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Feminisation due to *Wolbachia* in *Cotesia vestalis* (Haliday), a parasitoid of the Diamond back moth *Plutella xylostella* (Linnaeus)

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ABSTRACT

The endosymbiotic bacteria *Wolbachia,* associated with a number of hymenopteran parasitoids are known to play role in the metabolism, physiology and reproduction of their hosts. The impact on female progeny due to *Wolbachia* infection in the braconid *Cotesia vestalis* was investigated in the different geographic populations of the parasitoid. The populations cured of *Wolbachia* recorded a reduction in male progeny compared to those infected. The sex ratio skewed towards males in the *Wolbachia* eliminated populations, altered towards higher females, when there was infection. There was 36.6% increase in female progeny over the males. The exploitation of *Wolbachia* for the biological manipulations of the parasitoid for effective pest management is discussed.

Key Words: *Cotesia vestalis*, endosymbiont, fitness benefits, *Wolbachia.*

Symbiotic bacteria have been reported to be associated with a number of parasitoid species, and are known inflict various types of metabolic, physiological and reproductive alterations, with the sex regulators bacteria being the most frequent ones. Among the sex regulators, *Wolbachia* infects a large number of species including parasitic hymenoptera. About 2/3 of all insect species are infected with *Wolbachia* (Werren *et al*., 2000). To date *Wolbachia* have been detected in 31 genera and 70 species of parasitic hymenoptera, as well as three dipeteran parasitoids, 26% of parasitoid wasps are reported to have *Wolbachia* (Iturbe *et al*., 2007). *Wolbachia* spp. is maternally inherited obligate intracellular bacteria belonging to the α proteobacteria. The bacteria infect the reproductive tissues (ovaries and testes) of arthropods and are transmitted through the egg cytoplasm and alter reproduction in their hosts. *Wolbachia* arthropod relationships have variously been described as mutualistic (Girin *et al*., 1995, parasitic (Werren *et al.,* 1995), pathogenic (Min *et al*.,1997) and symbiotic (James *et al*., 2000). In arthropods, they have been implicated in several host reproductive modifications, including cytoplasmic incompatability, parthenogenesis, feminisation and male killing (Werren *et al.,*2008). These symbionts are the reproductive manipulators that promote their own spread in (*SRINIVASA MURTHY et al.)*a population by encouraging the production of female progeny or reducing reproduction of uninfected females. These have been investigated for their potential use to host control because of their ability to modulate sex ratio *Cotesia vestalis (Haliday) (=Cotesia plutellae* (Kurdjumov) (Hymenoptera: Braconidae), a solitary larval endoparasitoid, is one of the most important biological control agents of the diamondback moth, *Plutella xylostella* (Linnaeus) (Lepidoptera: Plutellidae), regarded as the most significant pest of *Brassica* crops. Parasitoids have developed a natural arsenal and a number of physiological mechanisms to enable them to successfully colonise the host and regulate host development to their own benefit. One of these is through their association with symbionts. Obligate symbionts are required for the successful parasitism and suppression of the host immune system, as well as for inducing physiological alterations in the parasitized host (Consoli *et al*., 2006). Molecular evidence for the presence of endosymbiotic bacteria Wolbachia in *Cotesia* populations has been well documented (Rattan *et al*., 2011). The impact of *Wolbachia* infection on the parasitoid *C. vestalis* was investigated to rationalise the use of the parasitoid in pest management programmes.

Seven populations of *C.vestalis* collected from different geographic locations of the country [Bangalore (Hoskote, Malur & Kolar), Bhubaneshwar, Varanasi, Salem, Shillong, Tirupathi and Hyderabad] were considered for the study. Individual cocoons of *C. vestalis* obtained from *P. xylostella* larvae collected from field-grown cauliflower plants. The colony of *P. xylostella* was maintained on potted mustard seedlings, *Brassica juncea* L. Czern for oviposition, in ventilated oviposition cages for the development of larval stages. Host larvae at early L3 stage were exposed to *C.vestalis* on mustard seedlings in ventilated cages and maintained on the plant until cocoon formation. Cocoons were collected and held in plastic cages until adult emergence. Adult wasps were fed on honey.

Ten adult parasitoids each from different populations were surface sterilised in a series of double distilled water and 70% ethanol washes, then were freeze-killed at -80°C and transferred to an Eppendoff tubes and homogenization was done by crushing the adult in 20µl of 5% Chelex 100 MB DNA extraction buffer (BIO-RAD) using a DNA free disposable polypropylene pestle (Walsh *et al*., 1991). This was followed by incubation for 3 h at 56°C and then at 100ºC for 10 min. Eight microliters of 2.5 mg/ml Proteinase K solution were added to the tubes. Solutions were incubated at 55 °C for 1 h, heated twice to 90 °C for 15 min, and centrifuged for 2 min at 14,000 rpm. The supernatant was refreshed by a 1 min 14,000 rpm centrifugation. The supernatant was collected and gently mixed with 0.2 volume of Na-acetate (3mM, pH5.2) and 2 volumes of 100% ethanol. After precipitation for 2 hours at -20 ^{0} C , the DNA was washed with Feminization due to *Wolbachia* 70% ethanol, air dried and finally re- suspended in 20 µl double distilled water.DNA sample of 0.3 µl was used for PCR assays.

A molecular diagnostic approach was adapted for the detection of *Wolbachia* infection, since *Wolbachia* cannot be cultured. The assay was based on PCR mediated amplification of and sequence determination of 16S rRNA gene. The presence of *Wolbachia* was verified by a PCR method based on the *Wolbachia* surface protein (wsp). Diagnostic PCR

using the *Wolbachia* specific primer set (forward:5′-CAT ACC TAT TCG AAG GGA TAG-3′; reverse: 5′-AGA TTC GAG TGAAAC CAA TTC-3′) was performed to determine the *Wolbachia* infection status of adults of the wasps. The PCR reaction was performed in a 500 µl PCR tube with a 25 µl reaction mixtures each containing 1 mM dNTPs mix (3 µl), 5 ng/ µl specific primer (5 µl), 2.0 U Taq Polymerase (MBI, Fermentas) and 2 µl of template DNA solution (30 ng) in Taq reaction buffer. The reaction was set in the Thermal Cycler (Biorad Laboratories). The temperature profile for *Wolbachia* specific PCR was a pre-denaturing step of 2 min at 94 °C, followed by 38 cycles of 30 s at 94 °C, 45 s at 55 °C, and 90 s at 72 °C, with a final extension step of 10 min at 72 °C. The amplified PCR- products were resolved by horizontal gel electrophoresis in 1.8 percent Agarose gel with a low range ladder (Fermentas Mass Ruler 1000bp), visualized under UV trans-illuminator and size of the amplified *Wolbachia* specific bands was estimated by comparison with a co-migrating molecular weight standard. The wsp gene fragments from *Wolbachia* bacteria in *C.vestalis were* sequenced.

To determine the role of *Wolbachia* in the fitness attributes of the parasitoid, curing of *Wolbachia* with antibiotic and feeding of *Wolbachia* to the populations free of *Wolbachia* was done. Heat and antibiotic treatments are both estimated methods of producing *Wolbachia* free individuals (Grenier *et al*., 2002). Antibiotic Tetracycline (0.02%) a potent inhibitor of DNA dependent RNA polymerase of bacteria was used to produce *Wolbachia* free hosts. Tetracycline treatment of adults was accomplished by introducing a solution of tetracycline (0.02 %) dissolved in 50% honey solution and fed to adult parasitoids. Feeding of the antibiotic was done for more than ten generations and each generation was checked for presence of *Wolbachia* by molecular methods until no detectable levels of wsp gene was amplified.

Wolbachia was isolated and pelleted from infected parasitoids. The protocol prescribed by Iturbe *et.al.,* (2011) was followed to obtain pure *Wolbachia.* Approximately 100 adults of the parasitoid were collected, surface sterilised for 3 minutes inb 70% ethanol followed by sterile water. The insects were homogenised using a 40 ml cold SPG buffer (218 mM Sucrose, 3.8mM KH2PO4, 7.2 mM KH2PO4, 4.9 mM L-Glutamate, pH 7.2). The extract was split in to 4 Falcon tubes containing another 20 ml SPG buffer each and centrifuged at 3200 X g for 15 minutes. The supernatant was subsequently filtered through syringe filters and *Wolbachia* were pelleted at 18,000 X g for 20 minutes in a Oakridge tube and re-suspended in 4 X 750 µl cold SPG Buffer in appendorf tubes. Intact *Wolabchia* were treated with 20 µl DNAseI for 30 min at 37^0C to remove host DNA contamination without disrupting the cells and resuspended in appendorf tubes. The pellet was fed to the cured population of *C.vestalis* by mixing the pellet with 50% honey and feeding. The feeding was done for over ten generations. Presence of *Wolbachia* was detected by assays for *wsp* genes. The PCR using *Wolbachia* specific primers for the wsp gene was performed to detect *Wolbachia* infection in the populations of *C.vestalis* from Bangalore, Bhubaneshwar, Hyderabad, Salem Shillong, Tirupathi, and Varanasi. All the populations revealed the presence of *Wolbachia* (Fig -1).

Infection with *Wolbachia* resulted in greater female progeny production. Observations indicated that the sex ratio that was skewed towards males in the population free of infection altered towards females when there was infection. There was 36.6% increase in female progeny over the males. (Table 1). *Wolbachia* can alter the normal pattern of sex determination in their host. The bacterium distorts host sex ratio via male killing, parthenogenesis induction or feminisation. *Wolbachia* cause genetic male embryos to develop phenotypically as functional females. *Wolbachia* induces feminisation by blocking the formation of androgenic glands, which produces the androgenic hormone responsible for male differentiation (Johanowicz *et al*., 1998).

Weeks *et al.,* (2002) indicated that the shift towards females will occur without the elevated mortality of males, if nuclear genes or meiotic drive genes are involved in the distortion. Sex reversion, changing genetic males in to functional neo females might occur due to re-allocation of maternal resources from dead male embryos to their sisters provides a direct physiological mechanism through which fitness compensation could favour male killing by cellular endosymbionts as in Harlequin beetle, *Acroeinus longimennus* (Zeh *et al.,* 2005). Sex ration distortion in transfected strain of Mediterranean flour moth *Ephestia kuehniella* was due to male killing (Hurst *et al.,* 2002., Fujii *et.al.,* 2001). The removal of *Wolbachia* resulted in the recovery of 1:1 sex ratio. In *Drosophila bifasciata,* the sex ratio distortion was a result of reduced male hatching rate compared to uninfected females, due to the arrest of male embryos during the stage of development (mitotic abnormalities occurring during blastoderm formation and gastrulation) and chromosome segregation (chromatin remodelling) defects within the spindle in male embryos. Histone-modifying enzymes or chromatin-remodeling complexes can be targeted to specific promoters by gene-specific or general transcription factors, *Wolbach.ia* may interfere with any of the transcription pathways that regulate some of these processes, Male killing is thought to benefit sibling females by eliminating competition. These illustrate the Feminization due to *Wolbachia*mutualism where both the host and bacteria are benefitted. Because of the higher production of female progeny the infected females are predicted to be more efficacious in pest control. The feasibility of such utilization depends heavily upon how the transmission of wolbachia and the genes being driven in to a population occurs (Iturbe *et al*., 2007). Maternally inherited endosymbionts spread through populations by increasing relative fitness of infected females. They achieve this by increasing the fecundity and or survival of infected females relative to uninfected females through metabolic processes (Douglas, 1994), thereby providing benefit to both the host and symbiont (in a mutualistic association).

The detection of *Wolbachia* in the parasitoid populations of *C.vestalis* may prove to be useful for biological manipulations of the parasitoid as possible transgene drivers. These bacteria can drive particular mtDNA haplotypes through populations and alter reproductive biology. They can be utilised as vectors for spreading desirable genetic modifications in pest populations or as microbial agents to enhance productivity of natural parasitoids (Saiful Islam, 2007). Infections can be manipulated by elimination, transfection or genetic modifications. However, effective exploitation of *Wolbachia* in pest management requires an in depth exploring of its role in insect biology and population dynamics.

Table 1 Impact of *Wolbachia* on sex ratio (percent females) in different populations of *C.vestalis*.

Sl.No	Population	Percentage of females		Sex ratio (Female : Male)*	
		Wolbachia fed	*Wolbachia* cured	*Wolbachia* fed	*Wolbachia* cured
1	Bhubaneshwar	68.9	55.86	1.79:1	1:1.45
2	Bangalore	70.9	60.60	1.65:1	1:1.41
3	Shillong	74.07	66.22	1.51:1	1:1.35
4	Tirupathi	74.07	58.17	1.69:1	1:1.35
5	Varanasi	70.42	61.72	1:62:1	1:1.42
6	Salem	72.99	68.44	1.44:1	1:1.37
7	Hyderabad	72.99	70.92	1.41:1	1:1.37
8	Control	68.3	71.42	1:1.4	1:1.2

* Mean of 10 replications

CD (P=0.01%) Populations (P) 16.4 NS
Treatments (T) 6 .6 Sig
P x T 2.9 NS

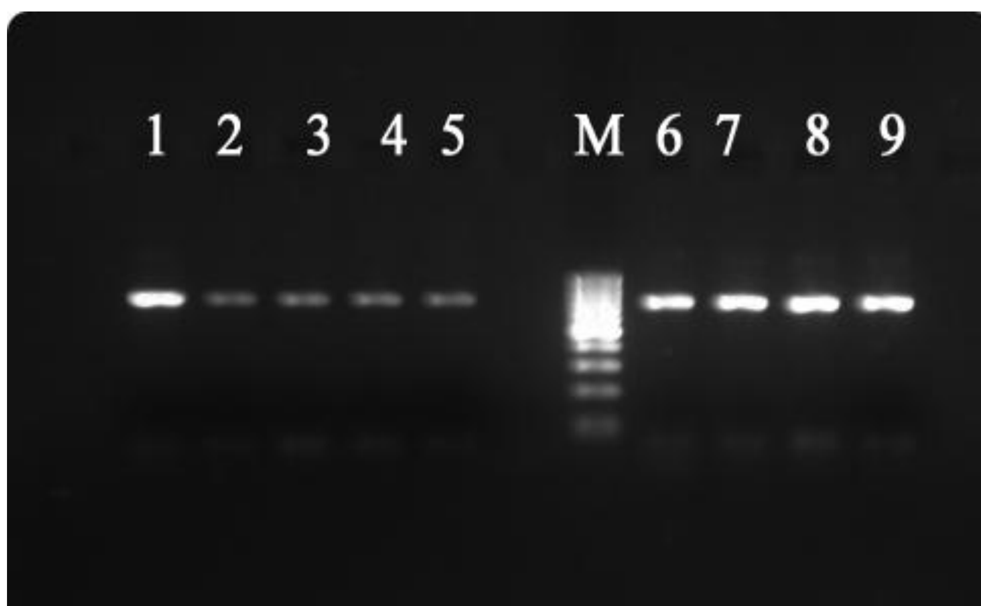

Fig. 1. PCR amplification of *wsp* gene of *Wolbachia* from *Cotesia vestalis*

Lane M: 100bp ladder, Lanes 1, 2 and 3: *C. vestalis* Bangalore (Hoskote, Malur & Kolar), Lanes 4 : *C. vestalis* (Varanasi), Lane 5: *C.vestalis* (Tirupathi), Lane 6: *C.vestalis* (Shillong), Lane 7: *C.vestalis* (Bhubaneshwar), Lane 8: *C.vestalis* (Salem) and Lane 9: *C.vestalis* (Hyderabad)

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