Evidence for a Genomic Imprinting Sex Determination Mechanism in *Nasonia vitripennis* (Hymenoptera; Chalcidoidea)

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ABSTRACT

Five different models have been proposed for the sex determination mechanism of Chalcidoidea (Hymenoptera). Except for the most recently proposed model (genomic imprinting sex determination; GISD), each of these models has required complicating additions to explain observed phenomena. This report provides the first experimental test of the GISD model while simultaneously examining the four previously proposed models of sex determination. This test utilizes the parasitic wasp *Nasonia vitripennis*, crossing polyploid females with males harboring the paternal sex ratio chromosome (PSR). The results of this study support the GISD model as the mechanism of sex determination in Chalcidoidea. Specifically, crosses demonstrate that sex determination is independent of embryonic heterozygosity, ploidy, and gametic syngamy but is directly correlated with the embryonic presence of correctly imprinted chromosomes of paternal origin. These crossing experiments also provide information about the poorly characterized mechanisms of PSR, a supernumerary chromosome that induces paternal autosome loss in early embryos. The results demonstrate that the poor transmission of PSR through females is not a result of the ploidy of the host but of an alternative sex-dependent process. Crossing data reveal that PSR consistently induces the loss of the entire paternal complement that it accompanies, regardless of whether this complement is haploid or diploid.

WITHIN Hymenoptera, the mode of sex determination is understood for relatively few species. Interest in these sex determination mechanisms stems from several areas, including the desire for a basic understanding of these mechanisms; the potential to manipulate economically important hymenopteran pests and beneficial parasitoids (Stouthamer *et al.* 1992); and insight into the evolution of haplodiploidy (Hartl and Brown 1970), sociality (Crozier 1977) and sex ratio distorters (Hurst 1991; Ebbert 1993) that are commonly observed within this order. Contributing to this lack of understanding are the complex and intriguing haplodiploid lifestyles of the Hymenoptera (Suomalainen and Saura 1993). Although many different variations of haplodiploidy occur, the most common is arrhenotokous parthenogenesis. In this system, fertilized embryos develop as diploid females and unfertilized embryos become haploid males (Figure 1A). Unlike the best-known plants and animals, sex determination in this system cannot be explained by heteromorphic sex chromosomes. Several alternative models have been proposed to explain sex determination in this order.

Complementary sex determination (CSD) has been previously shown to function in some arrhenotokous parthenogenic hymenopterans. With CSD, individuals that are heterozygous for sex-determining loci develop as diploid females and hemizygous or homozygous individuals develop as haploid or diploid males, respectively (Whiting 1943; Stouthamer *et al.* 1992; Cook 1993a,b). Although CSD has been shown to function in some hymenopteran species (Périquet *et al.* 1993 Cook and Crozier 1995), other species have defied numerous attempts to define their sex determination mechanism. Most notably, Chalcidoidea has confounded investigators, repeatedly requiring new models or complicating modifications as new phenomena were observed.

The genic balance sex determination (GBSD) and maternal effect sex determination (MESD) models were hypothesized as alternatives to CSD. With GBSD, sex is determined by the balance between male (M) and female (F) loci (da Cunha and Kerr 1957; Kerr and Nielsen 1967). The F loci are of a "cumulative" type that act synergistically in diploid individuals. The M loci are noncumulative. In haploid individuals, the noncumulative male loci outweigh the female loci ($M > F$), resulting in a male. In diploid individuals, the cumulative female loci outweigh the noncumulative male loci ($2F > M$), resulting in a female. The MESD model proposes that sex is determined by a ratio of nuclear-to-cytoplasmic products (Crozier 1977; Cook 1993a). A maternally derived cytoplasmic component induces male development in haploid embryos but is outweighed by feminizing loci in diploid individuals, resulting in a female.

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Fertilization sex determination (FSD) was initially hypothesized based on observations of the polyploid strain of *Nasonia vitripennis* (Chalcidoidea; Pteromalidae) (Whiting 1960). In this strain, females could be either diploid or triploid and males could be either haploid or diploid. Whiting noted that, as diagrammed in Figure 1B, the sex is correlated with fertilization of the embryo. This suggested that the individual's sex is determined by fertilization and not by the individual's ploidy. Specifically, unfertilized embryos become males, and fertilized embryos become females.

The genomic imprinting sex determination (GISD) model (Poiré *et al.* 1993; Beukeboom 1995) proposes that one or more loci are differentially imprinted in paternal versus maternal development. Unfertilized embryos contain only maternally derived autosomes with the "maternal imprint," and result in males. Fertilized embryos contain autosomes with the "paternal imprint" in addition to maternally derived autosomes, and the resulting individual develops as a female.

In this article, we focus on the parasitic wasp *N. vitripennis* by conducting crosses to simultaneously examine these five proposed mechanisms of sex determination. These crosses include both wasps from the polyploid strain and males that harbor the paternal sex ratio chromosome (PSR). PSR is a supernumerary chromosome carried by some males of *N. vitripennis*. These PSR males appear to fertilize embryos normally. Shortly after fertilization, however, all of the paternal autosomes abnormally condense prior to the first mitotic division and are eventually lost (Werren *et al.* 1987; Nur *et al.* 1988; Reed and Werren 1995; Dobson and Tanouye 1996). The resulting embryos develop as haploid PSR males harboring the maternally contributed autosome complement and the paternally contributed PSR chromosome (Figure 1C).

Our results support the GISD model. Crosses of triploid females with PSR males produced sons including males whose chromosomes consist of two maternally derived complements and the PSR chromosome. All models except GISD predict that these diploid, heterozygous, fertilized embryos should develop as females. The GISD model predicts that, because the paternal chromosomes are lost as a result of the action of PSR, these individuals should develop as males.

In addition to testing the mechanism of sex determination, these crossing experiments also examine the poorly understood PSR mechanisms of transmission and chromosome loss. Previous studies have shown that PSR is transmitted at high rates through males but poorly through females (Beukeboom and Werren 1993a,b). Our results demonstrate that this difference in transmission rates is because of a sex-specific process (*e.g.*, gametogenesis) and not the association of PSR with diploid versus haploid sets of autosomes. A comparison of PSR associated with haploid and diploid autosome complements reveals no difference in the transmission rate of PSR or in its ability to induce paternal loss.

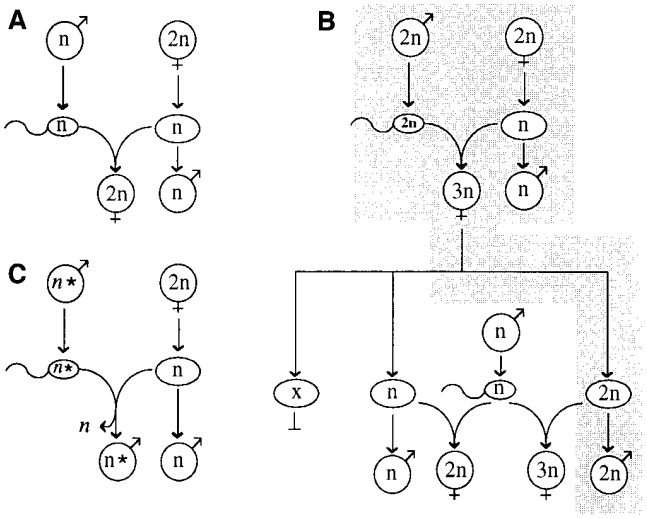

Figure 1.—Haplodiploidy, PSR and polyploid phenomena occurring in *N. vitripennis*. (A) Normally, haploid males produce haploid sperm via a nonreductive abortive meiosis (Hogge and King 1975). Diploid females produce haploid embryos via meiosis (King and Richards 1969; Bull 1982). Unfertilized embryos develop parthenogenetically as haploid males. Fertilized embryos develop as diploid females. (B) In the *N. vitripennis* polyploid strain (Whiting 1960), diploid males produce diploid sperm. Haploid eggs that are fertilized by these diploid sperm develop into triploid females. The fecundity of these triploid females is severely reduced because most of their eggs are aneuploid and inviable (Whiting 1960). These embryos are indicated as "x" embryos. The remaining viable eggs consist of haploid and diploid eggs. If unfertilized, these eggs develop as haploid and diploid males, respectively. If fertilized by haploid sperm, they develop as diploid and triploid females, respectively. The gray shading indicates the method of maintaining this polyploid strain. Diploid females (*st/st*) were crossed with diploid males (*st* +/+ *oy*). In the next generation, the resulting triploid daughters (*st* +/+ *oy/st* +) were allowed to produce sons parthenogenetically. Wild-type, diploid sons (*st* +/+ *oy*) were selected and used to repeat the first generation of maintenance crosses. (C) Males that harbor PSR (indicated as "*") produce haploid sperm bearing the paternal autosomes and PSR. Following fertilization, the paternal autosomes condense abnormally and are lost (Werren *et al.* 1987). The fertilized embryo develops as a haploid male carrying the maternal autosomes and PSR. Unfertilized eggs develop as non-PSR males.

MATERIALS AND METHODS

Nasonia strains and maintenance: In this study, three eye-color mutants are utilized: *scarlet* (*st*), *oyster* (*oy*) and *garnet* (*ga*) (Saul 1990). All three are recessive mutations that appear as wild-type eye color when heterozygous (*e.g.*, *st* +/+ *oy* or *st* +/+ *oy/st* +). The mutant phenotypes are apparent in hemizygous (*e.g.*, *st*), homozygous diploid (*e.g.*, *st/st*) and homozygous triploid (*e.g.*, *st/st/st*) individuals. The *scarlet* (*st*) and *oyster* (*oy*) mutant eye colors both occur at the segregation unit known as the "R locus" of *N. vitripennis*. Although both mutations occur at distinct loci, previous tests have detected no recombination between these loci in more than 25,000 F_2

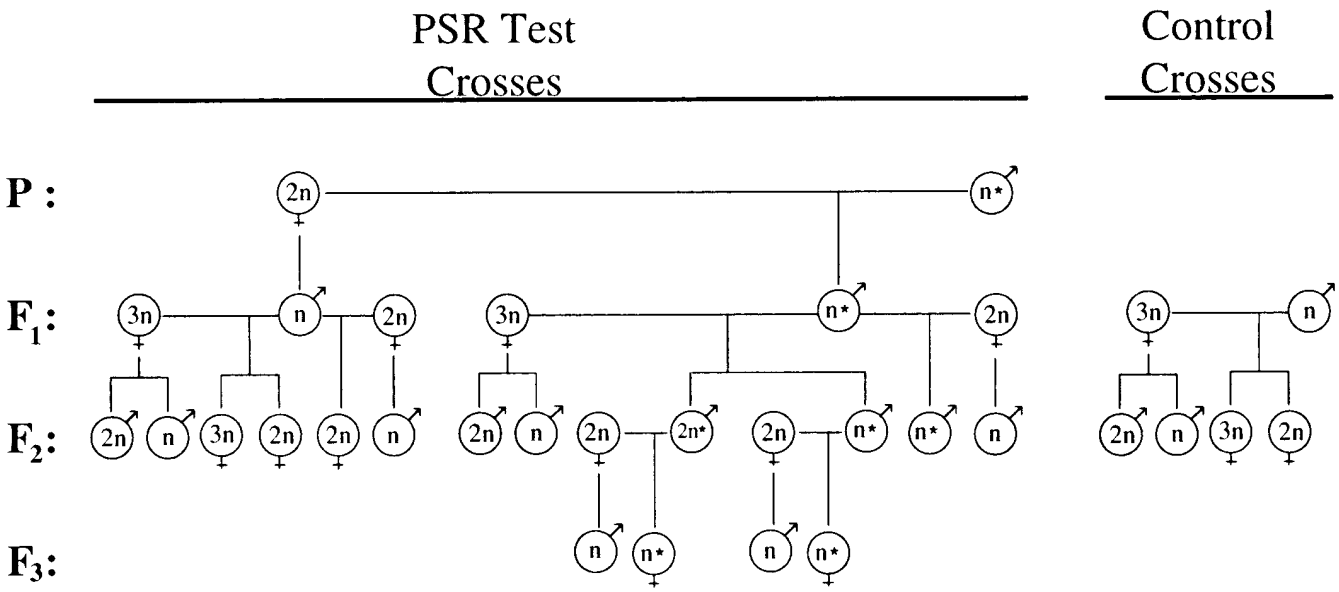

Figure 2.—Diagram of PSR test crosses and non-PSR control crosses. Only the sex, ploidy and presence of PSR ("*") are indicated. Eye color genotypes are not shown in this figure but are described in the text.

males (Whiting 1965). The polyploid strain has been previously described in detail (Whiting 1960). A summary of this strain and the typical maintenance crosses is diagrammed in Figure 1B. The PSR strain has also been previously described (Werren *et al.* 1987) and consists of males with and without PSR, resulting from fertilized and unfertilized eggs, respectively (Figure 1C). The PSR strain was maintained by crossing females of the wild-type Leiden Lab II strain (Saul *et al.* 1965) with random males from all-son PSR broods. Previous work has shown that immediately following mating, females produce mostly unfertilized embryos (van den Assem 1977). To promote sperm utilization, females were not placed on hosts until 24 hr after mating. For oviposition, female wasps were placed individually on three *Sarcophaga bullata* pupal hosts.

Experimental design and crosses: Crosses were conducted to examine the behavior of PSR when introduced into haploid versus diploid embryos (Figure 2). To provide a phenotypic marker for the paternal autosomes, PSR was first crossed into a *scarlet* background by pair mating (one male and one female) *scarlet* females (*st/st*) with PSR males (*+;PSR*). Because a majority of embryos are fertilized (Werren and van den Assem 1986), the resulting all-son broods (F_1) were expected to consist primarily of *scarlet* PSR males (*st;PSR*). The remainder were expected to be *scarlet* non-PSR males (*st*) resulting from unfertilized eggs. To determine which of these F_1 males harbored PSR and to examine the functionality of PSR, F_1 males were crossed ("test crosses") with a wild-type triploid female (*st +/+ oy/st +*) and a diploid *scarlet* female (*st/st*). "Control crosses" were also conducted of wild-type triploid females (*st +/+ oy/st +*) and haploid, non-PSR *scarlet* males (*st*).

The genotypes of the F_2 individuals resulting from the crosses of polyploid females were determined by observing the F_2 phenotypes or the sex ratio of their subsequent broods (F_3). *Oyster* and wild-type males were known to be haploid (*oy*) and diploid (*st +/+ oy*), respectively. The genotypes of females and *scarlet* males could not be determined from the phenotype and were not subsequently examined except to determine which males harbored PSR. The presence of PSR in these broods (F_2) was determined by mating diploid *garnet* females (*ga/ga*) with these males. Males (F_2) producing all-son broods were scored as harboring PSR. Males producing mostly daughter broods were scored as non-PSR males.

PCR amplification: The presence of PSR in diploid males was verified using a PSR-specific PCR reaction on diploid wild-type males. Amplification was identical to a previously described protocol (Dobson and Tanouye 1996). Immediately upon eclosion, males to be used for PCR amplification were pair-mated with a single *garnet* female for 24 hr. Each was then frozen, crushed and 500 μl of 5% CHELEX 100 resin solution (Bio-Rad, Hercules, CA) was added. Following 24 hr agitation at 57°, the solution was boiled for 15 min and pelleted. One microliter of supernatant was used as template for amplifications. PCR reactions were conducted in a 50 μl vol with final concentrations of 25 mm Tris-HCl (pH 8.3), 50 mm KCl, 0.2 mm dNTPs, 5 mm $MgCl_2$, 0.5 μmol primers (5′-ggC gAC Agg ACg gCg TTC-3′ and 5′-CAA gAT ggC ggC Cgg Ag-3′), and 2 units AMPLITAQ polymerase (Perkin-Elmer, Foster City, CA). PCR amplification consisted of 40 cycles of 94° (30 sec), 55° (30 sec), and 72° (30 sec), followed by extension at 72° (7 min). Reactions were analyzed on a 1% agarose gel. Figure 3 provides an example of the results obtained from this assay.

RESULTS

Introduction of PSR into diploid embryos: Crosses were conducted to examine sex determination in *N. vitripennis* and the behavior of PSR when introduced into diploid embryos (Figure 2). Fifty random males from *scarlet*, PSR broods (F_1) were each crossed with diploid *scarlet* (*st/st*) and triploid wild-type (*st +/+ oy/st +*) females. Matings with diploid females were made to determine the genotype of the males (*i.e.*, which males harbored PSR) and the functionality of PSR. The results of these crosses are shown in Table 1. Of the 45 successful crosses, 34 produced all-son broods; the fathers of these all-son broods were interpreted as PSR males (*st;PSR*). Two additional test crosses produced broods

TABLE 1

Determination of the presence and functionality of PSR

	Crosses with diploid female		Crosses with triploid female		
	Brood number	Brood sex ratio (%)	Brood number	Brood sex ratio (%)	F_1 male genotype
PSR test crosses	5	No progeny	1	66.7	Not used
			2	0.0	in study
			2	No progeny	
	34	0.0 ± 0.0	27	0.0	*st;PSR*
			7	No progeny	
	2	5.2 ± 5.7	2	0.0	*st;PSR*
	9	84.6 ± 9.1	8	68.2	*st*
			1	No progeny	
Non-PSR control crosses			26	72.1	*st*
			3	0.0	
			21	No progeny	

Fifty PSR test crosses and 50 non-PSR control crosses are categorized based on their resulting broods. The number of broods and brood sex ratio (percent daughters) are shown for each brood category. In diploid crosses, the brood sex ratio is calculated as the average and standard deviation observed in separate broods. Because of the small brood sizes, sex ratio in triploid crosses is expressed as the total females/total brood (all broods combined). Males in the PSR test crosses were crossed with both diploid and triploid females. Males in non-PSR control crosses were crossed only to triploid females.

with 9.2% daughters (11 daughters, 108 males) and 1.1% daughters (1 daughter, 87 males). These latter two crosses are interpreted as PSR males producing rare daughters. This phenomenon is reported as occurring in ~10% of PSR crosses and is likely attributed to mosaic germ tissue (Werren and van den Assem 1986; Beukeboom and Werren 1993b). Nine F_1 males produced broods with an average of 84.6% daughters when crossed to diploid females and were interpreted as not harboring PSR (*i.e.*, were *st* males resulting from unfertilized eggs). This demonstrates that 36/45 (80.0%) of the randomly selected F_1 males harbored PSR (*i.e.*, were *st;PSR* males). This transmission rate of PSR is equivalent to the fertilization rate (84.6%) observed in the nine F_1 test crosses of diploid females (*st/st*) with non-PSR males (*st*). This transmission rate of PSR also agrees with previous reports that show that the percentage of PSR males is equivalent to the fertilization rate (Werren and van den Assem 1986; Beukeboom and Werren 1993a,b; Dobson and Tanouye 1996).

Of the 36 males determined to harbor functional PSR chromosomes in crosses with diploid females, 29 also produced all-son broods when crossed with triploid females (Table 1). The remaining seven crosses produced no progeny. Reduced progeny numbers were expected because triploid females produce many inviable eggs as a result of aneuploidy (Whiting 1960; Conner 1966). Eight of the nine F_1 males determined to be devoid of PSR in crosses with diploid females also produced mostly daughter broods (average 68.2% daughters) when crossed to triploid females. The ninth cross produced no progeny. In control crosses of triploid females (*st +/+ oy/st +*) with non-PSR *scarlet* males (*st*), 21/50 failed to produce progeny. Of the remaining 29 control crosses, 26 produced mostly daughter broods (average 72.1% daughters). The three remaining crosses produced all-son broods. These all-son broods probably represent unfertilized females or an artifact of the small brood sizes produced by triploid females. These results demonstrate that both haploid and diploid eggs that are fertilized by PSR sperm develop as males, resulting in all-son broods; haploid and diploid eggs that are fertilized by non-PSR sperm develop as females.

When PSR is introduced into haploid eggs, it converts these fertilized embryos from females into males (Figure 1C). This conversion results from the loss of the paternal autosome complement (Werren *et al.* 1987). To determine if the same phenomenon occurs when PSR is introduced into diploid eggs, a simple model (appendix) was generated to describe the sex and phenotypes resulting from crosses of triploid females with PSR and non-PSR males. As shown in Table 2, the broods resulting from non-PSR crosses were similar to the predictions of this model. The broods resulting from PSR test crosses were also similar to the predicted percentages (Table 3). Independent tests of key assumptions used in the generation of this model demonstrated that these assumptions were valid. Alteration of these assumptions would require complicating modifications to produce the same

TABLE 2

Non-PSR broods resulting from F_1 crosses with triploid females

Sex: phenotype	Control	Test	Possible genotype(s)	Percent observed	Percent expected
♂; *oyster*	10	3	*oy*	4.6	5.9
♂; wild-type	10	3	*st* + / + *oy*	4.6	8.3
♂; *scarlet*	41	15	*st* or *st/st*	19.7	18.8
♀; *scarlet*	83	29	*st/st or st/st/st*	39.3	38.1
♀; wild-type	75	16	*st* + / + *oy* or *st* + / + *oy/st* +	31.9	28.9
Total progeny (F_2)	219	66			
Number of broods	29	8			

Broods resulting from F_1 crosses of triploid females (*st* + / + *oy/st* +) with haploid non-PSR males (*st*) are categorized by their sex and eye color phenotypes. These broods include the 29 control crosses of non-PSR males and the eight test crosses determined not to harbor PSR. The genotypes of these phenotypic categories are also shown. In some cases (*i.e.*, *scarlet* males and all females), multiple genotypes may be represented by these phenotypic categories. Each phenotype is also expressed as a percentage of the total progeny (combining non-PSR males from both test and control crosses). These percentages are compared with the percentages predicted by the model. These predictions use values of $m = 40\%$, $d = 40\%$ and $f = 67\%$ (see appendix). A comparison of the observed *vs.* the expected proportions yielded: $\chi^2 = 2.40$, df = 4, $P > 0.66$.

predictive power. These assumptions include: (1) that the transmission rate of PSR is equivalent to the fertilization rate, (2) the presence of PSR in sperm causes the loss of the entire paternal autosome complement(s) while PSR itself survives, (3) the maternal complement(s) are not affected by this paternal autosome loss, and (4) a GISD mechanism.

The transmission rate and survival of PSR are demonstrated by comparing the percentage of PSR sons resulting from PSR crosses with the percentage of females resulting from non-PSR crosses. As shown in Table 3, PSR was transmitted to an equivalent percentage of haploid (*oy;* 24/37 = 64.9%) and diploid (*st* +/+ *oy;* 26/38 = 68.4%) embryos. Both of these percentages are equivalent to the 71.2% females (203 females, 82 males) observed in non-PSR crosses (Table 2). This demonstrates that PSR is transmitted to and survives within all fertilized eggs, whether haploid or diploid.

The ratio of genotypes resulting from test crosses of triploid females with haploid PSR males demonstrates that PSR induces the specific loss of the entire paternal autosome set. Table 4 shows the genotypes expected to

TABLE 3

PSR broods resulting from F_1 crosses with triploid females

Sex: phenotype	Test (PSR)	Possible genotype(s)	(*n*)	Percent observed	Percent expected
♂; *oyster*	40	*oy*	13	7.1	5.9
		oy;PSR	24	13.1	12.1
		No progeny	3		
♂; wild-type	43	*st* + / + *oy*	12	6.9	8.3
		st + / + *oy;PSR*	26	14.9	16.9
		No progeny	5		
♂; *scarlet*	112	*st* or *st/st*	29	23.1	18.8
		st;PSR or *st/st;PSR*	44	35.0	38.1
		No progeny	5		
Total progeny (F_2)	195				
Number of broods	29				

Broods resulting from F_1 crosses of triploid females (*st* + / + *oy/st* +) and haploid PSR males (*st;PSR*) are categorized by their sex and eye color phenotypes. These broods resulted from the 29 test crosses where the F_1 male harbored a functional PSR. Multiple genotypes are possible for each of the phenotypic categories. Each of these F_2 categories is broken into subgroupings of PSR and non-PSR males based on observations of the broods (F_3) resulting from crosses of these males with *garnet* females as described in the text. Each phenotype is also expressed as a percentage of the total progeny. These percentages are compared with the percentages predicted by the model. These predictions use values of $m = 40\%$, $d = 40\%$ and $f = 67\%$ (see appendix). A comparison of the observed *vs.* the expected proportions yielded: $\chi^2 = 2.03$, df = 5, $P > 0.84$.

TABLE 4

Predicted genotypes and phenotypes resulting from crosses of triploid females with PSR and non-PSR males

	Haploid eggs		Diploid eggs	
	oy	*st*	*st/st*	*st/oy*
Unfertilized	♂; *oy* (oyster)	♂; *st* (scarlet)	♂; *st/st* (scarlet)	♂; *st* + / + *oy* (wild type)
st sperm	♀; *st* + / + *oy* (wild type)	♀; *st/st* (scarlet)	♀; *st/st/st* (scarlet)	♀; *st* + / + *oy/st* + (wild type)
st;PSR sperm	♂; *oy/PSR* (oyster; PSR)	♂; *st;PSR* (scarlet; PSR)	♂; *st/st;PSR* (scarlet; PSR)	♂; *st* + / + *oy;PSR* (wild type; PSR)

Rows are categorized by the possible sperm types that were introduced into eggs in control and test crosses (including unfertilized embryos). Columns are divided by the possible viable genotypes that can be produced by triploid females (*st* + / + *oy/st* +). The sex, genotype and phenotype (in parentheses) are indicated for the resulting individuals. These predicted sex and genotypes are based on several assumptions discussed in both the text and appendix.

result from control and test crosses. Aneuploid embryos produced by triploid mothers are inviable (Whiting 1960; Conner 1966). If PSR failed to cause paternal autosome loss, an increase would be expected in the percentage of wild-type PSR males as a result of the conversion of *oy* embryos to *st* +/+ *oy;PSR* males (*st;PSR* contributed by the father and *oy* contributed by the mother). If PSR caused the loss of one or both maternal complement(s), an increase would be expected in the percentage of *scarlet* PSR males. As shown in Tables 2 and 3, the percentages are as predicted for the specific loss of the paternal autosomes. Incomplete loss of a paternal or maternal complement caused by PSR would be expected to result in an increased incidence of aneuploidy and reduced brood sizes in PSR crosses relative to non-PSR crosses. A comparison of brood sizes resulting from crosses of triploid females with non-PSR males [7.7 ± 5.1 (SD); $n = 37$] and PSR males [7.0 ± 3.6 (SD); $n = 29$] demonstrates that a significant increase in aneuploidy does not occur in PSR crosses ($P > 0.25$; *t*-test).

A GISD mechanism is demonstrated by the correlation between the presence of the paternal autosomes in the embryo and feminization of the individual. As discussed above, crossing results show that PSR induces the specific loss of the paternal autosomes. This loss results in the individual's masculinization, resulting in haploid or diploid PSR males. The individual's sex is not affected by gamete syngamy or genotype (*i.e.*, heterozygosity or ploidy).

Diploid PSR males: F_1 crosses of PSR males with triploid females allowed the introduction of PSR into a diploid male background (*st* +/+ *oy;PSR*). Subsequent crosses allowed us to examine the ability of PSR to induce autosome loss and its transmission in this diploid background. These males were known to be diploid due to their wild-type eye color. The presence of PSR in these males was demonstrated by PCR amplification using a previously developed PSR-specific PCR amplification (Dobson and Tanouye 1996). Forty of the 43 wild-type males resulting in test crosses were PCR assayed (Figure 3). Twenty-seven of these males were shown to harbor PSR by the PCR assay. Of these, 22 produced all-son broods. An additional PSR-positive male produced a brood with one female and 90 males (1.1% female). This cross was interpreted as rare female production by PSR males as previously discussed. The remaining four PSR-positive males did not produce any progeny in crosses. Of the 13 males shown to not harbor PSR by

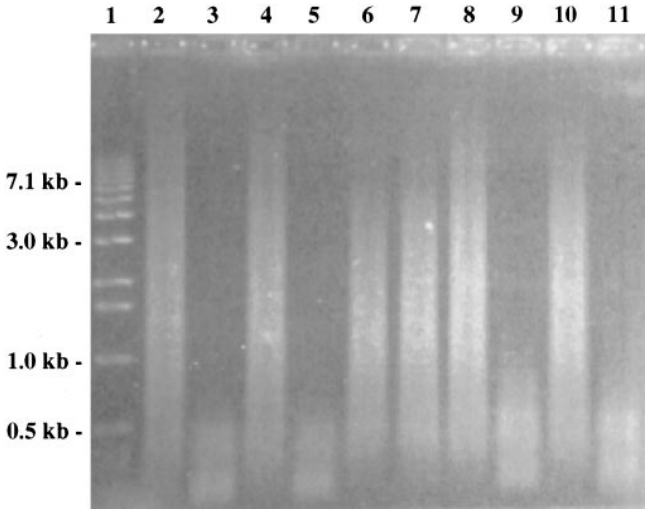

Figure 3.—An example of PCR amplification utilized to detect the PSR chromosome in diploid males. Ten males from PSR all-son broods (lanes 2–10) and one control non-PSR male (lane 11) were assayed. The high molecular weight smear (<4 kb) in lanes 2, 4, 6–8 and 10 is indicative of the presence of PSR in wasps. As previously discussed (Dobson and Tanouye 1996), this unusual amplification product (*i.e.*, smearing pattern instead of a distinct band) may be a result of the amplification within a repetitive region of the PSR chromosome (Nur *et al.* 1988) and the subsequent concatamerization of the multiple-repeat products. This smear is not observed in non-PSR wasps (lanes 3, 5, 9, 11). Lane 1 is a molecular size standard (1-kb ladder; Gibco BRL, Grand Island, NY).

the PCR assay, 11 produced broods with many daughters. Two produced all-son broods and were interpreted as fertilization failures of the males. Supporting this, one of these males was found dead <12 hr after pairing with a female. This demonstrates that 27 of the males (67.5%) harbored PSR (*i.e.*, were *st +/+ oy;PSR* males). The remaining 13 males did not harbor PSR (*i.e.*, were *st +/+ oy* males) and were interpreted as males developing from unfertilized, diploid embryos.

To examine the functionality of PSR in diploid males, the males used in the PCR assay were also crossed to diploid *garnet* females (*ga/ga*). These F_2 crosses produced all-son broods of the *garnet* phenotype, demonstrating that both paternal complements had been lost, leaving only the haploid maternal complement. Both sets of paternal chromosomes were completely lost, because partial loss would result in aneuploidy and reduced brood sizes. A comparison of the mean brood sizes in crosses of *garnet* females (*ga/ga*) with haploid PSR males [*oy;PSR*; 63.8 ± 23.8 (SD), $n = 24$] and diploid PSR males [*st +/+ oy;PSR*; 71.6 ± 25.3, $n = 26$] were not significantly different ($P > 0.15$; *t*-test).

The transmission rate of PSR through diploid males was examined by determining the percentage of their broods (F_3) that harbored PSR. This was accomplished by crossing random F_3 males from all-son broods with *garnet* females (*ga/ga*). Twelve of 15 crosses (80%) produced all-son broods demonstrating that the males harbored functional PSR chromosomes (*i.e.*, were *ga;PSR* males). The remaining crosses produced mostly daughter broods. This shows that PSR was transmitted from diploid PSR males to 80% of their sons. This is equivalent to the 80% fertilization rate observed in diploid female crosses (84.6%; Table 1) and demonstrates that PSR is transmitted through diploid males at rates equal to that of fertilization (identical to PSR in haploid background).

DISCUSSION

The crosses conducted in this report provided a test of the multiple hypotheses of sex determination that have been proposed for *N. vitripennis.* Of specific interest, these crosses produced diploid heterozygous eggs that were fertilized by sperm that harbored a haploid complement of autosomes and PSR. As a result of the action of PSR, these embryos lost the paternally contributed complement of autosomes leaving only the maternally contributed complements and the paternally contributed PSR. These embryos developed into diploid heterozygous males harboring a functional PSR chromosome. Of the five previously described sex-determination hypotheses, only the GISD model predicts that these fertilized, diploid, heterozygous embryos should develop as males. This is because of PSR's removal of the paternal "imprint" in the process of destroying the paternal chromosomes.

Complicated adjustments are needed to explain these crossing results using the four alternatively proposed sex determination models. The heterozygosity of these males demonstrates that a complementary sex determination (CSD) mechanism cannot function in these wasps. Because of heterozygous sex loci, CSD would predict that the diploid PSR wasps generated in this study should develop as daughters. Our results complement a previous study in which inbreeding experiments with *N. vitripennis* failed to generate diploid males (Skinner and Werren 1980). The absence of a CSD mechanism in Chalcidoidea is also demonstrated by observations of Wolbachia-induced gamete duplication in Trichogramma (Stouthamer and Kazmer 1994). Wolbachia is a clade of intracellular microorganisms that form inherited infections within numerous arthropod species including *N. vitripennis* (Werren 1997). In this system, the intracellular bacteria Wolbachia cause a segregation failure during the first mitotic division in unfertilized (haploid) embryos. The resulting nucleus contains two sets of identical chromosomes. According to CSD, this individual would be expected to be male because there is complete homozygosity at all loci. However, these individuals develop as diploid females.

The observation that males result from these diploid PSR sons also demonstrates that the genic balance sex determination (GBSD) and maternal effect sex determination (MESD) models do not operate in *N. vitripennis.* Both of these models predict that diploid PSR offspring generated in our crosses should be daughters. According to GBSD, the cumulative *F* loci in these diploids should outweigh the noncumulative *M* loci resulting in a female. According to MESD, the feminizing loci in these diploids should overcome the masculinizing cytoplasmic effects resulting in a female. These results show that feminization does not simply result from the individual having a diploid complement of chromosomes.

It has been hypothesized that a mutation in the polyploid strain may have altered the GBSD mechanism. This mutation may have inactivated an important *F* locus (Stouthamer and Kazmer 1994). In diploids with this inactive *F* locus, the *M* loci would outweigh the *F* loci and the individual would develop as a diploid male. We find this hypothesis untenable for the following reason: according to the definition of GBSD, the *F* loci must outweigh the *M* loci in a triploid females. When this female is allowed to parthenogenetically reproduce, the *F* loci would also be expected to outweigh the *M* loci in some of her diploid offspring resulting in females. No parthenogenetically produced females were observed in this study or have been previously reported. Nonrandom segregation of sex loci or other complicated adjustments would be required for GBSD to explain observations of *N. vitripennis.*

The fertilization sex determination (FSD) model is also not consistent with our observations of diploid PSR males. The presence of PSR in these diploid PSR sons demonstrates that they developed from fertilized eggs.

Therefore, sex determination is unlikely to be determined by the physical syngamy of sperm and egg.

The possibility that PSR itself is male-determining because of its encoding of one or more "masculinization genes" that are independent of its paternal autosome loss mechanism cannot be excluded. However, previous deletion experiments failed to generate PSR chromosomes that maintained the masculinization effect but that did not cause paternal loss (Beukeboom *et al.* 1992; Beukeboom and Werren 1993a). Nonfunctional PSR chromosomes were generated, but these did not cause masculinization or paternal loss. This suggests that if PSR encodes masculinization genes, they must be closely linked to the genes or chromosomal regions that cause paternal loss. We believe that a more likely explanation is that PSR is male determining, but that this masculinization results secondarily from paternal autosome loss and not as a direct result of male-determining genes present on PSR.

One report suggests that a maternal component may also play a role in masculinization (Friedler and Ray 1951). In this study, females were heavily irradiated and then mated to produce haploid, androgenic zygotes. These individuals developed as males. If feminization of the embryo were solely dependent on paternally contributed component(s), these individuals would be expected to be females. This suggests that both the paternal autosomes and an additional maternally contributed component are needed to produce daughters.

Also of interest, if GISD is a general mechanism of Chalcidoidea, then Wolbachia must have at least two mechanisms to explain the genome duplication phenomenon in Trichogramma (discussed above). In addition to causing genome duplication (Stouthamer and Kazmer 1994), Wolbachia would need to imprint at least one set of chromosomes to imitate the "paternal imprint." Otherwise the Wolbachia-induced genome duplication would result in diploid males. Considering the varied reproductive effects of Wolbachia on insects, many of which may involve chromosomal imprinting (Werren 1997), it is not unreasonable to hypothesize this dual ability of Wolbachia.

This report demonstrates that the poor transmission of PSR through females is a result of sex-specific processes and not because of the association of PSR with a diploid complement. Previous experiments showed that nonfunctional, PSR deletion chromosomes are transmitted at high rates through males and poorly through females (Beukeboom *et al.* 1992; Beukeboom and Werren 1993a). This suggests the selective advantage for PSR to develop and maintain its mechanism for converting females to males by inducing paternal autosome loss. The difference in transmission rates could be explained by the inability of PSR to function when associated with a diploid complement of autosomes instead of a haploid complement. Our results show that the mechanisms of PSR function equivalently in both haploid and diploid males, suggesting that a different sex-specific process or product participates in the differential transmission of PSR. A likely candidate for this process is the different forms of gametogenesis occurring in males and females (Beukeboom *et al.* 1992; Beukeboom and Werren 1993a). Oogenesis in *N. vitripennis* occurs by meiotic division, whereas spermatogenesis occurs via nonreductive, abortive meiotic divisions (Hogge and King 1975). This suggests that PSR survives nonreductive divisions (*i.e.*, spermatogenesis and mitosis) at high rates but is poorly transmitted through reductive meiotic divisions (*i.e.*, oogenesis).

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APPENDIX

A simple model was generated to predict the sex and genotype expected from crosses of triploid females (*st* +/+ *oy/st* +) and haploid PSR males (*st;PSR*). This model assumed sex determination by a genomic imprinting mechanism (GISD). In this system, fertilized embryos developing with a normal set of paternal autosomes become females; unfertilized embryos become males; embryos fertilized by PSR males also become males because the paternal autosomes are lost in the early embryo.

A previous experiment in which virgin, triploid females were allowed to reproduce parthenogenetically (Conner 1966) shows that the number of diploid males is consistently below 50% with an average of 41.8% diploid sons. Here we assumed that 40% of embryos produced by females were diploid (d). This same study also demonstrated that preferential segregation occurs in the alleles of triploid females. Triploid females contain one maternally contributed complement and two paternally contributed complements. This maternally derived complement is indicated by underlining (*e.g.*, *st*) throughout this appendix. The transmission of the maternal:paternal:paternal alleles was consistently demonstrated to be in a ratio of

$$\underline{m}: \frac{1-m}{2} : \frac{1-m}{2}$$

which is proportional to 4:3:3. We used this same ratio for the transmission of the autosomal complements from triploid females. The *scarlet* (*st*) and *oyster* (*oy*) mutant eye color markers were treated as a single segregation unit because recombination does not occur between these loci (Whiting 1965).

The model assumes that the behavior of PSR in a polyploid background is similar to its behavior in nonpolyploid background (Werren and van den Assem 1986; Beukeboom and Werren 1993b; Beukeboom 1994). Specifically, PSR causes the specific loss of all the autosomes (haploid or diploid complement) that it accompanies. This specific loss does not affect the maternal complement (*e.g.*, cause an increase in aneuploidy). The transmission rate of PSR is assumed to be equivalent to the fertilization rate (f) for crosses with both diploid and triploid females and regardless of whether PSR is associated with a haploid or diploid complement of paternal autosomes.

Triploid females used in F_1 crosses were of the *st* +/+ *oy/st* + genotype. These females could produce haploid ($1-d$) or diploid (d) gametes. Haploid gametes could of be three types: *st* +, with an expected proportion of $m(1-d)$, and *st* + or + *oy*, each with an expected proportion of

$$\frac{(1-m)}{2}(1-d).$$

Grouping these into the *scarlet* and *oyster* phenotypes yields an expected proportion of

$$\left[\frac{(1-m)}{2}+m\right](1-d)$$

and

$$\frac{(1-m)}{2}(1-d),$$

respectively.

Diploid gametes produced by $\underline{st\ +}/+\ oy/st\ +$ females could be of two phenotypes: wild type and *scarlet*. Wild-type eggs could be $st\ +/+\ oy$ in a proportion of

$$\left[\frac{(1-m)^2}{(1+m)}\right](d)$$

or $\underline{st\ +}/+\ oy$ in a proportion of

$$\left[\frac{m(1-m)}{(1+m)}+\frac{m}{2}\right](d).$$

This gives an expected proportion of phenotypically wild-type eggs of

$$\left[\frac{(1-m)}{(1+m)}+\frac{m}{2}\right](d).$$

Diploid *scarlet* eggs ($\underline{st\ +}/st\ +$) were expected in a proportion of

$$\left[\frac{m(1-m)}{(1+m)}+\frac{m}{2}\right](d).$$

Haploid and diploid gametes could be fertilized or remain unfertilized and develop parthenogenetically. Fertilized embryos could receive either the paternally donated PSR chromosome in PSR crosses, or the *st* complement in non-PSR crosses. Table A1 shows the formulae for the expected proportions for each of the phenotypic categories used in this study.

TABLE A1

Model predictions

Non-PSR crosses	PSR crosses	Expected proportion of broods	(%)
♂; *oy*	♂; *oy*	$\left(\frac{1-m}{2}\right)(1-d)(1-f)$	5.9
♂; *st +/+ oy*	♂; *st +/+ oy*	$\left[\left(\frac{1-m}{1+m}\right)+\frac{m}{2}\right]d(1-f)$	8.3
♂; *st*	♂; *st*	$\left(\frac{(1-m)}{2}+m\right)(1-d)(1-f)$	18.8
♂; *st/st*	♂; *st/st*	$\left(\frac{m(1-m)}{1+m}+\frac{m}{2}\right)d(1-f)$	
♀; *st/st*	♂; *st;PSR*	$\left[\left(\frac{1-m}{2}\right)+m\right](1-d)f$	38.1
♀; *st/st/st*	♂; *st/st;PSR*	$\left[\frac{m\ 1-m)}{1+m}+\frac{m}{2}\right]df$	
♀; *st +/+ oy*	♂; *oy;PSR*	$\left(\frac{1-m}{2}\right)(1-d)f$	12.1
♀; *st +/+ oy/st +*	♂; *st +/+ oy;PSR*	$\left[\left(\frac{1-m}{1+m}\right)+\frac{m}{2}\right]df$	16.9

Sex and genotypes expected from crosses of triploid females ($st\ +/+\ oy/st\ +$) with non-PSR (*st*) and PSR males (*st;PSR*). For each category, a formula representing the expected brood proportion is also shown. The final column indicates the percentage expected for each category. These predictions use values of $m = 40\%$, $d = 40\%$ and $f = 67\%$ (see appendix).