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Mortality in Interspecific Hybrids of Nasonia vitripennis and Nasonia giraulti

Bonnie Cobb

MORTALITY IN INTERSPECIFIC HYBRIDS OF NASONIA VITRIPENNIS AND NASONIA GIRAULTI

by

BONNIE COBB

(Under the Direction of Joshua D. Gibson)

ABSTRACT

Nasonia is a parasitoid wasp genus that serves as an emerging model for studying speciation due to an incompatibility between nuclear and mitochondrial genomes between sister taxa. Short generation times, easy rearing in a lab setting, producing large amounts of progeny, and whole genome sequencing make *Nasonia* is an excellent candidate for studying incomplete reproductive isolation. *Nasonia* have five chromosomes and exhibit haplo-diploid sex determination in which fertilized eggs develop into diploid females and unfertilized eggs develop into haploid males. Recessive phenotypes are hidden in diploid females but are laid bare in haploid males as there is no interaction between alleles. F2 male hybrids of *Nasonia vitripennis* and *Nasonia giraulti* experience high rates of mortality during development as compared to parent strains. Previous research has shown that 96% of F2 males with an incompatible allele on chromosome 5 combined with *N. giraulti* maternity die. F1 females experience low mortality in comparison to the high mortality that their sons, F2 males, experience. Large differences in mortality between F1 females and F2 males can be further investigated by studying F2 backcross females. I hypothesized that F1 females and F2 backcrossed heterozygous females experience similar levels of incompatibility to one another at the mortality locus as well as F2 backcrossed homozygous females and F2 males. I predicted that F2 backcross females would have intermediate mortality overall as compared to F1 females and F2 males. To test this, I conducted mortality experiments to determine differences in egg to adult survival between parent species, F2 males, and backcrossed F2 females. I found that F2 hybrids suffered greater mortality than both parent species. Egg to adult mortality was similar between F2 males

and F2 females. Adult F2 females survived more than adult F2 males, but sexes were not significantly different. I also found that F1 hybrids laid significantly fewer eggs than both parent species. However, F2 backcrossed females survive much less than previously expected and suffer similar mortality to F2 males. My findings therefore indicate that there must be other interactions on the recombinant F2 backcrossed chromosome contributing to mortality in these individuals.

INDEX WORDS: Nasonia, Hybrid mortality, Hybrid breakdown, Interspecific hybrids, Mortality, Egg to adult survival

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by

BONNIE COBB

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TABLE OF CONTENTS

Page

ACKNOWLEDGMENTS.....2

LIST OF TABLES.....4

LIST OF FIGURES.....5

CHAPTER

1 INTRODUCTION…….....6

Background…….....6

Study System…….....7

Purpose of the Study…….....9

2 METHODS…….12

Study Organisms and Stock Maintenance.....12

F1 Hybrid Production.....12

F2 Hybrid Production.....13

Mortality Experiments.....14

Statistical Methods.....16

3 RESULTS…….17

Means of egg number taken from four groups.....18

Means of adult number taken from four groups.....19

LS Means from multiple pairwise comparisons using Tukey's HSD.....20

LS Means plot for egg and adult counts.....20

4 DISCUSSION…….....21

REFERENCES 27

APPENDICES

A GENOTYPING PROTOCOL 31

B OXPHOS PROTOCOL.....33

LIST OF TABLES

Page

Table 1: Results are from regression and LS Means analysis including group, stage, group by stage, and female nested in group as a random variable.....18

Table 2: Results of Least Squared Means from multiple pair wise comparisons between groups and stages using Tukey's HSD.....20

LIST OF FIGURES

Page

Figure 1: F2 male mosaic chromosomes with either a *N. giraulti* or *N. vitripennis* allele at a previously identified locus located toward the end of chromosome 5.....8

Figure 2: Examples of an F1 female, F2 backcrossed females, and F2 males with *N. giraulti* or *N. vitripennis* alleles at a previously identified locus located toward the end of chromosome 5.....11

Figure 3: *N. vitripennis* males are crossed to *N. giraulti* females to produce F1 hybrid female 13

Figure 4: Structural design of individual female oviposition chambers.....15

Figure 5: Experimental design outlining mortality count methods.…….....16

Figure 6: Means of egg numbers taken from four groups, including *N. giraulti* parents, *N. vitripennis* parents, F2 females, and F2 males.....18

Figure 7: Means of adult numbers taken from four groups, including *N. giraulti* parents, *N. vitripennis* parents, F2 females, and F2 males.....19

Figure 8: Results of the Least Squares Means plot for egg and adult counts of parents and hybrids 20

CHAPTER 1

INTRODUCTION

Background

Speciation is responsible for the vast diversity of life that we see on earth. Speciation is a byproduct of evolution of populations that lack gene flow leading to reproductive isolation. The Biological Species Concept defines a species as "a group of actually or potentially interbreeding natural populations, which are reproductively isolated from other such groups" (Mayr 1942). However, this traditional definition of the Biological Species Concept poses constraints on the existence of hybrids, as it requires complete reproductive isolation. Therefore, Coyne and Orr modified the Biological Species Concept (2004), by adding that "distinct species are characterized by substantial but not necessarily complete reproductive isolation." This concept allows us to see hybrids in the process of speciation by studying incomplete reproductive isolation.

During the process of speciation, geneflow is greatly reduced between populations which causes species to begin to diverge and eventually leads to complete reproductive isolation. One mechanism of reduced gene flow is incompatible interactions between pairs of genes that lead to decreased fitness in hybrids. The Bateson-Dobzhansky-Muller (BDM) model illustrates how incompatible interactions can evolve between diverging populations without each population experiencing these incompatibilities (Bateson, 1909; Dobzhansky, 1937; Muller, 1942). Interacting genes continually evolve together in each lineage so that every generation within each lineage maintains compatible interactions. When these lineages hybridize, these coevolved gene pairs are shuffled and combinations of interacting partners are produced that have never been subjected to natural selection. These untested interactions may result in incompatibilities in these hybrid organisms.

Any interacting pair of genes between two populations can cause divergence just by changing slightly over long periods of time such as through accumulation of mutations or through selection pressures causing specific genes to evolve more quickly. Some genes or chromosomes evolve more

quickly than others, such as sex chromosomes evolving more quickly than autosomes, and mitochondrial DNA having more errors and mutating faster than nuclear DNA. Mitochondrial DNA of parasitoid wasps in the genus *Nasonia* has a substitution rate that is 30 times greater than that of its nuclear DNA (Oliviera et al. 2008). As deleterious mutations accumulate in mitochondrial DNA, nuclear genes can be selected to make up for the reduced function of its mitochondrial partner through a process known as compensatory coadaptation (Rand et al. 2004). As the probability of incompatible interactions grows much faster than the number of substitutions, incompatibilities evolve faster and faster as time goes by. This phenomenon is known as the snowball effect (Orr and Turelli 2001), which can lead to a loss of fitness.

In hybrids, one outcome of this reduction in fitness is hybrid breakdown. This concept is opposite to hybrid vigor in that hybrid breakdown doesn't affect the F1 generation but F2 generations suffer severe complications (Gibson et al. 2013). Hybrid vigor, or heterosis, explains that the F1 generation of offspring of two parent species inherits the most positive qualities of both parents and survives better than either parent (East 1936). In examples of hybrid breakdown, F1 individuals don't suffer serious complications from incompatibilities caused by deleterious mutations, as opposed to F2 individuals who suffer greatly. Therefore, hybrid breakdown may serve as a reproductive barrier between species and lead to speciation.

Study System

Nasonia is a parasitoid wasp genus that serves as an emerging model for studying speciation due to an incompatibility between nuclear and mitochondrial genomes between sister taxa. Short generation times, easy rearing in a lab setting, producing large amounts of progeny and whole genome sequencing of their five chromosomes make *Nasonia* an excellent candidate for studying incomplete reproductive isolation (Werren and Loehlin 2009). There are four species currently known in the genus; *N. vitripennis, N. longicornis, N. giraulti* and *N. oneida*. *N. giraulti* and *N. vitripennis* diverged as recently as 1 million years ago. Although the geographic distributions of these species have been found to be sympatric (with *N. vitripennis* found globally), these species experience a form of postzygotic isolation as a result of an infection of inherited cytoplasmic bacteria called *Wolbachia*. Each species of wild-type *Nasonia* are

infected with a Wolbachia which causes cytoplasmic incompatibilities in *Nasonia* hybrids (Bordenstein et al. 2001). In a lab setting, *Nasonia* can be cured of their *Wolbachia* infection when fed antibiotic infused sugar water over three generations (Breeuwer and Werren 1990). Once cured, *Nasonia* can hybridize and produce fertile, viable progeny.

Nasonia exhibit haplo-diploid sex determination in which fertilized eggs develop into diploid females and unfertilized eggs develop into haploid males. This system can be used to study speciation at the molecular level due to the sheer simplicity of male genetics. Genetic recombination is responsible for diversity within a species. During oogenesis in F1 hybrid mothers, crossing over occurs between paired chromosomes leading to further recombination, F2 males whose chromosome is a mosaic of both parents (Figure 1). Recessive phenotypes are hidden in diploid females but are laid bare in haploid males as there is no interaction between alleles. This hemizygosity in males acts similarly to homozygosity in other systems and allows for clearer relationships between genotype and observed phenotype. Lastly, the *Nasonia* genome has been sequenced and mapped so there are many genetic resources and databases available to study this system (The Nasonia Working Group 2010).

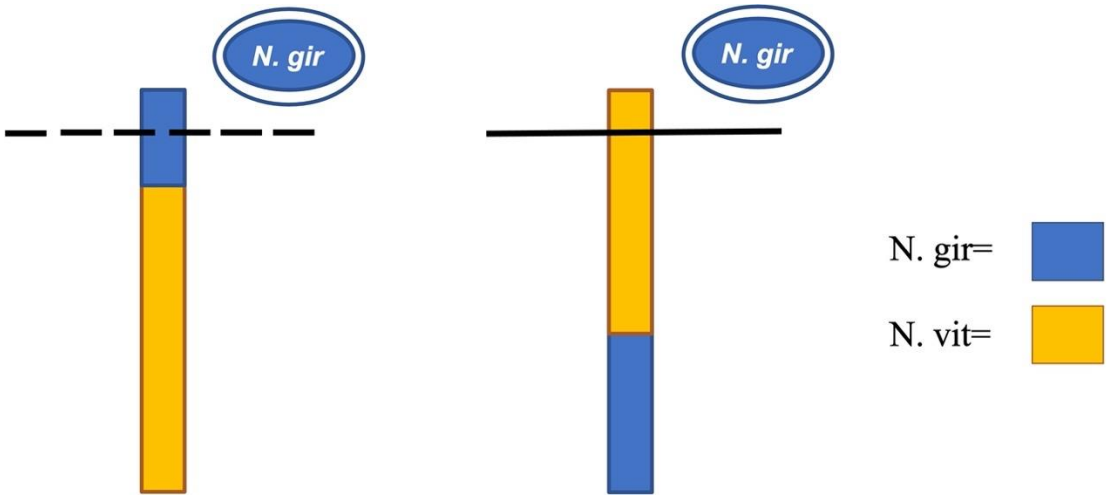


Figure 1. F2 male mosaic chromosomes with either a *N. giraulti* or *N. vitripennis* allele at a previously identified locus located toward the end of chromosome 5. Rectangles represent chromosomes and ovals in the top right corner of the chromosomes represent the mitochondrial background of each individual. Solid

line represents incompatible allele at locus interacting with mitochondrial background. Dashed line represents compatible allele at locus interacting with mitochondrial background.

Hybrid offspring of *Nasonia vitripennis* and *Nasonia giraulti* experience differential success within reciprocal crosses. F1 hybrid females with *N. giraulti* mitochondrial background suffer some mortality as compared to the parent species and produce fewer adult wasps (Breeuwer and Werren 1995). F1 hybrid females with *N. giraulti* mitochondrial background experience only 15% mortality. In contrast, F2 males with *N. giraulti* mitochondria die 80% of the time (Breeuwer and Werren 1995). There is a locus on chromosome 5, where Gibson et al. (2013) found that 98% of F2 males with the *N. vitripennis* allele and *N. giraulti* mitochondrial background died. Such significant mortality is not observed in the reciprocal cross (Niehuis et al, 2008). Given the mortality we see in individuals with *N. giraulti* maternity and *N. vitripennis* paternity, we are particularly interested in this side of the cross. This pattern of mortality and asymmetric incompatibility indicates an interaction between the *N. vitripennis* allele at the above mentioned locus on chromosome 5 and *N. giraulti* cytoplasm. Nuclear and mitochondrial incompatibilities greatly contribute to hybrid breakdown in F2 male hybrids of *N. giraulti* and *N. vitripennis* (Niehuis et al. 2008). Given that their mitochondrial genome has a far greater substitution rate than their nuclear genome, incompatibilities are expected to develop quickly between these hybrids.

Purpose of the study

F1 females experience low mortality in comparison to the high mortality that their sons, F2 males, experience. For the mortality locus located on chromosome 5, it is hypothesized that each allele interacts with that individual's mitochondrial background through a compatible or incompatible interaction. F2 males with an incompatible allele at this locus are those individuals that experience mortality. Although, F1 females and F2 males both have an *N. giraulti* background and one chromosome with a *N. vitripennis* allele at the mortality locus, only F2 males experience extreme amounts of mortality.

Heterozygosity in F1 females provides an interesting insight into these genic interactions through a possible rescue effect. A partial rescue in this case describes that one compatible and one incompatible allele could cause a female to have mitochondria that work less effectively than the parents' mitochondria but better than the F2 male mitochondria. These individuals could still be healthy enough to carry on a normal life cycle but be experiencing a degree of physiological dysfunction. In contrast, F2 males are haploid and therefore cannot experience this rescue effect. This could offer a possible explanation as to why F2 hybrid males die at a much higher rate than their mothers.

Large differences in mortality between F1 females and F2 males can be further investigated by studying F2 backcross females. F2 backcross females are produced by mating F1 hybrid females with *N. vitripennis* paternity and *N. giraulti* maternity, back to a *N. vitripennis* male. F2 backcross females therefore have, on average, 75% *N. vitripennis* and 25% *N. giraulti* chromosomes, with a *N. giraulti* mitochondrial background. In F2 backcross females, for alleles at a particular locus, females can either be heterozygous, having one *N. giraulti* and one *N. vitripennis* allele or homozygous, having two *N. vitripennis* alleles.

We hypothesize that F1 females and F2 backcrossed females experience similar levels of incompatibility to one another when the F2 female is heterozygous at the mortality locus, as they both have one *N. vitripennis* allele, and one *N. giraulti* allele, as well as a *N. giraulti* mitochondrial background. Due to this comparable incompatibility, we predict that F1 females and F2 backcrossed females that are heterozygous at this locus will have similar mortality. Both of these hybrids are diploid and have one compatible and one incompatible allele interacting with their mitochondrial backgrounds. We hypothesize that F2 backcrossed females that are homozygous at the mortality locus have similar levels of incompatibility to F2 hybrid males that have the *N. vitripennis* allele at the mortality locus. Both of these hybrids have only *N. vitripennis* alleles at this locus, with a *N. giraulti* background. Although, it is important to reiterate that homozygous F2 females are diploid and have two alleles at a particular locus and F2 males are haploid and have a single allele at a particular locus (Figure 2). Due to only having

putatively incompatible alleles we predict that F2 females that are homozygous at this locus will experience similar mortality to F2 hybrid males with the *N. vitripennis* allele at the same locus. Combining these predictions about their genotypes, we predict that F2 backcross females will have intermediate mortality overall as compared to F1 females and F2 males. More specifically, if F1 females experience 86% survival (Breeuwer and Werren 1995) and are comparable to heterozygous F2 females and F2 males with the *N. vitripennis* allele experience 4% survival and are comparable to homozygous F2 females, then we predict 45% survival in F2 backcross females. To test this, we conducted a series of crosses to estimate mortality and determine differences in egg to adult survival between parent species, F2 males, and backcrossed F2 females.

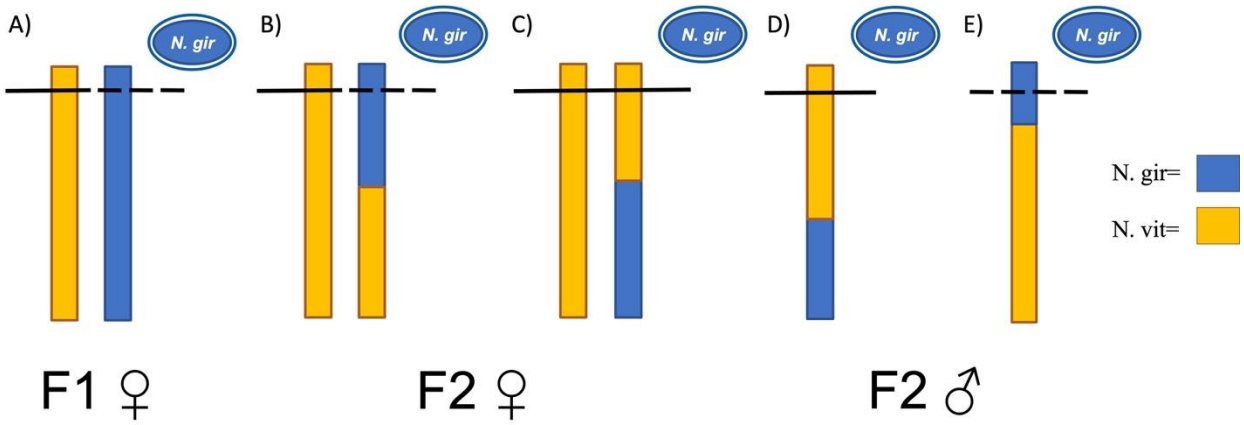


Figure 2. Examples of an F1 female, F2 backcrossed females, and F2 males with *N. giraulti* or *N. vitripennis* alleles at a previously identified locus located toward the end of chromosome 5. Rectangles represent chromosomes and ovals in the top right corner of the chromosomes represent the mitochondrial background of each individual. F1 females (A) are genetically comparable to heterozygous F2 females (B). Homozygous F2 females (C) are genetically comparable to F2 males with a *N. vitripennis* allele (D). F2 males with a *N. giraulti* allele are included for reference (E). Solid line represents incompatible allele at locus interacting with mitochondrial background. Dashed line represents compatible allele at locus interacting with mitochondrial background.

CHAPTER 2

METHODOLOGY

Study organisms and stock maintenance

Cured, isofemale strains for *N. vitripennis* and *N. giraulti* are AsymCX and RVCX2, respectively. *Nasonia* strains AsymCX and RV2X (U) were used for cross experiments. AsymCX is derived from the wild-type *N. vitripennis* strain LBii (LabII, Leiden, The Netherlands) and RV2X(U) is derived from the wild-type *N. giraulti* strain RV2 (Rochester, New York) (Breeuwer and Werren 1995). Both strains came from isofemale lines cured of Wolbachia infection. All *Nasonia* were cultured on flesh fly pupae *(Sarcophaga bullata)* in an incubator set at 25 degrees Celsius with constant light. Stocks were housed in Drosophila vials and provided 20 hosts per hosting and rehosted every 15 to 16 days.

F_1 Hybrid production

Cross experiments were performed by collecting virgin female and male pupae of each species at day 12 of development to accurately determine sex. Wasps were housed individually in glass vials plugged with cotton until eclosion. Single pairs of males and females were added into glass vials. *N. vitripennis* males were paired with *N. giraulti* females to produce F1 diploid females with one *N. giraulti* and one *N. vitripennis* chromosome and *N. giraulti* mitochondrial background (Figure 3). The pairs were given 48 hours to mate before providing the female with a feeder host to facilitate egg production. After 24 hours the feeder host was discarded and the female was provided two successive hosts, 24 hours apart. These hosts were collected and incubated for 12 days to collect female F1 hybrid pupae of each cross. F1 hybrid females were housed in glass vials and upon eclosion. F1 virgin females develop more slowly than the 14 to 15 day development period of either parent species.

F2 Hybrid production

To produce F2 progeny, F1 females were divided into two experimental groups: backcrossed (n=66) and unmated (n=78). Half of the F1 females were left unmated to lay unfertilized eggs, producing F2 male offspring (n=115). The other half of the F1 females were backcrossed to an *N. vitripennis* male to lay fertilized eggs, producing F2 female offspring (n=318).

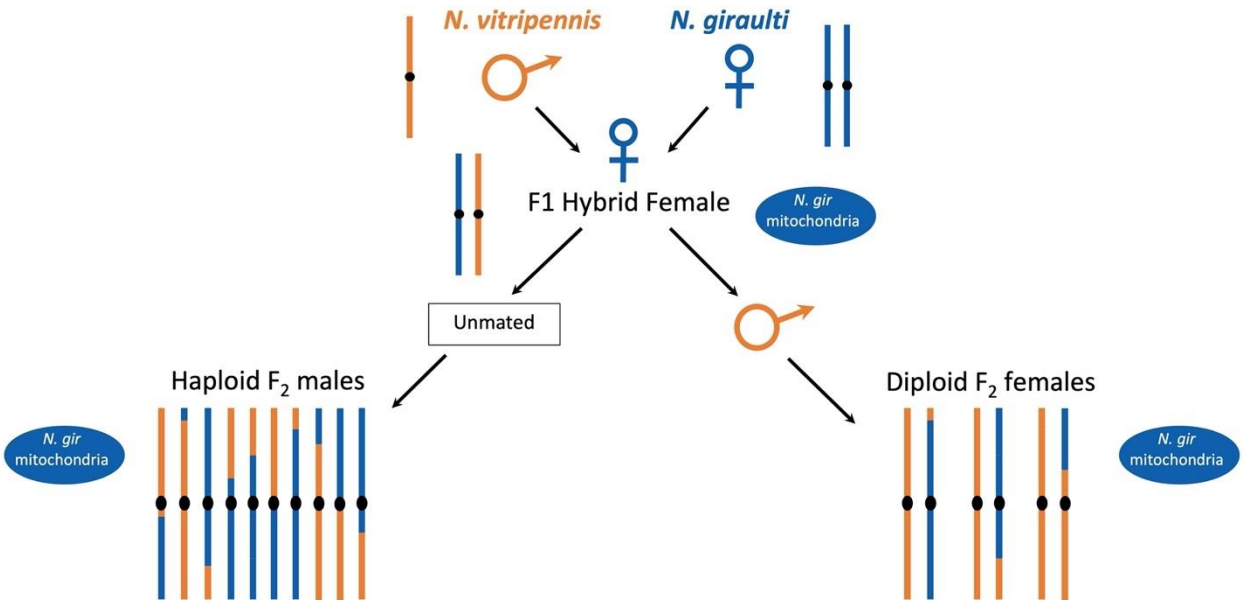


Figure 3. *N. vitripennis* males are crossed to *N. girault* females to produce F1 hybrid females. F1 hybrid females produced by this cross each have one *N. vitripennis* chromosome and one *N. girault* chromosome, with a *N. girault* mitochondrial background. F1 hybrid females are split into one of two groups: unmated (n=78) or backcrossed (n=66). Unmated F1 females lay unfertilized eggs that develop into haploid F2 males. Individual wasps are represented by a single (haploid males) or double (diploid females) chromosome image. F2 males are recombinant mosaics of both parents (n=115). Backcrossed F1 females lay fertilized eggs that develop into diploid F2 females (n=318). For each chromosomal pair in the F2 females, they have one completely *N. vitripennis* chromosome (inherited from their father) and one recombinant chromosome that is a combination of *N. vitripennis* and *N. girault* chromosomes (inherited from their mother). All hybrids in this study have *N. girault* mitochondrial backgrounds.

Mortality Experiments

To measure mortality, F1 female *Nasonia* (backcrossed and unmated) were housed individually in oviposition chambers (Figure 4). Individual housing for *Nasonia* females was designed to limit the females' access to the host to a single location. Fly pupae were inserted into the chamber headfirst in order to focus oviposition on one area, ensuring consistency in egg counts. Chambers were made by first inserting a cut 1000uL pipette tip into a second uncut pipette tip of the same volume. The first pipette was cut to allow a host to fit snugly in the bottom of the tip, allowing the female access to the host and providing a barrier to prevent the female from escaping. The bottom pipette tip was stuffed with enough cotton to seal the hole at the end of the pipette and provided the female a place to rest. In this experimental setup, a single host was inserted, head side down, into the first pipette and the top was sealed off with a cotton ball. Females were placed into the bottom of the second pipette, and the first pipette containing the host was placed into the second pipette, leaving about 3 cm of space for the female to spend her next 72 hours. In the first 24 hours, each female was given a single feeder host to facilitate egg production ensure she has enough energy to sting and parasitize her next two hosts. After 24 hours, the feeder host was removed and the female was then presented with her first experimental host to be collected and replaced with a second host at 48 hours, and the second experimental host is then collected at 72 hours (Figure 5).

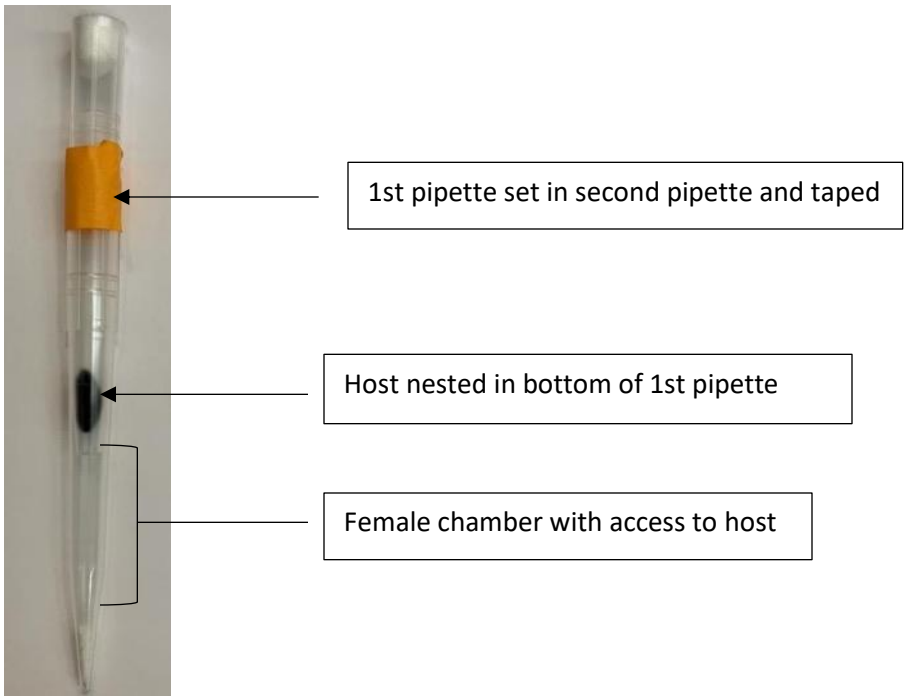


Figure 4. Structural design of individual female oviposition chambers. First pipette is trimmed at the tip to leave just enough room for female *Nasonia* to access a limited area of the host. Top of first pipette and bottom of second pipette is stuffed with cotton to prevent escape. First pipette is inserted into second pipette, creating a chamber for the female.

The experimental hosts for each female were assigned numbers and randomized into two groups of experimental data: egg number and adult number. Eggs were counted at 24 hours, the same day the host was collected. The head end of the host was perforated at 1/3 of the length of the host and the pupal casing was removed. Once the host is opened, eggs and large can't survive due to desiccation. This is a sacrificial technique that killed progeny that may have otherwise developed. The other half of the hosts were used for adult counts on day 18. Data noted on day 18 included sex and total number of eclosed, surviving adults. Egg production was assessed by comparing mean number of eggs across groups. Adult survival was assessed by comparing mean number of adults across groups. Mortality within each group was assessed by comparing mean egg production to the mean number of surviving adults within groups.

Differences in mortality rate were assessed by comparing the differences in mean egg and adult numbers across groups.

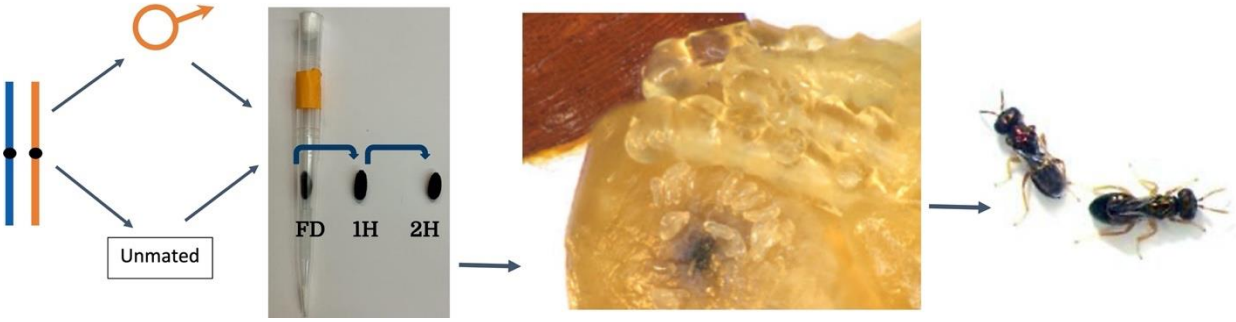


Figure 5. Experimental design outlining mortality experiment methods. F1 females were backcrossed to produce F2 females or left unmated to produce F2 males. Females were housed individually in a pipette chamber. Feeder hosts were given for the first 24 hours, the first experimental host at 48 hours, and the second experimental host at 72 hours. Hosts were randomly assigned to count eggs at 24 hours or count surviving adults at 18 days.

Statistical Methods

Mated females will still lay some unfertilized eggs to ensure male production, so corrections were made for F2 backcross offspring using sex ratios based on the parent counts. Predicted number of male eggs were subtracted from F2 backcross egg totals to compare female eggs more directly to female adult counts. To analyze mortality data, we used a regression model including group (F2 female, F2 male, *N. vitripennis* parent species, or *N girault*i parent species), stage (egg or adult), group by stage, and females nested within group as a random variable. We ran a regression and performed a Least Squared Means analysis to determine significance of effects within the model (Table 1). Differences in mean number of eggs, mean number of adults, egg to adult survival, and mortality rate were determined using Least Squared Means (LS Means) and Tukey's Honest Significant Difference test (Tukey HSD). All variables fit the assumptions of tests used in analyses. All statistical analyses were completed in JMP v. 16 (SAS Institute, Cary, USA).

CHAPTER 3

RESULTS

We found that there are significant effects coming from group (df= 3, F-ratio= 98.2375, p< 0.0001), life stage, (df= 1, F-ratio= 159.8732, p< 0.0001) and the interaction of group by stage (df= 3, F-ratio= 26.6134, p< 0.0001) (Table 1). There was no added effect of female nested within group as a random variable (df= 104, F-ratio= 0.8867, p= 0.7626).

Parent species *N. giraulti* and *N. vitripennis* parents laid a similar number of eggs. There was no significant difference between mean egg counts of parents (Egg G & Egg V, p= 0.9969). F1 hybrids laid fewer eggs than both parent species (Figure 6). *Nasonia giraulti* and *Nasonia vitripennis* mean egg numbers were significantly greater than F2 male mean egg numbers (Egg G & Egg F2M, p=0.0002; Egg V & Egg F2M, p=0.0099) and F2 female mean egg numbers (Egg G & Egg F2F, p=0.0005; Egg V & Egg F2F, p=0.0152). Hybrids laid a similar number of eggs. There was no significant difference between F2 male and F2 female mean egg counts (Egg F2F & Egg F2M, p=1.000).

We provide multiple pair wise comparisons between groups and stages using Tukey's HSD (Table 2). F2 female adult numbers are slightly higher but not significantly different from F2 male adult numbers (Adult F2F & Adult F2M, p= 0.1365) (Figure 7). Parent species experienced no significant mortality from egg to adult stage (Figure 8). Means of egg and adult numbers were similar in *N. giraulti* (Egg G & Adult G, p= 0.6551) and *N. vitripennis* (Egg V & Adult V, p= 0.8503). Hybrids suffered high mortality (Figure 8). There were significantly fewer adults than there were eggs in F2 females (Egg F2F & Adult F2F, p= 0.0001) and F2 males (Egg F2M & Adult F2M, p= 0.0001). F2 females and F2 males suffered similar mortality to one another, and less mortality than either parent species (Figure 8).

Table 1. Results are from regression and LS Means analysis including group, stage, group by stage, and female nested in group as a random variable. Significance level set at p=0.05, indicated in bold and by asterisk.

Source	SS	MS Num	DF	F Ratio	P-value
Group	11714.7	3904.91	3	98.2375	**<0.0001***
Stage	7026.42	7026.42	1	159.8732	**<0.0001***
Stage*Group	3772.67	1257.56	3	28.6134	**<0.0001***
Female[Group]&Random	4053.13	38.9724	104	0.8867	0.7626

Figure 6. Means of egg numbers taken from four groups, including *N. giraulti* parents, *N. vitripennis* parents, F2 females, and F2 males. Fewer hybrid eggs were laid than parent eggs. F2 female and F2 male means for egg number were similar to one another. Letters indicate significant differences between groups and stages from Tukey's HSD.

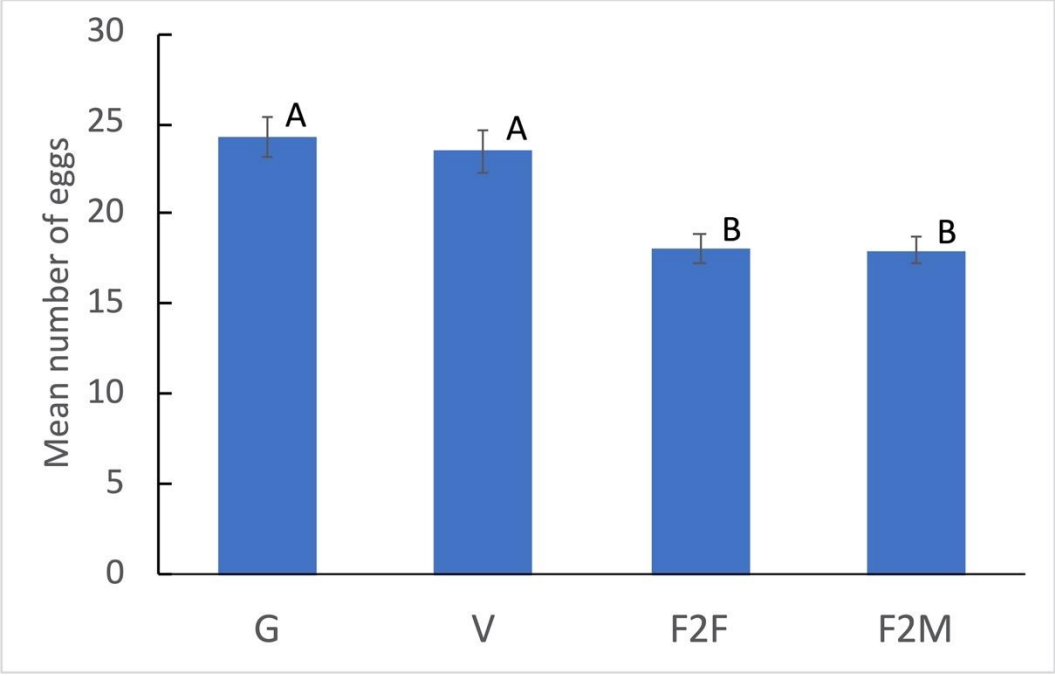


Figure 7. Means of adult numbers taken from four groups, including *N. giraulti* parents, *N. vitripennis* parents, F2 females, and F2 males. *N. giraulti* parents and *N. vitripennis* parents had similar means for adult numbers. F2 female and F2 male had similar means for adult numbers. Letters indicate significant differences between groups and stages from Tukey's HSD.

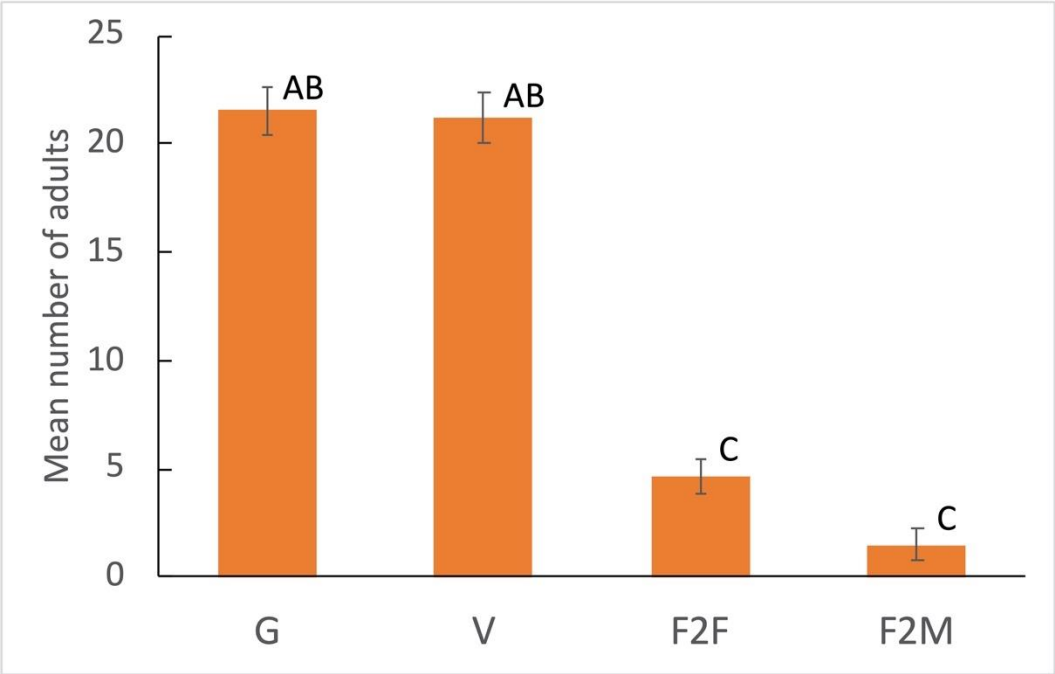
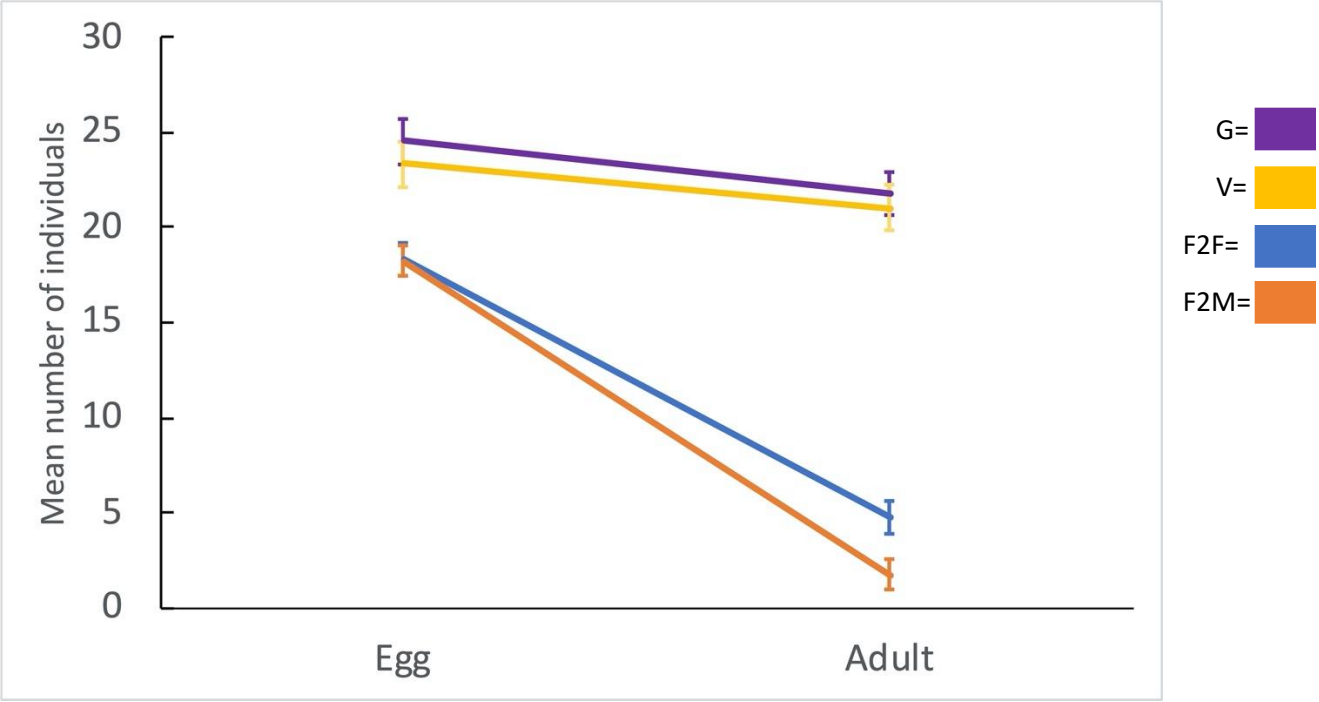


Table 2. Results of Least Squared Means from multiple pair-wise comparisons between different stage-group combinations using Tukey's HSD. V= *N. vitripennis*, G= *N. giraulti*, F2F= F2 female, and F2M= F2 male. Significance level set at p=0.05, indicated in bold and by asterisk.

Stage, Group	Stage, Group	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
Egg, V	Egg, G	1.17217	1.668349	-3.9193	6.26362	0.9969
Egg, V	Adult, V	2.34375	1.657369	-2.7142	7.40169	0.8503
Egg, G	Adult, G	2.77143	1.584748	-2.0649	7.60775	0.6551
Egg, G	Egg, F2M	6.29991	1.397308	2.0356	10.56420	**0.0002***
Egg, G	Egg, F2F	6.23203	1.432081	1.8616	10.60244	**0.0005***
Egg, V	Egg, F2M	5.12774	1.438248	0.7385	9.51697	**0.0099***
Egg, V	Egg, F2F	5.05985	1.472054	0.5675	9.55225	**0.0152***
Egg, F2F	Egg, F2M	0.06788	1.155865	-3.3596	3.59534	1.0000
Egg, F2F	Adult, F2F	13.49674	1.154044	9.9748	17.01864	**0.0001***
Egg, F2M	Adult, F2M	16.51282	1.061566	13.2731	19.75250	**0.0001***
Adult, F2F	Adult, F2M	3.08396	1.155865	-0.4435	6.61142	0.1365

Figure 8. Results of the Least Squares Means plot for egg and adult counts of parents and hybrids. Parent egg and adult count means are not significantly different. Hybrid adult count means are significantly lower than egg count means, indicating significant mortality. F2 female adult count means are slightly higher but not significantly different from F2 male adult means.



CHAPTER 4

DISCUSSION

I found that F1 hybrids laid significantly fewer eggs than both parent species (Figure 6). There were no significant differences in number of eggs laid within hybrids and within parents. Although F2 female adult means were slightly higher than F2 male adult means, they did not differ significantly (Figure 7). I found that F2 hybrids suffered greater mortality than both parent species (Figure 8). Egg to adult mortality was similar between F2 males and F2 females. I initially predicted that F2 backcross females would have intermediate mortality as compared to F1 females and F2 males. F1 females and F2 heterozygous backcrossed females each have one *N. vitripennis* allele, and one *N. giraulti* at the mortality locus as well as a *N. giraulti* mitochondrial background. I expected these individuals to be most similar to one another as they are both diploid and have one compatible and one incompatible allele interacting with their mitochondrial backgrounds. However, F2 backcrossed females survive much less than previously expected and suffer similar mortality to F2 males. I initially predicted that 45% of F2 backcross females survive, but instead I found that only 25.5% of F2 backcross females survive. The increased survival that I see in F1 females is likely due to them being heterozygous at every locus, whereas these F2 females are heterozygous at only a fraction of their loci.

Mortality in F2 females likely involves incompatible interactions at additional loci. There are two reasons to expect this. First, based on the principle of Mendelian inheritance of a gene with two alleles, if only a single allele at single locus influences mortality, it can only affect 50% of the individuals in the population resulting in a maximum of 50% mortality. The 74.5% mortality that we see in F2 hybrid females exceeds this 50% mortality and therefore indicates more than one incompatible locus. Second, previous research has shown that other loci can contribute to mortality in F2 hybrid males (Niehuis et al. 2008). Specifically for this direction of the backcross, there is a locus on chromosome 4 that can account for up to 32% of mortality in F2 hybrid males with *N. giraulti* mitochondrial background. Our findings

therefore indicate that there must be other interactions on the recombinant F2 backcrossed chromosomes contributing to mortality in hybrid females.

While multiple loci may explain the increased mortality that we observe in F2 hybrid females, the specific contributions of these two genotypes (heterozygous and homozygous) to mortality are still unknown. I suggest testing genotypes of F2 backcross female offspring in order to determine survival of each allele at the mortality locus on chromosome 5 (methods found in Appendix A). By comparing mortality rates of F2 female genotypes to those found in F1 females and F2 males, we can determine if these genotypes contribute to mortality differentially across hybrid types. Heterozygous genotype mortality can be compared to overall F1 mortality and homozygous genotype mortality can be compared to mortality of F2 males with the *N. vitripennis* allele at this locus.

Our data supports Breeuwer and Werren (1995) finding that egg yield doesn't seem to be related to females being mated or unmated. However, our results differ slightly from those of Breeuwer and Werren (1995) in that *N. vitripennis* parent species laid more eggs in their study, but I didn't see a difference in eggs laid in the parent species in my study. Previously, it was found that F1 hybrids laid eggs similar in number to *N. giraulti* but significantly fewer than *N. vitripennis* (Breeuwer and Werren 1995). F1 females produced fewer hybrid females than parent species produced nonhybrid females. In this previous study, egg counts were pooled between different females to obtain a mean of egg counts for each genotype of parent species or hybrid females. Egg production was not noted for every set of experiments under the assumption that females of the same genotype lay the same number of eggs whether mated or unmated. Our study presents unique data in that all of our egg and adult counts came from the same female. Although the above study was operating off of assumptions born out of their previous projects, there is value in a having a data set that doesn't rely on such assumptions. Additionally, their estimated survival of backcross F2 females is derived from a single datum and therefore cannot be tested statistically. While this doesn't undermine the validity of their findings, it does indicate the need of

measuring those important variables such as egg count per female, which is novel to our study and could account for their differences.

More recent findings continue to support that *Nasonia* have other noteworthy crosses in which hybrid breakdown results in mortality. F2 hybrid males produced by crosses of *N. vitripennis* males and *N. longicornis* females, as well as the reciprocal cross, suffer significant mortality as compared to parent species (Koevoets et al. 2012). F2 heterozygous males with a *N. longicornis* mitochondrial background experience slightly higher mortality than F2 heterozygous males with a *N. vitripennis* mitochondrial background. However, *Nasonia* is not the only system in which hybridization results in mortality. Early studies of *Drosophila* crosses resulted in the discovery that many hybrid combinations were lethal or resulted in the sterility of males and females (Sturtevant 1920). When *D. sechellia* males are crossed to *D. simulans* females, fertile females and sterile males are produced. Although the reciprocal cross can be done, it results in very few, weak hybrids that do not survive well (Lachaise et al. 1986). Additionally, Hadorn (1961) found that inviable female hybrids produced by *D. simulans* females and *D. melanogaster* males, died in the embryonic stage and in the reciprocal cross, inviable males died at the larval stage. Some species of fish also suffer mortality as a result of hybrid breakdown. F2 dwarf and normal whitefish backcross hybrids experience a rate of embryonic mortality that is 5.3 to 6.5 times higher than that of the parent species and F1 hybrids (Rogers and Bernatchez 2006).

While mortality is the most extreme way that hybrid breakdown can impact an organism, there are many other examples of how F2 hybrids can suffer. Hybrid breakdown can contribute to decreased viability, increases in development time, loss of fecundity, and metabolic deficits. For example, F2 hybrids of African haplochromine cichlids showed decreased viability as compared to parent species and F1 hybrids (Stelkens et al. 2015). F2 hybrids exhibited a 43% reduction in fitness as compared to parent species and a 21% reduction in fitness as compared to F1 hybrids. In copepods, F2 progeny produced from crosses of genetically differentiated populations of *Tigriopus californicus* showed increased developmental times as compared to F1 hybrids and parent species (Burton 1990). Edmands (1999)

studied outbreeding depression, otherwise known as hybrid breakdown, in crosses of intertidal copepods. For all three fitness components of hatching number, survivorship number, and metamorphosis number, F2 hybrids showed significant declines in fitness as genetic and geographic distance increased.

Although the effects of hybrid breakdown were evident in various model organisms, the possible mechanisms responsible for these deficits required further study. Studies across multiple organisms suggest evidence for cytonuclear incompatibility that disrupts regular mitochondrial functions, to which the degree of disruption positively correlates with an increase in divergence time (Rand et al. 2004). Metabolic studies were conducted within interspecific hybrids of *Drosophila* fruit flies (Sackton et al. 2003) as well as *Tigriopus californicus* copepods (Ellison & Burton 2006) to study the mechanics of the subunits making up the five complexes of the electron transport chain (ETC). These studies have been able to gauge these incompatibilities through measuring oxygen consumption of isolated mitochondria and running enzyme assays to test the efficacy of each complex in the ETC. Most recently, the insect genus *Nasonia* is emerging as an effective model to study hybrid mortality and mitochondrial physiology (Ellison & Burton, 2006; Ellison et al., 2008).

Interactions between divergent mitochondrial and nuclear genomes in hybrids can cause significant dysfunction and even prevent an organism from fully developing. One such pathway reliant on the close cooperation between these genomes is the OXPHOS (Oxidative Phosphorylation) pathway responsible for ATP production to fuel cellular processes. OXPHOS pathway contains 13 protein coding genes encoded by the mitochondrial genome that interact with approximately 70 other nuclear encoded genes to facilitate electron transport and the production of ATP (Rand et al. 2004). Four of five protein complexes in the OXPHOS pathway operate through direct interactions between nuclear and mitochondrial genomes, with one complex containing proteins encoded solely by the nuclear genome.

In the OXPHOS pathway in *Nasonia*, complex enzyme activity and ATP production was reduced in F2 hybrid males of *N. vitripennis* and *N. giraulti (*Ellison et al. 2008). While individuals with *N. giraulti* mitochondria are widely studied due to their high levels of mortality (Gibson et al. 2013) crosses

with *N. vitripennis* mitochondria are also a topic of interest (Neihuis 2008; Koevoets et al. 2012). Hybrids of both mitochondrial backgrounds have reduced Complex I activity relative to the parent strains, but do not differ significantly from each other. *N. giraulti* cytoplasm hybrids have lower overall ATP production and lower Complex III activity, but *N. vitripennis* cytoplasm hybrids have lower Complex IV activity (Ellison et al. 2008). Additionally, preliminary metabolic testing of mitochondrial oxygen consumption, (Gibson dissertation 2013; pg. 62) showed that F1 females with *N. giraulti* mitochondrial background had a reduced capacity to utilize oxygen in their mitochondria relative to the parent species, but the sample size was too small to statistically test it.

F2 backcross females could provide a better opportunity to study those individuals with incompatible alleles in greater numbers. If heterozygous and homozygous F2 females survive at a greater rate than F2 males with an *N. vitripennis* or *N, giraulti* allele, then F2 backcross females could be a viable option to study F2 progeny at the cellular level. While we don't know the allelic survival, if we see some differences between F2 male and F2 female survival, then we might be able to get more homozygous females than hemizygous males with the *N. vitripennis* allele at the mortality locus. Additionally, females have larger body size than males which would contribute more overall tissue to run complex efficacy tests (Whiting 1967). Physiological assays were previously run with groups of 40 F2 males per sample rather than individuals, which requires a minimum amount of tissue to be successful (Ellison et al. 2008). If F2 females have more tissue than F2 males, then assays could require fewer individuals per sample, allowing for a greater sample size with the same number of individuals. In future studies, we could measure the complex activity and ATP production of F2 backcross females to compare to F2 males (methods found in Appendix B).

This project explores the impacts of incompatible interactions between mitochondrial and nuclear DNA in hybrids of *Nasonia vitripennis* and *Nasonia giraulti.* Studying hybrids in *Nasonia* allow us a unique look into the process of speciation through observing the effects of incomplete reproductive isolation. In this study, I found that F1 hybrids laid significantly fewer eggs and there were no significant

differences in number of eggs laid within hybrids and within parents. F2 female adult means were slightly higher than F2 male adult means, but they did not differ significantly. F2 hybrids suffered greater mortality than both parent species and egg to adult mortality was similar between F2 males and F2 females. Our data supports the findings of Breeuwer and Werren (1995) that state that egg yield doesn't seem to be related to females being mated or unmated. However, our results differ slightly from those of Breeuwer and Werren (1995) in that *N. vitripennis* parent species laid more eggs in their study, likely due to the nature of our unique data in that all of our egg and adult counts came from the same female. From the initial prediction that 45% of F2 backcross females would survive, I found that only 25.5% of these F2 backcross females survive. Incompatible interactions on multiple loci could explain the increased mortality that we observe in F2 hybrid females and we can determine survival of each allele at the mortality locus on chromosome 5 by testing genotypes of F2 backcross female offspring, By comparing mortality rates of F2 female genotypes to those found in F1 females and F2 males, we can determine if these genotypes contribute to mortality differentially across hybrid types. Additionally, if heterozygous and homozygous F2 females survive at a greater rate than F2 males with an *N. vitripennis* or *N, giraulti* allele, then F2 backcross females could provide a better opportunity to study those individuals with incompatible alleles at the cellular level. Looking to future studies, we could measure the complex activity and ATP production of F2 backcross females and learn how they compare to F2 males to further shed light onto how incompatible interactions effect hybrids on a cellular level.

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APPENDIX A

GENOTYPING PROTOCOL

This protocol contains the methods to genotype *Nasonia vitripennis* and *Nasonia giraulti* in the Gibson lab at Georgia Southern University. There are three distinct steps: DNA extraction, PCR reactions, and gel imaging. DNA extractions can be done on *Nasonia* that have been previously frozen away. Freeze *Nasonia* as soon as possible after collection to avoid DNA degradation.

DNA Extraction

Extracted DNA from adult wasps and wasp eggs by using a Chelex protocol (Niehuis et al 2007). Individually homogenize wasps or eggs in a 1.5-ml centrifuge tube with plastic pestles fit to the tubes in 100 microliters of 5% Chelex suspension in TE buffer, pH 8.0. Add 1 microliter of proteinase K (5 mg/ml) to the centrifuge tube and incubate samples for 1 hour at 57 degrees Celsius and then for 5 minutes at 95 degrees Celsius. Lastly, centrifuge samples for 10 minutes at max speed and transferred the supernatant was into a sterile centrifuge tube. Quantity of DNA will then measured on a Biotek Synergy H1 Plate Reader.

Nasonia PCR Reaction

Species specific, length polymorphic identifying microsatellite markers were selected and will be amplified using polymerase chain reaction (PCR). The marker with the best band resolution and imaging potential was selected from Gibson et al. 2013. The marker to be used is Scaf14_13553, located on chromosome 5, associated with hybrid mortality. The *N.vitripennis* allele appears at 193 BP and the *N.giraulti* allele at 219 BP. This marker was selected because bands differed by 26 nucleotides and were easily discernable on standard agarose gel.

Gel Imaging

Run gel electrophoresis on a 3% Agarose gel containing SYBR Safe DNA Stain (10,000X concentrate in DMSO from Invitrogen). Run gels at 100V for 50 minutes and load samples with 100 BP ladder, an F1 hybrid female, and parent controls. Carefully remove gels from the gel rig and transfer to a Blue Light Base to be imaged with a E-Gel imager (Life Technologies). Be sure to take pictures of gel images in the program, annotate the pictures with order in which the gel was loaded, and save them on the lab computer for future reference.

APPENDIX B

OXPHOS PROTOCOL

This protocol contains the methods to isolate mitochondria and determine complex efficacy of each part of the electron transport chain (ETC), as well as ATP synthase, in the Gibson lab at Georgia Southern University. The first objective of this protocol is to isolate F2 female mitochondria to be used in physiological testing to compare components of the oxidative phosphorylation (OXPHOS) pathway. Once mitochondria have been isolated, you can use them to determine the productivity of each enzyme complex or to measure ATP production.

Mitochondrial fraction for electron transport chain (ETC) complex assays

Mitochondrial fraction procedure from Ellison and Burton (2008) will be used to prepare samples for physiological analyses in this set of experiments. F1 hybrid females will be reserved for physiological analysis and their bodies will be placed in 1mL of mitochondrial isolation buffer (comprised of 10 mM MOPS, pH 7.5, 55 mM KCl, and 500 microM EGTA) and kept on ice until mitochondrial preparations are started. Samples will then be incubated for 3 min in 1-mL of isolation buffer containing 0.25 mg mL^{-1} trypsin. The solution will be aspirated and replaced with 800 microliters of isolation buffer and 0.25 mg mL^{-1} of trypsin and then incubated on ice for 10 minutes. Upon conclusion of the incubation period, 200 microliters of 50 mg mL^{-1} albumin solution will be added. The solution will be aspirated and 800 microliters of isolation buffer will be added before it is homogenized with a plastic pestle in a microcentrifuge tube. Tubes will be centrifuged for 5 minutes at 600 X g at 4 degrees C, pelleting any cuticle or other cellular debris and the supernatant will be transferred to a fresh microcentrifuge tube. The supernatant will then be centrifuged at 11,000 X g for 10 min at 4 degrees C, resulting in a mitochondrial fraction pellet. The supernatant will be discarded and the pelleted mitochondria resuspended in 60 microliters of either isolation buffer (used to prepare ATP production assays) or storage buffer (for

measuring complex activity, comprised of 5mM HEPES with a pH of 7.5, 125 mM sucrose, 0.5 mM ATP, 40 microM ADP, 2.5 mM sodium succinate, 1 mM K2HPO4, and 0.5 mM DTT). Mitochondrial membrane integrity will be checked using a JC-1 assay (after Salvioli et al 1997) before running assays for ATP production or storage at -20 degrees C for later evaluation of specific electron transport complex activity. Two 5-microliter aliquot of each mitochondrial suspension will be removed and frozen at -20 degrees C for protein quantification using a NanoOrange Protein Quantification kit. Fluorescence measurements will be made in 96-well plate format on a Biotek Synergy H1 Plate Reader.

Measuring Enzyme Complex Activity

Enzyme activities will be measured according to Ellison & Burton (2008) and modified from Trounce et al. (1996) for the complexes I, II, I + III, and II + III and Rawson & Burton (2002) for complex IV. The mitochondrial preparations will be divided into three 15 microliters aliquots for each assay. All other protocol follows Ellison & Burton (2006). The previously frozen, mitochondria that was suspended in buffer will undergo a freeze-thaw process to break open mitochondrial membranes so ETS enzyme activities can be assayed (Ellison & Burton 2008). A plate reader will then be used to take spectrophotometric measurements to determine complex efficacy. Mitochondrial preparations will be divided into three 15-lL aliquots for each assay meaning that each assay will be performed in triplicate for a single individual (i.e. three measurements of enzyme complex I activity were taken for one individual and three measurements of enzyme complex II activity for another individual).

Complex I

Activity will be measured in a buffer containing 250 mM sucrose, 1.0 mM EDTA, 50 mM Tris-HCl (pH 7.4),1.0 M decylubiquinone, 2.0 mM KCN and the mitochondrial protein of a single individual. The reaction will be initiated by the addition of 50 M NADH and monitored for 1 min at 272 nm minus 247 nm (assuming an extinction coefficient of 8 mM1 cm1 for decylubiquinone*).*

Complex II.

In preparation for measuring complex II activity, a solution of 50 mM K2HPO4, 20 mM succinate, and mitochondrial protein will be incubated for 10 min at room temperature. After incubation, 2 g/ml antimycin A, 2 g/ml rotenone, 2 mM KCN, and 2 M DCPIP will be added to the well and a blank reading will be recorded. The reaction will then be initiated by the addition of 50 M decylubiquinone and monitored for 3 min at 600 nm minus 750 nm (assuming an extinction coefficient of 19.1 mM1 cm1 for DCPIP).

Complex IV.

Activity will be measured in a buffer containing 10 mM K2HPO4, pH 7.4, and 20 M reduced CYC. The reaction will be initiated by the addition of mitochondrial protein and monitored for 3 min at 550 nm minus 540 nm (assuming an extinction coefficient of 19.0 mM1 cm1 for reduced horse heart CYC). CYC for the complex IV assay will be reduced as follows: 100 mg/ml horse heart CYC (USB, Cleveland, OH) in 10 mM K2HPO4, pH 7.4 will be mixed with an equal volume of 0.1 M L-ascorbate. Ascorbate will then be removed using a PD-10 desalting column (Amersham Biosciences, Uppsala, Sweden) and the final product of reduced CYC will be collected and frozen at 80C until use. Concentration will be determined using an extinction coefficient of horse CYC of 27.8 mM1 cm1 at 550 nM (Rawson and Burton 2002).

Complex III.

The final two assays will be measured in comparison to one another to determine the degree of efficacy of **c**omplex III. The activity of complexes I and III together will be measured in a buffer containing 50 mM K2HPO4, pH 7.4, 80 M oxidized CYC (USB), 0.1 mM NADH, and 2 mM KCN. The reaction will be initiated by the addition of mitochondrial protein and monitored for 3 min at 550 nm minus 540 nm (assuming an extinction coefficient of 19.0 mM1 cm1 for horse heart CYC). Then we will measure

Complexes II and III **t**ogether in a buffer containing 40 mM K2HPO4, pH 7.4, 0.5 mM EDTA, 20 mM succinate, 2 mM KCN, and mitochondrial protein. The reaction will be initiated by the addition of 30 M CYC and monitored for 3 min at 550 nm minus 540 nm (assuming an extinction coefficient of 19.0 mM1 cm1 for horse heart CYC).

Extracting and storing whole mitochondria for ATP synthase assay.

In order to measure the efficacy of ATP synthase, mitochondria will be isolated from individual hybrid females and processed the same day it is extracted. Mitochondrial ATP production rate for mitochondrial preparations will be determined in an end-point assay following the protocol of Ellison & Burton (2006). Samples used to measure ATP production for ATP synthase with a dounce homogenizer to ensure mitochondria remain intact. To check for proper isolation, we will be measuring mitochondrial membrane integrity using a JC-1 assay. Individual samples that were suspended in storage buffer and frozen at -80 degrees C will be divided into two 20 microliter aliquots and five microliters of either substrate solution (1 mM ADP, 9 mM pyruvate, 4 mM malate in mitochondrial isolation buffer) or mitochondrial isolation buffer were added. Samples containing substrate solution will hereon be referred to as (+) and samples to which plain mitochondrial isolation buffer will be added referred to as (-).

Measuring ATP Production.

Samples will be incubated at 25 C for 5 minutes before performing an assay to measure ATP content using CellTiter-Glo ATP Quantification Kit (Promega, Madison, WI, USA). 20 samples of (+) and (-) will be assayed at a time in half-area 96-well plates. Each set of 20 will be independently paired with a series of ATP standards for quantification. ATP production rate will be determined by correcting the wells for background luminescence and subtracting the reading of each (-) sample from its corresponding (+) sample. Samples will be normalized for protein content.