Inheritance of gynandromorphism in the parasitic wasp *Nasonia vitripennis*

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ABSTRACT

The parasitic wasp *Nasonia vitripennis* has haplodiploid 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 feminisation 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 F$_2$ and F$_3$ progeny indicate a maternal effect locus (gyn1) which maps to chromosome IV. Coupled with previous studies, our results are consistent with a *Nasonia 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 the 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 over 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.

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 over 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. 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 Habrobracon juglandis (Clark et al. 1971). In the latter species the recessive mutant ebony (dark body colour) increases the frequency of gynandromorphs in fertilized eggs to about 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, like *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 for example been shown that the proportion of gynandromorphic individuals can be increased by short pulses of high temperature in Hymenoptera, such as *e.g. 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 *Nasonia 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 approximately 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, and 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* based on these data.

**MATERIALS AND METHODS**

*Nasonia biology and maintenance*: *Nasonia* are small (2-3 mm) parasitic wasps, which are easily cultured on *Sarcaphaga 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 25º or in diapause at 4º. Typically around 20 females were provided with around 50 hosts for life. Adult progeny emerged around 15 days later at 25º. All experiments below were conducted at 25º 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 25º or in diapause at 4º. *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 (wildtype lab strain cured from *Wolbachia*), OR123 (orange eyes), ST5219 (red eyes) and Stdr (red eyes). Furthermore, we used the 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 based on 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 hindwings 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, 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, that of the female pointed. These eleven 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 eleven landmarks described above were used to
determine the pattern of male and female external body parts (Figure 1). Based on the 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 three 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, independent of the ploidy effects of fertilization. This was accomplished by mating HiCD12 females to males carrying the Paternal Sex Ratio (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°C. 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: a) PCR amplification of Wolbachia specific genes (ZHOU et al. 1998), b) PCR amplification of 16S ribosomal DNA to detect the presence of any prokaryotic endosymbionts (LANE 1991), c) cytological examination of eggs (BREEUWER and WERREN 1990), and d) 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 20º. 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 five days at 20º. Then, the experiment was performed at 20º, 25º, 29º and 31º with three hosts per female. After three days the wasps were transferred to new vials and supplied with three fresh hosts. The emerging adults were scored for the eleven 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 31º for a restricted period of two hrs to minimize variation in age of the eggs within each age class. After various time intervals of four hrs, the parasitized hosts were transferred to 20º for further development.

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

**Effect of adult treatment on gynandromorph production:** The increase in gynandromorph production at 31º 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 31º as well as at 20º (control). Then, they were allowed to parasitize hosts at 20º. 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 eleven 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 the corresponding tables.

**The role of nuclear and cytoplasmic components:** An experiment was conducted to determine the role of nuclear versus 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$^{CYT}$x H (H control), 2) A$^{CYT}$ x A (wildtype control), 3) H$^{CYT}$x A (replacement of H nuclear genome with A in H cytoplasm) and 4) A$^{CYT}$ x H (replacement of A nuclear genome with H in A cytoplasm). Ten families were maintained per line. Each generation, five virgin females per family were backcrossed to three males from the indicated line, and then mass cultured. In addition, three 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: 5) B1 (H$^{CYT}$ x A) x H (a line established by taking F₁ females from the H$^{CYT}$ x A cross and subsequently backcrossing to H each generation), and 6) B6 (H$^{CYT}$ x A) x H (a line
established in the sixth generation of \(H^{CYT}\times A\), by backcrossing to \(H\) males each generation). The \(B6\) \((H^{CYT}\times A)\times H\) 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 the genotype of the zygote. Crosses were performed between the \(HiCD_{12}\) 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: \(Or_{123}\) (orange eyes), \(St_{5219}\) (red eyes) and \(Std\) (red eyes). The genes coding for the first two mutants are located on chromosome IV and \(St\) is located on chromosome V (based on chromosome numbering according to RÜTTEN et al., 2004). All crosses were performed reciprocally at 20º. The resulting \(F_1\) females were collected as virgins, aged for five days at 20º and subsequently individually allowed to parasitize hosts at 31º. 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 \(HiCD_{12}\) and \(Or_{123}\) males. Resulting heterozygous and homozygous \(F_2\) females with either the \(HiCD_{12}\) or the \(Or_{123}\) 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 \(\chi^2\) tests were performed by using Statistix 4.0 Analytical Software. Non-parametric statistics were used to compare strains (Mann Whitney U Test –MWU and Wilcoxon Matched Signs Ranks Test -WMSR).
RESULTS

Basic characterization

Gynandromorphism in *N. vitripennis* field strains: Following discovery of gynandromorphism in natural isolates of *N. vitripennis*, eleven 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 greater than 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 could either be derived from haploid eggs, or could result from non-disjunction 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 four-day-old larvae only show haploids, whereas 40% gynandromorphs and uniparental females were expected based on emerging control wasps (BEUKEBOOM *et al.* in press). This result shows that gynandromorphs and uniparental females are haploid and are not derived from non-disjunction 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 showed a decreasing trend in frequency of gynandromorphs. From generation one onwards, 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 are subsequently identified as HiCD$_{12}$ and LoCD$_{12}$, respectively.

After selection, the HiCD$_{12}$ line was maintained primarily by diapause at 4º resulting in approximately one generation per year. After nine years, the HiCD$_{12}$ 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 25º, which was similar to the result of selection at generation eight (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 Paternal Sex Ratio (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). HiCD$_{12}$ females (N=16) mated with PSR males produced a high proportion of gynandromorphs (mean=0.483, SE=0.033) at 31º. This value was similar to the offspring of HiCD$_{12}$ virgin females at that temperature (mean=0.458, SE=0.036; see also Table 3), indicating that fertilization *per sé* 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 HiCD$_{12}$ 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 ♂ x 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 about 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, et cetera. Lateral differences were much rarer and were generally restricted to one anterior/posterior unit (36 out of 39 cases). Of the three individuals with differences in more than one unit, two differed for two legs and one for three legs (rows 7, 9 and 11 in Table 2). The left/right difference of these latter three individuals was directional, while the one unit left/right differences of other individuals appeared to be randomly distributed. Although the fraction of gynandromorphs was significantly lower at 20º than at the other rearing temperatures (see below and Table 3A), the fraction of individuals with complete female external characters was significantly higher at 20º than at each of the other rearing temperatures ($\chi^2$ 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 versus 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. in press). Behavioural aspects of the different classes of gynandromorphs and the parthenogenetic females will be published in a separate paper.

Environmental effects

Temperature effects on gynandromorph production: Virgin females of the HiCD_{12} 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 3A). At 31°, a six-fold increase in the frequency of gynandromorphs was observed in comparison with 20° culturing 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 two hrs and then removed, after which hosts were placed back at 20° after different time periods of exposure to the higher temperature. After four hrs treatment at 31° (two hrs of oviposition followed by another two hrs at 31°), no increase in gynandromorph frequency was found compared to complete development at 20° (Table 3B). Treatment periods of 8, 12 and 24 hrs at 31° resulted in significant (\( P < 0.05 \)) increased gynandromorph frequencies,
which were somewhat (but not significant) lower than in the case of complete development at 31°C. These results suggest that the critical time period for gynandromorph production occurs between 4-8 hrs after egg-laying.

To investigate temperature effects further, an experiment was conducted where the eggs were laid at 20°C during a 4 hr oviposition period, kept at that temperature for different times and then exposed to 31°C for 4 hrs at various time points in development (Table 4). The proportion of gynandromorphs is significantly higher when the embryos were transferred to high temperature at a young age: exposure periods following laying of 0-4 and 4-8 hrs 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 hrs, embryos in the 0-4 hr exposure class were exposed to heat treatment at ages potentially ranging from 0 to 8 hrs, those from the 4-8 hr class for 4 to 12 hrs, et cetera. The results indicate that temperature-induction of gynandromorphism occurs in the zygote (not during egg development), and that the critical time period is between 0-8 hrs. Furthermore, based on the 0-4 and 4-8 exposure results, we believe that the critical period falls 4-8 hrs after egg-laying. Development from egg-deposition to gastrulation takes about 10 hrs at 25°C which encompasses about one-third of the egg stage (PULTZ and LEAF 2003), and is likely accelerated at 31°C. The older age classes of eggs at 31°C show gynandromorph frequencies similar to the control not exposed to high temperatures, indicating that high temperature induction of gynandromorph production only occurs 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°C (Table 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, independent of zygotic temperature effects. Females were exposed to 31°C for various periods without hosts, then moved to 20°C 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 four hrs 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 to 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 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 hrs adult treatment at 31°. In a second experiment, 40 virgin HiCD_{12} females were treated for 12 hrs at 31°, divided in two groups and allowed to parasitize hosts at 20° and 31°, respectively. A highly significant difference in offspring number was 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 twelve hrs without the possibility to parasitize hosts.

Taken together, the temperature experiments indicate that either exposure of 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 Or₁₂₃ on chromosome IV.

**Nuclear and cytoplasmic components**: A series of backcrossing experiments were performed at 25º C to investigate the role of nuclear and heritable cytoplasmic components (e.g. mitochondria) in the gynandromorph phenotype. Backcrosses were performed for 11 generations to (a) place the HiCD₁₂ cytoplasm in a wildtype nuclear background (HᶜYT x A) and (b) place the nuclear genotype in a wildtype cytoplasm (AᶜYT x H, Figure 3). Results show that production of gynandromorphs requires both the nuclear genome of the HiCD₁₂ (indicated as H) strain and a heritable cytoplasmic component (presumably mitochondrial). Both types of backcrosses show loss of gynandromorph production compared to the control HᶜYT x H, and the proportions of gynandromorphs are similar to AᶜYT x A (i.e. no gynandromorphs). At generation three, HᶜYT x A had lost gynandromorph production. But five generations of reintroduction of the H nuclear genome resulted in a significant increase in gynandromorph production (3.3%; N=21 families of B₆ [HᶜYTxA] xH), versus 0% (N=19) for HᶜYT x 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 HᶜYT x A F₁ females to H males (the B₁ (HᶜYT x A) x H line) resulted in the maintenance of a high level of gynandromorph production, as expected. However, the level was lower than the control HᶜYT x H (Wilcoxon, z=-2.501, N = 10 generations, P=0.012), whereas full restoration of gynandromorph production would be expected. Similarly, the B₆ (HᶜYT x A) x H line showed levels of gynandromorphism much lower than the control HᶜYT x H line (3.3%, N=21 families versus 13.4%,
N=20; MWU z=-3.269, P=0.001). This effect indicates a “memory” of exposure to the wildtype genotype. It may be due to retention of wildtype 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 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 significant higher frequency of gynandromorphs. In each case, the frequency was not significantly different for mutant versus wildtype F2 males.

**Mapping of a maternal gynandromorph (gyn1) locus:** An alternative, consistent with the temperature effects, is that maternal genotype determines gynandromorphism rather than zygotic genotype. 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 x Or123 male and the reciprocal cross – were backcrossed to HiCD12 and Or123 males. This yielded homozygous wildtype (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 similar conditions as 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 HiCD$_{12}$/HiCD$_{12}$. Thus, within each of the four backcrosses there is a significant effect of the maternal nuclear genotype on gynandromorph frequency in the F$_3$ progeny. There is also a significant difference between the haploid F$_3$ progeny of females with the same nuclear genotype originating from reciprocal grandparental crosses, either backcrossed with Or$_{123}$ or HiCD$_{12}$ males. For all four comparisons of F$_2$ females with a similar nuclear genotype and a different cytoplasm, those F$_2$ females with a HiCD$_{12}$ cytoplasm produce a significantly higher proportion of gynandromorphs. There also appears to be a mild effect of zygotic genotype at Or$_{123}$ on the phenotype. Individuals that were wildtype 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 F$_2$ mothers). This effect is significant for all four crosses pooled ($\chi^2$ 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 F$_2$ 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 Or$_{123}$ gene on chromosome IV. We propose to name this locus gyn1.

DISCUSSION

Gynandromorphs in this study are derived from unfertilized eggs which 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 strongly be elevated (about six-fold) by oviposition at high temperature. We have shown that high temperature induction of gynandromorph formation occurs about eight hrs after egg-laying. Probably in the blastoderm stage of the
egg (about 8 hrs 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 HiCD_{12} 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 feminisation. These observations show that gynandromorph induction also occurs through the mother but, just like 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 HiCD_{12} 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 pre-blastoderm 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 feminisation of gynandromorphs emerged from the first batch of eggs produced by females which 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 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 (HSPs) [MORIMOTO et al. 1994]. Through the functional relationships between 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 cytoplasmically 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 feminisation (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; HUIGEN et al. 2000). Possibilities for *Wolbachia* induced female or gynandromorph production in the *N. vitripennis* strain described in this paper 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 its magnitude 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 localisation 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 grand-parental 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, based on 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. in press).

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 paper originates from unfertilized eggs, and therefore excludes failures related to the fertilization process. This makes it more feasible that these *N. vitripennis* gynandromorphs are the result of an imperfect functioning sex determination system. In species with well understood sex determination system, like *Drosophila* and *Caenorhabditis elegans* (reviewed in Cline and Meyer 1996) and a number of insects (Saccone et al. 2002; Dubendorfer 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 paper 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 complementary sex determination (CSD), such as the Honey Bee (reviewed in Beye 2004). Yet, the basic pattern of haplodiploid 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: (a) inbreeding does not lead to diploid male production (SKINNER and WERREN 1980), (b) a diploid male/triploid female strain exists – triploid females routinely produce diploid males and haploid males from unfertilized eggs; however, diploid females are produced at a low frequency (<1%, BEUKEBOOM and KAMPING 2006), (c) a naturally occurring mutant line (described here) produces gynandromorphs and somatic females from unfertilized eggs – the 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, (a) genomic imprinting and (b) 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 based on 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 on 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)

ACKNOWLEDGEMENTS

We thank Royce Rogers for making the initial observations on gynandromorphs, Laas Pijnacker for cytological observations, Marina Louter for data on ovary and egg numbers and two anonymous
reviewers for critical comments. This research was supported by funds to JHW from the National Science Foundation and to LWB from a pioneer grant of the Dutch Scientific Organisation.
REFERENCES


Table 1. Frequencies of gynandromorphs in *N. vitripennis* field strains from different geographic origin.

<table>
<thead>
<tr>
<th>Field strain</th>
<th>Origin</th>
<th># mothers tested</th>
<th># mothers producing gynandrom.</th>
<th>% gynandro-morphs (N)</th>
<th>Mean # of offspring (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NV CD12</td>
<td>Canada</td>
<td>14</td>
<td>14</td>
<td>9.27&lt;sup&gt;c&lt;/sup&gt; (81)</td>
<td>62.4&lt;sup&gt;a&lt;/sup&gt; (6.5)</td>
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<td>NV XIDB433AP</td>
<td>Idaho</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>93.5&lt;sup&gt;bc&lt;/sup&gt; (6.3)</td>
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<tr>
<td>NV IN2217</td>
<td>Indiana</td>
<td>13</td>
<td>2</td>
<td>0.23&lt;sup&gt;a&lt;/sup&gt; (3)</td>
<td>100.4&lt;sup&gt;bc&lt;/sup&gt; (8.7)</td>
</tr>
<tr>
<td>NV IN226</td>
<td>Indiana</td>
<td>14</td>
<td>13</td>
<td>3.52&lt;sup&gt;b&lt;/sup&gt; (54)</td>
<td>109.6&lt;sup&gt;c&lt;/sup&gt; (7.0)</td>
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<td>NV MI003C</td>
<td>Michigan</td>
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<td>100.0&lt;sup&gt;bc&lt;/sup&gt; (8.7)</td>
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<td>75.6&lt;sup&gt;ab&lt;/sup&gt; (9.6)</td>
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<td>NV XUTC406A</td>
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<td>118.3&lt;sup&gt;c&lt;/sup&gt; (5.8)</td>
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Different superscript characters indicate a significant difference at the 5% level.
Table 2. Pattern of gynandromorphism in the HiCD_{12} strain of *N. vitripennis*.

<table>
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<tr>
<th>Female features</th>
<th>AN</th>
<th>AN</th>
<th>HW</th>
<th>HW</th>
<th>FL</th>
<th>FL</th>
<th>ML</th>
<th>ML</th>
<th>HL</th>
<th>HL</th>
<th>All ♀</th>
<th>N (Fraction)</th>
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<td>74 (0.359)</td>
</tr>
</tbody>
</table>

AN: antenna; FL: foreleg; ML: midleg; HL: hindleg; 1^{st}: one of the two body sides of a particular feature; 2^{nd}: both body sides of particular feature; √: feature present.
Table 3. **A.** Mean fraction (with standard error) of gynandromorphs in the offspring of virgin HICD\textsubscript{12} females cultured at four different temperatures. **B.** Mean fraction (with standard error) of gynandromorphs after different periods at 31°. Parasitizing occurred at 31° during two hrs and the hosts were subsequently left at 31° for additional hrs and then transferred to 20°, where further development was completed.

<table>
<thead>
<tr>
<th></th>
<th>Temperature</th>
<th>B</th>
<th>Period at 31° (hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20°</td>
<td>25°</td>
<td>29°</td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td>0.074\textsuperscript{a}</td>
<td>0.149\textsuperscript{b}</td>
<td>0.235\textsuperscript{c}</td>
</tr>
<tr>
<td><strong>S.E.</strong></td>
<td>0.009</td>
<td>0.015</td>
<td>0.017</td>
</tr>
<tr>
<td><strong># Females</strong></td>
<td>20</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td><strong># Offspring</strong></td>
<td>1198</td>
<td>550</td>
<td>529</td>
</tr>
</tbody>
</table>

Different superscript characters indicate a significant difference at the 5% level.
Table 4. Mean proportions of gynandromorphs among the offspring of HiCD$_{12}$ *N. vitripennis* virgin females, which parasitized hosts at 20° during four hrs and subsequently were placed at 31° after various intervals of four hrs.

<table>
<thead>
<tr>
<th>Period after egg-laying at 20°</th>
<th>Proportion of gynandromorphs</th>
<th>Number of offspring</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SE</td>
</tr>
<tr>
<td>0- 4</td>
<td>0.334$^b$</td>
<td>0.034</td>
</tr>
<tr>
<td>4- 8</td>
<td>0.307$^b$</td>
<td>0.025</td>
</tr>
<tr>
<td>8-12</td>
<td>0.147$^a$</td>
<td>0.032</td>
</tr>
<tr>
<td>12-16</td>
<td>0.119$^a$</td>
<td>0.032</td>
</tr>
<tr>
<td>16-20</td>
<td>0.129$^a$</td>
<td>0.037</td>
</tr>
<tr>
<td>20-24</td>
<td>0.113$^a$</td>
<td>0.023</td>
</tr>
<tr>
<td>Control 20°</td>
<td>0.097$^a$</td>
<td>0.032</td>
</tr>
</tbody>
</table>

Different superscript characters indicate a significant difference at the 5% level.
Table 5. Mean proportions of gynandromorphs (with standard error) after various hrs of adult treatment at 31° and subsequent host parasitizing at 20°. Data for each of the four successive periods of host parasitizing are given.

<table>
<thead>
<tr>
<th>Parasit. period</th>
<th>0 hrs at 31° Mean</th>
<th>SE</th>
<th>4 hrs at 31° Mean</th>
<th>SE</th>
<th>8 hrs at 31° Mean</th>
<th>SE</th>
<th>12 hrs at 31° Mean</th>
<th>SE</th>
<th>16 hrs at 31° Mean</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.074&lt;sup&gt;a1&lt;/sup&gt;</td>
<td>0.021</td>
<td>0.270&lt;sup&gt;b2&lt;/sup&gt;</td>
<td>0.055</td>
<td>0.387&lt;sup&gt;b3&lt;/sup&gt;</td>
<td>0.058</td>
<td>0.396&lt;sup&gt;b2&lt;/sup&gt;</td>
<td>0.051</td>
<td>0.389&lt;sup&gt;b2&lt;/sup&gt;</td>
<td>0.035</td>
</tr>
<tr>
<td>2</td>
<td>0.090&lt;sup&gt;a1&lt;/sup&gt;</td>
<td>0.018</td>
<td>0.104&lt;sup&gt;a1&lt;/sup&gt;</td>
<td>0.022</td>
<td>0.160&lt;sup&gt;a2&lt;/sup&gt;</td>
<td>0.025</td>
<td>0.096&lt;sup&gt;a1&lt;/sup&gt;</td>
<td>0.017</td>
<td>0.137&lt;sup&gt;a1&lt;/sup&gt;</td>
<td>0.025</td>
</tr>
<tr>
<td>3</td>
<td>0.063&lt;sup&gt;a1&lt;/sup&gt;</td>
<td>0.011</td>
<td>0.122&lt;sup&gt;a1&lt;/sup&gt;</td>
<td>0.024</td>
<td>0.094&lt;sup&gt;a12&lt;/sup&gt;</td>
<td>0.011</td>
<td>0.100&lt;sup&gt;a1&lt;/sup&gt;</td>
<td>0.022</td>
<td>0.083&lt;sup&gt;a1&lt;/sup&gt;</td>
<td>0.021</td>
</tr>
<tr>
<td>4</td>
<td>0.071&lt;sup&gt;a1&lt;/sup&gt;</td>
<td>0.022</td>
<td>0.094&lt;sup&gt;a1&lt;/sup&gt;</td>
<td>0.020</td>
<td>0.070&lt;sup&gt;a1&lt;/sup&gt;</td>
<td>0.017</td>
<td>0.092&lt;sup&gt;a1&lt;/sup&gt;</td>
<td>0.023</td>
<td>0.093&lt;sup&gt;a1&lt;/sup&gt;</td>
<td>0.019</td>
</tr>
</tbody>
</table>

A different superscript letter indicates a significant difference at the 5% level within a parasitizing period. A different superscript number indicates a significant difference at the 5% level within a treatment period.
Table 6. Gynandromorph frequencies among F₃ progeny of various backcrosses. Grandparental crosses between HiCD₁₂ and Or₁₂₃ yielded F₁ females that were backcrossed to either strain. Resulting F₂ females were bred as virgins at 31° and the resulting F₃ progeny were scored for gynandromorphs and eye-color. Number of F₃ individuals and families are given in brackets.

<table>
<thead>
<tr>
<th>Grandparental cross</th>
<th>F₁ backcross male</th>
<th>F₂ female genotype</th>
<th>Proportion gynandromorphs F₃</th>
<th>F₃ gynandromorph proportions from heterozygous mothers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cytoplasmic</td>
<td>or / or</td>
<td>or / +</td>
</tr>
<tr>
<td>HiCD₁₂ x Or₁₂₃</td>
<td>Or₁₂₃</td>
<td>Or</td>
<td>0.010</td>
<td>0.054*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(1026,17)</td>
<td>(989,17)</td>
</tr>
<tr>
<td>Or₁₂₃ x HiCD₁₂</td>
<td>Or₁₂₃</td>
<td>H</td>
<td>0.024</td>
<td>0.122*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(917,16)</td>
<td>(673,15)</td>
</tr>
<tr>
<td>HiCD₁₂ x Or₁₂₃</td>
<td>HiCD₁₂</td>
<td>Or</td>
<td>-</td>
<td>0.178</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(732,16)</td>
<td>(552,13)</td>
</tr>
<tr>
<td>Or₁₂₃ x HiCD₁₂</td>
<td>HiCD₁₂</td>
<td>H</td>
<td>-</td>
<td>0.225</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(451,12)</td>
<td>(659,16)</td>
</tr>
</tbody>
</table>

* indicates a significantly higher proportion of gynandromorphs in comparison to the other phenotype within the same cross.
FIGURE LEGEND

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

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.

Figure 3. Backcross experiment to determine the role of nuclear and cytoplasmic genotype on gynandromorphism. The gynandromorphic trait is lost if either the HiCD12 (H) genotype is replaced by the standard AsymC (A) genotype (filled circles) or the H cytotype is replaced by the A cytotype (filled squares). One and six generations after backcrossing the A genotype into the H cytoplasm gynandromorphism can be recovered although not completely (B₁ and B₆, respectively). Experiments were performed at 25°.
Kamping et al. figure 1
Proportion gynandromorphs

Generation 012345678

0.00 0.05 0.10 0.15 0.20 0.25

High line
Low line

Kamping et al figure 2
Kamping et al. figure 3