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Impacts of temperature and crowding on sex ratio, fecundity and *Wolbachia* infection intensity in the copepod, *Mesocyclops thermocyclopoides*

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ABSTRACT

Wolbachia are a group of intracellular bacteria that cause reproductive alterations in arthropods. Here, we describe the effects of two environmental factors (crowding and temperature) on phenotypic expression of feminization, the host's fecundity and *Wolbachia* infection intensity among life cycle stages in the naturally *Wolbachia*-infected copepod, *Mesocyclops thermocyclopoides*. The copepod was first found to be co-infected with *Wolbachia* A- and B-supergroups *Wolbachia* strains based on *wsp* primers. The relative *Wolbachia* infection intensity within individuals was determined using quantitative real-time PCR and was significantly higher in the B-supergroup than in the A-supergroup. Experimental results of temperature effect on bacterial density in each developmental stage revealed a significant decrease in *Wolbachia* infection intensity following exposure to high temperature (37 °C) in both sexes and implied that *Wolbachia* might survive in room temperature (25 °C) better than in high temperature. Experimental results of crowding effects on *Wolbachia* infection intensity suggested a negative correlation between copepod nauplii and *Wolbachia* infection intensity. No effect of rearing temperature on the sex ratio was reported although the fecundity was significantly decreased by high temperature. The results showed that *Wolbachia* infection intensity to be correlated with crowding conditions and was decreased following exposure of elevated temperature.

1. Introduction

Wolbachia are maternally-inherited bacterial endosymbionts that infect a diverse range of arthropods; up to 76% of arthropod species are estimated to be infected (Cordaux et al., 2012; Werren et al., 2008; Wiwatanaratanabutr, 2013). These bacteria affect the reproductive biology of their hosts, resulting in different abnormal reproductive phenotypes such as cytoplasmic incompatibility (CI), parthenogenesis, male-killing and feminization (Stouthamer et al., 1999). *Wolbachia* are distributed through their host populations under the action of CI by increasing the numbers of infected females. These bacteria have shown potential to be used as a biocontrol agent to reduce dengue virus transmission in mosquitoes by controlling the number of vector population using CI advantages (Werren et al., 2008). *Wolbachia* infection intensity has been shown to be affected by several factors including *Wolbachia* strain, host species, temperature and rearing density of the hosts (Clancy and Hoffmann, 1998; Wiwatanaratanabutr and Kittayapong, 2006, 2009). Feminization was first discovered in isopod crustaceans; infected males become morphologically functional females while their genetic traits remain male, causing sex ratio distortion (Rigaud et al., 1999). *Wolbachia* were reported to be a feminizing agent in the terrestrial isopod, *Armadillidium vulgare* (Rousset et al., 1992), and recently in copepod crustaceans (Wiwatanaratanabutr, 2013). Experimental transfer of feminizing *Wolbachia* among isopod and copepod hosts revealed the importance of host conditions for the expression of the feminizing property (Cordaux et al., 2012; Wiwatanaratanabutr, 2013).

Wolbachia infection, diversity and distribution have been studied in many arthropod groups such as isopod crustaceans (Cordaux et al., 2012), mosquitoes (Dutton and Sinkins, 2004), crickets (Jeong et al., 2012), fruit flies (O'Neill et al., 1992) and copepods (Wiwatanaratanabutr, 2013). The evolutionary relationships based on phylogenetic analyses of these endosymbionts in arthropod hosts wereprimarily determined by sequencing of 16S rRNA, *ftsZ*

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and *wsp* genes. Sequence analysis shows that *Wolbachia* are a monophyletic clade in the alpha-proteobacterial group and are closely related to the *Ehrlichia* assemblage (Ricci et al., 2002; Zhou et al., 1998) and are currently composed of up to 16 lineages or supergroups (Gerth, 2016). *Wolbachia* from terrestrial isopods belonged to the B supergroup (Cordaux et al., 2012; Verne et al., 2007), which, along with supergroup A, are widespread reproductive manipulators in arthropods (Glowska et al., 2015).

Copepods are small crustaceans and are among the predators of a variety of phytoplankton and zooplankton, as well as mosquito larvae. Freshwater copepods are an important component in aquatic ecosystems because they are food for planktivorous fishes and the young stages of several fish species. They are usually 1–2 mm in length and dominate the freshwater zooplankton, although most species cannot be considered truly planktonic. Free-living copepods are divided into three groups: calanoids, cyclopoids and harpacticoids (Alekseev, 1996). Different species of cyclopoid copepods suppress mosquito larvae under experimental conditions and are considered to be an important natural control against dengue fever and other diseases in tropical countries (Kay et al., 2002; Nam et al., 2000). Approximately 39 species of calanoids and 19 species of cyclopoids have been recorded from freshwater habitats in Thailand (Sanoamuang, 1999), including the common species *Mesocyclops thermocyclopoides* and *M. aspericornis* (Copepoda: Cyclopoida). *M. thermocyclopoides* harbors *Wolbachia* that induce feminization (genetic males are converted to functional females) consistent with infections in other crustacean hosts (Wiwatanaratanabutr, 2013). Both male and female *M. thermocyclopoides* are infected. Similar *Wolbachia* infection patterns have been reported in some terrestrial isopods such as *Porcellionides pruinosus* (Rigaud et al., 1997); however, *Wolbachia* do not appear to occur in some crustacean groups, including a freshwater ostracod (Bruvo et al., 2011) and the cladoceran, *Daphnia pulex* (Fitzsimmons and Innes, 2005).

Environmental factors can affect symbiont infection intensity (Mouton et al., 2006). The influence of temperature on *Wolbachia* infection intensity has been reported in many arthropods (Mouton et al., 2006) but not in copepods. This sensitivity to high temperature has also been reported for *Wolbachia* inducing parthenogenesis in *Trichogramma* (Louis et al., 1993) and male killing in *Drosophila* sp. (Hurst et al., 2000). Within a host infected with more than one strain, the infection intensity of each strain can differ under the same environmental conditions. The aims of this study were (I) to examine the effects of two environmental factors, crowding and temperature variations, on the *Wolbachia* infection intensity of two supergroups, A and B, for all life cycle stages of copepods (nauplii, copepodids and adults) using a real-time quantitative PCR assay and (II) to determine the effect of crowding and temperature on phenotypic expression of feminization in terms of sex ratio distortion and host fecundity.

2. Materials and methods

2.1. Hosts and rearing conditions for crowding effect tests

Wolbachia-infected *M. thermocyclopoides*, originally collected from natural breeding habitats in Bangkok, Thailand, were established and maintained in the laboratory. Fifty gravid females were reared in a glass beaker filled with 800 ml of culture medium containing the copepod prey, *Paramecium* sp. The stock culture of *Paramecium* sp. was mass reared in boiled rice straw water held for one week in order to increase numbers. The *Paramecium* sp. were fed to all copepodid stages. The presence of *Paramecium* sp. in the culture was confirmed by stereomicroscopy and the *Paramecium* sp. were tested for *Wolbachia* infection. Copepods were divided into different developmental stages: nauplius, copepodid (Co), adult male (Am) and adult female (Af).

After egg hatch, the first nauplius stage was counted into selected densities. The first nauplius stage crowding experiment consisted of three different treatments: uncrowded control (150 individuals/beaker), moderately crowded (300 individuals/beaker) and crowded (500 individuals/beaker). The nauplii were placed in glass beakers containing 800 ml of boiled rice straw water after cooling (without *Paramecium*) and reared at 25 °C. Twenty ml of *Paramecium* suspension was supplied daily. After 3–5 days, 15 individuals of the fourth nauplius stage were randomly selected from each treatment and DNA was extracted immediately from each individual. The fifth nauplius stage was allowed to develop to the first copepodid stage at which time 15 copepods from each treatment were selected and immediately prepared for DNA extraction. Copepodids were maintained in the treatments until molt to the adult stage. At 24–48 h post-molt, adults were collected and DNA extracted. The experiment was repeated three times and the results compared.

2.2. Hosts and rearing conditions for temperature effect tests

Naturally *Wolbachia*-infected *M. thermocyclopoides* were used in temperature control rearing experiments. Thirty gravid female copepods from the colony were placed in glass beakers filled with 800 ml of culture medium containing *Paramecium* sp. After egg hatch, approximately 200 individuals of the first nauplius stage were counted and divided into two treatment groups of different temperature conditions: room temperature (25 °C) and elevated temperature (37 °C). The nauplii were placed in glass beakers, 100 individuals each, containing 600 ml of cooled boiled rice straw water (without *Paramecium* sp.). One beaker was held 25 °C and another beaker was placed in a 37 °C water bath with a thermometer to monitor temperature. Twenty ml of *Paramecium* suspension was supplied daily. After 3–5 days, 15 individuals of the fourth nauplius stage from each treatment were selected randomly and the DNA was extracted immediately from each individual. Fifth stage nauplii were allowed to develop to the first copepodid stage at which time 15 individuals were selected from each treatment and immediately prepared for DNA extraction. Copepodids were maintained at the 25 °C and 37 °C temperatures until molt to the adult stage. At 24–48 h post-molt, adults were collected and DNA extracted. This experiment was repeated three times and the results compared.

2.3. DNA preparation

Total DNA from copepods (nauplius, copepodid and adult stages) was extracted, examined and confirmed for the presence of *Wolbachia* using both *wsp* (Zhou et al., 1998) and *groE* gene amplification primers (Wiwatanaratanabutr et al., 2009) in PCR amplification. For all life cycle stages of copepods, each individual was ground and homogenized in a hand-held polypropylene homogenizer in a 1.5 ml microcentrifuge tube filled with 100 µl of STE buffer (100 mM NaCl, 10 mM Tris HCl, 1 mM EDTA, pH 8.0) following the crude boiling DNA extraction procedure of O'Neill et al. (1992). The homogenate was heated at 95 °C for 10 min and then centrifuged at 1400*g* for 1 min. One and 2 µl of supernatant were used as DNA template in the PCR reactions and real-time quantitative PCR assays, respectively.

2.4. Polymerase chain reaction (PCR) amplification

DNA from *Wolbachia*-infected *M. thermocyclopoides* was used as a positive control for general *Wolbachia* screening of crustaceans. Contamination was checked using double-distilled water as a