# Maternal Control of Haplodiploid Sex Determination in the Wasp Nasonia

Eveline C. Verhulst, *et al.*

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To determine the minimum cost of signaling, $q$, necessary to ensure that the signal is honest, we introduced a third strategy: "liar," who may benefit by "turning on" the punishment process without paying the costs. During the first period, liars signal that they are punishers, incurring the signaling cost, and then cooperate so as to avoid punishment during the first period. However, they do not punish, and therefore avoid the associated costs. In subsequent periods, liars count the number of other group members that signaled in the first period and cooperate if the number of such signalers is greater than $\tau + 1$. Because liars never punish, after the first period they behave like nonpunishers and so receive the nonpunisher payoff. At equilibrium, punishers and nonpunishers have the same fitness, and thus liars can invade if their expected payoff during the first period is greater than the expected payoff of nonpunishers during the first period. This leads to a minimum cost of signaling, given in (*23*). The value of $q$ used in our calculations satisfies this condition for all results presented here.

Although punishment is evolutionarily stable in this model, so is nonpunishment. A complete account of the evolution of cooperation must explain how punishing strategies can increase when rare. In their classic work on pairwise reciprocity, Axelrod and Hamilton (*24*) showed that a small amount of nonrandom assortment, such as interaction between weakly related group members, destabilizes noncooperative equilibria but not cooperative equilibria. This principle holds in a wide range of pairwise cooperative interactions, but not in larger groups (*13*–*15*).

To explore the effects of genetic assortment, we dropped our assumption that groups are formed at random and assumed that the relatedness within groups is $r > 0$, so that individuals are more likely to interact with individuals similar to themselves than expected by chance. Figure 4 shows the equilibrium behavior assuming that $r = 0.07$, which is a rough estimate of the average relatedness within human foraging groups (*22*). For low thresholds ($\tau \leq 3$), the only stable equilibrium is a mixture of punishers and nonpunishers, which means that punishers invade when rare. And because of the population structure (between-group genetic differences), punishment may also be altruistic at the polymorphic equilibrium.

This result persists when groups are much larger ($n = 72$) and for lower levels of relatedness if the benefit-cost ratio is somewhat higher (*23*). However, modest assortment does not allow punishment strategies with higher thresholds to invade populations of punishers with lower thresholds, so there is no evolutionary process in this model that would ratchet up the threshold levels. Thus, consistent with ethnographic observation the model predicts that only some individuals will engage in punishment. However, even when $\tau = 3$—meaning that a minimum of four out of 18 individuals punish—groups achieve about two thirds of the maximum gains from cooperation attainable with higher thresholds (Fig. 3).

Unlike many models of the evolution of punishment, this one does not suffer from a "second-order free-rider" problem in which individuals who cooperate but do not punish out-compete the punishers. To see why, consider a new strategy: "contingent cooperators," who cooperate during the first period if there are $\tau + 1$ signaling individuals but do not punish. Contingent cooperators avoid punishment during the first period and otherwise behave like nonpunishers, and thus have higher fitness than nonpunishers. As a result, they invade the polymorphic punisher-nonpunisher equilibrium, replacing the nonpunishers. However, because they still respond to punishment, and punishment still benefits punishers, the population evolves to a stable equilibrium at which punishers and contingent cooperators coexist and that cannot be invaded by other second-order free-riding types. The frequency of punishers at this new equilibrium is approximately the same as in the original punisher-nonpunisher equilibrium (*23*).

In our model, the initial proliferation of punishment occurs under plausible levels of group genetic differences and results in persistent and high levels of cooperation. This result depends on the contingent nature of punishment and the existence of increasing returns to punishment. It differs from the model of Hauert *et al*. (*28*), in which the population cycles between periods of cooperation, defection, and opting-out of the interaction entirely, the latter strategy invading the all-defect phase of the cycle and subsequently being invaded by cooperators. Although their model applies to some forms of cooperation, the present model is a more realistic representation of the nature and dynamics of human cooperation (*29*, *30*).

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### Supporting Online Material

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Materials and Methods
Figs. S1 to S7
References

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# Maternal Control of Haplodiploid Sex Determination in the Wasp *Nasonia*

**Eveline C. Verhulst, Leo W. Beukeboom, Louis van de Zande***

All insects in the order Hymenoptera have haplodiploid sex determination, in which males emerge from haploid unfertilized eggs and females are diploid. Sex determination in the honeybee *Apis mellifera* is controlled by the *complementary sex determination* (*csd*) locus, but the mechanisms controlling sex determination in other Hymenoptera without *csd* are unknown. We identified the sex-determination system of the parasitic wasp *Nasonia*, which has no *csd* locus. Instead, maternal input of *Nasonia vitripennis transformer* (*Nvtra*) messenger RNA, in combination with specific zygotic *Nvtra* transcription, in which *Nvtra* autoregulates female-specific splicing, is essential for female development. Our data indicate that males develop as a result of maternal imprinting that prevents zygotic transcription of the maternally derived *Nvtra* allele in unfertilized eggs. Upon fertilization, zygotic *Nvtra* transcription is initiated, which autoregulates the female-specific transcript, leading to female development.

Mechanisms for sex determination are remarkably variable. In many insect species, a primary signal initiates one of two alternative routes of regulatory gene cascades (*1*). This cascade leads to sex-specific differential splicing of the gene *doublesex* (*dsx*) and the pro-

duction of either male- or female-specific DSX proteins (*2*–*11*). The splicing factor transformer (TRA) (*12*–*15*), termed feminizer (FEM) in *Apis mellifera* (*16*), mediates the primary sex-determining signal in females by regulating the female-specific splicing of *dsx* pre-mRNA. In males, no functional TRA/FEM protein is present because of sex-specific splicing of *tra*/*fem* pre-mRNA, leading to default male-specific splicing of *dsx* primary transcripts.

In diploid insects, sex is mostly signaled by components of sex chromosomes (for example, XY and ZW). In Hymenoptera, however, sex is usually regulated by the ploidy of the embryo (*17*, *18*): Males are haploid, developing from unfertilized eggs, whereas diploid females develop from fertilized eggs. In the honeybee *A. mellifera*, the *complementary sex determiner* (*csd*) gene (which exhibits homology to *tra*/*fem*) (*19*, *20*) initiates the female sex-determining route when the animal is heterozygous at this locus, whereas homozygosity or hemizygosity leads to maleness. A *csd* mechanism of sex determination can easily be determined because it results in predictable proportions of homozygous diploids that develop into males (*21*). Because a number of Hymenoptera, including *Nasonia*, do not produce diploid males upon inbreeding (*22*), it was surmised that another mechanism controls haplodiploid sex determination in these species.

We screened the *Nasonia* genome (*22*) for motifs matching the *Drosophila tra* and *Apis csd* genes, which resulted in the identification of a single gene (*16*, *22*) composed of nine exons and containing two Arg/Ser-domains (SR-domains), of which one is located entirely in exon one and the second spans exons four to seven. In exons seven and eight, a proline-rich (Pro) domain is present. Reverse-transcriptase polymerase chain reaction (RT-PCR) showed that female-specific splicing retains only the first part of exon two and yields a single transcript encoding a full-length protein, containing both SR domains and the Pro-rich domain. In male *Nasonia*, either the complete exon two or different 3′ parts of exon two can be retained by cryptic 3′ splice-site recognition to yield three different transcripts, all of which encode truncated proteins containing only the first SR domain (*22*). This gene was named *Nasonia vitripennis transformer* (*Nvtra*). *Nvtra* expression was knocked down by injecting double-stranded RNA (dsRNA) against a non–sex-specific part of *Nvtra* in 1- to 2-day-old female pupae (*23*) carrying the recessive eye color mutation STDR ($st^{DR}/st^{DR}$). After emergence, neither phenotypic nor behavioral changes were observed as compared with control uninjected females. *Nvtra* dsRNA-injected females were capable of mating and ovipositing and were fully fertile. The levels of *Nvtra* mRNA 5 days after dsRNA injection, when the females were in the late pupal stage, showed a 2.8-fold decrease in *Nvtra* expression [$t(16) = 3.86$, $P = 0.0007$, Fig. 1A] relative to uninjected controls.

In control females, only the female-specific *Nvtra* splice form was present. However, *Nvtra* dsRNA-injected females had a decreased amount of female-specific splice form and produced all three male-specific *Nvtra* splice forms (Fig. 1B). Apparently, repression of *Nvtra* also disrupted female-specific splicing of *Nvtra* pre-mRNA itself. For control females, *N. vitripennis doublesex* (*Nvdsx*) female-specific splicing along with very low quantities of a male-specific *Nvdsx* splice form (*11*) were observed. In *Nvtra* dsRNA-injected females, the expression of the predominant female splice form of *Nvdsx* decreased, whereas expression of the male-specific splice form increased (Fig. 1B). This indicates that, in *Nasonia*, an active NvTRA is necessary for female-specific splicing of *Nvdsx* mRNA. The presence of both male- and female-specific splice forms of *Nvtra* and *Nvdsx* was observed to be correlated with the degree of femaleness in haploid *Nasonia* gynandromorphs (*11*, *22*, *24*), indicating that these genes function in sex-specific phenotype establishment. The fact that a similar *Nvtra* and *Nvdsx* transcript composition in dsRNA-injected females nevertheless leads to complete morphological and functional females indicates either that the essential period of this *Nvtra*/*Nvdsx*-mediated phenotypic establishment is before the pupal stage or that the lower level of female-specific *Nvtra* is still sufficient to elicit female development.

To monitor the relative levels of *Nvtra* and *Nvdsx* during early and late embryonic development, we sampled embryos over time and determined the ratio of *Nvtra* and *Nvdsx* transcripts. In 0- to 1-hour-old embryos, an eightfold excess of *Nvtra* over *Nvdsx* was observed [$t(18) = 3.62$, $P = 0.0020$, table S1]. Because no appreciable zygotic gene expression occurs at this early stage (*25*), this relatively high level of *Nvtra* mRNA must be provided to the egg during oogenesis as a maternal factor and should be the female-specific splice variant only. RT-PCR confirmed this expectation, by showing only female-specific transcripts of *Nvtra* in 0- to 5-hour-old embryos from fertilized and unfertilized eggs (Fig. 2A).

As expected, virgin *Nvtra* dsRNA-injected STDR females produced only $st^{DR}$ males (fig. S1). When injected STDR females were mated to wild-type ($st^{+}$) males, they still produced only male offspring of which 44% had the $st^{DR}$ red-eye phenotype (representing unfertilized eggs) and 56% had wild-type eyes and must therefore be diploid ($st^{DR}/st^{+}$) (Table 1). Both haploid and diploid adult males had only the male-specific splice forms of both *Nvtra* and *Nvdsx* (Fig. 1B). Because neither intersex nor female offspring were observed, *Nvtra* dsRNA-injected females exhibit a complete sex reversal in their offspring. Flow cytometry confirmed the diploidy of the $st^{DR}/st^{+}$ males (fig. S2). We mated a subset of these diploid $st^{DR}/st^{+}$ males to STDR females.

Evolutionary Genetics, Centre for Ecological and Evolutionary Studies, University of Groningen, Netherlands.

*To whom correspondence should be addressed. E-mail: louis.van.de.zande@rug.nl

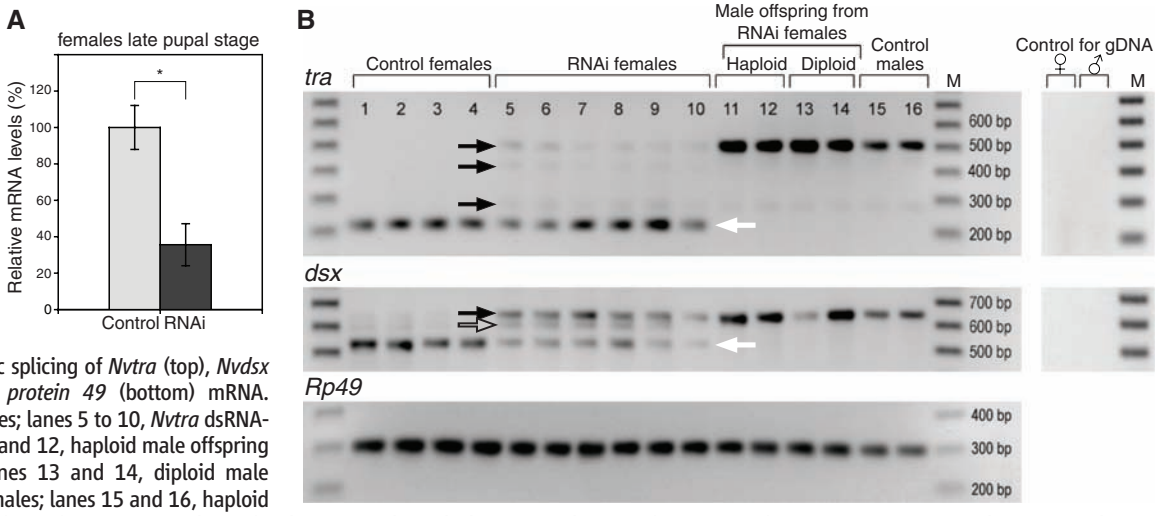


**Fig. 1.** Sex-specific differential splicing of *Nvtra* and the functional relationship of *Nvtra* and *Nvdsx*. (**A**) Relative levels of *Nvtra* mRNA after RNAi in control (light gray bar) and *Nvtra* dsRNA-injected (black bar) females in the late pupal stage. Error bars represent SE. *$P < 0.001$. (**B**) RT-PCR analysis of sex-specific splicing of *Nvtra* (top), *Nvdsx* (middle), and *Ribosomal protein 49* (bottom) mRNA. Lanes 1 to 4, control females; lanes 5 to 10, *Nvtra* dsRNA-injected females; lanes 11 and 12, haploid male offspring from injected females; lanes 13 and 14, diploid male offspring from injected females; lanes 15 and 16, haploid male offspring from control females. M is a 100-bp molecular size marker. Black arrows indicate male-specific splice forms, gray arrow indicates an unknown splice form, and white arrows indicate female-specific splice forms. A control for amplification from residual genomic DNA is present in the rightmost panel.

The female offspring of this cross all had wild-type eyes. Because male gametogenesis does not involve reduction division, we assume that these males had transmitted their complete diploid genome to generate triploid $st^{DR}/st^{DR}/st^{+}$ daughters, as reported earlier for diploid males from a triploid strain (*26*).

To assess whether *Nvtra* dsRNA-injected mothers provided lower amounts of *Nvtra* to the eggs, we measured the relative levels of *Nvtra* in the offspring of *Nvtra* dsRNA-injected and uninjected females. We found that very early embryos (0 to 3 hours old), in which zygotic gene expression has not yet started (*25*), resulting from both virgin and mated *Nvtra* dsRNA-injected females, had decreased levels of *Nvtra* mRNA to about 20% of that of early embryos from control noninjected females [$t(35) = -3.92$, $P = 0.0002$, Fig. 2B].

Our results suggest that a threshold level of maternally provided female-specific *Nvtra* mRNA is essential for female development of the fertilized egg, because knockdown of *Nvtra* in mothers leads to the production of diploid male offspring. They also indicate that female-specific *Nvtra* splicing depends on an autoregulatory loop. First, knockdown of *Nvtra* in the mother leads to the disruption of the female-specific splicing of both *Nvtra* and *Nvdsx* in these mothers. Second, the diploid male offspring from *Nvtra* dsRNA-injected mothers had only male-specific spliced *Nvtra* transcripts, indicating the dependence of a functional NvTRA protein for female-specific splicing. Third, the high sensitivity of the diploid embryos from the injected mothers to the lowered levels of female-specific *Nvtra* resulting in a full sex reversal indicates that sufficient NvTRA is needed for female-specific splicing. Fourth, eight putative TRA/TRA2 binding motifs (U/G)GAAGAU(U/A) in the *tra/fem*-regulated *dsx* and *fruitless* (*fru*) genes of *N. vitripennis* and *A. mellifera* (*27*) are located in the male-specific exon *2m1* (*22*) and in the intronic region between exons two and three of the *Nvtra* gene. Based upon similar arguments, *tra* autoregulatory loops have been proposed for the dipterans *Ceratitis capitata, Bactrocera oleae*, *Lucilia cuprina* and *A. mellifera* (*14*, *20*, *28*, *29*). We conclude that *Nvtra* is part of the *Nasonia* sex-determining cascade and is responsible for the sex-specific splicing of *Nvdsx.* In addition, sufficient levels of female-specific *Nvtra* transcripts are necessary to maintain the female-specific splicing pattern of *Nvtra* itself.

In diploid houseflies (*Musca domestica*), which lack haplodiploidy, the dominant male-determining M factor represses the sex-determining F factor, resulting in male development (*30*). In the absence of M, F, which is an ortholog of the *Ceratitis tra* gene (*13*), is activated, leading to female development. In *M. domestica*, the M factor can be located on the Y chromosome and/or on one of the autosomes. In other Diptera, such as *Ceratitis* and *Lucilia*, the M factor leads to male development by blocking the transcription or translation of female *tra* or by interfering with *tra* splicing (*13*, *29*). Only males can provide the M factor for the next generation. Therefore, M factors are incompatible with haplodiploid sex determination, where only unfertilized eggs develop into males. This implies that in *Nasonia*, a different mechanism is responsible for the development of males in the presence of maternally provided *Nvtra* mRNA or protein. Thus, we conclude that maternal *Nvtra* mRNA is most likely provided to all eggs as a means to start the female-specific autoregulatory loop.

Because fertilization per se had been ruled out as a sex-determining factor in *Nasonia* before (*31*), and because unfertilized eggs will develop as males, we asked whether the presence of a paternal genome together with a maternal genome explains why only fertilized eggs develop as females. Quantitative PCR (qPCR) showed that in 1- to 3-hour-old embryos from both fertilized and unfertilized eggs, the maternally provided *Nvtra* mRNA input gradually decayed (Fig. 3A). In embryos from unfertilized eggs, a low level of *Nvtra* mRNA was maintained throughout the 23 hours of embryonic development (Fig. 3A). In sharp contrast, a 15-times-higher expression of *Nvtra* in embryos from fertilized eggs was observed 7 hours after egg laying [$t(8) = 4.18$, $P = 0.0031$, Fig. 3A], which cannot be explained by the presence of two versus one *Nvtra* alleles in these embryos and calls for a regulatory explanation. After this peak expression, a significantly higher level ($F_{1,63} = 5.25$, $P < 0.0001$) of *Nvtra* mRNA was maintained as compared with embryos from unfertilized eggs (Fig. 3A). We used a Russian strain of *N. vitripennis* that harbors a deletion of 18 base pairs (bp) in the first exon of the *Nvtra* gene, which apparently does not affect the function of the gene, to monitor the paternal genome for the onset of zygotic *Nvtra* production. RT-PCR of *Nvtra* transcripts in these samples showed that in offspring from fertilized (diploid) eggs, zygotic *Nvtra* mRNA is transcribed from the paternal genome 5 hours after egg laying (Fig. 3B) and confirmed our assumption that, in early (0 to 3 hours) *Nasonia* embryos, no zygotic *Nvtra* transcription takes place. A reciprocal cross yielded identical results. Unfortunately, because of the repetitive nature of the indel and its flanking region, we were unable to design primers to perform qPCR to quantify the relative contributions of the paternal and maternal *Nvtra* alleles, respectively.

Because 1- to 5-hour-old embryos from fertilized and unfertilized eggs contained only female-specific *Nvtra* mRNA (Figs. 2A and 3C), we hypothesize that the absence of sufficient zygotic *Nvtra* expression to initiate the autoregulatory loop results in default male-specific splicing (Fig. 3C). However, in embryos from fertilized eggs, the female-specific splicing of *Nvtra* is maintained (Fig. 3C) because of the availability of zygotic *Nvtra* mRNA. The low levels of the male-specific splice forms observed in these pooled embryo samples most likely result from the unfertilized eggs laid by the mated STDR females (a typical brood contains 20% males). One explanation could be that only the paternal allele of *Nvtra* is transcribed in the early embryo, thus allowing the loop of autoregulatory splicing to take place. Alternatively, a trans factor necessary for the

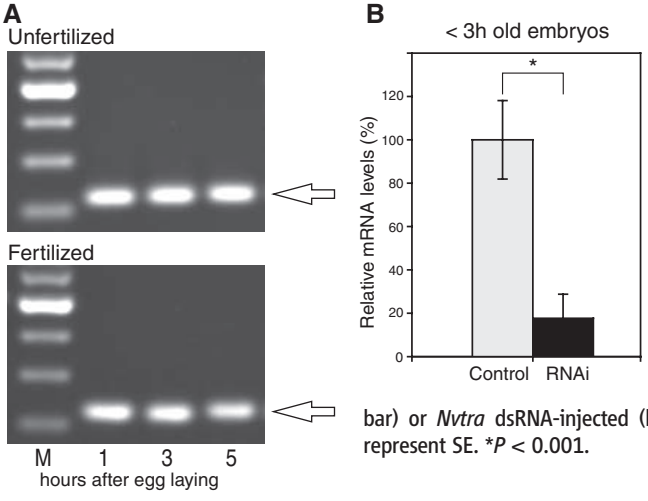


**Fig. 2.** Maternal input in early embryos. (**A**) Maternal input of female-specific *Nvtra* mRNA in early embryos from unfertilized (top) and fertilized (bottom) eggs shown 1, 3, and 5 hours after egg laying. Open arrows indicate female-specific *Nvtra* splice forms of 228 bp. M is a 100-bp size marker. (**B**) Relative *Nvtra* mRNA levels in equally mixed embryos from mated and unmated control (gray bar) or *Nvtra* dsRNA-injected (black bar) females. Error bars represent SE. *$P < 0.001$.

**Table 1.** *Nvtra* dsRNA-injected females and their offspring numbers. Number of *Nvtra* dsRNA-injected females [P: parental females (RNAi)] that produced offspring [P: parental females (fertile)] as virgin or as mated to AsymC males and the offspring they produced ($F_1$: haploid males; $F_1$: diploid females; and $F_1$: diploid males).

| | P: ♀ (RNAi) | P: ♀ (fertile) | $F_1$: haploid ♂ | $F_1$: diploid ♀ | $F_1$: diploid ♂ |
|---|---|---|---|---|---|
| Virgin | 60 | 17 | 418 | 0 | 0 |
| Mated | 60 | 26 | 295 | 0 | 379 |

timely onset of zygotic *Nvtra* transcription may be silenced in the maternal genome set of the embryo.

Our data show that maternal provision of *Nvtra* to all embryos, followed by sufficient early zygotic *Nvtra* expression, which occurs only in fertilized eggs, is necessary for female development in *Nasonia*. RNA interference (RNAi) treatment decreased the maternal provision of *Nvtra* to the eggs, which alone would be sufficient for the production of diploid males. It is possible that the resulting small interfering RNAs (siRNAs) were also transmitted to the eggs, resulting in a decrease in zygotic *Nvtra* transcript expression in addition to a decrease in maternal *Nvtra* input. Either way, the simplest explanation for the mechanism behind *Nasonia* sex determination appears to be maternal input of *Nvtra* mRNA combined with a form of maternal imprinting (*31*).

Several insects have maternal input of *tra* mRNA followed by an autoregulatory loop for the continuous production of female-specific *tra* (*13*, *20*, *29*). However, in *Nasonia*, male development does not result from disruption of the *Nvtra* autoregulatory loop by paternal repression (for example, an M factor) or a nonfunctioning CSD, but is most likely caused by maternal silencing of the *tra* gene. The presence of a paternal genome leads to zygotic expression of *Nvtra*, but maternally provided *Nvtra* mRNA is required to initiate female-specific splicing. Hence, in *Nasonia*, females regulate the sex of the offspring by providing a feminizing effect by maternal input of *Nvtra*, while at the same time preventing zygotic expression of *Nvtra* in haploid offspring. Pane *et al*. (*13*) suggested that the sensitivity of the *tra* autoregulation is evolutionarily important for the recruitment of upstream regulators. Indeed, in *A. mellifera*, *csd* originated as a duplication of *fem* (=*tra*) (*16*). The gregarious lifestyle of *Nasonia* implies potential high levels of inbreeding, so the evolution of a *csd* sex-determining mode is under constraint. Instead, a maternal imprinting event seems to be an upstream regulator, rendering the system dependent on zygotic expression. This is analogous to the observed evolutionary modulation of the maternal provision versus zygotic transcription of patterning determinants by Rosenberg *et al*. (*32*). The interplay of maternal and zygotic provision of sensitive sex-determination regulatory factors may facilitate the recurrent appearance of thelytokous reproduction in haplodiploid insects.

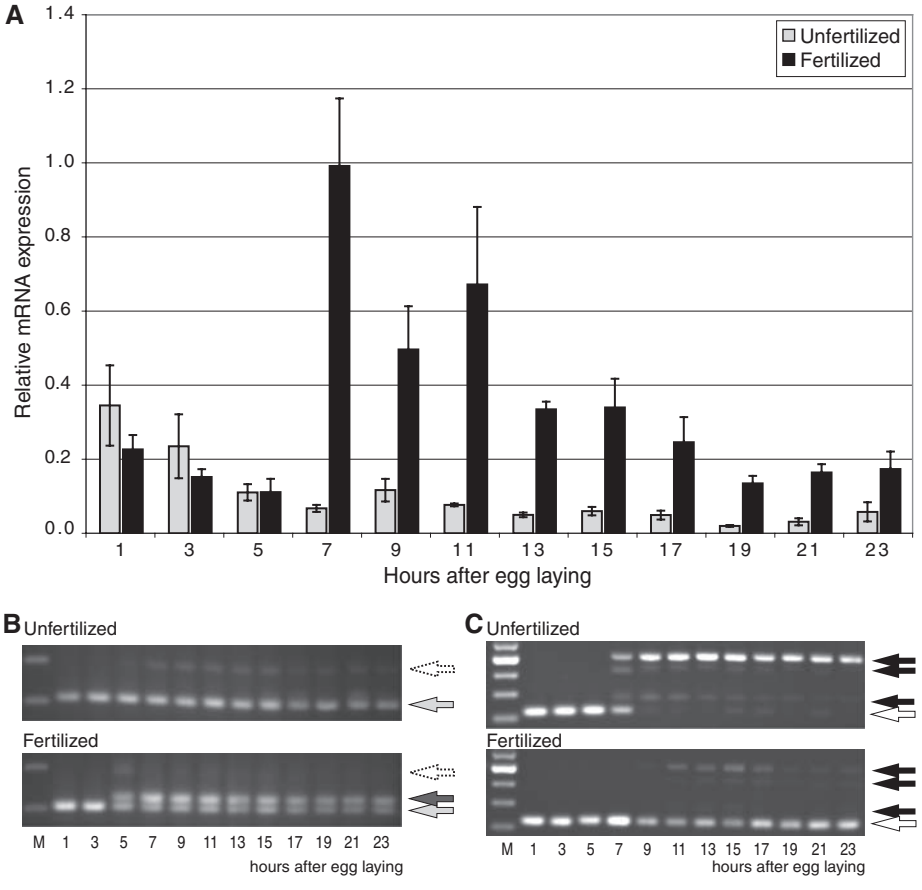


**Fig. 3.** Expression and splicing of *Nvtra* during embryonic development. (**A**) Relative *Nvtra* mRNA levels in embryos from unfertilized (gray bars) and fertilized (black bars) eggs at different developmental times, indicated as hours after egg laying. Error bars represent SE. (**B**) *Nvtra* mRNA originating as maternal input (light gray arrows) or transcribed from the paternal genome (dark gray arrows) in embryos from unfertilized (top) and fertilized (bottom) eggs. Open dotted arrow indicates amplification resulting from residual genomic DNA. M is a 100-bp size marker. (**C**) Temporal pattern of sex-specific splicing of *Nvtra* mRNA in embryos from unfertilized (top) and fertilized (bottom) eggs. Black arrows indicate male-specific splice forms. White arrows indicate female-specific splice form.


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**Supporting Online Material**

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References


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