

Inheritance of Gynandromorphism in the Parasitic Wasp *Nasonia vitripennis*

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ABSTRACT

The parasitic wasp *Nasonia vitripennis* has haplo-diploid sex determination. Males develop from unfertilized eggs and are haploid, whereas females develop from fertilized eggs and are diploid. Females and males can be easily distinguished by their morphology. A strain that produces individuals with both male and female features (gynandromorphs) is studied. We provide data on female/male patterning within and between individuals, on environmental effects influencing the occurrence of gynandromorphism, and on its pattern of inheritance. A clear anterior/posterior pattern of feminization is evident in gynandromorphic individuals that developed from unfertilized haploid eggs. The proportion of gynandromorphic individuals can be increased by exposing the mothers to high temperature and also by exposing embryos at early stages of development. Selection for increased gynandromorph frequency was successful. Backcross and introgression experiments showed that a combination of a nuclear and a heritable cytoplasmic component causes gynandromorphism. Analyses of reciprocal F₂ and F₃ progeny indicate a maternal effect locus (*gyn1*) that maps to chromosome IV. Coupled with previous studies, our results are consistent with a *N. vitripennis* sex determination involving a maternal/zygotic balance system and/or maternal imprinting. Genetics and temperature effects suggest a temperature-sensitive mutation of a maternally produced masculinizing product that acts during a critical period in early embryogenesis.

ALMOST all taxa contain species with two sexes: males and females. However, the genetic mechanisms underlying the establishment of the two sexes are quite diverse. From an evolutionary point of view, it is important to understand the genetics behind the various mechanisms. In many organisms sex determination relies on heteromorphic sex chromosomes. In mammals the presence of the Y chromosome is the primary determinant of maleness and in *Drosophila* the ratio of X chromosome to autosomes is the key factor for sex determination. Chromosomal sex determination also applies for birds and fish. This type of primary sex determination does not hold for the order Hymenoptera, which includes ants, bees, and wasps. These insects have a haplo-diploid sex determination system: haploid males arise from unfertilized eggs, while diploid females arise from fertilized eggs. However, diploid males and triploid females have also been reported (WHITING 1960), but never haploid females. It is unclear how this can be reconciled with the mechanism of sex determination.

The honeybee, a member of the Hymenoptera order, has single locus complementary sex determination (sl-CSD) (MACKENSEN 1955; LAIDLAW *et al.* 1956). BEYE *et al.*

(2003) characterized the *csd* gene and found many alleles with different amino acid sequences. Heterozygotes for this gene develop into females, whereas hemi- and homozygotes develop into males. Inactivation of the *csd* gene also leads to development of males. The consequence of this mode of sex determination is the presence of diploid males, which can easily be generated by inbreeding under laboratory conditions. This type of sex determination was originally demonstrated 60 years ago for the parasitic wasp *Bracon hebetor* (WHITING 1943) and has now been confirmed for >60 species of Hymenoptera (STOUTHAMER *et al.* 1992; COOK 1993a,b; PERIQUET *et al.* 1993; BUTCHER *et al.* 2000; VAN WILGENBURG *et al.* 2006). However, not all species generate diploid males by inbreeding and these therefore do not have sl-CSD. This has led to the development of alternative models for haplo-diploid sex determination, as discussed and reviewed by COOK (1993b), BEUKEBOOM (1995), and DOBSON and TANOUYE (1998).

Nasonia vitripennis, a small parasitic wasp, is one of the species that does not have sl-CSD (WHITING 1967; SKINNER and WERREN 1980), although, as in other Hymenoptera, it has a haplo-diploid system of sex determination. Some exceptional individuals have been found, such as fertile diploid males and triploid females (WHITING 1960), but these diploid males appear to have arisen by mutation rather than by homozygosity at the sex locus.

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Females and males of a particular species can generally be distinguished on the basis of their secondary external sexual traits. Occasionally, individuals with both female and male “external” characters occur. Such gynandromorphic individuals are widespread among taxa, but typically occur at very low frequencies. They have been reported from mammals, birds, fish, and insects (STERN 1968 and references therein), including >60 species of bees (reviewed by WCISLO *et al.* 2004).

Several distinctive female/male patterns within individuals have been found, including mosaic, bilateral, and anterior/posterior (A/P). The particular phenotype probably depends on which “failure” occurs in the early stages of development. The type of failure will depend on the sex determination system of the species involved. Bisexual morphs may originate from mitotic aberrations during early embryogenesis, the presence of two nuclei in some eggs, retarded fertilization, or aneuploidy (gain or loss of sex chromosomes) like the Klinefelter (XXY) and Turner (X0) syndromes in humans. Multiple types of gynandromorphism may occur within a species, *e.g.*, in the shrimp Anostraca (SASSAMAN and FUGATE 1997) and in the wasp *Habrobracon juglandis* (CLARK *et al.* 1971). Gynandromorphs may develop from fertilized as well as from unfertilized eggs, as shown for honeybees (ROTHENBUHLER *et al.* 1952) and the parasitic wasp *H. juglandis* (CLARK *et al.* 1971). In the latter species the recessive mutant *ebony* (dark body color) increases the frequency of gynandromorphs in fertilized eggs to ~5% (CLARK *et al.* 1968). Such a phenomenon has also been observed in *Drosophila simulans* (STURTEVANT 1929) and in *D. melanogaster* (SEQUEIRA *et al.* 1989) where the third chromosome recessive mutant *claret* (red eyes) induces the production of gynandromorphs by means of both maternal and paternal X chromosome elimination.

Gynandromorphs may also originate from an incorrect functioning of the sex determination system, for example, in *Drosophila*, where the primary signal for sex determination depends on the ratio of sex chromosomes and autosomes. Variants for each of the genes involved in the sex determination cascade, such as *Sex-lethal* (*Sxl*), *transformer* (*tra*), and *doublesex* (*dsx*), can lead to gynandromorphic individuals (CLINE and MEYER 1996 and references therein). Many of these variants are temperature sensitive. Indeed, environmental conditions appear to strongly affect the occurrence of gynandromorphism. It has been shown, for example, that the proportion of gynandromorphic individuals can be increased by short pulses of high temperature in Hymenoptera, such as *Habrobracon*, Trichogrammatids, and Encyrtids, by egg chilling or by an increase in egg-laying intensity in bees (reviewed in BERGERARD 1972).

Here we describe studies of gynandromorphism in *N. vitripennis* and relate our findings to the underlying genetic mechanisms of haplo-diploid sex determination. A natural *N. vitripennis* strain collected in Canada was found to produce gynandromorphs at ~5% among

unfertilized eggs. We investigated the genetic basis of this trait, possible influences of cytoplasmically inherited factors (*e.g.*, Wolbachia and mitochondria), pattern of gynandromorphism, ploidy of gynandromorphs, and effects of temperature at different stages of development on frequency of the trait. Data are discussed in relation to the origin and presence of gynandromorphism in other taxa, along with the possible role of gynandromorphism in unravelling the mode of sex determination in *Nasonia*. We propose adjustments to existing models for sex determination in *Nasonia* on the basis of these data.

MATERIALS AND METHODS

Nasonia biology and maintenance: *Nasonia* are small (2–3 mm) parasitic wasps, which are easily cultured on *Sarcophaga bullata* or *Calliphora vicina* pupae hosts under laboratory conditions. Infection with Wolbachia bacteria is an important mechanism of reproductive isolation in the *Nasonia* sibling species group (*N. vitripennis*, *N. longicornis*, and *N. giraulti*). The biosystematics of the *Nasonia* species complex has been extensively described by DARLING and WERREN (1990). Sex determination in *Nasonia* follows the haplo-diploid system: haploid males develop from unfertilized eggs, while diploid females develop from fertilized eggs. Virgin females can easily be collected from hosts parasitized by mated females by opening the host pupae before the wasps emerge. Strains are kept in mass culture at 25° or in diapause at 4°. Typically ~20 females were provided with ~50 hosts for life. Adult progeny emerged ~15 days later at 25°. All experiments discussed below were conducted at 25° unless stated otherwise.

Laboratory and field strains: *N. vitripennis* field strains were derived from single females collected from their natural habitat and maintained in the lab in mass culture at 25° or in diapause at 4°. *Nasonia* females were collected from Canada, Idaho, Indiana, Michigan, New York, Utah, and Wyoming. For the various experiments we used the following *N. vitripennis* lab strains: AsymC (wild-type lab strain cured from Wolbachia), OR₁₂₃ (orange eyes), ST₅₂₁₉ (red eyes), and Stdr (red eyes). Furthermore, we used the *paternal sex ratio* (PSR) strain—a strain with a supernumerary chromosome that is transmitted through sperm but then induces the loss of the paternal chromosomes (except itself) after fertilization of the egg (WERREN 1991).

Female and male external morphology: Males and females can be distinguished on the basis of the following external morphology (DARLING and WERREN 1990): (1) antennae—male antennae are thinner and yellow throughout, whereas female antennae are dark brown and thicker; (2) wings—male wings in *N. vitripennis* are rudimentary, narrow, and short, not reaching the abdomen tip, whereas females have full-sized wings that extend beyond the abdomen; (3) legs—male legs are yellow throughout, whereas the proximal region of female legs are dark brown; and (4) external genitalia—the distal abdominal tergites of males are continuous, whereas female abdominal tergites are interrupted medially to allow extrusion of the ovipositor. The tip of the male abdomen is round and that of the female is pointed. These 11 landmarks on the adult body can readily be scored for sex: two antennae, two hind wings, six legs, and the genital region (Figure 1, A and B).

Frequency, pattern, and fertility of gynandromorphs: Frequency and pattern of gynandromorphism were scored by placing virgin females on hosts and recording the number and pattern of gynandromorphism among their progeny. The 11

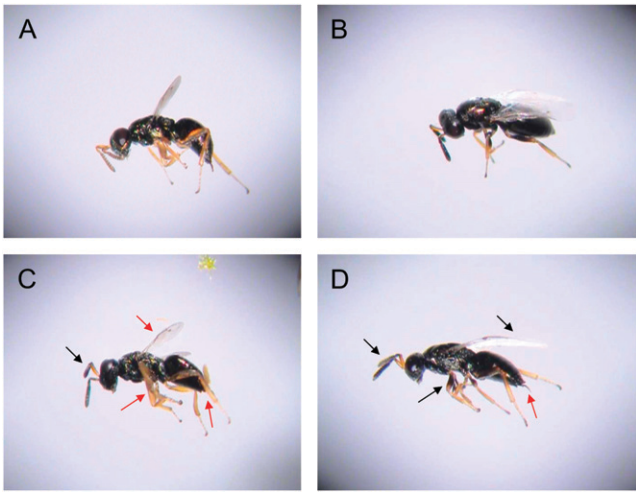


FIGURE 1.—Morphology of *N. vitripennis*. Male (A), female (B), gynandromorph with female antennae (C), gynandromorph with female antennae, wings, and legs (D). Black arrows indicate femaleness; red arrows indicate maleness.

landmarks described above were used to determine the pattern of male and female external body parts (Figure 1). On the basis of external morphology, a proportion of the parthenogenetic progeny exhibited purely female characteristics. These morphological females were set on hosts for life to determine whether they were capable of reproduction.

Selection for high and low frequency of gynandromorphs:

An experiment was conducted to determine whether the frequency of gynandromorphs could be increased and/or decreased by directional selection. Females from the CD₁₂ field strain were first set individually as virgins for 3 days on two hosts each and subsequently transferred to a new vial, mated, and provided with two new hosts. Frequencies of gynandromorphs were scored among the uniparental offspring of each female from the first setting. Six daughters of each of the five females producing the highest and each of the five females producing the lowest gynandromorph frequencies in the first setting were used to establish a high (HiCD₁₂) and a low (LoCD₁₂) selection line, respectively. Thereafter, in each generation, two sets of 30 families were maintained to select families for the next generation. After eight generations of selection, the selected HiCD₁₂ and LoCD₁₂ lines were maintained by standard culturing procedures without further selection.

The influence of fertilization on gynandromorph production:

The following experiment was conducted to determine whether the incidence of gynandromorphs is altered by fertilization, independently of the ploidy effects of fertilization. This was accomplished by mating HiCD₁₂ females to males carrying the PSR chromosome (WERREN 1991). PSR is a supernumerary chromosome transmitted through sperm that, after fertilization of the egg, causes condensation and loss of the paternal chromosomes (except itself). So, the mating above results in eggs that have been fertilized but which are effectively haploid, leading to all-male families. Paternal chromosome loss occurs at the first mitosis due to abnormal condensation of the paternal chromosomes. Controls for the experiment were virgin HiCD₁₂ females. Females were placed on hosts at 31°. Progeny of individual females were scored for family size and gynandromorph production.

Potential role of intracellular bacteria: Some intracellular bacteria such as *Wolbachia* are known to induce parthenogenesis in certain species of parasitoid wasps (STOUTHAMER *et al.* 1993). To test for the possible role of these or other

intracellular bacteria, tests were performed using (1) PCR amplification of *Wolbachia*-specific genes (ZHOU *et al.* 1998), (2) PCR amplification of 16S ribosomal DNA to detect the presence of any prokaryotic endosymbionts (LANE 1991), (3) cytological examination of eggs (BREEUWER and WERREN 1990), and (4) tetracycline treatment (BREEUWER and WERREN 1993).

Temperature effects on gynandromorphism: The goal of this experiment was to study the effect of environmental temperature on the production of gynandromorphic individuals. One generation prior to the test, inseminated females of the HiCD₁₂ strain were individually put in vials with three hosts at 20°. One virgin daughter of each female was used to parasitize hosts at a particular temperature. Before starting the experiment, the virgin daughters were collected in the pupal stage (inside the host pupae), individually put in small vials with three hosts each, and kept for 5 days at 20°. Then the experiment was performed at 20°, 25°, 29°, and 31° with three hosts per female. After 3 days, the wasps were transferred to new vials and supplied with three fresh hosts. The emerging adults were scored for the 11 distinguishing male and female characteristics. The fraction of gynandromorphs was calculated for each individual mother.

Timing of gynandromorph induction by high temperature: Since a strong effect of temperature on the proportion of gynandromorphs was found, we set up an experiment to determine whether there is a developmental stage sensitive to gynandromorph induction. Virgin *N. vitripennis* females of the HiCD₁₂ strain were individually allowed to parasitize hosts at 31° for a restricted period of 2 hr to minimize variation in age of the eggs within each age class. After various time intervals of 4 hr, the parasitized hosts were transferred to 20° for further development.

To test whether the developmental stage of the eggs influenced the induction of gynandromorphs at 31°, a similar experiment was performed. Whereas in the previous experiment the hosts were parasitized at 31°, now hosts were parasitized at 20° and then transferred to 31° after various time intervals of 4 hr. Various developmental stages of the eggs were obtained to acquire information about the embryonic stage that is sensitive to the induction of gynandromorphs.

Effect of adult treatment on gynandromorph production: The increase in gynandromorph production at 31° may result from influences either directly or indirectly acting on the early developmental processes in the egg. Therefore, we also performed an experiment to analyze the effects of preconditioning the adult virgin HiCD₁₂ females. Adults were kept for various periods at 31° as well as at 20° (control). Then they were allowed to parasitize hosts at 20°. Females were transferred to new hosts two times. Development of the offspring occurred at the parasitizing temperature. For these experiments, the emerging adults were scored for the 11 distinguishing male and female characteristics as described before. The proportion of gynandromorphs and the number of offspring were calculated for each individual female. Numbers of individual females and details of the other variables are given in Tables 3–5.

The role of nuclear and cytoplasmic components: An experiment was conducted to determine the role of nuclear *vs.* inherited cytoplasm (*e.g.*, mitochondria or intracellular bacteria) in gynandromorph production. Crosses were performed between the HiCD₁₂ line (designated H) and the *Wolbachia*-cured laboratory strain AsymC (designated A), to introgress the H nuclear genome into the A cytoplasm and the A nuclear genome into the H cytoplasm by repeated backcrossing. Four types of lines were established, with CYT indicating the cytoplasmic origin and females being indicated first: (1) H^{CYT} × H (H control), (2) A^{CYT} × A (wild-type control),

TABLE 1
Frequencies of gynandromorphs in *N. vitripennis* field strains from different geographic origins

Field strain	Origin	No. of mothers tested	No. of mothers producing gynandromorphs	% gynandromorphs (<i>N</i>)	Mean no. of offspring (SE)
NV CD12	Canada	14	14	9.27 (c) (81)	62.4 (a) (6.5)
NV XIDB433AP	Idaho	15	0	0 (0)	93.5 (b, c) (6.3)
NV IN2217	Indiana	13	2	0.23 (a) (3)	100.4 (b, c) (8.7)
NV IN226	Indiana	14	13	3.52 (b) (54)	109.6 (c) (7.0)
NV MI003C	Michigan	14	1	0.07 (a) (1)	100.0 (b, c) (8.7)
NV MI204	Michigan	13	0	0 (0)	119.5 (c) (8.3)
NV SP013	New York	15	2	0.14 (a) (2)	96.7 (b, c) (8.1)
NV R0020	New York	12	2	0.17 (a) (2)	96.4 (b, c) (9.6)
NV UTC402C	Utah	12	0	0 (0)	75.6 (a, b) (9.6)
NV XUTC406A	Utah	12	0	0 (0)	91.9 (b, c) (10.6)
NV WYC400G	Wyoming	15	3	0.23 (a) (4)	118.3 (c) (5.8)

Different lowercase letters in parentheses indicate a significant difference at the 5% level.

(3) $H^{CT} \times A$ (replacement of H nuclear genome with A in H cytoplasm), and (4) $A^{CT} \times H$ (replacement of A nuclear genome with H in A cytoplasm). Ten families were maintained per line. In each generation, 5 virgin females/family were backcrossed to three males from the indicated line and then mass cultured. In addition, 3 virgin females were collected per family and provided with one host each (30 females total) to assay for gynandromorph production. Two additional lines were established: (1) B1 ($H^{CT} \times A$) \times H (a line established by taking F_1 females from the $H^{CT} \times A$ cross and subsequently backcrossing to H each generation) and (2) B6 ($H^{CT} \times A$) \times H (a line established in the sixth generation of $H^{CT} \times A$ by backcrossing to H males each generation). The B6 ($H^{CT} \times A$) \times H line was established to test whether a heritable cytoplasmic component from the H line is retained after six generations of backcrossing to A.

Mapping of a locus for gynandromorphism: The goals of the following experiments are (1) to identify the linkage group(s) on which the nuclear gene(s) reside that cause gynandromorphism and (2) to determine whether the trait is due to the genotype of the mother or to the genotype of the zygote. Crosses were performed between the HiCD₁₂ strain and recessive eye-color marker strains from two different linkage groups of *N. vitripennis*. The linkage groups were chosen on the basis of preliminary data. The following Wolbachia-cured strains were used: Or₁₂₃ (orange eyes), St₅₂₁₉ (red eyes), and Stdr (red eyes). The genes coding for the first two mutants are located on chromosome IV and Stdr is located on chromosome V (based on the chromosome numbering of RÜTTEN *et al.* 2004). All crosses were performed reciprocally at 20°. The resulting F_1 females were collected as virgins, aged for 5 days at 20°, and subsequently allowed to individually parasitize hosts at 31°. The emerging F_2 's were scored for eye color and male and female external characteristics.

As the analyses of the progeny of the F_1 virgin females pointed to a maternal effect, F_1 females were backcrossed with HiCD₁₂ and Or₁₂₃ males. Resulting heterozygous and homozygous F_2 females with either the HiCD₁₂ or the Or₁₂₃ cytotype were bred as virgins, and their F_3 progeny were scored for eye color and gynandromorphism.

Statistical analyses: Prior to statistical analysis, the proportions of gynandromorphic individuals were angular transformed. ANOVAS, Tukey tests for multiple comparison of means, *t*-tests, and χ^2 tests were performed by using Statistix 4.0 Analytical software. Nonparametric statistics were used to

compare strains [Mann–Whitney *U*-test (MWU) and Wilcoxon matched-pairs signed ranks test].

RESULTS

Basic characterization

Gynandromorphism in *N. vitripennis* field strains: Following discovery of gynandromorphism in natural isolates of *N. vitripennis*, 11 field-collected strains were tested for gynandromorph production (Table 1). This was accomplished by setting females as virgins and scoring for gynandromorphs among their progeny. Of the two lines that produced >1% gynandromorphs (CD₁₂ from Canada and IN₂₂₆ from Indiana), all but one of the females tested produced some gynandromorphs among their progeny. The mean offspring number of the tested field strains is also shown in Table 1, where significant differences between strains are indicated. The lowest number of offspring and the highest proportion of gynandromorphs occurred in the CD₁₂ strain. The observed frequencies in the field strains suggest that gynandromorph production is not an unusual phenomenon in natural populations of *N. vitripennis*.

Gynandromorphism in the CD₁₂ field strain: We further characterized gynandromorphism in the CD₁₂ strain. Virgin females from the original CD₁₂ line were found to produce gynandromorphs (up to 10%) and males, whereas only males are normally expected from unfertilized eggs in this haplo-diploid insect. The gynandromorphs either could be derived from haploid eggs or could result from nondisjunction during meiosis in eggs, giving rise to diploid embryos for all or some chromosomes. Cytological examinations of 459 developing eggs and brain tissue from 99 4-day-old larvae show only haploids, whereas 40% gynandromorphs and uniparental females were expected on the basis of the emerging control wasps (BEUKEBOOM *et al.* 2007). This result

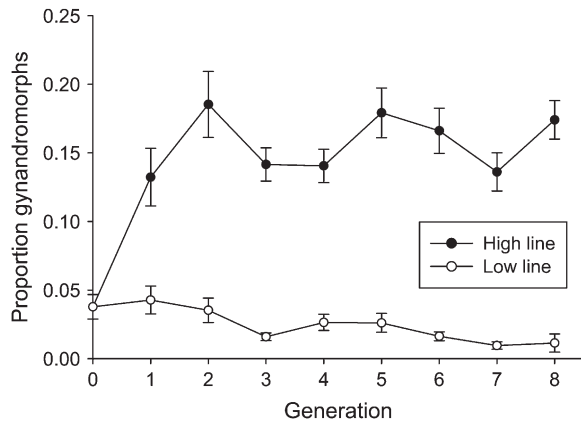


FIGURE 2.—Bidirectional selection for gynandromorph frequencies. Mean frequencies of gynandromorphs (with standard errors) among the uniparental offspring of 30 individual females during eight generations of selection for high and low frequencies of gynandromorphs.

shows that gynandromorphs and uniparental females are haploid and are not derived from nondisjunction of maternal chromosomes or other mechanisms, such as endoduplication, that could result in diploidization.

Selection experiment—production of high and low gynandromorph lines: Data on the CD₁₂ line suggested that it might contain genetic (or epigenetic) variation in gynandromorph production. We therefore initiated a selection experiment for high and low production of gynandromorphs (Figure 2). A significant response for high gynandromorph production was found after one generation of selection and the frequency remained high for seven more generations of selection without a significant increase. This shows that the original field strain was polymorphic for gynandromorphism and suggests that the genetics behind gynandromorph production is relatively simple. The “Low line” in Figure 2 shows a decreasing trend in frequency of gynandromorphs. From generation 1 onward, there was a significant difference in gynandromorph frequency between the “High line” and the “Low line” ($P < 0.001$ for each generation of selection). The selection lines were subsequently identified as HiCD₁₂ and LoCD₁₂, respectively.

After selection, the HiCD₁₂ line was maintained primarily by diapause at 4°, resulting in approximately one generation/year. After 9 years, the HiCD₁₂ line was again tested and still produced individuals exhibiting both male and female external characters. Each of the 20 tested virgin females produced both males and gynandromorphs from haploid unfertilized eggs. The mean percentage of gynandromorphs was 14.9% at 25°, which was similar to the result of selection at generation 8 (17.3%). This indicates that production of gynandromorphs remained in the selected line after selection-relaxation, probably as a result of homozygosity for alleles underlying gynandromorphism.

Taking advantage of the PSR chromosome in *Nasonia*, we could investigate whether fertilization of the egg

per se alters the probability of gynandromorphism, as opposed to the effects of diploidy restoration in normal fertilized eggs. Matings with PSR males lead to the transformation of diploid zygotes into haploid zygotes, due to improper condensation of the paternal chromosomes in the first mitosis, and generate sons only (WERREN 1991). HiCD₁₂ females ($N = 16$) mated with PSR males produced a high proportion of gynandromorphs (mean = 0.483, SE = 0.033) at 31°. This value was similar to the offspring of HiCD₁₂ virgin females at that temperature (mean = 0.458, SE = 0.036; see also Table 3), indicating that fertilization *per se* has no effect on the production of gynandromorphs.

Potential role of intracellular bacteria: Intracellular bacteria such as *Wolbachia* are known to alter sex determination in different insects, including parasitic wasps (WERREN 1997). Therefore, we investigated whether *Wolbachia* or some other intracellular bacteria were potentially involved in gynandromorph production by screening the HiCD₁₂ strain for *Wolbachia*, by cytological examination, and by antibiotic treatment. No amplification product was obtained from HiCD₁₂ using *Wolbachia*-specific primers, although controls amplified properly, indicating that this strain is free of *Wolbachia*. Since nearly all natural isolates of *N. vitripennis* carry *Wolbachia* (BORDENSTEIN and WERREN 1998), we suspect that the selected HiCD₁₂ strain lost its endosymbiont during prolonged diapause prior to testing, as has been observed in the past (PERROT-MINNOT and WERREN 1999). The absence of *Wolbachia* in the HiCD₁₂ strain was also confirmed in an indirect way: crosses with strains infected with *Wolbachia* proved to be incompatible (unidirectional: infected ♂ × HiCD₁₂ ♀). Further analysis showed no amplification product with general 16S ribosomal DNA derived from the HiCD₁₂ strain and hence indicated the absence of other prokaryotic endosymbionts. The positive controls showed an amplification product of ~1400 bp. Moreover, treatment with the broad-spectrum antibiotic tetracycline had no significant effect on the incidence of gynandromorphs.

Morphological pattern of gynandromorphism: The pattern of gynandromorphism was determined for 206 individuals (Table 2). Individuals were scored for antennae, wings, legs, and external genitalia. The data show a clear anterior/posterior patterning of femaleness. For example, gynandromorphic individuals that had one or two female wings always had two female antennae; and individuals with one or more female legs always had female antennae and female wings, etc. Lateral differences were much rarer and were generally restricted to one anterior/posterior unit (36 of 39 cases). Of the 3 individuals with differences in more than one unit, 2 differed for two legs and 1 for three legs (rows 7, 9, and 11 in Table 2). The left/right difference of these latter 3 individuals was directional, while the one-unit left/right differences of other individuals

TABLE 2
Pattern of gynandromorphism in the HiCD₁₂ strain of *N. vitripennis*

Female features												
Anterior → Posterior											All ♀ features	N (fraction)
AN		FW		FL		ML		HL		N (fraction)		
First	Second	First	Second	First	Second	First	Second	First	Second		features	
Yes											8 (0.039)	
Yes	Yes										58 (0.282)	
Yes	Yes	Yes									17 (0.083)	
Yes	Yes	Yes	Yes								22 (0.107)	
Yes	Yes	Yes	Yes	Yes							5 (0.024)	
Yes	Yes	Yes	Yes	Yes	Yes						3 (0.015)	
Yes	Yes	Yes	Yes	Yes	Yes	Yes					1 (0.005)	
Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes				3 (0.015)	
Yes	Yes	Yes	Yes	Yes	Yes	Yes		Yes			1 (0.005)	
Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes				2 (0.010)	
Yes	Yes	Yes	Yes	Yes	Yes	Yes		Yes			1 (0.005)	
Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes			3 (0.015)	
Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes		8 (0.039)	
Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	74 (0.359)	
1.00	0.961	0.680	0.597	0.490	0.456	0.451	0.421	0.422	0.398	0.359	206	

AN, antenna; FW, forewing; FL, foreleg; ML, midleg; HL, hindleg; “First,” one of the two body sides of a particular feature; “Second,” both body sides of particular feature; “Yes,” feature present.

appeared to be randomly distributed. Although the fraction of gynandromorphs was significantly lower at 20° than at the other rearing temperatures (see below and Table 3), the fraction of individuals with complete female external characters was significantly higher at 20° than at each of the other rearing temperatures (χ^2 tests, all comparisons $P < 0.05$).

All tested parthenogenetic individuals with complete female characters ($N = 17$) have ovaries, but the number of ovarioles (four on each abdominal side in normal females) is deviant and ranges between one and five on each side. The number of mature eggs per female is significantly lower than in normal females (4.2 ± 0.7 SE *vs.* 28.8 ± 1.8 SE; $P < 0.001$). Thus, although parthenogenetic females are able to lay eggs, both fecundity and

fertility are extremely low (see BEUKEBOOM *et al.* 2007). Behavioral aspects of the different classes of gynandromorphs and the parthenogenetic females will be published in a separate article.

Environmental effects

Temperature effects on gynandromorph production: Virgin females of the HiCD₁₂ strain were individually allowed to parasitize hosts at four different temperatures: 20°, 25°, 29°, and 31°. There was a striking and significant increase in the fraction of gynandromorphs with increasing culturing temperature (Table 3). At 31°, a sixfold increase in the frequency of gynandromorphs was observed in comparison with a 20° culturing

TABLE 3
Mean fractions of gynandromorphs in the offspring of virgin HiCD₁₂ females cultured at four different temperatures and after different periods at 31°

	Temperature				Period at 31°			
	20°	25°	29°	31°	4 hr	8 hr	12 hr	24 hr
Mean	0.074 (a)	0.149 (b)	0.235 (c)	0.426 (d)	0.109 (a)	0.401 (b)	0.341 (b)	0.299 (b)
SE	0.009	0.015	0.017	0.041	0.045	0.090	0.078	0.029
No. of females	20	14	14	13	8	8	8	16
No. of offspring	1198	550	529	382	48	47	53	99

Period at 31°: parasitizing occurred at 31° during 2 hr and the hosts were subsequently left at 31° for additional hours and then transferred to 20°, where further development was completed. Different lowercase letters in parentheses indicate a significant difference at the 5% level.

temperature. All four culturing temperatures showed a relatively low level of variation in the fraction of gynandromorphic individuals per mother. However, the variance in expression of male and female characters was high at all temperatures and also present within the offspring of a single mother. We further observed that developmental time and body size of the emerging adults were negatively correlated with rearing temperature. Moreover, the offspring number per mother was higher at 20° than at the other temperatures.

To determine whether a particular sensitive period exists at which gynandromorphism is induced, females were allowed to oviposit at high temperature for 2 hr and then removed, after which hosts were placed back at 20° after different time periods of exposure to the higher temperature. After a 4-hr treatment at 31° (2 hr of oviposition followed by another 2 hr at 31°), no increase in gynandromorph frequency was found compared to complete development at 20° (Table 3). Treatment periods of 8, 12, and 24 hr at 31° resulted in significant ($P < 0.05$) increased gynandromorph frequencies, which were somewhat (but not significantly) lower than in the case of complete development at 31°. These results suggest that the critical time period for gynandromorph production occurs between 4 and 8 hr after egg laying.

To investigate temperature effects further, an experiment was conducted in which the eggs were laid at 20° during a 4-hr oviposition period, kept at that temperature for different times, and then exposed to 31° for 4 hr at various time points in development (Table 4). The proportion of gynandromorphs was significantly higher when the embryos were transferred to high temperature at a young age: exposure periods 0–4 and 4–8 hr following egg laying of show significantly higher proportions of gynandromorphs than the older age classes. Because the eggs were laid over a 4-hr time period prior to exposure and then exposed for 4 hr, embryos in the 0- to 4-hr exposure class were exposed to heat treatment at ages potentially ranging from 0 to 8 hr, those from the 4- to 8-hr class for 4–12 hr, etc. The results indicate that temperature induction of gynandromorphism occurs in the zygote (not during egg development) and that the critical time period is between 0 and 8 hr. Furthermore, on the basis of the 0- to 4-hr and 4- to 8-hr exposure results, we believe that the critical period falls 4–8 hr after egg laying. Development from egg deposition to gastrulation takes ~10 hr at 25°, which encompasses about one-third of the egg stage (PULTZ and LEAF 2003) and is likely accelerated at 31°. The older-age classes of eggs at 31° show gynandromorph frequencies similar to the control not exposed to high temperatures, indicating that high-temperature induction of gynandromorph production occurs only in the early embryonic stages. No significant differences were found for the mean number of offspring per virgin female between the different egg-age classes of treatment at 31° (Table

TABLE 4
Mean proportions of gynandromorphs among the offspring of HiCD₁₂ *N. vitripennis* virgin females

Period after egg laying at 20°	Proportion of gynandromorphs		No. of offspring	
	Mean	SE	Mean	SE
0–4	0.334 (b)	0.034	6.24 (a)	0.85
4–8	0.307 (b)	0.025	7.65 (a)	0.72
8–12	0.147 (a)	0.032	7.23 (a)	0.88
12–16	0.119 (a)	0.032	7.35 (a)	0.86
16–20	0.129 (a)	0.037	7.46 (a)	0.91
20–24	0.113 (a)	0.023	8.86 (a)	1.11
Control 20°	0.097 (a)	0.032	9.10 (a)	1.42

Virgin females parasitized hosts at 20° during 4 hr and subsequently hosts were placed at 31° after various intervals of 4 hr. Different lowercase letters in parentheses indicate a significant difference at the 5% level.

4); however, because egg numbers were not scored, we do not know whether this is due to absence of mortality differences.

Maternal exposure to high temperature: A final set of experiments was conducted to determine whether higher temperature exposure of the female induces increased gynandromorphism, independently of zygotic temperature effects. Females were exposed to 31° for various periods without hosts, then moved to 20° for oviposition, and transferred to new hosts three times (Table 5). The data show that the first batches of eggs after heat treatment generate significantly higher proportions of gynandromorphs at 20° than the successive batches. This effect has been found for all treatment periods, and apparently a 4-hr adult treatment period at 31° is sufficient to significantly increase the gynandromorph frequency at 20°. However, this effect is lost in the later laid egg batches, where gynandromorph frequencies are similar to the complete developmental regime at 20° (see also Table 3). Therefore, even a 4-hr exposure of maturing eggs (inside the mother) to high temperature is sufficient to increase the frequency of gynandromorphism for eggs that subsequently develop at 20°. The results are consistent with a temperature-sensitive sex-determining factor that is maternally produced in the egg.

Despite the adult treatment at 31°, the mean number of offspring per female is not significantly different from that of the control, indicating that the high-temperature treatments had no negative effect on the overall productivity. The total number of offspring per female ranged from 126.80 ± 11.91 (SE) for the control to 110.57 ± 13.53 (SE) for the 16-hr adult treatment at 31°. In a second experiment, 40 virgin HiCD₁₂ females were treated for 12 hr at 31°, divided in two groups, and allowed to parasitize hosts at 20° and 31°, respectively. A highly significant difference in offspring number was

TABLE 5

Mean proportions of gynandromorphs after various periods of adult treatment at 31° and subsequent host parasitizing at 20°

Parasitizing period	0 hr at 31°		4 hr at 31°		8 hr at 31°		12 hr at 31°		16 hr at 31°	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
1	0.074 (a, 1)	0.021	0.270 (b, 2)	0.055	0.387 (b, 3)	0.058	0.396 (b, 2)	0.051	0.389 (b, 2)	0.035
2	0.090 (a, 1)	0.018	0.104 (a, 1)	0.022	0.160 (a, 2)	0.025	0.096 (a, 1)	0.017	0.137 (a, 1)	0.025
3	0.063 (a, 1)	0.011	0.122 (a, 1)	0.024	0.094 (a, 1, 2)	0.011	0.100 (a, 1)	0.022	0.083 (a, 1)	0.021
4	0.071 (a, 1)	0.022	0.094 (a, 1)	0.020	0.070 (a, 1)	0.017	0.092 (a, 1)	0.023	0.093 (a, 1)	0.019

Data for each of the four successive periods of host parasitizing are given. Different lowercase letters in parentheses indicate a significant difference at the 5% level within a parasitizing period, and different numbers in parentheses indicate a significant difference at the 5% level within a treatment period.

found between the two culturing temperatures (125.88 ± 9.80 at 20° *vs.* 48.25 ± 4.67 at 31°) due to lower egg production and/or higher juvenile mortality. The proportion of gynandromorphs at 20° is consistent with the data shown in Table 5. At 31° the proportion of gynandromorphs is high for all three consecutive parasitizing periods and is significantly higher than at 20° for all comparisons. Another remarkable observation is the significantly higher frequency of individuals with complete female external morphology from the first batch of eggs in comparison with the two following batches ($P < 0.001$). This significant effect is found at both culturing temperatures and is associated with adult treatment at high temperature during 12 hr without the possibility to parasitize hosts.

Taken together, the temperature experiments indicate that exposure of either the (nearly) mature egg in the mother's reproductive tract or the early developing embryo to high temperature can increase gynandromorph production. The results are consistent with a temperature-sensitive sex-determining factor that is maternally produced in the egg and acts during a sensitive period in early stages of embryogenesis.

Genetic analysis

We conducted a series of experiments to investigate the genetic basis of gynandromorph production. Results show an interaction between a heritable cytoplasmic (*e.g.*, mitochondrial) effect and a nuclear maternal locus that maps to visible mutation Or_{123} on chromosome IV.

Nuclear and cytoplasmic components: A series of backcrossing experiments were performed at 25° to investigate the role of nuclear and heritable cytoplasmic components (*e.g.*, mitochondria) in the gynandromorph phenotype. Backcrosses were performed for 11 generations to (1) place the $HiCD_{12}$ cytoplasm in a wild-type nuclear background ($H^{CYT} \times A$) and (2) place the nuclear genotype in a wild-type cytoplasm ($A^{CYT} \times H$, Figure 3). Results show that production of gynandromorphs requires both the nuclear genome of the $HiCD_{12}$ (indicated as H) strain and a heritable cytoplasmic

component (presumably mitochondrial). Both types of backcrosses show loss of gynandromorph production compared to the control $H^{CYT} \times H$, and the proportions of gynandromorphs are similar to $A^{CYT} \times A$ (*i.e.*, no gynandromorphs). At generation 3, $H^{CYT} \times A$ had lost gynandromorph production. But five generations of re-introduction of the H nuclear genome resulted in a significant increase in gynandromorph production [3.3%; $N = 21$ families of $B_6 (H^{CYT} \times A) \times H$] *vs.* 0% ($N = 19$) for $H^{CYT} \times A$ (MWU: $z = -4.649$, $P < 0.001$). This means that gynandromorph production involves an epistatic interaction between the nuclear and the cytoplasmic genotype of the gynandromorph-producing strain.

Backcrossing of $H^{CYT} \times A$ F₁ females to H males [the B₁ ($H^{CYT} \times A$) \times H line] resulted in the maintenance of a high level of gynandromorph production, as expected. However, the level was lower than the control $H^{CYT} \times H$ (Wilcoxon, $z = -2.501$, $N = 10$ generations, $P = 0.012$), whereas full restoration of gynandromorph

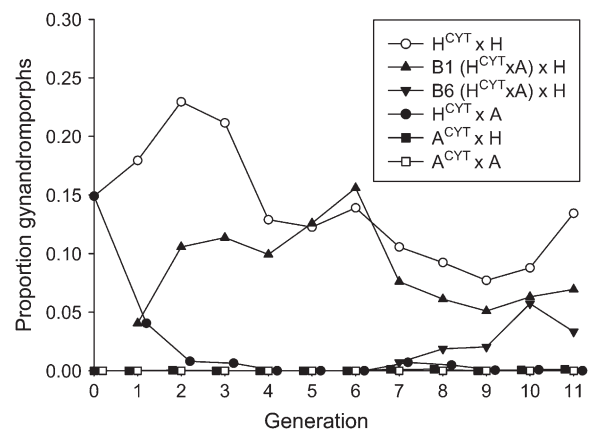


FIGURE 3.—Backcross experiment to determine the role of nuclear and cytoplasmic genotypes on gynandromorphism. The gynandromorphic trait is lost if either the $HiCD_{12}$ (H) genotype is replaced by the standard $AsymC$ (A) genotype (solid circles) or the H cytotypic is replaced by the A cytotypic (solid squares). One and six generations after backcrossing the A genotype into the H cytoplasm, gynandromorphism can be recovered although not completely (B_1 and B_6 , respectively). Experiments were performed at 25°.

TABLE 6
Gynandromorph frequencies among F₃ progeny of various backcrosses

Grandparental cross (male × female)	F ₁ backcross male	Cytoplasmic	Proportion of F ₃ gynandromorphs from F ₂ female genotypes			Proportion of F ₃ gynandromorphs from heterozygous mothers	
			Nuclear			Or	+
			Or/Or	Or/+	+/+		
HiCD ₁₂ × Or ₁₂₃	Or ₁₂₃	Or	0.010 (1026, 17)	0.054 ^a (989, 17)	—	0.047	0.061
Or ₁₂₃ × HiCD ₁₂	Or ₁₂₃	H	0.024 (917, 16)	0.122 ^a (673, 15)	—	0.113	0.131
HiCD ₁₂ × Or ₁₂₃	HiCD ₁₂	Or	—	0.178 (732, 16)	0.250 ^a (552, 13)	0.156	0.197
Or ₁₂₃ × HiCD ₁₂	HiCD ₁₂	H	—	0.225 (451, 12)	0.513 ^a (659, 16)	0.188	0.267

Grandparental crosses between HiCD₁₂ and Or₁₂₃ yielded F₁ females that were backcrossed to either strain. The resulting F₂ females were bred as virgins at 31° and the resulting F₃ progeny were scored for gynandromorphs and eye color. The number of F₃ individuals and families are given in parentheses.

^aA significantly higher proportion of gynandromorphs in comparison to the other phenotype within the same cross.

production would be expected. Similarly, the B₆ (H^{CYT} × A) × H line showed levels of gynandromorphism much lower than those of the control H^{CYT} × H line (3.3%, *N* = 21 families *vs.* 13.4%, *N* = 20; MWU: *z* = -3.269, *P* = 0.001). This effect indicates a “memory” of exposure to the wild-type genotype. It may be due to retention of wild-type alleles in the line by selection, perhaps due to increased mortality of H genotypic females. Alternatively, it could reflect an epigenetic effect (*e.g.*, imprinting).

Crosses to visible markers: To investigate the genetic basis of gynandromorphism, we initially performed reciprocal crosses between HiCD₁₂ and lines with visible markers on different linkage groups of *N. vitripennis*. The frequencies of gynandromorphism were scored among F₂ males from mothers of the reciprocal crosses at 31° (conducive to high frequencies). Results of these crosses show (1) a strong and significant strain effect, (2) a strong and significant effect of cytoplasm, and (3) no evidence that the zygotic genotype linked to any of these mutants is associated with gynandromorph production. The frequencies of gynandromorphism among F₂ males range from zero for heterozygous females with ST₅₂₁₉ cytoplasm to 22.2% for heterozygous OR₁₂₃ females with HiCD₁₂ cytoplasm and 42.9% in homozygous HiCD₁₂ females with HiCD₁₂ cytoplasm. In each reciprocal mutant strain cross, heterozygous females with HiCD₁₂ cytoplasm show a significantly higher frequency of gynandromorphs. In each case, the frequency was not significantly different for mutant *vs.* wild-type F₂ males.

Mapping of a maternal gynandromorph (*gynI*) locus:

An alternative, consistent with the temperature effects, is that the maternal genotype, rather than the zygotic genotype, determines gynandromorphism. Our preliminary data suggested a maternal-acting locus linked to the visible marker Or₁₂₃ located on chromosome IV (nomenclature according to RÜTTEN *et al.* 2004). To test for this, F₁ females from the cross HiCD₁₂ female ×

Or₁₂₃ male and the reciprocal cross were backcrossed to HiCD₁₂ and Or₁₂₃ males. This yielded homozygous wild-type (HiCD₁₂ alleles), heterozygous, and homozygous Or₁₂₃ females (Table 6). These females were set as virgins at 31° and the frequency of gynandromorphs in the progeny of each female was determined. These gynandromorph frequencies have been obtained under conditions similar to those among the F₂ progeny. The results show that females homozygous for the Or₁₂₃ allele produce a significantly lower frequency of gynandromorphs than do heterozygous HiCD₁₂/Or₁₂₃ females (Table 6). In turn, HiCD₁₂/Or₁₂₃ females produce a significantly lower frequency of gynandromorphs than do homozygous HiCD₁₂/HiCD₁₂. Thus, within each of the four backcrosses there is a significant effect of the maternal nuclear genotype on gynandromorph frequency in the F₃ progeny. There is also a significant difference among the haploid F₃ progeny of females with the same nuclear genotype originating from reciprocal grandparental crosses, backcrossed with either Or₁₂₃ or HiCD₁₂ males. For all four comparisons of F₂ females with a similar nuclear genotype and a different cytoplasm, those F₂ females with a HiCD₁₂ cytoplasm produce a significantly higher proportion of gynandromorphs. There also appears to be a mild effect of zygotic genotype at Or₁₂₃ on the phenotype. Individuals that were wild type for the allele have a slight but consistently higher tendency to be gynandromorphs in each cross (last column in Table 6: data derived from heterozygous F₂ mothers). This effect is significant for all four crosses pooled (χ^2 test, *P* < 0.01). Whether this represents the same locus as the maternal effect or a second linked locus is unclear.

In conclusion, the results of the F₂ analyses and the backcrosses (Figure 3, Table 6) show that the nuclear component of the trait for gynandromorphism is primarily a maternal-effect locus that is linked to the Or₁₂₃ gene on chromosome IV. We propose to name this locus *gynI*.

DISCUSSION

Gynandromorphs in this study are derived from unfertilized eggs that normally develop into haploid males. They show an anterior/posterior pattern of female/male external morphology. Gynandromorph frequencies can be elevated by selection to a limited level, but are also affected by environmental conditions, such as high temperature during oogenesis and early egg development. Moreover, maternal effects as well as heritable cytoplasmic effects (presumably mitochondrial) play a prominent role in the occurrence of gynandromorphism.

Environmental effects: The proportion of gynandromorphs can be strongly elevated (about sixfold) by oviposition at high temperature. We have shown that high-temperature induction of gynandromorph formation occurs ~8 hr after egg laying. Probably in the blastoderm stage of the egg (~8 hr after egg laying; PAK and PINTO 1976), genes involved in gynandromorph production can be induced and remain activated during further development at 20°. When high-temperature treatment was initiated in the late embryonic stage or in the larval stage, induction of gynandromorph production was absent.

In subsequent experiments, we investigated the effect of temperature during oogenesis and early egg development. High-temperature-treated HiCD₁₂ females that were subsequently allowed to parasitize hosts at 20° yielded two conspicuous observations: (1) the first batch of eggs generated a significantly higher proportion of gynandromorphs than the following batches and (2) the gynandromorphs from the first batch had a significantly higher level of feminization. These observations show that gynandromorph induction also occurs through the mother but, as for treatment after oviposition, the effect is lost after a certain period at 20°. This implies a sensitive period from late oogenesis to early embryogenesis. It may indicate that stability of a protein involved in male determination at this stage is temperature dependent in the HiCD₁₂ line or that translation of the protein is temperature dependent. It is unlikely to involve transcriptional effects because most insects show very little transcriptional activity in the preblastoderm stages of development. Targets for this product appear to be sensitive along the A/P axis of the embryo, possibly explaining the A/P patterning of female structures (KEISMAN *et al.* 2001). Alternatively, the product has an A/P gradient, which shifts during egg maturation, explaining the increase in feminization of gynandromorphs that emerged from the first batch of eggs produced by females that were unable to oviposit during high-temperature treatment. It remains unclear whether we are dealing with a hypomorph of a male-determining product, with female being the default somatic sex, or with a hypermorph of a female-determining product with male being the default. However, the sensitivity of the trait to high temperatures does suggest

the former (male-determining product), since mutant proteins are more likely to become unstable at higher temperatures.

The environmental and physiological influences on gynandromorph production perhaps are a general effect of stress. It is a well-known phenomenon that stress induces the synthesis of several heat-shock proteins (MORIMOTO *et al.* 1994). Through the functional relationships between heat-shock protein (*Hsp*) genes and hormone receptors (*e.g.*, PICARD *et al.* 1988), *Hsp* genes may directly influence developmental processes and could modify the balance between feminizing and masculinizing genes when embryonic stages are exposed to stressful conditions.

Influence of Wolbachia: We have checked the gynandromorph-producing *N. vitripennis* strain for Wolbachia infection. Wolbachia bacteria are found in reproductive tissues of many insects and are cytoplasmatically transmitted. These bacteria may manipulate physiology and reproduction of their hosts, including reproductive incompatibility, parthenogenesis by causing endoduplication of the haploid egg during mitosis, and feminization (reviewed in WERREN 1997). These bacteria are further known to alter early development and mitotic processes in their hosts (REED and WERREN 1995). Parthenogenesis-inducing Wolbachia bacteria may cause female wasps to produce daughters without mating (STOUTHAMER *et al.* 1990; HUIGENS *et al.* 2000). Possibilities for Wolbachia-induced female or gynandromorph production in the *N. vitripennis* strain described in this article can be excluded, as we observed no Wolbachia or other endosymbionts present in the *N. vitripennis* strain studied. Therefore, we can also exclude that the increase in gynandromorph production at high temperature is the cause of the partial elimination of Wolbachia under these conditions, as shown for the two-spotted spider mite (VAN OPIJNEN and BREEUWER 1999).

Inheritance and maternal influence: Analyses of the F₂ progeny from crosses between the gynandromorph-producing strain (HiCD₁₂) and various strains with visible mutants showed that gynandromorph production is heritable, but is not simply the result of a single segregating gene. The F₂ progeny showed a strong positive maternal effect for the production of gynandromorphs, but the magnitude of the effect was different for the various reciprocal F₂ progeny. The significant differences in gynandromorph frequencies between F₂ progeny of different mutant strains, coupled with the strong cross-direction effects, further support the importance of cytoplasmic background. The mutant strains used for crosses with the HiCD₁₂ strain also differ in nuclear genetic background, which can contribute to this variation.

Heritable cytoplasmic factors, such as mitochondria, appear to contribute to gynandromorph production. This is evidenced by the backcross experiments (Figure 3) and the Or₁₂₃ localization experiment (Table 6).

These are to be distinguished from maternal effects, which is the contribution of the maternal nuclear genotype to the zygotic phenotype. The maternal effect could result from the exclusive expression of maternally inherited alleles in the early development of the egg, either through differential expression of the grandparental alleles in the mother or through differential expression of the parental alleles in the egg. Examples of maternally expressed genes in early development are known from various organisms (reviewed in ROSSITER 1996). Very early expression coinciding with the early sensitive period for gynandromorph induction, which we observed in *Nasonia*, is known from, *e.g.*, *Drosophila* (SCOTT and O'FARRELL 1986), *Nasonia* (LYNCH *et al.* 2006), other parasitic wasps (GRBIC and STRAND 1998), and reptiles (SARRE *et al.* 2004). The regulatory mechanism behind the differences in expression of maternal and paternal genes possibly is DNA methylation and probably occurs when the maternal and paternal gametes are separately subjected to environmental influences, *i.e.*, during gametogenesis (MARX 1988).

Ploidy level: The gynandromorphs and putative females obtained from unfertilized eggs produced by diploid females should “normally” develop into haploid males. The question arises whether these individuals are haploid, diploid, or haploid/diploid mosaics. BEUKEBOOM and KAMPING (2006) have shown that unfertilized diploid eggs originating from triploid females occasionally develop into females or gynandromorphs. The uniparentally produced daughters from triploid females have normal fertility and proved to be diploid on the basis of the segregation of eye-color mutants in the F₁ and F₂ progeny. The “morphological females” from diploid virgin mothers of the gynandromorph-producing strain described in this study did (almost) not produce offspring. Our findings show a haploid genome of the gynandromorphs and uniparental females from diploid mothers (and see BEUKEBOOM *et al.* 2007). Apparently, at least a diploid level of chromosomes is a prerequisite for normal female germline function.

Gynandromorphism and sex determination: As mentioned in the Introduction, gynandromorphs may arise in various ways. The female/male patterning of *N. vitripennis* individuals described in this article originates from unfertilized eggs and therefore excludes failures related to the fertilization process. This makes it more plausible that these *N. vitripennis* gynandromorphs are the result of an imperfectly functioning sex determination system. In species with a well-understood sex determination system, such as *Drosophila* and *Caenorhabditis elegans* (reviewed in CLINE and MEYER 1996) and a number of insects (DÜBENDORFER *et al.* 2002; SACCONI *et al.* 2002), there is a hierarchy of regulatory genes involved in sex determination. These genes also control the different developmental processes and the sex-specific patterning (KEISMAN *et al.* 2001; SANCHEZ *et al.* 2001). Developmental processes and their interactions

in *Nasonia* highly parallel those present in *Drosophila* (PULTZ *et al.* 1999), and mutations in the regulatory sex determination genes in *Drosophila* (see Introduction) lead to the production of gynandromorphs. In *Drosophila*, the doublesex regulatory gene for sex determination also regulates the anterior/posterior development and is at the bottom of the sex determination hierarchy; functional homologs of this gene are found in distantly related species (WATERBURY *et al.* 1999). As we observed a typical anterior/posterior female/male patterning among the gynandromorphic individuals in *N. vitripennis*, it is tempting to consider gynandromorphs as resulting from a sex determination gradient in the egg (LYNCH *et al.* 2006). In this view, the gynandromorphs described in this article are the result of mutations in regulatory sex determination genes.

A model for *Nasonia* sex determination: It is clear that sex determination in *Nasonia* works differently from hymenopterans that have CSD, such as the honey bee (reviewed in BEYE 2004). Yet, the basic pattern of haplo-diploid sex determination still remains: typically, haploid males develop from unfertilized eggs and diploid females develop from fertilized eggs. Therefore, investigating the mechanisms of sex determination in *Nasonia* and contrasting it to CSD will be enlightening for our understanding of how sex determination systems evolve.

Any model of sex determination in *Nasonia* must account for the following key observations: (1) inbreeding does not lead to diploid male production (SKINNER and WERREN 1980); (2) a diploid male/triploid female strain exists—triploid females routinely produce diploid males and haploid males from unfertilized eggs, although diploid females are produced at a low frequency (<1%; BEUKEBOOM and KAMPING 2006); and (3) a naturally occurring mutant line (described here) produces gynandromorphs and somatic females from unfertilized eggs, and this effect is due to both cytoplasmic inputs and a maternal-effect locus (this study).

Several models exist, but none of them account for all of these observations. Here we contrast two key general models: (1) genomic imprinting and (2) maternal-effect sex determination. Genomic imprinting models invoke a differential imprinting of sex-determining alleles, either paternal or maternal, which determines sex in the developing zygote (BEUKEBOOM 1995). Maternal-effect sex determination invokes a ploidy “counting mechanism” that compares a maternal product to the number of zygotic chromosome complements (a maternal-zygotic balance model; COOK 1993b). This is somewhat analogous to the X:A balance model in *Drosophila* (CLINE and MEYER 1996), except the comparison is maternal to zygotic.

The imprinting model cannot readily explain how unfertilized eggs develop into gynandromorphs and females. Paternal imprinting requires input of chromosomes from the male for female development. Maternal

imprinting does not predict development of females from unfertilized eggs. The only counterpoint to this observation is the possibility that *gyn1* represents a defect in the maternal imprinting mechanism, resulting in haploid female development. However, the imprinting model does not explain the strong heritable cytoplasmic contribution to sex determination found in our study.

The maternal-zygotic model explains many observations of sex determination in *Nasonia*, including the polyploid strain. Since triploid females have a triple chromosome complement compared to the diploid zygote, one expectation is male production of diploids, but with the “threshold” for female development being “closer” in these diploids. The model is consistent with occasional production of diploid females in this line (BEUKEBOOM and KAMPING 2006), in contrast to previous claims (DOBSON and TANOUYE 1998). However, the model does not account for why diploids derived from unfertilized eggs from this strain typically develop into males, whereas diploids derived from haploid eggs from this strain that are fertilized with haploid sperm develop into females. This observation is best explained by genomic imprinting. The maternal-effect model is consistent with our observations of a maternal-effect locus influencing gynandromorph production. However, this model does not predict the influence of heritable cytoplasmic components, although it is not inconsistent with this observation.

We propose a “hybrid model” to account for the current observations. The simplest explanation consistent with the results of this study is that sex is determined through a balance between a maternal-effect gene and the number of chromosome sets in the zygote. However, some of these components are differentially imprinted on the basis of whether they are maternal or paternal in origin. In our gynandromorphic strain, an alteration of the counting mechanism occurs through a modification of the maternal product. To accommodate the earlier observations of the polyploid strain and other studies (TRENT *et al.* 2006), we maintain that the counting mechanism responsible for establishing zygotic ploidy is somehow sensitive to the parental origin of the chromosomal complements. Overlaid upon this mechanism, modification appears to be affected by a heritable cytoplasmic component (presumably mitochondria) because cytoplasm influences the level of gynandromorphism. Evolutionary theory predicts that mitochondria will be selected to favor female production (WERREN and BEUKEBOOM 1998), which may account for this input. Studies of the genetic and molecular basis of this gynandromorphic strain will help to elucidate the components of this sex determining system. Such studies will be facilitated by the nearly completed *Nasonia* genome project (WERREN *et al.* 2004).

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