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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, F_1 hybrids females are readily produced (due to haplodiploidy, males develop from unfertilized eggs and are therefore not hybrids). F_1 hybrid females are viable and fecund, but recombinant (haploid) F_2 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. F_2 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 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

(Breeuwer and Werren 1990; Breeuwer et al. 1992) or indirectly by altering sex ratio (Hurst 1993a; Stouthamer et al. 1990). Cytoplasmic incompatibility bacteria are widespread in insects and could play an important role in rapid speciation (Breeuwer and Werren 1990; Coyne 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 Coyne 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; F_1 males are derived from unfertilized eggs and receive the genetic complement only from the maternal species. There was, however, increased mortality among offspring of F_1 hybrid females, in particular among F_2 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: Asymc (N. *vitripennis)* and RV2T (N. giraulti). Both strains were derived from wild-type strains of each species, Labll (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, Asymc 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 F_1 hybrid (G [G] d X V [V] 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 $(1 - 1/2^5) \times 100\% \sim 97\%$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.

F, Hybrids.-Males and females of *N.* vitripennis and *N.* girault i 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.

F_2 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 haploid (male) offspring. Recombinant male hybrid breakdown was documented in independently replicated experiments.

Timing *of* F_2 Recombinant Male Mortality.-to determine whether offspring mortality occurs continuously throughout development or is associated with a particular developmental stage, for example, egg hatching or pupation, offspring of F, hybrid virgin females were observed at successive time points during development. Reciprocal F, hybrid females and females from both species (nonhybrid) were collected as virgins, and upon emergence each was provided with two hosts for 2 d. Females were transferred to a single plughost on the third day for 24 h. Next, females were removed and hosts were divided into three groups and placed in tissue culture plates (96-well plate, Corning Glassworks, USA). Hosts in the first group were opened immediately and egg numbers were recorded. After 48 h, the numbers of unhatched eggs were scored. Eggs typically hatched within 36 h after oviposition. The second group was opened 7 d after oviposition, which roughly corresponded with the prepupal stage. Numbers of wasp pupae were scored. Group 3 was allowed to emerge and adult offspring numbers were scored.

Fertility of F_2 Recombinant Males.-Fertility of recombinant males was assayed in two ways: (1) cytological observation of mature sperm in testes and (2) observation of ability to copulate and sire female offspring. For cytology, testes of reciprocal recombinant male pupae were dissected in Drosophila Ringer (6.5 gr NaCI; 0.14 gr KCI; 0.2 gr $NaHC0_3$; 0.01 gr $CaC1_2$; 0.01 gr NaH_2PO_4 in 1 L H_2O) on a slide. Testes were gently squashed under a cover slip and viewed under low magnification with a light microscope. The ability of males to sire female offspring was assayed as follows. Single recombinant males were combined with two virgin females and copulation was observed. Only G [G] females were used, because they mate more readily with males of both species than V [V] females. Females that did not copulate within 30 min were discarded. Females that did copulate were provided with two hosts for egg laying, and presence of females among subsequent offspring (indicating use of sperm) was scored.

Introgression Lines.-Negative interactions between genetic factors (nuclear or cytoplasmic) of two species that are combined in hybrid individuals lead to hybrid inferiority. The role of these factors in hybrid breakdown was further elucidated by introgression experiments. The nuclear genome of each species was introgressed into the cytotype of the other species by repeated backcrossing (fig. 1). Lines were started with reciprocal crosses between males and females of the parental species (V [V] d X G [G] 4 and G [G] d X V [V] Y). Resulting hybrid females were backcrossed to the paternal species for up to 15 generations. After 15 backcross generations, a line, G [V], was established from offspring of G [G] d X G16V [V] Y, and has been maintained by within strain mating without further backcrossing for over a year. Both morphological and behavioral characters of the G [V] line resemble *N.* girault i, indicating that this line contains a G nuclear genome. The reciprocal introgression of V genotype into a [G] cytotype was terminated after four backcross generations. Note that all crosses involved strains that were free of cytoplasmic incompatibility microorganisms.

Two simple predictions can be made based upon either nuclear-nuclear or nuclear-cytoplasmic interactions. If hybrid breakdown is caused by nuclear-nuclear interactions, progressive introgression should result in a decrease in hybrid breakdown and it is expected to be absent when the maternal species genome is completely replaced by the paternal species genome. On the other hand, if hybrid breakdown is caused by nuclear-cytoplasmic interactions, introgression does not necessarily reduce, and is likely to increase hybrid breakdown. Lineages backcrossed to the paternal species will continue to suffer from negative nuclear-cytoplasmic interactions.

Assumptions *of* the Introgression Process.-In theory, each backcross generation will replace half of the residual maternal nuclear genome with the paternal nuclear genome in the female (diploid) progeny (fig. 1). Thus, 97% of the original

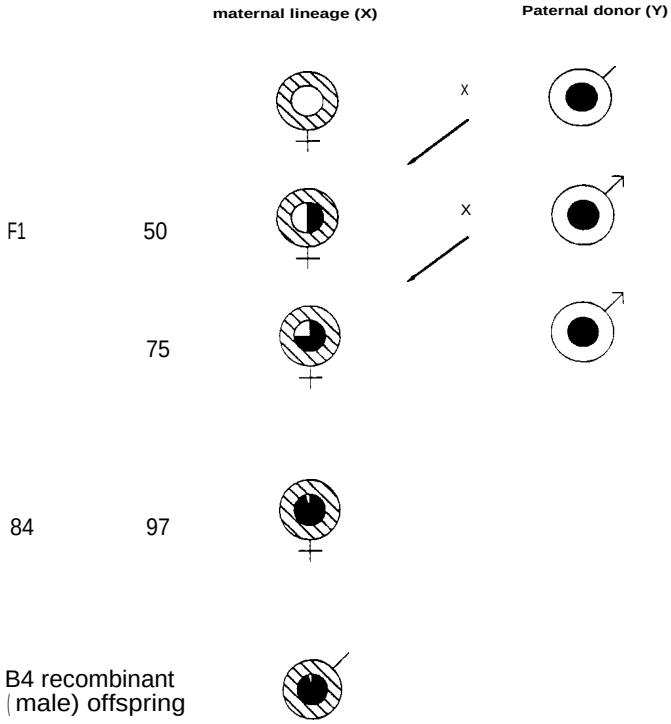

FIG. 1. Introgression scheme illustrating nuclear genome substitution by repeated backcrossing of a lineage derived from a female species X to males from species Y; P, paternal generation; F,, first filial generation; B1-B4, backcross generations. Inner circle represents nuclear genome, outer circle 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, $V_1 = p_{,2,}$ 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 (a) 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:

$$\text{var}(Z) = \text{var}(Y)/lLX + \text{var}(X)p,Y/l\sim X - 2\,\text{cov}(X, Y)p,y/[l,X, \quad (1)$$

where R_X is the mean egg number and $_{WY}$ 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

$$\text{var}(Z) = \text{var}(Y)/\sim,X + \text{var}(X)\sim,Y/l\sim X. \quad (2)$$

RESULTS

F, Hybrid Females.-Reciprocal crosses between Nasonia *vitripennis* and N. girault i lines cured of their cytoplasmic bacteria produced viable F_1 hybrid female progeny (Breeuwer and Werren 1990, 1993). Note that F_1 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 intraspecies 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] 4. 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) girault i 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 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 girault i (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.

Parental cross			Offspring			
			Sons	Daughters	Total offspring	Sex ratio
Female	Male	n	Mean I SD	Mean ± SD	Mean – SD	Mean I SD
	V	21	5.1 ± 1.9	31.7±5.2	37.0±5.3	.86±.05
	G	18	12.3±12.2	19.6±8.6	34.1±8.3	.64±.24
V[V]G[G]	G	24	3.3±2.5	29.1 ±8.9	34.8±9.2	.90±.08
	V	19	3.9± 1.8	21.8±7.7	27.6±7.0	.84±.08

TABLE 2. Recombinant F_2 male hybrid breakdown. Shown are the mean ± SD egg production, number of adult F_2 male offspring and estimated F_2 male survival (100 × mean numbers of adult offspring/mean number of eggs) produced by single virgin F_1 hybrid females and females of the parental species on single hosts. Each experiment was repeated (n) times and consisted of two groups, one for egg counts and one for adult offspring counts. Numbers of individual females on single hosts per group varied between 10 and 77. Means are based upon the means of each replicate experiment, that is, the result of each experiment is taken as a single datum. For a more complete treatment of the experiments measuring mortality in hybrid crosses, see Breeuwer (1993).

Genotype [cytotype] of mother	VG [G] *(n= 11)*	GV [V] *(n = 9)*	V [V] *(n = 10)*	G [G] *(n = 13)*
Mean egg production ± SD	28.7 ± 6.7	21.7±4.1	43.1±12.2	29.0±6.1
Mean adult off spring ± SD	4.9 ± 1.5	10.2±5.0	31.3±8.1	24.7±6.5
Mean % F2 male survival ± SD	18±6	47 ± 21	75 ± 10	88 ± 13

eggs laid (see tables 5, 6). The result indicates that F_1 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 intraspecies 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. girault males were often prematurely terminated (pers. obs.). This observation suggests that poor insemination may be a factor in excess male production. In summary, F_1 VG [G] females appear to suffer some increased mortality. Mortality among reciprocal F_1 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.* girault, but significantly lower than *N. vitripennis* females (for pairwise comparisons, see Breeuwer 1993). *N.* girault 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 F_1 hybrid females and parental (nonhybrid) females for the duration of the experiment (3-4 d).

F_2 Recombinant Male Breakdown.-Although F_1 hybrid females did not have abnormal fecundity, there were dramatic reductions in the numbers of emerging (adult) offspring from eggs produced by F_1 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] F_2 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 ($x = 0, n = 9, P < 0.01$).

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 girault 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 [V] cytotype cannot be ruled out, although they would be considerably weaker than in the reciprocal cross. Introgression experiments were conducted to determine whether maternal effects or cytoplasmically inherited elements are involved.

Timing of *F_2 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, F_2 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] F_2 recombinant males had an additional 49% mortality during the pupal stage. They

TABLE 3. Timing of mortality of F_2 recombinant males compared to non-hybrid males produced by virgin females. Shown are mean ± SD number of eggs oviposited per host per female on the third day of oviposition on a single host in group 1; percent (%) hatching after 48 h (group 1); mean ± SD number of pupating larvae per host (group 2); and mean ± SD number of eclosing adults per host (group 3). *n,* Numbers of individual females on single hosts.

Genotype [cytotype] of mother	Eggs			Pupating larvae		Eclosing adults	
	Mean – SD	*n*	% Hatching	Mean = SD	*n*	Mean = SD	*n*
V IV]	45.1 ± 15.4	19	98	28.6 ± 11.1	20	32.7 ± 12.8	19
G [G]	28.1±8.1	18	100	21.4±7.3	17	19.7-6.5	15
GV[V]	24.9±7.3	22	92	7.7±5.3	22	7.6±3.8	19
VG [G]	27.4±9.2	23	89	5.5±5.0	19	2.6±1.7	20

TABLE 4A. The effect of fertilization on rescue of recombinant eggs. Reciprocal Fl 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 hosts.

Parental cross		Offspring: Adult offspring				Sex ratio
Female	Male	Mean - SD	*n*	Student t		Mean - SD
GV [V]	—	11.2 ± 5.5	29	6.4***	1	—
	G	16.9 ± 6.4	39	-t	5.8***	.90 ± .23
	V	20.7 ± 7.4	45	2.5**	1	.98 ± .03
VG [G]	—	3.6 ± 2.5	30	8.6***	1	—
	G	18.5-9.0	34	-J'	11***	.97-.17
	V	7.4-3.4	50	7.8***	1	.93-.17

** 0.001 < 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.

either did not undergo metamorphosis or were unable to eclose.

Fertility of F_2 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 F_2 Recombinant Progeny.-F, hybrid females were backcrossed to males of either species to determine the effects of egg fertilization on hybrid breakdown. Hybrid F, 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 paternal species. Offspring survival from F, hybrid virgin females, which produced only haploid recombinant males, was compared with that of offspring from backcrossed (mated) F_1 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 F_2 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. F_2 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 F_2 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 F_2 recombinant males (table 3).

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

TABLE 413. The effect of sperm genotype on rescue of recombinant eggs. Reciprocal F, 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. 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 host.

Oviposition day	Parental cross: Female	Male	Eggs: Mean - SD	*n*	Offspring: Adult offspring: Mean - SD	*n*	Student t	Estimated % survival: Mean - SD	Sex ratio: Mean ± SD
3	GV[V]	G	28.8-9.6	35	13.2-7.1	18	1.4	46 ± 29	.99 ± .03
		V			16.4 ± 6.6	18	1	57-30	.98-.04
4		G	34.8± 12.9	36	17.5-8.9	16	1.5	50-32	.99-.04
		V			21.6 ± 6.0	17	1	62 ± 29	.99 ± .03
3	VG [G]	G	37.6 ± 8.7	42	19.3 ± 5.5	20	6.5***	51 ± 19	.96-.04
		V			9.5 ± 3.3	20	1	25-10	.96-.04
4		G	44.8±10.0	39	24.9-7.4	20	5.5***	56-21	.99-.02
		V			13.5 ± 5.0	19	1	30-12	.97-.06

*** P < 0.001 (Student t-value and level of significance for pairwise comparisons of offspring numbers).

TABLE 5. First (B1) introgression generation. The G2V [V] females are derived from GV [V] F, 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] F, 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.

Parental cross		Eggs		Offspring: Adult offspring				Estimated survival	Sex ratio
Female	Male	Mean ± SD	n	Mean ± SD	n	Student t		Mean ± SD	Mean ± SD
G2V [V]	—	30.5 ± 19.0	44	8.2 ± 4.2	12	3.3***	1	27 ± 18	—
	G			21.7 ± 13.1	14	1	4.5***	71 ± 54	.95 ± .08
	V			21.4 ± 9.0	14	0.1	1	70 ± 44	.99 ± .02
V2G [G]	—	29.8 ± 11.6	64	4.3 ± 1.8	15	5.8***	1	14 ± 8	—
	G			19.3 ± 9.4	12	1	4.1***	65 ± 40	.99 ± 0.3
	V			8.3 ± 3.6	12	3.6***	1	28 ± 16	.90 ± .13
G [G]	—	32.6±8.6	48	22.2±7.2	12	41.4		68±28	—
	G			26.6 ± 7.9	10	1		82 ± 32	diapause
V [V]	—	52.9 ± 14.4	60	34.8 ± 10.3	16	1.7		65 ± 26	
	V			41.3 ± 9.8	15	1		78 ± 28	.88 ± .04

*** P < 0.001. (Student t-value and level of significance for pairwise comparisons of offspring numbers). 'Lines indicate which paired means are being compared.

vitripennis cytoplasm (46% vs. 57% on day 3, 50% vs. 62% on day 4). In fact, offspring numbers of reciprocal F_1 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] cytoplasm are relatively 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

TABLE 6. B4 introgression generation. The G5V [V] female 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] are derived from F, hybrid VG [G] females 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.

Parental cross		Eggs		Offspring: Adult offspring				Estimated % survival	Sex ratio
Female	Male	Mean ± SD	n	Mean ± SD	n	Student t		Mean ± SD	Mean ± SD
G5V [V]	—	27.3 ± 7.3	57	14.5 7.2	17	1.0	1	53 ± 30	—
	G			16.9 6.7	19	1	0.1	62 ± 13	.96 ± .05
	V			14.8 8.3	10	0.7	1	54 ± 34	.88 ± .08
V5G [G]	—	26.4 ± 10.7	72	2.4 2.0	22	4.7***	1	9 ± 8	—
	G			7.0 2.5	6	1	4.9***	27 ± 14	.98 ± .04
	V			6.1 3.1	34	0.7	1	23 ± 15	.95 ± .10
G [G]	—	27.7±8.0	31	21.3 7.9	18	1.0		77 ± 36	—
	G			24.3 8.4	15	1		88 ± 39	.94 ± .02
V [V]	—	48.0 ± 19.7	47	37.3 10.9	21	1.0		78 ± 39	—
	V			34.1 9.9	21	1		71 ±36	.92±.04
G5V [V]	—	31.9 ± 10.4	54	16.8 10.8	18	1.5	1	53 ± 38	—
	G			21.9 9.1	20	1	0.1	69 ± 36	.96 ± .05
	V			16.3 14.1	14	1.4	1	51 ±47	.96±.04
V5G [G]	—	28.3 ± 11.2	56	2.8 2.5	10	3.2**	1	10 ± 10	—
	G			9.5 4.7	4	1	3.0**	34±21	.96±.04
	V			5.7 2.5	23	2.3 *	1	20 ± 12	.90 ± .17
G [G]	—	41.1 ± 9.2	27	29.3 10.5	19	0.8		77 ± 36	—
	G			26.2 11.9	18	1		88±39	.92±.04
V IV]	—	59.0 ± 19.5	40	40.8 15.1	24	0.1		78 ± 39	—
	V			41.1 11.4	28	1		71 ± 36	.93 ± .03

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.* girault 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 F_1 hybrid females (table 4). Haploid (male) offspring survival from virgin hybrid females was greatly depressed compared to male offspring survival from virgin nonhybrid 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 X G [G] Y versus G [G] d XG2V[V] Y:t=1.4,df=22,P=0.08,andV[V]d X G2 V [V] 4 : t = 1.3, df = 22, P = 0.1). As among F_2 hybrid eggs, fertilization raised survival of F_3 V2G [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 girault 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 ($t = .1$, df = 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 girault sperm (t = 3.6, df = 22, P < .001), indicating persistent negative interactions between *vitripennis* nuclear genes and girault 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.* girault morphology (J. H. Werren, D. Swank, and J. A. J. Breeuwer, unpubl. data). Egg production of backcrossed females and *N.* girault 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 G5V [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 predominantly G. Concordantly, the rescue effect of fertilization on offspring survival, that was so dramatic in crosses with F_1 and B 1 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.* girault wing morphology (J. H. Werren, D. Swank, and J. A. J. Breeuwer, unpubl. data), indicating that parts of their girault genome were being retained despite repeated introgression of V genes. This result further indicates that girault nuclear genes are being selectively retained in the presence of the girault 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 girault 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 girault 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 girault cytoplasmic factors (table 6). Introgression experiments therefore indicate that cytoplasmic interaction is not due to maternal effects,

TABLE 7. Effect of cytotype on hybrid breakdown among F2 male offspring with similar recombinant VG genotype but different cytotype ([V] or [G]) and control (G) males with different cytotypes ([V] or [G]) is shown. Numbers of individual females on single hosts are between parentheses. a, third oviposition day of the same females; b, fourth oviposition day of the same females.

Genotype [cytotype] of mother	VG [V] Mean – SD	*n*	VG [G] Mean – SD	*n*	G [G] Mean = SD	*n*	*G [V]* Mean – SD	
Eggs								
a	30.2 ± 10.9	80	22.3 ± 7.5	71	29.4±11.1	59	21.4±6.8	49
b	43.9 ± 14.3	80	39.0 ± 14.0	65	41.6 ± 15.8	53	25.6 ± 8.0	50
Adult offspring								
a	13.0 ± 6.2	73	5.3 ± 2.8	74	26.0 ± 7.3	53	20.1 ± 6.3	52
b	15.7 ± 10.3	77	6.3 ± 3.8	78	39.6 ± 14.2	51	26.0 ± 8.2	49
Estimated recombinant male survival								
a	43 ± 26		24 ± 15		88 ± 42		94 ± 42	
b	36 ± 26		16 ± 11		95 ± 50		102 ± 45	

but to cytoplasmically inherited factors (or cytoplasmic genes).

Complete Introgression Line.-A (nearly) complete introgression of girault nuclear genes into vitripennis cytoplasm was accomplished by 16 backcross generations. This strain (G [V]) was used to further document the incompatibility between girault 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 girault nuclear genome combined with vitripennis cytoplasmic genes. The only difference between the two types of F_t 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_2 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. girault 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.* girault produce hybrid F_t 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 girault and nuclear genes in vitripennis. This is observed at many different levels. First, there is an increase in mortality of F, females with girault 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, girault 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 girault males that in effect had 100% girault 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 girault females with girault cytoplasm.

Thus, it can be concluded that incompatibility between vitripennis nuclear genes and girault 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 heterogamety. Hurst and Pomiankowski (1991) argued 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 coevolved with the nuclear genome they are associated with. According to this model, epistatic interactions between nuclear 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 *girault*i and nuclear genes of *vitripennis.* Epistatic interactions between nuclear genes and mitochondria are likely because a number of mitochondrial proteins are encoded by the nucleus, and other nuclear genes are involved in mitochondrial interactions. However, it has not yet been established 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 haploid genome from either parental species by fertilization. Nuclear-nuclear incompatibility is further supported by the observation 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 *girault*i nuclear genome and *vitripennis* cytoplasm mated to *vitripennis* with *vitripennis* cytoplasm, showed a twofold increase in mortality of F_2 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 F, hybrids, but becomes apparent in the F_2 or backcross generations. Lethality supposedly results from incompatible combinations of genes or perhaps whole chromosome segments. Certain combinations must be imbalanced and disturb vital developmental processes, particularly in the haploid phase. Fertilization by a balanced gamete partially reverses this imbalance. In F, hybrid females there are two complete balanced sets of chromosomes and apparently there are few or no "dominant" negative interactions between genes or chromosome segments, since the F, is generally viable. However, recombination and assortment during meiosis in F, hybrids break up favorable gene arrangements present in the parental species, derailing normal epistatic interactions in F_2 hybrid males. Introduction of a complete species chromosome set can apparently override the "negative" epistatic

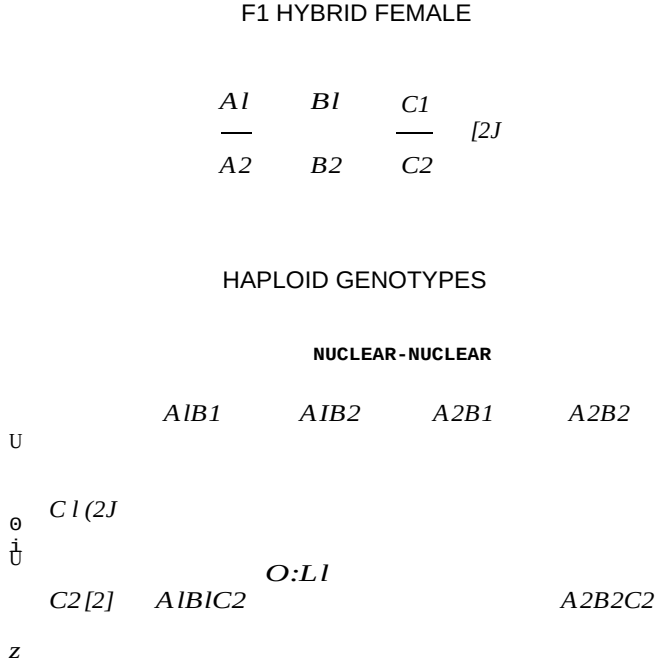

FIG. 2. Genetic model for nuclear-nuclear and nuclear-cytoplasmic interactions. Crosses between males of species 1 and females of species 2 produce hybrid F, females that are heterozygous at all three loci *A, B,* and C and inherit the cytoplasmic factors [21 from their mother. These F, hybrid females produce eight different gametes with different recombinant genotypes, but all *will* carry the [2] cytoplasmic factors. Suppose that *A* and *B* have epistatic interactions and that the presence of alleles from different species at these loci result in deregulation of these interactions causing mortality. 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, Cl nuclear allele and [21 cytoplasmic factors, recombinant eggs that receive the Cl allele *will* die, resulting in an additional 50% mortality (crossed cells). If nuclear/nuclear incompatibilities are asymmetric, then A1B2 die but A2B1 do not, giving 25% mortality from that interaction.

interactions of the recombinant complement (i.e., the negative epistasis is recessive), resulting in an increase in offspring survival.

Following Muller (1942), Orr (1993) developed a model showing that Haldane's rule results if hybrid lethality (or sterility) genes are primarily recessive. Although Haldane's rule does not directly apply to haplodiploids (the entire genome is like the X-chromosome in *Drosophila),* our data do indicate that the hybrid lethality genes are primarily recessive in this system, because of the "rescue" effect of backcrossing to either parental species. It has not been ruled out that the rescue is due to sex limited (male) expression of the hybrid breakdown genes rather than to their recessivity per se. However, although sterility genes are often sex limited, lethality genes are rarely so, suggesting that the sex limitation explanation for hybrid lethality is unlikely. A third formal explanation for the "rescue" effect is that hybrid lethal genes are sensitive to ploidy level and lethality is therefore greater in the hemizygous sex. However, somatic tissues of both males and females in *Nasonia* undergo polyploidization, and it is

unclear to what extent sex specific differences in somatic ploidy level exist.

Hybrid breakdown among grandsons of interspecific crosses between *N. vitripennis* and *N. giraulti* can be explained by as few as two sets of interacting genes; two negatively interacting nuclear genes *(A* and B), and a single nuclear gene (C) that interacts with a cytoplasmic element. Each *Nasonia* species is fixed for a particular set of coadapted alleles at each of these loci, *(A1 BI C1)* for N. *vitripennis* and *(A2B2C2)* for N. *giraulti* (fig. 2). Hybrid females are thus heterozygous for each locus, but carry [V] or [G] cytoplasm depending upon their mother species. Suppose that interactions between alleles of different species at locus *A* and *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 *A* and *B*. Half of those male will carry the G allele at locus C and the other half will carry the V allele. The latter males will die if they end up in a [G] cytoplasmic background, resulting in an additional 50% mortality among male offspring of F, hybrid females with [G] cytoplasm compared to reciprocals with [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., *A1* and *B2* are incompatible but *A2* and *B1* are the ancestral alleles and are compatible), each nuclear/nuclear interaction gives at most 25% mortality among 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 *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 *vitripennis* and *giraulti* are also involved in interactions with the third sibling species, *N. longicornis.*

The *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.

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LITERATURE CITED

Assem, J. van den, and J. H. Werren. 1994. A comparison of the courtship and mating behavior of three species of *Nasonia* (Hym., Pteromalidae). Journal of Insect Behavior 7:53-66.

Avise, J. C. 1991. Ten unorthodox perspectives on evolution prompted by comparative population genetic findings on mitochondrial DNA. Annual Review of Genetics 25:45-69.

Beeman, R. W., K. S. Friesen, and R. E. Denell. 1992. Maternal-effect selfish genes in flour beetles. Science 256:89-92.

Baird, S. E., M. E. Sutherlin, and S. W. Emmons. 1992. Reproductive isolation in Rhabditidae (Nematoda: Secernentea); mechanisms that isolate six species of three genera. Evolution 46:585-594.

Beukeboom, L. W. 1993. Genetics and population biology of the paternal sex ratio (PSR) chromosome. Ph.D. diss. University of Rochester, Rochester, N.Y.

Breeuwer, J. A. J. 1993. Postzygotic reproductive isolation in the genus *Nasonia* (Hymenoptera, Pteromalidae): the role of cytoplasmic microorganisms and other heritable cytoplasmic factors. Ph.D. disc. University of Rochester, Rochester N.Y.

Breeuwer, J. A. J., and J. H. Werren. 1990. Microorganisms associated with chromosome destruction and reproductive isolation between two insect species. Nature 346:558-560.

———. 1993. The effect of genotype on cytoplasmic incompatibility between two species of *Nasonia.* Heredity 70:428-436.

Breeuwer, J. A. J., R. Stouthamer, D. A. Burns, D. A. Pelletier, W. G. Weisburg, and J. H. Werren. 1992. Phylogeny of cytoplasmic incompatibility microorganisms in the parasitoid wasp genus *Nasonia* (Hymenoptera: Pteromalidae) based on 16S ribosomal DNA sequences. Insect Molecular Biology 1:25-36.

Brown, W. M., M. George, and A. C. Wilson. 1979. Rapid evolution of animal mitochondrial DNA. Proceedings of the National Academy of Sciences, USA 76:1967-1971.

Christie, P., and M. R. MacNair. 1984. Complementary lethal factors in two North American populations of the yellow monkey flower. Journal of Heredity 75:510-511.

———. 1987. The distribution of postmating reproductive isolating genes in populations of the yellow monkey flower. Evolution 41:571-578.

Clark, A. G. 1984. Natural selection with nuclear and cytoplasmic transmission. I. A deterministic model. Genetics 107:679-701.

Clark, A. G., and E. M. S. Lyckegaard. 1990. Natural selection with nuclear and cytoplasmic transmission. Ill. Joint analysis of segregation and mtdna in *Drosophila melanogaster.* Genetics 118:471-481.

Coyne, J. A. 1992. Genetics and speciation. Nature 355:511-515.

Coyne, J. A., and B. Charlesworth. 1986. Genetic analysis of X-linked sterility in hybrids between three sibling species of *Drosophila.* Heredity 62:97-106.

Coyne, J. A., and A. R. Orr. 1989. Two rules of speciation. Pp. 180-207 *in* D. Otte and J. A. Endler, eds. Speciation and its consequences. Sinauer, Sunderland, Mass.

Darling, D. C., and J. H. Werren. 1989. Biosystematics of *Nasonia* (Hymenoptera, Pteromalidae), two new species reared from

birds' nests in North America. Annals of the Entomological Society of America 83:352-369.
de Boer, R. 1981. Genetic affinities between spider mite *Tetranychus urticae* populations in a non-agricultural area 11. Entomologia Experimentalis et Applicata 30:63-67.
1982. Laboratory hybridization between semi-incompatible races of the arrhenotokous spider mite *Tetranychus urticae* Koch (Acari: Tetranychidae). Evolution 36:553-560.
Dobzhansky, Th. 1937. Genetics and the origin of species. Columbia University Press, New York.
. 1970. Genetics of the evolutionary process. Columbia University Press, New York.
Edwardson, J. R. 1970. Cytoplasmic male sterility. Botanical Review 36:341-420.
Ehrman, L. 1983. Endosymbiosis. Pp. 128-136 *in* D. J. Futuyma, ed. Coevolution. Sinauer, Sunderland, Mass.
Engels, W. R., and C. R. Preston. 1979. Hybrid dysgenesis in *Drosophila melanogaster:* the biology of male and female sterility. Genetics 92:161-174.
Frank, S. A. 1991. Divergence of meiotic drive suppression systems as an explanation for biased hybrid sterility and inviability. Evolution 45:262-267.
Fry, J. D. 1989. Nuclear-nuclear and nuclear-cytoplasmic interactions contribute to the reproductive incompatibility between two strains of the twospotted spider mite. Entomologia Experimentalis et Applicata 50:97-100.
Gerstel, D. U. 1954. A new lethal combination in interspecific cotton hybrids. Genetics 39:628-639.
Grun, P. 1976. Cytoplasmic genetics and evolution. Columbia University Press, New York.
Haldane, J. B. S. 1922. Sex-ratio and unisexual sterility in hybrid animals. Journal of Genetics 12:101-109.
Hollingshead, L. 1930. A lethal factor in *Crepis* effective only in an interspecific hybrid. Genetics 15:114-140.
Hurst, L. D. 1993a. The incidences, mechanisms and evolution of cytoplasmic sex ratio distorters in animals. Biological Review 68:121-193.
. 1993b. *scat+ Is* a selfish gene analogous to *Medea* of *Tribolium castaneum*. Cell 75:407-408.
Hurst, L. D., and A. Pomiankowski. 1991. Causes of sex ratio bias within species may also account for unisexual sterility in species hybrids: a new explanation for Haldane's rule and related phenomena. Genetics 128:841-858.
Hutter, P, and M. Ashburner. 1987. Genetic rescue of inviable hybrids between *Drosophila melanogaster* and its sibling species. Nature 327:331-333
Hutter, P, J. Roote, and M. Ashburner. 1990. A genetic basis for the inviability of hybrids between sibling species of *Drosophila.* Genetics 124:909-920.
Jablonka, E., and M. J. Lamb. 1989. The inheritance of acquired epigenetic variations. Journal of Theoretical Biology 139:69-83.
Kidwell, M. G. 1983. Evolution of hybrid dysgenesis determinants in *Drosophila melanogaster.* Proceedings of the National Academy of Sciences, USA 80:1655-1659.
Killias, G., and S. N. Alahiotis. 1982. Genetic studies on sexual isolation and hybrid sterility in long-term cage populations of *Drosophila melanogaster.* Evolution 36:121-131.
MacRae, A. F, and W. W Anderson. 1988. Evidence for non-neutrality of mitochondrial DNA haplotypes in *Drosophila pseudoobscura.* Genetics 120:485-494.
Mayr, E. 1973. Animal species and evolution. Harvard University Press, Cambridge, Mass.
Mood, A. M., E A. Graybill, and D. C. Boes. 1974. Introduction to the theory of statistics, 3d ed. McGraw-Hill, Singapore.
Moritz, C., T. E. Dowling, and W. M. Brown. 1989. Evolution of animal mitochondrial DNA: relevance for populations biology and systematics. Annual Review of Ecology and Systematics 18: 269-292.
Muller, H. J. 1942. Isolating mechanisms, evolution and temperature. Biological Symposia 6:71-125.
Naveira, H. F 1992. Location of X-linked polygenic effects causing sterility in male hybrids of *Drosophila simulans* and *D. mauritiana.* Heredity 68:211-217.
Nei, M., T. Maryama, and C.-I Wu. 1983. Models of evolution of reproductive isolation. Genetics 103:557-579.
Nigro, L. 1993. Nuclear background affects frequency dynamics of mitochondrial DNA variants in *Drosophila simulans.* Heredity 72:582-586.
Oka, H.-I. 1957. Genetic analysis for the sterility of hybrids between distantly related varieties of cultivated rice. Journal of Genetics 55:397-409.
O'Neill, S., R. Giordano, A. M. E. Colbert, T. L. Karr, and H. M. Robertson. 1992. 16s rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. Proceedings of the National Academy of Sciences, USA 89:2649-2702.
Orr, H. A. 1987. Genetics of male and female sterility in hybrids of *Drosophila pseudoobscura* and *D. persimilis.* Genetics 116: 555-563.
1989. Genetics of sterility in hybrids between two subspecies of *Drosophila.* Evolution 43:180-189.
Orr, H. A., and J. A. Coyne. 1989. The genetics of postzygotic isolation in the *Drosophila virilis* group. Genetics 121:527-537.
Otte, D., and J. A. Endler. 1989. Speciation and its Consequences. Sinauer, Sunderland, Mass.
Overmeer, W. P J., and A. Q. van Zon. 1976. partial reproductive incompatibility between populations of spider mites (Acarina: Tetranychidae). Entomologia Experimentalis et Applicata 20: 225-236.
Palopoli, M. E, and C.-I Wu. 1994. Genetics of hybrid male sterility between *Drosophila* sibling species: a complex web of epistasis is revealed in interspecific studies. Genetics 138: 329-341.
Perez, E. P., C.-I Wu, N. A. Johnson, and M.-L. Wu. 1993. Genetics of reproductive isolation in the *Drosophila simulans* clade: DNA marker-assisted mapping and characterization of a hybrid-male sterility gene, *Odysseus (Ods).* Genetics 133: 261-275.
Pontecorvo, G. 1943. Viability interactions between chromosomes of *Drosophila melanogaster* and *Drosophila simulans.* Journal of Genetics 45:51-66.
Powell, J. R. 1982. Genetic and nongenetic mechanisms of speciation. Pp. 67-74 *in* C. Barigozzi, eds. Mechanisms of speciation. Alan R. Liss, New York.
1983. Interspecific gene flow in the absence of nuclear gene flow: evidence from *Drosophila.* Proceedings of the National Academy of Sciences, USA 80:492-495.
Rose, M., and W. Doolittle. 1983. Molecular biological mechanisms of speciation. Science 20:157-162.
Siegel, S. 1965. Non-parametric statistics for the behavioral sciences. McGraw-Hill, London.
Stouthamer, R., R. E Luck, and W. D. Hamilton. 1990. Antibiotics cause parthenogenetic *Trichogramma* (Hymenoptera / Trichogrammatidae) to revert to sex. Proceedings of the National Academy of Sciences, USA 87:2424-2427.
Templeton, A. R. 1982. Genetic architectures of speciation. Pp. 105-121 *in* C. Barigozzi, ed. Mechanisms of speciation. Alan R. Liss, New York.
Thompson, J. N. 1987. Symbiont-induced speciation. Biological Journal of the Linnean Society 32:385-393.
van Damme, J. M. M., and W. van Delden. 1982. Gynodioecy in *Plantago lanceolata* L. I. Polymorphism for plasmon type. Heredity 49:303-318.
Wallace, B. 1981. Basic population genetics. Columbia University Press, New York.
Watanabe, T. K. 1979. A gene that rescues the lethal hybrids between *Drosophila melanogaster* and *D. simulans.* Japanese Journal of Genetics 52:1-8.
Whiting, A. R. 1967. The biology of the parasitic wasp *Mormoniella vitripennis [= Nasonia brevicornis]* (Walker). Quarterly Review of Biology 42:333-406.
Wu, C.-I. 1992. A note on Haldane's rule: hybrid inviability versus hybrid sterility. Evolution 46:923-931.
Wu, C.-I, and A. T. Beckenbach. 1983. Evidence for extensive genetic differentiation between the sex-ratio and the standard

arrangement of *Drosophila pseudoobscura* and *D. persimilis* and identification of hybrid sterility factors. Genetics 105:71-86.

Wu, C.-I, D. E. Perez, A. W. Davis, N. A. Johnson, E. L. Cabot, M. E Palopoli, and M.-L. Wu. 1992. Molecular genetic studies of postmating reproductive isolation in *Drosophila*. Pp. 199-212 *in* N. Takahata and A. G. Clark, eds. Mechanisms of molecular evolution. Sinauer, Sunderland, Mass.

Zeng, L.-W., and R. S. Singh. 1993. The genetic basis of Haldane's rule and the nature of asymmetric hybrid male sterility among *Drosophila simulans, Drosophila mauritiana* and *Drosophila sechellia*. Genetics 134: 251-260.

Zouros, E. 1981. The chromosomal basis of viability in interspecific hybrids between *Drosophila arizonensis* and *Drosophila mojavensis*. Canadian Journal of Genetics and Zoology 63:65-72.

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