Wolbachia infections in *Nasonia vitripennis*: A host-pathogen interaction study

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Dedicated to my family

Declaration

The work presented in this thesis has been carried out by me under the guidance of Dr. Rhitoban Raychoudhury at the Indian Institute of Science Education and Research Mohali. This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgment of collaborative research and discussions. This thesis is a bona fide record of original work done by me and all sources listed within have been detailed in the bibliography.

Alok Tiwary

Date:
Place:

In my capacity as the supervisor of the candidate's thesis work, I certify that the above statements by the candidate are true to the best of my knowledge.

Dr. Rhitoban Raychoudhury

Date:
Place:

Acknowledgments

I finally got to write this page after killing innumerable *Nasonia*, doing the fly room duties in the lab on all holidays (as I was the one who used to stay back), donning tons of plastic waste, and testing over 10,000 samples for COVID 19.

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Alok

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Introduction

Endosymbiosis

Endosymbionts are organisms residing in a symbiotic relationship with other cells or organisms. These organisms either can be found within the cell (intracellular), or attached to the cell surface (extracellular). Endosymbionts can be obligate or facultative in nature. These organisms devise mechanisms to evade host defense machinery and create a niche for their existence. Heritable bacterial endosymbionts are very common in insects, which have evolved to complement their host metabolism and provide nutritional benefits. These can have varying effects on their hosts ranging from the provision of essential nutrients and defense against natural enemies (Haine, 2008; Brownlie and Johnson, 2009) to alteration of the host's reproductive systems (Engelstädter and Telschow, 2009; Herbert and McGraw, 2018). Additionally, recent findings have also uncovered the fact that symbionts impose substantial detrimental effects for their maintenance on the host (Rigaud and Moreau, 2004; White *et al.*, 2011; Ross *et al.*, 2016) as well as impact the host's genetic diversity (Feldhaar, 2011a; Russell *et al.*, 2013).

One important feature of all these bacterial endosymbionts is their vertical transmission from the mother to offspring. Thus, bacterial endosymbionts not only influence the host biology but also affects the entire community and the interaction of the host with other organisms.

The dissertation thesis explores the association of one such bacterial endosymbiont named *Wolbachia* with its parasitoid host *Nasonia vitripennis*.

Wolbachia

Wolbachia was first discovered by Marshall Hertig and S. Burt Wolbach in 1924 as "tiny, rod-like or coccoid, gram-negative organisms in the smears of testis and ovaries of *Culex pipiens* (Hertig *et al.*, 1924). *Wolbachia* are members of the order Rickettsiales and are intracellular bacteria that show a mutualistic and parasitic relationship with their hosts. These gram-negative bacteria are inherited maternally by the offspring. In addition to vertical transmission, *Wolbachia* can also spread through horizontal transmission across species. *Wolbachia* has a very high incidence rate with almost 66% of terrestrial arthropods known to be infected by it (Hilgenboecker *et al.*, 2008). Arthropods are the most common group of animals and therefore, from a biodiversity perspective, *Wolbachia* is considered as one of "the great pandemics" of life.

Wolbachia act as reproductive parasites for the vast majority of their arthropod hosts. With stable integration, *Wolbachia* manipulates its host reproductive biology for its own benefit, so that infected females, which is the transmitting sex for *Wolbachia,* are at a selective advantage. Some of these reproductive manipulations are feminization (Kageyama *et al.*, 2002; Narita *et al.*, 2007), male-killing (Hurst and Majerus, 1993; Kageyama and Traut, 2004), parthenogenesis (Stouthamer and Kazmer, 1994; Pannebakker *et al.*, 2004), and cytoplasmic incompatibility (CI) (Werren, 1997; Landmann, 2019)

Such mechanisms of reproductive parasitism can lead to the evolution of host defenses and eventually, resistance against such infections. However, this has not been observed in *Wolbachia* and its association with its arthropod hosts. Theoretical models (Turelli, 1994; Driscoll *et al.*, 2020) suggest that for any such invasion to be sustained in a host for a long evolutionary time, the parasites should increasingly become benign against the host. However, this is not true for *Wolbachia*, which is still known to have a parasitic relationship with the vast majority of arthropod hosts. This raises questions on the maintenance of these *Wolbachia* infections in the arthropod hosts over a long evolutionary time scale. Another aspect of this association is how *Wolbachia* affects the physiological traits of its host? As *Wolbachia* behaves like a parasite and competes for nutritional benefits, whether *Wolbachia* infections have any negative effect on the host biology?

Nasonia: a model system to study host-*Wolbachia* interaction

One of the arthropod hosts, which *Wolbachia* infects, is a parasitoid wasp called *Nasonia*. Being a parasitoid, a major portion of its life cycle is spent inside the cyclorraphous fly pupa.

Nasonia has a haplodiploid sex-determination system where the males develop from unfertilized eggs through arrhenotokous parthenogenesis while the females develop from fertilized embryos (Beukeboom *et al.*, 2007). Four species of *Nasonia* namely *N. vitripennis, N. longicornis, N. giraulti,* and *N. oneida* have been reported till now (Darling *et al.*, 1990; Raychoudhury *et al.*, 2010). *N. vitripennis* has a cosmopolitan distribution while the other species are restricted to North America (Raychoudhury *et al.*, 2010).

Wolbachia in *Nasonia vitripennis*

N. vitripennis has two *Wolbachia* supergroup infections, one each from supergroup A and supergroup B (Perrot-Minnot *et al.*, 1996). These two infections are bidirectionally incompatible. Supergroup B and the double infection cause complete CI while supergroup A infection is known to cause incomplete CI (Perrot-Minnot *et al.*, 1996). *Wolbachia* leads to conversion CI in *N. vitripennis* (Bordenstein *et al.*, 2003).

Major Questions

What are the effects of the single and multiple *Wolbachia* infections on the various physiological and reproductive traits of the parasitoid host *N. vitripennis*?

Endosymbionts compete with the host for their nutritional demands (Kobayashi *et al*., 2009; Whittle *et al.*, 2021). This leads to the sharing of host nutrition between the host and *Wolbachia*, which can have serious negative effects on the host physiology. In the dissertation thesis, I have investigated the effects of single and multiple *Wolbachia* infections on both the sexes of *N. vitripennis*. The results show severe negative effects of the presence of single and multiple *Wolbachia* infections which in contrast to the previous reports suggesting positive fitness effects (Stolk *et al.*, 1996) and no fitness effects (Bordenstein *et al*., 2000) of *Wolbachia* on *N. vitripennis*. These negative effects are on both the sexes of the host where traits like longevity, copulation capability, sperm production, progeny family size, and fecundity are compromised in the infected individuals. I re-cured the single and double infection lines to ensure that the effects are because of the presence of *Wolbachia*. In the majority of the recently cured lines, the negative effects disappeared. I have established that the negative effects of these *Wolbachia* infections can also be specific to a particular supergroup. Supergroup B *Wolbachia* shows more severe negative effects on both the sexes of the host than supergroup A.

Why *Wolbachia* infections are being maintained in arthropod hosts over a long evolutionary time?

Another important question about the *Wolbachia*-host association is why these reproductive parasites are being maintained in the arthropod hosts over a long evolutionary time. While *Wolbachia* is known to provide resistance to the host against viral infections (Teixeira *et al.*, 2008; Chrostek *et al.*, 2013), it also mediates the host responses against adverse abiotic factors like high temperature (Hague *et al.*, 2020; Mazzucco *et al.*, 2020). Temperature changes in insects are considered a major driver of development (Reviewed in Ma *et al.*, 2021). Exposure to high temperatures has been reported to have implications on insect longevity, reproduction, and distribution (Mołoń *et al.*, 2020). Therefore, I investigated the role played by single and multiple *Wolbachia* infections in host responses against sub-lethal temperatures in *N. vitripennis*. I found that the presence of *Wolbachia* increases the thermal tolerance of both the male and female hosts against elevated temperatures. Single and multiple *Wolbachia* infected males and females were more tolerant towards elevated temperatures of 30°C, 32°C, and 37°C which was evident with their greater life span when compared to the uninfected males and females. The recently cured lines had decreased life span as compared to their infected counterparts at all the temperatures in females while in the case of males, this decrease was observed only at 30°C. I also found sex-specific variations where single *Wolbachia* infected females of supergroup A and supergroup B were more tolerant to higher temperatures of 32°C and 37°C, while in the case of males single *Wolbachia* infections were only tolerant to 30°C. The presence of *Wolbachia* positively affects the reproductive capability of males at high temperatures. *Wolbachia* infected males sired more daughters at all the high temperatures tested than the uninfected males. Thus, I predict that *Wolbachia* provides resistance against quicker sperm depletion at elevated temperatures.

My results also suggest temperature preference by particular *Wolbachia* supergroups. While the presence of single supergroup A *Wolbachia* provides tolerance at a temperature of 30°C, single supergroup B *Wolbachia* can provide tolerance to the host at even higher temperatures of 30°C, 32°C, and 37°C. The presence of both infections is the most beneficial factor for the host at all the sublethal temperatures tested.

Identification of the Developmental Stage of *Wolbachia*-induced sperm modification in *N. vitripennis*

Wolbachia causes CI in *N. vitripennis*. The male sperm undergoes “modification” which is “rescued” by the infected female to produce viable offspring. The genetic basis of these “modifications” in males and “rescue” in females remain poorly understood. I investigated the genetic basis of sperm modification in males. Using a single supergroup B infection line prospective stage where *Wolbachia* induces CI modification in males has been identified using stage-specific antibiotic treatment. Further experiments include stage-specific transcriptomes to identify candidate genes responsible for sperm modification in males.

Significance of the dissertation work

Wolbachia association with arthropod hosts has been previously studied for its effect on life-history traits (Stolk and Stouthamer, 1996; Bordenstein and Werren, 2000) and CI (Le Page *et al.*, 2017; Shropshire *et al.*, 2020). The dissertation work is the first empirical study to report supergroup-specific effects of *Wolbachia* in *N. vitripennis*. Moreover, the profound negative effects of *Wolbachia* infections on both the sexes of *N. vitripennis* have been investigated in the study, which has not been reported before.

The dissertation work also reports *Wolbachia* supergroup-specific tolerance of the host against elevated temperatures. These reports possess significant agronomic importance in the use of *Wolbachia* to control the spread of vector-borne diseases in tropical countries.

The dissertation work identifies the stage of CI sperm modification in *N. vitripennis* males. Stage-specific transcriptome studies on the CI stage can be done to get the candidate genes responsible for *Wolbachia*-induced sperm modification in males.

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- **Bacterial supergroup specific "Cost" of *Wolbachia* infections in *Nasonia vitripennis*.** *Ecology and Evolution* (Under Revision).

- ***Wolbachia* mediated tolerance against heat stress in *Nasonia vitripennis*.**

  (Manuscript under preparation)

- **Stage identification for *Wolbachia*-induced sperm modification in *Nasonia vitripennis*.**

  (Manuscript under preparation)

Co-authored Publications

- **Investigating the diversity, function and transmission of gut symbionts in *Coptotermes heimi***

  (Manuscript under preparation)

Abbreviations

Weights and measures

%	Percentage
°C	Degree centigrade
mm	millimetre
µg, g	microgram, gram
µl, mL	Microliter, milliliter
µM, mM, M,	micromolar, millimolar, molar

Symbols

~	Approximately
=	Equal to

Techniques

PCR	Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction

Chemicals

dNTPs	2'-deoxyadenosine 5'-triphosphate
EDTA	Ethylenediamine-tetra-acetic acid
LB	Luria Bertani
PCI	Phenol-Chloroform-Isoamyl alcohol

Miscellaneous

DNA	Deoxyribonucleic acid
rRNA	Ribosomal ribonucleic acid
NCBI	National Centre for Biotechnology Information

Chapter 1

An Introduction to Endosymbiosis, *Wolbachia and Nasonia vitripennis*

1.1 Endosymbiosis

Endosymbionts are organisms residing in a symbiotic relationship with other cells or organisms. These organisms can be found either within the cell (intracellular) or attached to the cell surface (extracellular). The origin of modern endosymbiotic theory is based on the concept of "symbiosis"(from the Greek words" living" and "together") which came into existence by the study of lichens by Simon Schwendener in 1867 (Archibald, 2015). De Bary (1878) defined symbiosis as an association of unlike organisms. He included the concepts of mutualism, parasitism, and commensalism under the common heading of symbiosis. Notable works by Sachs (1882) Schimper (1885) and Altmann (1890) suggested the symbiotic association of many organelles like plastids and mitochondria with plant and animal cells (Kutschera and Niklas, 2005). Sachs and Altmann also concluded the semi-autonomous existence of chloroplasts and mitochondria. Russian botanist Constantin Mereschkowsky's theory of plastids being reduced "foreign organisms"(cyanobacteria) evolving as a symbiont in eukaryotic cells (Martin and Kowallik, 1999), lead to the concept of "modern endosymbiont theory". Margulis (1993) reignited the debate of the endosymbiotic origin of organelles by proposing the concept of "Serial Endosymbiosis Theory" (Gray, 1992). She conveyed the idea that mitochondria and chloroplasts gained the symbiotic association with their hosts in two discrete historical "events" and not simultaneously. With breakthroughs in sequencing techniques, the sequencing of rRNA supported the endosymbiont theory for the origin of organelles.

Endosymbionts can be obligate or facultative in nature. These organisms devise mechanisms to evade host defense machinery and create a niche for their existence. Some microbes are resistant to host lysosomes while others can evade the lysosome-symbiont fusion to avoid lysosome exposure (Jeon, 2011). Some gram-negative bacterial endosymbionts can survive even with the phagolysosome as they contain enzyme-resistant components secreted by chromosomal or plasmid genes. For example, mycobacteria have a protective layer of mycolic acids, which resist digestion by lysosomal enzymes (Jeon, 2011).

1.2 Endosymbionts in Insects

Many bacterial, algal, and fungal endosymbionts are found in invertebrates. These endosymbionts have evolved to complement their host metabolism and provide nutritional benefits. For example, in algal-protozoan symbiosis (*e.*g., *Chlorella-Hydra* symbiosis) the algae are photosynthetic and hence benefit the host by providing photosynthetic products and oxygen. Bacterial and fungal endosymbionts in insects can provide dietary advantages to the host thereby enabling the host to venture into new habitats (José Gosalbes *et al.*, 2010).

Heritable bacterial endosymbionts are very common in insects. These can have varying effects on their hosts ranging from the provision of essential nutrients and defense against natural enemies (Haine, 2008; Brownlie and Johnson, 2009) to alteration of the host's reproductive systems (Engelstädter and Telschow, 2009; Herbert and McGraw, 2018). Additionally, recent findings have also uncovered the fact that symbionts impose substantial costs for its maintenance on the host (Rigaud and Moreau, 2004; White *et al.*, 2011; Ross *et al.*, 2016) as well as impact the host's genetic diversity (Feldhaar, 2011b; Russell *et al.*, 2013).

One important feature of all these bacterial endosymbionts is their vertical transmission from the mother to offspring. Thus, bacterial endosymbionts not only influence the host biology but also affects the entire community and the interaction of the host with other organisms.

The present study explores the association of one such bacterial endosymbiont named *Wolbachia* with its parasitoid host *Nasonia vitripennis*.

1.3 ***Wolbachia***

Wolbachia was first discovered by Marshall Hertig and S. Burt Wolbach in 1924 as "tiny, rod-like or coccoid, gram-negative organisms in the smears of testis and ovaries of *Culex pipiens* (Hertig and Wolbach, 1924). *Wolbachia* are members of the order Rickettsiales and are intracellular bacteria that show a mutualistic and parasitic relationship with their hosts. These gram-negative bacteria are maternally inherited from the mother by the offspring. In addition to vertical transmission, *Wolbachia* can also spread through horizontal transmission across species. *Wolbachia* has a very high incidence rate with almost 66% of terrestrial arthropods known to be infected by it (Hilgenboecker *et al.*, 2008). Arthropods are the most common group of animals and therefore, from a biodiversity perspective, *Wolbachia* is considered as one of "the great pandemics" of life.

With stable integration, *Wolbachia* influences the germline biology of its host to manipulate the reproductive outcome for its benefit. Thus, it can also have an impact on host evolution, immunity, and development (Werren *et al.*, 2008). During the initial years of its discovery, the focus remained mainly on the diversity of *Wolbachia* (Duron *et al.*, 2008; Arthofer *et al.*, 2009; Augustinos *et al.*, 2011; Salunke *et al.*, 2012) and the phenotypes induced by it on their hosts

(Reviewed in Werren *et al.*, 2008; Landmann, 2019). However, recently, *Wolbachia*-host interaction at the cellular and molecular level has gained immense importance owing to the potential of biomedical applications of *Wolbachia* to control vector-borne diseases like dengue, malaria, chikungunya (Hancock *et al.*, 2011; Iturbe-Ormaetxe *et al.*, 2011b; Caragata *et al.*, 2016), and other neglected tropical diseases like filariasis (Manoj *et al.*, 2021).

1.4 *Wolbachia* Diversity

Wolbachia has spread across many different arthropod and nematode communities with nineteen different supergroups (named A-S) (Wang *et al.*, 2016; Lefoulon *et al.*, 2020) reported till now. Using *16S* rDNA and multiple gene sequence information like *wsp*, *ftzZ*, *coxA*, and *groEL* (O'Neill *et al.*, 1992; Werren *et al.*, 1995; Zhou *et al.*, 1998; Baldo, Hotopp, *et al.*, 2006), *Wolbachia* has been categorized into Supergroups. Supergroup A and B are mainly found in arthropod hosts where *Wolbachia* behaves mostly as reproductive parasites (Werren *et al.*, 2008) whereas supergroup C and D are found to have a mutualistic relationship with their nematode hosts (Bandi *et al.*, 1998). Supergroup F is present both in arthropods and nematode hosts (Zimmermann *et al.*, 2021). Such a large diversity is generally attributed to the ease with which *Wolbachia* can be horizontally transferred across taxonomically unrelated hosts (Heath *et al.*, 1999; Cordaux *et al.*, 2001). This is also evident from the incongruency seen in the phylogeny of the host and its current resident *Wolbachia* (Ahmed *et al.*, 2013; Balvín *et al.*, 2018). *Wolbachia* also undergoes extensive recombination (Baldo, Bordenstein, *et al.*, 2006; Zhao *et al.*, 2021) between strains that affect various regions of the genome like surface proteins (wsp), housekeeping genes, and prophage genes (Baldo, Bordenstein, *et al.*, 2006). Such recombination events can help in bacterial adaptations as well as mediate their interactions with various hosts.

1.5 *Wolbachia* Genomes

Wolbachia genomes from arthropod hosts are relatively large (1.2 to 1.6 Mb) as compared to that found in filarial nematodes (0.9 to 1Mb) (Wu *et al.*, 2004; Foster *et al.*, 2005). This larger size has been attributed to a large number of coding proteins, the presence of repetitive elements like ankyrin repeat sequences, transposons, and prophages (Landmann, 2019). The presence of more effector proteins from arthropod *Wolbachia* (Rice *et al.*, 2017) suggests complex roles of *Wolbachia* than just being a symbiont to fulfill the basic demands of an intracellular lifestyle (Bi and Wang, 2020).

1.6 *Wolbachia* as a mutualist

Wolbachia has been found to have mutualistic interaction with their filarial nematode hosts. *Wolbachia* supergroup C has been reported from nematodes like *Brugia malayi* which causes lymphatic filariasis while supergroup D has been reported in Onchocerca and other related taxa which includes the one causing river blindness in humans (Fenn and Blaxter, 2004). *Wolbachia* is reported to be necessary for the survival of these hosts as antibiotic treatment of *Wolbachia* in these nematodes results in abnormal embryonic development (Slatko *et al.*, 2010a). The phylogenetic congruence between both the nematode and their endosymbionts also suggest a long-term co-evolution (Bandi *et al.*, 1998; Casiraghi *et al.*, 2001).

Wolbachia-induced positive fitness effects have also been observed in arthropod hosts. The presence of *Wolbachia* can lead to enhanced longevity (Dobson *et al.*, 2004) in *A. albopictus* and fecundity (Fry *et al.*, 2004; Weeks *et al.*, 2007) in *Drosophila* species. In addition to these, other benefits of *Wolbachia* comprise nutritional provisioning (Brownlie *et al.*, 2009; Hosokawa *et al.*, 2010; Newton and Rice, 2020) and protection against viral infections (Teixeira *et al.*, 2008).

1.7 *Wolbachia* as a parasite

Wolbachia act as reproductive parasites for the vast majority of their arthropod hosts. To ensure its transmission, *Wolbachia* manipulates its host reproductive biology so that infected females, which is the transmitting sex for *Wolbachia,* are at a selective advantage. Some of these parasitic effects of *Wolbachia* are:

Feminization: *Wolbachia*-infected males are hormonally sterile due to the inhibited proliferation of the androgenic glands. Consequently, genetic males develop as pseudo females with functional ovaries. Thus, more females, which is the transmitting sex for *Wolbchia* leads to higher *Wolbachia* transmission. Feminization has been reported in Isopods, Lepidoptera and Hemiptera (Kageyama *et al.*, 2002; Narita *et al.*, 2007).

Parthenogenesis: In some haplodiploid species, *Wolbachia*-infected unfertilized eggs develop into diploid zygotes due to defective anaphase during the cell cycle (Stouthamer and Kazmer, 1994; Pannebakker *et al.*, 2004). Thus, almost all the progenies are females, which is the transmitting sex for *Wolbachia*. Parthenogenesis has been reported in species with arrhenotokous development like hymenopterans, Thysanoptera, and Acari (Tagami *et al.*, 2001; Weeks and Breeuwer, 2001; Stouthamer *et al.*, 2006).

Male Killing: In the presence of *Wolbachia,* genetic males die during larval development (Hurst and Majerus, 1993; Kageyama and Traut, 2004). This reduces the competition for the infected females for the available resources. Thus, more females, which is the transmitting sex, benefits *Wolbachia* transmission and prevalence. Male killing has been reported in Coleoptera, Diptera, Lepidoptera, and Pseudoscorpiones (Landmann, 2019).

Cytoplasmic Incompatibility (CI): Cytoplasmic incompatibility (CI) is the inability of a *Wolbachia*-infected male to produce viable embryos with females that are either uninfected or are infected with a different *Wolbachia*. The key to understanding CI is the "modification" and a "rescue" hypothesis (Werren, 1997). Accordingly, *Wolbachia* modifies the male sperm during development and induces a rescue mechanism in the eggs to produce viable offspring. Such modified sperm can produce viable offspring only when it mates with a female infected by the same *Wolbachia* type and hence has the rescue function for the sperm modification. Thus, CI gives a selective advantage to the infected females to mate with both infected and uninfected males. CI has been observed in Diptera, Coleoptera, Hemiptera, Hymenoptera, Isopoda, Lepidoptera, Orthoptera, and Acari (Landmann, 2019)

CI can be of two types:

a. **Unidirectional CI**: When the male is infected with one *Wolbachia* type while the female is uninfected, the mating results in either embryonic death or development of haploid males in haplodiploid species (Serbus *et al.*, 2008). While the uninfected female, upon mating with an infected male, will not be able to produce viable offspring, the infected female can mate with both uninfected and infected males (as shown in figure 1.1).

b. **Bidirectional CI**: Bidirectional CI has been observed between *Wolbachia* strains having different modification and rescue mechanisms (Perrot-Minnot *et al.*, 1996). In such cases,

   males of one *Wolbachia* type are incompatible with the females of a different *Wolbachia* type infection (figure 1.2).

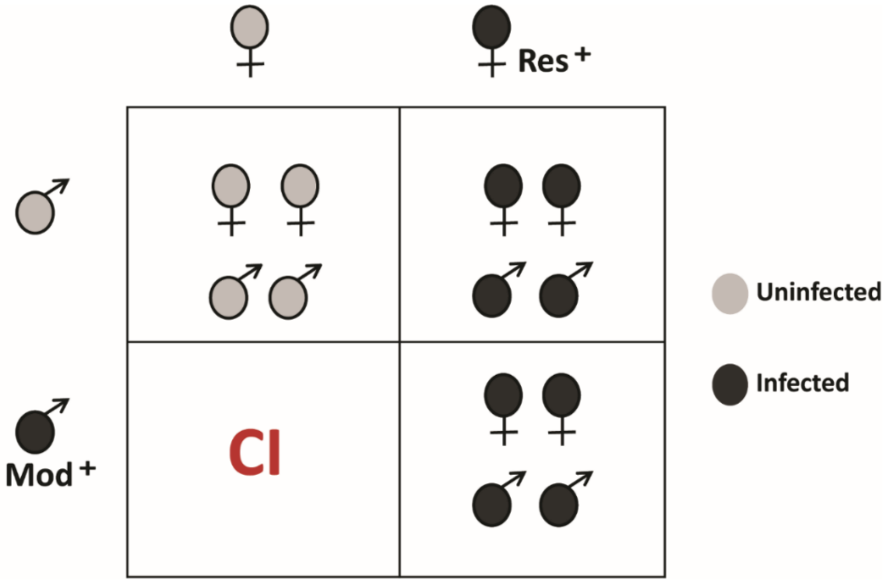


Figure 1. 1 Unidirectional CI

***Wolbachia*-infected males with sperm modification (Mod+) are incompatible with uninfected females (CI). The infected females have the rescue for sperm modification (Res+) and hence can produce viable offspring with infected males.**

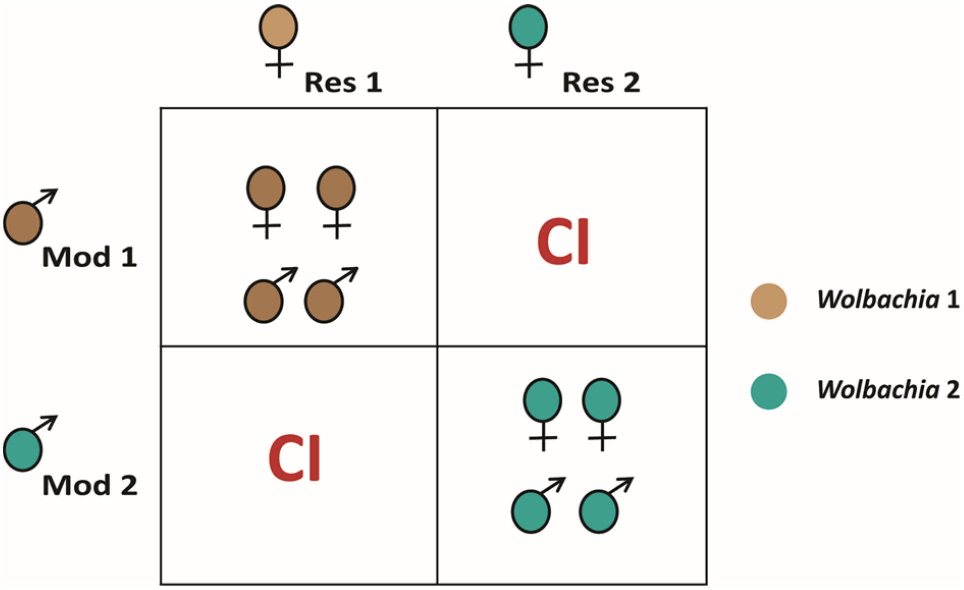


Figure 1. 2 Bidirectional CI

***Wolbachia* type 1 infected males (with a modification 1) are incompatible with *Wolbachia* type 2 infected females (with rescue 2) and vice versa.**

Reproductive parasitism can lead to the evolution of host defenses and resistance against such infections. However, this has not been empirically proven in the majority of arthropod hosts currently infected with *Wolbachia*. Theoretical models (Turelli, 1994; Driscoll *et al.*, 2020) suggest that for any invasion to be sustained in a host for a considerable period of time, the parasites should increasingly become benign against the host. However, this is not true for *Wolbachia*, which is still known to have a parasitic relationship with the vast majority of the arthropod hosts. This raises questions on the maintenance of these *Wolbachia* infections in the arthropod hosts over a long evolutionary time scale.

1.8 *Nasonia*: a model system to study host-*Wolbachia* interaction

One of the arthropod hosts which *Wolbachia* infects is *Nasonia*. *Nasonia* is a group of closely-related parasitoid wasps that are widely used for studies in genetics (Grillenberger *et al.*, 2008; Loehlin *et al.*, 2010), ecology (Mair and Ruther, 2019), behavior (van den Assem and Werren, 1994), developmental biology (Rosenberg *et al.*, 2014) and evolutionary biology (Breeuwer and Werren, 1993; Raychoudhury *et al.*, 2009). Being a parasitoid, a major portion of its life cycle is spent inside the cyclorraphous fly pupa. *Nasonia* lays its eggs inside the pupae where development to adulthood takes place and the adults emerge out after cracking open the fly pupae. With a small body size, a short generation time (2 weeks at 25°C), and relatively large family sizes, *Nasonia* serves as an easy model system to handle.

Nasonia has a haplodiploid sex-determination system where the males develop from unfertilized eggs through arrhenotokous parthenogenesis while the females develop from fertilized embryos (Beukeboom *et al.*, 2007). Four species of *Nasonia* namely *N. vitripennis*, *N. longicornis*, *N. giraulti,* and *N. oneida* have been reported till now (Darling and Werren, 1990; Raychoudhury *et al.*, 2010). *N. vitripennis* has a cosmopolitan distribution while the other species are restricted to North America (Raychoudhury *et al.*, 2010).

N. vitripennis serves as an ideal model system to study *Wolbachia*-host interaction. Its cosmopolitan distribution makes it easier to collect and rear. Both the sexes have visible morphological differences with the males having non-functional wings. The sexes can be separated at the pupal stages based on visible morphological differences (e.g., presence of ovipositor in females). *N. vitripennis* genome is sequenced (Werren *et al.* 2010), and advanced manipulation techniques like RNAi (Lynch et al., 2006), and CRISPR Cas systems (Chaverra-Rodriguez *et al.*, 2020) are also readily available.

1.9 *Wolbachia* in *Nasonia vitripennis*

N. vitripennis has two *Wolbachia* supergroup infections, one each from supergroup A and supergroup B (Perrot-Minnot *et al.*, 1996). These two infections are bidirectionally incompatible and the double infection causes complete CI (Perrot-Minnot *et al.*, 1996). Supergroup B infection causes complete CI while supergroup A infection is known to cause incomplete CI (Perrot-Minnot *et al.*, 1996). *Wolbachia* leads to conversion CI in *N. vitripennis* (Bordenstein *et al.*, 2003).

Wolbachia infections in *N. vitripennis* have been worked upon to answer a vast array of questions like the role of *Wolbachia* in host genome architecture (Kondo, Nikoh, *et al.*, 2002; Richardson *et al.*, 2012), effect on life-history traits (Stolk and Stouthamer, 1996; Bordenstein and Werren, 2000), effects of CI (Reviewed in Landmann, 2019; Chen *et al.*, 2020; Shropshire *et al.*, 2020), CI and *Wolbachia* density (Breeuwer and Werren, 1993), host genotype, and CI (Bordenstein *et al.*, 2006b), *Wolbachia* acquisition and horizontal transfer (Raychoudhury *et al.*, 2009) and the association of *Wolbachia*, host, and bacteriophage (Bordenstein *et al.*, 2006a; Le Page *et al.*, 2017).

1.10 *Wolbachia* infection in *N. vitripennis*: Questions

Wolbachia biology and its association with its host has been in the focus of cell biologists and molecular biologists in recent times (Reveillaud *et al.*, 2019; Krafsur *et al.*, 2020; Sanaei *et al.*, 2021). However, many aspects of this association remain poorly understood. The first among them is how *Wolbachia* affects the reproductive and life history traits of its host. As *Wolbachia* behaves like a parasite and competes for nutritional benefits, whether *Wolbachia* infections have any negative effect on the host biology, remains unresolved.

Chapter 2 details the effect of *Wolbachia* infection on the reproductive and physiological traits of *N. vitripennis*. The effect on both male and female hosts has been studied and the findings suggest a significant cost associated with the maintenance of these *Wolbachia* infections where traits like progeny family size, fecundity, longevity, and male copulation are compromised in *Wolbachia*-infected strains. The double-infected *Wolbachia* strain has the most detrimental impact on the host as compared to single infections. Moreover, there is a supergroup-specific negative impact on these wasps as the supergroup B infections elicit more pronounced negative effects than supergroup A. These findings raise important questions on the survival and maintenance of these reproductive parasites in arthropod hosts.

Wolbachia causes CI in *N. vitripennis*. The male sperm undergoes “modification” which is “rescued” by the infected female to produce viable offspring. The genetic basis of these “modifications” in males and “rescue” in females remain poorly understood. Chapter 3 investigates the genetic basis of sperm modification in males. A prospective stage where *Wolbachia* induces CI modification in males has been identified using stage-specific antibiotic treatment. Further experiments include stage-specific transcriptomes to identify candidate genes responsible for sperm modification in males.

Another question to be answered is how *Wolbachia* infection is being maintained in arthropod hosts for relatively long evolutionary time even though it alters its host reproductive biology for its benefit? The presence of *Wolbachia* has a wide range of implications on the host in surviving a variety of biotic and abiotic factors. While protection against viruses is considered one of the more important reasons for the maintenance of these infections, *Wolbachia* is also known to mediate host response against adverse temperature effects. Chapter 4 explains the effect of the presence of *Wolbachia* infections on host responses against elevated temperatures. The important findings of the dissertation work have been summarized in chapter 5.

Chapter 2

Bacterial supergroup specific "cost" of *Wolbachia* infections in *Nasonia vitripennis*

2.1 Introduction

Wolbachia are maternally-inherited, obligatory intracellular endosymbionts of the order Rickettsiales (Hertig and Wolbach, 1924) which are widely found in arthropods and filarial nematodes (Bandi *et al*,. 1992; Rousset *et al.*, 1992; Weinert *et al.*, 2015). To enhance their own transmission, these bacteria often alter host reproductive biology with mechanisms like male-killing, feminization, parthenogenesis, and cytoplasmic incompatibility (CI), (Werren *et al.*, 2008). While CI leads to an increase in the number of infected individuals in the population, male-killing, and feminization shifts the offspring sex ratio towards females, which is the transmitting sex for *Wolbachia*. Thus, *Wolbachia* increases the fitness of the infected hosts, over the uninfected ones, as it increases its own rate of transmission. The vast majority of *Wolbachia*-host association studies reveal many negative effects on the hosts. In addition to reproductive traits, many other life-history traits like longevity and developmental time are also known to be compromised. A review of such negative effects of *Wolbachia* on hosts where CI is prevalent is presented in Table 1. In *Trichogramma kaykai and T. deion,* the infected (thelytokous) line shows reduced fecundity and adult emergence rates than the antibiotically cured (arrhenotokous) lines (Hohmann *et al.*, 2001; Tagami *et al.*, 2001). *Leptopilina heterotoma*, a *Drosophila* parasitoid, has adult survival rates, fecundity, and locomotor performance, of both sexes, severely compromised in *Wolbachia* infected lines (Fleury *et al.*, 2000). Larval mortality has been observed in both sexes of insecticide-resistant *Wolbachia*-infected lines of *Culex pipiens* (Duron *et al.*, 2006). *Wolbachia* infections can also result in a range of behavioral changes and altered phenotypes in *Aedes aegypti* (Turley *et al.*, 2009). While these cases highlight a parasitic effect of *Wolbachia*, there are several examples where no such effect is discernible (Hoffmann *et al.*, 1996). Moreover, there are also examples where *Wolbachia* has now become a mutualist and offers specific and quantifiable benefits to its host. One such example of an obligate mutualism with *Wolbachia* has been reported in the common bedbug *Cimex lectularius* where *Wolbachia*, found to be localized in bacteriomes, provides essential B vitamins needed for growth and fertility (Hosokawa *et al.*, 2010). Such examples of arthropod-*Wolbachia* mutualism have now been reported from various arthropod taxa (Pike & Kingcombe, 2009; Miller *et al.*, 2010). This shift from parasitic to mutualistic effect can also happen in facultative associations as seen in *Drosophila simulans*, where within a span of just two decades, *Wolbachia* has evolved from a parasite to a mutualist (Weeks *et al.*, 2007).

The negative effects of *Wolbachia* on their hosts are not unexpected. The presence of bacteria within a host entails sharing of nutritional and other physiological resources (Kobayashi &

Crouch, 2009; Whittle *et al.,* 2021), especially with *Wolbachia*, as they are obligate endosymbionts and cannot survive without cellular resources derived from their hosts *(Foster et al., 2005., Slatko et al.,* 2010). Accordingly, *Wolbachia* is known to compete with the host for key resources like cholesterol and amino acids in *A. aegypti* (Caragata *et al.,* 2014). The precise molecular mechanisms of many of these negative effects have not been ascertained and are generally ascribed to partitioning off of host nutrients for its benefit, but what is clear is that *Wolbachia* infections can impose severe nutritional demands on their hosts (Ponton *et al.,* 2014). However, it is also known that *Wolbachia* can elicit antipathogenic responses from their hosts where the host resistance or tolerance to the infection increases (Zug & Hammerstein, 2015). For example, *Wolbachia* induces host methyltransferase gene *Mt2* towards antiviral resistance against Sindbis virus in *D. melanogaster* (Bhattacharya *et al.,* 2017). *Wolbachia* can utilize the Immune deficiency (IMD) and Toll pathways (Pan *et al.,* 2018) and increase Reactive Oxygen Species (ROS) levels in *Wolbachia*-transfected *A. aegypti* mosquitoes, inhibiting the proliferation of the Dengue virus (Pan *et al.*, 2012). Such immune responses require additional allocation of resources, which can further affect other physiological traits of the host. This concept of a "cost of immunity" is well-established and suggests a trade-off between immunity and other life-history traits (Zuk & Stoehr, 2002). For example, elevated ROS levels negatively affect many host traits like longevity, and fecundity (Dowling & Simmons, 2009; Monaghan *et al.*, 2009; Selman *et al.*, 2012; Moné *et al.*, 2014). Thus, there is sufficient evidence to conclude that *Wolbachia* can have substantial negative effects on the overall fitness of its host.

One of the arthropod hosts infected by *Wolbachia* is the parasitoid wasp *Nasonia vitripennis. N. vitripennis*, being cosmopolitan, has been used to study *Wolbachia* distribution, acquisition, spread, and *Wolbachia*-induced reproductive manipulations (Werren *et al.,* 2008; Landmann, 2019). However, the effect of the endosymbiont on the life-history traits of this wasp remains poorly understood with conflicting reports. *N. vitripennis* harbor two *Wolbachia* supergroup infections, one each from supergroup A and supergroup B (Perrot-Minnot *et al.*, 1996), and the presence of these two infections has been found in all lines of *N. vitripennis* from continental North America to Europe (Raychoudhury *et al.,* 2010), indicating that it has reached fixation across the distribution of its host. The two *Wolbachia* in *N. vitripennis* together cause complete CI, but single infections of supergroup A *Wolbachia* cause incomplete CI while supergroup B infections still show complete CI (Perrot-Minnot *et al.,* 1996). In some *N. vitripennis* lines, *Wolbachia* has been reported to cause enhanced fecundity (Stolk & Stouthamer, 1996), but a similar effect has not been observed in some other lines (Bordenstein & Werren, 2000). In this study, we investigate, what, if any, are the negative effects of CI-inducing *Wolbachia* infections in *N. vitripennis*? We investigate the effects of *Wolbachia* infections in a recently acquired line of *N. vitripennis* from the field. This line, like other *N. vitripennis* lines, has two *Wolbachia* infections, one each from the supergroup A and B. Sequencing of the five alleles from the well-established multi-locus strain typing (MLST) system (Baldo *et al.*, 2006) reveals no sequence variation with other *Wolbachia* strains done previously (Prazapati, personal communication) indicating, that this new *N. vitripennis* line is also infected by the same or very similar *Wolbachia* that are present across the distribution of *N. vitripennis* (Raychoudhury *et al.*, 2010b). To compare supergroup specific effects, these two infections were separated into singly infected wasp lines. A comparative analysis between the double infected, supergroup A infected, supergroup B infected, and uninfected lines revealed a consistent pattern of decreased longevity, quicker sperm depletion, and reduced family size for the infected individuals. While supergroup B infection had a more pronounced negative effect on most of the traits investigated, supergroup A infection on the other hand showed milder negative effects only for some of those traits. By testing for differential titer of *Wolbachia* by qRT-PCR, we also show a higher density of supergroup B and double infected *Wolbachia* strains, compared to the supergroup A infection, across the majority of the developmental stages of *N. vitripennis*.

2.2 Materials and Methods

2.2.1 *N. vitripennis* lines used, their *Wolbachia* infections, and nomenclature

The *N. vitripennis* NV-PU-14 strain was obtained from Mohali, Punjab, India, in 2015. NV PU-14 was cured of *Wolbachia* by feeding the females with 1 mg/ml tetracycline in 10 mg/ml sucrose solution for at least two generations (Breeuwer and Werren, 1990). The curing was confirmed by PCR using supergroup-specific *ftsZ* primers (Baldo *et al.*, 2006), and CI crosses between the infected and uninfected lines. NV PU-14 also served as the source strain for separating the two *Wolbachia* infections into single A and single B infected wasp lines.

To separate the *Wolbachia* supergroups, we utilized relaxed selection on the double infected females by repeatedly mating them with uninfected males which were obtained by antibiotic curing of the same NV PU-14 strain. Uninfected males do not have any sperm modification by *Wolbachia* which results in the removal of any selection pressure on the females to maintain their *Wolbachia* infections. Repeated mating with uninfected males was continued for ten generations till some of the progenies were found to be infected with either single A or single

B supergroup infections. The single infection status of these *N. vitripennis* lines was confirmed by using supergroup-specific *ftsZ* gene PCR primers (Baldo *et al.*, 2006). The single infections were tested for CI phenotype. Single supergroup A *Wolbachia* lines showed incomplete CI while single supergroup B *Wolbachia* infection line showed complete CI (Appendix Figure S1).

The preferred method of nomenclature of *Nasonia* lines and their *Wolbachia* infections includes information on supergroups as well as the host genotype. For example, [*w*NvitA *w*NvitB]V-PU14 indicates that the host species is *N. vitripennis,* with NV-PU14 as the host genotype, which has two *Wolbachia* infections, one each from supergroup A and supergroup B. However, since we used only *N. vitripennis* lines in this study, the nomenclature has been simplified by removing the species name. For example, the same line will now be denoted as *w*A*w*B(PU), and when cured of these infections, as 0(PU). The single *Wolbachia*-infected *N. vitripennis* lines used were designated as *w*A(PU) for the supergroup A-infected line while *w*B(PU) for the supergroup B-infected line. As the cured 0(PU) lines were in culture for three years, many of the infected lines were cured again to obtain 'recently cured' lines to minimize the effects of any host divergence that might have accumulated within them. These 'recently cured' lines were named 0(*w*A PU), 0(*w*B PU), and 0(*w*A*w*B PU).

Another *N. vitripennis* line NV-KA, obtained from Bengaluru, Karnataka, India, in 2016, was similarly named *w*A*w*B(KA). The MLST sequences of the two *Wolbachia* strains (one each from supergroups A and B), even in *w*A*w*B(KA), were found to be identical to *w*A*w*B(PU) and are also identical to all other *N. vitripennis* studied across the world (Prazapati, personal communication). *w*A*w*B(KA) was also cured of *Wolbachia* to obtain 0(*w*A*w*B KA).

All these wasp lines were raised on *Sarcophaga dux* fly pupae with a generation time of 14-15 days at 25ºC, 60% humidity, and a continuous daylight cycle.

2.2.2 Sequential mating and sperm depletion of the males

To test the effect of *Wolbachia* on male reproductive traits like mating ability, individual males were assayed for the number of copulations they can perform, as well as sperm depletion. As *N. vitripennis* is haplodiploid, every successful mating will result in both female and male progenies while an unsuccessful one will result in all-male progenies. The males used were obtained from virgin females hosted with one fly pupa for 24 hours and were not given any external sources of nutrition (usually a mixture of sucrose in water) before the experiment.

Each male was then mated sequentially with virgin females from the same line. At the first sign of a male not completing the entire mating behavior (Jachmann & Assem, 1996), it was given a rest for half an hour and was subjected to mating again until it stopped mating altogether. The mated females were hosted after a day with one fly pupa for 24 hours. The females were then removed and the offspring were allowed to emerge and then counted. The average number of copulations and the number of copulations, before sperm depletion, were compared using the Kruskal-Wallis test with a significance level of 0.05. Mann-Whitney U tests, with a significance level of 0.05, were used for comparisons between the two groups.

2.2.3 Host longevity, family size, and fecundity

To test whether the presence of *Wolbachia* has any influence on longevity, emerging wasps of both the sexes were kept individually in ria vials at 25ºC, without any additional nutrition. Survival following emergence was measured by counting the number of dead individuals every 6 hours. Kaplan-Meier analysis, followed by Log Rank Statistics, was used to identify differences between the strains with a significance level of 0.05.

To test for the effect of *Wolbachia* infections on the adult family size of virgin and mated females, each female was sorted at the pupal stage and separated into individual ria vials. To enumerate the brood size of mated females, some of these virgins were offered single males from the same strain and observed till mating was successful. All the females were then hosted individually with one fly pupa for 24 hours. These were kept at 25ºC for the offspring to emerge which were later counted for family size by randomizing the ria vials in a double-blind assay. The differences between groups were compared using the Kruskal - Wallis test with a significance level of 0.05. Mann-Whitney U test, with a significance level of 0.05, was used to compare two groups.

To investigate if *Wolbachia* affects the female fecundity, emerged females were hosted with one host for 24 hours. The host pupa was placed in a foam plug so that only the head portion of the pupa was exposed and available for the females to lay eggs. They were removed after 24 hours and the eggs laid were counted under a stereomicroscope (Leica M205 C). The differences in fecundity were compared between groups using the Kruskal -Wallis test with a significance level of 0.05. The fecundity difference between two groups was compared using the Mann-Whitney U test with a significance level of 0.05.

2.2.4 Estimation of relative density of *Wolbachia* infections across different developmental stages of *N. vitripennis*

To collect the different developmental stages, females were hosted for 4 hours, (instead of 24 hours in the previous experiments), with one host to narrow down the developmental stages of the broods. The larval and pupal stages (from Day 3 to Day 13 for males and from Day 8 to Day 14 for females) were collected every 24 hours. Larval stages for females were not done to avoid any DNA contamination from the males as the two sexes are virtually indistinguishable at the larval stage. Three replicates of ten larvae or pupae from the three strains, *w*A(PU), *w*B(PU), and *w*A*w*B(PU), were collected for each developmental stage. DNA extraction was done using the phenol-chloroform extraction method, where samples were crushed in 200 µl of 0.5 M Tris-EDTA buffer with 1% sodium dodecyl sulfate (SDS) and 2 µl of 22 mg/ml Proteinase K and incubated overnight at 37°C. DNA was purified with buffer saturated phenol and chloroform-isoamyl alcohol solution (24:1) and precipitated overnight with isopropanol at -20° C. The precipitated DNA pellet was dissolved in 60 µl nuclease-free water. The DNA concentration of the samples was measured using the Nanodrop 2000® spectrophotometer (Thermo Scientific). The extracted DNA was checked with *28S* primers to confirm the PCR suitability of the DNA. The concentrations of all the samples were normalized to 200 ng/µl across the different male and female developmental stages to be used for quantitative PCR. The CFX96 C1000® Touch Real-time qRT-PCR machine (BioRad) was used to assay the relative density of *Wolbachia* across the strains. Amplification was done for the *Wolbachia* hcpA gene (Forward Primer: 5’-CTTCGCTCTGCTATATTTGCTGC-3’, Reverse Primer: 5’-CGAATAATCGCAACCGAACTG-3’). The primers were tested to amplify both the *Wolbachia* supergroup A and B strains. *Nasonia* S6K was used as the control gene (Bordenstein and Bordenstein, 2011). Each reaction of 10 µl contained 5 µl of iTaq Universal SYBR® Green supermix (BIO-RAD), 0.05 µl each of 10 µM of forward and reverse primers, and 200 ng of template DNA. Uninfected *N. vitripennis* DNA was used as negative control while DNase-free water was used as a no-template control. Reaction conditions included an initial denaturation step of 95°C for 3 minutes followed by 39 cycles of 95°C for 10 seconds, annealing, and amplification at 52°C for 30 seconds. All the reactions were performed in triplicates and included a melt curve to check for non-specific amplification. The relative *Wolbachia* density was estimated by calculating the mean delta threshold cycle (ΔC_q), using the formula:

3. $\Delta C_q = 1/3 \sum_{j=1}^{3} [1/3 \sum_{i=1}^{3} hcpA - 1/3 \sum_{i=1}^{3} S6K]$
4. where i = number of technical replicates and j = number of biological replicates.

5. 1/ΔCq was calculated and plotted to show the *Wolbachia* density across different developmental stages. Mann Whitney U test was used to compare two different groups with a significance level of 0.05.

2.3 Results

2.3.1 The presence of *Wolbachia* reduces the lifespan of both males and females

Wolbachia can compete with the host for available nutrition which can increase nutritional stress resulting in a shortened lifespan for many hosts (McMeniman *et al.,* 2009; Caragata *et al.,* 2014). Therefore, we first investigated the effect of *Wolbachia* infections on the survival of both male and female wasps. As figure 2.1 (A), indicates, there is a significant difference in the longevity of the infected males across the three infection types. The double-infected line, *w*A*w*B(PU), starts to die off first and has a significantly shorter life span compared to the two single infected lines {Log Rank Test, χ^2=16.8, $p < 0.001$ for *w*A(PU) and χ^2=33.9, $p < 0.001$ for *w*B(PU)}. Males from the uninfected line, 0(PU), lived the longest and showed significantly longer life span compared to all the other infected lines {Log Rank Test: χ^2=76.3, $p < 0.001$ for *w*A*w*B(PU); χ^2=33.0, $p < 0.001$ for *w*A(PU) and χ^2=16.3, $p < 0.001$ for *w*B(PU)}. However, there was no significant difference in the life span of the two single infected lines of *w*A(PU) and *w*B(PU) {Log Rank Test, χ^2=3.84, $p = 0.05$}. Thus, the presence of *Wolbachia* leads to a significant reduction in the life span of the infected males. However, complex phenotypes like longevity can also be affected by the host genotype.

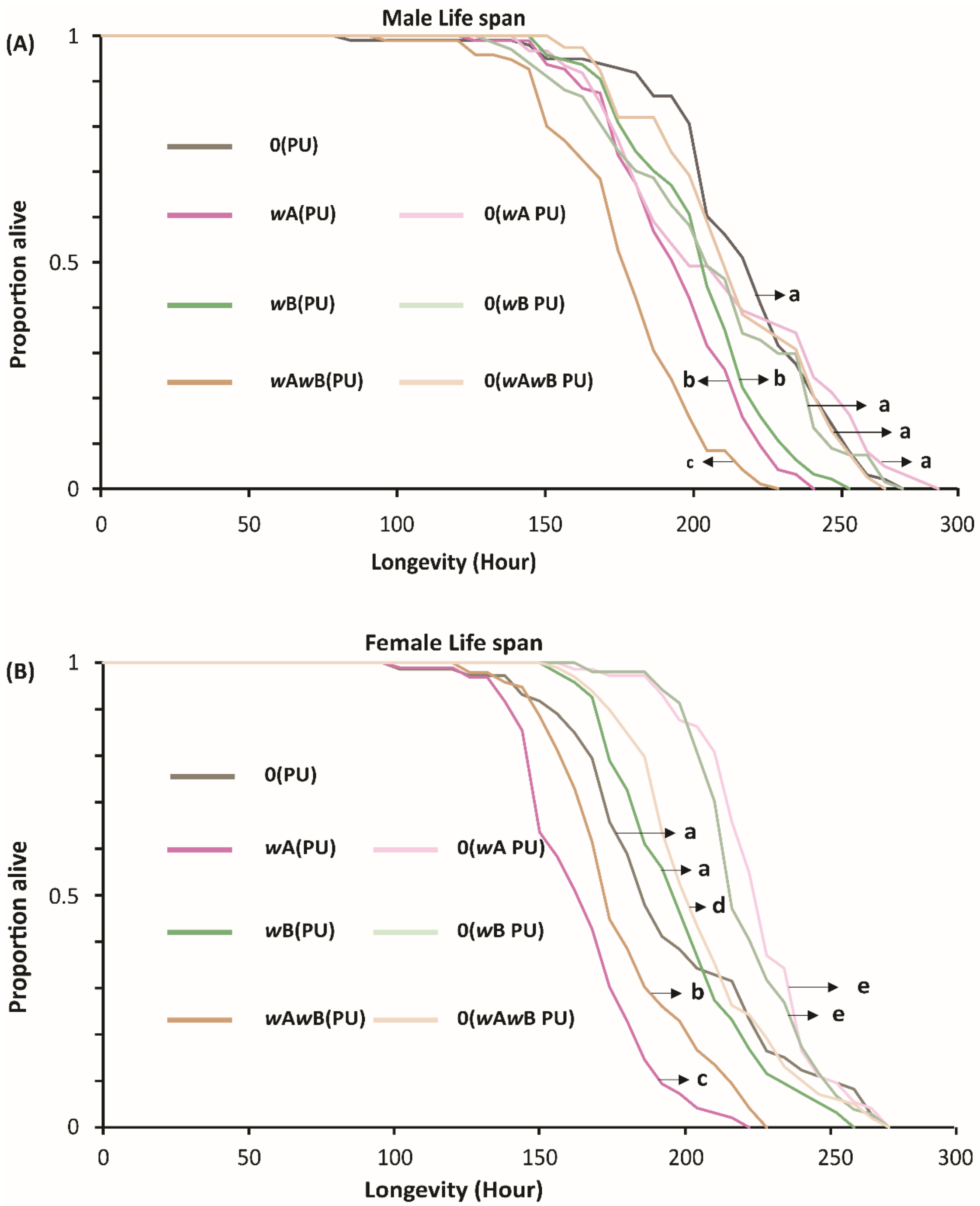


Figure 2. 1 *Wolbachia* infected males and females show reduced lifespan

(A): Life span of males. (B): Life span of females.

Statistical significance was tested using Log Rank statistics with $p < 0.05$.

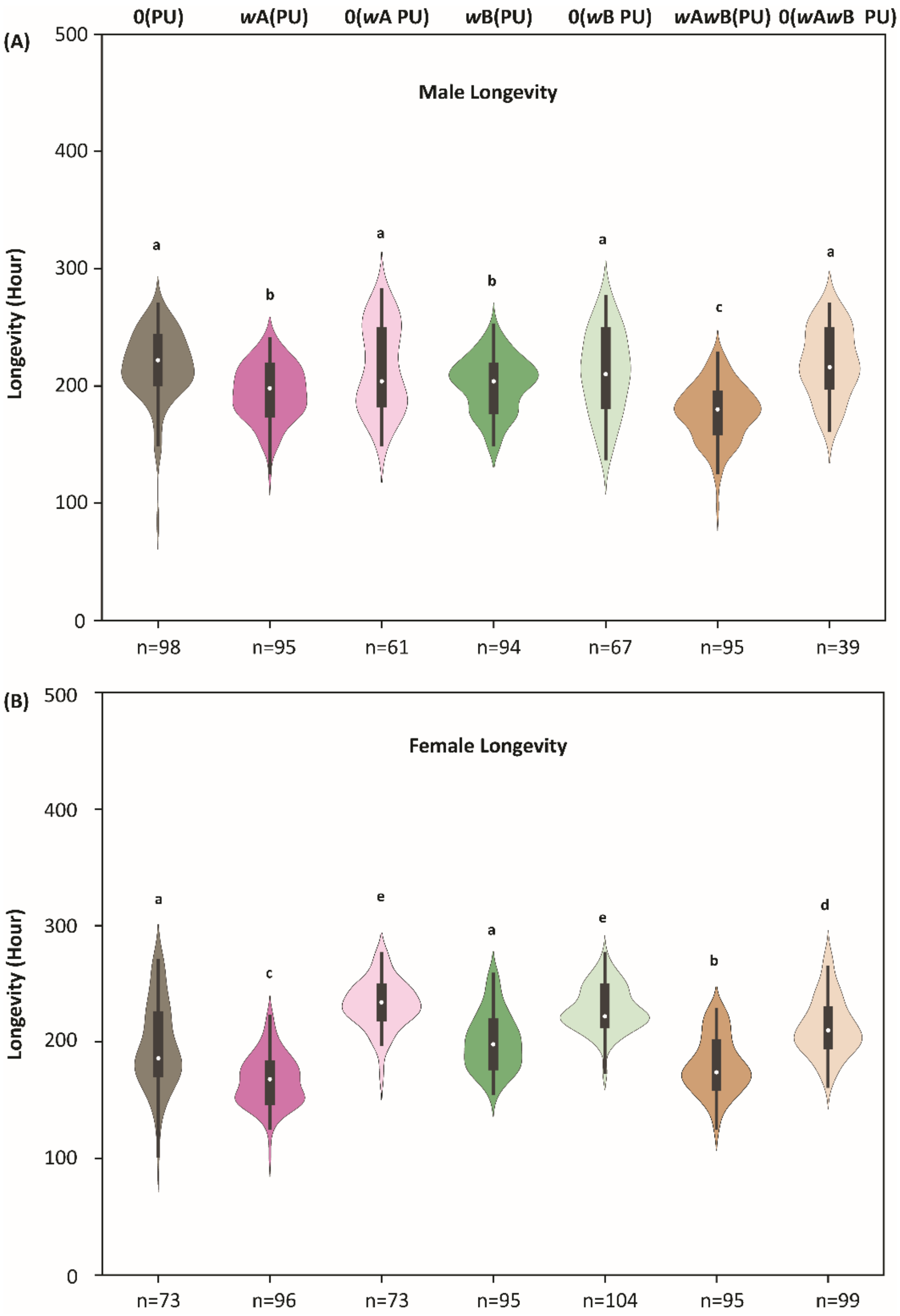


Figure 2. 2 The average life span and distribution for (A) males and (B) females.

A significant difference was observed between the average life span of the males of different N. vitripennis lines (Kruskal-Wallis: H=115.99, p < 0.001).

***w*A*w*B(PU) had shorter life span compared to the two single infected lines {MWU, U = 6130, p < 0.0001 for *w*A(PU) and U = 6604, p < 0.0001 for *w*B(PU)}. 0(PU) showed significantly longer life span compared to all the other infected lines {MWU, U = 2324, p < 0.05 for *w*A(PU); U = 6261, p < 0.0001 for *w*B(PU), and 8276.5, p < 0.0001 for *w*A*w*B(PU)}.**

The recently cured lines 0(*w*A PU), 0(*w*B PU) and 0(*w*A*w*B PU) showed significantly longer life span than their parental lines *w*A(PU) {MWU: U = 1957.5, $p < 0.01$}, *w*B(PU) (MWU: U = 2324.5, $p < 0.05$) and *w*A*w*B(PU) {MWU: U = 441.5, $p < 0.0001$} respectively, and were comparable to the uninfected line 0(PU) {MWU: U = 3256, p = 0.36 for *w*A(PU), MWU: U = 3484, p = 0.25 and MWU: U = 1808, p = 0.66 for *w*A*w*B(PU)}.

A significant difference was observed between the average life span of the females of different *N. vitripennis* lines (Kruskal-Wallis: H=298.09, $p < 0.001$).

The single A infected *w*A(PU) females, showed the shortest life span {MWU: U = 3112.5, $p < 0.0001$ for *w*A*w*B(PU), U = 1331, $p < 0.0001$ for *w*B(PU) and U = 5307, $p < 0.0001$, for 0(PU)} followed by *w*A*w*B(PU) { MWU: U = 6500, $p < 0.0001$ for *w*B(PU) and U = 4452.5, $p < 0.0001$ for 0(PU)}. 0(PU) and *w*B(PU) females showed similar life spans { MWU: U = 3027, p = 0.24}.

The recently cured lines of 0(*w*A PU), 0(*w*B PU) and 0(*w*A*w*B PU) showed significant increase in the life span when compared to their parent lines *w*A(PU) {MWU: U = 96, $p < 0.0001$}, *w*B(PU) {MWU: U = 1746.5, $p < 0.0001$} and *w*A*w*B(PU) {MWU: U = 1608, $p < 0.0001$} respectively. The recently cured lines showed longer life span than 0(PU) {MWU: U = 1012.5, $p < 0.0001$ for 0(*w*A PU), U = 1667, $p < 0.0001$ for 0(*w*B PU), U = 3112.5, $p < 2370.5$ for 0(*w*A*w*B PU)}.

Although all these four lines were derived from the same field-collected isofemale line, continuous culturing in the laboratory can fix specific alleles within them resulting in interline divergence. Moreover, it is also known that in *Nasonia* the effect of *Wolbachia*-induced phenotype is influenced by the hosts' genetic background (Raychoudhury *et al.*, 2012). Therefore, we cured all these infections again and tested whether the host genotype, rather than *Wolbachia*, is causing this reduction in life span. This was done by comparing the longevity of these newly cured lines back with the previously used uninfected line, 0(PU). The recently cured lines 0(*w*A PU), 0(*w*B PU) and 0(*w*A*w*B PU) showed significantly longer life span than their parental lines *w*A(PU) {Log Rank Test: χ^2=16.47, $p < 0.0001$}, *w*B(PU) (Log Rank Test: χ^2=9.36, $p < 0.01$) and *w*A*w*B(PU) {Log Rank Test: χ^2=35.04, $p < 0.0001$} respectively, and were comparable to the uninfected line 0(PU) {Log Rank Test: χ^2=0.76, p = 0.38 for *w*A(PU), χ^2=0.04, p = 0.8 and χ^2=0.475, p = 0.50 for *w*A*w*B(PU)}.

Similarly, infected females {(figure 2.1(B)} also showed a distinct reduction in life span when compared with the uninfected line. However, unlike the males, the single A infected *w*A(PU) females, showed the shortest life span {Log Rank Test: χ^2=11.2, $p < 0.001$ for *w*A*w*B(PU), χ^2=56.9, $p < 0.001$ for *w*B(PU) and χ^2=31.1, $p < 0.001$, for 0(PU)} followed by *w*A*w*B(PU)

{Log Rank Test: χ^2=20.4, $p < 0.001$ for *w*B(PU) and χ^2=12.9, $p < 0.001$ for 0(PU)}. Curiously, 0(PU) and *w*B(PU) females showed similar life spans {Log Rank Test: χ^2=0.24, p = 0.62}.

The recently cured lines of 0(*w*A PU), 0(*w*B PU) and 0(*w*A*w*B PU) showed significant increase in the life span when compared to their parent lines *w*A(PU) {Log Rank Test: χ^2=107.31, $p < 0.0001$}, *w*B(PU) {Log Rank Test: χ^2=39.02, $p < 0.0001$} and *w*A*w*B(PU) {Log Rank Test: χ^2=48.77, $p < 0.0001$} respectively. Surprisingly, the recently cured lines showed longer life span than 0(PU) {Log Rank Test: χ^2=19.31, $p < 0.0001$ for 0(*w*A PU), χ^2=16.57, $p < 0.0001$ for 0(*w*B PU), and χ^2=4.26, $p < 0.05$ for 0(*w*A*w*B PU)}.

These results indicate a sex-specific variation in longevity as the *w*A*w*B(PU) line shows the shortest life span among the males but *w*A(PU) shows the shortest among the females. Moreover, the effect of single infections on longevity also varied among the sexes as *w*A(PU) and *w*B(PU) males had similar life spans, but it was *w*B(PU) and 0(PU) who had similar life spans among the females. But what is unambiguous from these results is that the uninfected line always lived the longest, irrespective of the sex of the hosts. The increase in the life span of the recently cured lines indicates that the presence of *Wolbachia* is associated with the reduction in life span and is thus, costly for *N. vitripennis* to maintain.

2.3.2 The presence of *Wolbachia* reduces the number of copulations a male can perform

Wolbachia is known to be associated with a reduction in the number of mating a male can perform in *Ephestia kuehniella* (Sumida *et al.,* 2017). To test whether similar effects are seen in *N. vitripennis,* we enumerated the number of copulations an individual male can perform across the infection types. As figure 2.3 indicates, a significant difference was observed in the number of copulations performed by the males of different *N. vitripennis* lines (Kruskal-Wallis: H=23.06, $p < 0.001$). There is indeed a reduction in the capacity of infected males to mate. When compared with the uninfected line 0(PU), this reduction was most pronounced in *w*B(PU) (MWU, U=30, $p < 0.01$), followed by *w*A*w*B(PU) and *w*A(PU), which showed similar successful copulations (MWU: U=11, p = 0.49). The uninfected 0(PU) line produced males with the highest number of copulations {MWU: U= 32, $p < 0.05$ for *w*A(PU) and U= 27, $p < 0.05$ for *w*A*w*B(PU)}. Thus, the presence of *Wolbachia* substantially reduced the number of copulations that a male could perform. As figure 2, indicates, males from most of these re-cured lines showed a marked and significant increase in the number of copulations performed. This number in the re-cured double infected line, 0(*w*A*w*B PU), increased to similar levels as shown by 0(PU) (an increase of 29%, MWU: U=9.5, p = 0.2), while also showing a significant increase from its infected counterpart *w*A*w*B(PU) (from 73.5 ± 10.5 to 94.8 ± 15.39, MWU: U=3, $p < 0.05$).

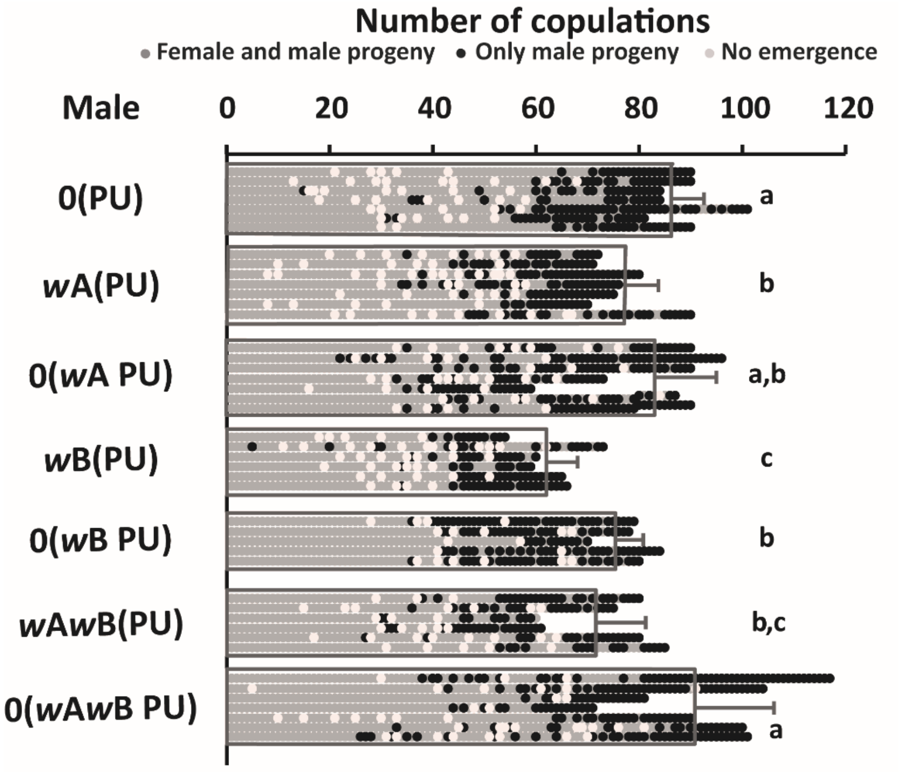


Figure 2. 3 *Wolbachia* infected males show a reduction in the number of copulations

Males from different *Wolbachia* infection status strains were mated sequentially until each of them stopped mating. Some of the matings had “no emergence” of progenies because of poor host quality (shown by white dots). The results show that the presence of *Wolbachia* is associated with the reduction in the number of copulations a male can perform. The figure also shows whether the progenies of these sequential copulations produce any daughters or not, as a measure of sperm depletion. The details of sperm depletion are shown in figure 3. Sample sizes for the lines 0(PU), *w*A(PU), 0(*w*A PU), *w*B(PU), 0(*w*B PU), *w*A*w*B(PU) and 0(*w*A*w*B PU) were n=7, n=7, n=7, n=6, n=5, n=6 and n=7 respectively.

Similarly, the number of copulations for the re-cured single A supergroup infected line, 0(*w*A PU), also increased to the levels of the uninfected line 0(PU) (an increase of 7%, MWU: U=20, p = 0.76). However, this increase (from 77.5 ± 6.3 to 83.5 ± 12.9) with its infected counterpart was not significant (MWU: U=23, p = 0.48).

The re-cured line from the single B supergroup infection, 0(*w*B PU), was the only line that did not revert to uninfected levels (MWU: U=22, $p < 0.05$) despite showing a marginal increase (from 62.8 ± 6.6 to 78.2 ± 5.1; MWU: U=1, $p < 0.05$). However, what is evident is that the presence of *Wolbachia* is also associated with a reduction in the capability of a male to mate. Furthermore, by curing the infected lines again, we showed that this decrease is not due to the host genotype but is an effect of the presence of *Wolbachia* in these lines.

2.3.3 *Wolbachia* infected males deplete their sperm reserves faster than the uninfected ones

N. vitripennis males are prospermatogenic (Boivin *et al.*, 2005), where each male emerges with their full complement of mature sperm and has not been reported to produce any more during the rest of their life span (Chirault *et al.*, 2016). Thus, if a single male is mated sequentially with as many females as it can mate with, it should eventually run out of this full complement of sperm and produce all-male broods even after successful copulation. As figure 2.4, indicates, each male did run out of sperm at the tail end of this continuous mating and produced only male progenies (shown by black dots). We looked at the number of mating done by these males before sperm depletion to see if *Wolbachia* affects the sperm production in the males. As shown in figure 3, the average number of daughter progenies reduced with the number of mating (shown by the primary Y-axis on the left), indicating sperm depletion. Similar to copulation numbers, a significant difference was observed in the number of matings before sperm depletion between the males of different *Wolbachia* infected lines (Kruskal-Wallis: H=21.48, $p < 0.01$). *w*B(PU) males were the quickest to deplete their sperm reserve {MWU: U=27.5, $p < 0.05$ for *w*A(PU) and U=30, $p < 0.01$ compared for 0(PU)}. This was followed by *w*A*w*B(PU) and *w*A(PU) (MWU: U=13, $p = 0.7$). However, the uninfected males from 0(PU) were the slowest to deplete their sperm reserve {MWU: U= 35, $p < 0.01$ for *w*A(PU) and U= 24, $p < 0.05$ for *w*A*w*B(PU)}.

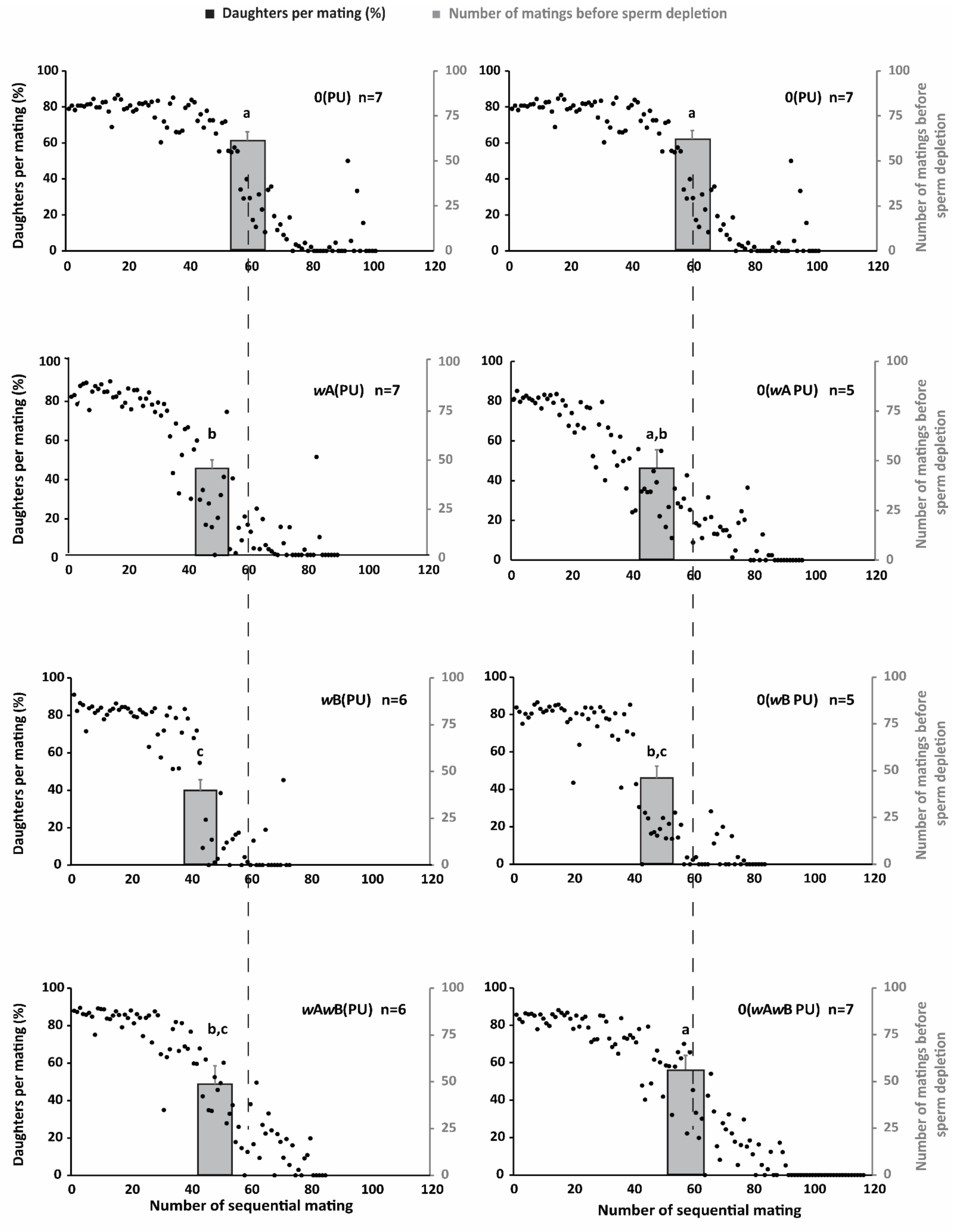


Figure 2. 4 *Wolbachia*-infected males deplete their sperm faster than uninfected males

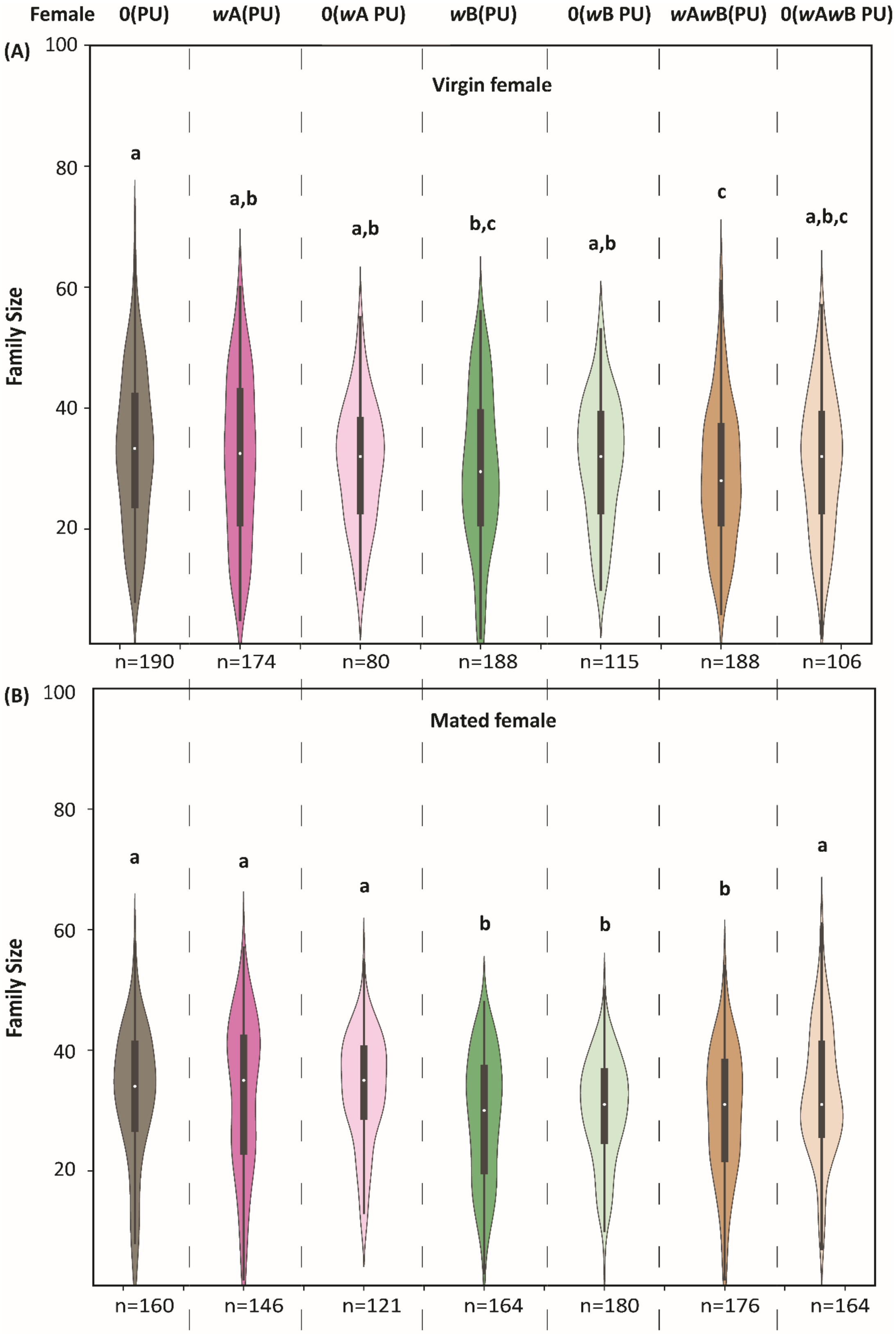
The Y-axis in black on the left of each figure represents the percentage of daughters produced for each mating. The black dots represent the average number of daughters produced for each sequential mating by the males of different *Wolbachia* infection statuses (detailed in figure 2). The number of daughters produced is taken as a measure of the number of sperm transferred during each mating. The Y-axis, in grey, on the right, for each figure tallies the average number of copulations that yielded at least one daughter. Thus, it measures the number of mating before a male is depleted of its sperm. The left panel shows the males from *Wolbachia* infected lines whereas the right panel shows their respective cured versions. Data for 0(PU) is repeated at the top for comparison. The statistical significance was tested using the Mann-Whitney U test with $p < 0.05$.

We again tested whether the host genotype, rather than *Wolbachia*, is causing this rate of sperm depletion, by comparing it with the recently cured lines. As shown in figure 2.4, the number of mating before sperm depletion increased for the recently cured 0(*w*A PU) line up to the levels of 0(PU) (an increase of 5%, MWU: U=30, $p = 0.06$). However, this increase (from 48.14 ± 4.94 to 50.57 ± 9.41) was not significantly different from their infected counterpart *w*A(PU) (MWU: U=16, $p = 0.8$). Rates of depletion for 0(*w*A*w*B PU) also increased up to the levels of 0(PU) (an increase of 15.2%, MWU: U=21, $p = 0.66$). Again, the cured line 0(*w*B PU), increased from *w*B(PU) (from 41 ± 1.67 to 47.6 ± 6.0,MWU: U=0, $p < 0.05$) but was still lower than 0(PU) (MWU: U=23, $p < 0.05$). These results indicate that the presence of *Wolbachia* has a significant negative impact on the number of sperm produced or utilized by the infected lines.

2.3.4 *Wolbachia*-infected females produce fewer offspring

Wolbachia is known to have a negative impact on the progeny family size of its host (Hoffmann *et al.*, 1990; Hohmann *et al.*, 2001). To test whether a similar effect is seen in *N. vitripennis*, we enumerated the family sizes for both virgins and mated females for the four different *Wolbachia* infected lines and their recently cured counterparts.

As figure 2.5 (A), indicates, there is a significant reduction in the average family sizes of all-male broods produced by the virgin females of the *Wolbachia* infected *N. vitripennis* lines (Kruskal-Wallis: H=12.6, $p < 0.05$). When compared with the uninfected line 0(PU), this reduction was most pronounced in *w*A*w*B(PU) (MWU: U=21151.5, $p < 0.01$) followed by *w*B(PU) (MWU: U=19880.5, $p < 0.05$). *w*B(PU) and *w*A*w*B(PU) showed similar family size (MWU: U=18582.5, $p = 0.29$). However, *w*A(PU) produced similar family sizes when compared with 0(PU) (MWU: U=17191, p = 0.39) and *w*B(PU) (MWU: U=17284, p = 0.26) but had larger all-male brood sizes than *w*A*w*B(PU) (MWU: U=18252, p < 0.05). We also



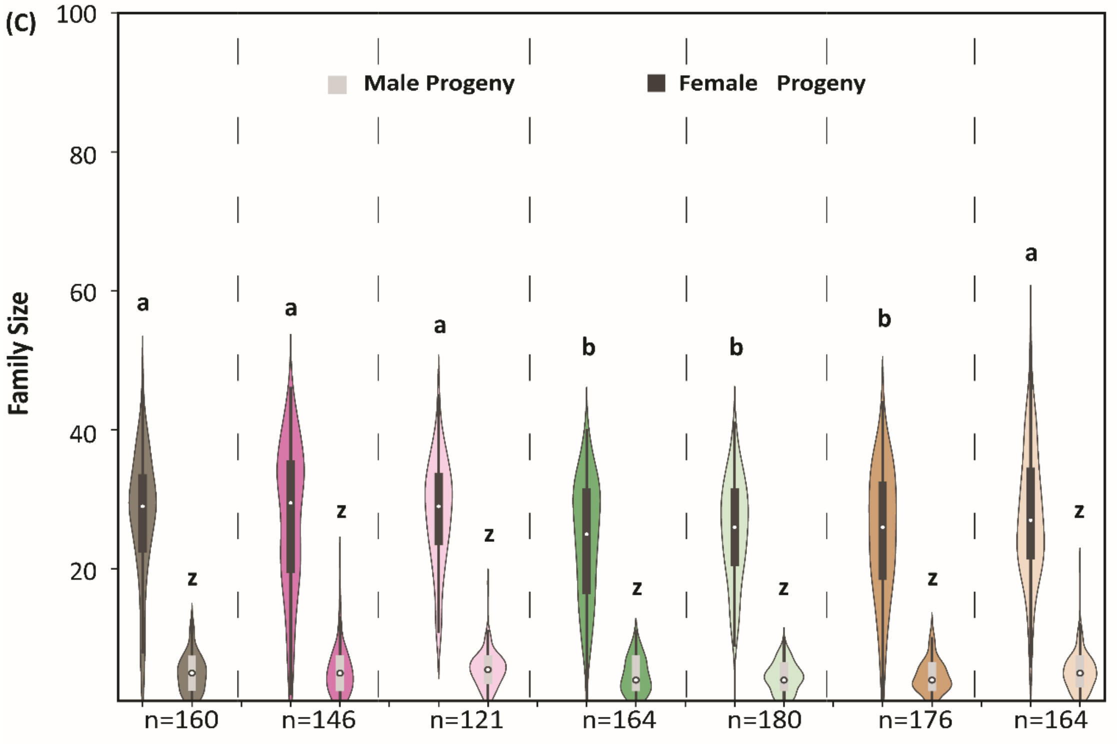


Figure 2. 5 *Wolbachia*-infected females produce fewer offspring

Family size is produced by females when hosted as virgins (A) and mated (B). The difference in the family size of mated females is due to the difference in the number of daughters (C) as there is no significant difference in the number of males produced. The statistical significance is tested using the Mann-Whitney U test with $p < 0.05$.

compared the recently cured single and double infected lines with the infected parental lines. 0(*w*B PU) and 0(*w*A*w*B PU) showed marginal increase in their family sizes which was comparable to the uninfected line 0(PU) {an increase of 1.5%, MWU: U=11554, p = 0.29 for 0(*w*B PU); an increase of 2%, MWU: U=10798, p = 0.21 for 0(*w*A*w*B PU)}. However this marginal increase {from 29.84 ± 12.94 to 31.44 ± 10.30 for 0(*w*B PU) and from 28.99 ± 11.60 to 30.97 ± 11.48 for 0(*w*A*w*B PU)} was not significantly different from their infected counterparts *w*B(PU) (MWU: U=9963.5, p = 0.34) and *w*A*w*B(PU) (MWU: U=8650.5, p = 0.09). The recently cured line 0(*w*A PU) did not show any increase in the family size and was comparable to *w*A(PU) (MWU: U=7085.3, p = 0.63) and 0(PU) (MWU: U=8161, p = 0.22). Similar to the virgin females, a reduction was also observed for average family sizes of mated females as shown in figure 2.5 (B) (Kruskal-Wallis: H=30.45, p < 0.0001). When compared with the uninfected line 0(PU), this reduction is most pronounced in *w*B(PU) (MWU: 15582, p < 0.01) and *w*A*w*B(PU) (MWU: U=16303, p < 0.01). However, *w*B(PU) and *w*A*w*B(PU) showed similar family sizes (MWU: 13732.5, p = 0.55). Interestingly, the *w*A(PU) line showed similar family sizes as 0(PU) (MWU: U=11396.5, p = 0.86) but had larger family sizes when compared to *w*B(PU) (MWU: U=14080, p < 0.01) and *w*A*w*B(PU) (MWU: U=14682, p < 0.05). Upon curing, the average family sizes of the recently cured 0(*w*A*w*B PU) reverted back to the levels of the uninfected line 0(PU) (an increase of 11.8%, MWU: U= 13295, p = 0.61) showing a significant increase from the infected counterpart *w*A*w*B(PU) (from 29.49 ± 10.67, MWU: U=12023, p < 0.05). The recently cured line 0(*w*A PU) did not show any significant increase from the infected counterpart *w*A(PU) (MWU: U=8385.5 p = 0.69) and was comparable to 0(PU) (MWU: U=9022.5, p = 0.5). However, 0(*w*B PU) line did not show an increase to the levels of the uninfected line 0(PU) (an increase of 4.3%, MWU: U=16782, p < 0.05). The marginal increase in the family sizes (from 28.72 ± 10.46 to 29.97 ± 8.59) was not significantly different from the parental line *w*B(PU) (MWU: U=13854, p = 0.47).

To understand whether this difference in the family size of the mated females is due to the production of fewer daughters or sons or both, we compared their numbers separately for the four lines {(figure 2.5 (C)}. No difference was observed in the number of sons produced by the mated females. However, significant differences were observed in the number of daughters produced. When compared to the uninfected line 0(PU), *w*B(PU) and *w*A*w*B(PU) showed the least number of daughters produced {MWU: 15964, p < 0.01 for *w*B(PU) and U=16283, p < 0.01 for *w*A*w*B(PU)} whereas *w*B(PU) and *w*A*w*B(PU) produced nearly equal number of daughters (MWU: 13392, p = 0.33). Again, *w*A(PU) line produced equal number of daughters compared to 0(PU) (MWU: U=11543, p = 0.98) but higher in number than *w*B(PU) and *w*A*w*B(PU) {MWU: U=14201, p < 0.01 for *w*B(PU) and MWU: U=14372, p < 0.05 for *w*A*w*B(PU)}. Upon curing, the recently cured 0(*w*A*w*B PU) reverted to the levels of the uninfected line 0(PU) (MWU: U= 13545, p = 0.42) showing a significant increase in the number of daughters from the infected counterpart *w*A*w*B(PU) (MWU: U=12331, p < 0.039). The recently cured line 0(*w*A PU) did not show any increase in the number of daughters produced from their infected counterpart *w*A(PU) (MWU: U=8468 p = 0.79) and was also comparable to 0(PU) (MWU: U=9330, p = 0.84). However, recently cured line 0(*w*B PU) did not increase to the levels of the uninfected line 0(PU) (MWU: U=16749.5, p < 0.01).

To determine if the negative effect on progeny family size in females is not limited to NV-PU14 *N. vitripennis*, we also checked the virgin and mated female progeny family size of another *N. vitripennis* line NV KA from Bangalore (India).

The double infected *w*A*w*B(KA) line was cured to generate recently cured 0(*w*A*w*B KA). In the average family sizes of all-male broods produced by the virgin females {(figure 2.6 (A)}, the recently cured line 0(*w*A*w*B KA) has more progenies as compared to *w*A*w*B(KA) (MWU: U=1534.5, $p < 0.05$). Similar to the virgin females, the mated females {(figure 2.6 (B)} of 0(*w*A*w*B KA) also produced more progenies as compared to *w*A*w*B(KA) (MWU: U=2568.5, $p < 0.05$). Thus, the negative effects of the presence of *Wolbachia* on the family sizes produced were confirmed in two different geographical lines of *N. vitripennis*.

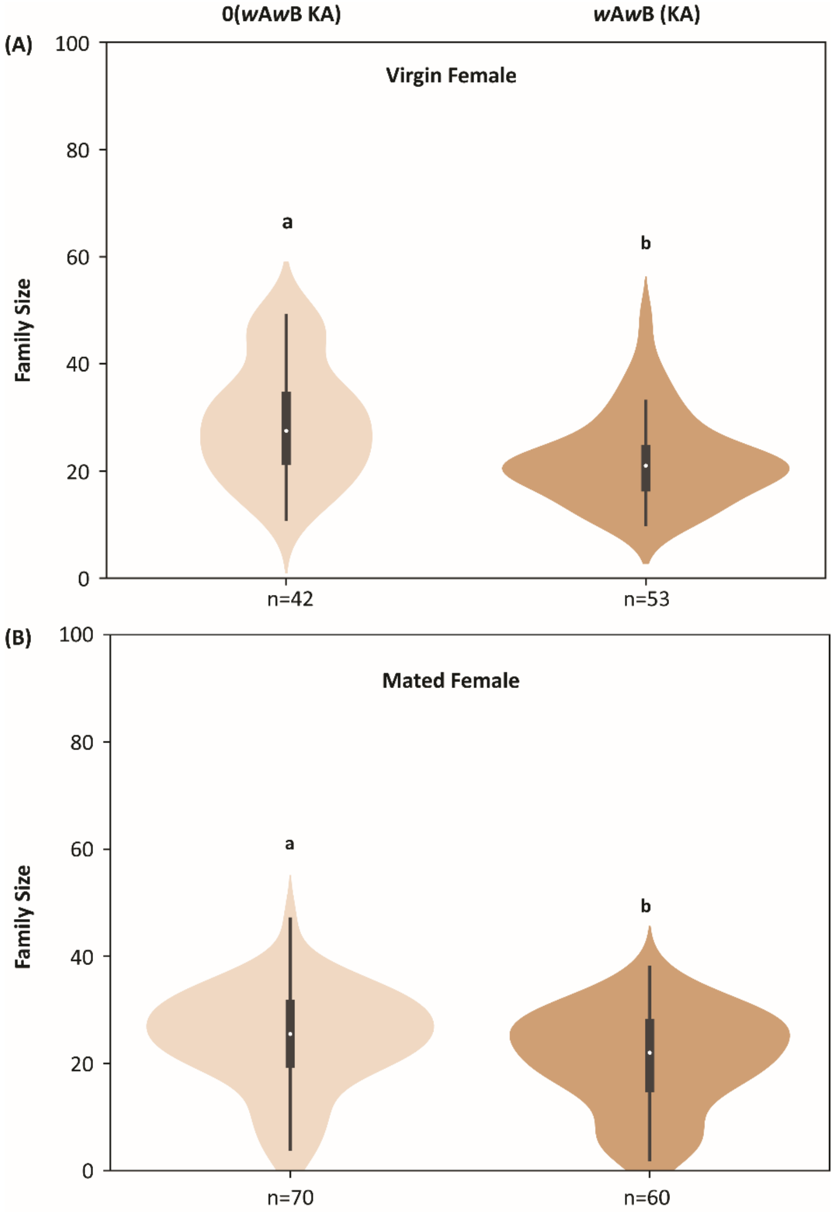


Figure 2. 6 Progeny family size of *N. vitripennis* line NV KA.

Family size is produced by females when hosted as virgins (A) and mated (B). The statistical significance is tested using the Mann-Whitney U test with $p < 0.05$.

2.3.5 *Wolbachia* negatively impacts the fecundity of infected females

To check whether the differences in the family sizes between the different infected lines of *N. vitripennis* are due to the number of eggs being laid by the females, we looked at the fecundity of both virgin and mated females across these lines. Among the virgin females {(figure 2.7 (A)} significant differences were observed in the fecundity of the different *N. vitripennis* lines (Kruskal-Wallis: H=28.8, p < 0.001). *w*A*w*B(PU) had the least fecundity (MWU: U=8424.5, p < 0.0001) when compared to the uninfected line 0(PU). Significant differences were observed between 0(PU) and *w*A(PU) (MWU: U=5383, p < 0.05), between 0(PU) and *w*B(PU) (MWU: U=3600.5, p < 0.05), and also between *w*A(PU) and *w*A*w*B(PU) (MWU: U=5155, p < 0.01). However, no difference was observed between *w*A(PU) and *w*B(PU) (MWU: U=2153.5, p = 0.46). Upon curing, the recently cured 0(*w*A*w*B PU) reverted to the levels of the uninfected line 0(PU) (MWU: U= 3404.50, p = 0.42) showing a significant increase in the number of eggs laid from the infected counterpart *w*A*w*B(PU) (MWU: U=2101.5, p < 0.05).

However, the recently cured line 0(*w*A PU) (MWU: U=3137.5, p < 0.01) and 0(*w*B PU) (MWU: U=3077, p < 0.05) did not increase to the levels of the uninfected line 0(PU).

Among the mated females {(figure 2.7 (B)} (Kruskal-Wallis: H=42.6, p < 0.001), *w*A*w*B(PU) again had the least fecundity (MWU: U=9410.5, p < 0.0001) when compared to the uninfected line 0(PU). *w*B(PU) had similar fecundity as that of *w*A*w*B(PU) (MWU: U=4731, p = 0.098) but had significantly lower fecundity than 0(PU) (MWU: U=7052.5, p < 0.01) and *w*A(PU) (MWU: U=6684, p < 0.05). However, *w*A(PU) showed higher fecundity than *w*A*w*B(PU) (MWU: U=8899, p < 0.01) and was similar to the uninfected line 0(PU) (MWU: U=10100, p = 0.5) . Upon curing, the recently cured 0(*w*A*w*B PU) line reverted to the levels of the uninfected line 0(PU) (MWU: U= 3415, p = 0.149), showing a significant increase in the number of eggs from the infected counterpart *w*A*w*B(PU) (MWU: U=1265, p < 0.0001). However, the recently cured line 0(*w*B PU) (MWU: U=3655, p < 0.05) did not increase to the levels of the uninfected line 0(PU) and was still comparable to the infected counterpart *w*B(PU) (MWU: U=1713.5, p = 0.79)

The results thus suggest a negative effect of *Wolbachia* on egg production in females. The assay also established that the difference in family sizes can be due to the differences in the fecundity of the females.

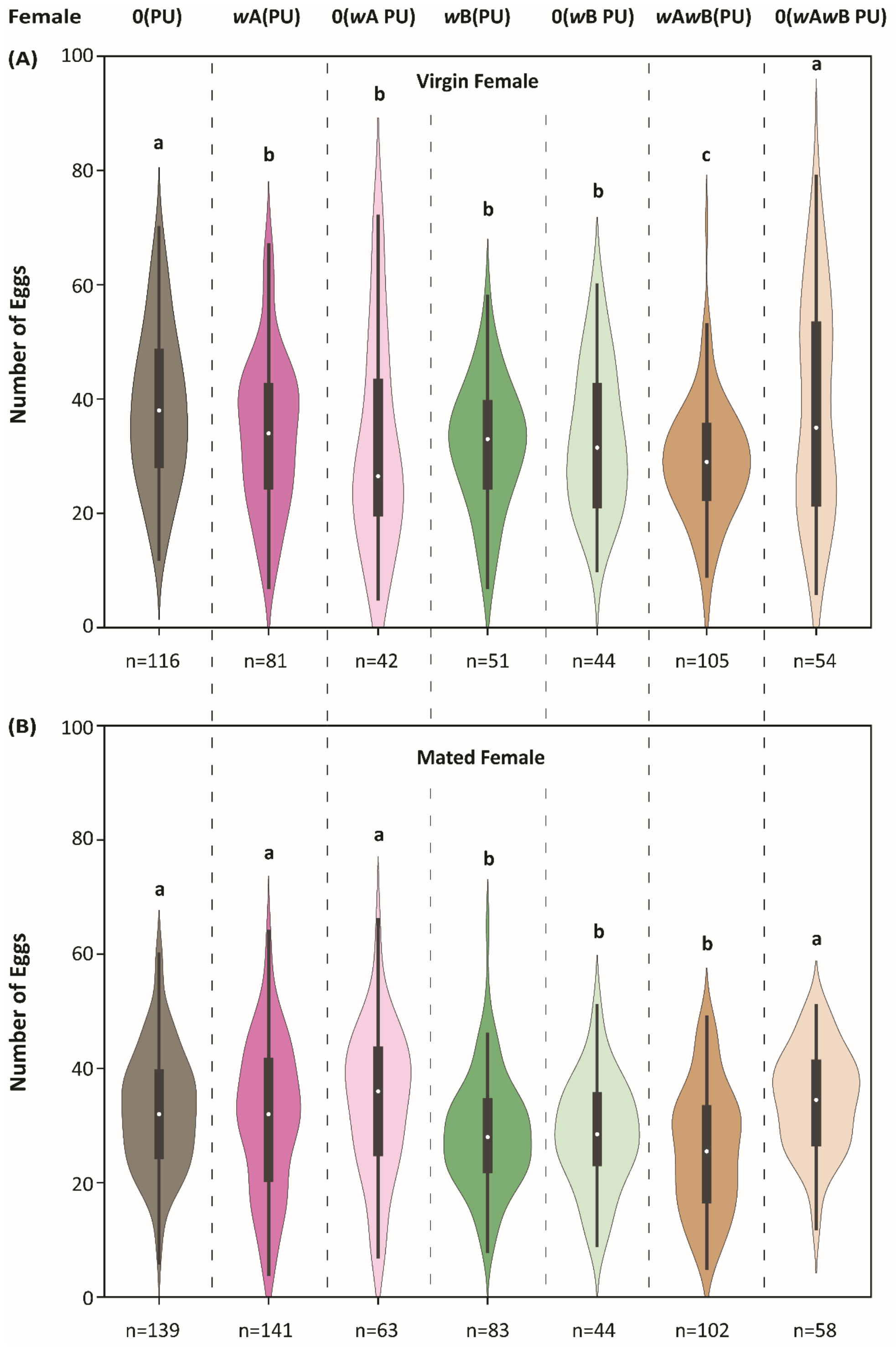


Figure 2. 7 *Wolbachia* infection reduces female fecundity

The measure of fecundity (number of eggs laid) by females of different *Wolbachia* infection status {virgin females (A) and mated females (B)}. The statistical significance is tested with the Mann-Whitney U test, $p<0.05$.

2.3.6 Relative *Wolbachia* density in single and multiple *Wolbachia* infection strains

Wolbachia density has a major role to play in expressing the effects of the infection on host biology (Hoffmann *et al.*, 1996; Min & Benzer, 1997).

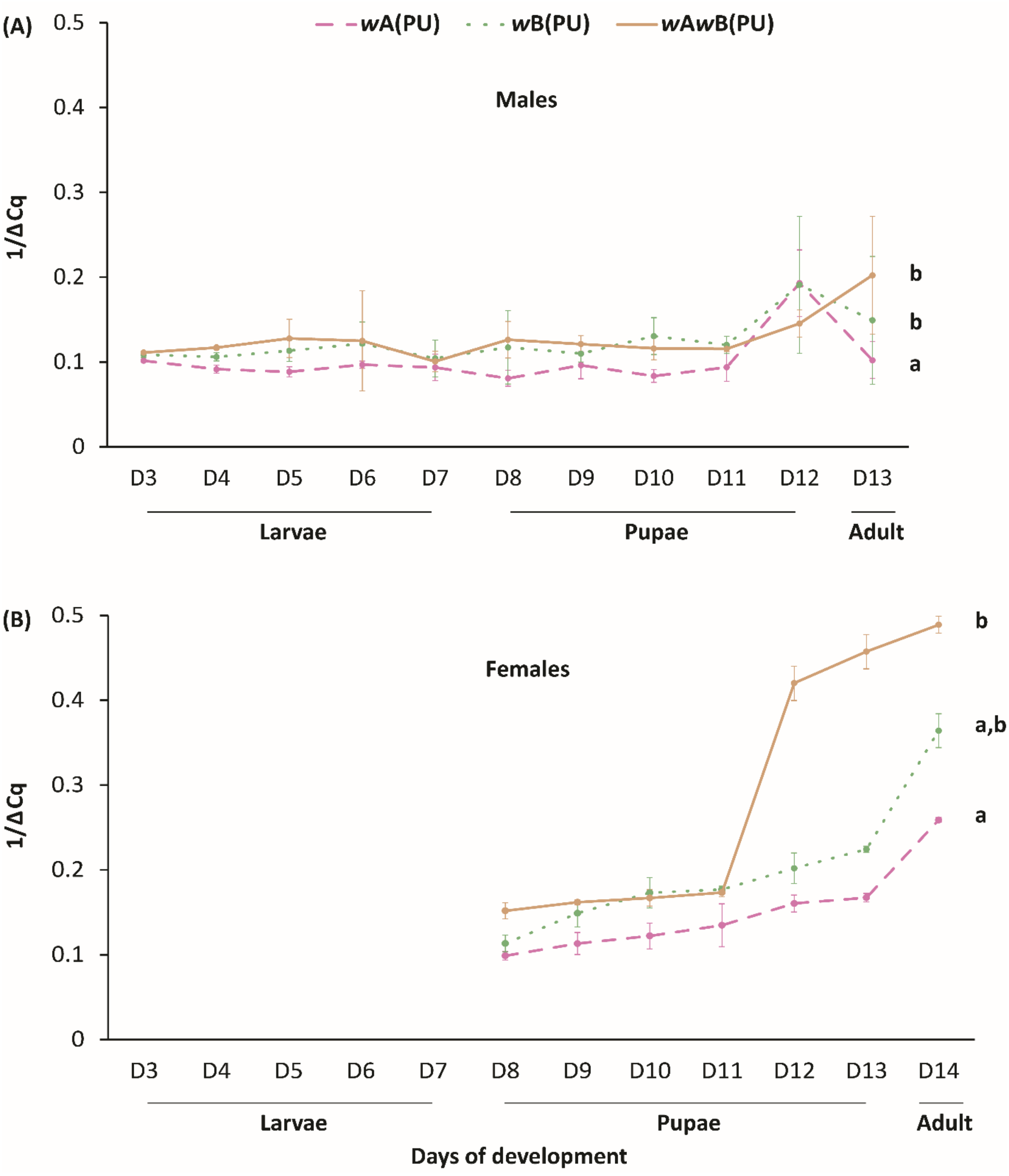


Figure 2. 8. Quantitative estimation of *Wolbachia* across different developmental stages of *N. vitripennis*

Males (A) and females (B). The statistical significance between groups is tested using the Mann-Whitney U test, p < 0.05.

An increase in cellular *Wolbachia* density is often associated with a greater expression of their effects (Breeuwer and Werren, 1993). Thus, we estimated *Wolbachia* titers across the different developmental stages of *N. vitripennis*. In the case of males {(figure 2.8 (A)} *w*A(PU) had the lowest *Wolbachia* density across different larval and pupal developmental stages when compared with *w*B(PU) (MWU: U=11, p < 0.01) and *w*A*w*B(PU) (MWU: U=12, p < 0.01). However, no such differences were found between *w*B(PU) and *w*A*w*B(PU) (MWU: U=51, p = 0.56).

In the case of females {(figure 2.8 (B)}, *w*A(PU) showed lower levels of *Wolbachia* when compared to *w*A*w*B(PU) (MWU: U=8, p < 0.05) again at the pupal and adult stages. However, no difference was observed between *w*A(PU) and *w*B(PU) (MWU: U=12, p = 0.12) and also *w*B(PU) and *w*A*w*B(PU) (MWU: U=19, p = 0.5).

2.4 Discussion

The results from this study (summarized in Table 2) demonstrate a sex-independent cost of the presence of single and multiple *Wolbachia* infections. Many phenotypes show a significant reduction across the sexes such as longevity (figure 2.1) where infected males and females show reduced life span. When compared with the uninfected line 0(PU), the *Wolbachia* infected lines *w*B(PU) and *w*A*w*B(PU) have reduced life spans. However, sex-specific variations have also been observed among the infected lines where *w*A(PU), in females, had the shortest life span, while in the case of males, had a greater life span than that of *w*A*w*B(PU). *Wolbachia* affects the reproductive capabilities of the infected males, reduces their copulation capability (figure 2.3), and also leads to quicker sperm depletion (figure 2.4). Such negative effects on reproductive traits were also observed in females where the infected females produce fewer progenies (figure 2.5). These differences are elicited at the level of female fecundity where the infected females lay fewer eggs (figure 2.7) indicating that the negative effects of *Wolbachia* manifest themselves even before the egg-laying stage. However, the egg to larval to pupal stage mortality could also have an effect on the brood sizes but these were not assayed. In most cases, these negative effects disappear with the removal of *Wolbachia*, indicating the role of *Wolbachia* in producing these negative effects and not the host genotype. In phenotypes like longevity, family sizes, and fecundity, the recently cured lines show a significant increase suggesting that the negative effects are due to the presence of *Wolbachia*. However, 0(*w*B PU) did not revert to the levels of 0(PU) in the number of copulations performed, sperm depletion assays, and female fecundity. A possible reason can be some residual effects of the parent genotype in 0(*w*B PU) but needs further empirical validation.

Our experiments indicate an additive or synergistic effect of the presence of the two different *Wolbachia* supergroups in the double infected line *w*A*w*B(PU). Evidence of such effects can be seen in traits like male longevity (additive effect) where the deficit in longevity for *w*A*w*B(PU) is equal to the total deficits caused by *w*A(PU) and *w*B(PU). Similarly, for traits like female longevity, virgin female family size, and female fecundity, the negative effects on the *w*A*w*B(PU) line appear to be a combined effect of both the *w*A(PU) and *w*B(PU) lines (i.e., a synergistic effect). Since the two supergroup infections are bidirectionally incompatible with each other, it is plausible that they are also competing for the host nutrition, which can further enhance the negative impacts of these infections.

Our results also demonstrate supergroup-specific negative effects on the host. Supergroup B *Wolbachia* is costlier to maintain in both the sexes than supergroup A. While the *w*B(PU) line shows strong effects of supergroup B *Wolbachia* on all the traits studied across the sexes, *w*A(PU) has significant negative effects of supergroup A *Wolbachia* only on the reproductive traits of the males and the longevity of females. *w*A(PU) females, as an exception, have their family sizes comparable to 0(PU). These observations are unique as no comprehensive data is available on the supergroup-specific cost of *Wolbachia* infections in most insect systems.

Previous reports have suggested a direct correlation between *Wolbachia* density and the level of CI (Breeuwer & Werren, 1993; Noda *et al.*, 2001; Ikeda *et al.*, 2003; Dutton & Sinkins, 2004; Ruang-Areerate & Kittayapong, 2006). Our results also suggest that the cost of *Wolbachia* maintenance is correlated with the density of *Wolbachia* strains present in the host. Thus, in the case of females, *w*A*w*B(PU), which shows a high bacterial load, has reduced fecundity and longevity. Similarly, in the case of males, the *w*A*w*B(PU) shows a reduced number of copulations and the number of sperm produced/transferred. *w*B(PU), shows substantial detrimental effects in both the sexes of the host which is similar to *w*A*w*B(PU). This again can be explained by the high *Wolbachia* supergroup B load in both the sexes of *w*B(PU). However, although *w*A(PU) males show a reduced number of copulations and number of sperm produced/transferred, *w*A(PU) females had progeny family sizes as well as the fecundity of mated females comparable to 0(PU) females. A possible explanation for this can be the relatively low density of supergroup A *Wolbachia* in *w*A(PU) across the different developmental stages (figure 2.8) as compared to the other infections. Many intracellular bacteria like *Wolbachia*, *Rickettsia*, and *Phytoplasma* are known to have mobile genetic elements in their genome (Kent and Bordenstein, 2010). *Wolbachia* in particular is known to be infected with Prophage WO (named after *Wolbachia*). PCR amplification of the capsid gene indicates the presence of Prophage WO in the majority supergroup A and some supergroup B *Wolbachia* in arthropods. While supergroup C and D *Wolbachia* does not contain phage infection (Gavotte *et al.,* 2004, 2007). Supergroup A infected *N. vitripennis* lines are known to have relatively higher levels of phage density (Bordenstein *et al.,* 2006) and according to the phage density model, this higher phage density has an inverse impact on the level of CI caused by supergroup A *Wolbachia*. This results in a significant reduction in the *Wolbachia* titer and hence shows a milder intensity of the effect of CI. Our results also confirm these previous reports of the positive correlation between *Wolbachia* abundance and the level of CI induced not only in *N. vitripennis* (Bordenstein *et al.,* 2006b) but in other insect taxa as well (Ijichi *et al.,* 2002; Kondo *et al.,* 2002). *w*B(PU) *Wolbachia* shows complete CI and has higher *Wolbachia* titers in both males and females, which is also comparable to the *Wolbachia* densities of *w*A*w*B(PU) males and females, whereas *w*A(PU) has the lowest *Wolbachia* titers among the three strains and shows incomplete CI (Appendix figure 1). Thus, higher levels of *Wolbachia* in *w*B(PU) than in *w*A(PU) can also explain the more severe effects in *w*B(PU) than *w*A(PU).

The negative fitness effects of CI-inducing *Wolbachia,* and nutritional competition raises important questions on the maintenance of these endosymbionts over long evolutionary time scales. Theoretical studies indicate that evolution toward mutualism can aid the long-term persistence of these maternally inherited reproductive parasites (Prout, 1994; Turelli, 1994). Moreover, if there are indeed some adverse effects of maintaining *Wolbachia,* then hosts would be under strong selection pressure to develop immunity against them. Evidence suggests that there are examples of such emergence of host genetic factors against *Wolbachia* infections in *Drosophila* and mosquitoes (Zug and Hammerstein, 2015). Host suppressor alleles have been identified which confer resistance against feminizing (Rigaud *et al.,* 1999) and male-killing *Wolbachia* (Hornett *et al.,* 2006). However, no such host genetic factors have been found for CI-inducing *Wolbachia,* especially in *N. vitripennis*. Therefore, a possible explanation for the maintenance of these multiple infections then comes from the high efficiency of transmission of these infections in *N. vitripennis,* which is nearly 100% (Breeuwer and Werren, 1990). Theoretical studies also suggest that even in the presence of selective pressures, multiple infections are maintained and transmitted owing to the fitness advantages conferred and CI (Vautrin *et al.,* 2008).

Another possibility can be that these *Wolbachia* infections in *N. vitripennis* are relatively recent, the evidence of which comes from the rapid spread of *Wolbachia* in populations of *N. vitripennis* across North America and Europe (Raychoudhury *et al.,* 2010b). These recent infections, although bearing a cost on the host at present, might eventually lead to the evolution of host resistance against them.

Our results indicate supergroup B to be a “stronger” *Wolbachia* than supergroup A and any competition for nutritional resources and niche habituation between them should drive out the supergroup A *Wolbachia*. Moreover, *w*A(PU) has milder effects on females with the reduction in longevity being the only pronounced negative effect. Therefore, the continuation of this supergroup infection is difficult to explain. One possibility could be the supergroup A infection conferring mutualistic effects on the host. This strain is closely related to other supergroup A *Wolbachia* strains like *w*Mel in *D. melanogaster* and *w*Ha, *w*Au, and *w*Ri in *D. simulans* (Díaz-Nieto *et al.,* 2021). *w*Mel in *D. melanogaster* and *w*Ha, *w*Au, and *w*Ri in *D. simulans* are known to provide defense against viral infections to their hosts (Teixeira *et al.,* 2008; Bhattacharya *et al.,* 2017; Pimentel *et al.,* 2021). The continued presence of supergroup A *Wolbachia* in *N. vitripennis* could be due to such defenses against viral infections but this hypothesis remains to be tested.

The higher cost of maintenance of supergroup B *Wolbachia* can be an attribute of the CI phenotype induced by supergroup B *Wolbachia*. Complete CI (i.e, nearly 100%) are rare events reported mainly for supergroup B *Wolbachia* in *Culex pipiens*, *Aedes aegypti* (Sinkins *et al.,* 2005; Xi *et al.,* 2005), and *N. vitripennis* (Appendix figure 1 and Bordenstein *et al.,* 2006a). This essentially means that nearly the entire sperm complement of each male has the *Wolbachia*-induced CI modification and correspondingly, nearly all the eggs from the females have the rescue effect (Werren *et al.,* 2008). Introducing 100% modification and rescue would necessitate relatively high *Wolbachia* titers to be maintained in both sexes which in turn can cause an elevated nutritional burden, eventually resulting in negative effects on the physiological traits of the host. This seems a plausible explanation for both the high negative effects as well as the relatively higher titers of *Wolbachia* seen in *w*B(PU).

CI-inducing *Wolbachia* is known to have negative effects on various physiological traits in the vast majority of its host population (summarized in Table 1). The present study also suggests such effects, or a “cost”, associated with the maintenance of *Wolbachia* infection in *N. vitripennis*. This is in contrast to the previous reports suggesting positive fitness effects (Stolk C & Stouthamer R, 1996) and no fitness effects (Bordenstein and Werren, 2000) of *Wolbachia* on *N. vitripennis*. However, the strain used are all from India and the negative effects seen can be unique to these lines. Although the lines used here have the same or very similar *Wolbachia* as far as sequence uniformity is concerned across the five MLST alleles, other lines from other continents need to be analyzed to confirm whether this effect is ubiquitous in *N. vitripennis*.

Chapter 3

Identification of the developmental stage of *Wolbachia*-induced sperm modification in *N. vitripennis*.

3.1 Introduction

Wolbachia has a wide continuum of interactions with their hosts ranging from mutualism to parasitism (Werren *et al.*, 2008). The ability to manipulate the host reproductive biology gives *Wolbachia* the capability to effectively control their own transmission. Cytoplasmic incompatibility (CI) is the most common *Wolbachia*-induced reproductive phenotype and has been observed in the orders Diptera, Coleoptera, Hemiptera, Hymenoptera, Isopoda, Lepidoptera, Orthoptera, and Acari (Landmann, 2019). In CI, the sperm produced by *Wolbachia*-infected males are incompatible with eggs produced by uninfected females or females infected with a different *Wolbachia*, which often leads to embryonic death (Zabalou *et al.*, 2008).

The most accepted hypothesis explaining the phenomenon of CI is that of the "modification" of the sperm and its corresponding "rescue" in the female egg with the same infection (Werren, 1997). Cytological evidence of CI identifies defects in paternal chromatin condensation leading to defective chromosomal segregation during embryonic mitosis resulting in embryonic lethality (Callaini *et al.*, 1997; Zabalou *et al.*, 2008). The incompatible crosses between a *Wolbachia*-infected male and uninfected female has a delay in the deposition of maternal H3.3 and H4 histones of the paternal pronucleus leading to a delay in the zygotic pronucleus formation and delayed or incomplete mitotic condensation of male chromosomes (Lassy and Karr, 1996; Callaini *et al.*, 1997; Tram *et al.*, 2006; Landmann *et al.*, 2009). When *Wolbachia* are present in the female egg, these defects in zygotic development are not observed. Thus, *Wolbachia* can modify the sperm through the potential secretion of some factor(s) responsible for the modification, leading to CI, and can rescue the embryonic development when present in the egg, through the potential action of some rescue factor(s) (Zabalou *et al.*, 2008; Atyame *et al.*, 2011, 2014).

Recent studies on these modification and rescue factors have identified genetic determinants of CI in a two-gene operon called CI-factors or Cifs. The term Cif is generally used to address all the CI-factors expressed by this *Wolbachia* two-gene operon (Ref). The upstream gene within each operon is denoted by A while the downstream gene is denoted by B. This two gene operon can be divided into two groups based on the enzymatic activity of the B gene. The Cid operon (with genes *cid A* and *cid B*) has deubiquitylase activity while the Cin operon (with gene *cin A* and *cin B*) has nuclease activity. The transgenic expression of functional *cifA* and *cifB* of *w*Mel from *D. melanogaster*, and their orthologs *cidA* and *cidB* of *w*Pip from the mosquito *C. pipiens*, have resulted in the recapitulation of CI traits which includes post-zygotic male sterility (Beckmann *et al.*, 2017; Le Page *et al.*, 2017). Moreover, the expression of *w*Mel *cidA* and *cidB* in females leads to the rescue of these defects (Shropshire *et al.*, 2018; Shropshire and Bordenstein, 2019). However, still, the CI modification and rescue remains poorly understood. In the present study, we use *N. vitripennis* males as a model system to study *Wolbachia*-induced sperm modification.

N. vitripennis males are prospermatogenic (Boivin *et al.*, 2005), where each male emerges with their full complement of mature sperm and has not been reported to produce any more during the rest of their lifespan (Chirault *et al.*, 2016). Hence, sperm development in *N. vitripennis* is synchronized. Thus, specific stages of sperm development of *N. vitripennis* males can be targeted to alter *Wolbachia* levels and hence its effect on *Wolbachia*-induced CI phenotype.

In the present study, *N. vitripennis* sperm synchrony has been used to alter *Wolbachia* levels at specific stages of male development using antibiotics. Antibiotic delivery at these developmental stages led to a reduction and/or removal of *Wolbachia* levels and hence on the intensity of the *Wolbachia*-induced sperm modification. Developmental stages where such sperm modification is introduced during *N. vitripennis* male development has been identified. Then, stage-specific transcriptome has been done to identify candidate genes responsible for CI modification in *N. vitripennis* males by comparing it with uninfected males from the same stage.

3.2 Materials and Methods

3.2.1 Lines used and their maintenance

Single supergroup B line *w*B(PU), was used for the experiments. Supergroup B *Wolbachia* causes complete CI in *N. vitripennis* (Perrot-Minnot et al., 1996, and the present study). Thus, identifying CI phenotype is easier with the observation of all-male brood progeny. The uninfected 0(PU) line was used for the CI crosses. All the lines were raised on *Sarcophaga dux* fly pupae with a generation time of 14-15 days @ 25°C.

3.2.2 *N. vitripennis* male developmental stages

The different developmental stages of *N. vitripennis* males, ranging from the early larval to pupal stages were collected (Day 5 to Day 13) (Figure 3.3). To identify the stages, virgin mothers were hosted with one host for 4 hours. This relatively short time span was used to restrict the egg-laying to reduce any temporal difference between the stages from a single hosting. The females were then removed and the parasitized pupae were kept at 25°C for completion of development. This parasitized pupa was termed Day 1 of the developmental stages.

3.2.3 Stage-specific delivery of antibiotics

To identify during which developmental stage the sperm of *N. vitripennis* is modified, stage-specific antibiotic delivery methods were adopted. The approach was aimed at administering optimal antibiotic concentrations to all the individual developmental stages of *N. vitripennis* males. Once administered, these individual stages were then allowed to develop into adults. The newly emerged adult males were then mated with uninfected females to check the effect of antibiotic on CI phenotype.

If the sperm modification happens at a particular developmental stage, and is administered with optimal antibiotic concentration such that *Wolbachia* is removed from that stage and onwards, then *Wolbachia*-induced sperm modification (CI modification) can also be inhibited. Thus, those males (upon emergence), when mated with uninfected females, will not show the CI phenotype. As *N. vitripennis* is haplodiploid, such crosses will have both sons and daughters in the progenies. Any developmental stage before the prospective sperm modifiction stage, when subjected to optimal antibiotic concentrations, will also show a reduction in CI phenotype. However, any stage after the prospective sperm modification stage even subjected to antibiotic treatment will still show CI, as the sperm modification must have happened before the antibiotic exposure. Thus, mating of such males with uninfected females will produce all-male progenies.

The antibiotic delivery was achieved by several methods like larval culture using *Nasonia* rearing medium (NRM), manual injection into the parasitized fly pupae, surfactant mediated antibiotic delivery and microinjection.

3.2.4 Antibiotic delivery in larval culture using *Nasonia* Rearing Medium (NRM)

The NRM preparation protocol was adapted from Brucker *et al*., (2012) and Shropshire *et al.*, (2016).

3.2.4.1. Preparing *Nasonia* Rearing Medium (NRM)

a. 55 grams of two-to-three-day old *Sarcophaga dux* fly pupae were collected in a beaker and filled with sterile water to remove any particulate matter. The pupae covered with water were agitated for 5 minutes using a spatula. Water was discarded and the pupae were rinsed one more time to remove any leftover particulates.
b. The pupae were then covered with sterile water and the beaker was placed in a water bath at 36°C for 25 minutes to soften the pupae.
c. The pupae were removed from the water bath and homogenized with a sterile mortar pestle at room temperature.
d. The homogenate was filtered through a 75µm nylon mesh to remove unhomogenized particulates. The filtrate was collected in a sterile 50ml conical flask.
e. The filtrate was centrifuged at 25000 X G for 5 minutes at 4°C. The filtrate got separated into three distinct layers. While the top and the bottom layer consisted of the insoluble lipids and pupal debris, the middle layer consisted of the proteins which were pipetted into another sterile beaker under a laminar flow (Thermo scientific 1300 Series A2). The protein extract was filtered using a syringe filter (0.22µm) and transferred to a sterile beaker under laminar flow.

   2:1 ratio of Schneider's *Drosophila* medium (HIMEDIA) was added to the protein extract.

   The media was then filtered through progressively smaller filters of mesh sizes 0.8µm, 0.45µm, and 0.22µm and stored at 4°C for up to 2 weeks and at -20°C for two months. The NRM was filtered with a 0.22µm filter before use.
f. Antibiotic tetracycline (5mg/ml) was freshly added to the NRM before using for the experiment.

3.2.4.2. Setting up the growth assay in NRM

NRM was brought to room temperature in a sterile laminar flow hood. *N. vitripennis* larvae of respective days were collected immediately before the experiment and placed into a Cell Culture transwell (HIMEDIA) basket with 3µm pore size polycarbonate membrane and were sterilized twice with 70µl of 10% bleach solution and once with 70µl of 70% ethanol solution. The larvae were then washed with sterile water. The transwell baskets were then placed into the wells with 250µl of NRM mixed with antibiotic (5mg/ml). The plates were stored in sterile plastic boxes at 25°C under constant light conditions for the duration of the experiment.

The developing larvae fed with the NRM, mixed with 5 mg/ml tetracycline. The transwell were moved to new wells every 24-48 hours (depending on contamination and evaporation of NRM) with 250 µl of fresh NRM. The transwell were then moved to dry wells once the larvae reached the late pupal stage (Day 10). The surrounding wells were filled with sterile water to maintain humidity. Separate plates were set for tests as well as controls. The test plates contained NRM mixed with 5mg/ml tetracycline. The control plate did not contain any antibiotic. The males once emerged were set up for CI crosses with 0(PU) virgin females.

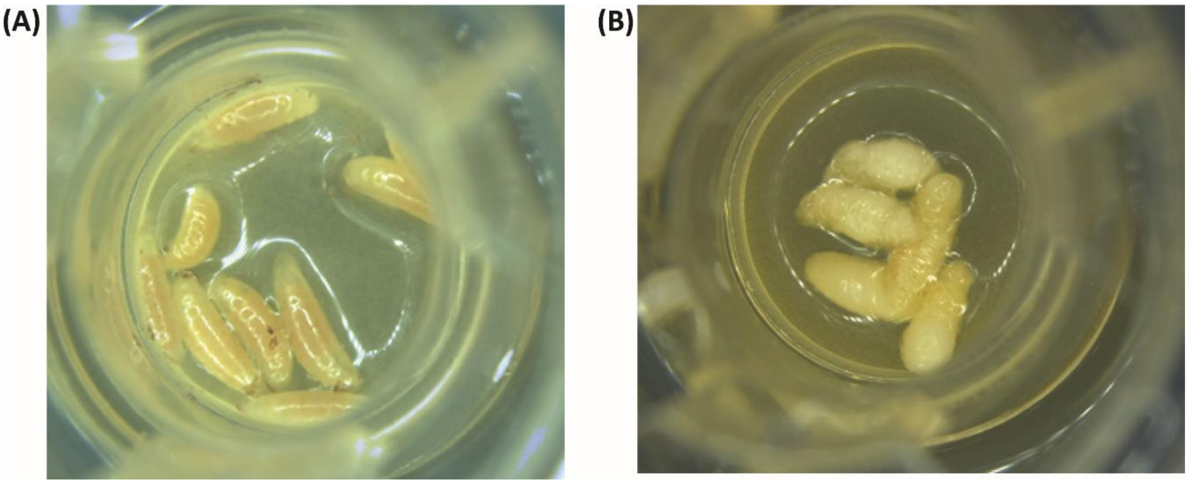


Figure 3. 1 Larval culture in *Nasonia* Rearing Medium (NRM)

(A) Larval culture in NRM with tetracycline. (B) Early pupal stage in NRM

3.2.5 Antibiotic delivery by injection into the parasitized pupae

The other method adopted to administer antibiotics to the larval stages of *N. vitripennis* males was by direct injection of antibiotic into the parasitized fly pupae. The fly pupae were placed in a foam plug so that only the posterior end of the pupae was available for parasitisation. The parasitized fly pupae containing the respective male developmental stages (figure 3.3) larvae were then injected with tetracycline solution (5mg/ml) using a sterile 1ml syringe. The antibiotic was mixed with a blue-coloured food dye (Chefmaster) in a 1:10 ratio, which worked as an indicator for the uptake of tetracycline by the larvae.

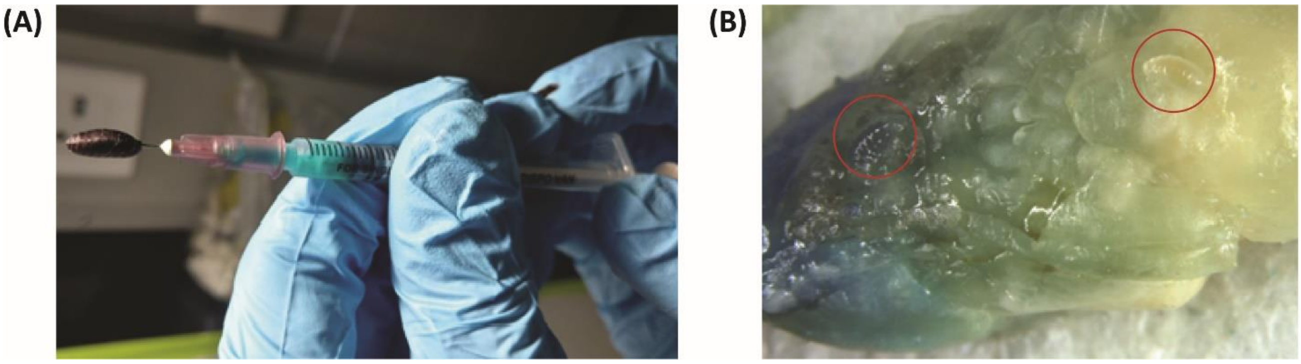


Figure 3. 2 Manual antibiotic injection into the parasitized fly pupae

(A) Injection of antibiotic mixed with coloured dye into the parasitized fly pupae. (B) Larvae that fed on an antibiotic (blue), larvae that did not feed on an antibiotic (yellow)

The antibiotic was pumped from the posterior end of the fly pupae until it started to ooze out. The hole in the pupae was then sealed using a liquid adhesive (Feviquick). All the developing larvae feeding on tetracycline turned blue while the larvae not feeding on tetracycline remained pale yellow. The blue-colored larvae were then separated at the late larval stage or early pupal stage (Day 7 or Day 8) and kept for emergence. These emerged males were used for CI crosses. The control did not contain any antibiotic along with the food dye.

3.2.6 Antibiotic treatment using surfactants

A surfactant named Silwet L-77 (Phytotech Labs) was used to deliver antibiotics at different stages of *N. vitripennis* male development. Silwet L-77 is used in agrobacterium-mediated transformation in plants (Clough *et al.* 1998). A concentration of 7.5 mg/ml tetracycline solution was mixed with 0.05 - 0.1% Silwet L-77. Different developmental stages of *N. vitripennis* male were collected and dipped into the antibiotic solution containing the surfactant for 30 seconds to one minute. The larvae/pupae were then taken out and kept at 25°C for emergence. The control did not contain antibiotics with the surfactant.

3.2.7 Antibiotic delivery by microinjection

The antibiotic delivery for the larval and the pupal stages was also done with microinjection. The microinjections were performed using the InjectMan Ni 2 from Eppendorf. The capillary needles (FHC, Borosil 1mm x 0.75mm inner diameter) were pulled to specific lengths using a microinjection puller from Shutter (model 1000). A concentration of 7.5 mg/ml tetracycline was used for both the larval and pupal stage injections. In the control set, the developmental stages were injected with sterile water.

3.2.8 DNA extraction and Quantitative estimation of *Wolbachia* in antibiotic-treated males

Quantitative estimation of *Wolbachia* titer was done for the males treated with antibiotics by microinjection. Individual males after CI cross with uninfected females were used for DNA extraction and quantitative PCR (qPCR). DNA extraction was done using the phenol-chloroform extraction method, where individual males were crushed in 100 µl of 0.5 M Tris-EDTA buffer with 1% sodium dodecyl sulfate (SDS), 1 µl of 22 mg/ml Proteinase K and incubated overnight at 37°C. DNA was purified with buffer saturated phenol and chloroform-isoamyl alcohol solution (24:1) and precipitated overnight with isopropanol at -20° C. The precipitated DNA pellet was dissolved in 40 µl nuclease-free water. The DNA concentration of the samples was measured using the Nanodrop 2000® spectrophotometer (Thermo Scientific). The concentrations of all the samples were normalized to 100 ng/µl across the different male developmental stages to be used for quantitative PCR. CFX96 C1000® Touch Real-time qRT-PCR machine (BioRad) was used to assay the presence of *Wolbachia* across the lines. Amplification was done for the *Wolbachia ftsZ* B gene (Forward Primer: 5’-AAAGATAGCCATATGCTCTTT-3’, Reverse Primer: 5’-CATTGCTTTACCCATCTCA-3’). *Nasonia* S6K was used as the control gene (Bordenstein and Bordenstein, 2011). The total reaction volume contained 5 µl of iTaq Universal SYBR® Green supermix (BIO-RAD), 0.05 µl each of 10 µM of forward and reverse primers, and 100 ng of template DNA for a total volume of 10 µl for each reaction. Uninfected *N. vitripennis* DNA was used as negative control while DNase-free water was used as non-template control. Reaction conditions included an initial denaturation step of 95°C for 3 minutes followed by 39 cycles of 95°C for 10 seconds, annealing, and amplification at 52°C for 30 seconds. All the reactions were performed in triplicates and included a melt curve to check for non-specific amplification. The presence of *Wolbachia* was estimated by the number of threshold cycles (Cq).

3.2.9 RNA extraction and mRNA enrichment for transcriptome analysis

After identifying the best candidate developmental stage for sperm modification, males from that stage were used for the transcriptome analysis to identify candidate genes responsible for CI. Males of the same stage from the uninfected 0(PU) line and *w*B(PU) were used as controls to identify the *Wolbachia* and host genes responsible for CI. 8-10 males were used for the total RNA extraction using SV Total RNA isolation kit from Promega (Z3105) following the manufacturer's protocol. The quality of the extracted RNA was determined using a NanoDrop 2000 spectrophotometer (Thermo Fischer Scientific).

The prokaryotic RNA pool was enriched using the MICROB*Enrich*™ Kit (Catalog number: AM1901) which provides modified oligo-nucleotides for separating prokaryotic RNA from a mixed pool of RNAs. The enriched bacterial RNA was obtained using the manufacturer's protocol.

The prokaryotic mRNA was further enriched by removing the rRNA from the mixed pool of prokaryotic RNA using MICROB*Express*™ Bacterial mRNA Enrichment Kit (Catalog number: AM1905). The quality of the enriched bacterial mRNA was determined using a NanoDrop spectrophotometer (Thermo Fischer Scientific). This enriched bacterial mRNA was used for cDNA synthesis and sequencing.

3.2.10 cDNA synthesis, Nanopore sequencing, and analysis of the transcriptome profile

The enriched prokaryotic mRNA was poly-Adenylated using the Poly(A) Tailing Kit (Invitrogen, Catalog number: AM1350)**.** Both the prokaryotic and the eukaryotic mRNA were then processed for cDNA synthesis. The double-stranded cDNA synthesis, barcoding, and Nanopore sequencing of the samples were done using the Direct cDNA Native barcoding kit (SQK-DCS 109 with EXP-NBD 104) from Oxford Nanopore technologies. 500ng of the poly-Adenylated mRNA was used to synthesize the double-stranded cDNA using the VN primer (VNP), strand switching primer (SSP), and PR2 primer following the manufacturer's protocol. The double-stranded cDNA for both the prokaryotic and the eukaryotic samples were then end prepared using the Ultra II end preparation reaction mix (Oxford Nanopore Technologies). These were then cleaned using the AMPure XP beads and eluted in sterile nuclease-free water. The barcode adaptors were ligated for the addition of unique barcodes using the EXP-NBD 104 barcoding kit (Oxford Nanopore Technologies). These barcoded cDNAs were again purified using the QIAquick PCR purification kit. All the barcoded samples were pooled in equimolar concentrations to prepare a final pooled library amounting to 1µg.

Sequencing adaptors were added to the library and cleaned using the AMPure XP beads. The samples were then sequenced on the MinION Flow cell (FLO-MIN106) R9 version with the protocol NC 48Hr sequencing “FLO-MIN106 SQK-DCS 109 plus Basecaller” for 48 hours.

The raw sequencing reads obtained were first converted to FASTQ format and separated according to barcodes (demultiplexed) using the Guppy program (Oxford Nanopore Technologies). High-quality reads (average score ≥10) were sorted using the Nanofilt program (Oxford Nanopore).

The resultant reads were identified by comparing them with the reference “nr” database of the National Center for Biotechnology Information (NCBI) using local BLAST tool., The “nr” database is a protein database containing non-identical sequences from GenBank, Protein Data Bank (PDB), Swiss-Prot, Protein Information Resource (PIR), and Protein Research Foundation (PRF).

3.2.11 Statistical analysis

The effect of antibiotics on CI phenotype was estimated by the number of daughters produced by the antibiotic-treated males. The increase in the number of daughters was compared between the treated and untreated males by the Mann -Whitney U test with $p < 0.05$.

3.3 Results

The antibiotic treatment was done at the mid and late larval stages and all the pupal stages of *N. vitripennis* male development (Figure 3.3).

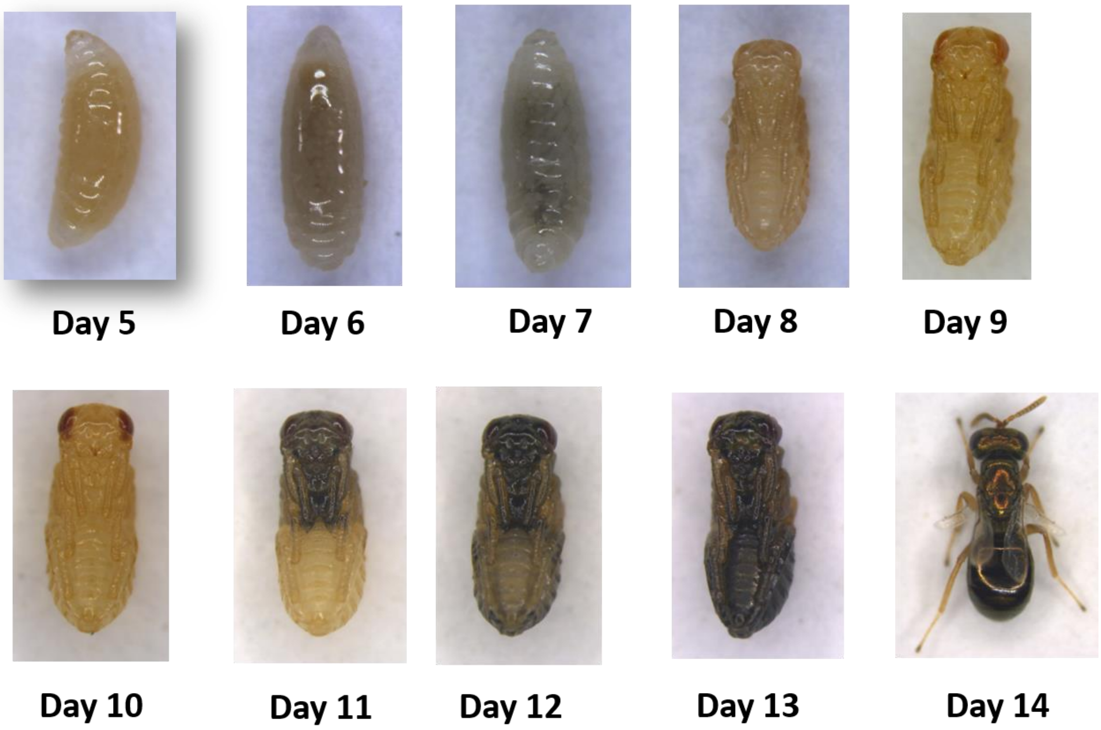


Figure 3. 3 ***N. vitripennis*** **male developmental stages**

3.3.1 Antibiotic delivery by NRM and manual injection methods

NRM was used to deliver antibiotics to the mid and late larval stages of *N. vitripennis* males. Day 5 to Day 7 larvae were grown in NRM along with antibiotic concentrations ranging from 1 mg/ml to 10 mg/ml (data not shown) out of which 5 mg/ml concentration was selected to be used for the study owing to higher effectivity and lesser toxicity. As shown in figure 3.2, the antibiotic-treated Day 5 and Day 6 larvae produced daughters upon mating with 0(PU) females, while the untreated (control) males did not produce any daughters (MWU: U=0, $p < 0.0001$ for Day 5; MWU: U=0, $p < 0.0001$ for Day 6). Thus, antibiotic treatment removed *Wolbachia* from that particular stage of development which resulted in no CI. However, Day 7 larvae treated with the antibiotic did not produce any daughters. A plausible explanation can be that the antibiotic treatment might not have worked for Day 7 as the particular stage larvae may not have fed on the NRM. Day 7 to Day 8 is the transition stage between larval and pupal stages in *N. vitripennis* male development. By Day 7, the larvae may have stopped feeding, and hence the antibiotic treatment did not work.

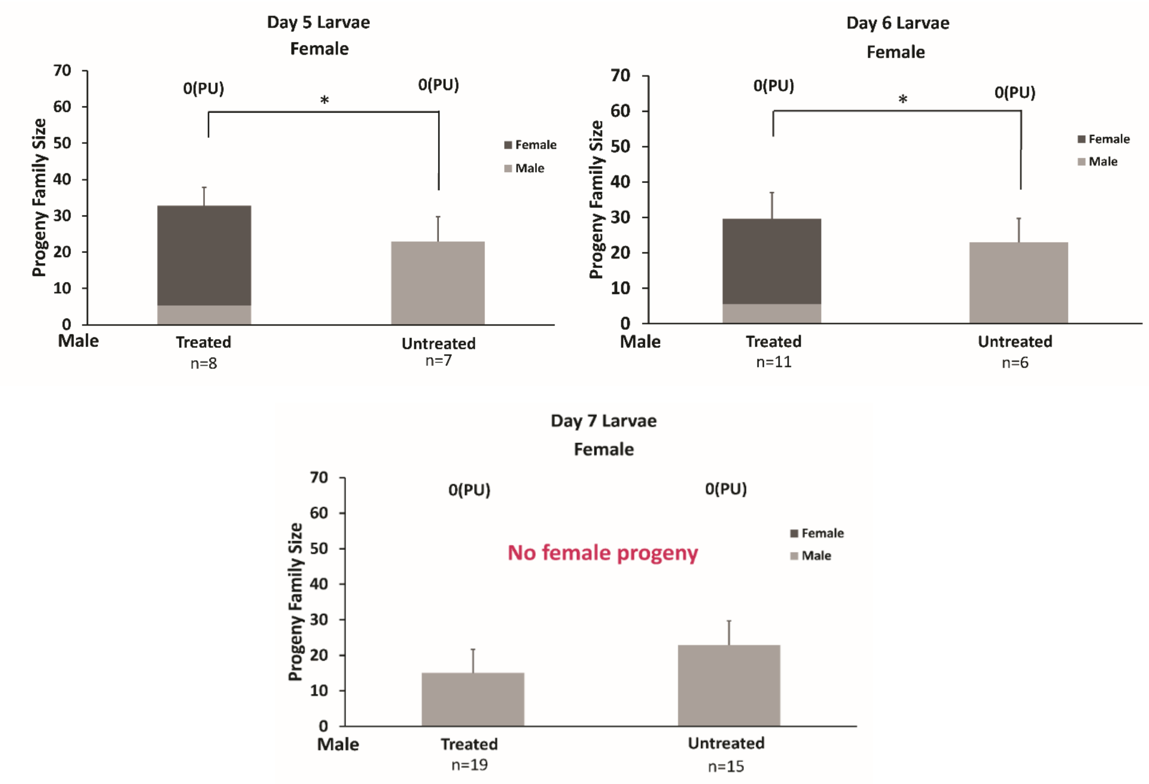


Figure 3. 4 Antibiotic delivery by *Nasonia* Rearing Medium (NRM)

CI crosses for Day 5, Day 6, and Day 7 larvae. The number of daughters produced by the treated males was compared to the controls using Mann-Whitney U with a significance level of 0.05.

The antibiotic treatment to the larval stages was also done by manual injection into the parasitized fly pupae. The male larvae feeding on the fly pupae also ingest antibiotics. The antibiotic was mixed with a colored (blue) food dye (figure 3.3 A). Thus, the male larvae ingesting antibiotics turned blue (figure 3.3 B). The blue-colored larvae were selected and upon emergence, were mated with 0(PU) females. As shown in figure 3.3 (C), again, the antibiotic-treated larvae of Day 5 produced daughters as progenies while untreated (control) males produced all-male broods (MWU: U=0, $p < 0.0001$) upon mating with 0(PU) females. A significant increase in the number of daughters was observed for the antibiotic-treated larvae of Day 6 when compared to the untreated controls (MWU: U=368, $p < 0.001$). This showed that antibiotic treatment removed *Wolbachia* from that particular stage of development which resulted in no CI. However, on Day 7, the larvae treated with antibiotics did not show any increase in the number of daughters upon mating with 0(PU) females, when compared to the control males (MWU: U=131, $p = 0.96$). This could be due to the Day 7 larvae not feeding on the antibiotic.

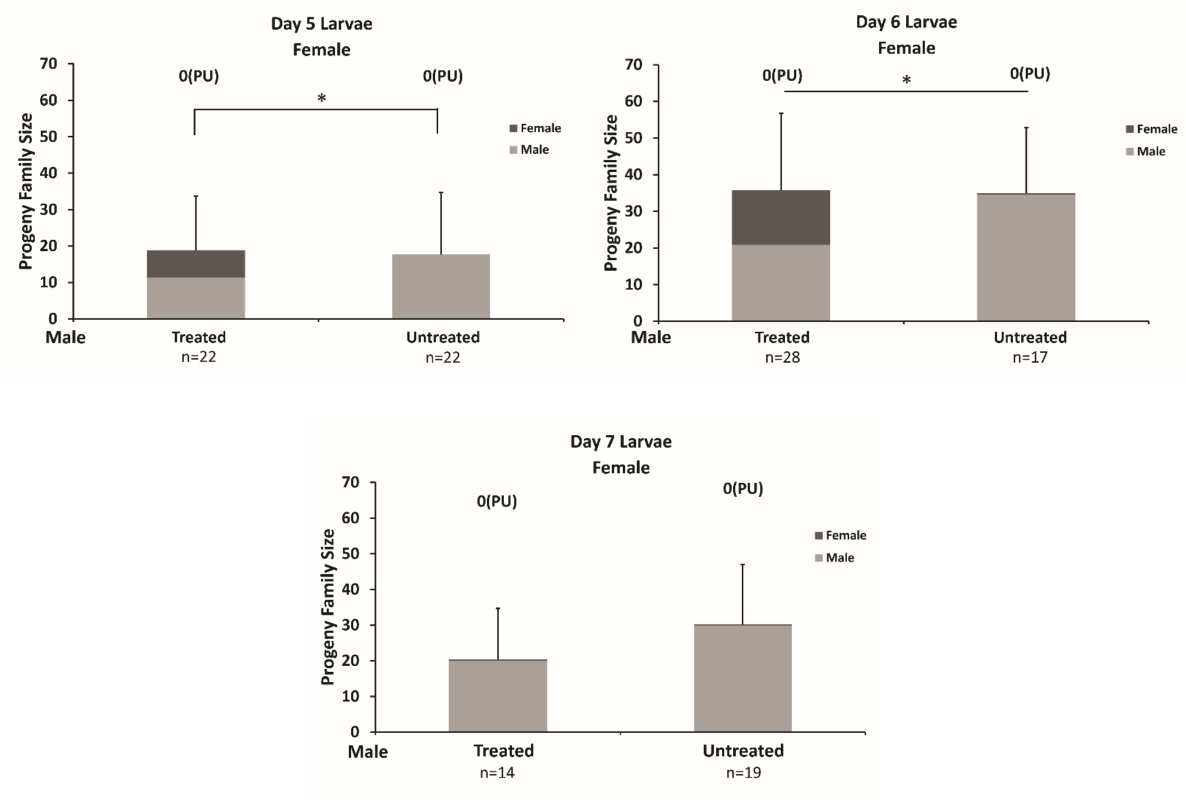
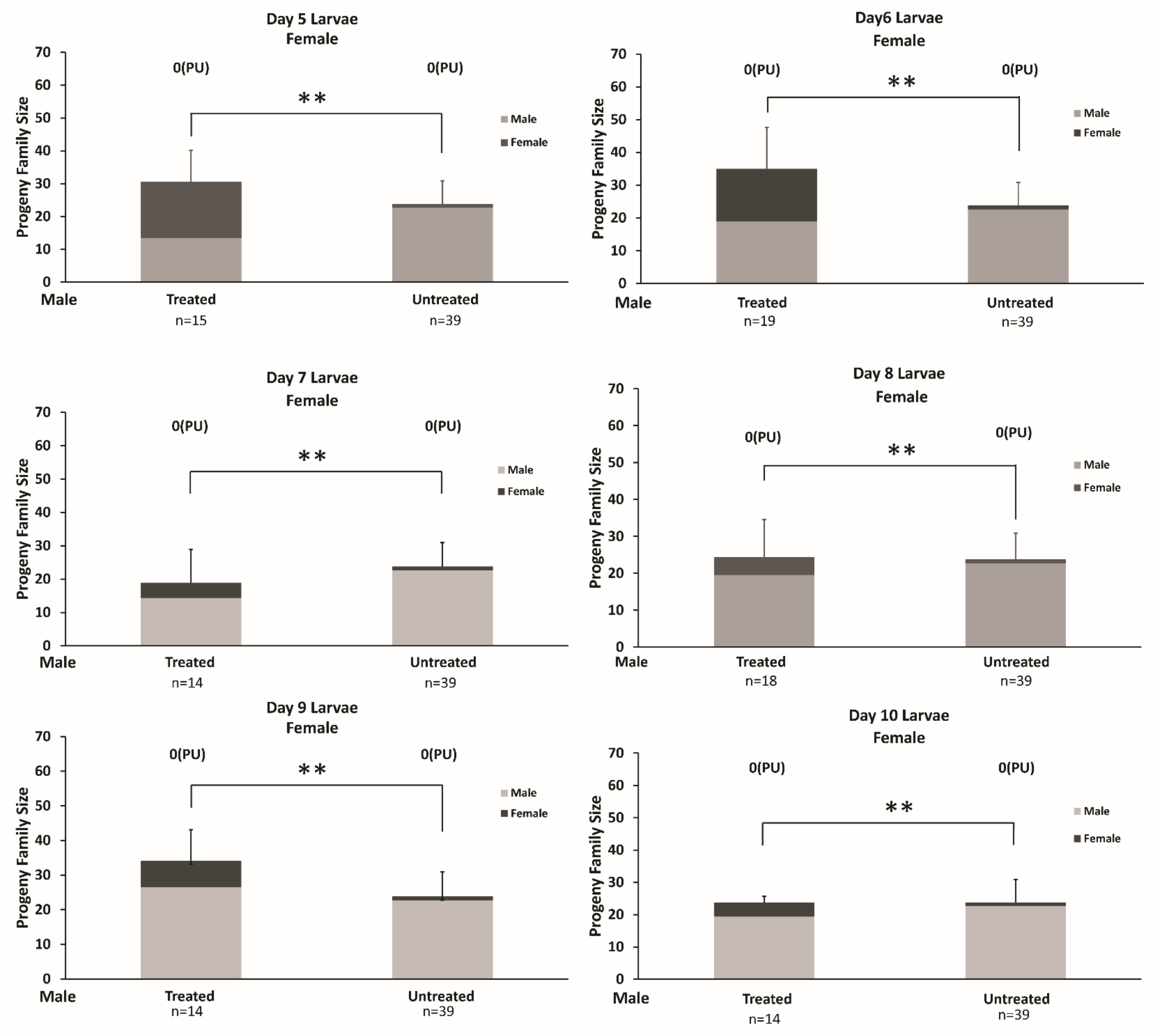


Figure 3. 5 Antibiotic delivery by manual injection into the parasitized fly pupae

CI crosses for Day 5, Day 6, and Day 7 larvae. The number of daughters produced by the treated males was compared to the controls using the Mann-Whitney U with a significance level of 0.05.

3.3.2 Antibiotic treatment using surfactants

Silwet L-77 (SKU 30630216-1) was used to deliver antibiotics to both the larval and the pupal stages of *N. vitripennis* males. This method was adapted from the Agrobacterium-mediated transformation of *Arabidopsis* and other plants via floral dip methods (Clough and Bent, 1998). Different concentrations of the surfactant ranging from 0.05% to 0.2% were tested (data not shown) out of which 0.01% was selected for the experiment. Antibiotic concentrations of 5mg/ml to 10mg/ml tetracycline were tested (data not shown) out of which 7.5mg/ml tetracycline was selected as the concentration to be used for the experiments owing to lesser toxicity and higher efficacy. Day 5 to Day 13 stages were dipped in a solution of 7.5mg/ml tetracycline mixed with 0.01% of the surfactant for 30 seconds. The stages (larvae/pupae) were then filtered out from the solution and kept for emergence. For the controls, no antibiotic was mixed in the solution. As shown in figure 3.4, Day 5 to Day 7 larval stages showed a significant increase in the number of daughters as compared to the untreated males (MWU: U=592, $p < 0.0001$ for Day 5; U=314, $p < 0.0001$ for Day 6; U=238, $p < 0.0001$ for Day 7). Among the pupal stages, Day 8 to Day 12 treatments also showed a significant increase in the number of daughters when compared to the control males (MWU: U=324, $p < 0.0001$ for Day 8; U=366, $p < 0.0001$ for Day 9, U=338, $p < 0.0001$ for Day 10, U=181, $p < 0.0001$ for Day 11, and U=37, $p < 0.0001$ for Day 12). However, Day 13, which is the last pupal stage before the adults emerge, did not show any significant increase in the number of female progenies (U=173, $p = 0.94$).


Day 5 Larvae
Female
Progeny Family Size
70
60
50
40
30
20
10
0
Male
0(PU)
0(PU)
**
Male
Female
Treated
n=15
Untreated
n=39
Day6 Larvae
Female
Progeny Family Size
70
60
50
40
30
20
10
0
Male
0(PU)
0(PU)
**
Male
Female
Treated
n=19
Untreated
n=39
Day 7 Larvae
Female
Progeny Family Size
70
60
50
40
30
20
10
0
Male
0(PU)
0(PU)
**
Male
Female
Treated
n=14
Untreated
n=39
Day 8 Larvae
Female
Progeny Family Size
70
60
50
40
30
20
10
0
Male
0(PU)
0(PU)
**
Male
Female
Treated
n=18
Untreated
n=39
Day 9 Larvae
Female
Progeny Family Size
70
60
50
40
30
20
10
0
Male
0(PU)
0(PU)
**
Male
Female
Treated
n=14
Untreated
n=39
Day 10 Larvae
Female
Progeny Family Size
70
60
50
40
30
20
10
0
Male
0(PU)
0(PU)
**
Male
Female
Treated
n=14
Untreated
n=39

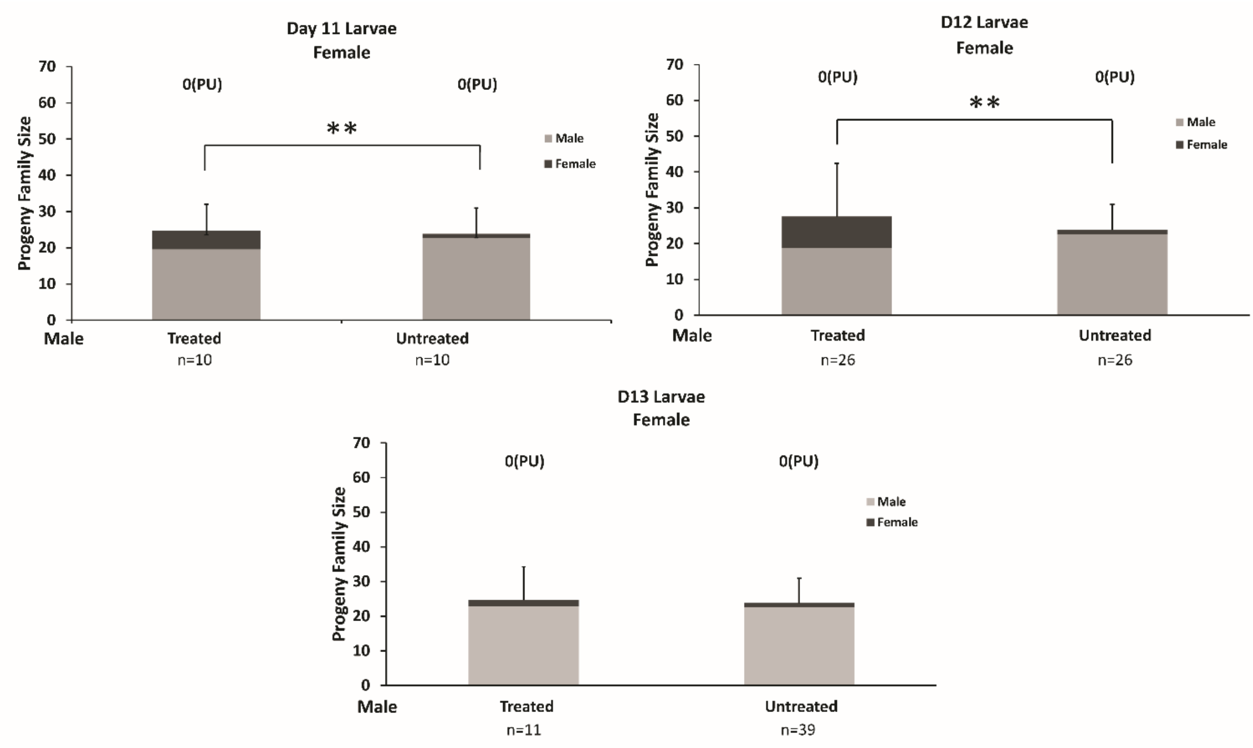


Figure 3. 6 Surfactant mediated antibiotic delivery

CI crosses for Day 5 to Day 13 developmental stages. The number of daughters produced by the treated males was compared to the controls using Mann-Whitney U with a significance level of 0.05.

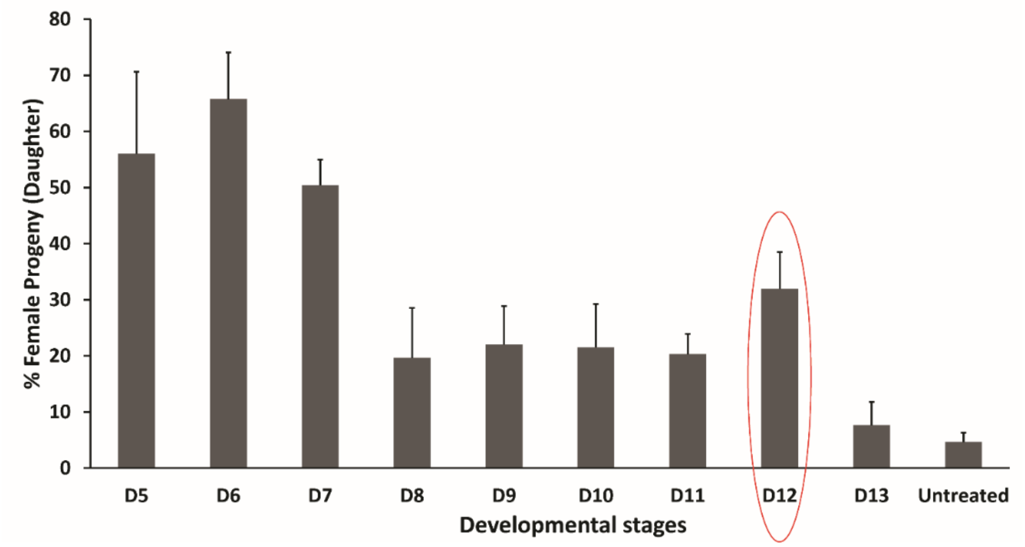
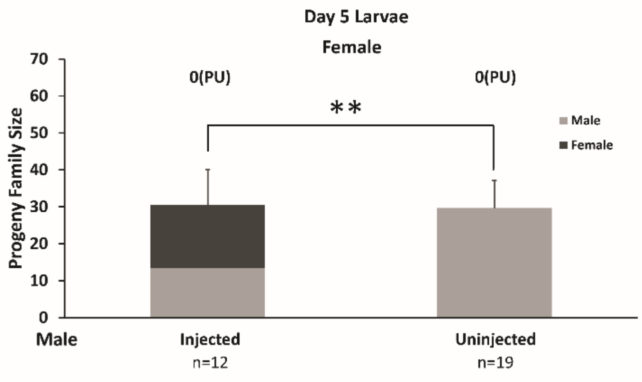


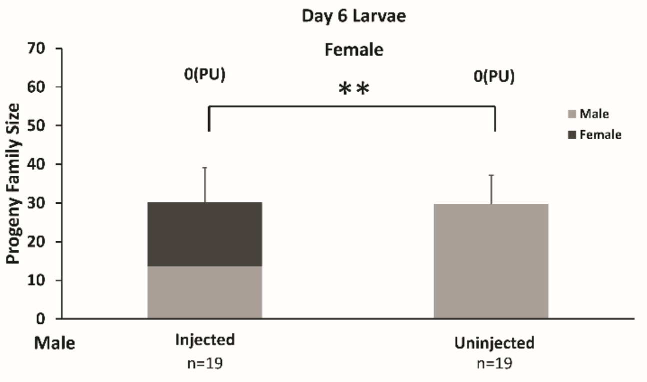
Figure 3. 7 Percentage of female progeny (daughters) produced by the different developmental stages on surfactant mediated antibiotic treatment

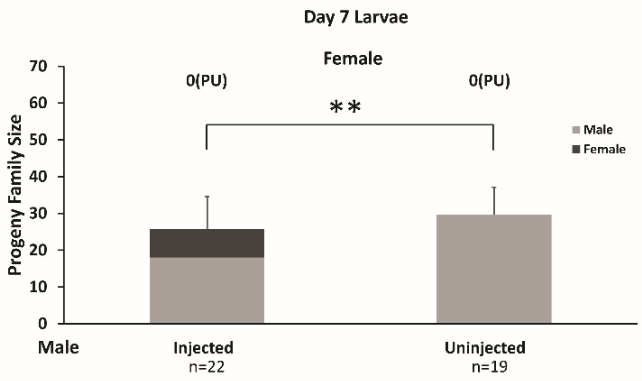
Thus Day 13 males showed CI phenotype even after antibiotic treatment. As Day 13 did not show any significant increase in the number of daughters compared to the untreated control (also shown in figure 3.5), the antibiotic treatment did not have any effect on the CI phenotype. Thus, the developmental stage before Day 13, which is Day 12, can be the prospective stage of *Wolbachia*-induced sperm modification.

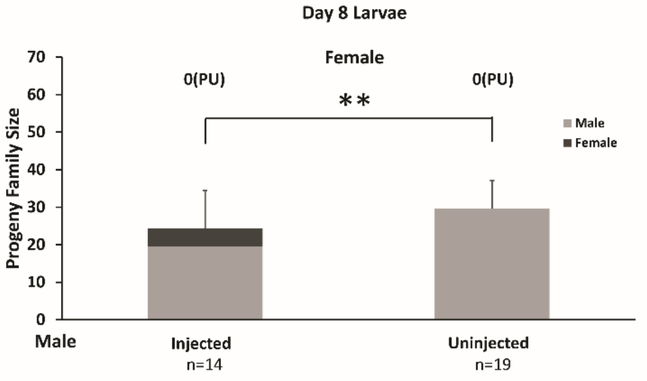
3.3.3 Antibiotic delivery by microinjection

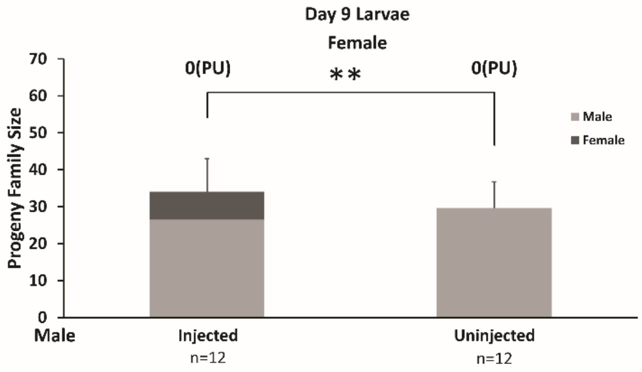
Microinjection was used to deliver antibiotic to both the larval and the pupal stages of *N. vitripennis* males. Day 5 to Day 13 stages were injected with antibiotics ranging from a concentration of 5mg/ml to 10mg/ml (data not shown), out of which 7.5mg/ml was selected as the concentration for the experiments owing to lesser toxicity and higher efficacy. As shown in figure 3.6, Day 5 to Day 7 larval stages showed a significant increase in the number of daughters as compared to the untreated males (MWU: U=436, $p < 0.0001$ for Day 5; MWU: U=592, $p < 0.0001$ for Day 6; U=238, $p < 0.0001$ for Day 7).

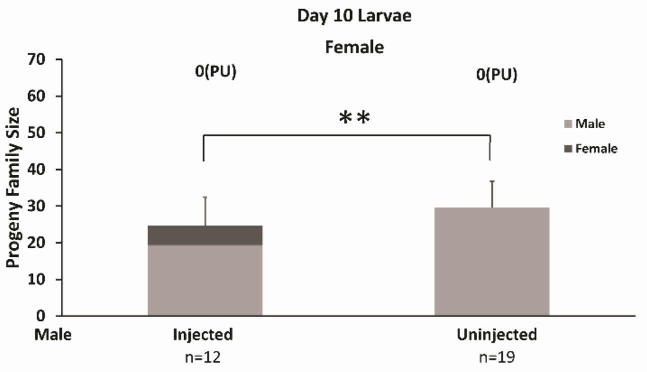

Day 5 Larvae
Female
70
60
50
40
30
20
10
0
Progeny Family Size
0(PU)
0(PU)
**
Male
Female
Male
Injected
n=12
Uninjected
n=19


Day 6 Larvae
Female
70
60
50
40
30
20
10
0
Progeny Family Size
0(PU)
0(PU)
**
Male
Female
Male
Injected
n=19
Uninjected
n=19


Day 7 Larvae
Female
70
60
50
40
30
20
10
0
Progeny Family Size
0(PU)
0(PU)
**
Male
Female
Male
Injected
n=22
Uninjected
n=19


Day 8 Larvae
Female
70
60
50
40
30
20
10
0
Progeny Family Size
0(PU)
0(PU)
**
Male
Female
Male
Injected
n=14
Uninjected
n=19


Day 9 Larvae
Female
70
60
50
40
30
20
10
0
Progeny Family Size
0(PU)
0(PU)
**
Male
Female
Male
Injected
n=12
Uninjected
n=12


Day 10 Larvae
Female
70
60
50
40
30
20
10
0
Progeny Family Size
0(PU)
0(PU)
**
Male
Female
Male
Injected
n=12
Uninjected
n=19

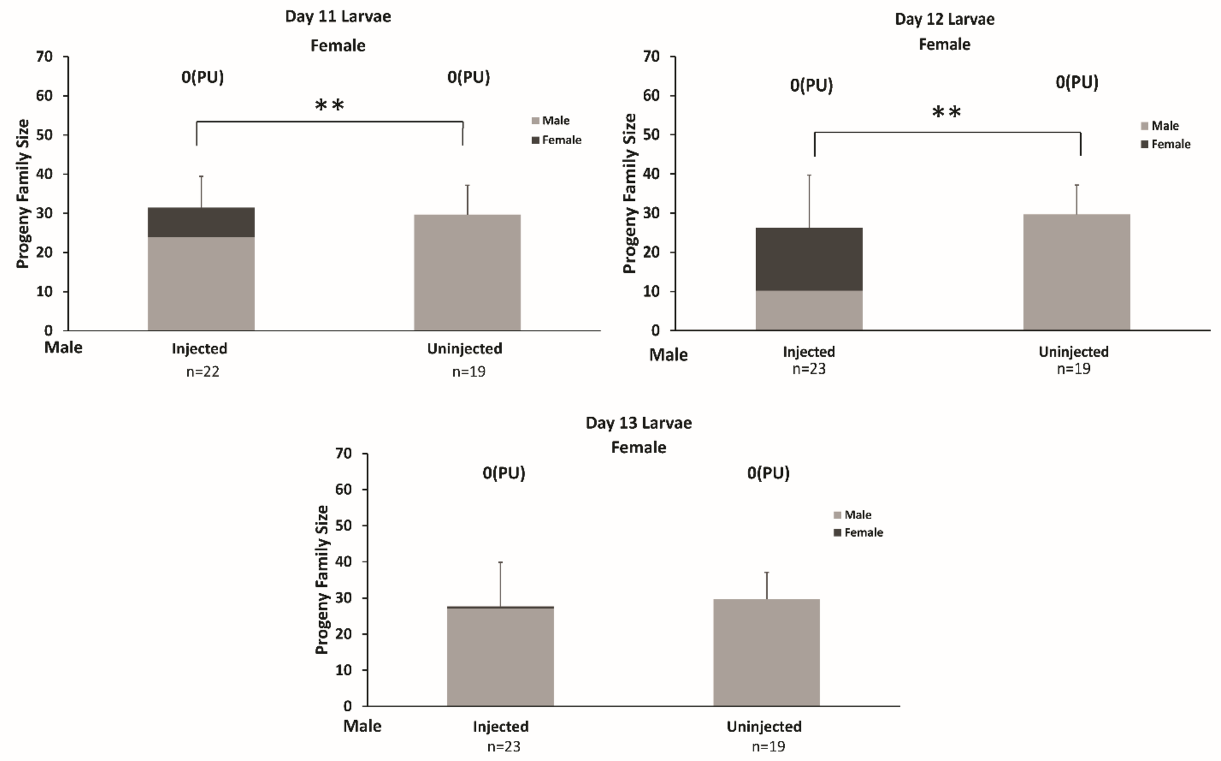


Figure 3. 8 Antibiotic delivery by microinjection

CI crosses for Day 5 to Day 13 developmental stages. The number of daughters produced by the treated males was compared to the controls using Mann-Whitney U with a significance level of 0.05.

For the pupal stages, Day 8 to Day 12 also showed a significant increase in the number of female offspring when compared to the untreated males (MWU: U=312, $p < 0.0001$ for Day 8; MWU: U=344, $p < 0.0001$ for Day 9, U=288, $p < 0.0001$ for Day 11 and U=372, $p < 0.0001$ for Day 12). However, Day 13, which is the last pupal stage before the adult emergence, again did not show any significant increase in the number of daughters (U=152, $p = 0.11$). Thus, Day 13 males showed CI phenotype even after antibiotic treatment.

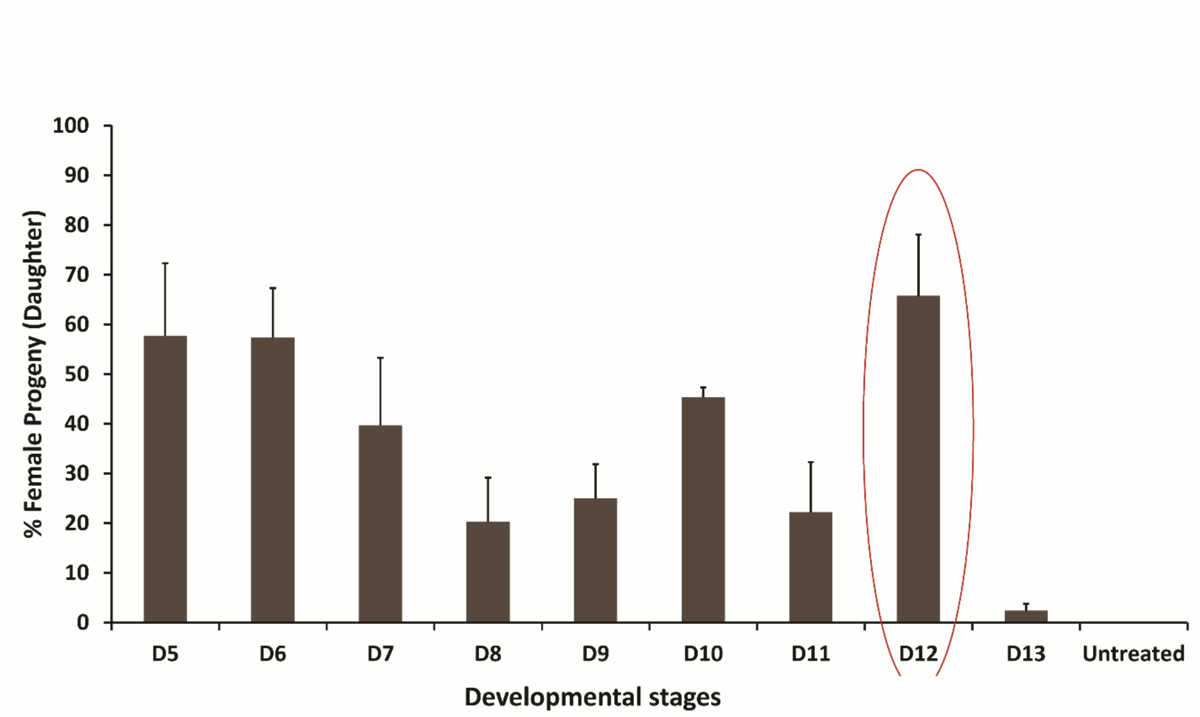


Figure 3. 9 Percentage of female progeny (daughters) produced by the different developmental stages on microinjection mediated antibiotic treatment

To check if the antibiotic treatment has depleted *Wolbachia* density in these males, quantitative estimation of *Wolbachia* levels in Day 5 to Day 13 males were also done, which showed undetectable *Wolbachia* levels even after 35 cycles of qPCR (figure 3.7).

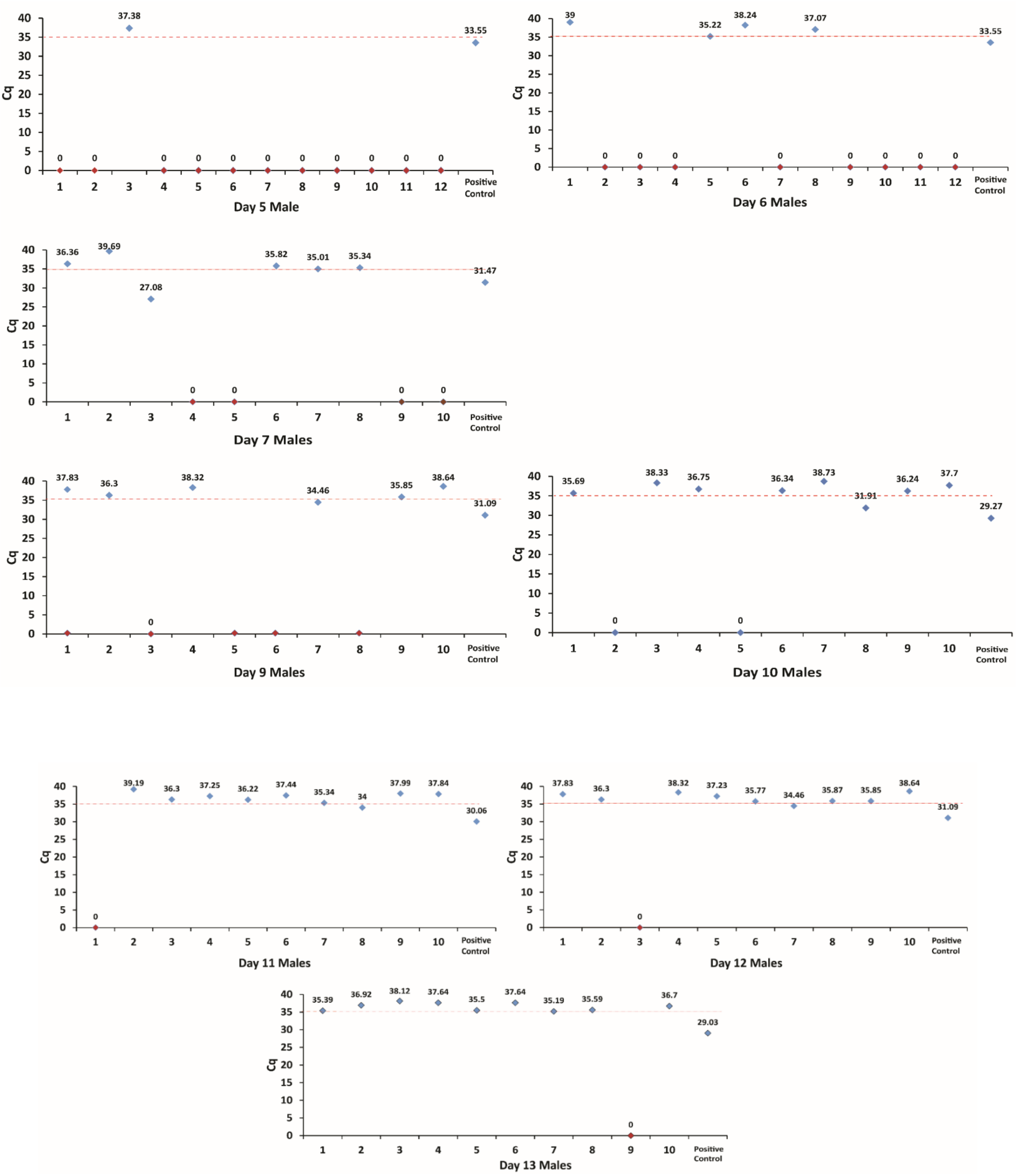


Figure 3. 10 ***Wolbachia*** **titer in the antibiotic-treated males using Quantitative Real-Time PCR (qPCR)**

As Day 13 did not show any significant increase in the number of daughters compared to the untreated control (also shown in figure 3.8), the antibiotic treatment did not have any effect on CI phenotype. Thus, the developmental stage before Day 13, which is Day 12, can be the prospective stage of *Wolbachia*-induced sperm modification.

3.3.4 Transcriptome analysis of the prospective sperm modification stage

Transcriptome profiling of the sperm modification stage was done using Nanopore sequencing and local BLAST tool with the “nr” database of the National Center for Biotechnology Information (NCBI), which is a protein database containing non-identical sequences from GenBank, Protein Data Bank (PDB), Swiss-Prot, Protein Information Resource (PIR) and Protein Research Foundation (PRF). The raw reads were processed for maximum identity (90-100%). The prokaryotic transcriptome of the sperm modification stage, (Day 12), was done for *w*B(PU). Day 13 pupae of *w*B(PU) were used as the control, while for the eukaryotic transcriptome, Day 12 pupae of 0(PU) were used as the control.

In both the test and control samples, the number of reads obtained after processing was very low (as shown in figure 3.8). Hence, no clear conclusion could be drawn from the transcriptome sampling of the CI stage.

Sample	**Total reads (raw)**	**Reads after processing**
D12 *w*B(PU) Prokaryotic	**396**	**254**
D13 *w*B(PU) Prokaryotic	**810**	**438**
D12 *w*B(PU) Eukaryotic	**875**	**705**
D12 0(PU) Eukaryotic	**188**	**139**

Table 3. 1 Number of reads for the transcriptome samples

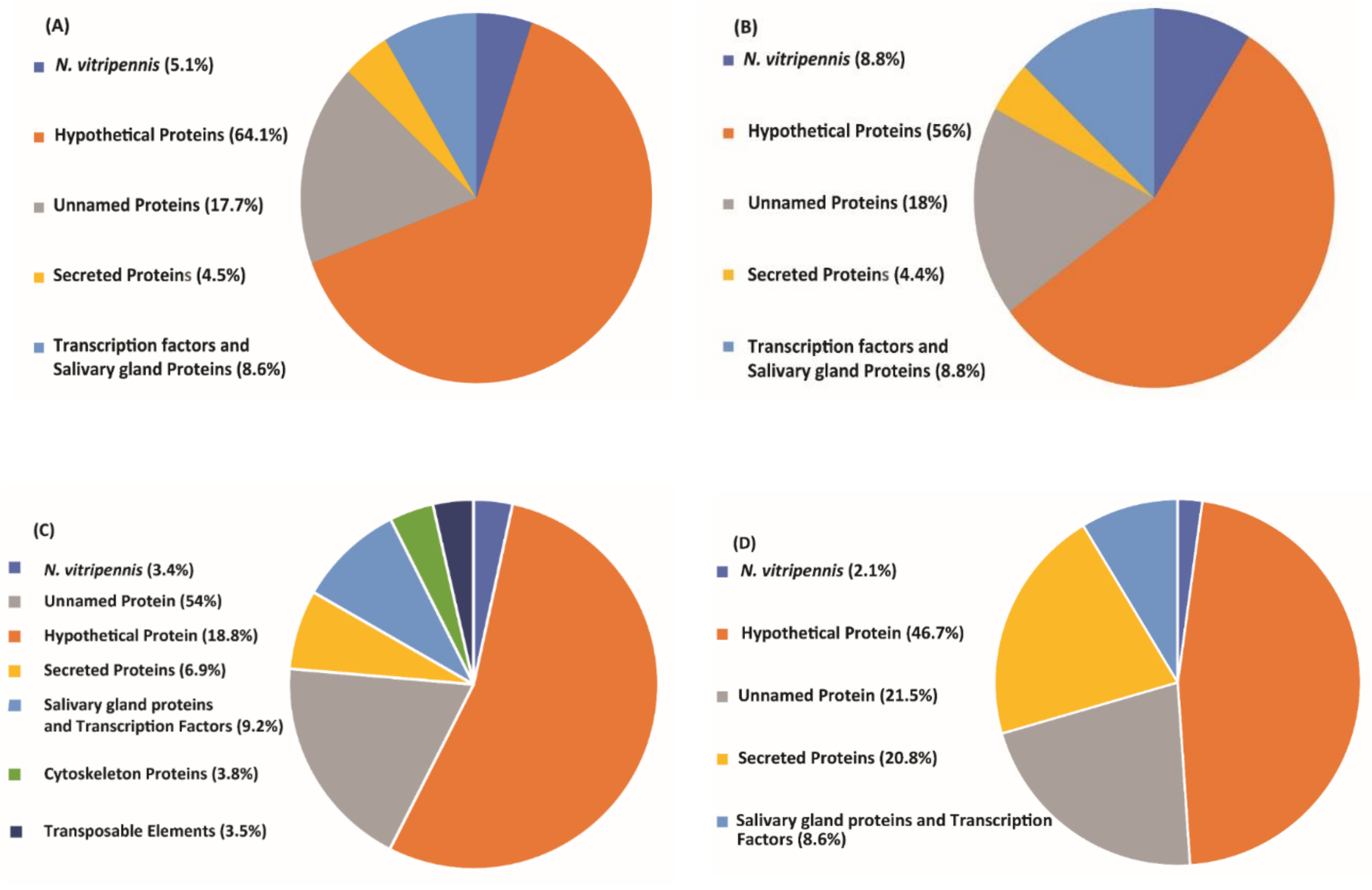


Figure 3. 11 Transcriptome profile of the prospective sperm modification stage

(A) Prokaryotic transcriptome profile of D12 males of *w*B(PU).

(B) Prokaryotic transcriptome profile of D13 males of *w*B(PU).

(C) Eukaryotic transcriptome profile of D12 males of *w*B(PU).

(D) Eukaryotic transcriptome profile of D12 males of 0(PU).

There can be multiple reasons for the unprecedented low number of reads produced in the transcriptome profiling. One probable reason can be the lack of double-stranded cDNA products produced from single-stranded cDNA. Another probable explanation can be the excessive loss of cDNA products during the multiple steps of sample preparation.

3.4 **Discussion**

Wolbachia-induced modification leads to sperm-egg incompatibility and hence inviable offspring. The approach in this study aimed to identify the stage where sperm modification happens in *N. vitripennis* males. *N. vitripennis*, as a model system gives the advantage of doing stage-specific investigations, which has been done in a few other systems earlier (Narita *et al.*, 2007). *N. vitripennis* males have their spermatogenic index as "one" (Boivin *et al.* 2005), which essentially means that the males are prospermatogenic. These males have their entire sperm complement matured on the first day of emergence and the males do not produce any sperm during their adulthood. Thus, sperm development in males is synchronized. The study uses this phenomenon of sperm synchrony to remove *Wolbachia* from specific stages of sperm development using antibiotics.

The stage identification for sperm modification paves the way to identify candidate genes responsible for sperm modification. A stage-specific transcriptome analysis, with the uninfected line of the same stage as control, was performed to identify both *Wolbachia* and host genes that are responsible for sperm modification. However, a sufficient number of reads were not obtained to make any meaningful conclusions. A probable reason can be due to the unavailability of sufficient double-stranded cDNA for adapter ligation and sequencing reaction. Another possibility can be an excessive loss of double-stranded cDNA while barcoding and adapter ligation resulting in the insufficient number of reads produced.

Multiple approaches were adopted to deliver antibiotics at different developmental stages. The aim was to reciprocate the results using different mechanisms of antibiotic delivery. At first, for the larval stages, *N. vitripennis* male larvae were cultured *in vitro* using NRM with antibiotics. Various concentrations of antibiotics were tested before deciding on the optimal concentration of 5 mg/ml, which produced higher efficacy of antibiotic action and least mortality. This was followed by the manual injection of antibiotics into the parasitized fly pupae, for the parasitoid larvae to feed. Again, multiple antibiotic concentrations were tested and 5 mg/ml was selected as the working concentration for the experiments. In both the methods, Day 5 and Day 6 larvae showed the effect of antibiotics with a significant increase in the number of female progenies than the untreated males. However, the Day 7 larvae did not show any effect of antibiotics in both the methods of antibiotic delivery. A possible explanation for this observation can be that Day 7 is the transition stage between the larval and pupal stages. These larvae in transition do not feed and hence the antibiotic treatment did not work.

For the pupal stages, surfactant-mediated antibiotic delivery and microinjection approaches were used. These methods were also extended up to the mid and late larval stages. A concentration of 7.5 mg/ml tetracycline was selected for both methods after titrating different antibiotic concentrations. In both the methods, all the developmental stages until Day 12 showed the effect of antibiotics on CI and hence had a significant increase in the number of daughters in the population as compared to the controls. However, the Day 13 pupal stage, which is the last stage before male emergence did not show any effect of the antibiotic and hence showed CI phenotype.

The successful antibiotic treatment was checked by quantifying *Wolbachia* levels once the respective day males emerged into adults. No *Wolbachia* was detected in all these antibiotic-treated males, which shows the effectiveness of the antibiotic treatment. Thus, Day 12, which shows negligible *Wolbachia* levels, still produced significantly more number of daughters when compared to the control males. However, Day 13 males, even after successful antibiotic treatment did not produce daughters as progenies. Thus, Day 12 can be the developmental stage where sperm modification occurs in *N. vitripennis* males.

N. vitripennis males have two *Wolbachia* supergroup infections, one each from supergroup A and supergroup B. After strain separation, supergroup B infection strain was used to identify the stage for sperm modification. Supergroup B infection in *N. vitripennis* shows complete CI, which results in an all-male brood (Bordenstein *et al.*, 2006b). Thus, the effect of antibiotics on CI can be easily identified, by the presence of daughters in the progeny population. This is however not feasible using supergroup A *Wolbachia* as it is known to cause incomplete CI in *N. vitripennis*.

The stage-specific antibiotic delivery approach identifies Day 12 as the developmental stage of sperm modification in *N. vitripennis* males. This is evident in the production of female progenies by D12 males even after successful antibiotic delivery.

I do agree with the reviewer's comment that the sperm modification induced by *Wolbachia* can be a phenomenon spread over more than one developmental stage. However, to identify candidates responsible for the sperm modification, Day 12 serves as the best possible candidate stage as possible sperm modification gets terminated at Day 12. Day 13 exposure to antibiotics does not have any effect on CI phenotype which is can be used as a control to get the candidate genes responsible for sperm modification.

Different *Wolbachia* supergroups are known to have varied effects on the host. For example, some *Wolbachia* supergroup A members are known to have positive fitness effects on the host

(Fry *et al.*, 2004; Weeks *et al.*, 2007) while most supergroup B *Wolbachia* is known to have a parasitic effect (Almeida *et al.*, 2011; Ross *et al.*, 2020). Thus, two different *Wolbachia* supergroups can also have independent mechanisms of CI induction in their hosts. Our results identify the developmental stage of sperm modification for supergroup B *Wolbachia* in *N. vitripennis*. A similar approach can be adapted to identify the stage of sperm modification for supergroup A *Wolbachia* and double infection strains as well. Thus, using such stage-specific transcriptome approaches, supergroup-specific studies can also be done to decipher the genetic basis of sperm modification by different *Wolbachia* supergroups.

Chapter 4

Wolbachia mediated tolerance against heat stress in *Nasonia vitripennis*

4.1 Introduction

Insects as poikilotherms (May, 1979), need to adapt to the changes in their environment to survive in a wide variety of temperature ranges. While the vast majority of the insects are ectotherms, some are considered heterotherms (May, 1979; Stone *et al.,* 1992). Ectothermic insects are susceptible to large changes in temperature regimes which affect their physiology and behavior (Deutsch *et al.*, 2008). Deviation from the optimum temperature range can lead to heat or cold stress which can have a detrimental impact on insect physiology and metabolism (Kingsolver *et al.*, 2013; Nyamukondiwa *et al.*, 2018; Chen *et al.*, 2019).

Heat stress has gained considerable importance owing to its agronomic usage such as pollinators and application in pest control strategies. Insects being small in size are susceptible to changes in abiotic factors, especially temperature (Colinet *et al.*, 2015). The effects of heat stress have been widely studied in a variety of insect species. Changes in environmental temperature are known to have direct effects on behaviors like courtship, oviposition, mating (Wilkes, 1959; Nguyen *et al,*. 2013; Moiroux *et al.*, 2014) and fitness components like fecundity, life span and rate of development (Taylor, 1979; Carroll *et al.*, 1993; Ju *et al.*, 2011) across the sexes. In the vast majority of insects, heat stress is also known to negatively impact metabolism, respiration and the endocrine system (Neven, 2000). For example, heat stress during development results in lower egg to adult viability, life span, and fertility of the adults in *Drosophila suzukii* (Green *et al.* 2019). Exposure to temperatures of 40°C and 42°C leads to decreased survival, prolonged development, decreased longevity, and egg production in *Bactrocera tau* (Huang *et al.*, 2020). High temperature leads to decreased life span in both the sexes of *D. melanogaster*. A high metabolic rate leads to increased aging in males and females (Mołoń *et al.*, 2020).

The negative impacts of extreme temperatures on insects depend on the duration of the stress (Chen *et al.*, 2018). Heat tolerance also depends on the sex and developmental stage of the individual (Enriquez *et al.*, 2017). For example, the adult stage of beetle species *Ophraella communa* show more tolerance of temperatures of 40°C to 44°C degrees with high survival rates when compared to the egg, larval and pupal stages (Chen *et al.*, 2019). Treated adults showed high fecundity and adult life span under heat stress (Chen *et al.*, 2019). However, significant detrimental effects were observed in survival, reproduction, life span, and egg hatch rates (Chen *et al.*, 2019). In the beetle *Serangium japonicum*, survival, predation capacity was significantly compromised (Yao *et al.*, 2019). Again, the egg and first instar larval stages were less tolerant than the fourth instar, pupae, and adults (Yao *et al.*, 2019). In *D. suzukii*, males are found to be less tolerant than the females at 34°C, 35°C, and 37°C (Enriquez *et al.*, 2017).

Symbionts are known to play a major role in insect physiology and nutrition (Douglas, 2009; Klepzig *et al.*, 2009; Ferrari *et al.*, 2011). In addition, many symbionts are known to mediate the thermal tolerance of the host (Feldhaar, 2011b). For example, Rickettsia-free strains of *Bemisia tabaci* show significantly higher mortality rates than the infected strains (Brumin *et al.*, 2011) at elevated temperatures. A single nucleotide mutation in the *ibp A* gene of *Buchnera*, the obligate symbiont of aphid *Acyrthosiphon pisum*, leads to the loss of thermal tolerance of the aphid (Dunbar *et al.*, 2007). The absence of the facultative symbionts of *A. pisum* leads to a decrease in the number of offspring produced (Montllor *et al.*, 2002). However, a particular fitness effect against heat stress can be conferred by the presence of a particular symbiont (Russell *et al.*, 2006). Thus, both the host and symbiont genotype have a crucial role to play in the response against heat stress (Cayetano *et al.*, 2013).

Wolbachia being an endosymbiont, is also known to modulate the host response against sub-lethal temperature exposure. *Wolbachia* infected *D. simulans* males exposed to heat stress sire more viable offspring than the uninfected males (Feder *et al.*, 1999). The effect of *Wolbachia* also varies with the genetic background of the strains. *wMelCS* genotype of *Wolbachia* shows increased resistance against heat stress in terms of higher survival rates of the individuals in *D. melanogaster* while *wMelPop* shows decreased survival in comparison to the controls (Gruntenko *et al.* 2017). The presence of *Wolbachia* has positive effects on the egg hatch rates in *D. suzukii* (Saeed *et al.* 2018).

The effect of heat stress in parasitoid wasps has an ecological and agronomic interest because of their use to control invasive species or agricultural pests naturally (Chirault *et al.*, 2015; Skendžić *et al.*, 2021). For example, in *Anisopteromalus calandrae*, a parasitoid wasp, the males suffer from decreased sperm count after getting exposed to high temperature during the early pupal stage of its development (Chevrier et al. 2019). In the parasitoid *Aphidius colemani*, heat stress during immature stages of development leads to detrimental effects on emergence rate, sex ratio, egg load, antennal symmetry, and courtship behavior (Jerbi-Elayed *et al.*, 2015). In the *Bradysia odoriphaga* and B. *difformis*, high temperatures of 36°C and 38°C resulted in decreased survival rate, longevity, and reproductive capacities (Zhu *et al.*, 2017).

Heat stress effects have also been studied on the parasitoid wasp *N. vitripennis*. Male *N. vitripennis* suffered a significant decrease in their stock of sperm once they were exposed to a sub-lethal temperature of 36°C at the first pupal stage of development (Chirault et al. 2015). However, no effect was observed on traits like longevity and emergence rates. *N. vitripennis* has a cosmopolitan distribution (Whiting, 1967). Thus, there can be locally adapted populations capable of eliciting variable responses against temperature-stress. Hence, the response against heat stress can vary between populations of different geographical locations and can be dependent on the genotype (Hidalgo *et al.,* 2019).

This study investigates the effect of *Wolbachia* on the heat stress-induced physiological and behavioral responses of the parasitoid host *N. vitripennis*. The study investigates the role of a particular *Wolbachia* supergroup in the responses of the host against heat stress. Single infection lines of supergroup A and supergroup B infections along with uninfected and double infection lines were exposed to high temperatures of 30°C, 32°C, and 34°C at the early pupal stage (Day 7 for males and Day 8 for females) for 24 hours. The control populations were maintained at 25°C. The effect of the presence of *Wolbachia* infections in the heat-stressed males and females for traits like life span and sperm depletion in males were investigated. The results suggest that the presence of *Wolbachia* increases the tolerance of the host against elevated temperatures.

4.2 Materials and methods

4.2.1 *N. vitripennis* lines used, their *Wolbachia* infections, and nomenclature

The four different infection lines 0(PU), *w*A(PU), *w*B(PU), and *w*A*w*B(PU) and the recently cured lines 0(*w*A PU), 0(*w*B PU), and 0(*w*A*w*B PU) were used in the study. All these lines were raised on *Sarcophaga dux* fly pupae with a generation time of 14-15 days at 25ºC, 60% humidity, and a continuous daylight cycle.

4.2.2 Heat stress treatment

The lines were subjected to heat stress at three different temperatures of 30°C, 32°C, and 37°C for 24 hours at the last larval stage of their development (Day 7 for males and Day 8 for females after oviposition at 25°C). After 24 hours, the lines were kept at 25°C for development. The control sets for all the lines were maintained continuously at 25°C.

4.2.3 Effect on Cytoplasmic incompatibility (CI)

The effect of heat stress on *Wolbachia*-induced CI phenotype between the infected males and uninfected females was estimated. The heat-stressed males of the three infected lines *w*A(PU), *w*B(PU), and *w*A*w*B(PU) were mated with 0(PU) females. Untreated males from the three infected lines were mated with the same line females and 0(PU) females and used as controls. Mating was observed and then the males were removed. All the females were hosted with one *Sarcophaga* fly pupae for 24 hours. The females were removed after 24 hours and the parasitized pupae were kept for emergence at 25°C. The progeny family sizes were counted by randomizing the vials in a double-blind assay. The differences were compared with the controls using the Mann-Whitney U test with a significance level of 0.05.

4.2.4 Longevity and sperm depletion after heat stress

To test whether the presence of *Wolbachia* has any influence on the tolerance of the host against heat stress, traits like longevity and the number of daughters produced were compared between the heat-stressed lines and the controls.

For longevity, emerging adult wasps of both the sexes (for the heat-stressed and the controls) were kept individually in ria vials at 25°C, without any additional nutrition. Survival following emergence was measured by counting the number of dead individuals every 6 hours. Log Rank Statistics was used to identify differences between lines with a significance level of 0.05.

To assess the effect of heat stress on sperm depletion of the males, heat-stressed males were mated with females of the same line. Mating was observed and all the females were then hosted individually with one fly pupa for 24 hours. These were kept at 25°C for the offspring to emerge which were later counted for progeny family size and number of female offspring (daughters) produced by randomizing the ria vials in a double-blind assay. The differences between groups were compared using the Kruskal-Wallis test with a significance level of 0.05. Mann-Whitney U test, with a significance level of 0.05, was used to compare two groups.

4.3 Results

4.3.1 Heat stress does not have any effect on *Wolbachia* induced CI phenotype in *N. vitripennis*

To test whether the sub-lethal temperature has any effect on *Wolbachia*-induced CI phenotype, heat-stressed males at 32°C and 37°C of the three infection lines *w*A(PU), *w*B(PU), and *w*A*w*B(PU) were mated with 0(PU) females. The control males from the three infection lines (not exposed to heat stress) were mated with 0(PU) females and same line females.

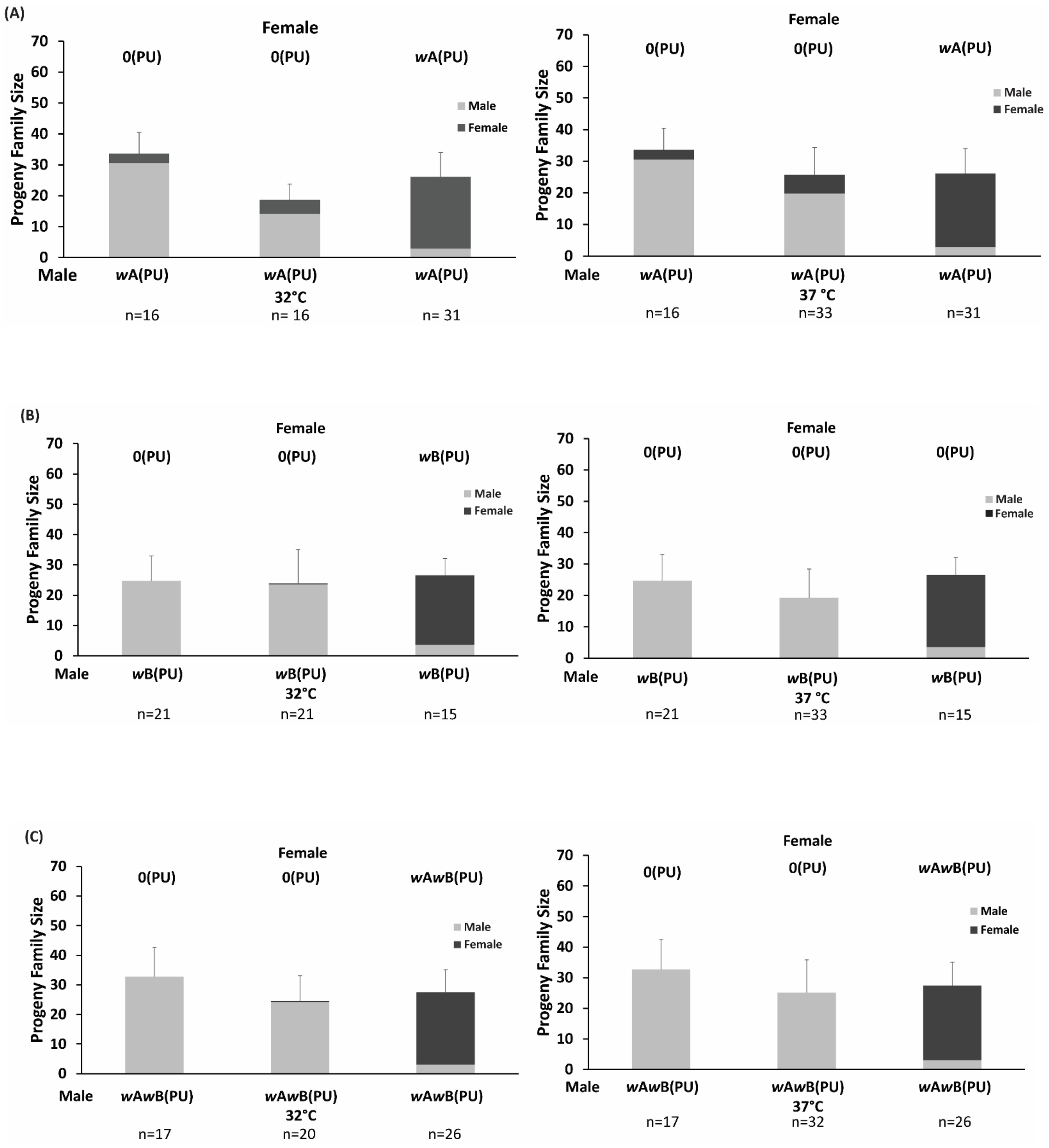


Figure 4. 1 CI cross for the heat-stressed males

The heat-stressed males were mated with 0(PU) females. Unstressed *w*A(PU), *w*B(PU), and *w*A*w*B(PU) males were used as controls. The number of female progenies was compared between the crosses using the Mann-Whitney U test, p < 0.05.

(A): Heat-stressed males at 32°C and 37°C of the line *w*A(PU)

(B): Heat stressed males at 32°C and 37°C of the line *w*B(PU)

(C): Heat-stressed males at 32°C and 37°C of the line *w*A*w*B(PU)

As shown in figure 4.1, the heat-stressed *w*B(PU) and *w*A*w*B(PU) males showed complete CI with 0(PU) females. This was evident with all-male brood as progenies. *w*A(PU) similar to the controls, still showed incomplete CI with 0(PU).

4.3.2 The presence of *Wolbachia* increases the heat tolerance of both males and females

I first investigated the effect of *Wolbachia* infections on the survival of heat-stressed male and female wasps. The heat stress was given at three different temperatures, 30°C, 32°C, and 37°C. as figure 4.2 (A) indicates, females exposed to 30°C, the infected *Wolbachia* lines had a significantly longer life span when compared to the uninfected line. *w*A*w*B(PU) and *w*B(PU) had longer life span when compared to 0(PU) {χ^2=6.58, p < 0.05 for *w*A*w*B(PU) and χ^2=3.95, p < 0.05 for *w*B(PU)}.

However, *w*A(PU), as an exception, had a life span comparable to that of 0(PU) (χ^2=0.032, p = 0.85). I tested if the phenotype is influenced by the host genetic background by comparing the life span of the recently cured lines with their infected counterparts. The three recently cured lines 0(*w*A PU) {χ^2=5.035, p < 0.05 for *w*A(PU)}, 0(*w*B PU) {χ^2=22.71, p < 0.01 for *w*B(PU)}, and 0(wAwB PU) {χ^2=37.83, p < 0.001 for *w*A*w*B(PU)} showed significant decrease in their life spans when compared to their infected counterparts.

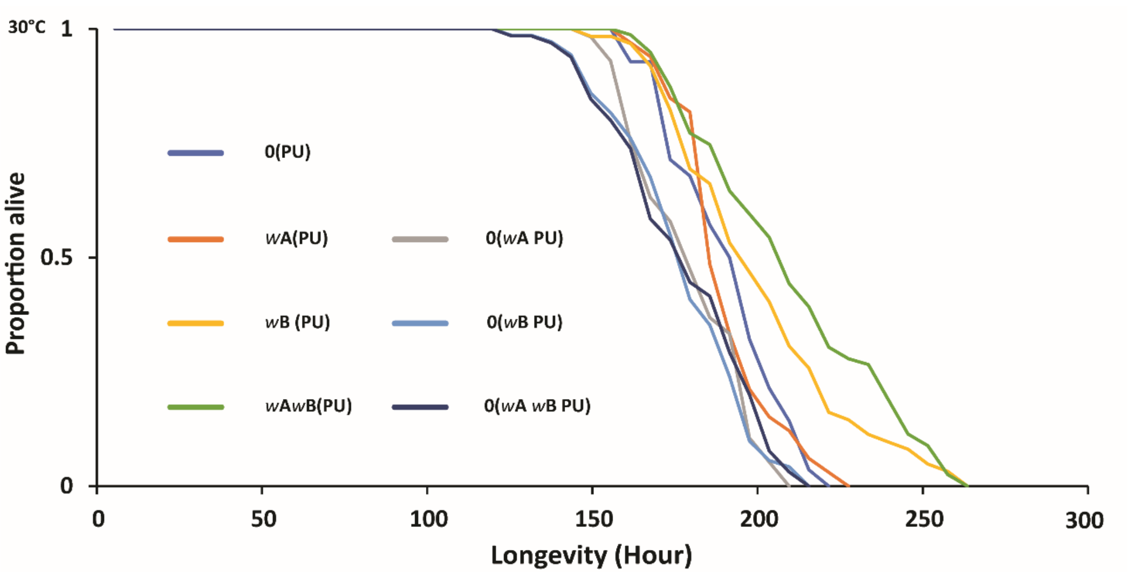


Figure 4. 2. Lifespan of females given heat shock at 30°C

Sample sizes for the four infections 0(PU), *w*A(PU), *w*B(PU), *w*A*w*B(PU) [n=28, n=33, n=62 and n=79 respectively] and the recently cured lines 0(*w*A PU), 0(*w*B PU), 0(*w*A*w*B PU) [n=57, n=71, and n=65 respectively].

Strain	Compared with	Strain	Result	χ^2value	p value
0(PU)		*w*A(PA)	0(PU) = *w*A(PA)	$\chi^2 = 0.032$	$p = 0.85$
0(PU)		*w*B(PU)	*w*B(PU) > 0(PU)	$\chi^2 = 3.95$	*$p < 0.05$
0(PU)		*w*A*w*B(PU)	*w*A*w*B(PU) > 0(PU)	$\chi^2 = 6.58$	*$p < 0.05$
*w*A(PU)		0(*w*A PU)	*w*A(PU) > 0(*w*A PU)	$\chi^2 = 5.0$	*$p < 0.05$
*w*B(PU)		0(*w*B PU)	*w*B(PU) > 0(*w*B PU)	$\chi^2 = 22.7$	***$p < 0.001$
*w*A*w*B(PU)		0(*w*A*w*B PU)	*w*A*w*B(PU) > 0(*w*A*w*B PU)	$\chi^2 = 37.83$	***$p < 0.001$

Table 4. 1 Life span comparison of females given heat shock at 30°C

The significance was calculated using Log Rank statistics with a significance level of 0.05.

Similarly, at 32°C (figure 4.2 B), the *Wolbachia* infected lines also had a significantly longer life span when compared to the uninfected line. *w*A*w*B(PU) and *w*B(PU) had longer life span when compared to 0(PU) {χ^2=14.8, $p < 0.001$ for *w*A*w*B(PU) and χ^2=14.84, $p < 0.001$ for *w*B(PU)}. This was followed by *w*A(PU) which also has a significantly longer life span when compared to 0(PU) (χ^2=19.0, $p < 0.01$).

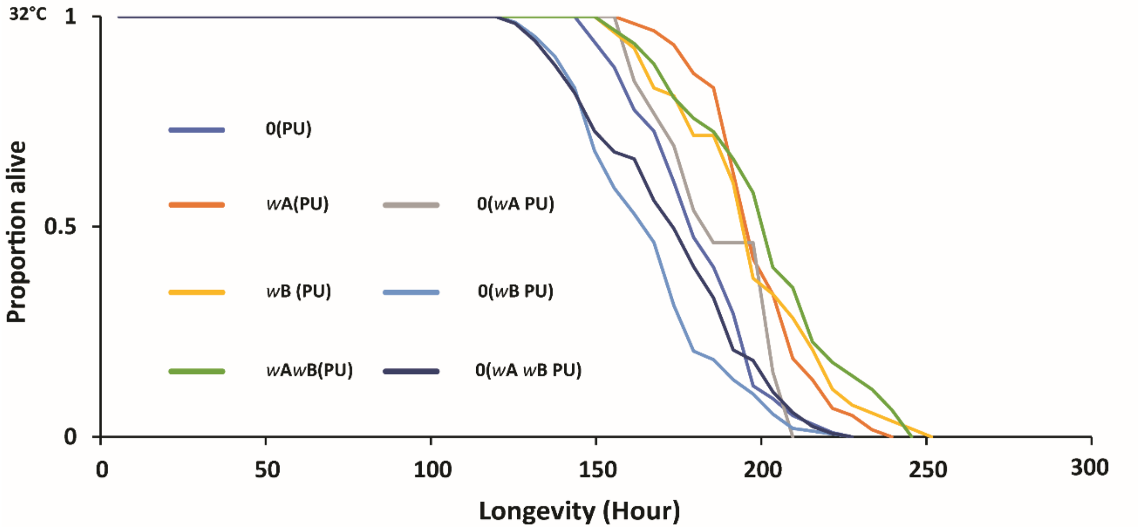
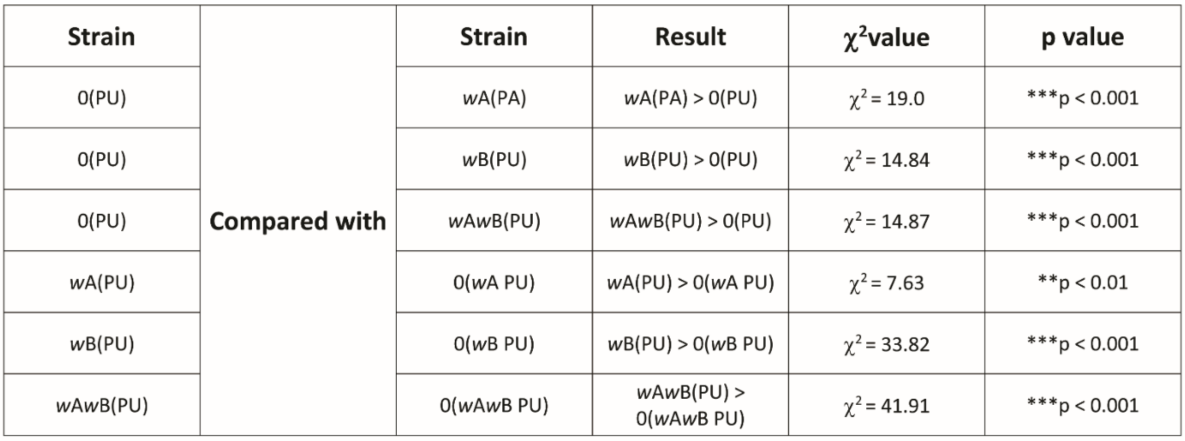


Figure 4. 3. Lifespan of females given heat shock at 32°C

Sample sizes for the four infections 0(PU), *w*A(PU), *w*B(PU), *w*A*w*B(PU) [n=99, n=59, n=53 and n=62 respectively] and the recently cured lines 0(*w*A PU), 0(*w*B PU), 0(*w*A*w*B PU) [n=13, n=147, and n=121 respectively].



Strain		Strain	Result	χ^2value	p value
0(PU)	**Compared with**	*w*A(PA)	*w*A(PA) > 0(PU)	$\chi^2 = 19.0$	***p < 0.001
0(PU)		*w*B(PU)	*w*B(PU) > 0(PU)	$\chi^2 = 14.84$	***p < 0.001
0(PU)		*w*A*w*B(PU)	*w*A*w*B(PU) > 0(PU)	$\chi^2 = 14.87$	***p < 0.001
*w*A(PU)		0(*w*A PU)	*w*A(PU) > 0(*w*A PU)	$\chi^2 = 7.63$	**p < 0.01
*w*B(PU)		0(*w*B PU)	*w*B(PU) > 0(*w*B PU)	$\chi^2 = 33.82$	***p < 0.001
*w*A*w*B(PU)		0(*w*A*w*B PU)	*w*A*w*B(PU) > 0(*w*A*w*B PU)	$\chi^2 = 41.91$	***p < 0.001

Table 4. 2 Life span comparison of females given heat shock at 32°C
The significance was calculated using Log Rank statistics with a significance level of 0.05.

The recently cured lines 0(*w*A PU) {(χ^2=7.63, p < 0.001 for *w*A(PU)}, 0(*w*B PU) {(χ^2=33.82, p < 0.001 for *w*A(PU)} and 0(*w*A*w*B PU) {(χ^2=41.91, p < 0.001 for *w*A(PU)} showed a significant decrease in their life span from their parent infection lines.

Even at 37°C, as shown in figure 4.2 (C), *w*A*w*B(PU) and *w*B(PU) had longer life span when compared to 0(PU) {χ^2=14.44, p < 0.001 for *w*A*w*B(PU) and χ^2=14.85, p < 0.001 for *w*B(PU)}.

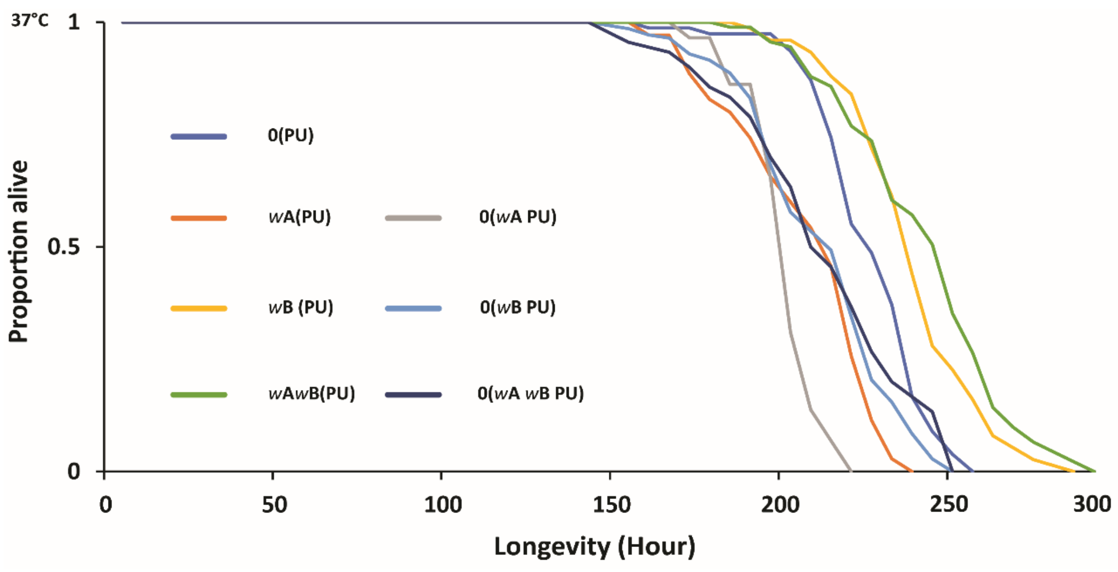


Figure 4. 4. Lifespan of females given heat shock at 37°C

Sample sizes for the four infections 0(PU), *w*A(PU), *w*B(PU), *w*A*w*B(PU) [n=78, n=35, n=75 and n=91 respectively] and the recently cured strains 0(*w*A PU), 0(*w*B PU), 0(*w*A*w*B PU) [n=29, n=142, and n=90 respectively].

Strain		Strain	Result	χ^2value	p value
0(PU)	**Compared to**	*w*A(PA)	*w*A(PA) > 0(PU)	$\chi^2 = 19.91$	***$p < 0.001$
0(PU)		*w*B(PU)	*w*B(PU) > 0(PU)	$\chi^2 = 14.85$	***$p < 0.001$
0(PU)		*w*A*w*B(PU)	*w*A*w*B(PU) > 0(PU)	$\chi^2 = 14.4$	***$p < 0.001$
*w*A(PU)		0(*w*A PU)	*w*A(PU) > 0(*w*A PU)	$\chi^2 = 10.5$	**$p < 0.01$
*w*B(PU)		0(*w*B PU)	*w*B(PU) > 0(*w*B PU)	$\chi^2 = 64.4$	***$p < 0.001$
*w*A*w*B(PU)		0(*w*A*w*B PU)	*w*A*w*B(PU) > 0(*w*A*w*B PU)	$\chi^2 = 52.36$	***$p < 0.001$

Table 4. 3 Life span comparison of females given heat shock at 37°C

The significance was calculated using Log Rank statistics with a significance level of 0.05.

However, *w*A(PU) was an exception where 0(PU) had a longer life span than *w*A(PU (χ^2=19.91, $p < 0.01$). Again, what is consistent is the decrease in life span of the recently cured lines 0(*w*A PU)

{(χ^2=10.50, $p < 0.001$ for *w*A(PU)}, 0(*w*B PU) {(χ^2=64.47, $p < 0.001$ for *w*B(PU)} and 0(*w*A*w*B PU) {(χ^2=52.36, $p < 0.001$ for *w*A*w*B(PU)} from their parental infected lines.

In the case of males, at 30°C, again, the infected *Wolbachia* strains had significantly longer life span when compared to the uninfected line.

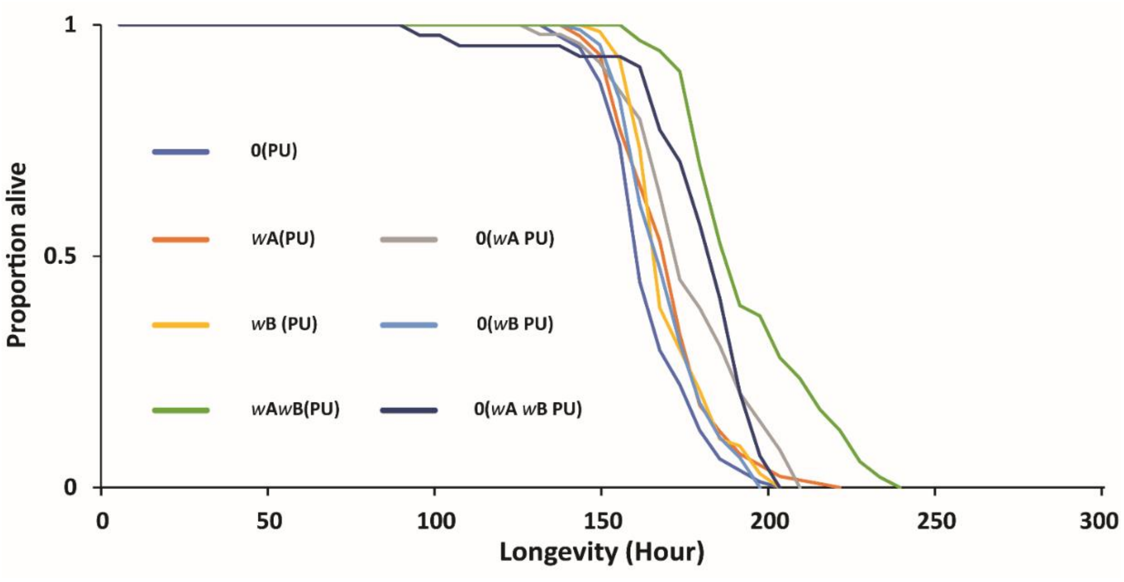


Figure 4. 5. Lifespan of males given heat shock at 30°C

Sample sizes for the four infections 0(PU), *w*A(PU), *w*B(PU), *w*A*w*B(PU) [n=81, n=124, n=67 and n=89 respectively] and the recently cured strains 0(*w*A PU), 0(*w*B PU), 0(*w*A*w*B PU) [n=49, n=93, and n=44 respectively].

Strain	Compared with	Strain	Result	χ^2value	p value
0(PU)		*w*A(PA)	*w*A(PA) > 0(PU)	χ^2 = 4.9	*p < 0.05
0(PU)		*w*B(PU)	*w*B(PU) > 0(PU)	χ^2 = 4.34	*p < 0.05
0(PU)		*w*A*w*B(PU)	*w*A*w*B(PU) > 0(PU)	χ^2 =70.15	***p < 0.001
*w*A(PU)		0(*w*A PU)	*w*A(PU) > 0(*w*A PU)	χ^2 = 11.9	*p < 0.05
*w*B(PU)		0(*w*B PU)	*w*B(PU) = 0(*w*B PU)	χ^2 = 3.35	p = 0.07
*w*A*w*B(PU)		0(*w*A*w*B PU)	*w*A*w*B(PU) > 0(*w*A*w*B PU)	χ^2 = 11.06	*p < 0.05

Table 4. 4 Life span comparison of males given heat shock at 30°C

The significance was calculated using Log Rank statistics with a significance level of 0.05.

*w*A(PU), *w*B(PU) and *w*A*w*B(PU) had longer life span when compared to 0(PU) { χ^2=4.90, p < 0.05 for *w*A(PU), χ^2=4.34, p < 0.05 for *w*B(PU) and χ^2=70.15, p < 0.001 for *w*A*w*B(PU)}. The recently cured lines 0(*w*A PU) {χ^2=11.9, p < 0.05 for *w*A(PU)}, and 0(*w*A*w*B PU) {χ^2=11.06, p < 0.05 for *w*A*w*B(PU)} showed significant decrease in life span when compared to the parent infection lines. However, 0(*w*B PU) (χ^2=3.25, p = 0.07) as an exception was still comparable to the parent line *w*B(PU).

Interestingly, at 32°C {figure 4.3 (B)} the infection lines of *w*A(PU) and *w*B(PU) had shorter life span as compared to 0(PU) {(χ^2=16.73, p < 0.001 for *w*A(PU) and χ^2=54.63, p < 0.001 for *w*B(PU)}. The recently cured lines 0(*w*A PU) (χ^2=2.25, p = 0.13) and 0(*w*B PU) (χ^2=2.25, p = 0.13) also had comparable life span to that of their parental infected lines *w*A(PU) and *w*B(PU) respectively.

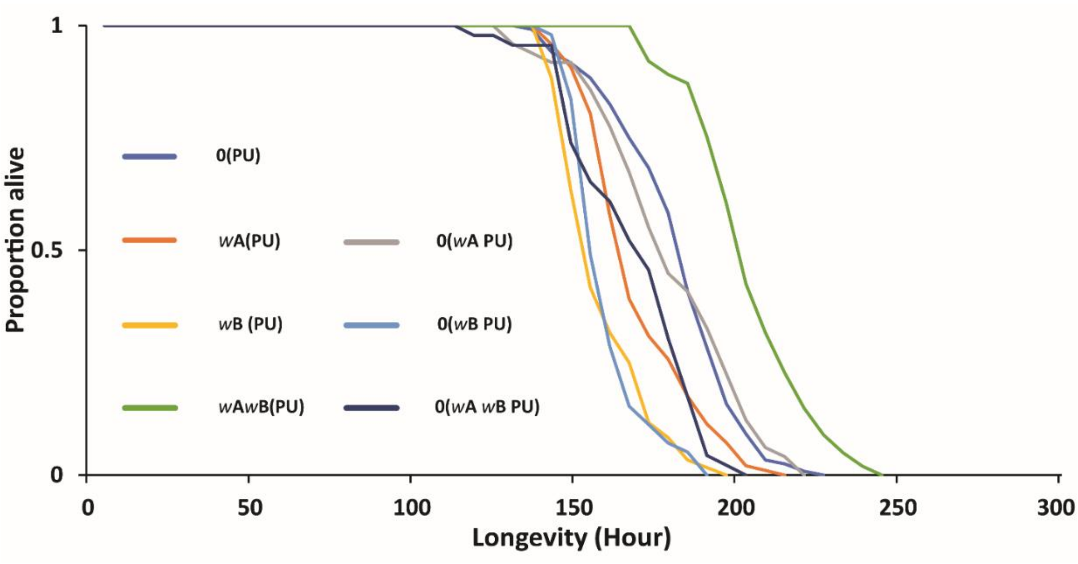


Figure 4. 6. Lifespan of males given heat shock at 32°C.

Sample sizes for the four infections 0(PU), *w*A(PU), *w*B(PU), *w*A*w*B(PU) [n=120, n=97, n=60 and n=101 respectively] and the recently cured lines 0(*w*A PU), 0(*w*B PU), 0(*w*A*w*B PU) [n=49, n=98, and n=46 respectively].

Strain		Strain	Result	χ^2value	p value
0(PU)	Compared with	*w*A(PA)	0(PU) > *w*A(PU)	$\chi^2 = 16.73$	*p < 0.05
0(PU)		*w*B(PU)	0(PU) > *w*B(PU)	$\chi^2 = 54.63$	*p < 0.05
0(PU)		*w*A*w*B(PU)	*w*A*w*B(PU) > 0(PU)	$\chi^2 = 26.7$	***p < 0.001
*w*A(PU)		0(*w*A PU)	*w*A(PU) = 0(*w*A PU)	$\chi^2 = 2.25$	p = 0.13
*w*B(PU)		0(*w*B PU)	*w*B(PU) = 0(*w*B PU)	$\chi^2 = 2.25$	p = 0.13
*w*A*w*B(PU)		0(*w*A*w*B PU)	*w*A*w*B(PU) > 0(*w*A*w*B PU)	$\chi^2 = 26.63$	*p < 0.05

Table 4. 5 Life span comparison of males given heat shock at 32°C

The significance was calculated using Log Rank statistics with a significance level of 0.05.

However, the double infection line *w*A*w*B(PU) (χ^2=26.70, p < 0.001) showed longer life span than 0(PU). The recently cured line of 0(*w*A*w*B PU) also showed a significant reduction in its life span compared to *w*A*w*B(PU)
(χ^2=26.63, p < 0.001).

Even at 37°C {figure 4.3 (C)}, the infection lines of *w*A(PU) and *w*B(PU) had shorter life span as compared to 0(PU) {(χ^2=49.79, p < 0.001 for *w*A(PU) and χ^2=71.48, p < 0.001 for *w*B(PU)}. The recently cured line 0(*w*A PU) (χ^2=24.22, p < 0.001) had longer life span than *w*A(PU). 0(*w*B PU) (χ^2=3.74, p = 0.053) also had comparable life span to that of their parent infection lines *w*B(PU). However, the double infection line *w*A*w*B(PU) (χ^2=19.19, p < 0.001) showed longer life span than 0(PU). The recently cured line of 0(*w*A*w*B PU) also showed significant reduction in its life span when compared to *w*A*w*B(PU) (χ^2=16.09, p < 0.001).

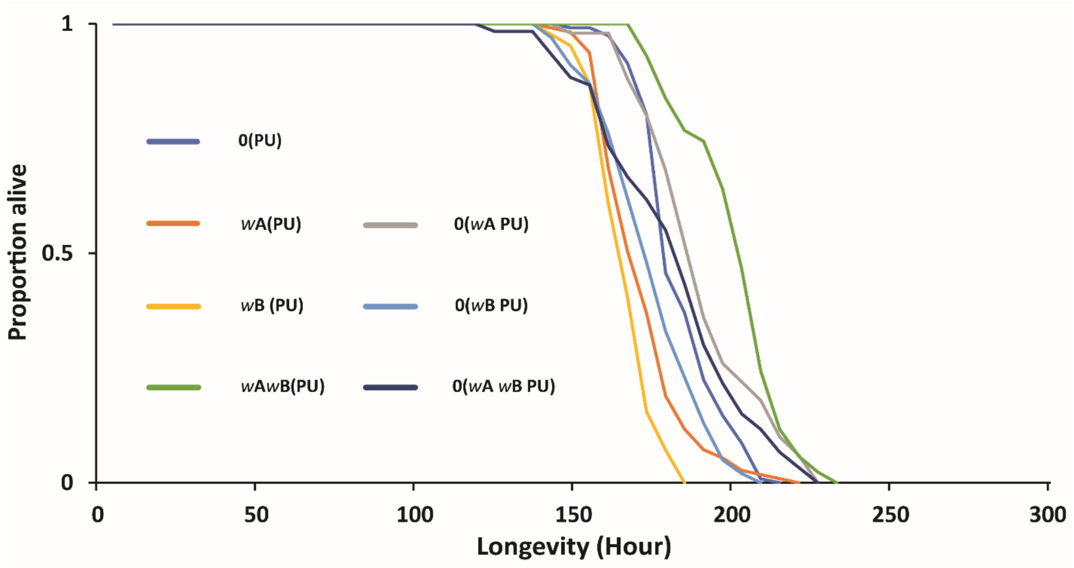


Figure 4. 7. Lifespan of females given heat shock at 37°C

Sample sizes for the four infections 0(PU), *w*A(PU), *w*B(PU), *w*A*w*B(PU) [n=116, n=111, n=84 and n=86 respectively] and the recently cured lines 0(*w*A PU), 0(*w*B PU), 0(*w*A*w*B PU) [n=50, n=100, and n=60 respectively].

Strain	Compared with	Strain	Result	χ^2value	p value
0(PU)		*w*A(PA)	0(PU) > *w*A(PU)	$\chi^2 = 49.79$	***$p < 0.001$
0(PU)		*w*B(PU)	0(PU) > *w*B(PU)	$\chi^2 = 71.48$	***$p < 0.001$
0(PU)		*w*A*w*B(PU)	*w*A*w*B(PU) > 0(PU)	$\chi^2 = 19.19$	***$p < 0.001$
*w*A(PU)		0(*w*A PU)	0(*w*A PU) > *w*A(PU)	$\chi^2 = 24.22$	***$p < 0.001$
*w*B(PU)		0(*w*B PU)	0(*w*B PU) = *w*B(PU)	$\chi^2 = 3.74$	$p = 0.053$
*w*A*w*B(PU)		0(*w*A*w*B PU)	*w*A*w*B(PU) > 0(*w*A*w*B PU)	$\chi^2 = 16.09$	***$p < 0.001$

Table 4. 6 Life span comparison of males given heat shock at 37°C

The significance was calculated using Log Rank statistics with a significance level of 0.05.

4.3.3 Heat stressed *Wolbachia* infected males sire more daughters

To test whether heat stress affects the reproductive capability of the males, we enumerated the number of female offspring (daughters) sired by the heat-stressed males which were mated with an untreated female of the same genotype. The males of different lines were exposed to elevated temperatures at 30°C, 32°C, and 37°C.

At 30°C {figure 4.4 (A)**}** a significant difference was observed between the different *N. vitripennis* lines for the number of daughters produced (Kruskal-Wallis: H=13.02, $p < 0.05$). Males of the *Wolbachia* infected lines *w*B(PU) (MWU: U=981.5, $p < 0.01$), and *w*A*w*B(PU) (MWU: U=416.5, $p < 0.05$), sired more daughters than 0(PU). *w*A(PU) however had similar number of daughters produced when compared to 0(PU) (MWU: U=749.5, $p = 0.4$).

The recently cured 0(*w*A*w*B PU) line showed significant reduction in the number of daughters produced as compared to the infected counterpart *w*A*w*B(PU) (MWU: U=804, $p < 0.05$). while 0(*w*A PU) {MWU: U=599, $p = 0.89$ for *w*A(PU)}, and 0(*w*B PU) {MWU: U=504.5, $p = 0.87$ for *w*B(PU)} did not show any difference from their infected counterparts.

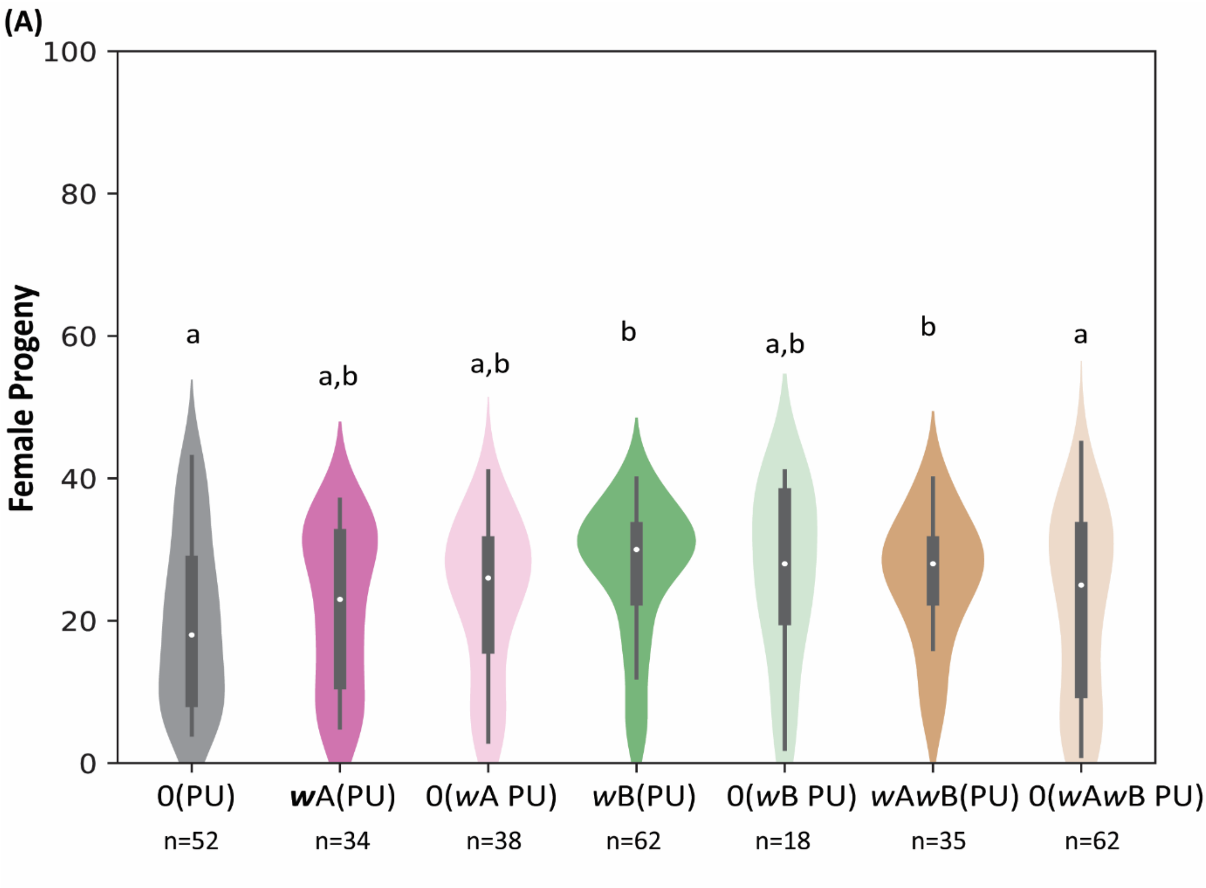


Figure 4. 8. Number of daughters produced by the males given heat shock at 30°C The differences were calculated using the Mann-Whitney U test with a significance level of 0.05.

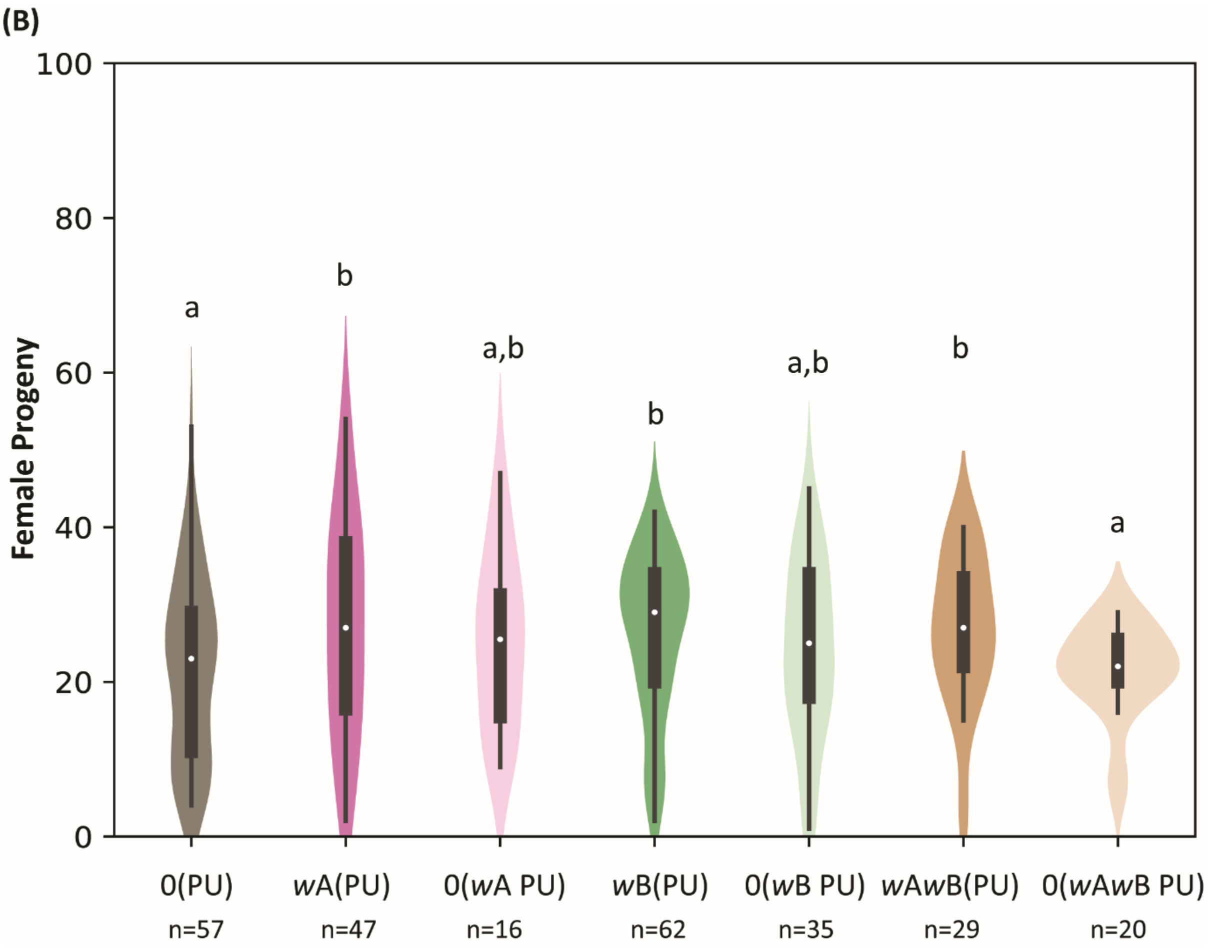


Figure 4. 9. Number of daughters produced by the males given heat shock at 32°C

The differences were calculated using the Mann-Whitney U test with a significance level of 0.05.

At 32°C {figure 4.4 (B)**}** *w*A(PU) (MWU: U=1014, $p < 0.05$) *w*B(PU) (MWU: U=1310, $p < 0.05$) and *w*A*w*B(PU) (MWU: U=625, $p < 0.05$), males produced more daughters than 0(PU).

The recently cured 0(*w*A*w*B PU) strain showed significant reduction in the number of daughters produced as compared to the infected counterpart *w*A*w*B(PU) (MWU: U=789, $p < 0.05$). while 0(*w*A PU) {MWU: U=558, p = 0.89 for *w*A(PU)}, and 0(*w*B PU) {MWU: U=523.5, p = 0.87 for *w*B(PU)} did not show any difference from their infected counterparts.

However, at 37°C {figure 4.4 (C)} *w*A(PU) (MWU: U=1383, p = 0.7) and *w*B(PU) (MWU: U=1418.5, p = 0.21), had similar number of daughters when compared to 0(PU). Only *w*A*w*B(PU) (MWU: U=994, $p < 0.05$) had number of daughters produced than 0(PU). Also, upon removal of *Wolbachia*, only 0(wAwB PU) (MWU: U=919, $p < 0.05$) line showed significant decrease in the number of daughters produced.

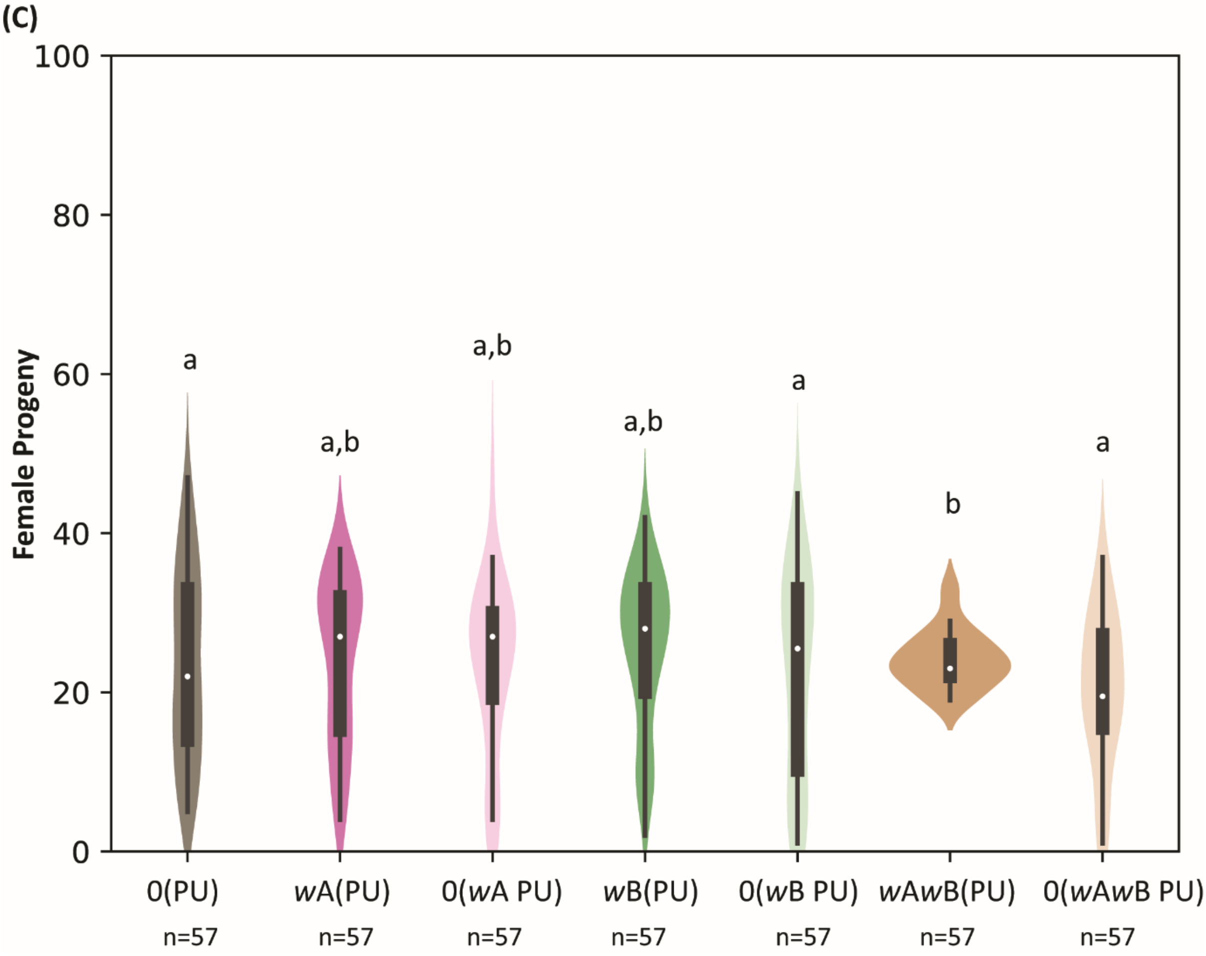


Figure 4. 10. Number of daughters produced by the males' given heat shock at 37°C.

The differences were calculated using the Mann-Whitney U test with a significance level of 0.05.

4.4 Discussion

Thermal stress negatively regulates the physiology and reproduction of host insects (Marshall and Sinclair, 2010; González-Tokman *et al.*, 2020). The presence of endosymbionts is known to influence such host responses against sub-lethal temperatures (Montllor *et al.* 2002; Russell and Moran, 2006; Su *et al.* 2013). Similarly, *Wolbachia* has been reported to increase the host tolerance against high-temperature stress effects (Hague *et al.*, 2020; Mazzucco *et al.*, 2020). The results suggest that temperature is an important factor mediating *Wolbachia* interactions with its host *N. vitripennis*. I observed that for most of the cases, the *Wolbachia* infected lines are more tolerant towards elevated temperatures than the uninfected line.

However, there were sex-specific variations in the phenotype where, in the case of females, the *Wolbachia* infected lines *w*B(PU) and *w*A*w*B(PU) had longer life spans than the cured 0(PU) at all the high temperatures exposures. However, *w*A(PU) had a longer life span only at 32°C. In the case of males, *w*A*w*B(PU) showed a longer life span in all the temperature treatments, while *w*A(PU) and *w*B(PU) showed decreased lifespan than 0(PU) at 32°C and 37°C. Both the single infection lines had a longer life span than 0(PU) only at 30°C. The recently cured lines 0(*w*A PU), 0(*w*B PU), and 0(*w*A*w*B PU) showed a decrease in the life span from their parent lines at all the elevated temperatures in the females. This indicates that the tolerance towards elevated temperature in terms of longer life span is because of the presence of *Wolbachia* and is not influenced by the host genotype.

The data suggests that the presence of *Wolbachia* leads to resistance against quicker sperm depletion at elevated temperatures. *w*B(PU) and *w*A*w*B(PU) males produced more daughters than 0(PU) at all the elevated temperatures. *w*A(PU) as an exception had more daughters than 0(PU) at 32°C only. As only males were given heat shock, a decrease in the number of female progenies for 0(PU) can be a consequence of sperm depletion. The heat-stressed 0(PU) males possibly had fewer spermatozoa to fertilize the females as compared to the infected males. As *N. vitripennis* males are prospermatogenic (Boivin *et al.*, 2005) and *Wolbachia* is not found in mature sperm (Bressac *et al.*, 1993; Snook *et al.*, 2000; Clark *et al.*, 2002), I also infer that *Wolbachia* provides resistance against heat at the stage of sperm production.

These experiments suggest a higher tolerance of supergroup B *Wolbachia* infection than supergroup A infection. Supergroup B provides tolerance against heat at all the three temperatures for both the sexes of the host while supergroup A infections does so only for 30°C. The results of temperature preference for a particular *Wolbachia* supergroup are similar to one another report in *Drosophila* species (Hague *et al.*, 2020) where *w*Ri and *w*HA in *D. simulans* was observed to prefer a lower temperature while *w*Mau in *D. mauritiana* preferred a warmer temperature.

The study also answers the question of the maintenance of these *Wolbachia* as single and multiple supergroup infections within one host. While protection against viruses (Ye *et al.*, 2013; Gong *et al.*, 2020; Pimentel *et al.*, 2021) remains one of the most prominent reasons for the maintenance of these infections, *Wolbachia*-induced modulation of host fitness at elevated temperatures can also be an important aspect of *Wolbachia*-host interaction.

Chapter 5

Conclusions and prospects of the dissertation thesis

5.1 Preface

Endosymbionts and their interaction with different hosts have been an area of investigation among the scientific community for a long time. From the initial investigations of the origin of organelles like mitochondria and plastids (De Bary et al. 1879, Sagan et al. 1967) to eukaryotic cell evolution (Reviewed in Archibald et al. 2015), the role of endosymbionts has been widely studied in host physiology and evolution. In this dissertation work, I have investigated the role of one of the most prevalent endosymbionts in insects named *Wolbachia* and its interactions with one of its parasitoid hosts *Nasonia vitripennis*. The dissertation work extensively studies the effect of *Wolbachia* infections on *N. vitripennis* physiology and reproduction. The dissertation also tries to address the question as to how single and multiple *Wolbachia* infections are being maintained in the parasitoid host over a long evolutionary time.

To address these questions, I separated the two *Wolbachia* infections found in *N. vitripennis* and generated stable lines of single *Wolbachia* supergroup A and supergroup B. I have extensively investigated the role of single and multiple *Wolbachia* infections in *N. vitripennis* physiology and reproduction, the role of *Wolbachia* in host responses against abiotic factors like temperature, and the genetic basis of *Wolbachia*-induced reproductive alteration in *N. vitripennis* males.

In general, the dissertation thesis adds significant information about endosymbiont-host interaction and their maintenance. The dissertation thesis adds valuable information on the ecological and evolutionary impacts of the presence of *Wolbachia* in arthropod hosts. These infections, even though bear a cost, are still being maintained in arthropod hosts. *Wolbachia* impact on host physiology has been studied extensively in systems like *Drosophila*. However, *Wolbachia* association with parasitoid hosts has been less studied. Parasitoids hold great significance in insect population ecology and pest management. Therefore the dissertation thesis on *Wolbachia* associations with parasitoid *N. vitripennis* also adds important information on *Wolbachia*-parasitoid host interaction and parasitoid biology.

5.2 Negative effects of the presence of *Wolbachia* infections on the host

Wolbachia being an endosymbiont competes with the host for its nutritional demands. This leads to the sharing of host nutrition between the host and *Wolbachia* (Kobayashi and Crouch, 2009; Whittle *et al.*, 2021). Thus, lack of nutrition can impart serious negative effects on host physiology. In Chapter 2 of the dissertation thesis, I have explained the profound negative effects of the presence of single and multiple *Wolbachia* infections on the host. The results are in contrast to the previous reports suggesting positive fitness effects (Stolk and Stouthamer, 1996) and no fitness effects (Bordenstein and Werren, 2000) of *Wolbachia* on *N. vitripennis*. These negative effects are on both the sexes of the host where traits like longevity, copulation capability, and sperm production are compromised in the infected males while longevity, number of progenies, and fecundity are compromised in the infected females. I have also observed sex-specific variations in these effects of *Wolbachia*. I recured the single and double infection lines to ensure that the effects are because of the presence of *Wolbachia*. In the recently cured strains of 0(*w*A PU), 0(*w*B PU), and 0(*w*A*w*B PU) the negative effects disappeared and these strains revert to the levels of 0(PU) in most of the phenotypes confirming the role of *Wolbachia* in eliciting the negative effects.

The results also establish a strong correlation between *Wolbachia* titers and the cost associated with their maintenance. *w*B(PU) and *w*A*w*B(PU) strains to have higher *Wolbachia* density also show more profound negative effects on both the sexes of the host than *w*A(PU).

5.3 First report of supergroup-specific “cost” of *Wolbachia* infections

The supergroup-specific effects of *Wolbachia* have not been studied before. With the help of the single supergroup A and supergroup B infection lines, I have established that the negative effects of these *Wolbachia* infections can also be specific to a particular supergroup. Supergroup B *Wolbachia* is costlier to both the sexes than supergroup A. While *w*B(PU) shows strong effects on all the traits studied in both the sexes, *w*A(PU) had significant negative effects on the reproductive traits of the males and the longevity of females. Thus, supergroup B *Wolbachia* behaves more like a parasite for both male and female hosts while supergroup A *Wolbachia* had detrimental effects mostly in the case of males. I also predicted additive and synergistic effects of the two *Wolbachia* infections of supergroup A and B which has not been reported before.

5.4 Maintenance of single and double *Wolbachia* supergroup infections in *N. vitripennis*

The negative effects on the host and nutritional burden raise questions on the maintenance of these infections in *N. vitripennis* over a long evolutionary time scale. Based on my results and previous studies I have explained the maintenance of these single and multiple *Wolbachia* infections in *N. vitripennis*. In our results, *w*A(PU) shows less severe effects on the females with longevity being the only factor to have any negative effect. Moreover, in the case of female hosts, *w*A(PU) shows no effect on the reproductive capability of the host. Supergroup A *Wolbachia* is known to have undergone such change in other hosts (Poinsot and Merçot, 1997; Olsen *et al.*, 2001; Dobson *et al.*, 2004; Fry *et al.*, 2004; Weeks *et al.*, 2007). I predicted a similar change in the nature of supergroup A *Wolbachia* which assists its maintenance in *N. vitripennis*. On the other hand *w*B(PU) which shows strong deleterious effects can be maintained because of the high efficiency of transmission in *N. vitripennis*, where these infections also show nearly 100% transmission as well as CI (Breeuwer and Werren, 1990). Thus, my work provides deep insights into the complex relationship between *Wolbachia*-induced physiological effects and its maintenance in arthropod hosts like *N. vitripennis*.

5.5 Stage-specific transcriptome study to decipher the genetic basis of sperm modification by *Wolbachia*

Wolbachia-induced sperm modification leads to CI. Using *N. vitripennis* male reproductive biology, I have identified a prospective stage of sperm modification. Using different methods of antibiotic delivery, I have been able to achieve a reduction in CI phenotype upon antibiotic exposure for all the stages before Day 12 of *N. vitripennis* male development. Transcriptome analysis of this sperm modification stage can lead to the identification of the genes responsible for sperm modification in *N. vitripennis* males.

5.6 *Wolbachia*-mediated host response against heat stress

Temperature changes in insects are considered a major driver of development (Ma *et al.*, 2021). Exposure to high temperatures has been reported to have implications on insect longevity, reproduction, and distribution (Mołoń *et al.*, 2020). With my results, I found that the presence of *Wolbachia* increases the thermal tolerance of both the male and female hosts. Single and multiple *Wolbachia* infected males and females were more tolerant toward elevated temperatures of 30°C, 32°C, and 37°C which was evident in their greater life span when compared to the uninfected males and females. The recently cured strains had decreased life span as compared to their infected counterparts at all the temperatures in females while in the case of males, this decrease was observed only at 30°C.

Wolbachia-infected males subjected to sub-lethal temperatures sired a greater number of female progenies at all the temperatures tested. Thus, I predicted that the presence of *Wolbachia* provides resistance against the detrimental effects of elevated temperature on sperm production, where the infected males produce more sperm or lead to better sperm transfer than the uninfected males.

5.7 Prospects of the dissertation thesis

The current study can serve as the basis for a more comprehensive investigation of such supergroup-specific effects of *Wolbachia* in arthropod hosts. In my findings, supergroup A *Wolbachia* shows a more benign effect on both the male and female hosts as compared to supergroup B *Wolbachia*. A comprehensive investigation of such supergroup-specific effects in hosts like *N. vitripennis* and *Drosophila* species across the globe can lead to a better understanding of such change in the nature of supergroup A *Wolbachia*, evolving to have less severe effects on arthropod hosts, which assists its maintenance over long evolutionary time.

The current study proposes positive effects of the presence of *Wolbachia* in host responses at elevated temperatures. *Wolbachia* has been used for insect pest control and disease vector control (Zabalou *et al.*, 2004; Brelsfoard and Dobson, 2009; Saridaki and Bourtzis, 2010; Mateos *et al.*, 2020). The present findings also hold agronomic importance and can be useful in pest management at elevated temperatures. Moreover, similar studies can be done to understand the effect of *Wolbachia* infections on the host at cold temperatures.

Transcriptome analysis of the heat-stressed infected males and females, compared to the uninfected strain as control can provide the genetic basis for such resistance of the infected host at elevated temperature.

Wolbachia-induced CI has been used to control the spread of many vector-borne diseases like dengue, malaria, and chikungunya(Turley *et al.*, 2009; Iturbe-Ormaetxe *et al.*, 2011a; Ye *et al.*, 2013). However, the genetic basis of sperm modification remains poorly understood. The stage of sperm modification identified in this study can be a breakthrough to understand sperm modification induced by *Wolbachia*. Stage-specific transcriptome can be done to identify both host and *Wolbachia* genes responsible for sperm modification. With RNAi (Lynch and Desplan, 2006) and CRiSPR(Chaverra-Rodriguez *et al.*, 2020) based gene silencing methods readily available in *Nasonia*, the genetic basis of sperm modification can be understood better.

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Appendix

Figure S1. CI crosses for single *Wolbachia* infection strains *w*A(PU) and *w*B(PU).

*w*A(PU) males show incomplete CI with 0(PU) females as the cross produces both male (son) and female (daughter) progenies (top panel). *w*B(PU) males show complete CI with 0(PU) females. The cross leads to the production of an all-male brood (bottom panel).

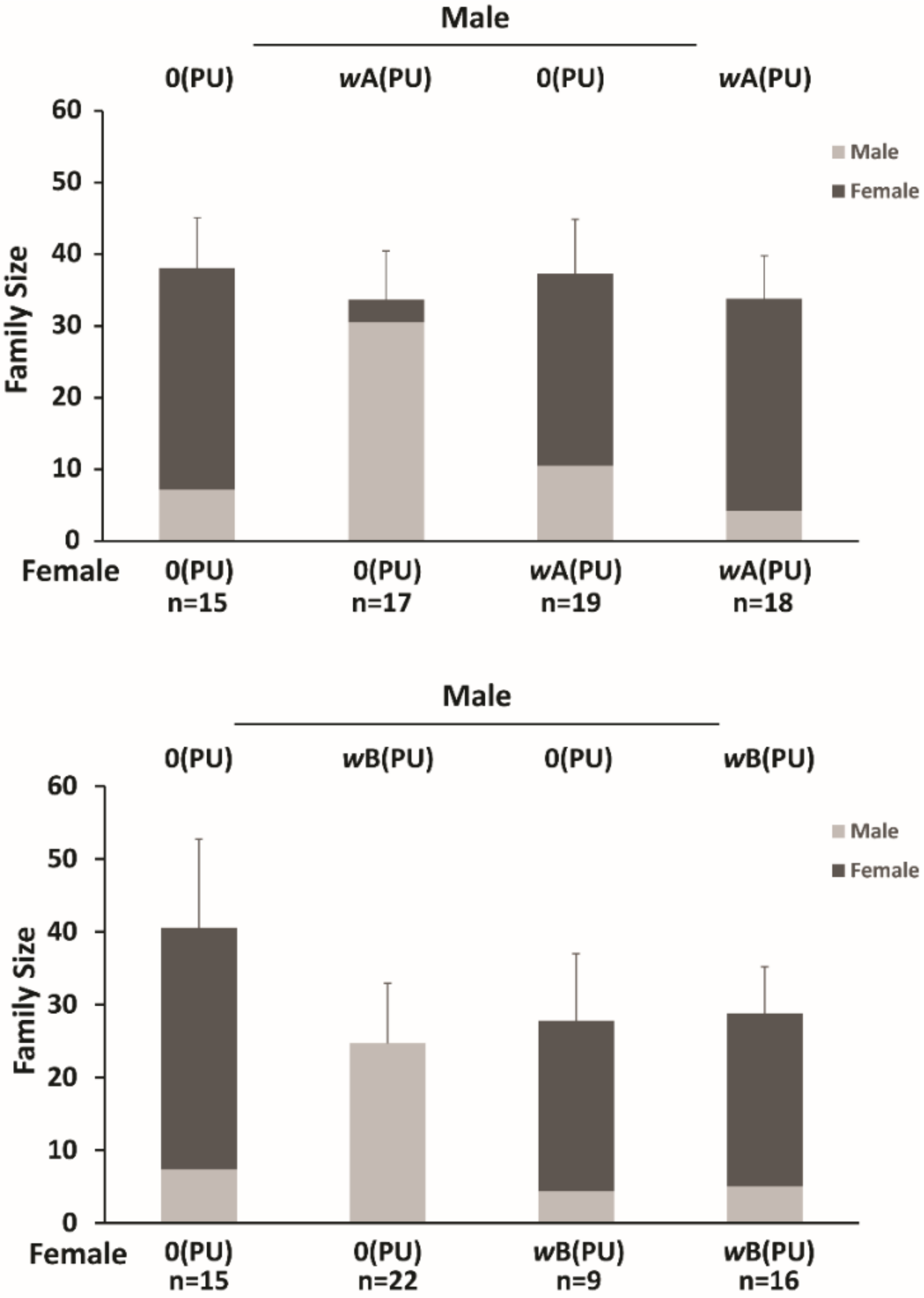
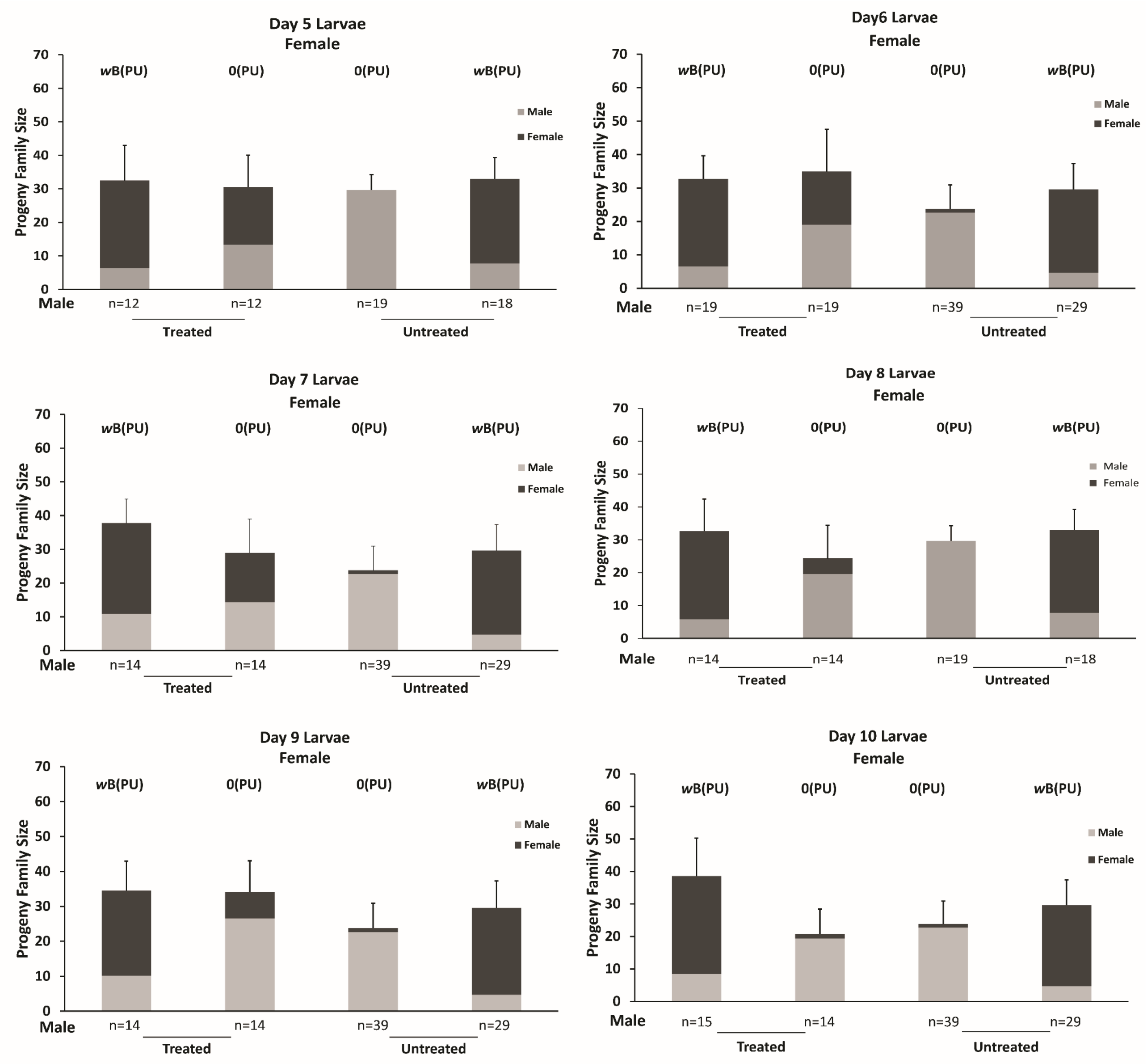
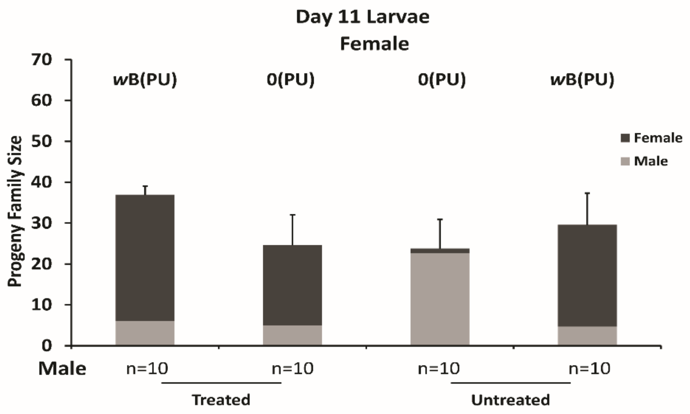
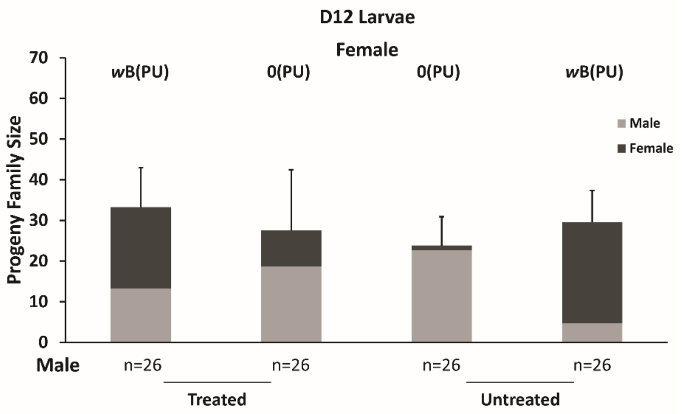


Figure S2. Surfactant mediated antibiotic delivery

CI crosses for Day 5 to Day 13 developmental stages. The number of daughters produced by the treated males was compared to the controls using Mann-Whitney U with a significance level of 0.05.




Day 11 Larvae
Female
Progeny Family Size
0
10
20
30
40
50
60
70
wB(PU)
0(PU)
0(PU)
wB(PU)
■ Female
■ Male
Male
n=10
n=10
n=10
n=10
Treated
Untreated


D12 Larvae
Female
Progeny Family Size
0
10
20
30
40
50
60
70
wB(PU)
0(PU)
0(PU)
wB(PU)
■ Male
■ Female
Male
n=26
n=26
n=26
n=26
Treated
Untreated

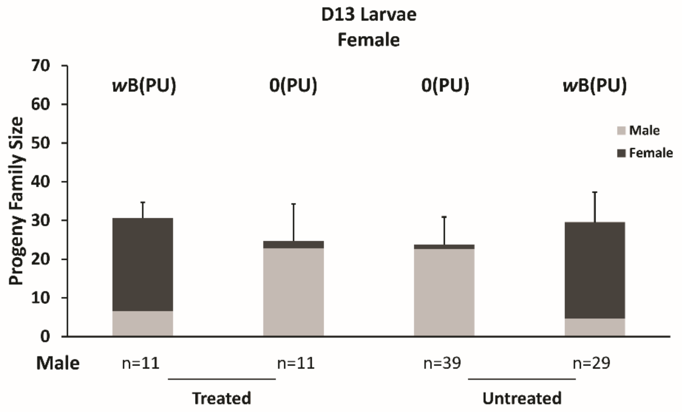

D13 Larvae
Female
Progeny Family Size
0
10
20
30
40
50
60
70
wB(PU)
0(PU)
0(PU)
wB(PU)
■ Male
■ Female
Male
n=11
n=11
n=39
n=29
Treated
Untreated

Figure S3. Antibiotic delivery by microinjection

CI crosses for Day 5 to Day 13 developmental stages. The number of daughters produced by the treated males was compared to the controls using Mann-Whitney U with a significance level of 0.05.

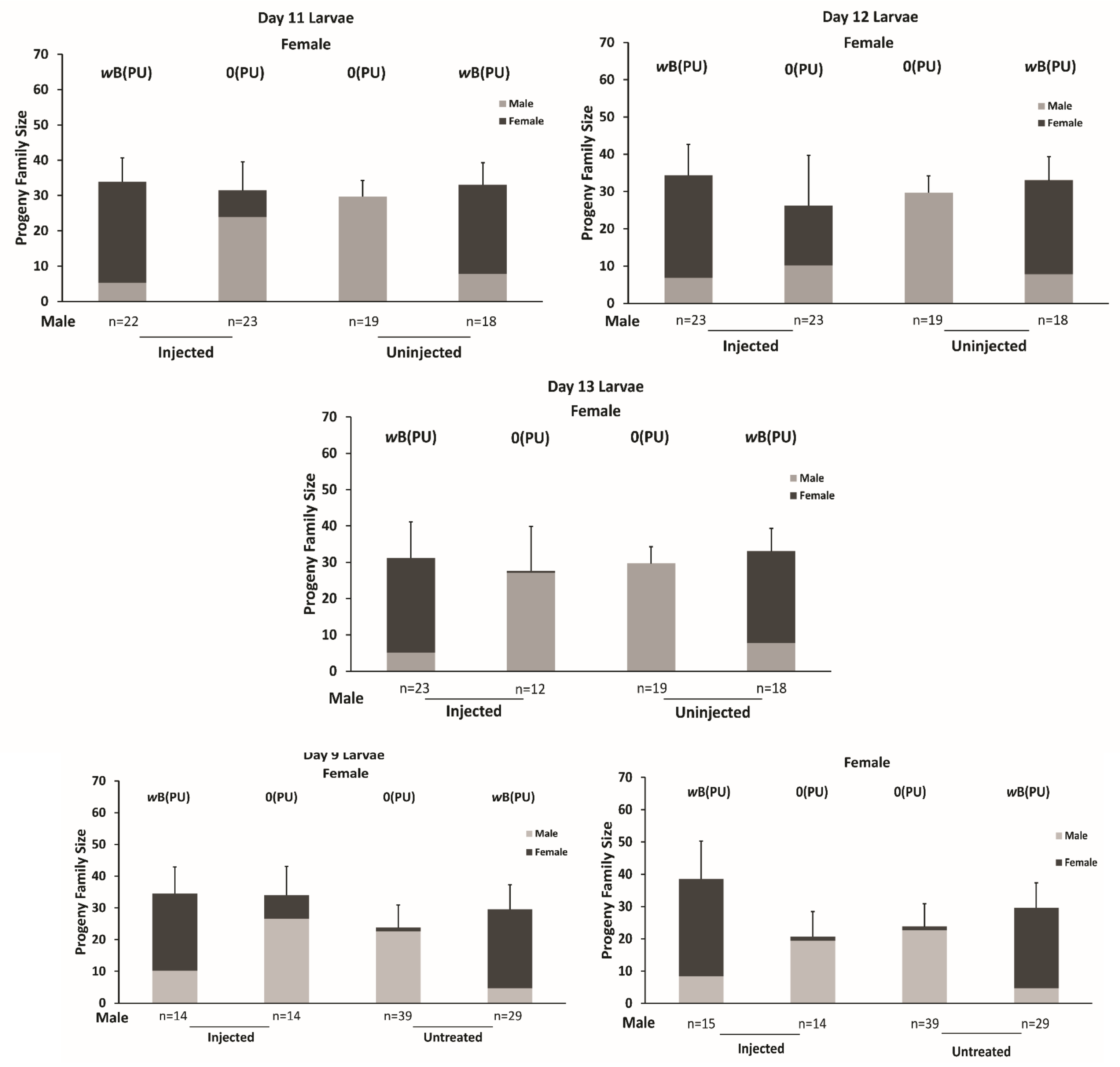


Table S1. Negative fitness effects of CI-inducing *Wolbachia*

***Wolbachia* Supergroup**	**Genera**	**Species**	***Wolbachia* Strain**	**Host Sex**	**Negative effect**	**Reference**
A	*Drosophila*	*D. melanogaster*	A-*w*MelPop	Female/Male	Tissue degeneration, reduced lifespan	Min et al. 1997; Reynolds et al. 2003
			A-*w*MelPop	Female/Male	Decreased response to food cues	Peng et al. 2008
			A-*w*Mel	Female	Reduced body size	Hoffmann et al. 1997
			A-*w*Mel	Female	Reduced fecundity after a dormancy period	Kriesner et al. 2016
		D simulans	A-*w*Ri	Female	Reduction in fecundity	Hoffmann et al., 1988, 1990
			A-*w*Ri	Female	Reduction in fecundity	Snook et al. 2000
			A-*w*Ri	Male	Lesser sperm cysts, reduced fertility	Snook et al. 2000
			A-*w*Ha	Female	Reduction in fecundity	Fytrou et al. 2006
			A-*w*Ha	Female/Male	Reduced thorax length, reduction in an immune response against parasitoid infection	Fytrou et al. 2006
			A-*w*Ri	Male	Reduced sperm competition	Crespigny et al. *2006*
		D. suzukii	A-*w*Suz	Female	Reduced progeny family size	Hamm et al. 2014
B	*Aedes*	*A. albopictus*	A-*w*Alb, B-*w*Alb	Female	Reduced lifespan, reduction in fecundity	Islam et al. 2006; Sun et al. 2009
		A. aegypti	A-*w*MelPop	Female	Reduced lifespan, reduction in fecundity	Ross et al. 2019
			A-*w*MelPop	Female/Male	Reduced lifespan	Mcmeniman et al. 2009
			A-*w*MelPop	Female	Reduction in fecundity, reduced blood-feeding success	Turley et al. 2009, 2013; Allman et al. 2020
	Culex	*C. pipiens*	B-*w*Pip	Female/Male	Embryonic Mortality	Duron et al. 2005
		C. quinquefasciatus	B-*w*Pip	Female	Reduced fecundity	Almeida et al. 2011

Table S2. Effect of *Wolbachia* infections on *N. vitripennis* (Summary)

Phenotype	**Host Sex**	**Effect of *Wolbachia***	**"Cost" compared to 0(PU)**
Lifespan	Male	0(PU) > *w*B(PU) = *w*A(PU) > *w*A*w*B(PU)	*w*A(PU) = 11.1%, *w*B(PU) = 6.5%, *w*A*w*B(PU) = 15.5%
	Female	0(PU) = *w*B(PU) > *w*A*w*B(PU) > *w*A(PU)	*w*A(PU) = 17.7%, *w*A*w*B(PU) = 15.5%
Number of copulations	Male	0(PU) > *w*A(PU) *w*B(PU) and *w*A*w*B(PU) *w*A(PU) = *w*A*w*B(PU), *w*A(PU)>*w*B(PU), *w*A*w*B(PU) = *w*B(PU)	wA(PU) = 12.4%, wB(PU) = 28.8%, wAwB(PU) = 16.7%
Sperm depletion	Male	0(PU) > *w*A(PU) *w*B(PU) and *w*A*w*B(PU) *w*A(PU) = *w*A*w*B(PU), *w*A(PU) > *w*B(PU), *w*A*w*B(PU) = *w*B(PU)	*w*A(PU) = 19.4%, *w*B(PU) = 31.3%, *w*A*w*B(PU) = 15.7%
Progeny family size	Female		
	a. Virgin	0(PU) > *w*B(PU) and *w*A*w*B(PU), 0(PU) = *w*A(PU), *w*A(PU) > *w*A*w*B(PU), *w*A(PU) = *w*B(PU), *w*B(PU) = *w*A*w*B(PU)	*w*B(PU) = 10%, *w*A*w*B(PU) = 12.4%
	b. Mated	0(PU) = *w*A(PU) > *w*A*w*B(PU) = *w*B(PU)	*w*B(PU) = 11.5%, *w*A*w*B(PU) = 9.1%
Fecundity	Female		
	a. Virgin	0(PU) = *w*A(PU) = *w*B(PU), 0(PU) > *w*A*w*B(PU)	*w*A*w*B(PU) = 14.1%
	b. Mated	0(PU) = *w*A(PU) > *w*B(PU) = *w*A*w*B(PU)	*w*B(PU) = 9.1%, *w*A*w*B(PU) = 18.2%
***Wolbachia* density**	Male	*w*A*w*B(PU) > *w*B(PU) = *w*A(PU)	
	Female	*w*A*w*B > *w*A(PU) = *w*B(PU)	

Table S3: Male Longevity

	0(PU)			***w*A(PU)**			***w*B(PU)**			***w*A*w*B(PU)**		
n=98	**Dead**	**Survival(X)**	**Proportion=(X/98)**	**Dead**	**Survival(X)**	**Proportion=(X/95)**	**Dead**	**Survival(X)**	**Proportion=(X/94)**	**Dead**	**Survival(X)**	**Proportion=(X/95)**
6 hours(Day 1)	0	98	1	0	95	1	0	94	1	0	95	1
12 hours	0	98	1	0	95	1	0	94	1	0	95	1
18 hours	0	98	1	0	95	1	0	94	1	0	95	1
24 hours	0	98	1	0	95	1	0	94	1	0	95	1
30 hours(Day 2)	0	98	1	0	95	1	0	94	1	0	95	1
36 hours	0	98	1	0	95	1	0	94	1	0	95	1
42 hours	0	98	1	0	95	1	0	94	1	0	95	1
48 hours	0	98	1	0	95	1	0	94	1	0	95	1
54 hours(Day 3)	0	98	1	0	95	1	0	94	1	0	95	1
60 hours	0	98	1	0	95	1	0	94	1	0	95	1
66 hours	0	98	1	0	95	1	0	94	1	0	95	1
72 hours	0	98	1	0	95	1	0	94	1	0	95	1
78 hours(Day 4)	0	98	1	0	95	1	0	94	1	0	95	1
84 hours	1	97	0.989795918	0	95	1	0	94	1	0	95	1
90 hours	0	97	0.989795918	0	95	1	0	94	1	0	95	1
96 hours	0	97	0.989795918	0	95	1	0	94	1	1	94	0.989473684
102 hours(Day 5)	0	97	0.989795918	0	95	1	0	94	1	0	94	0.989473684
108 hours	0	97	0.989795918	0	95	1	0	94	1	0	94	0.989473684
114 hours	0	97	0.989795918	0	95	1	0	94	1	0	94	0.989473684
120 hours	0	97	0.989795918	0	95	1	0	94	1	0	94	0.989473684
126 hours(Day 6)	0	97	0.989795918	1	94	0.989473684	0	94	1	3	91	0.957894737
132 hours	0	97	0.989795918	0	94	0.989473684	0	94	1	0	91	0.957894737
138 hours	0	97	0.989795918	0	94	0.989473684	0	94	1	1	90	0.947368421
144 hours	1	96	0.979591837	0	94	0.989473684	0	94	1	2	88	0.926315789
150 hours(Day 7)	3	93	0.948979592	5	89	0.936842105	4	90	0.957446809	12	76	0.8
156 hours	0	93	0.948979592	1	88	0.926315789	1	89	0.946808511	3	73	0.768421053
162 hours	0	93	0.948979592	4	84	0.884210526	1	88	0.936170213	4	69	0.726315789
168 hours	1	92	0.93877551	1	83	0.873684211	3	85	0.904255319	4	65	0.684210526
174 hours(Day 8)	1	91	0.928571429	13	70	0.736842105	9	76	0.808510638	15	50	0.526315789
180 hours	1	90	0.918367347	6	64	0.673684211	6	70	0.744680851	10	40	0.421052632
186 hours	5	85	0.867346939	10	54	0.568421053	4	66	0.70212766	11	29	0.305263158
192 hours	0	85	0.867346939	6	48	0.505263158	3	63	0.670212766	6	23	0.242105263
198 hours(Day 9)	6	79	0.806122449	8	40	0.421052632	6	57	0.606382979	8	15	0.157894737
204 hours	20	59	0.602040816	10	30	0.315789474	15	42	0.446808511	7	8	0.084210526
210 hours	5	55	0.56122449	5	25	0.263157895	9	33	0.35106383	0	8	0.084210526
216 hours	5	50	0.510204082	10	15	0.157894737	12	21	0.223404255	4	4	0.042105263
222 hours(Day 10)	10	40	0.408163265	6	9	0.094736842	6	15	0.159574468	3	1	0.010526316
228 hours	9	31	0.316326531	5	4	0.042105263	5	10	0.106382979	1	0	0
234 hours	4	27	0.275510204	1	3	0.031578947	4	6	0.063829787	N/A		
240 hours	7	20	0.204081633	3	0	0	3	3	0.031914894	N/A		
246 hours(Day11)	6	14	0.142857143	N/A			1	2	0.021276596	N/A		

252 hours	6	8	0.081632653	N/A			2	0	0	N/A		
258 hours	5	3	0.030612245	N/A			N/A			N/A		
264 hours	0	2	0.020408163	N/A			N/A			N/A		
270 hours(Day12)	2	0	0	N/A			N/A			N/A		

Table S4. Female Longevity

	0(PU)			***w*A(PU)**			***w*B(PU)**			***w*A*w*B(PU)**		
N=73	**Dead**	**Survival(X)**	**Proportion=(X/73)**	**Dead**	**Survival(X)**	**Proportion=(X/96)**	**Dead**	**Survival(X)**	**Proportion=(X/95)**	**Dead**	**Survival(X)**	**Proportion=(X/96)**
6 hours(Day 1)	0	73	1	0	96	1	0	95	1	0	96	1
12 hours	0	73	1	0	96	1	0	95	1	0	96	1
18 hours	0	73	1	0	96	1	0	95	1	0	96	1
24 hours	0	73	1	0	96	1	0	95	1	0	96	1
30 hours(Day 2)	0	73	1	0	96	1	0	95	1	0	96	1
36 hours	0	73	1	0	96	1	0	95	1	0	96	1
42 hours	0	73	1	0	96	1	0	95	1	0	96	1
48 hours	0	73	1	0	96	1	0	95	1	0	96	1
54 hours(Day 3)	0	73	1	0	96	1	0	95	1	0	96	1
60 hours	0	73	1	0	96	1	0	95	1	0	96	1
66 hours	0	73	1	0	96	1	0	95	1	0	96	1
72 hours	0	73	1	0	96	1	0	95	1	0	96	1
78 hours(Day 4)	0	73	1	0	96	1	0	95	1	0	96	1
84 hours	0	73	1	0	96	1	0	95	1	0	96	1
90 hours	0	73	1	0	96	1	0	95	1	0	96	1
96 hours	0	73	1	0	96	1	0	95	1	0	96	1
102 hours(Day 5)	1	72	0.986301	1	95	0.989583	0	95	1	0	96	1
108 hours	0	72	0.986301	0	95	0.989583	0	95	1	0	96	1
114 hours	0	72	0.986301	0	95	0.989583	0	95	1	0	96	1
120 hours	0	72	0.986301	0	95	0.989583	0	95	1	0	96	1
126 hours(Day 6)	1	71	0.972603	2	93	0.96875	0	95	1	2	94	0.979167
132 hours	0	71	0.972603	0	93	0.96875	0	95	1	0	94	0.979167
138 hours	0	71	0.972603	5	88	0.916667	0	95	1	2	92	0.958333
144 hours	3	68	0.931507	6	82	0.854167	0	95	1	1	91	0.947917
150 hours(Day 7)	1	67	0.917808	21	61	0.635417	0	95	1	6	85	0.885417
156 hours	2	65	0.890411	5	56	0.583333	2	93	0.978947	7	78	0.8125
162 hours	3	62	0.849315	7	49	0.510417	2	91	0.957895	8	70	0.729167
168 hours	4	58	0.794521	8	41	0.427083	3	88	0.926316	11	59	0.614583
174 hours(Day 8)	10	48	0.657534	12	29	0.302083	13	75	0.789474	16	43	0.447917
180 hours	5	43	0.589041	7	22	0.229167	6	69	0.726316	6	37	0.385417
186 hours	8	35	0.479452	8	14	0.145833	11	58	0.610526	8	29	0.302083
192 hours	5	30	0.410959	5	9	0.09375	5	53	0.557895	4	25	0.260417
198 hours(Day 9)	2	28	0.383562	2	7	0.072917	9	44	0.463158	3	22	0.229167
204 hours	3	25	0.342466	3	4	0.041667	9	35	0.368421	6	16	0.166667
210 hours	1	24	0.328767	1	3	0.03125	9	26	0.273684	3	13	0.135417
216 hours	1	23	0.315068	1	2	0.020833	4	22	0.231579	4	9	0.09375
222 hours(Day 10)	6	17	0.232877	2	0	0	6	16	0.168421	5	4	0.041667
228 hours	5	12	0.164384				5	11	0.115789	4	0	0
234 hours	1	11	0.150685				2	9	0.094737			
240 hours	2	9	0.123288				2	7	0.073684			
246 hours(Day11)	1	8	0.109589				2	5	0.052632			
252 hours	1	7	0.09589				2	3	0.031579			

258 hours	1	6	0.082192				3	0	0			
264 hours	4	2	0.027397									
270 hours(Day12)	2	0	0									

Table S5. Number of Copulations

Male No.	0(PU)	*w*A(PU)	0(*w*A PU)	*w*B(PU)	0(*w*B PU)	*w*A*w*B(PU)	0(*w*A*w*B PU)
1	90	72	90	54	79	80	81
2	90	71	73	73	70	75	71
3	84	90	90	60	78	60	104
4	84	80	59	59	84	80	117
5	101	76	96	65	80	61	100
6	81	75	87	66		85	101
7	90	79	90				90

Table S6. Male and Female progeny (sons and daughters) per mating

Mating Number	0(PU)																										
	Male 1				Male 2				Male 3				Male 4				Male 5				Male 6				Male 7		
	Female	Male	Total		Female	Male	Total		Female	Male	Total		Female	Male	Total		Female	Male	Total		Female	Male	Total		Female	Male	Total
1	21	7	28		28	4	32		26	4	30		30	9	39		33	10	43		36	12	48		21	7	28
2	20	8	28		33	6	39		29	5	34		14	2	16		40	13	53		34	4	38		20	8	28
3	22	8	30		38	8	46		30	9	39		25	4	29		34	5	39		13	6	19		22	8	30
4	21	4	25		28	4	32		34	7	41		27	16	43		36	8	44		32	7	39		21	4	25
5	24	7	31		29	3	32		46	6	52		32	4	36		26	17	43		31	7	38		24	7	31
6	41	10	51		30	9	39		35	5	40		31	12	43		25	5	30		31	7	38		41	10	51
7	24	5	29		32	6	38		36	7	43		35	8	43		33	7	40		39	15	54		24	5	29
8	22	8	30		37	7	44		37	7	44		33	6	39		29	6	35		24	3	27		22	8	30
9	14	2	16		36	5	41		34	10	44		31	7	38		32	9	41		22	2	24		14	2	16
10	13	3	16		34	9	43		32	14	46		28	6	34		29	9	38		32	6	38		18	3	21
11	24	7	31		22	4	26		35	7	42		30	11	41		28	7	35		24	5	29		24	7	31
12	26	6	32		25	4	29		25	7	32		37	5	42		21	7	28		37	5	42		26	6	32
13	23	3	26				0		33	14	47		32	5	37		35	10	45		29	5	34		23	3	26
14	19	10	29		37	7	44		42	6	48		30	5	35		25	7	32		34	11	45		19	10	29
15	20	4	24		17	9	26		0	45	45		23	5	28		41	12	53		25	3	28		22	4	26
16	20	3	23		21	3	24				0		22	4	26		30	6	36		29	8	37		20	3	23
17	18	2	20		38	4	42				0		38	12	50		28	5	33		39	5	44		18	2	20
18	28	5	33		23	4	27		41	6	47				0		29	6	35		12	3	15		28	5	33
19	28	7	35		26	7	33				0		32	14	46		30	6	36		29	7	36		28	7	35
20	23	8	31		28	10	38		33	5	38		21	6	27		34	8	42		22	3	25		23	8	31
21			0		27	9	36		33	4	37		39	8	47		31	10	41		28	5	33		24	7	31
22	17	10	27		20	6	26		25	4	29		27	2	29		24	7	31		29	6	35		17	10	27
23	28	9	37		30	8	38		10	3	13		26	4	30		23	5	28		36	13	49		28	9	37
24	18	5	23				0		39	6	45		34	6	40		24	8	32		38	5	43		18	5	23
25	16	4	20		14	2	16		31	9	40				0		26	8	34		37	6	43		19	4	23
26	19	5	24		15	3	18		40	7	47		36	8	44		38	5	43		8	2	10		19	5	24
27	25	6	31		22	6	28		33	11	44		28	8	36		24	3	27		22	4	26		25	6	31
28			0		27	6	33		35	4	39		48	9	57				0		40	12	52		22	5	27
29	14	6	20				0		5	2	7				0		17	6	23		35	6	41		14	6	20
30			0		23	6	29		26	4	30		38	7	45				0				0				0
31	31	10	41				0				0		34	8	42		23	10	33		0	34	34		31	10	41
32	28	7	35		33	9	42		8	28	36		26	4	30		24	7	31		37	10	47		28	7	35
33			0		33	8	41		43	7	50		32	4	36		28	4	32		0	48	48				0
34	18	5	23		32	7	39				0		46	6	52		37	8	45				0		18	5	23
35	23	6	29		30	7	37		38	6	44		1	0	1		28	4	32		24	5	29		23	6	29
36	21	5	26		24	8	32		40	8	48		0	8	8		7	4	11		34	9	43		21	5	26
37	21	6	27		36	9	45		33	8	41		0	48	48		26	7	33				0		21	6	27

38	22	5	27		8	2	10		34	9	43		0	52	52		44	11	55		25	13	38		22	5	27
39	21	6	27		35	8	43		12	3	15				0		32	9	41		32	7	39		21	6	27
40	26	4	30		34	9	43		46	6	52		28	16	44		28	3	31		21	8	29		26	4	30
41	20	3	23		28	6	34		46	8	54		17	3	20		29	6	35		39	11	50		20	3	23
42	18	3	21				0		38	8	46		12	1	13				0		2	1	3		18	3	21
43			0		2	2	4		46	6	52		26	15	41		30	11	41				0		19	3	22
44	28	10	38				0				0		23	10	33		29	7	36		27	6	33		28	10	38
45	29	10	39		11	22	33		40	8	48				0		21	11	32		20	5	25		29	10	39
46	25	11	36		9	2	11		35	7	42		5	1	6		40	10	50				0		25	11	36
47	6	2	8		7	21	28		46	7	53		30	9	39		29	6	35		26	4	30		6	2	8
48	36	9	45		19	8	27		20	4	24		2	1	3		26	9	35		22	19	41		36	9	45
49	34	10	44		37	5	42		0	35	35		26	10	36		30	12	42		26	11	37		34	10	44
50	12	12	24		22	14	36		39	10	49		0	48	48		22	11	33		8	2	10		12	12	24
51	29	5	34		12	8	20		33	10	43		30	15	45		26	9	35		1	1	2		29	5	34
52	26	8	34				0		34	12	46		25	16	41				0				0		26	8	34
53	24	8	32		24	16	40		25	6	31				0		0	58	58		18	24	42		24	8	32
54	19	16	35		6	48	54		10	0	10		29	9	38		22	4	26		1	28	29		19	16	35
55	25	2	27		5	32	37				0		22	2	24		0	41	41		13	11	24		25	2	27
56	28	13	41		2	3	5		19	3	22		39	10	49		14	17	31		0	33	33		28	13	41
57	14	12	26		1	12	13		27	15	42		13	39	52				0		0	27	27		14	12	26
58	3	1	4		2	36	38		4	38	42				0		2	18	20		0	21	21		3	1	4
59	25	11	36		2	28	30		18	10	28		25	29	54		3	10	13		0	12	12		25	11	36
60	25	9	34		0	32	32		0	43	43		28	20	48		0	44	44		0	12	12		25	9	34
61	6	12	18		23	24	47		2	41	43		0	51	51		0	22	22		0	10	10		6	12	18
62	1	9	10				0		0	45	45		0	52	52		15	10	25		0	12	12		1	9	10
63	10	32	42		0	41	41		9	41	50		45	12	57		0	15	15		6	2	8		10	32	42
64	17	5	22		0	22	22		3	38	41		36	11	47		0	22	22		0	22	22		0	5	5
65	0	28	28		27	16	43		0	5	5		4	35	39		0	41	41		0	15	15		0	28	28
66	14	3	17		0	34	34		0	48	48		40	15	55		0	48	48		0	5	5		14	3	17
67	12	8	20		22	12	34		0	8	8		29	15	44		0	38	38		0	6	6		12	8	20
68	2	9	11				0		10	16	26		29	20	49		0	30	30		0	9	9		0	9	9
69	4	17	21		0	4	4		2	45	47		16	25	41		0	58	58		0	18	18		4	17	21
70	11	21	32		0	34	34		0	50	50		33	15	48		0	57	57		0	23	23		0	21	21
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85	0	5	5		0	17	17										0	43	43						0	5	5
86	0	16	16		0	28	28										2	23	25						0	16	16
87	0	15	15		0	18	18										0	28	28						0	15	15
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91																	0	12	12								
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Mating Number	*w*A(PU)																				
	Male 1			**Male 2**			**Male 3**			**Male 4**			**Male 5**			**Male 6**			**Male 7**		
	Female	**Male**	**Total**	**Female**	**Male**	**Total**	**Female**	**Male**	**Total**	**Female**	**Male**	**Total**	**Female**	**Male**	**Total**	**Female**	**Male**	**Total**	**Female**	**Male**	**Total**
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86									0	21	21																

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Mating Number	***w*B(PU)**																						
	Male 1				**Male 2**				**Male 3**				**Male 4**				**Male 5**				**Male 6**		
	Female	**Male**	**Total**		**Female**	**Male**	**Total**		**Female**	**Male**	**Total**		**Female**	**Male**	**Total**		**Female**	**Male**	**Total**		**Female**	**Male**	**Total**
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Mating Number	*w*A*w*B(PU)																						
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	Female	**Male**	**Total**		**Female**	**Male**	**Total**		**Female**	**Male**	**Total**		**Female**	**Male**	**Total**		**Female**	**Male**	**Total**		**Female**	**Male**	**Total**
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Mating Number	0(*w*A PU)																										
	Male 1				Male 2				Male 3				Male 4				Male 5				Male 6				Male 7		
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66	27	6	33		0	18	18		20	30	50						2	22	24		0	12	12		0	28	28
67	23	16	39		0	1	1				0						0	18	18		3	38	41		0	38	38
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81	0	21	21						0	36	36						0	7	7		5	17	22		0	28	28
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91																	0	16	16								
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93																	0	17	17								
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Mating Number	0(*w*B PU)																		
	Male 1				**Male 2**				**Male 3**				**Male 4**				**Male 5**		
	Female	**Male**	**Total**		**Female**	**Male**	**Total**		**Female**	**Male**	**Total**		**Female**	**Male**	**Total**		**Female**	**Male**	**Total**
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3	23	7	30		8	5	13		25	6	31		25	6	31		19	6	25
4	22	4	26		28	4	32		20	6	26		18	6	24		21	6	27
5	24	4	28		22	5	27		15	5	20		15	5	20		15	5	20
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7	25	4	29		21	4	25		28	4	32		22	4	26		22	4	26
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23	28	6	34		22	4	26		21	6	27		21	6	27		21	6	27
24	28	9	37		21	5	26		28	4	32		28	4	32		28	4	32
25	35	6	41		2	2	4		32	6	38		32	6	38		32	6	38
26	24	6	30		23	6	29		25	4	29		25	4	29		25	4	29
27	24	3	27		24	9	33		22	5	27		22	5	27		22	5	27
28			0		31	9	40		21	8	29		21	8	29		21	8	29
29	24	2	26		18	6	24		32	6	38		32	6	38		32	6	38
30	26	5	31		9	4	13		23	4	27		23	4	27		23	4	27
31	37	14	51		24	10	34		21	5	26		29	5	34		21	5	26
32	13	3	16		18	6	24		20	6	26		20	6	26		20	6	26
33	21	4	25		19	6	25		11	7	18		11	7	18		11	7	18
34	19	5	24		24	12	36		18	3	21		19	3	22		18	3	21
35	15	4	19		19	4	23		4	3	7		4	3	7		4	3	7
36	0	25	25		24	11	35		15	10	25		32	10	42		0	10	10
37			0		3	1	4		28	6	34		30	6	36				0
38	10	38	48		3	1	4		24	4	28		28	4	32		24	4	28
39			0		17	3	20		23	4	27		24	4	28		23	4	27
40	0	36	36		22	3	25		21	3	24		16	3	19		21	3	24
41	0	22	22		24	4	28				0				0				0

42	0	31	31		19	7	26		0	28	28		0	28	28		16	4	20
43	0	4	4				0		0	25	25		0	25	25		0	25	25
44	0	25	25		19	4	23				0		0	12	12				0
45	0	21	21		8	2	10		2	12	14		2	12	14		2	12	14
46	0	12	12		9	2	11		0	47	47		0	47	47		0	47	47
47	0	16	16		36	6	42		0	32	32		0	32	32		0	32	32
48	0	18	18		13	4	17		0	36	36		0	36	36		0	36	36
49	0	6	6		25	12	37		0	11	11		4	11	15		0	11	11
50	0	50	50		25	13	38				0		2	22	24				0
51	0	40	40		9	4	13		0	38	38		0	38	38		0	38	38
52	0	56	56		5	1	6		0	35	35		3	35	38		7	35	42
53	0	21	21		19	9	28		0	41	41		0	41	41		0	41	41
54			0		14	4	18		0	36	36		6	36	42		8	36	44
55	0	34	34		5	2	7		0	19	19		0	19	19		0	19	19
56	0	22	22		8	7	15		0	21	21		9	21	30		6	21	27
57	0	13	13		0	3	3		0	16	16		0	16	16		0	16	16
58	0	12	12		0	38	38		0	36	36		8	36	44		0	36	36
59	0	8	8		0	22	22		0	12	12		0	12	12		0	12	12
60	0	35	35		0	18	18		1	24	25		1	24	25		1	24	25
61	3	13	16		0	6	6		0	36	36		0	36	36		0	36	36
62	0	22	22		0	1	1		0	22	22		0	22	22		0	22	22
63	0	47	47		0	14	14		0	14	14		0	14	14		0	14	14
64	0	24	24		0	15	15		0	18	18		0	18	18		0	18	18
65	0	36	36		0	9	9				0				0				0
66	0	26	26		0	6	6		24	10	34		0	10	10		24	10	34
67	0	30	30		0	1	1				0		6	12	18				0
68	4	24	28		0	21	21		14	28	42		0	28	28		14	28	42
69	0	29	29		0	30	30		0	22	22		0	22	22		0	22	22
70	0	12	12		0	16	16		4	8	12		4	8	12		4	8	12
71	0	51	51						0	29	29		0	29	29		0	29	29
72	0	7	7						0	35	35		0	35	35		0	35	35
73	6	8	14						2	21	23		0	21	21		2	21	23
74	0	32	32						0	18	18		0	18	18		0	18	18
75	0	14	14						0	11	11		2	11	13		0	11	11
76	0	5	5						0	39	39		0	39	39		0	39	39
77	0	26	26						0	34	34		0	34	34		3	34	37
78	0	21	21						0	41	41		0	41	41		0	41	41
79	0	45	45										0	17	17		0	32	32
80													0	13	13		0	13	13
81													0	22	22				
82													0	23	23				
83													0	11	11				
84													0	4	4				

Mating Number	0(*w*A*w*B PU)																										
	Male 1				**Male 2**				**Male 3**				**Male 4**				**Male 5**				**Male 6**				**Male 7**		
	Female	**Male**	**Total**		**Female**	**Male**	**Total**		**Female**	**Male**	**Total**		**Female**	**Male**	**Total**		**Female**	**Male**	**Total**		**Female**	**Male**	**Total**		**Female**	**Male**	**Total**
1	39	6	45		12	2	14		19	1	20		31	7	38		34	7	41		26	2	28		21	7	28
2	36	4	40		31	8	39		42	6	48		45	6	51		46	8	54		43	10	53		20	8	28
3	38	6	44		2	2	4		24	2	26		23	3	26		29	5	34		30	1	31		22	8	30
4	26	4	30		25	5	30		46	3	49		20	5	25		34	4	38		36	5	41		21	4	25
5	39	7	46		29	3	32				0		6	1	7		41	4	45		30	5	35		24	7	31
6	23	5	28		17	4	21		34	6	40		27	3	30		13	1	14		33	3	36		41	10	51
7	22	4	26		10	4	14		47	2	49		24	2	26		41	4	45		28	8	36		24	5	29
8	16	5	21		31	8	39		49	4	53		30	3	33		45	2	47		3	5	8		22	8	30
9	34	7	41		30	7	37		39	6	45		26	4	30		54	7	61		34	5	39		14	2	16
10	32	6	38		23	6	29		34	6	40		26	4	30		42	4	46		3	1	4				0
11	37	6	43		34	4	38		18	27	45		22	3	25		13	1	14		30	2	32		24	7	31
12	19	31	50		16	2	18		44	7	51		20	2	22		43	7	50		39	6	45		26	6	32
13	12	4	16		18	3	21		16	4	20		34	4	38		42	4	46		22	2	24		23	3	26
14	9	1	10		21	3	24		26	3	29		24	5	29		37	6	43		26	3	29		19	10	29
15	22	2	24		24	3	27		24	4	28		18	2	20		52	7	59		22	4	26				0
16	30	7	37		20	3	23		32	6	38		19	3	22		31	2	33		21	3	24		20	3	23
17	28	5	33		28	4	32		34	5	39		9	2	11		34	7	41		25	5	30		18	2	20
18	29	3	32		39	8	47		33	9	42		34	4	38		29	3	32		45	5	50		28	5	33
19	24	6	30		22	4	26		32	6	38		23	2	25		25	4	29		38	11	49		28	7	35
20	26	3	29		21	4	25		37	6	43		32	12	44		36	4	40		1	1	2		23	8	31
21	31	7	38		29	5	34		36	6	42		12	2	14		27	4	31		42	7	49				0
22	37	9	46		19	3	22		32	5	37		25	5	30		37	7	44		20	8	28		17	10	27
23	32	3	35		30	7	37		40	8	48		34	7	41		39	6	45		20	4	24		28	9	37
24	20	5	25		35	6	41		30	4	34		15	4	19		37	5	42		14	1	15		18	5	23
25	22	5	27		30	5	35		32	5	37		20	4	24		15	21	36		16	1	17				0
26	26	8	34		31	6	37		29	4	33		32	6	38		38	6	44		0	19	19		19	5	24
27	30	4	34		33	7	40		48	11	59		49	11	60		12	1	13		0	36	36		25	6	31
28	35	4	39		40	12	52		34	9	43		35	4	39		42	4	46		0	22	22		22	5	27
29	28	2	30		29	2	31		29	4	33		40	11	51		38	6	44		41	6	47		14	6	20
30	29	4	33		22	4	26		36	5	41				0		32	7	39		24	5	29				0
31	29	6	35		38	4	42		32	6	38		32	9	41		29	7	36				0		31	10	41
32	8	1	9		34	8	42		28	4	32		33	7	40		20	2	22		0	24	24		28	7	35
33	27	6	33		18	1	19		27	7	34		38	6	44		0	35	35				0				0
34	32	5	37		47	7	54		26	4	30		23	14	37		38	7	45		1	24	25		18	5	23
35	40	8	48		31	8	39		20	4	24		14	20	34		41	6	47		0	29	29		23	6	29
36	35	9	44		37	5	42		28	9	37		40	2	42		26	4	30		17	4	21		21	5	26
37	4	1	5		43	8	51		15	1	16		28	3	31		28	4	32		0	42	42		21	6	27
38	40	6	46		25	5	30		6	1	7		0	11	11		33	4	37		40	8	48		22	5	27
39	40	7	47		28	6	34		16	13	29		22	28	50		28	3	31		24	3	27		21	6	27
40	37	5	42		30	4	34		27	5	32		0	38	38		40	12	52		41	5	46		26	4	30
41	31	5	36		24	1	25				0		0	37	37		29	5	34				0		20	3	23

42	30	5	35		29	5	34		35	4	39		13	31	44		27	4	31		30	6	36		18	3	21
43	35	7	42		15	5	20		0	28	28		8	13	21		29	3	32		0	42	42				0
44	41	9	50		0	17	17		10	2	12		0	32	32		1	38	39				0		28	10	38
45	36	5	41		18	13	31		32	2	34		31	11	42				0		36	5	41		29	10	39
46	30	5	35		0	40	40		18	8	26		11	23	34		0	28	28		18	3	21		25	11	36
47	31	9	40		10	28	38		35	6	41		0	12	12		31	7	38		12	2	14		6	2	8
48	36	8	44				0		12	22	34		11	23	34		19	3	22		5	1	6		36	9	45
49	31	10	41		14	31	45		3	1	4		0	40	40		23	6	29		10	2	12		34	10	44
50	33	12	45		0	21	21				0		0	33	33		33	1	34		12	27	39		12	12	24
51	39	15	54				0		21	5	26		8	19	27		1	3	4				0		29	5	34
52	36	10	46		30	2	32		17	3	20		14	5	19		0	19	19		0	37	37		26	8	34
53	36	6	42		0	41	41		0	13	13		0	42	42				0				0		24	8	32
54	39	12	51		0	38	38		19	1	20				0				0		25	14	39		19	16	35
55	27	5	32		11	2	13		4	22	26		21	1	22		0	34	34		28	4	32		25	2	27
56	36	8	44		22	4	26		26	3	29		3	3	6				0		0	29	29		28	13	41
57	25	5	30		32	4	36		0	44	44		24	3	27		42	5	47		19	3	22		14	12	26
58	0	52	52		9	32	41		0	20	20		3	28	31		4	2	6		20	15	35		0	1	1
59	23	4	27		12	22	34		11	26	37		18	7	25		9	3	12		25	2	27		25	11	36
60	36	4	40		0	6	6		0	8	8		2	1	3		42	6	48		0	18	18		25	9	34
61	8	32	40		0	8	8				0		0	21	21		36	7	43		23	1	24		0	12	12
62	0	9	9		0	9	9		0	39	39		0	39	39		32	4	36		3	3	6		0	9	9
63	0	14	14		0	12	12		15	5	20		7	26	33		0	29	29		28	3	31		10	32	42
64			0		0	22	22		0	36	36		0	38	38		0	14	14		0	26	26		0	5	5
65	16	25	41		0	14	14		30	6	36		0	43	43		26	4	30		22	3	25		0	28	28
66			0		0	9	9				0				0		12	3	15				0		14	3	17
67	0	12	12		0	11	11		2	16	18		5	23	28		8	4	12		27	6	33		12	8	20
68	0	25	25		0	10	10		0	39	39		0	31	31				0		12	1	13		0	9	9
69	0	11	11		0	21	21		3	14	17		5	28	33				0		4	21	25		0	17	17
70	0	30	30		0	24	24		0	21	21		21	2	23		3	1	4				0		0	21	21
71	0	23	23		0	22	22		10	7	17		28	4	32				0		0	2	2		0	4	4
72	0	21	21						0	39	39		44	8	52		37	7	44		9	26	35		0	26	26
73	0	42	42						0	35	35		30	8	38		18	15	33		0	40	40		0	4	4
74	0	27	27						0	12	12		22	4	26		0	33	33		4	14	18		0	3	3
75	0	22	22						0	26	26		9	19	28		0	3	3		0	7	7		0	11	11
76	0	13	13						0	53	53		28	7	35				0		0	31	31		0	1	1
77	0	4	4						0	46	46		0	32	32		25	3	28		8	1	9		0	9	9
78	0	21	21						0	38	38		21	3	24		1	27	28		0	20	20		0	22	22
79	0	18	18						0	32	32		8	26	34		28	4	32		0	32	32		0	21	21
80	0	26	26						0	35	35		2	21	23		11	15	26		0	25	25		4	22	26
81	0	8	8						0	24	24		0	10	10				0		0	31	31		0	45	45
82									0	26	26		0	23	23		12	26	38		5	5	10		0	39	39
83									0	36	36		0	26	26		10	27	37		0	18	18		0	22	22
84									0	12	12		0	32	32		0	34	34		0	14	14		0	26	26
85									0	18	18		0	38	38		4	22	26		0	45	45		0	5	5
86									0	8	8		0	3	3		26	16	42		0	41	41		0	16	16

87									0	18	18		0	5	5		0	36	36		0	12	12		0	15	15
88									0	52	52		0	33	33		0	42	42		0	6	6		0	16	16
89									0	22	22		0	40	40		19	3	22		0	41	41		0	3	3
90									19	12	31		0	37	37		0	35	35		0	38	38		0	18	18
91											0		0	29	29		4	22	26		0	19	19				
92									0	29	29		0	18	18		0	9	9		0	51	51				
93									0	6	6		0	27	27		0	29	29		0	12	12				
94									0	9	9		0	18	18		0	32	32		0	28	28				
95									0	15	15		0	19	19		0	8	8		0	5	5				
96									0	6	6		0	20	20		0	33	33		0	22	22				
97									0	18	18		0	14	14		0	33	33		0	34	34				
98									0	28	28		0	11	11		0	15	15		0	2	2				
99									0	12	12		0	5	5		0	16	16		0	31	31				
100									0	12	12		0	45	45		0	8	8		0	19	19				
101									0	28	28		0	38	38						0	5	5				
102									0	5	5		0	16	16												
103									0	8	8		0	31	31												
104									0	23	23		0	11	11												
105													0	26	26												
106													0	38	38												
107													0	29	29												
108													0	12	12												
109													0	5	5												
110													0	18	18												
111													0	36	36												
112													0	21	21												
113													0	8	8												
114													0	9	9												
115													0	6	6												
116													0	10	10												
117													0	22	22												

Table S7. Progeny Family Size (Virgin Female)

No.	**0(PU)**	***w*A(PU)**	**0(*w*A PU)**	***w*B(PU)**	**0(*w*B PU)**	***w*A*w*B(PU)**	**0(*w*A*w*B PU)**
1	33	14	21	9	43	39	33
2	33	27	22	15	44	15	33
3	23	13	11	25	25	11	23
4	13	55	29	21	52	28	13
5	19	37	34	20	52	23	19
6	24	22	45	22	31	30	24
7	19	21	29	20	35	26	19
8	12	32	25	24	41	23	12
9	21	24	38	27	42	31	21
10	10	32	14	36	34	12	10
11	27	16	33	26	37	27	27
12	23	16	19	32	37	16	23
13	16	13	30	25	34	15	16
14	12	23	33	28	12	15	12
15	24	25	31	7	18	15	24
16	25	27	50	19	44	22	25
17	24	14	30	28	22	22	24
18	26	14	40	24	31	22	26
19	22	16	35	25	43	21	22
20	17	40	23	27	22	10	17
21	35	17	37	17	49	14	35
22	32	39	31	22	45	24	32
23	32	36	40	2	12	24	32
24	33	27	33	8	41	23	33
25	18	17	30	23	39	35	18
26	15	13	17	11	36	13	15
27	16	25	10	20	38	21	16
28	34	21	19	28	29	17	34
29	33	11	20	21	37	14	33
30	33	18	46	22	37	24	33
31	17	11	38	28	39	13	17
32	49	37	21	26	30	31	49
33	57	43	42	24	42	26	57
34	40	47	30	20	43	32	40
35	45	52	45	28	32	22	45
36	36	47	26	27	48	16	36
37	35	8	39	29	53	20	35
38	48	49	26	37	40	27	48
39	30	35	55	42	34	24	30
40	43	27	19	43	36	24	43
41	31	42	32	29	30	17	31
42	54	29	36	39	37	11	54
43	21	11	35	31	37	16	21
44	39	5	29	39	23	21	39
45	43	40	47	29	29	28	43
46	39	30	28	22	11	28	39
47	45	42	36	25	31	37	45
48	30	31	22	54	17	26	30
49	43	48	35	33	14	35	43
50	34	49	33	39	40	29	34
51	48	25	29	17	29	13	48
52	30	8	32	51	17	29	30
53	27	36	41	47	32	15	27
54	36	14	32	31	30	22	36
55	29	26	39	35	21	45	29
56	18	31	32	47	31	35	18
57	33	49	25	48	16	33	33
58	31	45	22	39	12	32	31
59	44	33	10	30	20	32	44
60	28	47	52	41	27	43	28
61	35	46	23	35	16	39	35
62	12	11	26	24	27	38	12
63	32	34	40	16	24	40	32

64	42	47	23	20	42	28	42
65	17	38	26	30	13	34	17
66	37	51	22	31	35	32	37
67	39	21	38	26	28	32	39
68	38	41	17	37	19	41	38
69	24	28	14	31	10	17	24
70	23	26	44	19	38	27	23
71	12	30	35	18	21	30	12
72	11	7	40	23	32	18	11
73	15	38	37	4	29	31	15
74	38	52	20	21	23	37	38
75	34	30	44	39	23	20	34
76	30	30	32	26	28	33	30
77	39	15	36	52	30	8	39
78	19	31	38	13	40	48	19
79	32	33	14	30	39	37	32
80	10	50	39	7	34	26	10
81	40	48		32	21	36	40
82	39	25		13	33	50	39
83	24	43		21	41	38	24
84	52	13		42	12	18	52
85	30	44		51	27	30	30
86	37	31		50	18	16	37
87	41	38		25	23	7	41
88	20	17		49	17	23	20
89	46	8		49	52	13	46
90	38	34		17	23	36	38
91	42	45		10	44	32	42
92	39	34		3	22	44	39
93	44	41		44	30	9	44
94	31	41		41	40	23	31
95	25	47		48	37	14	25
96	41	33		52	20	6	41
97	8	34		31	27	23	2
98	24	23		42	31	17	24
99	32	41		19	24	12	32
100	46	27		29	35	33	46
101	30	48		28	36	23	30
102	36	25		2	33	25	36
103	50	21		45	36	28	50
104	48	50		10	42	30	48
105	51	10		7	31	16	51
106	29	43		18	32	11	29
107	51	19		32	36	36	
108	50	55		33	42	43	
109	46	44		22	36	49	
110	24	54		31	13	63	
111	16	29		32	44	44	
112	37	48		23	27	25	
113	50	25		16	31	24	
114	22	23		35	43	46	
115	45	40		43	43	29	
116	37	17		38		49	
117	29	35		11		47	
118	58	52		18		42	
119	39	32		42		18	
120	31	16		43		54	
121	39	47		36		50	
122	43	48		50		30	
123	13	57		55		23	
124	10	35		41		25	
125	37	10		21		48	
126	17	45		31		37	
127	46	59		38		18	
128	50	44		16		12	
129	14	47		27		36	
130	50	22		25		22	
131	39	11		48		40	

132	46	36		36		42	
133	33	20		32		34	
134	21	38		43		39	
135	53	50		36		22	
136	58	26		41		37	
137	46	35		37		48	
138	59	21		44		25	
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141	50	22		45		51	
142	22	33		21		50	
143	14	42		27		16	
144	66	10		48		28	
145	29	20		25		45	
146	18	40		32		59	
147	35	35		15		20	
148	28	60		40		30	
149	41	33		47		24	
150	26	17		42		38	
151	51	25		36		25	
152	46	33		39		21	
153	47	32		35		61	
154	29	38		35		33	
155	48	56		50		33	
156	34	11		31		37	
157	28	24		28		26	
158	20	47		22		33	
159	35	13		56		40	
160	47	20		33		32	
161	56	11		32		58	
162	29	45		19		41	
163	13	34		31		32	
164	37	41		46		41	
165	21	27		44		38	
166	33	11		34		37	
167	33	29		31		13	
168	35	48		36		14	
169	41	60		50		10	
170	49	18		20		32	
171	33	40		26		22	
172	69	44		19		31	
173	34	33		45		28	
174	40	34		51		32	
175	16			48		23	
176	25			50		28	
177	49			45		39	
178	26			51		14	
179	53			27		26	
180	15			45		23	
181	16			14		38	
182	30			17		39	
183	25			21		39	
184	39			3		37	
185	30			2		38	
186	20			2		37	
187	29			6		37	
188	43			5		37	
189	40						
190	23						

Table S8. Progeny Family Size (Mated Female)

	0(PU)			*w*A(PU)			0(*w*A PU)			*w*B(PU)			0(*w*B PU)			*w*A*w*B(PU)			0(*w*A*w*B PU)		
No.	**Female Progeny**	**Male Progeny**	**Total**	**Female Progeny**	**Male Progeny**	**Total**	**Female Progeny**	**Male Progeny**	**Total**	**Female Progeny**	**Male Progeny**	**Total**	**Female Progeny**	**Male Progeny**	**Total**	**Female Progeny**	**Male Progeny**	**Total**	**Female Progeny**	**Male Progeny**	**Total**
1	27	7	34	35	4	39	12	2	14	16	1	17	35	6	41	23	2	25	34	10	44
2	29	8	37	31	7	38	32	6	38	16	1	17	28	7	35	23	5	28	25	2	27
3	30	12	42	4	0	4	34	8	42	9	2	11	30	8	38	32	4	36	10	0	10
4	29	4	33	22	1	23	26	4	30	29	6	35	21	3	24	26	5	31	31	4	35
5	36	7	43	25	6	31	35	7	42	16	2	18	35	8	43	30	11	41	22	2	24
6	23	3	26	40	3	43	23	6	29	24	2	26	30	7	37	25	5	30	22	6	28
7	23	3	26	34	4	38	29	5	34	22	3	25	17	2	19	28	7	35	31	5	36
8	40	5	45	20	2	22	32	7	39	18	3	21	35	5	40	13	4	17	33	7	40
9	37	6	43	46	8	54	19	2	21	17	5	22	28	5	33	2	0	2	31	3	34
10	38	9	47	36	8	44	14	6	20	33	7	40	30	8	38	23	6	29	26	6	32
11	30	8	38	27	2	29	28	10	38	12	2	14	28	6	34	31	3	34	34	9	43
12	19	2	21	39	8	47	26	3	29	32	6	38	34	8	42	21	3	24	27	4	31
13	29	5	34	18	2	20	30	5	35	37	6	43	24	4	28	10	3	13	7	4	11
14	30	4	34	15	1	16	27	10	37	26	3	29	17	7	24	23	3	26	24	5	29
15	39	8	47	20	5	25	17	2	19	20	3	23	40	7	47	11	10	21	23	4	27
16	29	5	34	41	6	47	23	2	25	15	3	18	11	2	13	17	3	20	17	6	23
17	39	6	45	32	6	38	24	5	29	11	4	15	30	7	37	21	2	23	22	2	24
18	28	5	33	16	2	18	30	6	36	23	3	26	31	3	34	13	3	16	29	7	36
19	31	7	38	33	5	38	27	4	31	30	3	33	31	3	34	44	6	50	28	6	34
20	29	3	32	22	3	25	27	4	31	18	1	19	27	4	31	19	3	22	21	6	27
21	30	9	39	28	5	33	32	4	36	37	8	45	35	6	41	34	6	40	28	5	33
22	25	5	30	33	3	36	25	5	30	1	2	3	11	0	11	13	3	16	28	2	30
23	30	4	34	20	3	23	18	5	23	22	2	24	35	5	40	31	6	37	23	2	25
24	37	6	43	35	5	40	19	5	24	23	3	26	26	3	29	5	1	6	18	5	23
25	24	2	26	40	4	44	36	6	42	9	5	14	10	2	12	20	5	25	23	3	26
26	15	2	17	10	2	12	16	5	21	36	6	42	27	6	33	23	3	26	10	2	12
27	32	2	34	41	6	47	29	5	34	28	6	34	41	2	43	13	3	16	29	7	36
28	28	4	32	34	6	40	24	3	27	23	4	27	38	7	45	17	5	22	35	7	42
29	7	1	8	34	6	40	26	7	33	24	4	28	19	1	20	23	6	29	19	1	20
30	16	2	18	14	4	18	11	0	11	28	7	35	31	5	36	20	3	23	30	6	36
31	36	6	42	8	1	9	23	2	25	27	7	34	28	4	32	33	6	39	22	6	28
32	28	3	31	18	2	20	21	7	28	25	8	33	29	3	32	7	1	8	23	5	28
33	29	6	35	21	5	26	21	4	25	18	2	20	24	4	28	34	6	40	28	7	35
34	28	3	31	9	2	11	24	6	30	36	9	45	28	5	33	37	5	42	26	2	28
35	37	5	42	31	4	35	20	3	23	33	8	41	17	1	18	26	6	32	43	10	53
36	9	2	11	41	5	46	27	6	33	22	4	26	27	8	35	7	0	7	30	1	31
37	18	1	19	34	4	38	30	6	36	21	7	28	15	2	17	21	3	24	36	5	41
38	7	3	10	33	8	41	36	8	44	28	6	34	12	4	16	17	4	21	30	5	35

39	12	2	14	40	5	45	30	8	38	24	4	28	13	1	14	19	3	22	33	3	36
40	30	4	34	20	4	24	10	2	12	30	5	35	11	2	13	16	3	19	28	8	36
41	10	2	12	31	3	34	30	11	41	16	2	18	27	6	33	29	12	41	3	5	8
42	17	2	19	32	4	36	32	7	39	18	3	21	29	7	36	24	3	27	34	5	39
43	32	4	36	22	7	29	24	5	29	22	4	26	14	6	20	18	4	22	24	5	29
44	22	1	23	7	1	8	19	2	21	16	2	18	12	2	14	5	0	5	30	2	32
45	12	1	13	32	5	37	26	6	32	33	7	40	32	2	34	30	7	37	39	6	45
46	25	4	29	40	6	46	14	3	17	14	2	16	33	4	37	16	5	21	22	2	24
47	30	4	34	18	4	22	32	5	37	13	1	14	35	4	39	24	3	27	26	3	29
48	17	1	18	23	3	26	27	5	32	16	4	20	18	8	26	29	5	34	22	4	26
49	29	2	31	35	6	41	28	5	33	33	4	37	32	7	39	35	6	41	21	3	24
50	14	2	16	25	2	27	39	7	46	15	2	17	26	5	31	31	8	39	25	5	30
51	25	4	29	21	2	23	35	7	42	27	5	32	37	1	38	38	9	47	45	5	50
52	29	4	33	12	3	15	20	0	20	16	3	19	39	3	42	19	2	21	38	11	49
53	2	1	3	22	2	24	37	5	42	23	3	26	23	3	26	31	3	34	41	6	47
54	4	0	4	18	2	20	12	1	13	30	3	33	17	2	19	22	7	29	42	7	49
55	4	0	4	38	1	39	21	5	26	25	7	32	18	2	20	22	2	24	20	8	28
56	25	3	28	44	6	50	22	8	30	16	3	19	33	9	42	4	0	4	20	4	24
57	29	7	36	33	16	49	31	9	40	32	4	36	9	1	10	41	10	51	40	8	48
58	38	8	46	18	2	20	38	6	44	31	5	36	34	7	41	28	4	32	41	5	46
59	29	5	34	37	6	43	40	5	45	20	1	21	14	1	15	34	7	41	21	7	28
60	19	4	23	11	0	11	31	3	34	38	8	46	27	5	32	24	4	28	20	8	28
61	33	8	41	23	4	27	26	5	31	20	1	21	21	3	24	15	5	20	22	8	30
62	29	3	32	39	9	48	26	6	32	19	3	22	21	4	25	1	1	2	21	4	25
63	28	2	30	21	14	35	26	6	32	25	2	27	31	6	37	44	10	54	24	7	31
64	31	3	34	27	8	35	31	7	38	25	5	30	35	5	40	10	4	14	41	10	51
65	3	2	5	6	0	6	21	18	39	28	5	33	31	4	35	29	4	33	24	5	29
66	27	6	33	2	0	2	34	5	39	34	6	40	17	4	21	20	4	24	22	8	30
67	33	4	37	3	0	3	27	3	30	4	0	4	26	5	31	24	1	25	14	2	16
68	26	4	30	2	0	2	34	10	44	15	5	20	24	5	29	15	5	20	24	7	31
69	23	3	26	24	3	27	24	5	29	17	0	17	26	5	31	32	9	41	26	6	32
70	27	2	29	16	0	16	28	3	31	16	1	17	27	7	34	30	5	35	23	3	26
71	38	5	43	21	0	21	36	7	43	23	7	30	25	5	30	23	3	26	19	10	29
72	34	8	42	11	3	14	32	10	42	31	9	40	39	3	42	22	3	25	20	3	23
73	41	6	47	31	7	38	28	4	32	39	5	44	31	4	35	36	3	39	18	2	20
74	31	5	36	38	9	47	18	1	19	34	9	43	33	8	41	22	9	31	28	5	33
75	33	5	38	25	6	31	24	7	31	23	10	33	25	9	34	36	8	44	28	7	35
76	33	7	40	34	7	41	24	8	32	18	2	20	30	3	33	32	4	36	23	8	31
77	43	11	54	21	3	24	17	3	20	30	8	38	27	3	30	16	1	17	17	10	27
78	26	2	28	25	4	29	39	8	47	31	10	41	13	2	15	24	3	27	28	9	37
79	34	6	40	32	5	37	26	4	30	26	2	28	28	4	32	37	2	39	18	5	23
80	32	10	42	43	8	51	23	4	27	32	4	36	19	2	21	35	7	42	19	5	24
81	34	7	41	36	9	45	32	7	39	13	0	13	14	1	15	35	3	38	25	6	31
82	32	11	43	13	2	15	31	6	37	32	4	36	23	4	27	22	2	24	22	5	27
83	38	8	46	20	3	23	33	7	40	12	3	15	30	3	33	31	4	35	14	6	20

84	34	3	37	23	8	31	38	3	41	33	11	44	18	5	23	10	4	14	31	10	41
85	30	7	37	35	5	40	38	7	45	38	6	44	32	5	37	35	11	46	28	7	35
86	32	7	39	45	12	57	34	7	41	33	7	40	29	7	36	36	3	39	18	5	23
87	30	11	41	40	5	45	37	7	44	21	3	24	26	4	30	37	4	41	23	6	29
88	31	6	37	25	3	28	12	2	14	27	9	36	31	5	36	33	2	35	21	5	26
89	31	11	42	41	12	53	21	3	24	40	8	48	33	6	39	36	7	43	21	6	27
90	23	8	31	17	6	23	33	6	39	28	3	31	15	1	16	24	6	30	22	5	27
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92	38	5	43	26	2	28	31	6	37	32	7	39	20	1	21	33	6	39	26	4	30
93	36	4	40	34	9	43	34	6	40	32	9	41	17	2	19	33	7	40	34	7	41
94	40	4	44	31	13	44	36	6	42	22	2	24	37	5	42	35	4	39	46	8	54
95	27	3	30	38	6	44	31	8	39	34	7	41	30	4	34	35	8	43	29	5	34
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97	22	6	28	34	7	41	37	5	42	26	6	32	27	8	35	32	4	36	41	4	45
98	37	6	43	40	8	48	24	7	31	27	5	32	21	3	24	32	3	35	13	1	14
99	21	1	22	33	8	41	12	0	12	21	5	26	23	4	27	36	9	45	41	4	45
100	38	8	46	14	2	16	34	10	44	28	4	32	35	2	37	36	5	41	45	2	47
101	47	4	51	36	8	44	36	6	42	8	1	9	33	1	34	28	6	34	54	7	61
102	8	0	8	26	3	29	29	6	35	34	9	43	22	2	24	37	6	43	42	4	46
103	39	8	47	42	8	50	38	8	46	28	3	31	22	4	26	27	4	31	13	1	14
104	41	6	47	39	7	46	32	4	36	38	6	44	33	5	38	6	3	9	43	7	50
105	32	5	37	18	3	21	34	7	41	29	5	34	35	9	44	18	5	23	42	4	46
106	35	10	45	35	5	40	34	9	43	31	8	39	35	7	42	32	5	37	37	6	43
107	38	13	51	33	11	44	30	5	35	11	1	12	18	1	19	30	6	36	52	7	59
108	37	4	41	35	9	44	34	7	41	19	9	28	19	1	20	27	7	34	31	2	33
109	31	8	39	40	10	50	32	4	36	29	11	40	32	3	35	40	5	45	34	7	41
110	22	7	29	27	6	33	32	5	37	29	4	33	21	2	23	42	9	51	29	3	32
111	40	12	52	26	5	31	11	7	18	28	6	34	13	2	15	29	8	37	25	4	29
112	24	7	31	36	6	42	37	7	44	23	5	28	13	1	14	35	4	39	36	4	40
113	29	5	34	30	7	37	42	6	48	32	7	39	37	5	42	19	4	23	27	4	31
114	14	2	16	34	8	42	32	3	35	22	7	29	27	1	28	29	3	32	37	7	44
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117	36	6	42	38	9	47	27	4	31	21	5	26	23	2	25	36	6	42	15	21	36
118	26	1	27	40	8	48	28	4	32	28	7	35	19	1	20	37	3	40	38	6	44
119	29	8	37	26	10	36	45	4	49	32	5	37	36	8	44	32	10	42	12	1	13
120	29	6	35	37	4	41	28	2	30	8	3	11	27	3	30	19	4	23	42	4	46
121	46	12	58	9	22	31				32	9	41	29	4	33	26	6	32	38	6	44
122	26	3	29	12	2	14				34	11	45	22	4	26	34	4	38	32	7	39
123	30	5	35	42	5	47				30	5	35	26	5	31	39	6	45	29	7	36
124	36	6	42	34	12	46				27	7	34	19	6	25	35	4	39	20	2	22
125	10	0	10	20	5	25				30	5	35	21	6	27	27	1	28	38	7	45
126	38	6	44	32	5	37				22	4	26	15	5	20	29	3	32	41	6	47
127	32	6	38	16	3	19				32	6	38	23	5	28	28	6	34	26	4	30
128	34	10	44	29	4	33				32	5	37	22	4	26	40	3	43	28	4	32

129	25	2	27	22	3	25				30	3	33	21	3	24	36	7	43	33	4	37
130	10	0	10	28	1	29				11	3	14	14	1	15	38	7	45	28	3	31
131	31	4	35	30	6	36				23	2	25	31	8	39	16	2	18	40	12	52
132	22	2	24	34	7	41				13	2	15	31	6	37	30	4	34	31	7	38
133	22	6	28	30	9	39				9	2	11	20	4	24	25	6	31	45	6	51
134	31	5	36	6	3	9				20	5	25	13	2	15	27	5	32	23	3	26
135	33	7	40	16	4	20				10	1	11	24	4	28	27	3	30	20	5	25
136	31	3	34	21	4	25				28	10	38	24	6	30	10	1	11	6	1	7
137	26	6	32	26	2	28				16	1	17	11	3	14	13	4	17	27	3	30
138	34	9	43	33	4	37				32	7	39	21	4	25	21	5	26	24	2	26
139	27	4	31	17	4	21				28	4	32	22	8	30	14	1	15	30	3	33
140	7	4	11	20	4	24				33	8	41	16	5	21	26	2	28	26	4	30
141	24	5	29	35	7	42				31	3	34	1	4	5	10	2	12	26	4	30
142	23	4	27	24	5	29				13	1	14	22	6	28	32	5	37	22	3	25
143	17	6	23	8	2	10				31	6	37	21	6	27	27	5	32	20	2	22
144	22	2	24	23	3	26				11	0	11	28	4	32	18	3	21	34	4	38
145	29	7	36	35	5	40				26	7	33	32	6	38	31	4	35	24	5	29
146	28	6	34	32	11	43				22	4	26	25	4	29	28	7	35	18	2	20
147	21	6	27							24	4	28	22	5	27	20	2	22	19	3	22
148	28	5	33							32	3	35	21	8	29	28	3	31	9	2	11
149	28	2	30							27	3	30	32	6	38	25	5	30	34	4	38
150	23	2	25							9	2	11	23	4	27	25	6	31	23	2	25
151	18	5	23							25	4	29	24	6	30	12	3	15	32	12	44
152	23	3	26							13	4	17	25	5	30	15	3	18	12	2	14
153	10	2	12							17	2	19	23	7	30	30	4	34	25	5	30
154	29	7	36							7	1	8	22	4	26	20	3	23	34	7	41
155	35	7	42							16	6	22	24	4	28	27	7	34	15	4	19
156	19	1	20							32	9	41	26	4	30	21	4	25	20	4	24
157	30	6	36							37	8	45	25	4	29	20	3	23	32	6	38
158	22	6	28							27	3	30	28	5	33	18	2	20	49	11	60
159	23	5	28							33	7	40	30	7	37	22	4	26	35	4	39
160	28	7	35							18	3	21	29	2	31	15	2	17	40	11	51
161										12	3	15	40	10	50	18	3	21	32	9	41
162										8	2	10	21	5	26	35	6	41	33	7	40
163										25	5	30	31	5	36	21	10	31	38	6	44
164										27	7	34	33	6	39	30	5	35			
165													32	4	36	8	3	11			
166													34	4	38	23	4	27			
167													26	4	30	25	3	28			
168													30	7	37	13	2	15			
169													27	5	32	16	1	17			
170													29	5	34	28	8	36			
171													26	3	29	21	2	23			
172													29	5	34	27	4	31			
173													28	6	34	23	5	28			

174													28	9	37	28	4	32			
175													35	6	41	18	2	20			
176													24	6	30	13	0	13			
177													24	3	27						
178													24	2	26						
179													26	5	31						

Table S9. Virgin Female Fecundity (Egg Count)

No.	0(PU)	*w*A(PU)	*w*B(PU)	*w*A*w*B(PU)
1	22	34	22	23
2	28	51	31	30
3	51	43	30	45
4	24	47	31	33
5	41	22	32	36
6	20	46	24	19
7	45	41	34	12
8	27	27	23	30
9	15	44	35	18
10	40	27	41	38
11	31	28	23	30
12	28	50	22	30
13	29	22	39	41
14	44	41	34	38
15	35	29	32	24
16	37	45	47	22
17	47	57	46	32
18	22	35	34	27
19	47	23	37	36
20	32	32	38	27
21	26	35	50	23
22	36	35	35	33
23	39	32	39	31
24	16	26	31	36
25	31	22	27	13
26	27	41	13	27
27	25	34	7	33
28	53	39	26	33
29	30	16	33	21
30	31	39	37	38
31	17	43	40	29
32	40	31	43	36
33	50	21	35	26
34	39	40	28	21
35	55	20	28	48
36	63	20	40	34
37	55	36	25	27
38	34	22	51	32
39	37	16	42	35
40	31	12	16	39
41	43	20	42	32
42	40	7	29	33
43	48	44	58	24
44	32	12	25	36
45	44	22	16	23
46	27	34	16	28
47	30	34	15	28
48	20	12	35	28
49	15	28	34	24
50	18	45	47	37
51	32	13	8	19
52	40	31		39
53	56	25		45
54	25	37		35
55	30	45		13
56	15	26		15
57	66	44		16
58	38	44		29

59	21	41		28
60	30	20		33
61	30	11		26
62	41	27		14
63	31			24
64	24			20
65	23			11
66	45			34
67	34			52
68	23			16
69	29			14
70	45			19
71	27			27
72	36			31
73	40			22
74	21			21
75	26			20
76	52			9
77	34			4
78	13			27
79	32			29
80	22			36
81				26
82				51
83				25
84				25
85				44
86				71
87				30
88				4
89				53
90				25
91				25
92				21
93				25
94				41
95				20
96				32
97				23
98				39
99				27
100				32
101				32
102				41
103				33
104				31
105				38

Table S10. Mated Female Fecundity (Egg Count)

No.	**0(PU)**	***w*A(PU)**	***w*B(PU)**	***w*A*w*B(PU)**
1	29	45	25	13
2	19	11	33	34
3	33	20	22	31
4	42	28	8	9
5	35	63	21	21
6	22	19	33	10
7	35	11	23	30
8	34	28	26	39
9	25	4	31	28
10	40	35	31	21
11	19	37	21	31
12	36	16	38	33
13	33	32	20	17
14	25	36	23	20
15	24	29	25	13
16	27	25	29	17
17	47	37	24	29
18	28	8	25	16
19	31	43	35	20
20	38	29	29	37
21	38	30	18	35
22	16	31	16	43
23	59	59	35	45
24	37	40	31	32
25	44	15	45	46
26	32	16	46	43
27	49	43	65	30
28	21	53	9	24
29	37	64	31	15
30	11	22	28	34
31	24	51	36	22
32	41	16	35	29
33	20	31	41	19
34	24	27	19	32
35	28	33	30	37
36	41	31	14	24
37	12	39	31	7
38	31	15	30	30
39	21	43	52	9
40	60	48	28	22
41	31	37	34	22
42	22	29	13	15
43	29	17	30	32
44	40	41	24	28
45	26	38	11	49
46	41	21	26	14
47	19	15	40	
48	31	7	44	
49	33	16	37	
50	46	41	35	
51	48	33	43	
52	40	35	45	
53	32	32	32	
54	37	30	31	
55	30	44	21	
56	37	33	30	
57	26	21	24	
58	39	30	15	
59	46	26	24	
60	29	36		

61	21	45		
62	41	33		
63	25	51		
64	20	36		
65	48	44		
66	27	48		
67	30	21		
68	33	16		
69	48	7		
70	28	21		
71	25	20		
72	43	35		
73	28	48		
74	47	31		
75	39	29		
76	36	31		
77	24	15		
78	36	10		
79	18	33		
80	39	39		
81	24	28		
82	30	58		
83	25	38		
84	32	48		
85	22	35		
86	21	52		
87		35		
88		31		

Table S11. *Wolbachia* titer (Cq) across different developmental stages of males

Developmental stage	***w*A(PU)**				***w*B(PU)**				***w*A*w*B(PU)**		
	S6K				***S6K***				***S6K***		
	Set 1	**Set 2**	**Set 3**		**Set 1**	**Set 2**	**Set 3**		**Set 1**	**Set 2**	**Set 3**
D3	25.62	24.5851	25.19332		25.45893	23.60937	24.6213		25.7	23.31672	25.60641
D4	25.8004	24.64186	26.3058		24.86544	25.4871	24.34773		26.92489	23.80929	24.31675
D5	25.27781	23.88815	24.63468		24.08132	22.7529	23.21743		22.59961	23.59816	24.45718
D6	26.29015	26.09548	26.77797		25.63496	23.14518	27.59994		22.79241	18.84822	22.80429
D7	22.06233	20.63956	21.82839		25.25836	24.75595	24.89159		24.93303	23.889	23.47183
D8	21.03566	21.02998	21.15824		24.13356	25.47012	24.4736		24.42823	24.47719	23.93583
D9	21.96073	21.77478	20.938		24.98492	23.43743	22.918		23.28449	25.70919	23.433
D10	21.93945	21.04829	21.18799		24.98758	24.50368	24.92166		23.8945	23.88199	24.47572
D11	23.97996	22.5321	22.38478		23.83598	23.812	22.76497		25.11599	25.138	25.68892
D12	23.32867	23.65705	22.90795		22.70601	23.13436	22.80641		26.02955	24.89488	25.52808
D13	21.89482	20.99336	23.96322		23.34722	23.638	22.40473		23.66698	25.93386	24.2751

Developmental stage	***w*A(PU)**				***w*B(PU)**				***w*A*w*B(PU)**		
	hcp				***hcp***				***hcp***		
	Set 1	**Set 2**	**Set 3**		**Set 1**	**Set 2**	**Set 3**		**Set 1**	**Set 2**	**Set 3**
D3	35.53929	34.31881	35.09201		34.36482	33.08302	33.87508		34.76331	32.29583	34.54757
D4	37.07841	35.92609	36.62297		34.81632	34.67953	33.53977		35.35316	32.43891	32.92643
D5	35.98486	36.11324	35.74786		31.89484	32.17702	32.67602		31.93541	31.64852	31.03577
D6	36.13435	36.73024	37.32527		32.26028	32.73426	36.72206		34.18513	29.47912	27.98753
D7	31.12556	28.38522	30.63027		36.28116	35.58404	35.08103		37.49379	34.84742	34.57188
D8	32.00976	27.2437	27.84877		36.99517	34.04236	32.65376		38.08319	37.93615	33.29256
D9	30.80369	30.31887			35.6096	33.57364			35.57645	34.49366	
D10	32.92325	27.58239	28.87973		36.97277	32.25734	33.61477		37.06005	33.00958	34.17317
D11	33.90877	31.3412	30.79617		37.20561	31.0367	31.63083		34.5803	34.0341	34.41043
D12	27.80904	27.17405	29.10867		29.43855	29.93574	29.66825		30.86263	31.95027	33.29066
D13	29.91395	25.2308	27.56439		35.65286	33.52065	29.50348		33.63446	34.9974	29.58879

Table S12. *Wolbachia* titer (Cq) across different developmental stages of females

Developmental stage	***w*A(PU)**				***w*B(PU)**				***w*A*w*B(PU)**		
	S6K				***S6K***				***S6K***		
	Set 1	**Set 2**	**Set 3**		**Set 1**	**Set 2**	**Set 3**		**Set 1**	**Set 2**	**Set 3**
D8	25.38504	25.97545	27.76942		22.97483	25.43075	26.68546		23.99019	25.50333	26.35483
D9	23.92207	25.12964	25.93591		23.01352	25.81046	26.74724				
D10	24.59859	25.29622	25.08671		23.95695	23.77794	24.85397		23.60871	24.12484	25.61057
D11	23.69833	25.71491	26.07241		25.00602	24.30477	25.90782		24.39678	25.31063	26.49862
D12	26.08995	25.29487	26.20547		26.01317	25.29722	26.66603		26.31712	25.27218	25.993
D13	25.22499	26.37663	27.88541		25.22499	26.37663	27.88541		25.22499	26.37663	27.88541
D14	25.57807	25.74308	25.51024		25.78073	25.07879	25.10245		25.80735	25.43079	25.90536

Developmental stage	***w*A(PU)**				***w*B(PU)**				***w*A*w*B(PU)**		
	hcp				***hcp***				***hcp***		
	Set 1	**Set 2**	**Set 3**		**Set 1**	**Set 2**	**Set 3**		**Set 1**	**Set 2**	**Set 3**
D8	34.98307	36.09445	38.47498		32.73557	33.65908	35.32293		30.44138	31.78292	33.44211
D9	32.86063	33.01992	35.8536		29.77809	31.85176	34.24305				
D10	32.44046	32.76323	34.61302		29.15245	29.6738	31.22292		29.99889	29.8273	31.53088
D11	31.25265	31.9201	35.10608		30.54584	29.94384	31.70709		32.24982	29.83164	32.34123
D12	32.32405	30.24738	27.42467		32.32405	30.24738	27.42467		32.32405	30.24738	27.42467
D13	31.20442	30.83544	29.40666		31.20442	30.83544	29.40666		31.20442	30.83544	29.40666
D14	29.44112	28.48959	26.90127		29.41168	28.89592	26.92724		28.11235	29.95922	26.27236

Table S13. Male longevity - 30°C

	0(PU)			**_w_A(PU)**			**_w_B(PU)**			**_w_A_w_B(PU)**			**0(_w_A PU)**			**0(_w_B PU)**			**0(_w_A_w_B PU)**		
	Dead	**Survival(X)**	**Fraction (X/81)**	**Dead**	**Survival(X)**	**Fraction (X/124)**	**Dead**	**Survival(X)**	**Fraction (X/81)**	**Dead**	**Survival(X)**	**Fraction (X/81)**	**Dead**	**Survival (X)**	**Fraction (X/49)**	**Dead**	**Survival(X)**	**Fraction (X/81)**	**Dead**	**Survival(X)**	**Fraction (X/44)**
0 hours(Day 1)	0	81	1	0	124	1	0	67	1	0	89	1	0	49	1	0	93	1	0	44	1
6 hours	0	81	1	0	124	1	0	67	1	0	89	1	0	49	1	0	93	1	0	44	1
12 hours	0	81	1	0	124	1	0	67	1	0	89	1	0	49	1	0	93	1	0	44	1
18 hours	0	81	1	0	124	1	0	67	1	0	89	1	0	49	1	0	93	1	0	44	1
24 hours(Day 2)	0	81	1	0	124	1	0	67	1	0	89	1	0	49	1	0	93	1	0	44	1
30 hours	0	81	1	0	124	1	0	67	1	0	89	1	0	49	1	0	93	1	0	44	1
36 hours	0	81	1	0	124	1	0	67	1	0	89	1	0	49	1	0	93	1	0	44	1
42 hours	0	81	1	0	124	1	0	67	1	0	89	1	0	49	1	0	93	1	0	44	1
48 hours(Day 3)	0	81	1	0	124	1	0	67	1	0	89	1	0	49	1	0	93	1	0	44	1
54 hours	0	81	1	0	124	1	0	67	1	0	89	1	0	49	1	0	93	1	0	44	1
60 hours	0	81	1	0	124	1	0	67	1	0	89	1	0	49	1	0	93	1	0	44	1
66 hours	0	81	1	0	124	1	0	67	1	0	89	1	0	49	1	0	93	1	0	44	1
72 hours(Day 4)	0	81	1	0	124	1	0	67	1	0	89	1	0	49	1	0	93	1	0	44	1
78 hours	0	81	1	0	124	1	0	67	1	0	89	1	0	49	1	0	93	1	0	44	1
84 hours	0	81	1	0	124	1	0	67	1	0	89	1	0	49	1	0	93	1	0	44	1
90 hours	0	81	1	0	124	1	0	67	1	0	89	1	0	49	1	0	93	1	0	44	1
96 hours(Day 5)	0	81	1	0	124	1	0	67	1	0	89	1	0	49	1	0	93	1	1	43	0.977272727
102 hours	0	81	1	0	124	1	0	67	1	0	89	1	0	49	1	0	93	1	0	43	0.977272727
108 hours	0	81	1	0	124	1	0	67	1	0	89	1	0	49	1	0	93	1	1	42	0.954545455
114 hours	0	81	1	0	124	1	0	67	1	0	89	1	0	49	1	0	93	1	0	42	0.954545455
120 hours(Day 6)	0	81	1	0	124	1	0	67	1	0	89	1	0	49	1	0	93	1	0	42	0.954545455
126 hours	0	81	1	0	124	1	0	67	1	0	89	1	0	49	1	0	93	1	0	42	0.954545455
132 hours	0	81	1	0	124	1	0	67	1	0	89	1	1	48	0.979591837	0	93	1	0	42	0.954545455
138 hours	2	79	0.975308642	0	124	1	0	67	1	0	89	1	0	48	0.979591837	0	93	1	0	42	0.954545455
144 hours(Day 7)	2	77	0.950617284	3	121	0.975806452	1	66	0.985074627	0	89	1	1	47	0.959183673	1	92	0.989247312	1	41	0.931818182
150 hours	6	71	0.87654321	5	116	0.935483871	4	62	0.925373134	0	89	1	2	45	0.918367347	3	89	0.956989247	0	41	0.931818182
156 hours	11	60	0.740740741	20	96	0.774193548	13	49	0.731343284	0	89	1	3	42	0.857142857	11	78	0.838709677	0	41	0.931818182
162 hours	24	36	0.444444444	15	81	0.653225806	23	26	0.388059701	3	86	0.966292135	3	39	0.795918367	21	57	0.612903226	1	40	0.909090909
168 hours	12	24	0.296296296	15	66	0.532258065	6	20	0.298507463	2	84	0.943820225	8	31	0.632653061	13	44	0.47311828	6	34	0.772727273
174 hours	6	18	0.222222222	25	41	0.330645161	6	14	0.208955224	4	80	0.898876404	9	22	0.448979592	15	29	0.311827957	3	31	0.704545455
180 hours	8	10	0.12345679	19	22	0.177419355	7	7	0.104477612	18	62	0.696629213	3	19	0.387755102	12	17	0.182795699	6	25	0.568181818
186 hours	5	5	0.061728395	7	15	0.120967742	1	6	0.089552239	15	47	0.528089888	4	15	0.306122449	7	10	0.107526882	7	18	0.409090909
192 hours	2	3	0.037037037	6	9	0.072580645	4	2	0.029850746	12	35	0.393258427	5	10	0.204081633	4	6	0.064516129	9	9	0.204545455
198 hours	2	1	0.012345679	3	6	0.048387097	2	0	0	2	33	0.370786517	3	7	0.142857143	6	0	0	6	3	0.068181818
204 hours	1	0	0	3	3	0.024193548				8	25	0.280898876	3	4	0.081632653				3	0	0
210 hours				1	2	0.016129032				4	21	0.235955056	4	0	0						
216 hours				1	1	0.008064516				6	15	0.168539326									
222 hours				1	0	0				4	11	0.123595506									
228 hours										6	5	0.056179775									
234 hours										3	2	0.02247191									
240 hours										2	0	0									
246 hours																					
252 hours																					
258 hours																					

264 hours																					
270 hours																					
276 hours																					
282 hours																					
288 hours																					
294 hours																					
300 hours																					

Table S14. Male longevity - 32°C

	0(PU)			***w*A(PU)**			***w*B(PU)**			***w*A*w*B(PU)**			**0(*w*A PU)**			**0(*w*B PU)**			**0(*w*A*w*B PU)**		
	Dead	**Survival(X)**	**Fraction (X/120)**	**Dead**	**Survival(X)**	**Fraction (X/97)**	**Dead**	**Survival(X)**	**Fraction (X/81)**	**Dead**	**Survival(A)**	**Fraction (A/120)**	**Dead**	**Survival(X)**	**Fraction (X/49)**	**Dead**	**Survival(X)**	**Fraction (X/81)**	**Dead**	**Survival(X)**	**Fraction (X/46)**
0 hours(Day 1)	0	120	1	0	97	1	0	60	1	0	101	1	0	49	1	0	98	1	0	46	1
6 hours	0	120	1	0	97	1	0	60	1	0	101	1	0	49	1	0	98	1	0	46	1
12 hours	0	120	1	0	97	1	0	60	1	0	101	1	0	49	1	0	98	1	0	46	1
18 hours	0	120	1	0	97	1	0	60	1	0	101	1	0	49	1	0	98	1	0	46	1
24 hours(Day 2)	0	120	1	0	97	1	0	60	1	0	101	1	0	49	1	0	98	1	0	46	1
30 hours	0	120	1	0	97	1	0	60	1	0	101	1	0	49	1	0	98	1	0	46	1
36 hours	0	120	1	0	97	1	0	60	1	0	101	1	0	49	1	0	98	1	0	46	1
42 hours	0	120	1	0	97	1	0	60	1	0	101	1	0	49	1	0	98	1	0	46	1
48 hours(Day 3)	0	120	1	0	97	1	0	60	1	0	101	1	0	49	1	0	98	1	0	46	1
54 hours	0	120	1	0	97	1	0	60	1	0	101	1	0	49	1	0	98	1	0	46	1
60 hours	0	120	1	0	97	1	0	60	1	0	101	1	0	49	1	0	98	1	0	46	1
66 hours	0	120	1	0	97	1	0	60	1	0	101	1	0	49	1	0	98	1	0	46	1
72 hours(Day 4)	0	120	1	0	97	1	0	60	1	0	101	1	0	49	1	0	98	1	0	46	1
78 hours	0	120	1	0	97	1	0	60	1	0	101	1	0	49	1	0	98	1	0	46	1
84 hours	0	120	1	0	97	1	0	60	1	0	101	1	0	49	1	0	98	1	0	46	1
90 hours	0	120	1	0	97	1	0	60	1	0	101	1	0	49	1	0	98	1	0	46	1
96 hours(Day 5)	0	120	1	0	97	1	0	60	1	0	101	1	0	49	1	0	98	1	0	46	1
102 hours	0	120	1	0	97	1	0	60	1	0	101	1	0	49	1	0	98	1	0	46	1
108 hours	0	120	1	0	97	1	0	60	1	0	101	1	0	49	1	0	98	1	0	46	1
114 hours	0	120	1	0	97	1	0	60	1	0	101	1	0	49	1	0	98	1	0	46	1
120 hours(Day 6)	0	120	1	0	97	1	0	60	1	0	101	1	0	49	1	0	98	1	1	45	0.97826087
126 hours	0	120	1	0	97	1	0	60	1	0	101	1	0	49	1	0	98	1	0	45	0.97826087
132 hours	0	120	1	0	97	1	0	60	1	0	101	1	2	47	0.959183673	0	98	1	1	44	0.956521739
138 hours	1	119	0.991666667	0	97	1	0	60	1	0	101	1	1	46	0.93877551	0	98	1	0	44	0.956521739
144 hours(Day 7)	6	113	0.941666667	4	93	0.958762887	7	53	0.883333333	0	101	1	1	45	0.918367347	2	96	0.979591837	0	44	0.956521739
150 hours	3	110	0.916666667	5	88	0.907216495	15	38	0.633333333	0	101	1	0	45	0.918367347	14	82	0.836734694	10	34	0.739130435
156 hours	4	106	0.883333333	10	78	0.804123711	13	25	0.416666667	0	101	1	3	42	0.857142857	34	48	0.489795918	4	30	0.652173913
162 hours	7	99	0.825	22	56	0.577319588	6	19	0.316666667	0	101	1	4	38	0.775510204	20	28	0.285714286	2	28	0.608695652
168 hours	9	90	0.75	18	38	0.391752577	4	15	0.25	0	101	1	5	33	0.673469388	13	15	0.153061224	4	24	0.52173913
174 hours	8	82	0.683333333	8	30	0.309278351	8	7	0.116666667	8	93	0.920792	6	27	0.551020408	4	11	0.112244898	3	21	0.456521739
180 hours	12	70	0.583333333	5	25	0.257731959	2	5	0.083333333	3	90	0.891089	5	22	0.448979592	4	7	0.071428571	7	14	0.304347826
186 hours	21	49	0.408333333	8	17	0.175257732	3	2	0.033333333	2	88	0.871287	2	20	0.408163265	2	5	0.051020408	6	8	0.173913043
192 hours	15	34	0.283333333	6	11	0.113402062	1	1	0.016666667	12	76	0.752475	4	16	0.326530612	5	0	0	6	2	0.043478261
198 hours	15	19	0.158333333	4	7	0.072164948	1	0	0	15	61	0.60396	5	11	0.224489796				1	1	0.02173913
204 hours	8	11	0.091666667	5	2	0.020618557				18	43	0.425743	5	6	0.12244898				1	0	0
210 hours	7	4	0.033333333	1	1	0.010309278				11	32	0.316832	3	3	0.06122449						

216 hours	1	3	0.025	1	0	0				9	23	0.227723	3	0	0						
222 hours	2	1	0.008333333							8	15	0.148515									
228 hours	1	0	0							6	9	0.089109									
234 hours										4	5	0.049505									
240 hours										3	2	0.019802									
246 hours										2	0	0									
252 hours																					
258 hours																					
264 hours																					
270 hours																					
276 hours																					
282 hours																					
288 hours																					
294 hours																					
300 hours																					

Table S15. Male longevity - 37°C

	0(PU)			***w*A(PU)**			***w*B(PU)**			***w*A*w*B(PU)**			**0(*w*A PU)**			**0(*w*B PU)**			**0(*w*A*w*B PU)**		
	Dead	**Survival(X)**	**Fraction (X/116)**	**Dead**	**Survival(X)**	**Fraction (X/111)**	**Dead**	**Survival(X)**	**Fraction (X/81)**	**Dead**	**Survival(X)**	**Fraction (X/116)**	**Dead**	**Survival(X)**	**Fraction (X/81)**	**Dead**	**Survival(X)**	**Fraction (X/81)**	**Dead**	**Survival(X)**	**Fraction (X/60)**
0 hours(Day 1)	0	116	1	0	111	1	0	84	1	0	86	1	0	50	1	0	100	1	0	60	1
6 hours	0	116	1	0	111	1	0	84	1	0	86	1	0	50	1	0	100	1	0	60	1
12 hours	0	116	1	0	111	1	0	84	1	0	86	1	0	50	1	0	100	1	0	60	1
18 hours	0	116	1	0	111	1	0	84	1	0	86	1	0	50	1	0	100	1	0	60	1
24 hours(Day 2)	0	116	1	0	111	1	0	84	1	0	86	1	0	50	1	0	100	1	0	60	1
30 hours	0	116	1	0	111	1	0	84	1	0	86	1	0	50	1	0	100	1	0	60	1
36 hours	0	116	1	0	111	1	0	84	1	0	86	1	0	50	1	0	100	1	0	60	1
42 hours	0	116	1	0	111	1	0	84	1	0	86	1	0	50	1	0	100	1	0	60	1
48 hours(Day 3)	0	116	1	0	111	1	0	84	1	0	86	1	0	50	1	0	100	1	0	60	1
54 hours	0	116	1	0	111	1	0	84	1	0	86	1	0	50	1	0	100	1	0	60	1
60 hours	0	116	1	0	111	1	0	84	1	0	86	1	0	50	1	0	100	1	0	60	1
66 hours	0	116	1	0	111	1	0	84	1	0	86	1	0	50	1	0	100	1	0	60	1
72 hours(Day 4)	0	116	1	0	111	1	0	84	1	0	86	1	0	50	1	0	100	1	0	60	1
78 hours	0	116	1	0	111	1	0	84	1	0	86	1	0	50	1	0	100	1	0	60	1
84 hours	0	116	1	0	111	1	0	84	1	0	86	1	0	50	1	0	100	1	0	60	1
90 hours	0	116	1	0	111	1	0	84	1	0	86	1	0	50	1	0	100	1	0	60	1
96 hours(Day 5)	0	116	1	0	111	1	0	84	1	0	86	1	0	50	1	0	100	1	0	60	1
102 hours	0	116	1	0	111	1	0	84	1	0	86	1	0	50	1	0	100	1	0	60	1
108 hours	0	116	1	0	111	1	0	84	1	0	86	1	0	50	1	0	100	1	0	60	1
114 hours	0	116	1	0	111	1	0	84	1	0	86	1	0	50	1	0	100	1	0	60	1
120 hours(Day 6)	0	116	1	0	111	1	0	84	1	0	86	1	0	50	1	0	100	1	0	60	1
126 hours	0	116	1	0	111	1	0	84	1	0	86	1	0	50	1	0	100	1	1	59	0.983333333
132 hours	0	116	1	0	111	1	0	84	1	0	86	1	0	50	1	0	100	1	0	59	0.983333333
138 hours	0	116	1	0	111	1	0	84	1	0	86	1	0	50	1	0	100	1	0	59	0.983333333
144 hours(Day 7)	0	116	1	1	110	0.990990991	2	82	0.976190476	0	86	1	0	50	1	3	97	0.97	3	56	0.933333333
150 hours	1	115	0.99137931	1	109	0.981981982	2	80	0.952380952	0	86	1	1	49	0.98	6	91	0.91	3	53	0.883333333
156 hours	0	115	0.99137931	5	104	0.936936937	7	73	0.869047619	0	86	1	0	49	0.98	4	87	0.87	1	52	0.866666667

162 hours	2	113	0.974137931	28	76	0.684684685	22	51	0.607142857	0	86	1	0	49	0.98	11	76	0.76	8	44	0.733333333
168 hours	7	106	0.913793103	20	56	0.504504505	17	34	0.404761905	0	86	1	5	44	0.88	14	62	0.62	4	40	0.666666667
174 hours	13	93	0.801724138	15	41	0.369369369	21	13	0.154761905	6	80	0.930232558	4	40	0.8	14	48	0.48	3	37	0.616666667
180 hours	40	53	0.456896552	20	21	0.189189189	7	6	0.071428571	8	72	0.837209302	6	34	0.68	15	33	0.33	4	33	0.55
186 hours	10	43	0.370689655	8	13	0.117117117	6	0	0	6	66	0.76744186	8	26	0.52	10	23	0.23	7	26	0.433333333
192 hours	17	26	0.224137931	5	8	0.072072072				2	64	0.744186047	8	18	0.36	10	13	0.13	8	18	0.3
198 hours	9	17	0.146551724	2	6	0.054054054				9	55	0.639534884	5	13	0.26	8	5	0.05	5	13	0.216666667
204 hours	7	10	0.086206897	3	3	0.027027027				15	40	0.465116279	2	11	0.22	3	2	0.02	4	9	0.15
210 hours	9	1	0.00862069	1	2	0.018018018				19	21	0.244186047	2	9	0.18	2	0	0	2	7	0.116666667
216 hours	1	0	0	1	1	0.009009009				11	10	0.11627907	4	5	0.1				3	4	0.066666667
222 hours				1	0	0				5	5	0.058139535	2	3	0.06				2	2	0.033333333
228 hours										3	2	0.023255814	3	0	0				2	0	0
234 hours										2	0	0									
240 hours																					
246 hours																					
252 hours																					
258 hours																					
264 hours																					
270 hours																					
276 hours																					
282 hours																					
288 hours																					
294 hours																					
300 hours																					

Table S16. Female longevity - 30°C

	0(PU)			***w*A(PU)**			***w*B(PU)**			***w*A*w*B(PU)**			**0(*w*A PU)**			**0(*w*B PU)**			**0(*w*A*w*B PU)**		
	Dead	**Survival(X)**	**Fraction (X/28)**	**Dead**	**Survival(X)**	**Fraction (X/33)**	**Dead**	**Survival(X)**	**Fraction (X/62)**	**Dead**	**Survival(X)**	**Fraction (X/62)**	**Dead**	**Survival(X)**	**Fraction (X/57)**	**Dead**	**Survival(X)**	**Fraction (X/81)**	**Dead**	**Survival(X)**	**Fraction (X/81)**
0 hours(Day 1)	0	28	1	0	33	1	0	62	1	0	79	1	0	57	1	0	71	1	0	65	1
6 hours	0	28	1	0	33	1	0	62	1	0	79	1	0	57	1	0	71	1	0	65	1
12 hours	0	28	1	0	33	1	0	62	1	0	79	1	0	57	1	0	71	1	0	65	1
18 hours	0	28	1	0	33	1	0	62	1	0	79	1	0	57	1	0	71	1	0	65	1
24 hours(Day 2)	0	28	1	0	33	1	0	62	1	0	79	1	0	57	1	0	71	1	0	65	1
30 hours	0	28	1	0	33	1	0	62	1	0	79	1	0	57	1	0	71	1	0	65	1
36 hours	0	28	1	0	33	1	0	62	1	0	79	1	0	57	1	0	71	1	0	65	1
42 hours	0	28	1	0	33	1	0	62	1	0	79	1	0	57	1	0	71	1	0	65	1
48 hours(Day 3)	0	28	1	0	33	1	0	62	1	0	79	1	0	57	1	0	71	1	0	65	1
54 hours	0	28	1	0	33	1	0	62	1	0	79	1	0	57	1	0	71	1	0	65	1
60 hours	0	28	1	0	33	1	0	62	1	0	79	1	0	57	1	0	71	1	0	65	1
66 hours	0	28	1	0	33	1	0	62	1	0	79	1	0	57	1	0	71	1	0	65	1
72 hours(Day 4)	0	28	1	0	33	1	0	62	1	0	79	1	0	57	1	0	71	1	0	65	1
78 hours	0	28	1	0	33	1	0	62	1	0	79	1	0	57	1	0	71	1	0	65	1
84 hours	0	28	1	0	33	1	0	62	1	0	79	1	0	57	1	0	71	1	0	65	1
90 hours	0	28	1	0	33	1	0	62	1	0	79	1	0	57	1	0	71	1	0	65	1
96 hours(Day 5)	0	28	1	0	33	1	0	62	1	0	79	1	0	57	1	0	71	1	0	65	1
102 hours	0	28	1	0	33	1	0	62	1	0	79	1	0	57	1	0	71	1	0	65	1

108 hours	0	28	1	0	33	1	0	62	1	0	79	1	0	57	1	0	71	1	0	65	1
114 hours	0	28	1	0	33	1	0	62	1	0	79	1	0	57	1	0	71	1	0	65	1
120 hours(Day 6)	0	28	1	0	33	1	0	62	1	0	79	1	0	57	1	1	70	0.985915493	1	64	0.984615385
126 hours	0	28	1	0	33	1	0	62	1	0	79	1	0	57	1	0	70	0.985915493	0	64	0.984615385
132 hours	0	28	1	0	33	1	0	62	1	0	79	1	0	57	1	1	69	0.971830986	1	63	0.969230769
138 hours	0	28	1	0	33	1	0	62	1	0	79	1	0	57	1	2	67	0.943661972	2	61	0.938461538
144 hours(Day 7)	0	28	1	0	33	1	1	61	0.983870968	0	79	1	1	56	0.98245614	6	61	0.85915493	6	55	0.846153846
150 hours	0	28	1	0	33	1	0	61	0.983870968	0	79	1	3	53	0.929824561	3	58	0.816901408	3	52	0.8
156 hours	2	26	0.928571429	1	32	0.96969697	1	60	0.967741935	1	78	0.987341772	10	43	0.754385965	4	54	0.76056338	4	48	0.738461538
162 hours	0	26	0.928571429	1	31	0.939393939	3	57	0.919354839	3	75	0.949367089	7	36	0.631578947	6	48	0.676056338	10	38	0.584615385
168 hours	6	20	0.714285714	3	28	0.848484848	6	51	0.822580645	6	69	0.873417722	3	33	0.578947368	9	39	0.549295775	3	35	0.538461538
174 hours	1	19	0.678571429	1	27	0.818181818	8	43	0.693548387	8	61	0.772151899	6	27	0.473684211	10	29	0.408450704	6	29	0.446153846
180 hours	3	16	0.571428571	11	16	0.484848485	2	41	0.661290323	2	59	0.746835443	6	21	0.368421053	4	25	0.352112676	2	27	0.415384615
186 hours	2	14	0.5	5	11	0.333333333	8	33	0.532258065	8	51	0.64556962	2	19	0.333333333	8	17	0.23943662	8	19	0.292307692
192 hours	5	9	0.321428571	4	7	0.212121212	4	29	0.467741935	4	47	0.594936709	13	6	0.105263158	10	7	0.098591549	6	13	0.2
198 hours	3	6	0.214285714	2	5	0.151515152	4	25	0.403225806	4	43	0.544303797	3	3	0.052631579	3	4	0.056338028	8	5	0.076923077
204 hours	2	4	0.142857143	1	4	0.121212121	6	19	0.306451613	8	35	0.443037975	3	0	0	1	3	0.042253521	3	2	0.030769231
210 hours	3	1	0.035714286	2	2	0.060606061	3	16	0.258064516	4	31	0.392405063				3	0	0	2	0	0
216 hours	1	0	0	1	1	0.03030303	6	10	0.161290323	6	24	0.303797468									
222 hours				1	0	0	1	9	0.14516129	2	22	0.278481013									
228 hours							2	7	0.112903226	2	21	0.265822785									
234 hours							1	6	0.096774194	5	15	0.189873418									
240 hours							1	5	0.080645161	6	9	0.113924051									
246 hours							2	3	0.048387097	2	7	0.088607595									
252 hours							1	2	0.032258065	5	2	0.025316456									
258 hours							2	0	0	2	0	0									
264 hours																					
270 hours																					
276 hours																					
282 hours																					
288 hours																					
294 hours																					
300 hours																					

Table S17. Female longevity - 32°C

	0(PU)			***w*A(PU)**			***w*B(PU)**			***w*A*w*B(PU)**			**0(*w*A PU)**			**0(*w*B PU)**			**0(*w*A*w*B PU)**		
	Dead	**Survival(X)**	**Fraction (X/99)**	**Dead**	**Survival(X)**	**Fraction (X/59)**	**Dead**	**Survival(X)**	**Fraction (X/53)**	**Dead**	**Survival(X)**	**Fraction (X/62)**	**Dead**	**Survival(X)**	**Fraction (X/81)**	**Dead**	**Survival(X)**	**Fraction (X/81)**	**Dead**	**Survival(X)**	**Fraction (X/81)**
0 hours(Day 1)	0	99	1	0	59	1	0	53	1	0	62	1	0	13	1	0	147	1	0	121	1
6 hours	0	99	1	0	59	1	0	53	1	0	62	1	0	13	1	0	147	1	0	121	1
12 hours	0	99	1	0	59	1	0	53	1	0	62	1	0	13	1	0	147	1	0	121	1
18 hours	0	99	1	0	59	1	0	53	1	0	62	1	0	13	1	0	147	1	0	121	1
24 hours(Day 2)	0	99	1	0	59	1	0	53	1	0	62	1	0	13	1	0	147	1	0	121	1
30 hours	0	99	1	0	59	1	0	53	1	0	62	1	0	13	1	0	147	1	0	121	1
36 hours	0	99	1	0	59	1	0	53	1	0	62	1	0	13	1	0	147	1	0	121	1
42 hours	0	99	1	0	59	1	0	53	1	0	62	1	0	13	1	0	147	1	0	121	1
48 hours(Day 3)	0	99	1	0	59	1	0	53	1	0	62	1	0	13	1	0	147	1	0	121	1
54 hours	0	99	1	0	59	1	0	53	1	0	62	1	0	13	1	0	147	1	0	121	1

60 hours	0	99	1	0	59	1	0	53	1	0	62	1	0	13	1	0	147	1	0	121	1
66 hours	0	99	1	0	59	1	0	53	1	0	62	1	0	13	1	0	147	1	0	121	1
72 hours(Day 4)	0	99	1	0	59	1	0	53	1	0	62	1	0	13	1	0	147	1	0	121	1
78 hours	0	99	1	0	59	1	0	53	1	0	62	1	0	13	1	0	147	1	0	121	1
84 hours	0	99	1	0	59	1	0	53	1	0	62	1	0	13	1	0	147	1	0	121	1
90 hours	0	99	1	0	59	1	0	53	1	0	62	1	0	13	1	0	147	1	0	121	1
96 hours(Day 5)	0	99	1	0	59	1	0	53	1	0	62	1	0	13	1	0	147	1	0	121	1
102 hours	0	99	1	0	59	1	0	53	1	0	62	1	0	13	1	0	147	1	0	121	1
108 hours	0	99	1	0	59	1	0	53	1	0	62	1	0	13	1	0	147	1	0	121	1
114 hours	0	99	1	0	59	1	0	53	1	0	62	1	0	13	1	0	147	1	0	121	1
120 hours(Day 6)	0	99	1	0	59	1	0	53	1	0	62	1	0	13	1	2	145	0.986394558	2	119	0.983471074
126 hours	0	99	1	0	59	1	0	53	1	0	62	1	0	13	1	5	140	0.952380952	5	114	0.94214876
132 hours	0	99	1	0	59	1	0	53	1	0	62	1	0	13	1	7	133	0.904761905	7	107	0.884297521
138 hours	0	99	1	0	59	1	0	53	1	0	62	1	0	13	1	11	122	0.829931973	8	99	0.818181818
144 hours(Day 7)	6	93	0.939393939	0	59	1	0	53	1	0	62	1	0	13	1	22	100	0.680272109	11	88	0.727272727
150 hours	6	87	0.878787879	0	59	1	2	51	0.962264151	2	60	0.967741935	0	13	1	13	87	0.591836735	6	82	0.67768595
156 hours	10	77	0.777777778	1	58	0.983050847	2	49	0.924528302	2	58	0.935483871	2	11	0.846153846	9	78	0.530612245	2	80	0.661157025
162 hours	5	72	0.727272727	1	57	0.966101695	5	44	0.830188679	3	55	0.887096774	1	10	0.769230769	10	68	0.462585034	12	68	0.561983471
168 hours	12	60	0.606060606	2	55	0.93220339	1	43	0.811320755	5	50	0.806451613	1	9	0.692307692	22	46	0.31292517	8	60	0.495867769
174 hours	13	47	0.474747475	4	51	0.86440678	5	38	0.716981132	3	47	0.758064516	2	7	0.538461538	16	30	0.204081633	11	49	0.404958678
180 hours	7	40	0.404040404	2	49	0.830508475	0	38	0.716981132	2	45	0.725806452	1	6	0.461538462	3	27	0.183673469	9	40	0.330578512
186 hours	11	29	0.292929293	12	37	0.627118644	6	32	0.603773585	4	41	0.661290323	0	6	0.461538462	7	20	0.136054422	15	25	0.20661157
192 hours	17	12	0.121212121	12	25	0.423728814	12	20	0.377358491	5	36	0.580645161	0	6	0.461538462	5	15	0.102040816	3	22	0.181818182
198 hours	3	9	0.090909091	5	20	0.338983051	2	18	0.339622642	11	25	0.403225806	4	2	0.153846154	7	8	0.054421769	9	13	0.107438017
204 hours	4	5	0.050505051	9	11	0.186440678	3	15	0.283018868	3	22	0.35483871	2	0	0	5	3	0.020408163	6	7	0.05785124
210 hours	2	3	0.03030303	3	8	0.13559322	4	11	0.20754717	8	14	0.225806452				1	2	0.013605442	4	3	0.024793388
216 hours	2	1	0.01010101	4	4	0.06779661	5	6	0.113207547	3	11	0.177419355				1	1	0.006802721	2	1	0.008264463
222 hours	1	0	0	1	3	0.050847458	2	4	0.075471698	2	9	0.14516129				1	0	0	1	0	0
228 hours				2	1	0.016949153	1	3	0.056603774	2	7	0.112903226									
234 hours				1	0	0	1	2	0.037735849	3	4	0.064516129									
240 hours							1	1	0.018867925	4	0	0									
246 hours							1	0	0												
252 hours																					
258 hours																					
264 hours																					
270 hours																					
276 hours																					
282 hours																					
288 hours																					
294 hours																					
300 hours																					

Table S18. Number of female progeny (daughters) - 30°C

No.	**0(PU)**	***w*A(PU)**	***w*B(PU)**	***w*A*w*B(PU)**	**0(*w*A PU)**	**0(*w*B PU)**	**0(*w*A*w*B PU)**
1	40	21	31	32	16	35	29
2	6	5	20	29	40	30	44
3	43	6	35	28	23	39	38
4	36	27	27	29	37	29	39
5	33	16	26	34	29	23	1
6	17	19	29	38	21	10	35
7	25	6	16	40	6	16	2
8	8	15	35	4	30	23	13
9	34	17	25	31	27	19	27
10	17	31	24	18	41	24	32
11	22	36	32	6	38	32	18
12	42	32	23	34	32	27	22
13	10	32	24	30	8	9	6
14	18	23	3	30	39	37	26
15	5	10	38	35	38	28	1
16	36	29	41	16	2	33	8
17	20	34	37	32	24	28	1
18	13	32	4	29	20	17	18
19	42	16	4	32		6	6
20	37	6	10	6		25	24
21	42	21	35	23		31	27
22	29	32	17	33		40	32
23	4	34	21	30		30	36
24	8	10	13	37		26	29
25	8	32	26	30		30	5
26	4	37	7	6			22
27	13	31	25	23			16
28	11	7	26	12			38
29	24	36	4	33			2
30	15	5	33	33			38
31	7	31	32	34			31
32	17	6	31	28			17
33	26	31	30	38			30
34	18	23	27	34			31
35	5		6	28			16
36	20		12	40			8
37	7		30	30			39
38	22		29	7			2
39	27			32			45
40	6			28			30
41	12			39			2
42	17			37			39
43	25			28			34
44	8			22			27
45	28			29			33
46	32			15			11
47	9			36			24
48	5			2			27
49	12			29			3
50	34			20			33
51	24			32			33
52	24			32			8
53				25			22
54				13			6
55				40			31
56				18			11
57				32			34
58				29			19
59				28			25

60				18			10
61				31			36
62				32			

Table S19. Number of female progeny (daughters) - 32°C

No.	0(PU)	*w*A(PU)	*w*B(PU)	*w*A*w*B(PU)	0(*w*A PU)	0(*w*B PU)	0(*w*A*w*B PU)
1	7	27	16	41	38	28	16
2	31	36	30	31	20	40	22
3	24	37	41	28	19	22	20
4	7	32	47	24	28	37	23
5	53	20	35	20	45	22	25
6	25	42	27	30	21	22	27
7	27	23	37	20	18	39	29
8	24	39	20	29	3	32	21
9	18	9	24	25	19	24	6
10	31	37	11	29	11	15	20
11	7	24	14	35	3	22	27
12	15	32	29	6	29	4	21
13	7	29	29	31	14	27	28
14	11	4	24	34	38	34	22
15	26	12	12	27	25	39	8
16	6	39	9	35	43	25	20
17	42	17		2	19	37	28
18	34	3		20	1	17	22
19	35	21		9	29	16	24
20	39	27		29	18	30	20
21	8	30		36	36	19	
22	5	53		32	36	22	
23	35	27		34	33	26	
24	35	11		37	35	40	
25	25	11		31	13	2	
26	40	16		37	19	29	
27	40	10		31	27	38	
28	32	25		31	37	31	
29	4	19		29	27	27	
30	22	12		32	7	30	
31	22	45		6	27		
32	13	2		8	22		
33	23	54		42	36		
34	25	17		21	28		
35	7	16		21	16		
36	16	39		18			
37	10	37		34			
38	22	40		37			
39	25	51		26			
40	28	23		41			
41	11	54		21			
42	14	3		38			
43	14	33		8			
44	6	43		31			
45	4	34		15			
46	9	44		35			
47	36	20		30			
48	26			18			
49	24			28			
50	29			32			
51	18			8			
52	22			32			
53	27			19			
54	9			28			
55	23			38			
56	31			22			
57	28			7			
58				17			
59				5			
60				38			

61				38			
62				28			

Table S20. Number of female progeny (daughters) - 37°C

No.	0(PU)	*w*A(PU)	*w*B(PU)	*w*A*w*B(PU)	0(*w*A PU)	0(*w*B PU)	0(*w*A*w*B PU)
1	36	30	48	17	29	33	20
2	26	26	17	34	44	19	19
3	8	15	4	8	38	23	28
4	45	37	30	37	39	20	21
5	40	35	28	2	1	26	18
6	40	36	6	10	35	21	3
7	47	19	32	28	2	23	19
8	14	6	28	42	13	25	11
9	7	19	15	20	27	27	3
10	43	28	4	27	32	20	29
11	6	37	20	22	18	19	14
12	34	4	32	26	22	23	25
13	31	31	4	25	6	23	19
14	8	38	5	42	26	27	1
15	29	4	25	29	1	33	29
16	8	12	31	38	8	26	18
17	14	24	26	29	1	29	33
18	14	18	24	9	18	23	35
19	19	21	27	36	6	24	13
20	38	13	28	22	24	23	19
21	18	26	30	33	27	23	27
22	16	31	37	33	32	25	37
23	8	26	28	29	36	28	27
24	33	9	21	7	29	19	7
25	29	32	28	31	5	22	27
26	25	37	21	20	22	22	22
27	27	30	27	36	16	26	28
28	20	34	33	37	38	24	16
29	17	32		34	2		
30	13	8		29	38		
31	38	27		34	31		
32	33	7		21	17		
33	5	33		32	30		
34	43	36		26	31		
35	26	5		14	16		
36	18	16		33	8		
37	21	31		24	39		
38	18	29		8	2		
39	35	24		34	45		
40	16	14		11	30		
41	10	34		35	2		
42	36	32		34	39		
43	22	31		32	34		
44	6	17		32	27		
45	10	32		27	33		
46	18	13		28	11		
47	30	13		33	24		
48	30	32		30	27		
49	29	27		32	3		
50	32	32		27	33		
51	19	35		26	33		
52	18	32		26	8		
53	31	17		25	22		
54	7	14		9	6		
55	38			29	31		
56				29	11		
57				24	34		
58				8	19		
59				11	25		
60				10	10		

61				18	36		
62					33		