Mortality in Interspecific Hybrids of *Nasonia vitripennis* and *Nasonia girault* (Hymenoptera: Pteromalidae)[1]

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Abstract Barriers to gene flow can result in populations evolving into separate species, and understanding how these barriers evolve is a key step in understanding the process of speciation. This study investigates a major barrier to gene flow, mortality during development, of two parasitoid wasp species, *Nasonia vitripennis* Walker and *N. girault* Darling. Previous work has demonstrated high mortality in haploid hybrid males of these species and has shown potential rescue from this mortality in diploid hybrid females through backcrossing. However, this previous work did not directly measure egg clutch sizes in hybrids and also did not account for male eggs in these clutches. Here, we measure female-only egg and adult clutch sizes of both parent species and F_2 hybrids, as well as F_2 male egg and adult clutch sizes, in order to determine the impact of backcrossing on mortality in these hybrids. We find significant egg-to-adult mortality in diploid F_2 hybrid females with a *N. girault* cytotype when backcrossed to *N. vitripennis* males; yet, these hybrids also experience less mortality than haploid F_2 males. These results confirm the mortality and rescue in these F_2 hybrid females and indicate that there is likely a combination of nuclear-cytoplasmic and nuclear-nuclear incompatibilities in these hybrids that lead to this mortality. This work provides a foundation for future studies to investigate the genetic basis of this mortality.

Key Words hybrid breakdown, *Nasonia*, speciation, postzygotic isolation

A major contributing factor to the process of speciation is the evolution of barriers to gene flow between populations. Intrinsic postzygotic isolation represents such a barrier and can potentially play an important role early in the speciation process (Coughlan and Matute 2020). One mechanism of this isolation is incompatible interactions between pairs of genes that lead to decreased fitness in hybrids. The Bateson-Dobzhansky-Muller (BDM) model illustrates how interacting genes can evolve in two diverging lineages while coevolving to maintain proper interactions, yet they can result in incompatible interactions in hybrids and, therefore, act as barriers to gene flow (Bateson 1909, Dobzhansky 1937, Muller 1942; see also Johnson 2010, Presgraves 2010). Interacting pairs of genes may be more likely to be incompatible in hybrids if one of the pair accumulates mutations more quickly than the other, requiring its partner to adapt in order to overcome deleterious mutations (Rand et al. 2004). This relationship is hypothesized to occur in nuclear-mitochondrial interactions due to the typically higher evolutionary rate of mitochondrial-encoded genes

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while maintaining very tight interactions between the proteins they encode (Blier et al. 2001, Hatefi 1985, Rand et al. 2004). For example, *Nasonia* Ashmead (Hymenoptera: Pteromalidae) parasitoid wasps have been shown to have mitochondrial synonymous nucleotide substitution rates that are over 30 times higher than their nuclear substitution rates (Oliveira et al. 2008), and hybridization between *Nasonia* species results in varying levels of hybrid mortality, some of which is associated with mitochondrial dysfunction (Beukeboom et al. 2015, Breeuwer and Werren 1995, Brucker and Bordenstein 2013, Gadau et al. 1999, Gibson et al. 2013, Koevoets et al. 2012, Niehuis et al. 2008, Saulsberry et al. 2017).

There are four species of *Nasonia* currently described: *N. vitripennis* Walker, *N. giraulti* Darling, *N. longicornis* Darling, and *N. oneida* Raychoudhury. *Nasonia vitripennis* is the most distantly related species, having diverged from its common ancestor with the other species approximately 1 million years ago (Werren et al. 2010). These species are completely reproductively isolated in nature due to incompatible *Wolbachia* Hertig (Rickettsiales: Ehrlichiaceae) infections, but they can successfully produce hybrids once they are cured of these infections (Breeuwer and Werren 1990). Crosses between cured *N. vitripennis* and *N. giraulti* result in F_1 hybrid female offspring that have generally comparable viability to the parental species, though hybrids with *N. giraulti* mothers had fewer offspring than pure *N. vitripennis* females (Breeuwer and Werren 1995). Note that, due to haplodiploidy, males develop from unfertilized eggs and, therefore, the first hybrid males that are produced are the result of these F_1 hybrid's unfertilized eggs developing into males, referred to as F_2 males (Breeuwer and Werren 1995). Unlike their F_1 mothers, these F_2 males suffer considerable mortality during development, ranging from approximately 50 to 80% depending on the direction of the cross, with the highest mortality in crosses with *N. giraulti* females (Breeuwer and Werren 1995). When F_1 females were backcrossed to either parent species, they produced significantly more adult F_2 offspring than unmated F_1 females, and females mated to males matching their cytotype produced more total adult offspring (males and females combined) than the reciprocal mating. However, they did not record egg production for this experiment so they could only estimate mortality by assuming that egg production in this experiment was comparable with other experiments. Based on this assumption, they estimated that survival of F_2 hybrids with *N. vitripennis* cytotype increased from 47% to ∼48%/∼60%, and those with *N. giraulti* cytotype from 18% to ∼28%/∼54%, depending on the species of male used in the backcross. These asymmetries in survival are a common feature of hybrid incompatibilities (Turelli and Moyle 2007). In both cases, the higher survival range was in the offspring of F_1 females mated to males matching their cytotype.

These previous results in F_2 males and F_2 females indicate a key role of cytoplasmic factors influencing survival in *Nasonia* hybrids. It should be noted that Brucker and Bordenstein (2013), using microbe-free rearing techniques, showed that the gut microbiome plays a critical role in this hybrid mortality. They found that many of the nuclear loci showing biased recovery in surviving hybrids with intact microbiomes were no longer biased in the microbe-free conditions. However, one locus that previously was shown to lose approximately 98% of the *N. vitripennis* alleles when in hybrids with the *N. giraulti* cytotype still retained a biased recovery in the microbe-free conditions (Gibson et al. 2013). Given the persistence of the

mortality in these *N. giraulti*-cytotype hybrids across experiments and the lack of explicitly measured egg-to-adult survival in F_2 hybrid females, we aimed to measure and compare egg and adult clutch sizes of *N. vitripennis*, *N. giraulti*, and two *N. giraulti*-cytotype hybrids; F_2 males and the F_2 hybrid females that are most likely to suffer mortality, those resulting from the backcross of F_1 hybrid females to *N. vitripennis* males.

Materials and Methods

Species studied and stock maintenance. All crosses were performed with strains of two *Nasonia* species, *N. vitripennis*, strain AsymCX, and *N. giraulti*, strain RV2X(U) (Breeuwer and Werren 1995). Both strains were from isofemale lines cured of *Wolbachia* infection. All *Nasonia* were cultured on flesh fly pupae (*Sarcophaga bullata* Parker [Diptera: Sarcophagidae]) in an incubator set at 25°C with constant light. Stocks were housed in 25-mm diameter Drosophila vials (VWR International, Radnor, PA), provided 20 hosts per hosting, and were rehosted every 15 to 16 d.

Hybrid crosses. All cross experiments were performed by collecting virgin pupae at day 12 of development to accurately determine sex. Pupae were housed individually in glass vials plugged with cotton until emergence. Single pairs of males and females were combined in glass vials. A single direction of the cross was performed; *N. vitripennis* males were mated to *N. giraulti* females. The pairs were given 48 h to mate before providing the female with a feeder host for 24 h to facilitate egg production. The feeder host was discarded and each female was then provided two successive hosts, 24 h apart, to facilitate mass F_1 hybrid production. Individual virgin F_1 hybrid female pupae were housed in glass vials and upon eclosion were either backcrossed with a *N. vitripennis* male to produce F_2 hybrid females ($n = 66$) or were left as virgins to produce F_2 hybrid males ($n = 78$). Virgin females of each parental species were crossed with their own species' males as a control (*N. giraulti* $n = 35$, *N. vitripennis* $n = 32$).

Mortality inference. F_1 female *Nasonia* (backcrossed and virgin) as well as mated females of the two parent species were housed individually in oviposition chambers modeled after those in Breeuwer and Werren (1995). These restrict oviposition to the anterior end of the host, facilitating egg clutch measurements. Each female was given a single feeder host for 24 h to facilitate egg production. Due to *Nasonia* development occurring within the host puparium, opening the host puparia to count eggs can result in increased artificial mortality and, therefore, egg-to-adult mortality cannot be directly measured (Breeuwer and Werren 1995). To circumvent this problem, *Nasonia* egg-to-adult mortality was inferred by providing each female with two experimental hosts in succession, for 24 h each and one was randomly designated as the egg host and one as the adult host. Egg hosts were opened immediately after removal from the chamber and the egg clutch size was counted, while adult hosts were maintained at 25°C for 18 d, at which point they were opened and all eclosed adults were counted and their sex was noted. Both hosts were discarded if either one was inviable for offspring counts (e.g., hosts died or host damage prevented egg counting). Finally, mated females will also produce a small proportion of unfertilized eggs (resulting in male offspring), so to accurately assess female offspring survival we used the sex ratios of the offspring in the pure species to calculate the expected number of male eggs in each

female's clutch. There was no difference in the mean number of adult males from *N. vitripennis* and *N. giraulti*; therefore, we used the combined mean proportion of males of both species (*N. vitripennis* male proportion = 0.082, *N. giraulti* male proportion = 0.086; *t* test: df = 62.72, $t = -0.68$, $P = 0.50$; mean male proportion = 0.084). We subtracted this expected number of male eggs from the total eggs from each mated female, providing a more accurate measure of female egg-to-adult mortality.

Statistical methods. A Kruskal-Wallis test was used to determine if there are any differences in the mean clutch size of eggs/adults in our experiment. A Games-Howell *post hoc* test was used to determine differences in mean clutch size of eggs and adults in each cross due to unequal variance and sample sizes (Games and Howell 1976, Sauder and DeMars 2019). All pairwise comparisons of cross type and developmental stage (egg/adult) were tested. Statistical analyses were completed in JMP v.16, additionally utilizing the Games-Howell Test V1.3 add-in (SAS Institute, Cary, NC). Within the JMP Games-Howell add-in, the iterations to sweep for the connecting letter report was set to 100 (Piepho 2004).

Results

The total number of F_2 hybrid male eggs and surviving adults were 1,403 and 115, respectively. The total number of female eggs (corrected value) and surviving adults were, respectively, 1,198.6 and 303 for F_2 hybrid females, 779.9 and 713 for *N. giraulti*, and 689.1 and 649 for *N. vitripennis*. The Kruskal-Wallis test indicated that there are significant differences in the mean clutch sizes of eggs/adults in our experiment ($\chi^2 = 256.95$, df = 7, $P < 0.001$).

The Games Howell *post hoc* test indicated that the clutch sizes of eggs did not differ for any pairs except between *N. giraulti* and the F_2 females (Fig. 1; $P = 0.02$, 95% confidence interval [CI] = 0.07, 8.17). The only significant differences between egg and adult clutch size, indicating mortality, were in the F_2 hybrid females (Fig. 1; $P < 0.0001$, 95% CI = 10.54, 16.60) and in the F_2 hybrid males (Fig. 1; $P < 0.0001$, 95% CI = 9.43, 17.36). The two hybrids laid the same number of eggs (Fig. 1; $P > 0.05$, 95% CI = −4.46, 4.81); however, there were significantly fewer surviving F_2 male adults than F_2 female adults (Fig. 1; $P < 0.0001$, 95% CI = 1.64, 4.59). We ran this same test using the uncorrected egg clutch sizes for the mated females; the only difference in the pattern was that the egg clutches of F_2 hybrid males (no egg correction applied) were significantly smaller than the two parent species (F_2 male mean = 17.99; *N. vitripennis*: mean = 23.5, $P = 0.001$, 95% CI = 0.73, 10.29; *N. giraulti*: mean = 24.31, $P = 0.005$, 95% CI = 1.38, 11.27, data not shown).

Discussion

We found that egg clutch sizes were largely consistent across our crosses, with the exception that *N. giraulti* clutches were slightly, though significantly, larger than the F_2 female clutches (Fig. 1). This is in contrast to previous studies in which *N. vitripennis* females had egg clutches that were significantly larger than the clutches of the other *Nasonia* parent species and their hybrids (Breeuwer and

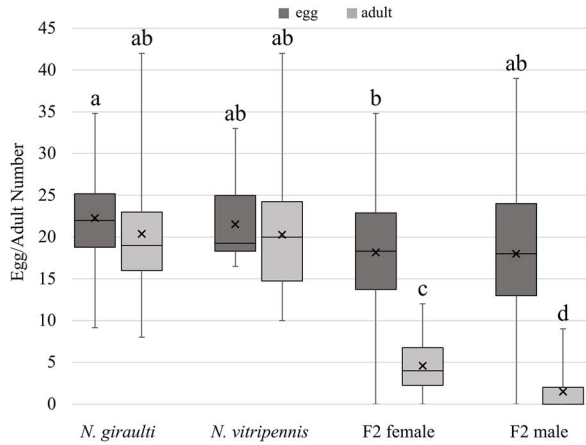

Fig. 1. Number of eggs/adults of each cross type. Boxes enclose the 25th to 75th percentiles and whiskers designate minimum and maximum values. The center horizontal line marks the median value and the X marks the mean value. Different letters over the boxes denote significant differences ($P < 0.05$). Sample sizes: *Nasonia girault* = 35, *N. vitripennis* = 32, F_2 female = 66, F_2 male = 78.

Werren 1995, Koevoets et al. 2012). It is unclear why this difference is observed, but it could be due to changes in the strain between study years or host quality differences (Rivers and Denlinger 1995). The increased egg clutch size of *N. girault* relative to the F_2 females that we detected was relatively small in effect and the difference in significance between our study and Breeuwer and Werren (1995) may be due to the much larger sample sizes in our study (*N. girault* N: 35 versus 13, F_2 females N: 66 versus 11; Fig. 1). Our comparison of egg clutch size between the mated and unmated F_2 hybrid females indicates that mating does not alter egg laying, whether considering all eggs (data not shown) or only hybrid eggs (Fig. 1). The F_2 female mortality that we inferred (~75%) confirms the 70–75% mortality estimated by Breeuwer and Werren (1995); however, the F_2 male mortality that we inferred was higher (~92% versus ~82%; Fig. 1). Importantly, while these F_2 females still suffer considerable and significant mortality during development, our results also indicate a significant decrease in mortality of F_2 females versus F_2 males (Fig. 1).

Previous work has identified a locus at the end of Chromosome V that results in approximately 98% mortality in males with an *N. vitripennis* allele in combination with an *N. girault* cytoplasm (Gibson et al. 2013). This locus indicates a very strong nuclear-cytoplasmic incompatibility, but other loci have also been implicated in these cytoplasmic incompatibilities in this cross (Ellison et al. 2008, Gadau et al. 1999, Niehuis et al. 2008). However, these other loci were not found in a study using microbe-free *Nasonia* (Brucker and Bordenstein 2013). While this finding does not rule out cytoplasmic incompatibilities, it implicates an additional environmental interaction or epistatic interactions with genes associated with the microbiome.

Despite the extreme mortality attributed to this locus, overall mortality in these hybrids is very likely due to more than one locus. There are two reasons to suspect

this. First, a single-locus incompatibility is not consistent with our results on F_2 male mortality. Haploid males are hemizygous at all loci, meaning they would either have the incompatible allele or not, so this would only account for approximately 49% total mortality in these males and cannot account for the additional approximately 43% mortality that we observed. Second, previous work has identified multiple incompatibility loci in these F_2 hybrid males (Niehuis et al. 2008). It is unknown whether these loci are biased in F_2 female or later backcross generations as they are in F_2 males, but it seems likely that multiple loci could be impacting these F_2 females as well. If this is the case, it is possible that the incompatible allele is recessive (accounting for approximately 98% mortality of homozygous females, approximately 49% of the total offspring) and the remaining mortality is due to incompatible interactions at these other loci. Regardless of the number of loci, our results exclude the incompatible allele being dominant, as that would predict approximately 98% overall morality in the F_2 females. Interestingly, the reduced mortality in these diploid females relative to the haploid males is consistent with nuclear-nuclear incompatibilities occurring in the males that are alleviated in the females by increasing the proportion of the nuclear genome coming from one parent species (F_2 males have 50% *N. vitripennis* alleles while these F_2 females have 75% *N. vitripennis* alleles). It is possible that this reduced mortality is due to either sex-specific effects or to ploidy differences; however, previous work in a cross with *N. longicornis* indicated that neither of these factors played a major role in altering mortality (Beukeboom et al. 2015).

Overall, we have confirmed the level of F_2 female mortality in these hybrids with *N. giraulti* cytoplasm previously estimated by Breeuwer and Werren (1995) using an experimental design that measures a large sample of individual female egg and adult clutch sizes while controlling for male offspring. Additionally, we have noted a novel difference in parental species egg production, though further work will be required to determine the cause. This work provides a strong foundation to begin investigating allelic bias in these F_2 hybrid females, which will greatly hone our understanding of the genetic mechanisms and loci underlying this mortality as well the factors contributing to its reduction.

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