

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

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Abstract

Wolbachia are the 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 upstream 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.

eLife assessment

This **valuable** study, which implicates a specific *Wolbachia* gene in driving the male-killing phenotype in a moth, contributes to a growing body of literature from the authors in which they have nicely teased apart the loci responsible for male killing across diverse insects. **Solid** evidence supports the conclusions, though improvements to the statistical analysis for certain assays would strengthen the inferences further.

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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 seemingly 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 and Ishikawa, 2012).

More recently, a protein produced in *Wolbachia* wFur strain named Oscar (Osgoroshi protein containing CifB C-terminus-like domain and many Ankyrin Repeats; Osgoroshi translates to male killing in Japanese) was shown to recapitulate the *Wolbachia*-induced MK phenotype in *Ostrinia* (Katsuma et al., 2022). The Oscar protein degrades and interacts with 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 protein (Arai et al., 2023b; 2024a). Furthermore, Oscar homologs are evolutionarily dynamic, with highly variable sequences and structures (classified as Type I and Type II Oscar) (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 (approximately 300 aa) 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, 2020, 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 an MK-associated prophage region that underlies the evolutionary transition from a non-MK *Wolbachia* (wHm-c) to an MK *Wolbachia* (wHm-t) in the tea tortrix moth *Homona magnanima* (Tortricidae) (Arai et al., 2023b; Arai et al., 2024b). The MK-associated prophage element encodes four *wmk* homologs (*wmk*-1 to *wmk*-4) as well as an *oscar* homolog (Hm-Oscar, 1181 aa), which differs significantly in sequence length and structure from the wFur-encoded Oscar (1830 aa). 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 (Arai et al., 2023b), suggesting that their combined action is similar to that observed in *Wolbachia*-induced CI (CifA and CifB, Beckmann et al., 2017; LePage et al., 2017). However, 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 ($85.6\% \pm 11.1\%$; 42 males and 218 females in total, 15 replicates), which was in sharp contrast with the ratios observed in the GFP-injected ($50.9\% \pm 2.08\%$; 126 females and 123 males in total, 10 replicates) and non-injected (NSR) ($48.6\% \pm 3.08\%$; 201 females and 215

males in total, 9 replicates) groups ($P = 0.001$ and 0.002 , respectively, Steel–Dwass test, **Fig. 1** [↗](#)). Compared with the GFP-injected group, the sex ratio was not biased by the overexpression of *wmk-1* ($48.9\% \pm 7.27\%$, 6 replicates, $P = 0.999$), *wmk-2* ($46.8\% \pm 4.77\%$, 6 replicates, $P = 0.810$), *wmk-3* ($46.7\% \pm 7.91\%$, 5 replicates, $P = 0.999$), and *wmk-4* ($47.1\% \pm 3.47\%$, 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 ($42.1\% \pm 7.67\%$, 5 replicates, $P = 0.704$). Similarly, the dual expression of the tandemly arrayed *wmk-1* and *wmk-2* did not bias the sex ratio ($50.6\% \pm 2.82\%$, 5 replicates) compared with the value in the GFP-injected group ($P = 1.000$).

Males are killed mainly during 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. 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, that of unhatched mature embryos was male-biased (18 females and 38 males, $P = 0.005$), suggesting that males died mainly during the embryonic stage. Likewise, the sex ratios of hatched larvae (34 females and 6 males) and unhatched embryos (27 females and 47 males) of the MK *wHm-t* infected line (W^{T12}) were also female- and male-biased, respectively ($P = 0.013$ and 0 , respectively). 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, *wHm-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 *wHm-t*-infected and *Hm-oscar*-expressed embryos induced female-type *dsx* splicing regardless of the presence or absence of W chromatin (**Fig. 2b** [↗](#)).

Hm-Oscar impairs dosage compensation in male embryos

RNA-seq analysis revealed that, in *Hm-oscar*-injected embryos, Z-linked genes (homologs on the *Bombyx 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. (20-23a), high levels of Z-linked gene expression were also observed in *wHm-t*-infected males, but not in NSR males (**Figure 3c,d** [↗](#)).

Hm-Oscar suppresses the masculinizing functions of lepidopteran Masc

To confirm whether Hm-Oscar suppresses the functions of the upstream male sex determinant Masc, their interactions were assessed by transfection using BmN-4 cells, as described in Katsuma et al. (2022) [↗](#). BmN-4 cells, which are derived from the female ovaries of *B. mori* (Grace, 1967 [↗](#)), exhibit a female-type default sex determination (Kiuchi et al., 2014 [↗](#)). 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 *Hm-masc*-inserted pIZ/V5-His vector exhibited the male-type sex determination, as evidenced by the increased expression levels of the male-specific

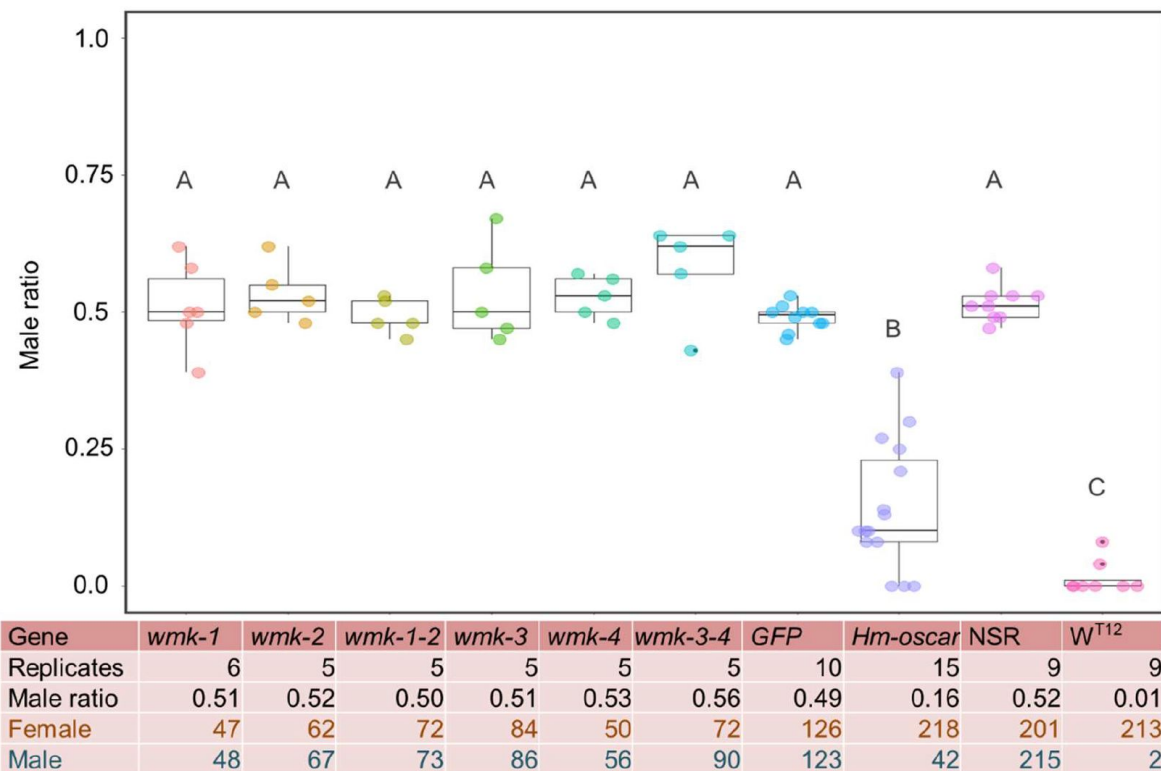


Figure 1.

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

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 *wHm-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.

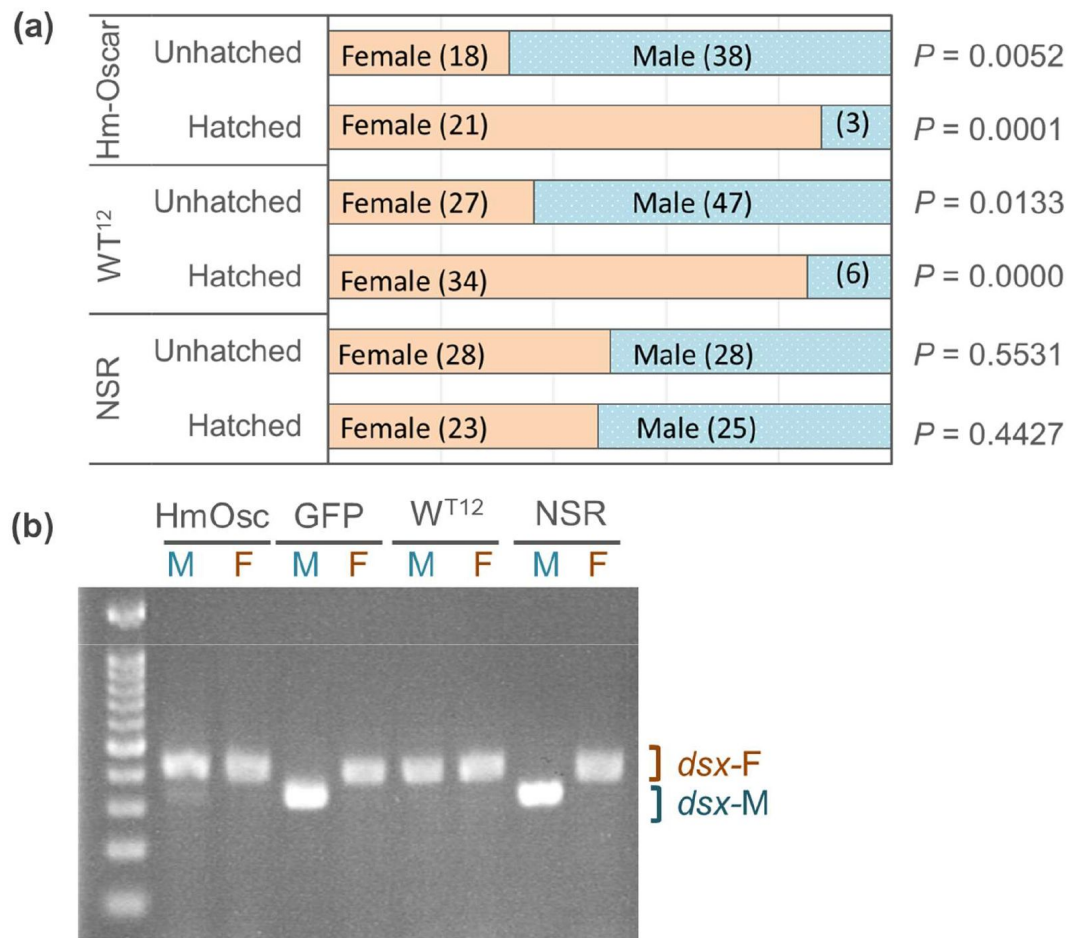


Figure 2.

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, *wHm-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; WT¹², *wHm-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.

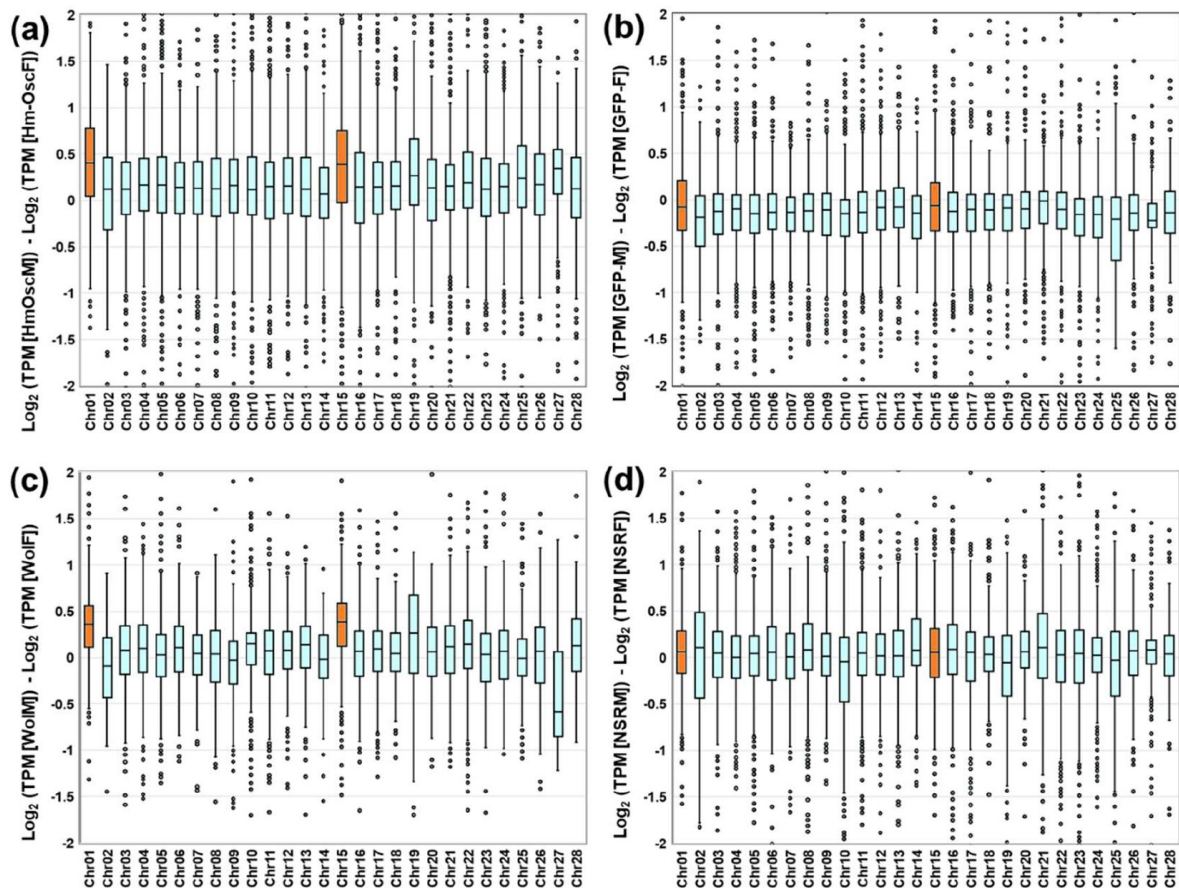


Figure 3.

***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 [\[1\]](#)).

splicing of the sex-determining gene *BmImp^M* (Fig. 4). Furthermore, the masculinizing function of *Hm-masc* was suppressed by co-transfecting the *Hm-masc/Hm-oscar*-inserted pIZ/V5-His vectors, as manifested by the low expression levels of *BmImp^M*. While type I *oscar* of the MK wFur strain also suppressed *Hm-masc*, the function of this gene was less active than that of *Hm-oscar*. *Hm-oscar* also suppressed the masculinizing function of *masc* genes derived from various lepidopteran insects (i.e., *Ostrinia furnacalis*, *Spodoptera frugiperda*, *Bombyx mori*, *Trichoplusia ni*, and *Papilio machaon*), suggesting its broad spectrum of action 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 wHm-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 wHm-t-induced MK function in *H. magnanima*. The *Wolbachia*-encoded Oscar homologs identified so far are classified as type I and type II (Arai et al., 2024a). Although long ankyrin repeat sequences are postulated to be critical for the function of wFur-encoded Oscar (1830 aa, type I) (Katsuma et al., 2022a), our study revealed that the functions of Hm-Oscar (1181 aa, type II), which encodes fewer ankyrin repeats, were comparable with those of Oscar carried by wFur. Interestingly, type II Oscar is also present in the feminizing *Wolbachia* wFem in butterflies belonging to genus *Eurema* (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., 2022b). 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 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 wFur 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. An intense evolutionary arms race between *Wolbachia* and their hosts may have increased the diversity of MK-inducing genes in the *Wolbachia* genome.

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 wHm-t-encoded Hm-Oscar suppresses the function of Hm-Masc in *H. magnanima* more efficiently than the wFur-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 the expression of the MK phenotype. For example, the type I Oscar-bearing wCauA

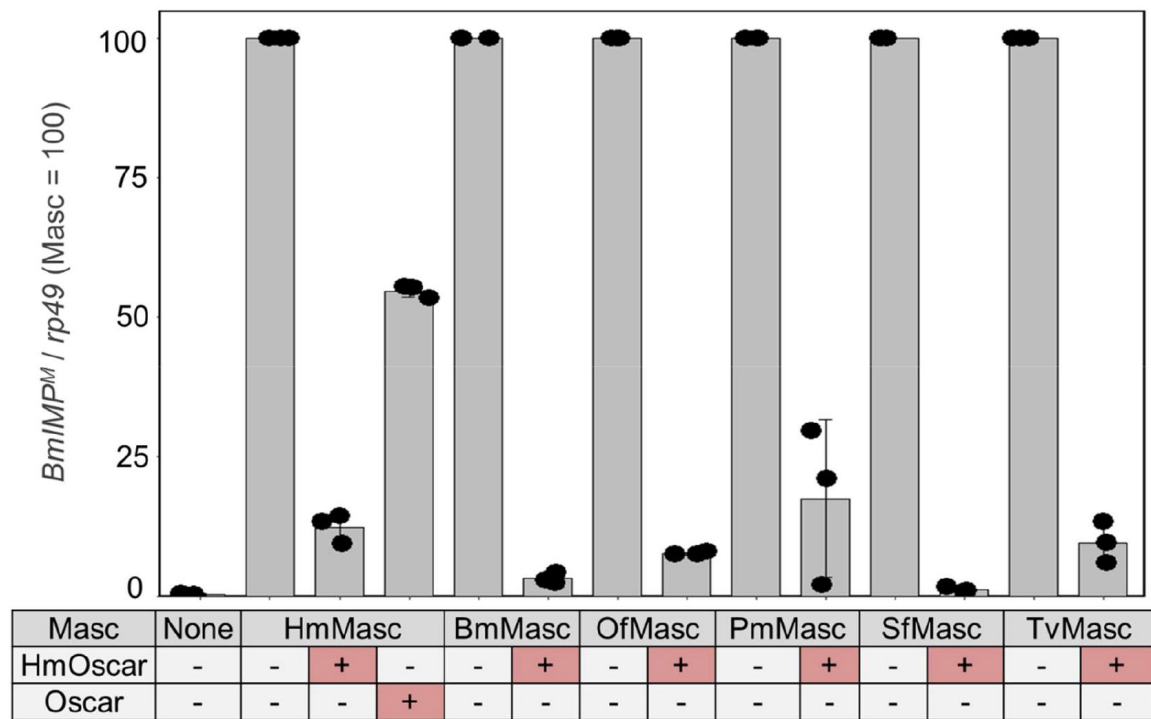


Figure 4.

Hm-Oscar suppressed the masculinizing function of lepidopteran Masc

Relative expression levels of the male-specific *BmImp^M* variant in transfected BmN-4 cells. The expression of *BmImp^M* was normalized using the housekeeping gene *rp49*. The expression levels of *BmImp^M* in the *Masc* and *Hm-Oscar/Oscar* co-transfected cells were normalized by setting each *Masc*-transfected cell 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, *Spodoptra frugiperda*; and Tv, *Trilocha varians*.

strain did not induce MK in *Ephesia (Cadra) cautella* collected around 2000, although it did induce MK when transferred to the closely related host *Ephesia kuehniella* (Sasaki et al., 2002 [↗](#)). However, an MK phenotype, presumably induced by *wCauA*, was recorded in *E. cautella* around the 1970s (Takahashi & Kuwahara, 1970 [↗](#)). These findings suggested the emergence of suppressor(s) against MK induced by Oscar-bearing *wCauA* in *E. cautella*. 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 [↗](#), 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) [\[link\]](#) 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) [\[link\]](#) (i.e., pIZ-F-T7: 5'-TAATACGACTCACTATAGGGAGACAGTTGAACAGCATCTGTTC-3' and pIZ-R: 5'-GACAATACAACTAAGATTAGTCAG-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 (Nikon, 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 *wHm-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) [\[1\]](#). 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) [\[2\]](#). In brief, 0.4 times the volume of UltraPure distilled water (Invitrogen) was added to the ISOGEN II homogenates, which were centrifuged at $12000 \times g$ 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 $17,900 \times g$ 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) [\[2\]](#). 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'-TGCTAAAGTGAAAACGCCGAGGAGCC-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.

RNA-seq-based 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) [\[3\]](#) and Arai et al. (2023a) [\[2\]](#). A total of 1.0 µg of the total RNA extracted from *Hm-oscar* or *GFP*-overexpressed mature embryos (108 hpo) 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) [\[4\]](#). The trimmed reads were mapped to the previously assembled transcriptome database for *H. magnanima* (Arai et al., 2023a) [\[2\]](#) using Kallisto (Bray et al., 2016) [\[5\]](#) to generate the normalized read count data (transcripts per million, TPM).

The binary logarithms of the TPM differences between males and females belonging to each *H. magnanima* group (i.e., *Oscar*/GFP-expressed) were calculated to assess the fold-changes in gene expression levels between sexes. As described in Arai et al. (2023a) [\[2\]](#), the transcriptome data of *H. magnanima* were annotated using the *B. mori* gene sets obtained from KAIKOBASE (<https://kaikobase.dna.affrc.go.jp>) [\[6\]](#). The binary logarithms of the TPM differences between males and

females in the *B. mori* chromosomes 1 to 28 were plotted. 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) [\[1\]](#).

Transfection assays and quantification of *BmIMP*

The coding sequence of *Hm-masc* 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_HindIII F: 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 *wFur* (Katsuma et al. 2022 [\[2\]](#)) was also cloned into the pIZ/V5-His vector using the KpnI and NotI enzymes (TaKaRa).

To verify the masculinizing function of *Hm-masc*, BmN-4 cells (4×10^5 cells per dish, diameter 35 mm) were transfected with 1 µg of plasmid DNA (pIZ/V5-His having *Hm-masc*) using FuGENE HD (Promega, WI, USA), as described in Katsuma et al. (2022) [\[2\]](#). To clarify whether *Hm-oscar* suppressed the function of *Hm-masc*, 1 µg of plasmid DNA (pIZ/V5-His bearing either *Hm-masc* or *Hm-oscar*) was co-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*, which is involved in male-specific sex determination cascades, using primers rp49_F: 5'-CCCAACATTGGTTACGGTTC-3' and rp49_R: 5'-GCTCTTTCCACGATCAGCTT-3'; BmIMP_F: 5'-ATGCGGGAAGAAGGTTTATG-3' and BmIMP_R: 5'-

TCATCCGCCTCAGACGATTG-3', as described in Fukui et al. (2015) [\[3\]](#). Further interactions between Hm-Oscar and Masc proteins derived from lepidopteran insects [i.e., *Trilocha varians* Masc (TvMasc), *Spodoptra frugiperda* Masc (SfMasc), *B. mori* Masc (BmMasc), *O. furnacalis* Masc (OfMasc), and *Papilio machaon* Masc (PmMasc)] (Katsuma et al., 2022a) were assessed using the same procedures.

Quantification and statistical analysis

The number of surviving *H. magnanima* injected with either *Hm-oscar*, *wmks*, 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.

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 supporting files. Further information and requests for resources and reagents are accessible from the lead contact, Hiroshi Arai (dazai39papilio@gmail.com/HARai@liverpool.ac.uk).

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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.

Declaration of interests

The authors declare no competing interests.

Inclusion and diversity

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

Benefit-Sharing

The wHm-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.

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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:

- (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 silkworm 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 (see recommendations).

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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.

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.

(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.

(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*.

(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.

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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) They 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 the use of a "masculinization" assay in Lepidopteran cell lines to determine the impact of interactions between specific masc and oscar homologs.

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.

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?

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?

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.

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.

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.

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.

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