HYBRID BREAKDOWN BETWEEN TWO HAPLODIPLOID SPECIES: THE ROLE OF NUCLEAR AND CYTOPLASMIC GENES

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Abstract.—A central question in evolutionary biology concerns the population and genetic processes by which new species arise. Here, the genetic basis of hybrid breakdown between two haplodiploid species, Nasonia vitripennis and N. giraulti is investigated. Hybridization between the two species is normally prevented by microorganisms that cause bidirectional incompatibility. However, after elimination of microorganisms, F1 hybrids females are readily produced (due to haplodiploidy, males develop from unfertilized eggs and are therefore not hybrids). F1 hybrid females are viable and fecund, but recombinant (haploid) F2 male offspring suffer from severe hybrid breakdown (larval and pupal mortality). This is typically interpreted as evidence for the existence of different coadapted gene complexes in the two species, which are broken up by recombination. F2 recombinant eggs were rescued by fertilization with the complete chromosome complement from either species, supporting the view that hybrid lethality genes tend to be recessive. Negative epistatic interactions occur between nuclear genes of the two species, and between cytoplasmically inherited factors (cytoplasmic genes) of giraulti and nuclear genes of vitripennis. Interactions between nuclear genes and cytoplasmic genes are asymmetric. Experiments clearly demonstrate that the latter incompatibility is not due to maternal-effect genes, but to cytoplasmically inherited elements. Nuclear-mitochondrial interactions are possibly involved.

Key words.—Cytoplasmic inheritance, haplodiploidy, hybrid breakdown, speciation, Nasonia, nuclear-cytoplasmic incompatibility.

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The problem of speciation—how new species arise—is a central question in evolutionary biology. A key element in speciation is the development of barriers against gene flow between populations. Most theoretical and empirical studies of speciation have focused on the role of conventional nuclear genes. The classical view of the evolution of postzygotic reproductive isolation under the biological species concept (see Otte and Endler 1987) argues that reproductive isolation is achieved by fixation of a set of interacting genes or incompatibility genes in different populations (Dobzhansky 1937; Muller 1942). This view is supported by a number of empirical studies in plants (e.g., Gerstel 1954; Oka 1957; Christie and MacNair 1984, 1987; Hollingshead 1930). Drosophila (e.g., Pontecorvo 1943; Watanabe 1979; Hutter and Ashburner 1987; Hutter et al. 1990; Perez et al. 1993; Zeng and Singh 1993) and nematodes (Baird et al. 1992). Apparently, such genes are dysfunctional in hybrid development, but are perfectly functional in individuals within each species (Nei et al. 1983). It is unclear whether reproductive isolation initially evolves from the accumulation of many changes of small effect (i.e., whether it is polygenic) or by a few genes of major effect (Mayr 1973; Dobzhansky 1970; Zouros 1981; Kilias and Alahiotis 1982; Templeton 1982; Wu and Beckenbach 1983; Coyne and Charlesworth 1986; Hutter et al. 1990; Coyne 1992; Naveira 1992). Coyne (1992) pointed out that genetic changes causing negative epistatic effects in hybrids may continue to accumulate after speciation is complete, and do not necessarily represent the genes that are or were involved in speciation itself. Therefore, the process should be studied in species that have only recently diverged.

In recent years, it has also been hypothesized that reproductive isolation may involve a variety of novel genetic processes, such as effects of tandemly repetitive DNA, transposon release, meiotic drive release, cytoplasmic incompatibility bacteria, and sex-ratio distorters (Ehrman 1983; Engels and Preston 1979; Powell 1982; Kidwell 1983; Rose and Doolittle 1983; Thompson 1987; Clark and Lyckegaard 1988; Jablonka and Lamb 1989; Frank 1991; Hurst and Pomiankowski 1991; Beeman et al. 1992).

Possible effects of cytoplasmically inherited factors on speciation have not been widely explored. It is important to distinguish, in this regard, between maternal-effect genes and true cytoplasmically inherited elements. Maternal effects are in cases in which the (nuclear) genotype of the mother affects the phenotype of the offspring. Many maternal-effect genes are expressed early in embryogenesis, and represent maternal products placed in the egg. An example of maternal-effect genes possibly involved in speciation is a new class of dominant, maternal-effect embryonic lethal factors described in the flour beetle Tribolium (Beeman et al. 1992) and in mammals (Hurst 1993b). These so-called M factors arrest development of embryos that do not inherit a copy of the factor. M factors differ between geographic populations and may cause genetic isolation (Beeman et al. 1992).

In contrast to maternal-effect genes, cytoplasmically inherited elements are actually inherited from one generation to the next through the maternal (egg) cytoplasm. Cytoplasmic elements are diverse and include heritable microbes, viruses and cytoplasmic organelles (e.g., mitochondria and chloroplasts). Heritable microbes constitute a large group of nonnuclear factors that can have dramatic effects on their hosts. For example, cytoplasmically inherited microorganisms can impair hybridization between insect species or populations directly by causing cytoplasmic incompatibility.
Cytoplasmic incompatibility bacteria are widespread in insects and could play an important role in rapid speciation (Breeuwer and Werren 1990; Cooney 1992; O’Neill et al. 1992). In other systems, nuclear-cytoplasmic interactions have been reported to cause hybrid inferiority, for example, the spider mite *Tetranychus urticae* (de Boer 1981, 1982; Fry 1989; Overmeer and van Zon 1976), *Drosophila* (Engels and Preston 1979; MacRae and Anderson 1988), and plants (Edwardson 1970; van Damme and van Delden 1982). In these studies, mitochondria, chloroplasts, and transposable elements have been implicated.

Much research on the genetics of speciation is devoted to elucidating the underlying genetics of Haldane’s rule (Haldane 1922): the observation that in interspecific crosses the sterile or inviable sex is nearly always the heterogametic sex (see Cooney and Orr 1989; Wu et al. 1992). So far, genetic studies on hybrids between *Drosophila* species have demonstrated that sex chromosomes, and in particular the X chromosome, play a large role in hybrid sterility and viability (Orr 1987, 1989; Orr and Coyne 1989; Wu 1992; Perez et al. 1993; Zeng and Singh 1993). Following Muller (1942), Orr (1993) has recently proposed that Haldane’s rule is a simple function of the recessivity of incompatibility alleles in hybrids.

Although Haldane’s rule applies to asymmetrical sterility or viability between homogametic and heterogametic hybrids in most diploid species, it is not directly relevant to species with other sex determination systems simply because they lack sex chromosomes and a heterogametic sex. However, if Haldane’s rule is a consequence of recessivity of hybrid incompatibility genes, then Orr’s model (1993) may also apply to haplodiploids, because males are haploid and will express such recessive incompatibility alleles. An alternative explanation was offered by Hurst and Pomiankowski (1991) for unisexual hybrid disruption in crosses between species that lack sex chromosomes. They suggested that sex-ratio-distorting cytoplasmic genes are responsible for male hybrid sterility (or viability).

It is evident that more information is needed on the genetic basis of reproductive isolation in different systems to understand the evolution of postzygotic reproductive isolation and speciation. Haplodiploids (haploid males, diploid females) make up a large number of arthropod species, but little is known about the genetic basis of reproductive isolation in haplodiploid species. Haplodiploids may provide new insights concerning the genetic basis of reproductive isolation, as well as Haldane’s rule because of male hemizygosity.

In the parasitoid wasp *Nasonia*, it was previously demonstrated that cytoplasmically inherited microorganisms prevent hybridization between *N. vitripennis* and a closely related, sympatric species *N. giraulti*, by causing bidirectional incompatibility (Breeuwer and Werren 1990, 1992). Hybrid offspring are produced only after elimination of the microorganisms by antibiotic treatment. Removal of the cytoplasmic bacteria allows study of the genetic basis of hybrid breakdown. Preliminary studies revealed that hybrid (female) offspring from both reciprocal interspecific crosses were viable and fertile. Note that only female progeny are hybrids in haplodiploid species; F₁ males are derived from unfertilized eggs and receive the genetic complement only from the maternal species. There was, however, increased mortality among offspring of F₁ hybrid females, in particular among F₁ haploid (recombinant) male offspring, indicating hybrid breakdown. This study investigates the genetic basis of hybrid breakdown in the absence of cytoplasmic incompatibility bacteria. The interactions between nuclear and cytoplasmic genetic factors of the two species resulting in hybrid breakdown is further investigated by introgression crosses. Both nuclear/nuclear and cytoplasmic/nuclear incompatibilities were found to be involved in hybrid breakdown.

**MATERIALS AND METHODS**

The general biology of *Nasonia* is described by Whiting (1967). *Nasonia* are ectoparasitoids of pupae of a variety of calliphorid flies that occur in birds’ nests and carcasses. Under laboratory conditions (25°C, constant light) *Nasonia* were cultured on fleshfly pupae, *Sarcophaga bullata*, and generation time was approximately 14 d. All experiments were carried out under these conditions.

Strains.-Two uninfected (i.e., free of the cytoplasmic incompatibility microorganism) strains were used in crossing experiments: Asy whole (*N. vitripennis*) and RV2T (*N. giraulti*). Both strains were derived from wild-type strains of each species, Labil (laboratory strain Leiden) and RV2 (collected in Rochester N.Y., USA, 1986) respectively, by feeding females an antibiotic (1 mg/ml tetracycline in 10% sucrose) before egg laying for three generations (Breeuwer and Werren 1990). This eliminated the cytoplasmic microorganisms and altered the incompatibility type of the wasps (Breeuwer and Werren 1990, 1992). These cured strains have been maintained free of cytoplasmic incompatibility microorganisms ever since.

Terminology.-An individual is designated by genotype, with cytotype indicated between brackets. Analogous to an individual’s genotype, the term cytotype refers to the heritable cytoplasmic factors, such as mitochondria and cytoplasmic microorganisms, that reside in the cytoplasm of an individual. A cytotype is typically inherited only via the mother. For both genotype and cytotype, *V* stands for *N. vitripennis* and *G* for *N. giraulti*. Thus, Asy whole is designated as *V* [V], whereas RV2T is *G* [G]. Hybrids are identified by the genotype of father, followed by that of mother and her cytotype between brackets; *VG* [G] hybrid females are offspring from *V* [V] d *X G* [G] 4 and *GV* [V] hybrid females are offspring from the reciprocal interspecies cross *G* [G] d *X V* [VI 4. The genotype of males is described by the genotype of their mother, but note that males are haploid and inherit a haploid genome derived from their mothers’ via recombination and segregation. The genotype of individuals from introgression lines is described by the original paternal genotype, the number of introgression generations followed by the original maternal genotype. For example, *GSV* [V] is derived from an original cross between *F₁* hybrid (*G* [G] d *X V* [V] 4) 4 backcrossed to *G* [G] d for an additional four generations. Assuming absence of selection and normal meiosis, its nuclear genome is expected to be (1 − 12%) × 100% ~97% G.
Experimental Procedures.-For all experimental crosses males and females were collected as virgin pupae. Upon emergence, females were either mass mated to males (4 d X 10 4) or left virgin, unless otherwise noted. Virgin females produce only male progeny, due to haplodiploidy. After 24 h, males were removed, and females were individually placed in vials and provided with a single host. Females were transferred to fresh hosts every day for 4 d. On experimental days, hosts were offered to the females in a foam plug, which exposed only the anterior tip of the host to the female. This was done to facilitate localization and counting of the wasp eggs. In most experiments, plug hosts were provided only on days 3 and 4.

Parasitized plug hosts from experimental days were divided into two groups: the hosts from one group were cracked open and eggs were counted, the other group was left alone and subsequent emerging offspring number and sex ratio were recorded. Survival probability from egg to adult was estimated by dividing offspring numbers by egg numbers. Note that this is an indirect way of estimating survival (see below). Direct measurement of egg-to-adult survival is problematic for two reasons: (1) once hosts are cracked open, the host and the wasp larvae that feed on it are subject to desiccation, which increases mortality, and (2) hosts are easily damaged upon opening and eggs occasionally stick to the puparium wall, introducing additional error sources.

F1 Hybrids.-Males and females of N. vitripennis and N. giraulti were combined in single pairs in four possible combinations. Copulations were observed to exclude all male offspring as a result of female virginity. Because comparisons were between like females, which were assumed to produce similar egg numbers, only offspring numbers were recorded. Note that only F, female offspring of interspecies crosses are hybrid and receive a complete chromosome complement from both parental species. F, males develop from unfertilized eggs and receive only the maternal genome. Therefore, they are not hybrid.

F2 Recombinant Male Breakdown.-Hybrid males first appear as offspring from F, hybrid females. Because of meiosis, their genome is a recombination of the two chromosome complements of their mother and hereafter they are referred to as recombinant males. Recombinant male hybrid breakdown was assayed by comparing egg and adult offspring numbers of virgin F, hybrid and nonhybrid females. Because of haplodiploidy, virgin or unmated females will produce only male progeny, due to haplodiploidy. After 24 h, males were removed, and females were individually placed in vials and provided with a single host. Females were transferred to fresh hosts every day for 4 d. On experimental days, hosts were offered to the females in a foam plug, which exposed only the anterior tip of the host to the female. This was done to facilitate localization and counting of the wasp eggs. In most experiments, plug hosts were provided only on days 3 and 4.

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maternal lineage (X)  Paternal donor (Y)

F1  50  75

84  97

B4 recombinant (male) offspring

FIG. 1. Introgresion scheme illustrating nuclear genome substitution by repeated backcrossing of a lineage derived from a female species X to males from species Y; P, paternal generation; F1, first filial generation; B1-B4, backcross generations. Inner circle represents nuclear genome, outer circle represents cytotype. Empty and solid areas refer to nuclear genes from species X and Y respectively. Numbers refer to the accumulated fraction of genes expected to be contributed by species Y to the descendants of X, assuming that there is no selection for and against paternal nuclear DNA and strict maternal inheritance of cytotype.

maternal genome will be substituted by the paternal genome after five introgression generations (or four backcross generations), assuming normal recombination and segregation. On the other hand, heritable cytoplasmic factors are only maternally inherited; the male sperm does not contribute cytoplasm to the egg. Therefore, introgression lines will carry the substituted paternal genome in a cytoplasmic background that contains the original maternal heritable cytoplasmic factors. Typically, three assumptions are made about the backcross process (Grun 1976): (1) there is no preferential retention of maternal genes among recombinant eggs, (2) the heritable cytoplasmic factors do not change during the introgression process, and (3) sperm does not contribute cytoplasm to the egg.

Statistics.-The Student t-test was used for testing the null hypothesis for no differences, that is, $\eta_1 = \eta_2$, assuming that samples follow a normal distribution. In the case of ratio comparisons, the Mann-Whitney U test (MWU) was employed (Siegel 1965). The level of significance ($\alpha$) was set at 0.05. Because of the biology of Nasonia (see above), egg-to-adult survival could not be measured directly, and was measured by comparing egg counts in one sample to adult emergences in another. Variance in survival was estimated as follows (Mood et al. 1974): Suppose $Z$ is a function of the random variables $X$ and $Y$, where $X$ is number of eggs and $Y$ is the number of adult offspring. In our case, $Z$ is survival probability and equal to $Y/X$. Then the variance of $Z$ is approximately equal to:

$$\text{var}(Z) = \frac{\text{var}(Y)}{\text{mean}(X)} + \frac{\text{mean}(Y)}{\text{mean}(X)^2} \cdot \text{var}(X)+\text{mean}(Y)$$

where $\text{mean}(X)$ is the mean egg number and $\text{mean}(Y)$ is the mean offspring number. If $X$ and $Y$ are independent, and thus are not correlated, the $\text{cov}(X, Y) = 0$. This reduces (1) to:

$$\text{var}(Z) = \frac{\text{var}(Y)}{\text{mean}(X)} + \frac{\text{mean}(Y)}{\text{mean}(X)^2} \cdot \text{var}(X)+\text{mean}(Y)$$

RESULTS

F, Hybrid Females.-Reciprocal crosses between Nasonia vitripennis and N. giraulti lines cured of their cytoplasmic bacteria produced viable F1 hybrid female progeny (Breeuwer and Werren 1990, 1993). Note that F1 males were not hybrids, because they developed parthenogenetically from unfertilized eggs and thus only inherited maternal genes and cytoplasmic factors. Offspring sex ratios (percentage females) of both reciprocal interspecies crosses were significantly lower than those of the corresponding control intraspecific crosses (table 1). In addition, both interspecies crosses had fewer emerging offspring than the controls, but the difference was only significant in crosses between V [V] X d G [G] Y versus G [G] d' X G [G] Y. Male production of V [V] d X G [G] Y did not differ significantly ($t = 0.86$, df = 41, $P = 0.2$), whereas production of females was significantly lower ($t = 2.8$, df = 41, $P < 0.01$). Poor insemination is not a likely explanation for the lower number of daughters because (1) giraulti females mated equally well with vitripennis males as with their own males, (2) less sperm should result in more sons which is not the case, and (3) mating does not affect the number of

<table>
<thead>
<tr>
<th>Parental cross</th>
<th>Offspring</th>
<th>Sex ratio</th>
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<tbody>
<tr>
<td></td>
<td>Sons</td>
<td>Daughters</td>
</tr>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td></td>
<td>Male n</td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>21</td>
<td>5.1 ± 1.9</td>
</tr>
<tr>
<td>G</td>
<td>18</td>
<td>12.3 ± 12.2</td>
</tr>
<tr>
<td>G</td>
<td>24</td>
<td>3.3 ± 2.5</td>
</tr>
<tr>
<td>V</td>
<td>19</td>
<td>3.9 ± 1.8</td>
</tr>
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</table>

TABLE 1. F, female hybrid viability. Shown are mean ± SD of sons, daughters, total offspring (sons + daughters + diapause larvae) and sex ratio (% females) of inter- and intraspecific crosses of Nasonia giraulti (G) and N. vitripennis (V) from eggs laid on the third oviposition day by a single female on one host. Female cytotype is between brackets. Note sons are not hybrids. n, number of replicates.
eggs laid (see tables 5, 6). The result indicates that F1 hybrid VG [G] female progeny had reduced viability compared to nonhybrid G [G] females. In contrast, the reciprocal cross G [G] d X V [V] 4 produced significantly more male offspring than the interspecies cross V [V] d X V [V] 4 (t = 2.5, df = 37, P = 0.009), although offspring numbers were not significantly lower. There are at least two possible explanations: (1) poor insemination, and (2) sperm-egg incompatibility. Although copulations were observed in these crosses, interspecific copulations between N. vitripennis females and N. giraulti males were often prematurely terminated (pers. obs.). This observation suggests that poor insemination may be a factor in excess male production. In summary, F1 VG [G] females appear to suffer some increased mortality. Mortality among reciprocal F1 GV [V] females could not be detected because of confounding effects of difficulties in sperm transmission in copula between G males and V [V] females.

As seen in table 2, egg production of virgin hybrid females on experimental days was similar to N. giraulti, but significantly lower than N. vitripennis females (for pairwise comparisons, see Breeuwer 1993). N. giraulti females laid significantly fewer eggs than N. vitripennis Darling and Werren 1989). Egg production of the four female types can be ranked as follows, V [V] > G [G] = VG [G] > GV [V]. No differences in adult survival were observed between F1 hybrid females and parental (nonhybrid) females for the duration of the experiment (3-4 d).

F2 Recombinant Male Breakdown.-Although F1 hybrid females did not have abnormal fecundity, there were dramatic reductions in the numbers of emerging (adult) offspring from eggs produced by F1 hybrid females versus nonhybrid females, as seen in all the replicated experiments (table 2). In addition, VG [G] virgin females laid consistently more eggs per host per day than reciprocal GV [V] virgins, but the number of adult recombinant male offspring was always significantly lower from VG [G] virgins. VG [G] F2 recombinant males had much lower survival probability than GV [V] recombinants (respectively, 18 ± 6 [n = 11] and 47 ± 21 [n = 9]; table 2). Using estimated survival percentage of each experiment as a single datum (table 2), matched-pairs comparison of survival estimates of reciprocal recombinant males using the Sign test (Siegel 1965) was highly significant (x = 0.9, n = 9, P < 0.01).

Although reciprocal male offspring are expected to inherit on average the same recombinant genotype (50% G and 50% V genes), they did not inherit the same cytotype, which was either of giraulti or vitripennis origin. Differences in performance of reciprocal hybrids typically indicate that either maternal-effect genes or heritable cytoplasmic factors are involved (Grun 1976). The direction of asymmetry suggests strong negative interactions between V nuclear genes and [G] cytotype. Negative interactions between G nuclear genes and [VI] cytotype cannot be ruled out, although they would be considerably weaker than in the reciprocal cross. Introgresion experiments were conducted to determine whether maternal effects or cytoplasmically inherited elements are involved.

Timing of F2 Recombinant Male Mortality.-The crosses above clearly demonstrated hybrid breakdown among recombinant males. The next experiment investigated whether hybrid mortality was associated with a specific developmental stage. As table 3 shows, F2 recombinant male mortality was not due to hatching failure. The most dramatic mortality was observed during larval development, reducing offspring numbers by 66%–77%. Control larval mortality in these experiments was between 25% and 35%. Control mortality was higher than observed in most other experiments and may have been due to larval crowding. VG [G] F2 recombinant males had an additional 49% mortality during the pupal stage. They
either did not undergo metamorphosis or were unable to eclose.

Fertility of F2 Recombinant Males.—Fertility of recombinant males was assayed by cytological examination of testes, that is, presence of mature sperm, and by testing their ability to sire female offspring. Testes of both VG [G] (n = 10) and GV [V] (n = 12) males contained mature sperm that appeared normal compared to testes of nonhybrid V [V] (n = 8) and G [G] (n = 8) males, based upon light microscopic examination. All GV [V] males (n = 15) copulated with at least one female and sired female offspring (sex ratios were not scored). Reciprocal VG [G] males (n = 19) were unable to court N. giraulti and N. vitripennis females and consequently sired no female offspring. These males suffered from a lack of motor skills and behaved in an uncoordinated and sluggish fashion.

Rescue of F1 Recombinant Progeny.—F1 hybrid females were backcrossed to males of either species to determine the effects of egg fertilization on hybrid breakdown. Hybrid F1 females readily mated with males of both species. As a result of fertilization the recombinant egg genome was combined with a complete (nonrecombinant) haploid complement of either parental species. Offspring survival from F1 hybrid virgin females, which produced only haploid recombinant males, was compared with that of offspring from backcrossed (mated) F1 hybrid females.

Reciprocal F, hybrid females mated to either N. giraulti or N. vitripennis males had significantly more emerging offspring than unmated F, hybrid females (table 4a). Egg production was not recorded in this particular experiment. However, other experiments have shown that virgin and mated females with the same genotype (cytotype) lay the same number of eggs (see tables 5, 6; Breeuwer 1993; Beukeboom 1993). Extrapolating between experiments (e.g., tables 2, 4b), it can be concluded that fertilization of recombinant VG [G] eggs increased survival from around 18% to 30% when fertilized by V [V] sperm and 51% to 56% when fertilized with G [G] sperm. Survival of reciprocal F2 recombinant GV [V] eggs increased from around 47% to 50% when fertilized by V [V] sperm and 57% to 62% when fertilized with G [G] sperm. F2 recombinant (haploid) eggs were partially “rescued” by receiving a complete set of chromosomes from either species. Apparently, an intact genome from either species can compensate for the negative epistatic interactions in the recombinant haploid complement. This suggests that negative epistatic interactions between nuclear genes of the two species in a recombinant genome are recessive.

The giraulti chromosomal complement provided a greater rescue effect than the vitripennis complement in F2 hybrids with giraulti cytoplasm (51% vs. 25% at day 3, 56% vs. 30% at day 4, table 4b). This is reflected in the significant differences in adult offspring of these two crosses (because estimated survival for each cross is a single datum, it is not used directly for statistical analysis). The effect is unlikely to be due to greater fertility of giraulti males because numbers of offspring sired by each male species were higher on the fourth oviposition day after mating compared to third, indicating that sperm depletion was not occurring. The result is consistent with negative interactions between the paternal vitripennis genome and the giraulti cytoplasm, as was also seen with the asymmetry in hybrid breakdown between reciprocal F2 recombinant males (table 3).

There was a less pronounced positive effect of the vitripennis genome relative to the giraulti genome in hybrids with

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**Table 4A.** The effect of fertilization on rescue of recombinant eggs. Reciprocal F1 hybrid females were kept virgin or mated to V or G males. Mean ± SD number of offspring and sex ratio (% females), based on third day of oviposition, are shown. Numbers of eggs were not measured. *n* Numbers of individual females on single host.

<table>
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<tr>
<th>Parental cross</th>
<th>Adult offspring</th>
<th>Sex ratio</th>
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<tbody>
<tr>
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<td>Male</td>
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<td>G</td>
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<td>V</td>
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* ** P < 0.01; *** P < 0.001. (Student t-value and level of significance for pairwise comparisons of offspring numbers). Lines indicate which paired means are being compared.

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**Table 4B.** The effect of sperm genotype on rescue of recombinant eggs. Reciprocal F1 hybrid females were kept virgin or mated to V or G males. Mean ± SD number of eggs, offspring and sex ratio (% females), based on third and fourth day of oviposition, are shown. Numbers of individual females on single host.

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</table>

*** P < 0.001 (Student t-value and level of significance for pairwise comparisons of offspring numbers).
vitripennis cytoplasm (46% vs. 57% on day 3, 50% vs. 62% on day 4). In fact, offspring numbers of reciprocal F₁ hybrid GV [V] females mated to different male species were not significantly different (Breeuwer 1993). This suggests that negative interactions between G nuclear genes and [V] cytoplasmic factors is weak or absent.

Introgression and Hybrid Breakdown.—The role of cytoplasmic factors was further assessed by repeated backcrossing of hybrids to the paternal species (introgression). In theory, this method should eventually combine the male parental species genome with the original maternal species cytotype. Progressive introgression by backcrossing should gradually eliminate negative nuclear-nuclear interactions between genes from the two species, because of the replacement of a maternal species cytotype.

Table 5: First (B1) introgression generation. The G2V [V] females are derived from GV [V] F₁ hybrids backcrossed to G males and are presumed to contain 75% G and 25% V nuclear genes in a [V] cytotype; V2G [G] females are derived from VG [G] F₁, hybrid females that are backcrossed to V males and are presumed to contain 75% V and 25% G nuclear genes in a [G] cytotype. Mean egg numbers are based on all crosses with like females, because no differences were observed between like females from different crosses (t-test not shown). n, Numbers of individual females on single hosts.

<table>
<thead>
<tr>
<th>Offspring</th>
<th>Parental cross</th>
<th>Eggs</th>
<th>Adult offspring</th>
<th>Estimated survival</th>
<th>Sex ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Female × Male</td>
<td>Mean ± SD</td>
<td>n</td>
<td>Mean ± SD</td>
<td>n</td>
</tr>
<tr>
<td>G2V [V]</td>
<td>—</td>
<td>30.5 ± 19.0</td>
<td>44</td>
<td>8.2 ± 4.2</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>21.7 ± 13.1</td>
<td>14</td>
<td>0.1</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>V</td>
<td>21.4 ± 9.0</td>
<td>12</td>
<td>5.8***</td>
<td>12</td>
</tr>
<tr>
<td>V2G [G]</td>
<td>—</td>
<td>29.8 ± 11.6</td>
<td>64</td>
<td>4.3 ± 1.8</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>19.3 ± 9.4</td>
<td>12</td>
<td>1</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>V</td>
<td>8.3 ± 3.6</td>
<td>12</td>
<td>3.6***</td>
<td>12</td>
</tr>
<tr>
<td>G [G]</td>
<td>—</td>
<td>32.6 ± 8.6</td>
<td>48</td>
<td>22.2 ± 7.2</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>26.9 ± 7.9</td>
<td>10</td>
<td>1</td>
<td>82 ± 32</td>
</tr>
<tr>
<td></td>
<td>V</td>
<td>52.9 ± 14.4</td>
<td>60</td>
<td>34.8 ± 10.3</td>
<td>16</td>
</tr>
<tr>
<td>V [V]</td>
<td>—</td>
<td>41.3 ± 9.8</td>
<td>15</td>
<td>1</td>
<td>78 ± 28</td>
</tr>
</tbody>
</table>

*** p < 0.001. (Student t-value and level of significance for pairwise comparisons of offspring numbers). Lines indicate which paired means are being compared.

Table 6: B4 introgression generation. The G5V [V] females are derived from F₁ hybrid GV [V] females backcrossed to G males for four generations and are presumed to contain 97% G and 3% V nuclear genes in a [V] cytotype; V5G [G] females are derived from VG [G] F₁ hybrid females that are backcrossed to V males for four generations and are presumed to contain 97% V and 3% G nuclear genes in a [G] cytotype. Top and bottom table show data from third and fourth day of oviposition respectively. Mean egg numbers are based on all crosses with like females, because no differences were observed between like females from different crosses (t-test not shown). Numbers of individual females on single hosts.

<table>
<thead>
<tr>
<th>Offspring</th>
<th>Parental cross</th>
<th>Eggs</th>
<th>Adult offspring</th>
<th>Estimated % survival</th>
<th>Sex ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Female × Male</td>
<td>Mean ± SD</td>
<td>n</td>
<td>Mean ± SD</td>
<td>n</td>
</tr>
<tr>
<td>G5V [V]</td>
<td>—</td>
<td>27.3 ± 7.3</td>
<td>57</td>
<td>14.5 ± 7.2</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>16.9 ± 6.7</td>
<td>19</td>
<td>6.9</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>V</td>
<td>14.8 ± 8.3</td>
<td>10</td>
<td>0.7</td>
<td>10</td>
</tr>
<tr>
<td>V5G [G]</td>
<td>—</td>
<td>26.4 ± 10.7</td>
<td>72</td>
<td>2.4 ± 2.0</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>7.0 ± 2.5</td>
<td>6</td>
<td>4.9***</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>V</td>
<td>6.1 ± 3.1</td>
<td>34</td>
<td>0.7</td>
<td>34</td>
</tr>
<tr>
<td>G [G]</td>
<td>—</td>
<td>27.7 ± 8.0</td>
<td>31</td>
<td>21.3 ± 7.9</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>24.3 ± 8.4</td>
<td>15</td>
<td>1</td>
<td>88 ± 39</td>
</tr>
<tr>
<td></td>
<td>V</td>
<td>48.0 ± 19.7</td>
<td>47</td>
<td>37.3 ± 10.9</td>
<td>21</td>
</tr>
<tr>
<td>V [V]</td>
<td>—</td>
<td>34.1 ± 9.9</td>
<td>21</td>
<td>71 ± 36</td>
<td>.92±0.04</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>16.3 ± 14.1</td>
<td>14</td>
<td>1.4</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>V</td>
<td>21.9 ± 9.2</td>
<td>20</td>
<td>0.5</td>
<td>20</td>
</tr>
<tr>
<td>G5V [V]</td>
<td>—</td>
<td>31.9 ± 10.4</td>
<td>54</td>
<td>16.8 ± 10.8</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>21.9 ± 9.2</td>
<td>20</td>
<td>0.5</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>V</td>
<td>16.3 ± 14.1</td>
<td>14</td>
<td>1.4</td>
<td>14</td>
</tr>
<tr>
<td>V5G [G]</td>
<td>—</td>
<td>28.3 ± 11.2</td>
<td>56</td>
<td>2.8 ± 2.5</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>9.5 ± 4.7</td>
<td>4</td>
<td>7.4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>V</td>
<td>5.7 ± 2.5</td>
<td>23</td>
<td>2.3*</td>
<td>23</td>
</tr>
<tr>
<td>G [G]</td>
<td>—</td>
<td>41.1 ± 9.2</td>
<td>27</td>
<td>29.3 ± 10.5</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>26.2 ± 11.9</td>
<td>18</td>
<td>1</td>
<td>88±39</td>
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<tr>
<td></td>
<td>V</td>
<td>59.0 ± 19.5</td>
<td>40</td>
<td>40.8 ± 15.1</td>
<td>24</td>
</tr>
<tr>
<td>V [V]</td>
<td>—</td>
<td>41.1 ± 11.4</td>
<td>28</td>
<td>71 ± 36</td>
<td>.93±0.03</td>
</tr>
</tbody>
</table>

Lines indicate which paired means are being compared.
one genome with the other. Consequently, the component of hybrid breakdown caused by nuclear gene interactions should gradually disappear, and male viability should increase. On the other hand, the component of hybrid breakdown resulting from negative interactions between nuclear genes of one species and cytoplasmically inherited factors of the other species will still be present.

Introgression lines were assessed for hybrid breakdown after one (75% nuclear substitution) and four (97% nuclear substitution) backcross generations of hybrid females to the respective paternal species. Introgression of G genes in a [V] cytoplasmic background was continued for 15 backcross generations. The reciprocal introgression, V genes into [G] cytoplasm, was terminated after four backcross generations because of severe hybrid breakdown.

Table 5 presents the results from crosses with females from the first backcross (B1) generation. In theory, 75% of the genome of their recombinant eggs should be of paternal origin. There were no significant differences in egg production of virgin versus mated females of the same type and therefore egg numbers were pooled for like females. Pairwise comparisons of egg numbers produced by B1 and N. giraulti females also showed no significant differences (see Breeuwer 1993).

In contrast to similar egg numbers produced by backcross-derived females, adult offspring numbers differed considerably between mated and unmated females, and between females mated to males from different species. The pattern was similar to what was observed among offspring from unmated and mated F1 hybrid females (table 4). (male) offspring survival from virgin hybrid females was greatly depressed compared to male offspring survival from virgin non-hybrid females. In both reciprocal introgression lines, fertilization with nonrecombinant genomes greatly increased survival of recombinant progeny up to levels similar to survival estimates in pure species (table 5). Direct comparison of adult offspring numbers of mated G2V [V] and G [G] females were not significantly different (G [G] d × G [G] V versus G [G] d × G2V [V] Y: t = 1.4, df = 22, P = 0.08, and V [V] d × G V [V] 4 : t = 1.3, df = 22, P = 0.1). As among F2 hybrid eggs, fertilization raised survival of F1 G2V [G] eggs from 14% to 65% when G [G] d sperm was used and to 28% when V [V] d sperm was used. Survival of reciprocal G2V [V] eggs increased from 27% to 70% (V [V] d sperm) and 70% (G [G] d sperm). This supports the hypothesis that hybrid breakdown is due, in part, to recessive negative nuclear-nuclear interactions between genes of the two species.

The results are also consistent with earlier observations that giraulti cytoplasmic factors have negative interactions with vitripennis nuclear genes: (1) Offspring numbers of V2G [G] virgin females were again significantly lower than those of G2V [V] females (t = 1.25, P < 0.01), and (2) rescue effect of vitripennis sperm of recombinant eggs with a [G] cytotype was significantly lower than the rescue effect of giraulti sperm (t = 3.6, df = 22, P < .001), indicating persistent negative interactions between vitripennis nuclear genes and giraulti cytoplasmic factors. No such male effect was found in the reciprocal G2V [V] line.

After four backcross generations, 97% of the paternal species genome is expected to have been substituted into the maternal species cytoplasmic background. Successful introgression of the G nuclear genome into a [V] cytotype was indicated by the wing and antennal morphology of males and forewing bristles of females; all which resembled the N. giraulti morphology (J. H. Werren, D. Swank, and J. A. J. Breeuwer, unpubl. data). Egg production of backcrossed females and N. giraulti females (mated and unmated) were similar on day 3 (table 6). As in previous experiments, egg production of nonhybrid V [V] females was significantly higher than that of nonhybrid G [G] females, but estimated survival rates of the two nonhybrid species were similar.

Survival estimates of recombinant GSV [V] males were approximately twice as high as G2V [V] males, indicating that GSV [V] haploid males suffered less from negative epistatic interactions between nuclear genes of the two species. This result was expected if hybrid inviability was due primarily to nuclear/nuclear incompatibilities, because their nuclear genetic make-up was now predominately G. Concurrently, the rescue effect of fertilization on offspring survival, that was so dramatic in crosses with F, and B1 females, was absent in the eggs of B4 GSV [V] females. There were no significant differences in offspring numbers of mated versus unmated GSV [V] females (table 6). However, GSV [V] estimated offspring survivals were lower than those of pure species, which was supported by significant lower offspring numbers from virgin GSV [V] versus G [G] females on day 3 (t = 2.6, df = 33, P = 0.007).

Introgression of V nuclear genes into a [G] cytotype gave dramatically different results. First, recombinant male survival showed no improvement after four backcross generations (9%-10% for VSG [G] vs. 14% for V2G [G] recombinant males). In addition, a large proportion of these males still showed N. giraulti wing morphology (J. H. Werren, D. Swank, and J. A. J. Breeuwer, unpubl. data), indicating that parts of their giraulti genome were being retained despite repeated introgression of V genes. This result further indicates that giraulti nuclear genes are being selectively retained in the presence of the giraulti cytoplasm in these hybrids. It violates the assumptions of introgression and indicates that the percent nuclear substitution was probably lower than predicted. However, it provides very strong evidence that hybrid breakdown involves giraulti cytoplasmic factors that are independent of the nuclear genome and stably inherited. Persistence of hybrid breakdown made it difficult to continue introgression of V nuclear genes into [G] cytotype and introgression was therefore terminated at this point.

As in the V2G [G] generation (table 5), fertilization increased survival of recombinant VSG [G] offspring, but in this case rescue was independent of the father’s species. The result is difficult to explain, but may indicate a cumulative effect of negative interactions between giraulti cytoplasmic genes and vitripennis genes. For example, mitochondrial replication may be disrupted in the wrong nuclear background, since mitochondria replication and expression relies on close interaction with nuclear genes (Moritz et al. 1987).

Results of crosses with reciprocal B4 females clearly illustrated the strong persistent antagonistic interactions between vitripennis nuclear genes and giraulti cytoplasmic factors (table 6). Introgression experiments therefore indicate that cytoplasmic interaction is not due to maternal effects,
but to cytoplasmically inherited factors (or cytoplasmic genes).

Complete Introggression Line.-A (nearly) complete introgression of giraulti nuclear genes into vitripennis cytoplasm was accomplished by 16 backcross generations. This strain (G [V]) was used to further document the incompatibility between giraulti cytoplasmic factors and vitripennis nuclear factors. The level of hybrid breakdown among F$_2$ offspring of (V d X G [G] 4) females and (V d X G [V] 4) females was measured. The G [V] introgression line has a (nearly) complete giraulti nuclear genome combined with vitripennis cytoplasmic genes. The only difference between the two types of F$_1$ hybrid females in the cross above is their heritable cytoplasmic factors. They have the same nuclear VG genotype and the same maternal effects, that is, both females had genotypic G mothers. Thus, any difference in survival of reciprocal F$_2$ recombinant males can be attributed to their different cytoplasmic factors. The results of this experiment are shown in table 7.

Survival of F$_1$, recombinant males with V cytoplasm (from VG [V] females) was around 50% lower than survival of control G [G] and G [V] males. Survival was reduced another 40%-50% among F$_2$ recombinant males with G cytoplasm, further indicating a strong incompatibility between G cytoplasm and V nuclear genes and ruling out nuclear grandmaternal effects. Both results were consistent with previous survival estimates of recombinant male hybrid breakdown (table 2).

There is also a significantly lower egg production of G [V] females compared to G [G] females, suggesting that interactions between V cytoplasmic genes and G nuclear genes negatively affect fecundity (day 3: t = 15, df = 106, P < 0.0001; day 4: t = 6.4, df = 101, P < 0.0001). However, estimated survival of haploid offspring of G [V] females and pure G [G] females were similar, indicating that neither nuclear-nuclear nor nuclear-cytoplasmic interactions were acting in the G [V] line that adversely affected haploid male survival.

Results support the hypothesis that hybrid breakdown between N. vitripennis and N. giraulti is the result of two types of negative interactions: nuclear-nuclear interactions and asymmetrical (nonreciprocal) interactions between nuclear genes and cytoplasmic genes of the two species.

**DISCUSSION**

Results show that Nasonia vitripennis and N. giraulti produce hybrid F$_1$ females (once cured of their cytoplasmic incompatibility bacteria), and that these females are healthy and fecund. Therefore, the genomes of these two species have not diverged to the point that dominant negative epistatic interactions result in F$_1$ hybrid lethality or sterility. Significant levels of F$_2$ hybrid breakdown occur, although F$_2$ hybrids are produced and these are fertile. This permits an analysis of the genetic basis of hybrid breakdown, and indicates that genetic divergence between these species is at the incipient stages.

F$_2$ hybrid mortality occurs in the larval and pupal stages. Our results clearly show that hybrid mortality involves a strong negative interaction between cytoplasmically inherited factors in giraulti and nuclear genes in vitripennis. This is observed at many different levels. First, there is an increase in mortality of F$_1$ females with giraulti cytoplasm, relative to the reciprocal cross. Second, F$_2$ male progeny of VG [G] females suffer considerably higher mortality than do the reciprocal males from VG [V] females. Third, introgression of V nuclear genes into G cytoplasm shows a persistent high level of offspring mortality, up to the fifth generation. Mortality was sufficiently severe that it was difficult to maintain the line. In addition, even after five generations of V nuclear introgression, giraulti phenotypes were still evident in the hybrid males, indicating selective retention of G nuclear genes in the presence of G cytoplasm. The reciprocal backcrosses (G nuclear genes into V cytoplasm) showed no such difficulties, and survival rebounded to normal levels by the fifth generation. Finally, a strain was produced by 16 generations of backcrossing to giraulti males that in effect had 100% giraulti nuclear genes but was vitripennis with respect to heritable cytoplasmic elements. Females of this strain mated to vitripennis males gave 50% the mortality among F$_2$ hybrid males relative to those using giraulti females with giraulti cytoplasm.

Thus, it can be concluded that incompatibility between vitripennis nuclear genes and giraulti cytoplasmically inherited elements results in hybrid breakdown. This pattern would appear to corroborate the postulate of Hurst and Pomiankowski (1991) that cytoplasmic elements are responsible in
cases in which hybrid breakdown is limited to males but not
linked to heterogamy. Hurst and Pomiankowski (1991) ar-
gued that these cytoplasmic elements are likely to be sex-
ratio distorters that cause female-biased sex ratios because
their exclusive maternal transmission. These elements are
supposed to be suppressed in their own nuclear background,
but suppression is released when in a novel genetic (hybrid)
background. However, we favor an alternative interpretation,
that the effect is due to cytoplasmic elements that have coe-
volved with the nuclear genome they are associated with.
According to this model, epistatic interactions between nu-
clear and cytoplasmic genes are disrupted in hybrids, causing
hybrid breakdown. The most likely candidates in this scenario
are the mitochondria. Indeed, an increasing number of studies
report on the nonneutrality of mitochondrial haplotypes
(Brown et al. 1979; Clark and Lyckegaard 1988; MacRae and
Anderson 1988; Avise 1991, Nigro 1994; see also Clark
1984). In the Nasonia system, hybrid breakdown is probably
due to negative epistatic interactions between mitochondria of
giraulti and nuclear genes of vitripennis. Epistatic inter-
actions between nuclear genes and mitochondria are likely
due to a number of mitochondrial proteins encoded by the
nucleus, and other nuclear genes are involved in mito-
chondrial interactions. However, it has not yet been estab-
lished that the cytoplasmic elements involved in Nasonia
hybrid breakdown are mitochondria.

Nuclear-cytoplasmic incompatibilities are not the only
cause of hybrid breakdown. Nuclear-nuclear interactions
were found in both reciprocal hybrid crosses. These negative
epistatic interactions appear to be primarily recessive, since
hybrids were "rescued" by incorporation of a complete hap-
lod genome from either parental species by fertilization.
Nuclear-nuclear incompatibility is further supported by the ob-
servation that progressive introgression of G nuclear genes
gradually increased survival of haploid (male) eggs up to
levels comparable to pure species male survival (fig. 2). In
addition, hybridization using the strain with giraulti nuclear
and vitripennis cytoplasm showed a twofold increase in mortality of
F1 males relative to controls. This finding is consistent
with nuclear-nuclear induced hybrid breakdown.

Hybrid breakdown is generally believed to be caused by
disruption of normal epistatic interactions between nuclear
genes that are organized in coadapted (or cooperating) gene
systems (Wallace 1981). Typically, it does not affect the F1,
hybrids, but becomes apparent in the F2 or backcross gen-
erations. Lethality supposedly results from incompatible combinations of genes or perhaps whole chromosome seg-
ments. Certain combinations must be imbalanced and disturb vital development processes, particularly in the haploid phase. Fertilization by a balanced gamete partially reverses
this imbalance. In F1, hybrid females have two complete balanced sets of chromosomes and apparently there are few or no "dominant" negative interactions between genes or
chromosome segments, since the F1 is generally viable. How-
ever, recombination and assortment during meiosis in F1, hy-
brids break up favorable gene arrangements present in the
parental species, derailing normal epistatic interactions in F2
hybrid males. Introduction of a complete species chromo-
some set can apparently override the "negative" epistatic

\begin{verbatim}
F1 HYBRID FEMALE
\begin{align*}
A1 & B1 & C1 \\
A2 & B2 & C2 \\
\end{align*}
\end{verbatim}

HAPLOID GENOTYPES
\begin{verbatim}
NUCLEAR–NUCLEAR
\begin{align*}
\end{align*}
\end{verbatim}

\begin{verbatim}
NUCLEAR–CYTOPLASMIC
\begin{align*}
A1B1C2 & A1B2C2 \\
A2B1 & A2B2C2 \\
\end{align*}
\end{verbatim}

\begin{verbatim}
FIG. 2. Genetic model for nuclear-nuclear and nuclear-cytoplasmic interactions. Crosses between males of species 1 and females of
species 2 produce hybrid F, females that are heterozygous at all three loci \(A, B, \) and \(C\) and inherit the cytoplasmic factors \([2]\) from
their mother. These F, hybrid females produce eight different gam-
etes with different recombinant genotypes, but all will carry the
[2] cytoplasmic factors. Suppose that \(A\) and \(B\) have epistatic inter-
actions and that the presence of alleles from different species at
these loci result in deregulation of these interactions causing mor-
tality. Then, eggs with recombinant genotype for \(A\) and \(B\) (striped
cells) will die, resulting in 50% mortality. If, in addition, negative
nuclear-cytoplasmic interactions exist between, for example, \(C\)
nuclear allele and [2] cytoplasmic factors, recombinant eggs that
receive the \(C\) allele will die, resulting in an additional 50% mor-
tality (crossed cells). If nuclear/nuclear incompatibilities are asym-
metric, then A1B2 die but A2B1 do not, giving 25% mortality from
that interaction.
\end{verbatim}
unclear to what extent sex specific differences in somatic ploidy level exist.

Hybrid breakdown among grandsons of interspecific crosses between \textit{N. vitripennis} and \textit{N. giraulti} can be explained by as few as two sets of interacting genes; two negatively interacting nuclear genes (\textit{A} and \textit{B}), and a single nuclear gene (\textit{C}) that interacts with a cytoplasmic element. Each \textit{Nasonia} species is fixed for a particular set of coadapted alleles at each of these loci, (\textit{A1 BI CI}) for \textit{N. vitripennis} and (\textit{A2B2C2}) for \textit{N. giraulti} (fig. 2). Hybrid females are thus heterozygous for each locus, but carry [\textit{V}] or [\textit{G}] cytoplasm depending upon their mother species. Suppose that interactions between alleles of different species at locus \textit{A} and \textit{B} result in mortality. This would confer a 50\% mortality among male offspring of hybrid females; all recombinants would die. Surviving male offspring are expected to be homozygous at locus \textit{A} and \textit{B}. Half of those male will carry the \textit{G} allele at locus \textit{C} and the other half will carry the \textit{V} allele. The latter males will die if they end up in a [\textit{G}] cytoplasmic background, resulting in an additional 50\% mortality among male offspring of \textit{F}, hybrid females with [\textit{G}] cytoplasm compared to reciprocals with [\textit{V}] cytoplasm. Actual mortality rates of reciprocal recombinant males (tables 2, 7; Breeuwer 1993) are very similar to the predictions of the above three-locus model.

Asymmetrical negative nuclear/nuclear interactions between genetic factors of two species are expected to arise before the evolution of symmetric interactions (Muller 1942, Nei et al. 1983; Wu and Beckenbach 1983). Symmetric interactions, on the other hand, may represent a more advanced stage in the process of species divergence, when separation has been long enough to allow accumulation of additional allelic substitutions and incompatibility interactions (Wu and Beckenbach 1983). Applying asymmetric interactions to the model above (e.g., \textit{A1} and \textit{B2} are incompatible but \textit{A2} and \textit{B1} are the ancestral alleles and are compatible), each nuclear/nuclear interaction gives at most 25\% mortality among \textit{F}$_2$ males. Then a minimum of two to three nuclear/nuclear and one nuclear/cytoplasmic incompatibility are needed for the observed levels of mortality.

Although this model is likely to be an oversimplification of hybrid breakdown between the two \textit{Nasonia} species, it provides a working hypothesis for characterization and identification of the genes involved in hybrid breakdown. It is important to keep in mind, however, that complex epistatic interactions between multiple factors may be involved in hybrid breakdown, rather than simply sets of two factor interactions (Palopoli and Wu 1994). It will be most interesting to determine whether the same genes involved in hybrid breakdown between \textit{vitripennis} and \textit{giraulti} are also involved in interactions with the third sibling species, \textit{N. longicornis}.

The \textit{Nasonia} system is likely to be a rich one for genetic studies of speciation. Three species are known that show partial hybrid breakdown but produce fertile hybrids, thus facilitating genetic analysis (Breeuwer 1993). There are clear advantages of haplodiploidy, which allows studies of epistatic interactions in recombinant hybrids without the complications of dominance interactions. This permits the rapid screening of the entire nuclear genome for sets of interacting "speciation" genes. Visible mutant markers are available but limited and we are currently developing molecular markers for this purpose. These markers will allow not only the study of hybrid breakdown, but also the study of the genetic basis of distinct morphological (Darling and Werren 1989; Werren, Swank, and Breeuwer, in prep.) and behavioral (Assem and Werren 1994) differences that exist between these species.

ACKNOWLEDGMENTS

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