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.

Keywords: symbiosis, *Wolbachia*, Oscar, Wmk, microinjection

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* *w*Fur 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 *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* (*w*Hm-c) to an 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 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 *w*Fur-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% ± 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% ± 2.08%; 126 females and 123 males in total, 10 replicates) and non-injected (NSR) (48.6% ± 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% ± 7.27%, 6 replicates, P = 0.999), *wmk*-2 (46.8% ± 4.77%, 6 replicates, P = 0.810), *wmk*-3 (46.7% ± 7.91%, 5 replicates, P = 0.999), and *wmk*-4 (47.1% ± 3.47%, 5 replicates, P = 0.773). Although the dual expression of *wmk*-3 and *wm*k-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% ± 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% ± 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 *w*Hm-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, *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**).

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 *w*Hm-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 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 *w*Fur 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 *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*. 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 *w*Fur-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 *w*Fur. Interestingly, type II Oscar is also present in the feminizing *Wolbachia* *w*Fem 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 *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. 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 *w*Hm-t-encoded Hm-Oscar suppresses the function of Hm-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 the expression of the MK phenotype. For example, the type I Oscar-bearing *w*CauA strain 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). However, an MK phenotype, presumably induced by *w*CauA, 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 *w*CauA 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) 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′-

321 GACAATACAAACTAAGATTTAGTCAG-3′) under the following PCR conditions: 20
322 cycles at 98°C for 10 s, 62°C for 5 s, and 68°C for 15 s. The amplicons were purified
323 using the QIAquick PCR purification kit (Qiagen, Hilden, Germany), and 100–200 ng
324 DNA was used for mRNA synthesis. The capped mRNA (cRNA) with poly(A) tail was
325 synthesized using the mMESSAGE mMACHINE® T7 Ultra kit (Invitrogen, MA, USA)
326 following the manufacturer's protocol with some modifications. In brief, the assembled
327 transcription reaction (10 μL of T7 2X NTP/ARCA, 2 μL of 10X T7 reaction buffer, 2 μL
328 of T7 enzyme mix, and 6 μL of linear DNA template diluted with nuclease-free water)
329 was incubated at 37°C for at least 10 h to maximize RNA yields. After poly(A) tailing,
330 cRNA was purified using ISOGEN II (Nippongene, Tokyo, Japan) and dissolved in up
331 to 10 μL of nuclease-free water to achieve an RNA concentration of approximately
332 1500–2000 ng/μL. The synthesized cRNA was preserved at −80°C until further use.
333
334 **(ii) Preparation of *H. magnanima* eggs**
335 A total of 15 males and 10 females of *H. magnanima* were mated in a plastic box (30
336 cm × 20 cm × 5 cm) for 3–4 days. The collection of egg masses began the day after
337 oviposition was confirmed. In brief, newly oviposited egg masses were collected at 30-
338 min intervals during the dark period using red light (which the moths cannot perceive).
339 Females started to oviposit eggs at least 4 h into the dark period and, within less than
340 30 min post oviposition (mpo), the egg masses were collected. The collection lasted
341 until the start of the light period. The egg masses were then subjected to microinjection
342 assays.
343
344 **(iii) Inoculum preparation, microinjection, and maintenance of the injected**
345 **embryos**
346 A glass needle for microinjection was prepared using glass capillary GD-1 (Narishige,
347 Tokyo, Japan) with a PC-10 puller (Narishige). The glass capillaries were pulled at two
348 temperatures (first stage: 75°C, second stage: 65°C) using two heavy weights and one
349 light weight. The movement position during the second heating stage was set to 3 mm
350 (range 1–10 mm).
351 The cRNA solution was diluted in a buffer (100 mM potassium acetate, 2 mM
352 magnesium acetate, and 30 mM HEPES-KOH; at pH 7.4) containing 0.2% (W/V)
353 Brilliant Blue FCF (Wako, Osaka, Japan) to obtain an RNA concentration of 1000 ng/μL.
354 Approximately 1–4 μL of dye-containing mRNA solution was aspirated into the glass
355 needles (capillaries), and the edge of each needle was ground using Micro Grinder
356 EG-402 (Narishige) at an angle of 20 degrees for 2 s.
357 The fresh egg masses of *H. magnanima* (collected at less than 30 mpo as
358 described above) were put on a double-sided sticky tape (15 mm × 5 m T4612, Nitoms,
359 Tokyo, Japan) and placed on glass slides. Under a Nikon SMZ1270 microscope (Nicon,
360 Tokyo, Japan), the RNA solution (30–100 fL) was injected into individual eggs using
361 an microinjector IM-400 (Narishige) (balance pressure set at 0.010–0.030; injection
362 pressure set at 0.050-0.100) and a QP-3JOY-2R electric micromanipulator
363 (MicroSupport, Shizuoka, Shizuoka).
364 The injected egg masses were maintained in a 90-mm plastic Petri dish fitted
365 with a slightly wet filter paper. As high humidity interfered with the development of the
366 embryos, the injected eggs (egg mass) were first maintained at a relative humidity of
367 30%–50% (0–3 days post injection) and then at a higher humidity until hatching (4–6
368 dpo, 60% relative humidity). The hatched larvae were reared separately with 1/2

369 ounces of SilkMate 2S (Nosan Co.) until eclosion. The adult *H. magnanima* moths that
370 emerged from the cRNA-injected embryos were sexed based on their external
371 morphology.
372
373 **Sexing of embryos/neonates and RNA extraction**
374 To verify the effect of the transient expression of *Hm-oscar* on sex determination in *H.*
375 *magnanima*, *Hm-oscar*/GFP-expressed, non-injected, and *w*Hm-t-infected mature
376 embryos showing black head capsule (1 day before hatching, 5–6 days post
377 oviposition, dpo) were dissected on glass slides using forceps, as described in Arai et
378 al. (2022). Their Malpighian tubules were fixed with methanol/acetic acid (50% v/v) and
379 stained with lactic acetic orcein for W chromatin observations. The remaining tissues
380 not used for sexing were stored in ISOGEN II (Nippon Gene) at −80°C until subsequent
381 extraction. In total, 12 males or females (confirmed based on the presence or absence
382 of W chromatin) were pooled and homogenized in ISOGEN II to extract RNA as
383 described in Arai et al. (2023a). In brief, 0.4 times the volume of UltraPure distilled
384 water (Invitrogen) was added to the ISOGEN II homogenates, which were centrifuged
385 at 12000 × *g* for 15 min at 4°C to pellet proteins and DNAs. The resulting supernatant
386 was mixed with the same volume of isopropanol to precipitate RNAs; then, the resulting
387 solutions were transferred to EconoSpin columns (Epoch Life Science) and centrifuged
388 at 17,900 × *g* and 4°C for 2 min. The RNAs captured in the column were washed twice
389 with 80% ethanol and eluted in 20 μL of UltraPure distilled water (Invitrogen).
390
391 ***Hmdsx* detection**
392 Sex-specific *dsx* splicing variants of *H. magnanima* were detected as described in Arai
393 et al. (2023a). In brief, total RNA (100–300 ng) extracted from sex-determined mature
394 embryos was reverse-transcribed using PrimeScript™ II Reverse Transcriptase
395 (TaKaRa, Shiga, Japan) at 30°C for 10 min, 45°C for 60 min, and 70°C for 15 min.
396 Then, cDNA was amplified using KOD-FX Neo (Toyobo Co., Ltd.) with the following
397 two primers: Hmdsx_long3F (5′-TGCCTAAAGTGAAAACGCCGAGGAGCC-3′) and
398 Hmdsx_Mrev (5′-TGGAGGTCTCTTTTCATCCGG-3′). The PCR conditions used were
399 as follows: 94°C for 2 min, followed by 45 cycles of 98°C for 10 s, 66°C for 30 s, and
400 68°C for 30 s. The amplicons were subjected to electrophoresis on 2.0% agarose Tris-
401 borate-EDTA buffer (89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA) gels.
402
403 **RNA-seq-based quantification of Z chromosome-linked genes**
404 The effects on dosage compensation were assessed by measuring differences in gene
405 expression, as described in Fukui et al. (2015) and Arai et al. (2023a). A total of 1.0 μg
406 of the total RNA extracted from *Hm-oscar* or *GFP*-overexpressed mature embryos (108
407 hpo) was used to prepare mRNA-seq libraries via the NEBNext Poly (A) mRNA
408 Magnetic Isolation Module (New England Biolabs) and the NEBNext Ultra II RNA
409 Library Prep kit for Illumina (New England Biolabs) following the manufacturer's
410 protocol. The adaptor sequences and low-quality reads (Qscore <20) were removed
411 from the generated sequence data [150 bp paired-end (PE150)] using Trimmomatic
412 (Bolger et al., 2014). The trimmed reads were mapped to the previously assembled
413 transcriptome database for *H. magnanima* (Arai et al., 2023a) using Kallisto (Bray et
414 al., 2016) to generate the normalized read count data (transcripts per million, TPM).
415 The binary logarithms of the TPM differences between males and females
416 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), 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. The expression data of the *w*Hm-t-infected and non-infected groups were also calculated based on the transcriptome data included in Arai et al. (2023a).

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_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 *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). 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′-ATGCGGGAAGAAGGTTTTATG-3′ and BmIMP_R: 5′-TCATCCCGCCTCAGACGATTG-3′, as described in Fukui et al. (2015). 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 *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.

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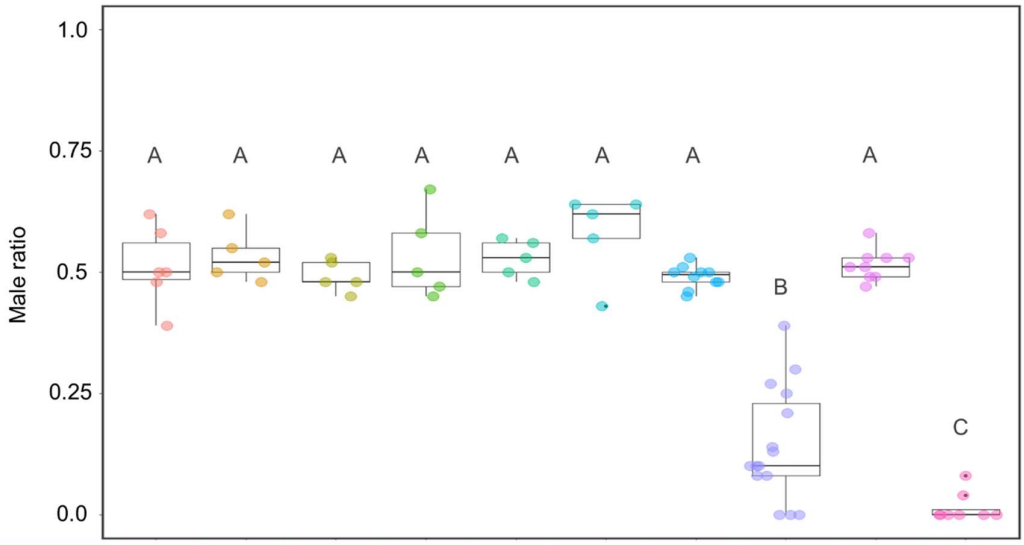
672 **Figures and figure legends**



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

673
674 **Figure 1 Transient expressions of the *Hm-oscar* gene resulted in female-biased**
675 **sex ratios**
676 Male ratio of adult progeny obtained from cRNA-injected groups (*wmk*-1, *wmk*-2, T2A-
677 brigged *wmk*-1 and *wmk*-2, *wmk*-3, *wmk*-4, T2A-brigged *wmk*-3 and *wmk*-4, GFP, and
678 *Hm-oscar*; n = 5–15 independent replicates using different egg masses), the non-
679 injected NSR line, and the MK *w*Hm-t-positive W^{T12} line. The total numbers of adult
680 females and males are shown at the bottom. Different letters indicate significant
681 differences (Steel–Dwass test, $P < 0.05$). The dot plots show all data points individually.
682

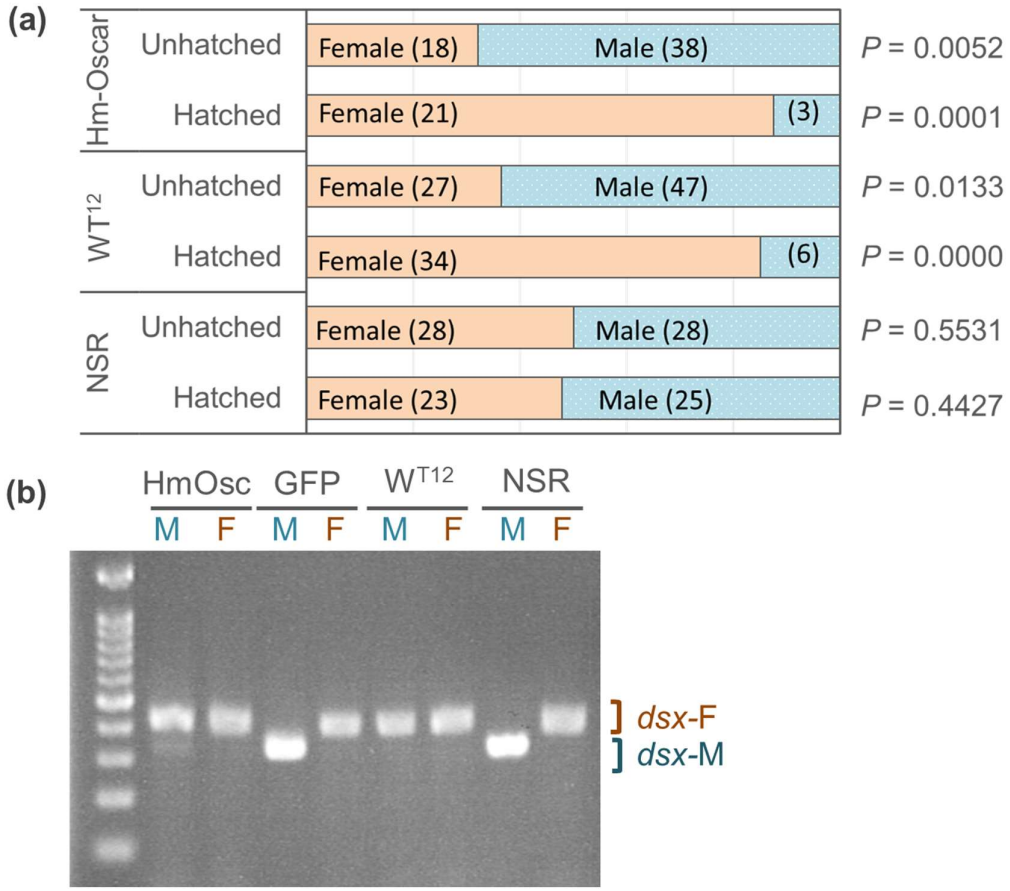


Figure 2 Hm-Oscar induced lethality in male embryos with the female-type sex determination

(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.

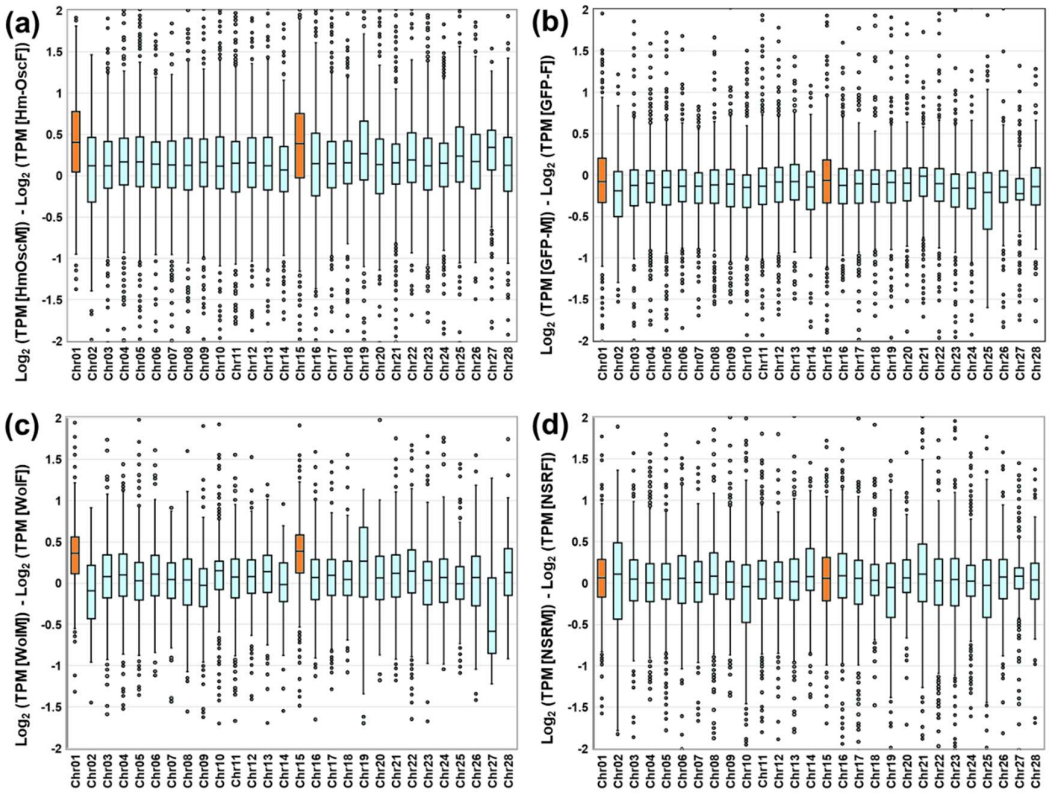


Figure 3 *Hm-oscar*-overexpressed male embryos showed higher levels of Z-linked gene expression

(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).

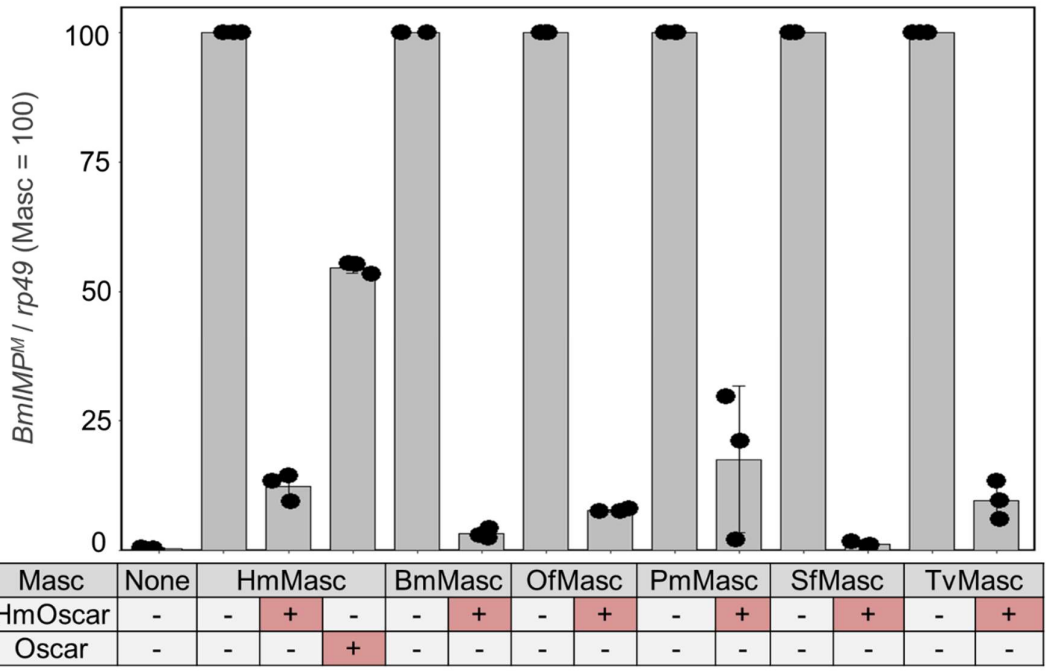


Figure 4 Hm-Oscar suppressed the masculinizing function of lepidopteran Masc Relative expression levels of the male-specific *BmImp*^{M} variant in transfected BmN-4 cells. The expression of *BmImp*^{M} was normalized using the housekeeping gene *rp49*. The expression levels of *BmImp*^{M} in the *Masc* 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.*