary aspects of Wolbachia-induced MK.*

We would like to thank you for evaluating our manuscript.

> *Strengths/weaknesses:*
>
> *(1) The authors found that transient overexpression of Hm-oscar, but not wmk-1-4, in Wolbachia-free H. magnanima embryos induces female-biased sex ratios. These results are striking and mirror the phenotype of the wHm-t infected line (WT12). However, Table 1 lists the "male ratio," while the text presents the "female ratio" with standard deviation. For consistency, the calculation term should be uniform, and the "ratio" should be listed for each replicate.*

We have revised the first results section (*Hm-oscar* induces female-biased sex ratios, starting from line 147) accordingly to maintain the consistency in the calculation term. In the revised manuscript, the 'male ratio' is now consistently used, in alignment with Fig. 1. In addition, we have included all sex ratio information (number of males and females) in the supplementary data file for transparency and clarity.

> *(2) The error bars in Figure 3 are quite large, and the figure lacks statistical significance labels. The authors should perform statistical analysis to demonstrate that Hm-oscar-overexpressed male embryos have higher levels of Z-linked gene expression.*

The large error bar on each chromosome (Fig.3a-d) likely reflect the overall variation in expression levels across different transcripts. Accordingly, we have included statistical data for Figure 3 based on the Steel-Dwass test for expression levels. However, displaying statistical significance directly on the whisker plots would make the figure too cluttered due to the numerous combinations. Instead, we have provided all the statistical data in the supplementary data file. To further support the claim that Z-linked genes are more highly expressed in wHm-t-infected/Hb-Oscar-injected embryos, we have included the expression data for a Z-linked gene tpi, along with its statistical data in the revised manuscript (Fig. 3e, lines 210-212).

> *(3) The authors demonstrated that Hm-Oscar suppresses the masculinizing functions of lepidopteran Masc in BmN-4 cells derived from the female ovaries of Bombyx mori. They should clarify why this cell line was chosen and its biological relevance. Additionally, they should explain the rationale for evaluating the expression levels of the male-specific BmIMP variant and whether it is equivalent to dsx.*

Thank you for your suggestion. We selected BmN-4 cell line because previous studies have established it as a reliable model for investigating the functions of lepidopteran *masc* genes and the interactions between *masc* and *Oscar* genes (Katsuma et al., 2019; 2022). In addition, $BmIMP^{M}$ is a male-specific regulator of the male-type *dsx*, making it an ideal target for assessing the 'maleness' induced by transfection of the *masc* gene in female-derived BmN-4 cells (Suzuki et al., 2010; Katsuma et al., 2015). We have included more detailed background information in the revised manuscript and have thoroughly revised this section (*Hm-oscar* suppresses the masculinizing functions of lepidopteran *masc* genes, starting at line 214) and Figure 4 for better clarity.

> *(4) Although the authors show that Hm-oscar is involved in Wolbachia-induced MK in Homona magnanima and interacts with the sex determination system in lepidopteran insects, the precise molecular mechanism of Hm-oscar-induced MK remains unclear. Further studies are needed to elucidate how Hm-oscar regulates Homona magnanima genes to induce MK, though this may be beyond the scope of the current manuscript.*

Based on our findings and previous studies in *Homona*, *Ostrinia* and *Bombyx* (Arai et al., 2023a; Katsuma et al., 2023; Kiuchi et al., 2014), we hypothesize that the molecular mechanisms underlying _w_Hm-induced MK are likely linked to impaired dosage compensation caused by the inhibition of Masc function by the Hm-Oscar protein. While the precise mechanisms remain unclear, unbalanced Z-linked gene expression due to the impaired dosage compensation (i.e., 2-fold higher Z-linked gene expression compared to normal males) is known to be lethal for lepidopteran males (Kiuchi et al., 2014; Fukui et al., 2015; Visser et al., 2021). We have outlined this hypothesis in the Discussion section (lines 245-254).

> ***Reviewer #3 (Public review):***
>
> *Summary:*
>
> *Overall, this is a clearly written manuscript with nice hypothesis testing in a non-model organism that addresses the mechanism of Wolbachia-mediated male killing. The authors aim to determine how five previously identified male-killing genes (encoded in the prophage region of the wHm Wolbachia strain) impact the native host, Homona magnanima moths. This work builds on the authors' previous studies in which:*
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> *(1) They tested the impact of these same wHm genes via heterologous expression in Drosophila melanogaster.*

> *(2) They examined the activity of other male-killing genes (e.g., from the wFur Wolbachia strain in its native host: Ostrinia furnacalis moths).*
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> *Advances here include identifying which wHm gene most strongly recapitulates the male-killing phenotype in the native host (rather than in Drosophila), and the finding that the Hm-Oscar protein has the potential for male-killing in a diverse set of lepidopterans, as inferred by the cell-culture assays.*
>
> *Strengths:*
>
> *Strengths of the manuscript include the reverse genetics approaches to dissect the impact of specific male-killing loci, and the use of a "masculinization" assay in Lepidopteran cell lines to determine the impact of interactions between specific masc and oscar homologs.*

We would like to thank you for evaluating our manuscript.

> *Weaknesses:*
>
> *My major comments are related to the lack of statistics for several experiments (and the data normalization process), and opportunities to make the manuscript more broadly accessible.*

Thank you for your suggestions. We have thoroughly revised the manuscript to provide clearer explanations for non-experts. In addition, we have included more detailed statistical data for Figure 3 and Figure 4 based on the Steel-Dwass tests. For Figure 3a-d, displaying statistical significance directly on the whisker plots would make the figure too cluttered due to the numerous combinations. Therefore, we have provided all the statistical data in the supplementary data file. To further support the claim that Z-linked genes are more highly expressed in _w_Hm-t-infected/Hm-Oscar-injected embryos, we have included the expression data for a Z-linked gene *tpi*, along with its statistical data in the revised manuscript (Fig.3e, lines 210-212). Regarding Figure 4, we have revised the Figure based on the reviewer's suggestions, and provided more detailed information on how the expression data were analyzed (Transfection assays and quantification of $BmIMP^{M}$, lines 495-520). We have also included more detailed background information on the assay system (*Hm-oscar* suppresses the masculinizing functions of lepidopteran *masc* genes, lines 215-237). Although we did not observe statistical significance based on the Steel-Dwass test, likely due to limited replicates, the observed changes in the *IMP* gene expression remain clearly evident.

> *The manuscript I think would be much improved by providing more details regarding some of the genes and cross-lineage comparisons. I know some of this is reported in previous publications, but some summary and/or additional analysis would make this current manuscript much more approachable for a broader audience, and help guide readers to specific important findings. For example, a graphic and/or more detail on how the wmk/oscar homologs (within and across Wolbachia strains) differ (e.g., domains, percent divergence, etc) would be helpful for contextualizing some of the results. I recognize the authors discuss this in parts (e.g., lines 223-227), but it does require some bouncing between sections to follow. Similarly, the experiments presented in Figure 4 indicate that Hm-oscar has broad spectrum activity: how similar are the masc proteins from these various lepidopterans? Are they highly conserved? Rapidly evolving? Do the patterns of masc protein evolution provide any hints at how Oscar might be interacting with masc?*

Thank you for your valuable suggestion. To address this, we have included a visualization of the structural differences between the Oscar and wmk homologs in Figure 1a of the revised

manuscript. In addition, we have included more detailed information for these genes and revised the introduction (lines 110-114; 124-137) and discussion (lines 255-266) to provide a clearer and more comprehensive overview. We have also described the similarity of the Masc proteins and Oscar proteins that we used, which is now reflected in the revised Figure 4b and 4d. More detailed information on these proteins is available in the supplementary data. Notably, Masc proteins exhibit high sequence variability with conserved domains (Figure 4d). Previous study identified the N-terminal region of Masc as crucial for the Oscar function (Katsuma et al., 2022). The wide spectrum of the actions of Hm-Oscar likely stems from these conserved structures of Masc, but the effects might have undergone evolutionary tuning through interactions with the native host as discussed in lines 293-294.

> *It is clear from Figure 1 that the combinations of wmk homologs do not cause male killing on their own. Did the authors test if any of the wmk homologs impact the MK phenotype of oscar? It looks like a previous study tested this in wFur (noted in lines 250-252), but given that the authors also highlight the differences between the wFur-oscar and Hm-oscar proteins, this may be worth testing in this system. Related to this, what is the explanation for why there would be 4 copies of wmk in Hm?*

Thank you for your valuable suggestion. Unfortunately, we have not yet tested the effects of co-expression of wmk and Oscar. Due to a technical issue, the mixing of multiple constructs results in a reduced amount of mRNA (i.e. mixing wmk-3 and Hm-Oscar at the same concentration results in a 2-fold lower concentration in mRNA for both genes compared to mono-injected groups). In addition, we have previously tested injecting mRNA at the twofold higher concentration (i.e. 2 ug/ul mRNA), which resulted in very low hatchability regardless of the genes. Katsuma et al (2022) tested the effect of *wmk* on the sex determination system, but did not test the effect of co-injection/transfection of wmk and Oscar. Considering the results of this and previous studies (Katsuma et al., 2022; Arai et al., 2023), it is likely that the targets of the *wmk* and *oscar* genes are different (as discussed in lines 267-289). Co-injection of *wmk* and *oscar* may not produce additive effects. Nevertheless, we would like to test the results in future studies using the *Drosophila* system as well.

As you point out, it is an interesting point that the moth-derived MK *Wolbachia* _w_Hm-t encodes four *wmk* genes, although they have no apparent effect on host survival. The exact functional relevance of these *wmk* homologs remains unclear. However, they may play a role in *Wolbachia* biology as transcriptional regulators, given that they encode HTH domains. *Wolbachia* generally encode several *wmk* homologs in their genome, regardless of whether they induce MK. This suggests that the functions of the *wmk* genes may be 'suppressed' in certain *Wolbachia*-host systems. The *wmk* and *Hm-oscar* genes are located within a prophage region, and some *wmk* genes are tandemly arrayed (as described in Arai et al., 2023). These *wmk* homologs may have increased in number by horizontal phage transfer, and the region containing *wmk* and adjacent sequences may act as a genomic island for virulence. So far, the function of *wmk* homologs has only been tested in *D. melanogaster* and *H. magnanima*, and further studies in other *Wolbachia*-host systems are highly warranted to test whether *wmk* exerts MK effects in other insect models. These points have been briefly discussed in the revised manuscript (lines 301-306; 318-320).

> *Why are some of the broods male-biased (2/3) rather than ~50:50? (Lines 170-175, Figure 2a). For example, there is a strong male bias in un-hatched oscar-injected and naturally infected embryos, whereas the control uninfected embryos have normal 50:50 sex ratios. It is difficult to interpret the rate of male-killing given that the sex ratios of different sets of zygotes are quite variable.*

The observed male-biased sex ratios in unhatched embryos are due to the occurrence of MK during embryogenesis. In the unhatched groups, the skew towards males reflects that fact

that the male embryos were targeted and killed by *Wolbachia*/*Oscar*, leading to a surplus of unhatched male embryos. Conversely, hatched individuals show a higher proportion of females because many of the males were eliminated during embryogenesis. Thus, the unhatched embryos are more male-biased, while the hatched individuals are more female-biased in the *Hm-oscar*/_w_Hm-t treated groups. We have revised the relevant section (Males are killed mainly at the embryonic stage, lines 179-186) and provided more detailed information to clarify this explanation.

> *Figure 2b - it appears there are both male and female bands in the HmOsc male lane. I think this makes sense (likely a partial phenotype due to the nature of the overexpression approach), but this is worth highlighting, especially in the context of trying to understand how much of the MK phenotype might be recapitulated through these methods. Related, there is no negative control for this PCR.*

Thank you for your suggestion. As you noted, a faint *dsx-M* band is visible in the *Hm-oscar* treated group in Figure 2b. This is consistent with previous findings by Arai et al. (2023), which reported that male embryos with low-density _w_Hm-t showed double bands of *dsx-M* and *dsx-F*, similar to what we observed in this study. This information has been included in the revised manuscript in lines 196-198, as follows:

“Notably, male embryos expressing *Hm-oscar* also exhibited weak male-type *dsx* splicing in addition to the female-type splicing, resembling the previously observed pattern in male embryos infected with low-titer _w_Hm-t (Arai et al., 2023a).”

Also, we appreciate your comment regarding the missing of negative control. The figure has now been revised as we realised that the negative control lane had been lost during the preparation of the figure. We also included the relevant molecular marker information in both the figure legends and Figure 2b.

> *It appears the RNA-seq analysis (Figure 3) is based on a single biological replicate for each condition. And, there are no statistical comparisons that support the conclusions of a shift in dosage compensation. Finally, it is unclear what exactly is new data here: the authors note "The expression data of the wHm-t-infected and non-infected groups were also calculated based on the transcriptome data included in Arai et al. (2023a)" - So, are the data in Figure 3c and 3d a re-print of previous data? The level of dosage compensation inferred by visually comparing the control conditions in 3b and 3d does not appear consistent. With only one biological replicate library per condition, what looks like a re-print of previous data, and no statistical comparisons, this is a weakly supported conclusion.*

Thank you for your suggestion. In this study, we generated the RNA-seq data for the *Hm-oscar*/*GFP*-injected groups, but did not sequence the _w_Hm-t-infected/NSR lines. Instead, the previously generated RNA-seq data of _w_Hm-t-infected/NSR (Arai et al., 2023) were re-analyzed (rather than simply reprinted) to evaluate whether the expression patterns of *Hm-oscar*-injected and _w_Hm-t-infected groups are similar. We have revised the Results section (*Hm-oscar* impairs dosage compensation in male embryos, lines 200-212), the Materials and methods section (Quantification of Z chromosome-linked genes, lines 454-456), and the figure legends to provide more precise information about this analysis.

Although we did not perform replicates for the RNA-seq comparisons, it is important to note that each RNA-seq sample contains 50-60 male/female individuals. We believe the results are still robust and clearly indicative of the trends we observe. This was further supported by the quantification of *Hmtpi* gene expression, which we have visualized in Figure 3e (and lines 210-212). As you noted, the expression patterns in Figure 3b (*GFP* injected) and Figure 3d (NSR) are not completely identical. This discrepancy may be due to the differences between

injection treatments and natural infections. Nevertheless, both treatments are consistent in showing that gene expressions on the Z chromosome (Chr01 and Chr15) are not upregulated.

We have also added more detailed statistical data for Figure 3 based on the Steel-Dwass tests. For Figure 3a-d, however, showing the statistical significance directly on the whisker plots would create excessive clutter due to the numerous combinations of chromosomes. Instead, we have provided the full statistical data in the supplementary data file. Furthermore, to support/strengthen our conclusion that Z-linked genes are highly expressed in _w_Hm-t-infected/*Hm-Oscar*-injected embryos, we have included expression data for the Z-linked gene *tpi*, along with statistical data, in the revised manuscript (Fig. 3e, lines 210-212).

> *In Figure 4: There are no statistics to support the conclusions presented here. Additionally, the data have gone through a normalization process, but it is difficult to follow exactly how this was done. The control conditions appear to always be normalized to 100 ("The expression levels of BmImpM in the Masc and Hm-Oscar/Oscar co-transfected cells were normalized by setting each Masc-transfected cell as 100"). I see two problems with this approach:*
>
> *(1) This has eliminated all of the natural variation in BmImpM expression, which is likely not always identical across cells/replicates.*
>
> *(2) How then was the percentage of BmImpM calculated for each of the experimental conditions? Was each replicate sample arbitrarily paired with a control sample? This can lead to very different outcomes depending on which samples are paired with each other. The most appropriate way to calculate the change between experimental and control would be to take the difference between every single sample (6 total, 3 control, 3 experimental) and the mean of the control group. The mean of the control can then be set at 100 as the authors like, but this also maintains the variability in the dataset and then eliminates the issue of arbitrary pairings. This approach would also then facilitate statistical comparisons which is currently missing.*

Thank you for your suggestion. As you pointed out in (1), the previous analysis did indeed eliminate the natural variation in *BmIMP-M* expression. In the revised manuscript and Figure 4, we have reanalyzed the data following your suggestion and have described the variation across replicates.

For (2), the data shown in the previous manuscript were normalized to 100 for each Masc-treated group. In doing so, each replicate sample was arbitrarily paired with a control sample from the same cell lot to account for variations that might occur due to differences in cell lots. However, following your recommendation, we have revised the figure to set the average of the *Hm-masc* treated group to 100, rather than using arbitrary pairings. More detailed normalization procedures have been provided in the section 'Transfection assays and quantification of *BmIMP*' (lines 483-520). Additionally, we have provided more detailed background information on the assay system in lines 218-223. Although we did not observe statistical significance based on the Steel-Dwass test, likely due to the limited number of replicates, the differences in *IMP* gene expression between the *Masc*-treated and *Masc*&*Hm-oscar*-treated groups remain evident.

> ***Recommendations for the authors:***
>
> ***Reviewer #1 (Recommendations for the authors):***
>
> *Line 38: change to: 'Wolbachia are maternally transmitted'.*

Revised accordingly (line 38).

> *Line 69: remove 'seemingly'.*

Revised accordingly (line 69).

> *Paragraph starting line 123: I don't think this is so clear to a reader who is not familiar with the work and system. It would be helpful to more clearly explain that candidate male-killing genes from Wolbachia that infect Homona were inserted into Drosophila melanogaster, and that their expression was then induced, with interesting patterns (and that it can be a bit difficult to interpret the transgenic expression of genes from a moth male-killer that are inserted into a fly). Also, the sentence about the combined action of cifA and cifB in Drosophila cytoplasmic incompatibility is also confusing to a non-expert. I would suggest removing it.*

Thank you for your suggestion. We have revised the paragraph (lines 124-139) to provide clearer background information, making it easier for non-experts to follow. We have also removed the sentence regarding the combined effect of *cifA* and *cifB* to improve the flow and overall clarity.

> *Line 170: what is the explanation for the male-biased sex ratio instead of 50-50?*

The male-biased sex ratio occurs because MK happens during embryogenesis. Unhatched embryos include males that were killed by *Wolbachia*/*Oscar*, resulting in a higher proportion of unhatched male embryos. Conversely, the hatched individuals display a female bias, as most of the males were eliminated during embryogenesis. Thus, the unhatched embryos are more male-biased, while the hatched individuals are more female-biased in the *Hm-oscar*/_w_Hm-t treated groups. We have revised the section "Males are killed mainly at the embryonic stage" (lines 170-186) to include more detailed information explaining this phenomenon.

> *Line 190: please explain what are the Z chromosomes in Bombyx and Homona and Lepidoptera in general (chromosomes 1 and 15?), as this is not so clear for a non-expert.*

Thank you for your suggestion. I have revised the section (lines 200-212) to include more precise background information about the chromosome constitutions in lines 202-204 as follows:

"Unlike other lepidopteran species, Tortricidae, including *H. magnanima*, generally possess a large Z chromosome that is homologous to *B. mori* chromosomes 1 (Z) and 15 (autosome)."

> *Line 222: please explain oscar diversity and classification in more detail, as this is not so clear for a non-expert.*

Thank you for your suggestion. We have revised the sentences to provide clearer background information on the diversity of *oscar* genes (lines 255-264).

> *Figure 4: I found this difficult to follow. Why are there 2 rows (HmOscar and Oscar)? Does oscar here refer to oscar from Ostrinia? I am also a bit confused about the baseline control of Masc in these cell lines. If I understand Lepidoptera sex determination, then these cell lines are expressing high levels of female-specific piRNAs that suppress Masc. How specific are these piRNAs (i.e. do Bombyx piRNAs suppress Mascs from other Lepidoptera)? How much extra Masc will override endogenous piRNA? Information is lost by setting Masc expression to 100% in each separate comparison.*

Yes, the *Oscar* indicates the _w_Fur-encoded *oscar* (*Oscar* from *Ostrinia*) that was tested to compare function with the *Homona*-derived *Hm-oscar* gene. In addition, following the reviewer's suggestions, we have revised the figure and included more detailed information on how we adjusted the expressions in the M&M section.

A previous study (Shoji et al., 2017, *RNA* 23:86–97) demonstrated that the *Fem* piRNA (29 bp) in *Bombyx mori* requires a 17 bp complementary sequence from its 5' region for its function. However, in species other than *B. mori*, no significant homology (i.e., over 17 bp matches) was found between the *B. mori Fem* piRNA and the *masc* genes analyzed in this study. Therefore, it is likely that the *Fem* piRNA expressed in BmN-4 cells is unable to suppress the masculinizing function driven by *masc* genes in other lepidopteran species. In addition, we did not quantify the levels of piRNA in this system, but the expression levels of *masc* are probably too high to be suppressed.

> *Figure 4 legend: spelling of Spodoptera.*

Revised accordingly.

> ***Reviewer #2 (Recommendations for the authors):***
>
> *In Figure 2, what is the dsx splicing type for the hatched male in the Hm-oscar-injected group and the wHm-t infected line? Dsx-F or dsx-M?*

Thank you for your suggestion. Unfortunately, we have not tested splicing in the hatched male neonates (1st instar larvae), partly due to difficulties in obtaining sufficient material for RNA extraction. Based on the previous publication in the *Ostrinia* system, where *Oscar*-bearing _w_Sca induces MK, the hatched males (ZZ) exhibit female type *dsx* as observed in the male embryos (Herran et al., 2022). The hatched *Homona* males may show double bands for *dsx-M* and *dsx-F* as observed in this study.

> *The size of the markers (in kilobase pairs) should be indicated in Figure 2.*

We have accordingly included the marker information in the revised Figure 2b and the figure legends.

> *In Figure 3, could the authors identify which genes exhibit higher expression levels in the Hm-oscar-injected group and the wHm-t infected line? Could they provide hints for the possible mechanism of male-killing?*

In the RNA-seq data shown in Figure 3a-d, we observed that both the *Hm-oscar*-injected and _w_Hm-infected groups generally exhibited upregulated expression of Z-linked genes. Rather than the upregulation or downregulation of a specific gene, we consider that global upregulation of Z-linked genes, caused by improper dosage compensation, is lethal for males. The Z chromosome contains various genes involved in key biological processes such as endocrine function and detoxification, and disruption of these processes may contribute to male lethality. Additionally, in this revised manuscript, we have provided more detailed information on the expression level of the Z-linked gene *tpi*. We have also discussed the potential mechanisms of MK in the Discussion section (lines 245-254).

> *The format of the references should be consistent. Gene and species names should be italicized.*

We have accordingly formatted.

> ***Reviewer #3 (Recommendations for the authors):***
>
> *The authors use the term "upstream" (e.g., Oscar suppressed the function of masculinizer, the upstream male sex determinant...), which was sometimes confusing. In many cases, it reads as though the masculinizer was upstream of oscar, but what I think the authors are trying to convey is that masculinizer is a primary sex-determining factor.*

Thank you for your suggestion. We have accordingly revised the term.

> *Line 101: which insect is wFur from?*

It is from *Ostrinia furnacalis* - line 104 has been revised.

> *Figure 1: it would be helpful to indicate the statistical results on the figure.*

Accordingly, we have added statistical data (binominal test) for Figure 1. The data for the Steel-Dwass test have been included in the supplementary data.

> *Figure 2b: please label the ladder on the gel.*

Thank you for your suggestion. We have accordingly labeled the DNA ladder on the gel.

https://doi.org/10.7554/eLife.101101.2.sa0