Investigating the effects of the two *Wolbachia* supergroups infecting *Nasonia vitripennis*

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Certificate of Examination

This is to certify that the dissertation titled "***Investigating the effects of the two Wolbachia supergroups infecting Nasonia vitripennis***", submitted by Mr. Rahul Babu (Reg. No. MS15030) for the partial fulfilment of BS-MS dual degree program of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

Dr. Rhitoban Ray Choudhury
(Supervisor)

Dated: 15 June 2020

Declaration

The work presented in this dissertation has been carried out by me under the guidance of Dr. Rhitoban Ray Choudhury 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 the contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgements of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all the sources listed within have been detailed in the bibliography.

Rahul Babu
Date: 15 June 2020

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

Dr. Rhitoban Ray Choudhury (Supervisor)
Date: 15 June 2020

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Thank You,
Rahul Babu

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Abstract

The endosymbiotic bacteria *Wolbachia* is estimated to infect around 66% of insect species in existence. This widespread infection is a result of many effects the endosymbiont has on its host biology, especially reproductive biology. They are a class of maternally inherited endosymbionts and they try to increase the number of infected females in a population to ensure their spreading. This is carried out by phenotypic effects induced by *Wolbachia* like cytoplasmic incompatibility, parthenogenesis, feminization of genetic males, male-killing etc. (Werren *et al*. 2008).

The parasitoid wasp genus *Nasonia* is infected by 11 different strains of *Wolbachia*. *Nasonia vitripennis* is a species which has two strains of *Wolbachia* infection. The haplodiploid sex determination of *Nasonia vitripennis* makes it a very good model system to study the genetics of *Wolbachia* effects on its host. The purpose of this study is to investigate the consequences of single and multiple *Wolbachia* infections in its host *Nasonia vitripennis*. Different assays like effect on progeny size, mating potential etc. are studied. The study also aims to understand the reasons behind these consequences that are observed

1. Introduction

1.1 Basic Theory

The Endosymbiont – *Wolbachia*

An organism is termed an endosymbiont when it lives inside the body or the cells of another organism (host). This relationship is not necessarily mutualistic and can vary greatly with organisms. They may be facultative where the host and the endosymbiont can survive without the other (e.g.: nitrogen-fixing bacteria (*rhizobia sp.*) and certain leguminous plants) or obligatory where either the host or the endosymbiont cannot survive without the other (e.g.: pea aphid (*Acyrthosiphon pisum*) and its bacterial endosymbiont *buchnera sp*.). *Wolbachia*, *Rickettsia*, and *Cardinium* are some of the bacterial endosymbionts commonly found in several arthropods and nematodes. It is estimated that around 66% of arthropod species are infected by *Wolbachia*, making it one of the most widespread endosymbionts in the biosphere (Hilgenboecker *et al*. 2008).

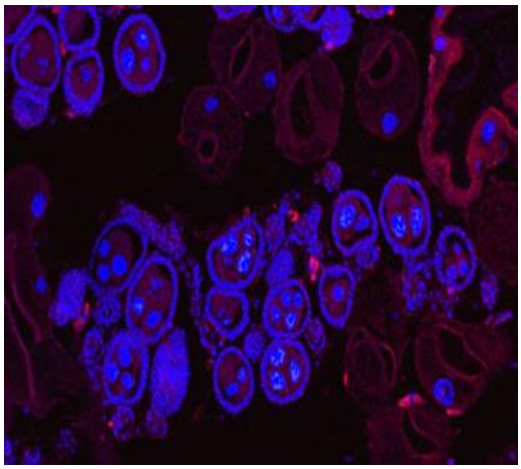

Figure 1.1.a: A cross-section of an Anopheles mosquito that has been injected with *Wolbachia* (red). The blue area represents mosquito DNA.
IMAGE: JASON RASGON/PENN STATE

Wolbachia is a genus of gram-negative endosymbiotic bacteria that have the ability to manipulate the host reproductive biology (Werren *et al*. 2008). It comes under the order of Rickettsiales and is involved in complex interactions with their host. In some cases, the

parasitic relations have evolved into mutualistic interactions where the host species need *Wolbachia* colonization in-order to reproduce or even survive (Comandatore *et al.* 2013). They are known to infect the testes and ovary of the host organism but are ubiquitous in mature egg and not in mature sperm. Therefore, *Wolbachia* is transmitted to the host offsprings through maternal inheritance. As they are maternally inherited, *Wolbachia* manipulates the host biology such that there are a larger number of infected females in the population as this enables them to be more vastly spread. *Wolbachia* are divided into different supergroups (A, B, C, etc.) on the basis of 16S rRNA sequences and clustering patterns in ftsZ based phylogenetic trees (O'Neill *et al*. 1992, Werren *et al*. 1995, Bandi *et al*. 1998). The supergroups C, D are known to infect nematodes whereas the other supergroups are primarily found in arthropods. *Wolbachia* infection in a host can either be by a single supergroup or by multiple supergroups although single infections of *Wolbachia* are rarely found in the wild (Perrot-Minnot *et al.* 1996). *Wolbachia* are also seen as a potential eco-friendly bio-control agent as they can manipulate the host reproductive biology of the host organism. It has attracted attention in recent years, mainly owing to the effects they induce on their host reproductive biology that includes

1. Cytoplasmic Incompatibility
2. Parthenogenesis
3. Feminization of genetic males
4. Male-killing

Each of these phenotypic effects on the host is beneficial for *Wolbachia* as this enhances the production of infected females and thus enhances their spread and survival (Werren *et al.* 1997, 2008).

Wolbachia induced host phenotypes

An important feature of *Wolbachia* is the ability to induce cellular and reproductive alterations in the host organism. Given below are some of the known effects of *Wolbachia* infection.

1. Cytoplasmic Incompatibility (CI)

Cytoplasmic Incompatibility is one of the most prominent effects *Wolbachia* induces on its host. It is a form of embryonic lethality that results in sperm and an egg not being able to form a viable offspring after fertilisation. The sperm of a *Wolbachia*-infected male is incompatible with the eggs of a female that is not infected by the same *Wolbachia* strain (or strains). This incompatibility arises due to the asynchronous formation of male and female pronuclei at the first embryonic mitotic division after fertilization (Lassy, C. W. & Karr, T. L., 1996, Tram, U. & Sullivan, W., 2002, Reed, K. M. & Werren, J. H., 1995). The exact molecular mechanisms that govern CI are still not clearly known. The two distinct components of CI include the modification of sperm during spermatogenesis and rescue of this modification in the eggs by the same *Wolbachia* supergroup (Werren *et al.* 2008).

2. Parthenogenesis

Parthenogenesis being induced by *Wolbachia* is much less common than *Wolbachia* induced CI. Parthenogenesis is the process by which reproduction and growth of offspring happen without fertilization in organisms. *Wolbachia* induce female parthenogenesis i.e. daughters are produced from unfertilized eggs of the infected females as females are able to transmit the bacteria to their offspring. This effect is only documented in species having arrhenotokous development (males develop from unfertilized eggs, e.g. hymenopterans) (Stouthamer *et al.* 2002). Like CI, this is also a result of cell cycle disruption during early embryonic development. In *Wolbachia* infected species, a particular type of parthenogenesis termed thelytoky is observed in which only females are produced from unfertilized eggs.

3. Feminization of genetic males

This *Wolbachia* induced phenotype causes genetic males to develop into females. This is caused by the proliferation of *Wolbachia* inside the androgenic gland which leads to the

hyperinflation of the androgenic gland and ultimately the inhibition of the gland. These genetic males then develop as females (Beladjal, Lynda, *et al*. 2003). Currently, only two host species- *Eurema hecabe* (Order Lepidoptera) and *Zyginidia pullula* (Order Hemiptera) are known to show *Wolbachia* induced feminization.

4. Male-Killing

Wolbachia induces selective killing of male progenies of the host organism in order to enhance the survival of female progenies which can transmit the bacteria to further generations. This phenomenon mainly occurs during embryogenesis as this result in more nutrition for the surviving female progenies (Werren *et al*. 2008).

The Host - The parasitoid wasp genus, *Nasonia*

The genus *Nasonia* consists of four closely related species of wasps- *Nasonia vitripennis, Nasonia oneida, Nasonia girault i and Nasonia longicornis* (Darling and Werren 1990, Raychoudhury et al. 2008). They are small parasitoid insects which lay their eggs in the pupae of various flies (e.g. Sarcophaga dux).

Figure 1.1.b: *Nasonia vitripennis* male mounting on female
IMAGE: BABITA RONSA, EVOGEN LAB

Figure 1.1.c: *Sarcophaga dux* adult and pupa
IMAGE: ALOK TIWARY, EVOGEN LAB

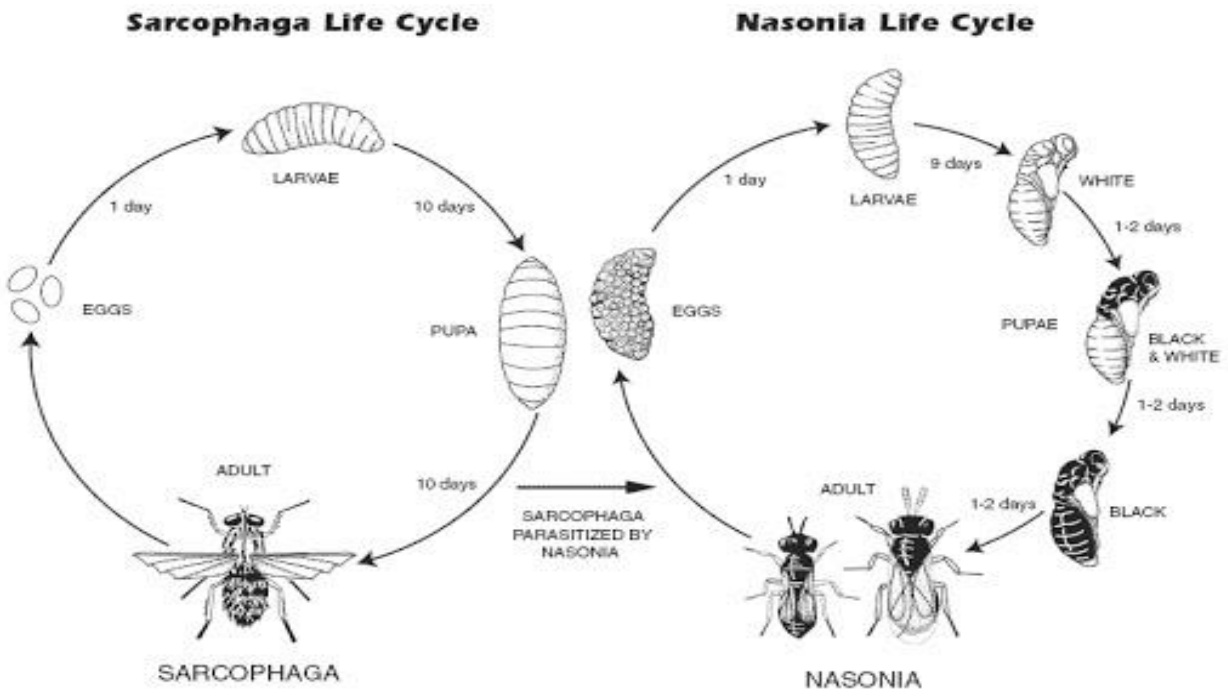

Figure 1.1.d: Life cycles of *Nasonia vitripennis* and its host *Sarcophaga*
SOURCE: VERHULST-LAB

Nasonia has haplodiploid sex determination which makes it a good model system suited for genetic studies. The males are haploid and only develop from unfertilized eggs parthenogenetically whereas the females are diploid and only develop from fertilized eggs. The females are often larger in size and have the capability of flight. *Nasonia* has a short generation time of two weeks at 25°C making it easy to handle and work within a laboratory (Whiting, 1967).

Aim

Investigating the effects of the *Wolbachia* supergroups infecting *Nasonia vitripennis*

The four species of the genus *Nasonia* together harbour 11 different *Wolbachia* infections (Raychoudhury *et al.* 2009).

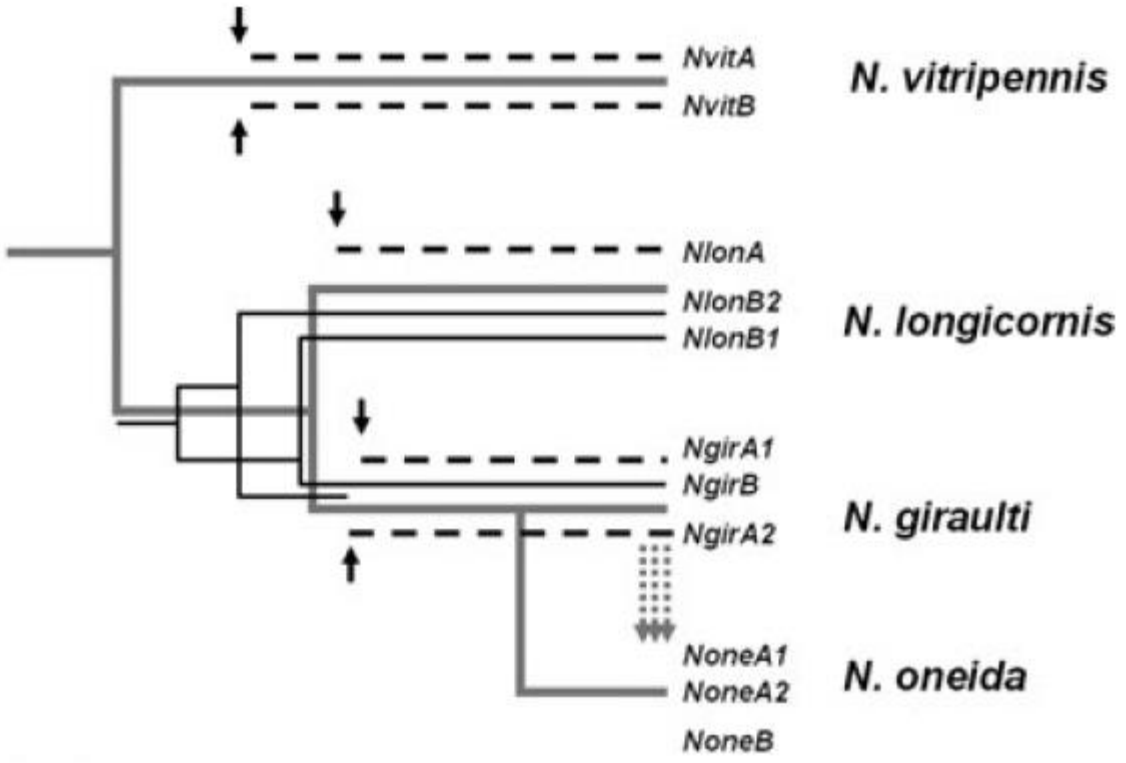

Figure 1.1.e: A diagrammatic representation of the history of the different *Wolbachia* infections superimposed on the phylogeny of *Nasonia*.
SOURCE: RAYCHOUDHURY ET AL. 2009

The experiments conducted were using *Nasonia vitripennis* as a model organism. It harbors only two *Wolbachia* infections namely supergroup A and supergroup B making it easier to compare the effects of different infection strains on the host. As mentioned earlier, *Wolbachia* infections can be either multiple or single, resulting in four different infection

strains in *N. vitripennis*- Single-A infection, Single-B infection, Double-AB infection and Uninfected.

The objective of these experiments is to understand the consequences of having two *Wolbachia* infections in a single host and to find possible explanations for it. This can be done by studying

1. The effect of single and multiple *Wolbachia* infections on the progeny size of *N. vitripennis.*

2. The effect on the mating potential of a male *N. vitripennis*

The bacterium is an endosymbiont and cannot be cultured on plates outside the hosts. The single infections are rare in nature and have to be generated by separating from the wild type double infections. The wild type strain of *N. vitripennis* carrying *Wolbachia* AB infection were separated and single supergroup A, single supergroup B infections and uninfected strains were generated in the lab (Alok Tiwary, Evogen Lab).

1.2. Materials and Methods

All the experiments were done with stocks reared in the lab. *Nasonia vitripennis* stocks were maintained in the lab using *Sarcophaga dux* pupae as the host which was reared on chicken liver in the lab itself. The experimental stocks were maintained at 25°C in incubators with constant light.

The effect of *Wolbachia* on the progeny size of *Nasonia vitripennis*

As mentioned earlier, *Nasonia vitripennis* is a haplodiploid organism. When they are hosted as virgins, they produce only males and when hosted after mating they can produce both males and females as females only develop from fertilized eggs.

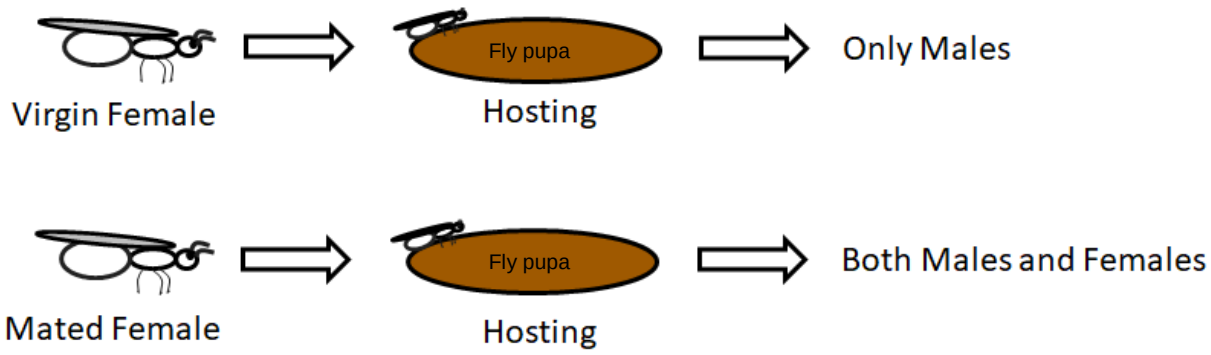

Figure 1.2.a: Schematical representation of two types of hosting

The experiments conducted were using only virgin females. Experimental stocks were derived from the stock (separated and maintained by Alok Tiwary, Evogen Lab) by generating isofemale sublines for all the four infection strains. To make the isofemale sublines, a single mated female was taken from each of the stocks and was rehosted over generations to produce the required number of individuals for the experiments as a separate stock.

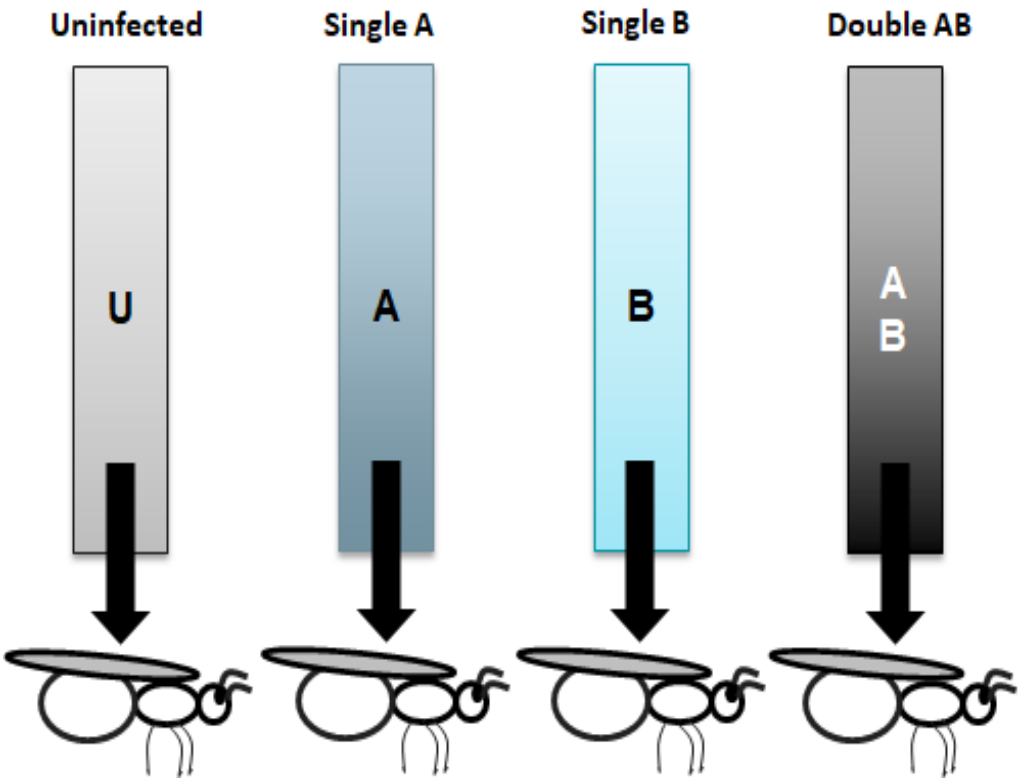

Figure 1.2.b: Schematical representation of Isofemale subline generation

The virgin females used for the experiment were collected from this experimental stock. Virgins can be collected from day 8 to day 13 when they are in the immobile pupal stage of their development. Female pupae can be identified by the presence of larger wing pads and ovipositor at the abdomen of the pupae.Experimental hostings with the collected virgins were then set up for 48 hours with two *Sarcophaga dux* pupae as hosts in separate vials. No added nutrition from outside were given to any of the virgins. All the tubes were randomized in order to eliminate biasing while counting the progenies. It took 14 days for the progenies (only males) to emerge and another 4 days for them to die. The number of progenies emerged were then counted. All the data points without two healthy hosts and dead females before 48 hours were not taken into account as it may have an influence on the results obtained.

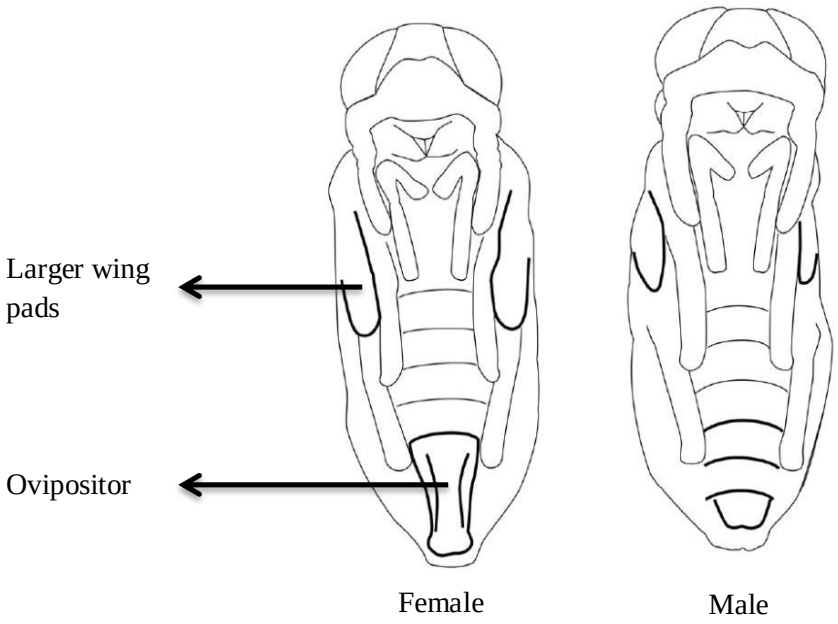

Figure 1.2.c: Diagram of *Nasonia vitripennis* pupae with the characteristics used to distinguish males and females (wing size, ovipositor) highlighted.
SOURCE: WERREN ET AL. 2009

The effect on the mating potential of a male *N. vitripennis*

Nasonia vitripennis females mate only once during their lifetime. They can produce progenies without mating as they can lay unfertilized eggs which gives rise to viable males. This is in stark contrast with the males as they can mate multiple times till they die. The males emerge a day earlier than the females emerge and are observed to stick around the host pupa waiting for the females to emerge to mate. As the males do not have the capability of flight, they have to mate before the female flies away after emerging.

The experiments conducted used Uninfected and Double infected virgin females from the experimental stock derived from the Isofemale sublines. The males used for the experiment were from separate virgin hostings from the same experimental stock. One healthy male was selected from the stock and was given a female to mate with. After the mating is observed the female is transferred to a separate vial. The same male is then given another female to mate

and then transferred after mating. This process is repeated till the male stops mating or dies. The number of matings done by a male is recorded. All the matings were done continuously and no external nutrition was provided. All the mated females were provided hosts to lay eggs after a gap of 24 hours.

The females were taken out of the vials 24 hours after giving them a host each. The progenies were counted after their emergence. Both the number of males and females were recorded separately. Only the vials with live females and healthy hosts were taken into account as this may influence the results.

2. Results

2.1 Effect of *Wolbachia* on the progeny size of *Nasonia vitripennis*

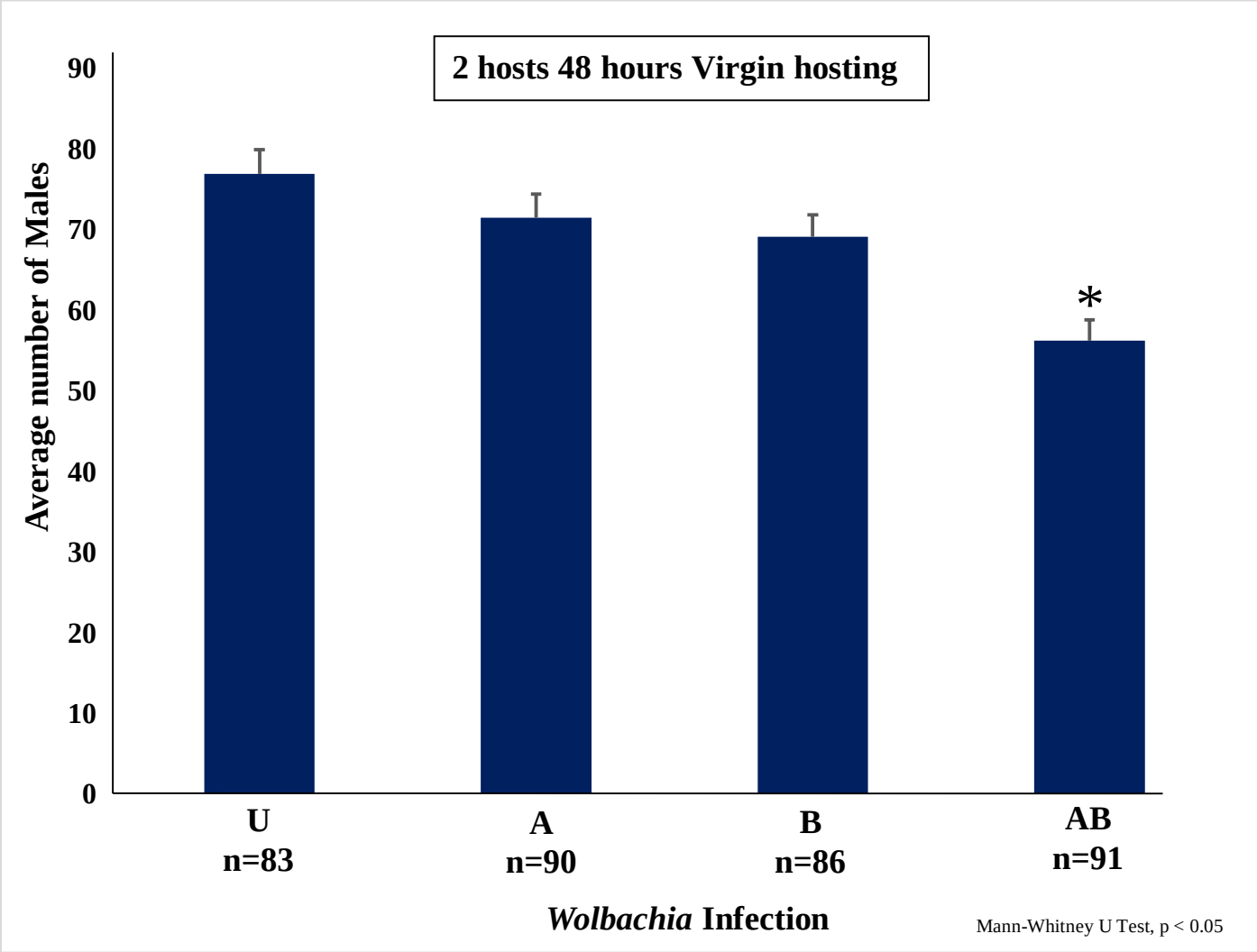

Figure 2.1.a: Graphical representation of average progeny size varying across different *Wolbachia* infections

In the graph, Y axis is average progeny size, X axis is the infection strain type and the sample sizes are above 80 for each infection strains. It was observed that uninfected strains (U) produced the maximum number of progenies compared to single and multiple infections. In

fact, double infected (AB) produced the least number of progenies and showed significant statistical difference from all the other infection strains.

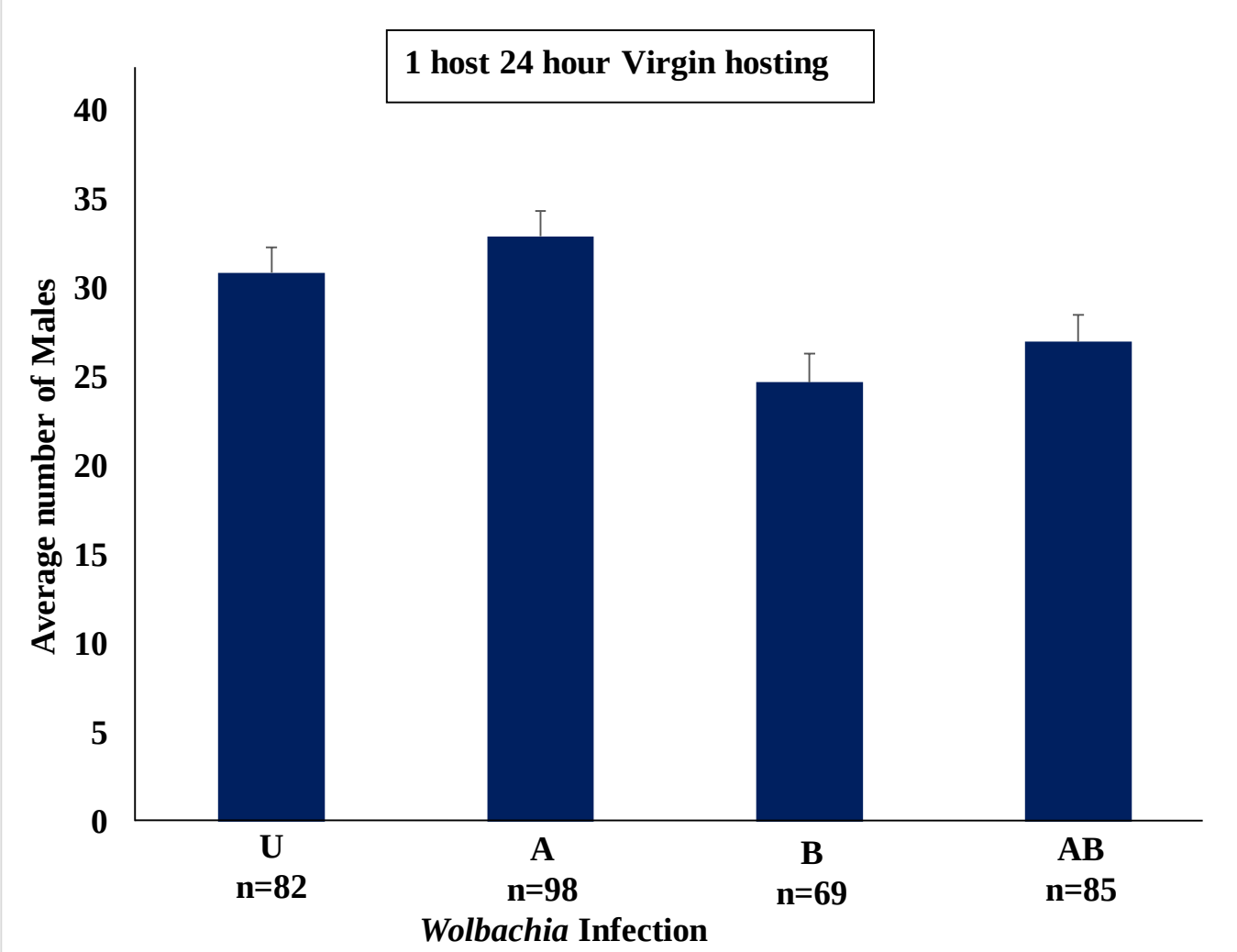

Figure 2.1.b: Graphical representation of average progeny size varying across different *Wolbachia* infections

The same general trend where uninfected strains produced more number of progenies than double infections was observed when the experiment was modified to hosting the virgins for 24 hours with a single host.

2.2 Effect on the mating potential of a male *Nasonia vitripennis*

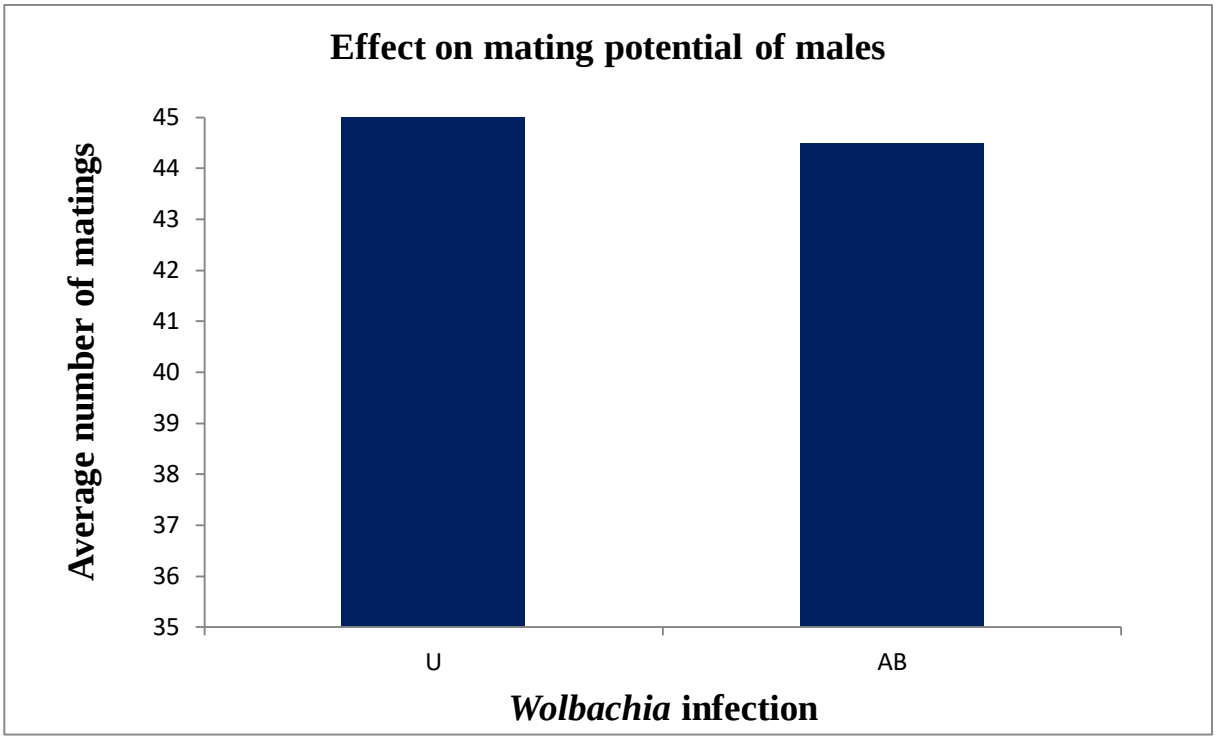

Figure 2.2.a: Graphical representation of average matings per male across U and AB *Wolbachia* infections

The results showed no significant difference between the number of matings done by a male from Uninfected and Double infected strains.

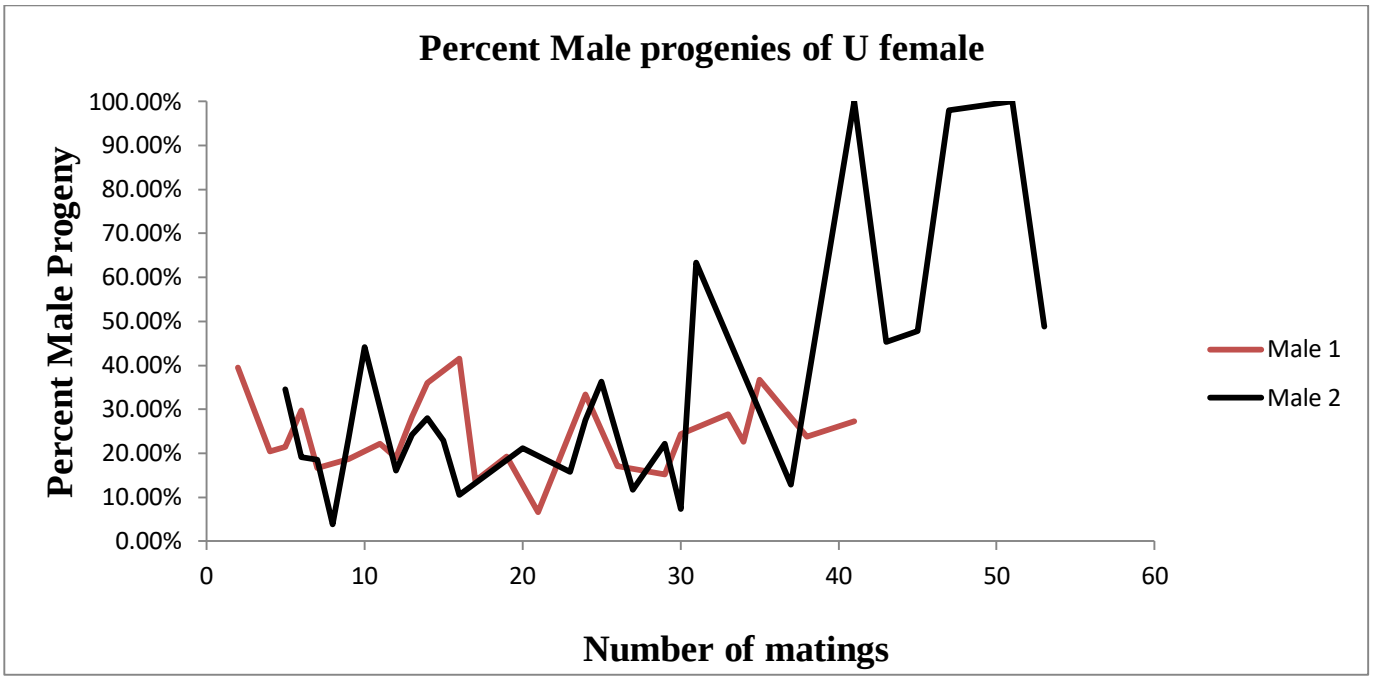

Figure 2.2.b: Graphical representation of percent male progeny produced by Uninfected *N. vitripennis* females

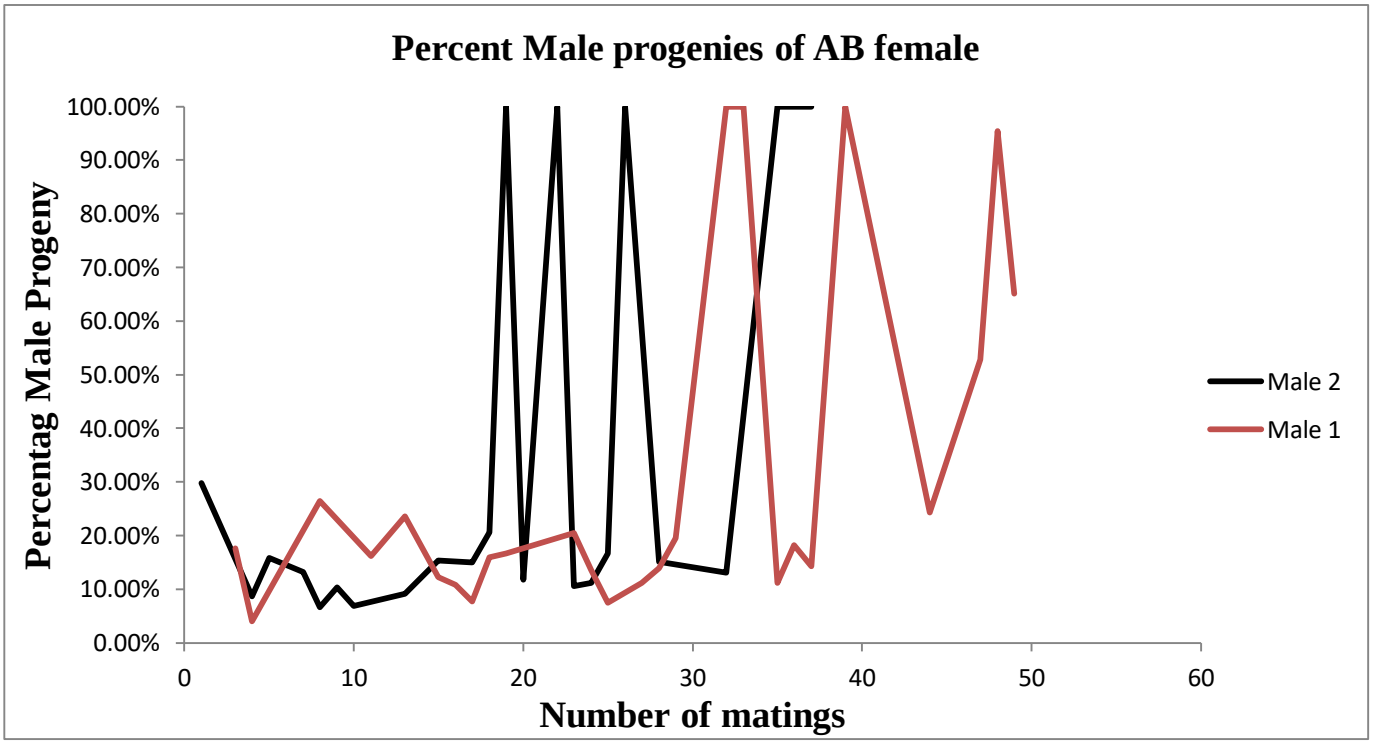

Figure 2.2.c: Graphical representation of percent male progeny produced by Double infected *N. vitripennis* females

3. Discussions and Conclusions

In the previous studies, it was observed that the type of *Wolbachia* infection had no effect on the progeny size of *Nasonia vitripennis* (Bordenstein *et al.* 2000). Our observations indicate that there is a significant effect on the progeny size of *Nasonia vitripennis* depending on the type of *Wolbachia* infection it harbours.

The double infected strain AB showed a significant decrease in the number of progenies (males) produced compared to all the other *Wolbachia* infection strains. The single infections A and B showed results somewhere in between the double infection and uninfected strains. This maybe a consequence of the presence of multiple *Wolbachia* supergroups infecting the same host as this may increase the taxation on the host resources. It is also observed that the progeny size of single A infection is not significantly different than the progeny size of uninfected strains. This can be due to a phage infection present in the *Wolbachia* supergroup A. There were some observable changes when the experiment was modified to 24 hour hostings with only 1 host but the general trend stood up. The uninfected strains showed increased progeny sizes compared the double infected strains.

The mating potential of males from uninfected and double infected strains showed no significant difference from the data obtained. This is inconclusive as the data obtained may be too small to observe any difference. Further experiments and data points are required to be conclusive of this effect. However there is a slight indication about the exhaustion of sperm stored in the males. As they are prospermatogenic, the males do not produce more sperm after reaching adulthood (Whiting *et al.* 1968). In figures 2.2.b and 2.2.c, we can observe that there are more number of male progenies produced as the number of matings reaches a certain level. This is more noticeable in the double infections. This may be an indication of the males running low on the pre-produced sperm before they reached adulthood. Further experimentation is required to be conclusive of this effect.

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