Dynamics of multiple *Wolbachia* infections in *Nasonia vitripennis*

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Certificate of Examination

This is to certify that the dissertation titled "**Dynamics of multiple *Wolbachia* infections in *Nasonia vitripennis***" submitted by Mr. Yogesh M Bangal (Reg. No.MS10114) for the partial fulfilment of BS-MS dual degree programme. The work has been found satisfactory by the thesis committee duly appointed by the Institute which recommends that the report be accepted.

Dr. Rhitoban Ray Choudhury (Supervisor)

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Dated: April 22, 2016

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 contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a confide record of original work done by me and all sources listed within have been detailed in the bibliography.

Yogesh M. Bangal

April 22, 2016

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)

April 22, 2016

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I must thank Dr. Rhitoban Ray Choudhury, who, apart from being my supervisor for this thesis, has also been my mentor during the project. He introduced me to a new model system to work with and taught me how to design and carry out experiments.

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Contents

List of figures

List of Table

Abstract

Wolbachia are cellular bacteria harboured by wide number of arthropod and nematode species. These are maternally inherited bacteria like mitochondria. In order to increase their own transmission, these bacteria alter the reproductive biology of the host by inducing different phenotypes like feminization, parthenogenesis, male killing and cytoplasmic incompatibility.

Till date, *Wolbachia* have been divided into 8 (A-H) super groups. About 66% of terrestrial arthropods have been found to be *Wolbachia* infected. Some of them carry single infection while others carry two or more.

The present study explores the potential intra-host competition between A and B super group of *Wolbachia* within single host, *Nasonia vitripennis*, in various developmental stages. Our data shows higher super group-B density than super group-A in different developmental stages of *Nasonia vitripennis* males and is in accordance with results in previous studies. Nature of this difference needs to be investigated further.

Chapter 1

Introduction

1.1 General introduction about *Wolbachia*

Endosymbiosis is a mutually beneficial relationship between an organism residing in another organism (Host). The relationship can either be facultative or obligatory, where the host and endosymbiont cannot survive without each other. The most common examples of obligate endosymbionts are mitochondria and Chloroplast. *Wolbachia, Cardinium* and *Spiroplasma* are few common intracellular bacteria that are found in arthropods and nematodes. Around 66% of terrestrial arthropods have been found to be *Wolbachia* infected (Hilgenboecker *et al*., 2008).

Wolbachia are the member of the order *Rickettsiales* which is a diverse group of intracellular bacteria that contains species in parasitic, mutualistic and commensal relationship with their host. The first species of the genus *Wolbachia* is *W. pipientis*, described in the mosquito *Culex pipiens* (Hertig and Wolbach, 1924). Based on 16S ribosomal sequence and other sequence information, *Wolbachia* have been divided into 8 supergroups (A-H) (O'Neill *et al*., 1992; Werren *et al.*, 1995b; Zhou *et al.*, 1998; Lo *et al.*, 2002; Bordenstein and Rosengans, 2005; Lo *et al*., 2007)., out of which only A and B are found in 66% of land arthropods. C & D super groups are found to be mutualistic of filarial nematodes. Researchers are also looking for *Wolbachia* as a pest and disease vector control (Werren *et al*., 2008).

The last decade has seen *Wolbachia* biology being extensively studied because of their prevalence in nature (Werren *et al*., 2008). *Wolbachia* is transmitted vertically and induces reproductive alterations in the host, giving rise to different phenotypes such as parthenogenesis, male killing, cytoplasmic incompatibility and feminization (Stouthamer *et al*., 1990; Hurst *et al*., 1999; Yen and Barr, 1971; Hoffman *et al*., 1986; Breeuwer and Werren, 1990; O'Neill and Karr, 1990; Rigaud *et al*., 1991).

1.2 *Wolbachia* induced Phenotypes

1) Parthenogenesis: In this phenotype, *Wolbachia* infected females can produce female offspring without fertilization of eggs by sperm. *Wolbachia* induced parthenogenesis is caused by disruption of cell cycle during embryonic development causing diploid development of unfertilized eggs (thelytoky) (Stouthamer *et al*., 1994).

Currently, *Wolbachia*-induced parthenogenesis is known from thrips, mites and wasps (Weeks *et al*., 2001)

2) Feminization: In this phenotype, majority of males are converted to females. Found in the isopod *Armadillidium vulgare*. Here *Wolbachia* are able to manipulate the endocrine gland secretions of insects which is responsible for sexual differentiation. (Reviewed by Rigaud, 1997). Till date feminization has been reported only in Lepidoptera and Hemiptera (Negri *et al*., 2006).

3) Male Killing: Majority of males are killed in this phenotype. It is hypothesized that *Wolbachia*- infected females benefit by getting access to more resources. To date, male-killing *Wolbachia* infections have been described in four different Arthropod orders. Within insects, these include Diptera, Coleoptera and Lepidoptera (Dyson *et al*., 2001). Outside of Insecta, male killing has been reported in Pseudoscorpiones (Class:Arachnida) (Zeh DW *et al*.,2005).

4) Cytoplasmic incompatibility (CI): This remains one of the most common of the many different reproductive alterations produced by *Wolbachia* (Werren *et al*., 2008). When *Wolbachia* infected males mate with the females lacking the same *Wolbachia* strain, cytoplasmic incompatibility results (Yen and Barr 1971; Hoffman *et al*., 1986; Breeuwer and Werren 1990; O'Neill and Karr 1990). Here the offspring do not develop beyond the embryonic stage resulting in high mortality and reduction in offspring number. Currently known from eight different arthropod orders of Acari, Coleoptera, Diptera, Isopoda, Lepidoptera, Hymenoptera, Hemiptera and Orthoptera (Werren *et al*., 2008).

Here, the punnet square shown below elaborates the basic features of CI.

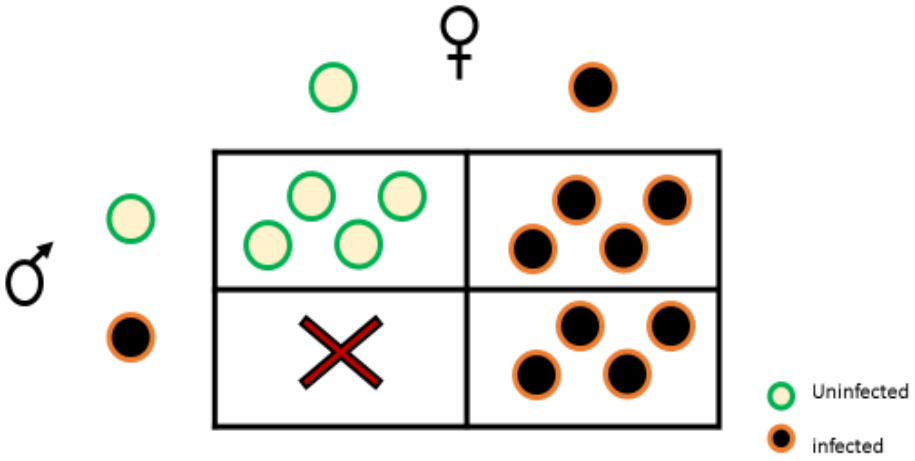
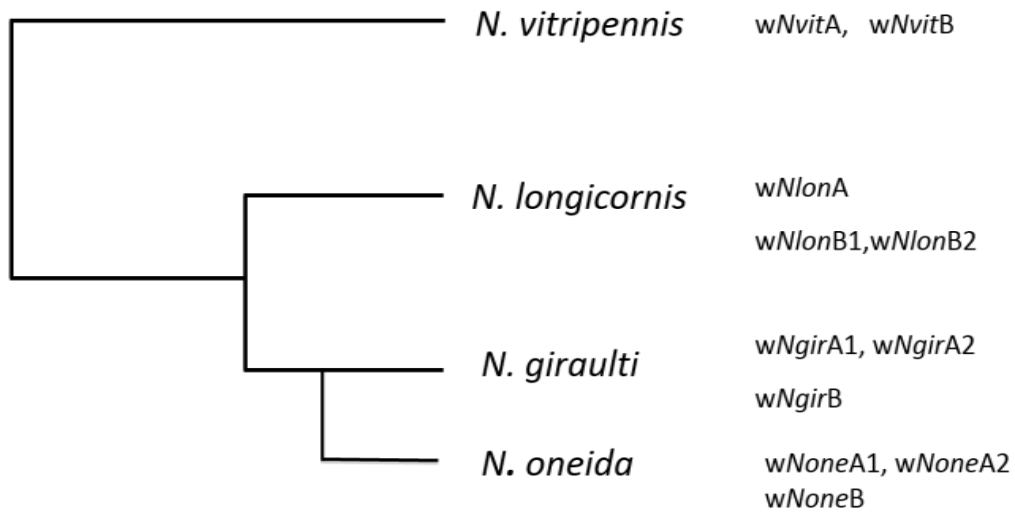


Figure 1.1 Cytoplasmic Incompatibility by *Wolbachia*

As shown in the figure 1.1, mating between both *Wolbachia* uninfected individuals give *Wolbachia* uninfected progeny while mating between both *Wolbachia* infected individuals give rise to *Wolbachia* infected progeny but when *Wolbachia* uninfected males mates with the *Wolbachia* infected female it give rise to *Wolbachia* infected progeny because *Wolbachia* is a maternally inherited bacteria. When *Wolbachia* infected males mate with the females which does not have any *Wolbachia* or same *Wolbachia* strain, both results in cytoplasmic incompatibility because these females do not have the rescue system for the sperm modified by the *Wolbachia* in males.

Multiple *Wolbachia* infections has been reported from various species of arthropod hosts and some examples of such multiple infections are from ant species (Reuter *et al.*, 2002), mites which is found to be infected with two different endosymbionts (Zhao *et al*., 2013) and many others (Werren *et al*., 2008).The major question that such multiple infections posit is how they are being maintained across generations. One key feature of this long-term maintenance is cytoplasmic incompatibility where multiple infected males select for the presence of all its infections in the females. However, this does not explain the competitions that multiple *Wolbachia* infections undergo with each other within each host. Do multiple infections compete for host resources? If yes, then why one strain does not displace the other? One of the problems of answering such questions is the lack of a proper model systems. One needs enough information about the host biology as well as the multiple infections therein to posit proper scientific experiments. One such system is the parasitoid genus of *Nasonia*.

Previous study by Raychoudhury *et al*. (2009, 2010b) on *Nasonia vitripennis* from two continents, North America and Europe, found both *Wolbachia* super group-A and B infections within single host.



Raychoudhury *et al*., 2009, 2010b

Figure 1.*2: Wolbachia* infections in *Nasonia* spp.

Dr. Rhitoban Raychoudhury collected stains of *Nasonia vitripennis* from IISER Mohali Campus, which was confirmed as *Nasonia vitripennis*. (Prazapati & Raychoudhury unpublished). Moreover, it was also found that these strains are multiply infected with A and B Super groups of *Wolbachia*, like all other collected strains of *Nasonia spp.* (Raychoudhury *et al*. (2010b).

Introduction to *Nasonia vitripennis* Biology

Nasonia vitripennis is a parasitoid wasp that lays eggs in the pupae of flesh flies.

The diagram below depicts the lifecycle of *Nasonia* reared on flesh fly pupae.

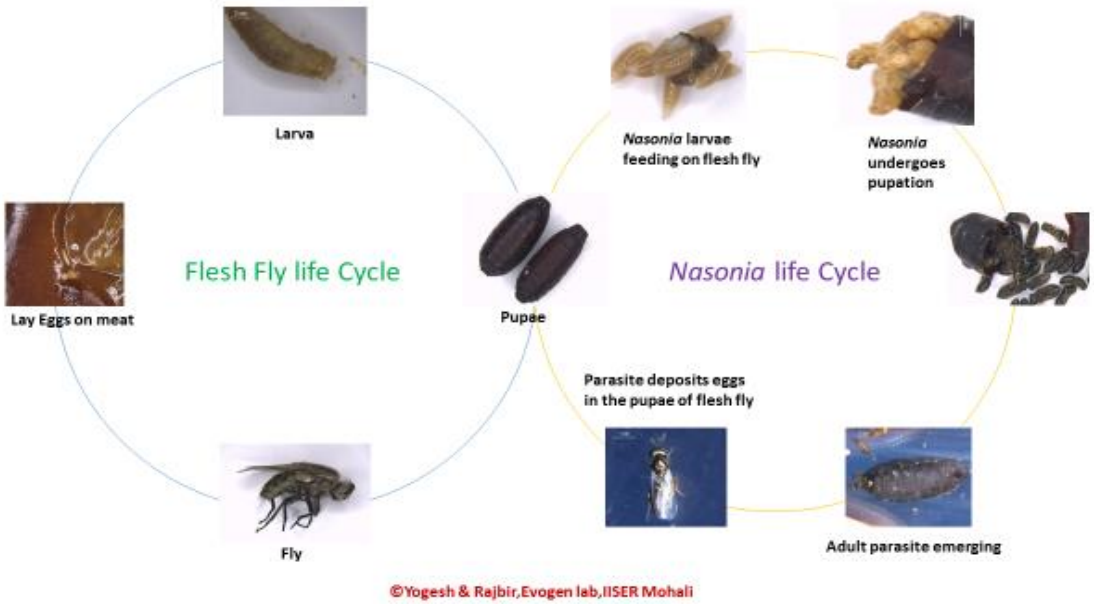


Figure: 1.3 *Nasonia* life cycle

Nasonia wasps, consists of four closely related species, *N. vitripennis, N. giraulti, N. longicornis,* and *N. oneida* (Darling and Werren, 1990; Raychoudhury *et al.,* 2009). *Nasonia* females are diploid whereas haploid males arise from unfertilized eggs. *Nasonia* haplodiploidy and available genome sequence makes it a good model system to study genetics and evolutionary biology. *Nasonia vitripennis* are very easy to handle in laboratory and have a short generation time (2 weeks at 25 ºC) (Whiting, 1967).

The four closely related species are inter-fertile if we remove *Wolbachia* and hence we can make hybrids between them. By using such system we can find out the genes responsible for particular phenotype too (Desjardins *et al*., 2010).

Previous studies have shown that if *Wolbachia* super group-A and B strains are separated in *Nasonia vitripennis* (which carries both the *Wolbachia* infections) (Perrot *et al*., 1996) and then mating experiment between males of super group-A and uninfected females is done, it results in incomplete CI. However, males which have B super group *Wolbachia* when mates with the

females which does not carry any *Wolbachia* infection, it leads to complete CI i.e. no female progeny (Bordenstein *et al*., 2006).

Therefore, to understand the dynamics of multiple *Wolbachia* infections within single host, the questions addressed are:

a) How does a host maintain multiple *Wolbachia* infections?
b) Is there any competition between them? If so, which strain is dominant over the other?

Therefore, I am looking for the intra-host competition between A and B super group *Wolbachia* in various developmental stages of *Nasonia vitripennis*.

This includes,

1) Primer designing (for qPCR analysis)
2) Primer quality control
3) DNA extraction from different developmental stages of *Nasonia vitripennis* males
4) *Wolbachia* cured *Nasonia vitripennis* (as control)
5) qPCR analysis

Chapter 2

Materials and Methods

2.1 qPCR primer designing

To design primers, *Wolbachia* A and B super group specific gene fragments were aligned. These primers were designed from previously sequenced genetic fragments sequenced for multi locus strain typing of the world-wide *Wolbachia* strains.

Figure 2.1 Alignment of *Wolbachia* super group-A and B gene fragments

Region variable in both groups was chosen as the basis of primer designing. 5'-3' primers for *Wolbachia* super group-A and B are as follows,

Table: 2.1 qPCR primers list

fbpA	1	*fbpA*_A_QPCRF1	GTTCCAACTCTTTACATTCAAAGGATC
	2	*fbpA*_A_QPCRR1	TTGGCTTCAGCTATGATTCCACGG
	1	*fbpA*_B_QPCRF1	GCTAACTCCTTGCACTCGAAAAAC
	2	*fbpA*_B_QPCRF1	GCAAGACTTAGCCTCAGCTATAATTTCG
hcpA	1	*hcpA*_A_QPCRF1	ACGAGGAAATACAATATGAAGGTCAT
	2	*hcpA*_A_QPCRR1	GTTTCTCCCAAGTTTCCACCC
	1	*hcpA*_B_QPCRF1	AGAAATACAATATGAAGGCTGCGGA
	2	*hcpA*_B_QPCRR1	ACTTCTAATTCAATTCCATAGTTA
ftsZ	1	*ftsZ*_A_QPCRF1	GCCGCAGTTAAGGATAGAGCGC
	2	*ftsZ*_A_QPCRR1	CAGCAAGTTTAAATGCATCAGAAAATG
	1	*ftsZ*_B_QPCRF1	GCGGTAGTTAAAGATAAAGGAG
	2	*ftsZ*_B_QPCRR1	GGCGAGTTGAAATGCGTCAGC

***hcpA*:**	**conserved hypothetical protein, NCBI COG0217 (WD_0484)**
ftsZ:	cell division protein (WD_0723)
fbpA:	fructose-bisphosphate aldolase (WD_1238)

2.2 To check whether designed primers are amplifying the product that they are supposed to and they are not cross reacting, gradient PCR was done to standardise the primers and the product obtained was sent for sequencing to confirm.

2.3 Genomic DNA was extracted from various developmental stages of *Nasonia vitripennis* males by using Hi-media DNA extraction purification kit.

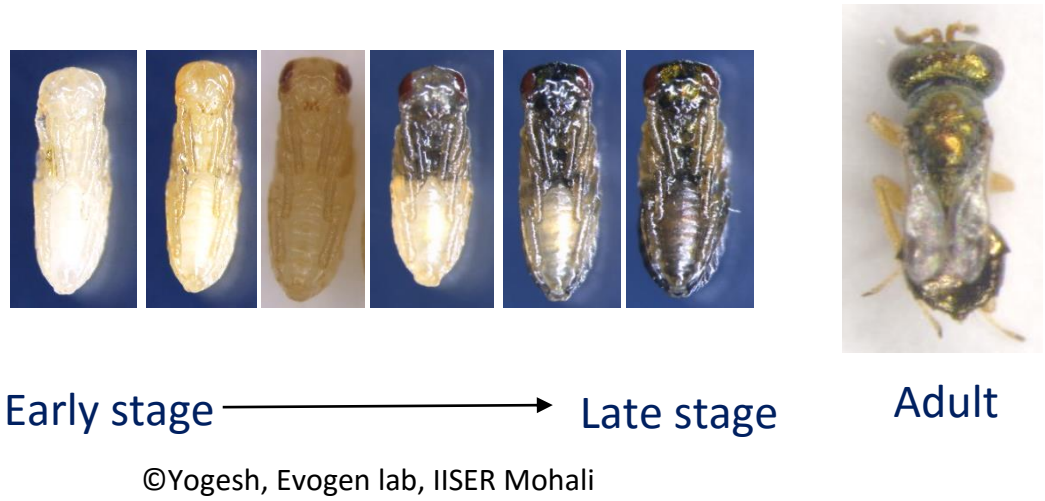


Figure: 2.2 *Nasonia vitripennis* males at various developmental stages

2.4) INSECT DNA Extraction Protocol:

Procedure

a) 180 µl of Lysis Solution and 20 µl of the Proteinase K solution (20 mg/ml) was added to capped 2.0ml micro centrifuge tube containing the insect sample. The sample was crushed and mixed thoroughly by vortexing and incubated at 55°C overnight in water bath.

b) Added 20 µl of RNase-A Solution to the sample containing tube and incubated for 2 minutes at room temperature (25°C)

c) Added 200 µl of Lysis Solution to the sample and vortexed thoroughly for 15 seconds. Incubated at 70°C for 10 minutes.

d) Added the lysate onto HiShredder placed in an uncapped 2.0 ml collection tube and centrifuged for 2 minutes at 13,000 x g (≈14,000 rpm). Transferred the flow-through fraction to a new 2.0 ml collection tube without disturbing the cell debris pellet.

e) Added 200 µl of ethanol (96-100 %) to the lysate and mixed thoroughly by vortexing for 5-10 seconds.

f) Loaded lysate onto HiElute Miniprep Spin Column and centrifuged at ≥6,500 x g (≈10,000 rpm) for 1 minute. Discarded the flow-through liquid and placed the column in a same 2.0 ml collection tube.

g) Added 500 µl of diluted wash solution to the column and centrifuged at (≈10,000 rpm) for 1 minute. Discarded the flow-through liquid and re-used the same collection tube with the column.

h) Added another 500 µl of diluted Wash Solution to the HiElute Miniprep Spin Column (Capped) and centrifuge at (≈13,000 rpm) for 3 minutes to dry the column. Discarded the flow-through. Centrifuged the column for another minute for residual ethanol removal. Discarded the collection tube and placed the column in a new 2.0 ml uncapped collection tube.

i) To increase the elution efficiency, 120µl of the elution buffer was added into the column and incubated for 5 minutes at room temperature. Centrifuged (≈10,000 rpm) for 1 minute to elute the DNA.

j) Samples stored at -20°C for long term storage.

2.5 Curing of *Wolbachia*

Wolbachia can be eliminated from the host by feeding them on antibiotic called tetracycline (Casiraghi *et al*., 2002).

a) Procedure to prepare antibiotics

1) To create 1% tetracycline in 10% sugar water solution

   a) 1 ml double distilled H_2O
   b) 0.1gm sugar
   c) 0.01gm tetracycline

2) Vortexed solution until all sugar crystals had dissolved.

(Werren & Loehlin, 2009)

b) For curing *Wolbachia*, the protocol followed is as follows,

1) Newly emerged 5 mated females of *Nasonia vitripennis* were taken from the tube which had larger number of males. Kept them on starvation for one day.

2) Single female taken in individual tube, made 5 such tubes.

3) Added antibiotic (1% tetracycline).

4) Next day added host (1 more than the number of females).

5) Re-hosted after every 48 hrs (3-4 times).

6) Females were removed after 3-4 of re-hosting, extracted the DNA, and performed PCR with *Wolbachia* specific primers.

2.6 Curing confirmation

The curing is confirmed by doing PCR using *Wolbachia* specific primers and by performing genetic crosses.

1) Chosen the cured strain.

2) Collected virgin females and males by cracking open pupae near eclosion (13 day old).

3) Mated the virgin females with the original infected males and vice-versa.

4) A total of 10 replicates were maintained.

5) Mating was observed in each replicate for at least 15 minutes.

6) Host were given to the mated females for 48 hours

7) After 48 hrs females were removed.

8) Waited till the *Nasonia vitripennis* emerged from the host, counted the family size of the CI cross.

9) Confirmed curing and maintained the cured strain in 25ºC incubator.

2.7 DNA extraction

Genomic DNA was isolated from single adult males (pupal stages) using Hi-media DNA Extraction purification kit using the protocol mentioned above.

2.8 PCR reaction

For PCR, a reaction volume of 20µl containing 13.9µl of sterile water, 2.0µl PCR buffer, 0.6µl dNTPs, 0.3µl $MgCl_2$, 0.5µl each of 10µM forward and reverse primers and 0.2 µl of Taq polymerase was used to amplify the target DNA.

PCR product clean-up was done by using Exonuclease- I -SAP (Shrimp Alkaline phosphatase) in following quantities:

SAP 0.6µl,

Exo-I 0.15µl,

10X SAP Buffer 0.1µl,

double distilled H_2O 2.85µl,

PCR Product 6µl

Protocol followed is:

1) Incubation of reaction mix at 37 ºC for 1 hour for enzyme activation.

2) Enzyme deactivation by keeping the reaction mix at 90 ºC for 10 minutes.

3) Sample were then sent for sequencing to Integrated DNA Technologies.

2.9 Real -Time PCR

a) *Wolbachia* infection status was confirmed by PCR with *Wolbachia* specific primers.
b) RT-PCR was performed in C1000Touch Thermal cycler system (Bio-Rad, CFX-96 Real Time system). Reaction volume of 20µl contained 10µl of S4438 SIGMA SYBR Green JumpStart Taq Ready Mix Super mix, 7µl of sterile water, 0.5µl each of 10µM forward and reverse primer, and 2µl of Target DNA in single well. For the selective amplification of the *Nasonia S6 Kinase gene* (133 bp), *fbpA_A* (172bp), *fbpA_B* (~170bp), the following primers were used.

.

No.	Primer name	5'-3' primers	Annealing Temperature
1	*fbpA*_A_QPCRF1	GTTCCAACTCTTTACATTCAAAGGATC	51ºC
2	*fbpA*_A_QPCRR1	TTGGCTTCAGCTATGATTCCACGG	51ºC
3	*fbpA*_B_QPCRF1	GCTAACTCCTTGCACTCGAAAAAC	51ºC
4	*fbpA*_B_QPCRF1	GCAAGACTTAGCCTCAGCTATAATTTCG	51ºC
5	*NvS6KQTF2*	GGCATTATCTACAGAGATTTG	55ºC
6	*NvS6KQTR2*	GCTATATGACCTTCTGTATCAAG	55ºC

qPCR Basics and Data Analysis

In the classical PCR, at the end of the amplification, the product can be run on a gel for detection of its specific products. In real time PCR this step can be avoided because of the advanced technology used, this results in amplification of product with immediate detection in a single tube. This technique is highly beneficial because it removes significant contamination which may be caused by opening of the tubes for post PCR manipulations. And it is also consumes less time than gel based analysis and can give quantitative result.

The main advantage of real time PCR over PCR is that real time PCR allows us to determine the initial number of copies of template DNA with accuracy and with high sensitivity over a wide range.

Real time PCR results can either be qualitative (the presence or absence of sequence) or quantitative (copy number). In qPCR the (1) Amplified DNA is fluorescently labelled (usually with Cyanine based fluorescent dyes) and (2) the amount of fluorescence released during amplification is directly proportional to the amount of amplified DNA.

How Does Real-Time PCR work?

To understand how real time PCR works, for e.g.it is easy to understand by using the amplification plot .shown below,

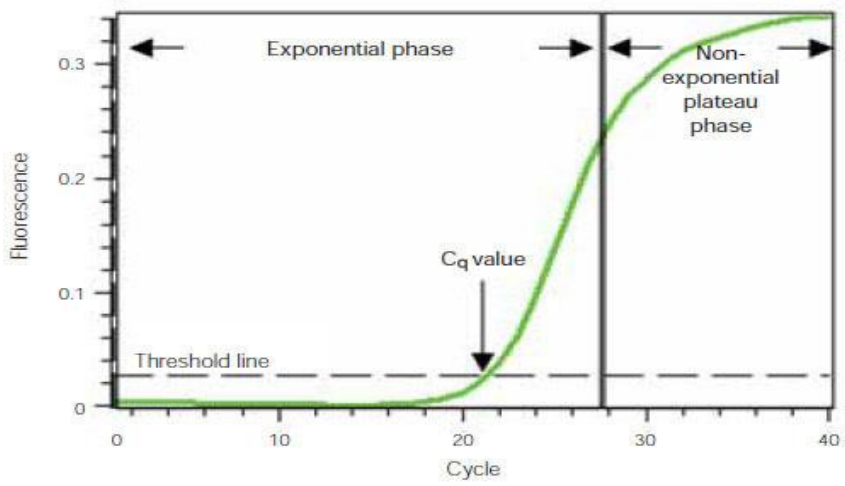


Figure 2.3: Plot depicting C_q value with increase in no. of cycles

In this plot, the number of PCR cycles shown on X-axis, and fluorescence from amplification reaction, which is proportional to the amplification product in the tube is shown on Y-axis.

The amplification plot show two phases, an exponential phase followed by non-exponential phase. During the exponential phase, the amount of PCR product approximately doubles in each cycle. As the reaction proceeds, at some particular point the reaction components are consumed, and ultimately one or more components become limiting, at this point the reaction enters in non-exponential phase.

In the beginning the fluorescence remains at background level and the increase in the fluorescence are not detectable (cycle1-18) even though product accumulate exponentially. Eventually enough amplified product accumulates to yield a detectable fluorescence signal, and the cycle number at which this occurs is called quantification cycle or C_q.

The C_q value of a reaction is determined by the amount of template DNA present at the beginning of the amplification reaction. If large amounts of template is present during the start of the reaction, relatively few amplification cycles are required to get desired product to give a fluorescence signal above background therefore the reaction will have minimum C_q if a small amount of templates present during the start of the reaction, more amplification cycles required for fluorescence signal to rise above background therefore the reaction will have a higher C_q. Therefore this relationship forms the basis for the quantitative aspect of Real -Time PCR.

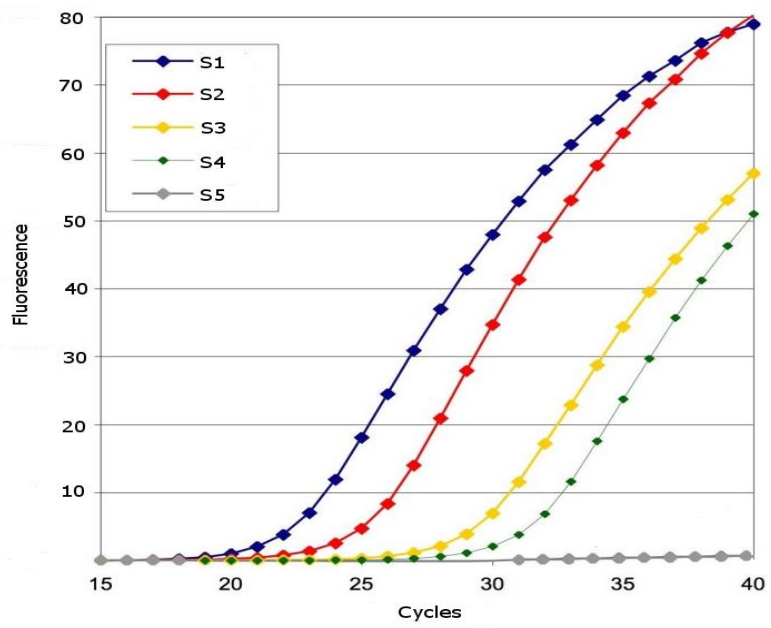


Figure: 2.4 An example of Amplification plots

Fig. Amplification plot of five samples (S1-S5). As the target DNA in each cycle is amplified the fluorescence increases. Sample S1 contains the highest amount of target DNA molecule which shows fastest amount of increase in fluorescence while sample four shows lowest amount of target DNA molecule and sample S5 contains no target DNA molecule.

Chapter 3

Results

1) For the qPCR primer quality control, gradient PCR was performed with *Wolbachia* super group-A and B specific primers.

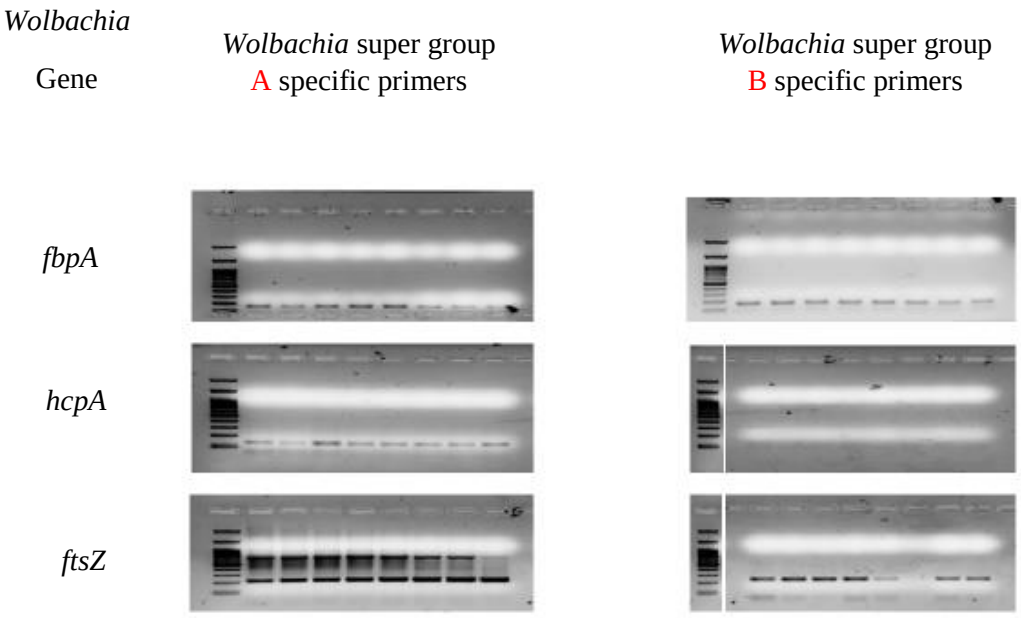


Figure: 3.1 Gradient PCR for primer check

Sequenced PCR product from the potential primers were also found to be super group specific for *fbpA_A* and *fbpA_B* after NCBI Blast.

Wolbachia can be eliminated from the host by feeding them on antibiotics like tetracycline. As *Nasonia vitripennis* was cured from *Wolbachia*, it showed progressively reduced sex ratio over time i.e. increasing proportion of males over time. Due to reduced *Wolbachia* levels in the eggs produced after antibiotic treatment, females from the later hostings were found to produce higher proportion of males as the antibiotic treatment may have reduced the *Wolbachia* load (Werren and Loehlin, 2009). Repeated curing experiments were done for 6-7 generations, to cure the *Wolbachia* infection permanently. Curing of *Nasonia vitripennis* from *Wolbachia* was checked by doing PCR using *Wolbachia* specific primers for 10 individual females.

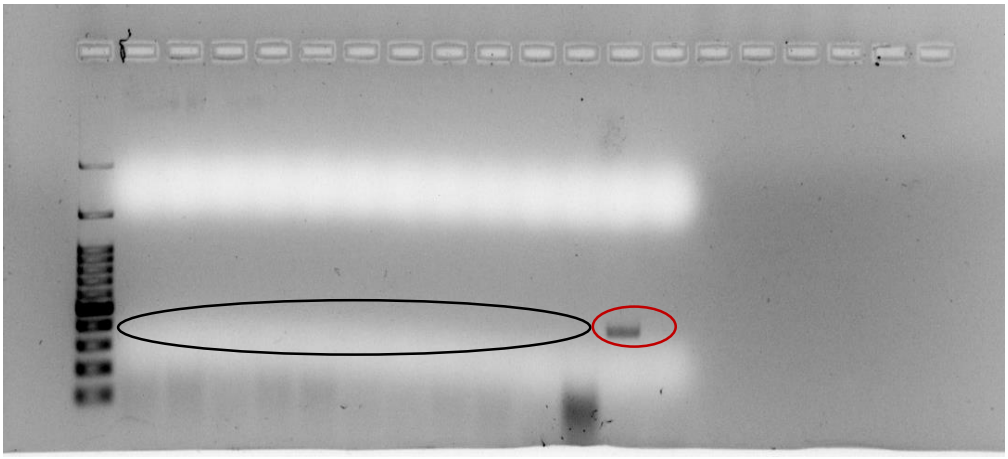


Figure 3.2 PCR with *Wolbachia* specific primers to confirm curing

As shown in figure 3.2 confirmatory PCR did not show any specific band for *Wolbachia* for the DNA samples of 10 females, compared to a positive control (infected *Nasonia vitripennis*) shown in red circle.

Progeny produced by the females which did not show any specific band for *Wolbachia* after PCR with *Wolbachia* specific primers, were screened for virgins at the pupal stage. Various CI crosses for cured males and females were put with *Wolbachia* infected males and females of *Nasonia vitripennis* and mating was observed. The results of these crosses are shown below.

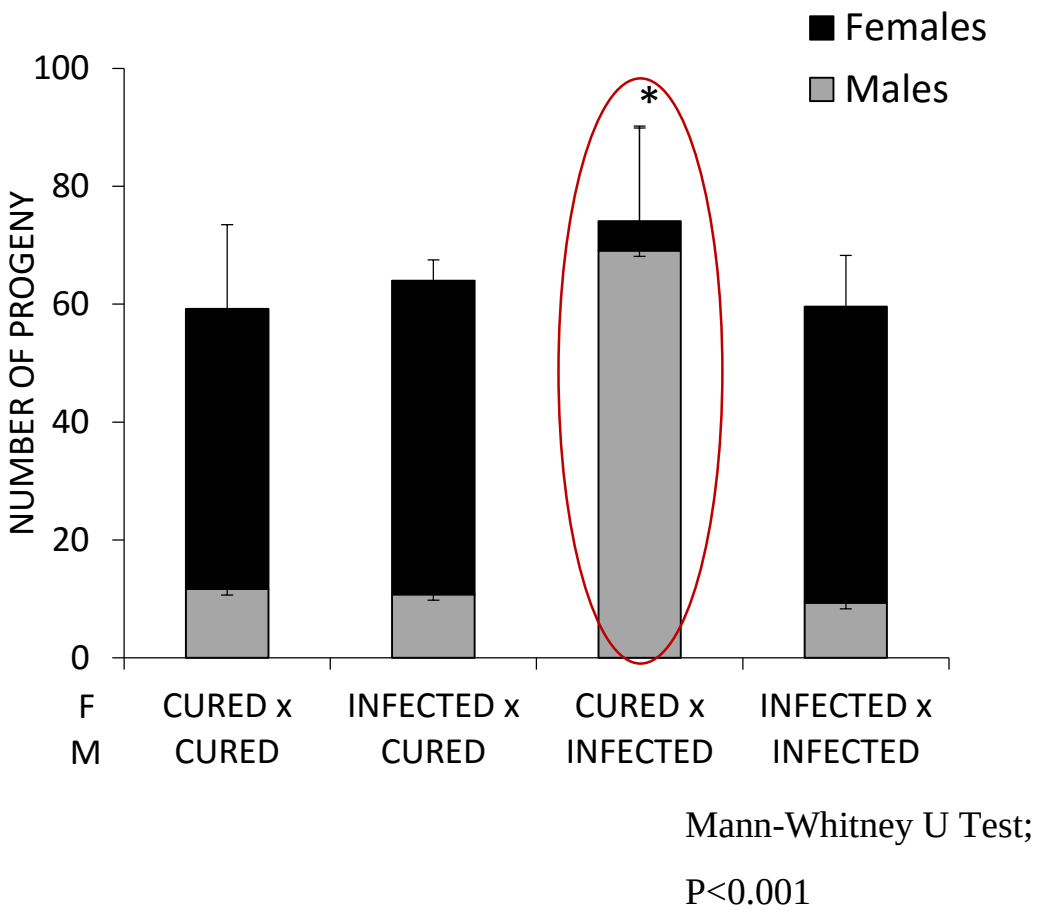


Figure: 3.3 Cytoplasmic incompatibility (CI) crosses

Progeny number were counted after doing the CI crosses. When *Wolbachia* infected males mate with the uninfected females (as discussed in figure1.1) it does not produce any progeny because females does not have the rescue system for the sperm modified by *Wolbachia*. But in case of *Nasonia vitripennis*, which is a haplodiploid system, unfertilized eggs give rise to progeny which are mostly haploid males. Hence, when infected *Nasonia* male mates with cured female (3rd CI cross), the number of male progeny is significantly higher than in the other 3 crosses. This shows that the cured strain is producing conversion CI (Bordenstein *et al*., 2003).

We confirmed the curing through PCR and genetic crosses. Cured *Nasonia* from *Wolbachia* served as negative control to confirm that the *Wolbachia* specific primers were not amplifying any region from the host eukaryotic genome.

qPCR data Analysis:

To perform qPCR reaction, primers specific for *Wolbachia* supergroup A and supergroup B *fbpA_A* and *fbpA_B*, respectively, were used in addition to *NvS6K* (*Nasonia S6 kinase gene)* which was a primer used as a control (Bordenstein *et al*., 2006). Details of the qPCR reaction are discussed in section 2.9.

To know the density difference between the two supergroups, we calculated ΔC_q i.e. the differene between C_q values of [(*NvS6K*)- (*fbpA_A*)] and [(*NvS6K*)- (*fbpA_B*)], respectively .

Table: 3.1 C_q values of *fbpA_A, fbpA_B* and *NvS6K*

	fbpA_A(C_q)	*fbpA_B*(C_q)	*NvS6K*(C_q)	*NvS6K*-*fbpA_A*	*NvS6K*-*fbpA_B*
S1	29.91	36.33	33.18	5.27	-3.15
S3	28.13	36.50	33.03	4.9	-3.47
S4	26.42	29.61	30.08	3.66	0.47
S5	27.06	34.87	32.11	4.51	-2.76
S6	27.35	33.91	32.26	4.91	-1.65
S7	27.15	33.93	33.42	6.27	-0.51
S8	24.57	31.02	33.12	8.55	2.1

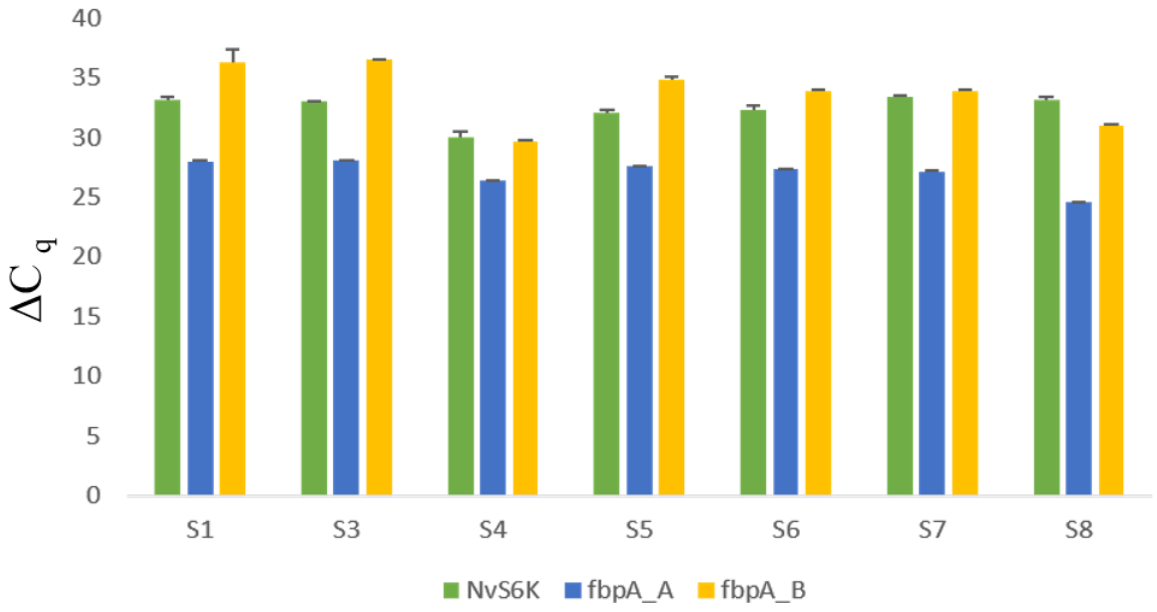


Figure: 3.4 Bar graph for C_q values

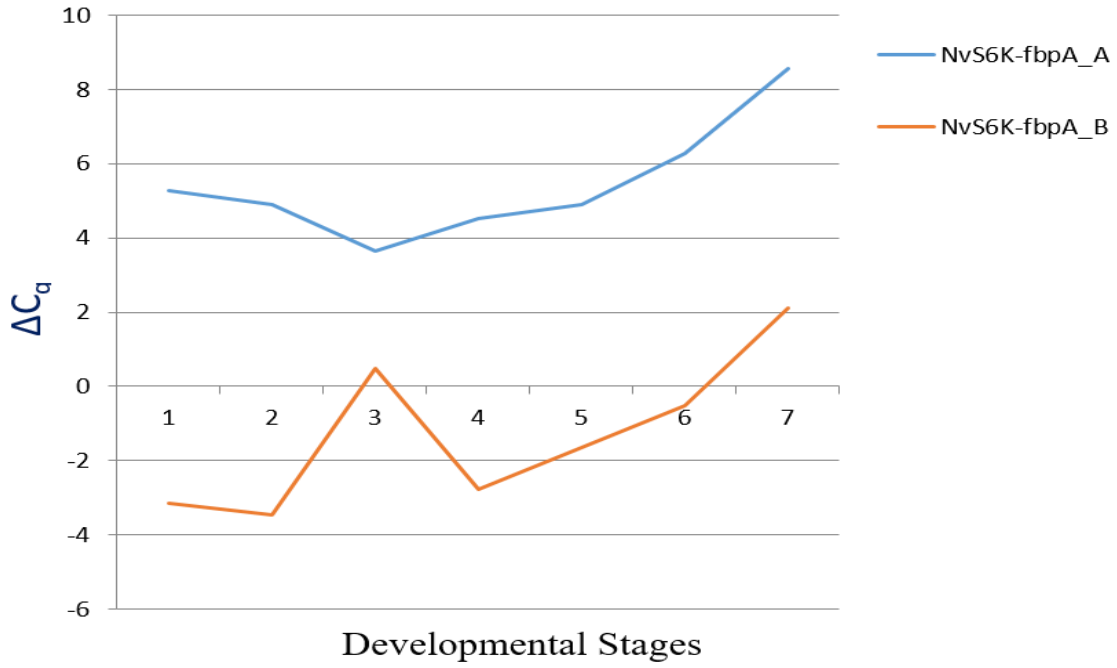


Figure: 3.5 Comparison of A and B super group of *Wolbachia* at different developmental stages of *Nasonia vitripennis*

The non-parametric Wilcoxon match pair test was performed for the comparison of A and B super group of *Wolbachia* in S1-S8 stages. The test is used for comparing two related samples or repeated measurement on single sample to check whether there is any rank difference in the population.

Here, the null hypothesis was of no difference between the two densities and alternative hypothesis was that there is a difference between the two densities.

Table: 3.2 Wilcoxon match pair test

N	T	Z	P
7	**0**	**2.366**	**0.018**

Wilcoxon match pair test shows that there is a difference between the densities of two super groups within single sample with significant P value of 0.018.

Discussion and Conclusion

Previous studies have shown that, if we separate the strains of *Wolbachia* super group-A and B in *Nasonia vitripennis* (which carries both the *Wolbachia* infections) (Perrot-Minnot *et al*., 1996), the mating between males of super group-A and uninfected females results in incomplete CI, whereas when males which have B super group *Wolbachia* mates with the females which does not carry any *Wolbachia* infection, it produces compete CI i.e. no female progeny (Bordenstein *et al*., 2006).This has been hypothesized to be due to the higher density of super group-B in the host compared to the A super group infection (Bordenstein *et al*., 2006). Our data corroborates this across the different pupal stages investigated. However, what remains uncertain is whether there are any detectable density differences in the earlier larval stages. The equilibrium density in the different pupal stages found here might be too late a developmental stage to detect any such variation in densities of the two *Wolbachia* infections.

In males, no differential density was observed between two *Wolbachia* super groups except at stage S4, which has some variation than other stages for both the super groups.

However, a complete picture will emerge only when we do the same experiment with eggs, different larval, pupal, and adult stages of both males and females of *Nasonia vitripennis*. Nature of this difference needs to be investigated further.

From our experiments we concluded that there is no major difference in the density of *Wolbachia* super group A or B across various developmental stages of *Nasonia vitripennis* males. However there is a significant difference between super group A and B infections in *Nasonia vitripenni*s males.

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