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 \( t + 1 \). 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 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 (\( r = 72 \)) 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 \( t + 1 \) 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).

References and Notes

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.

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Fig. 51 to 57
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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 embryonic (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 homogygosity 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 haplo-diploid 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 STDTR (stDR/stDR). 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 femalelessness 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 [f(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 STDTR females produced only stDR males (Fig. S1). When injected STDTR females were mated to wild-type (st) males, they still produced only male offspring of which 44% had the stDR red-eye phenotype (representing unfertilized eggs) and 56% had wild-type eyes and must therefore be diploid (stDR/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 stDR/st males (Fig. S2). We mated a subset of these diploid stDR/st males to STDTR females.

**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.
The female offspring of this cross all had wildtype eyes. Because male gametogenesis does not involve reduction division, we assume that these males had transmitted their complete diploid genome to generate triploid \( D^{DR}_s/ s^{DR}/ s \) 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)GAAGA(U/A) in the tra/fem-regulated \( d sx \) and \( fruitless \) (\( fru \)) genes of \( N. vitripennis \) and \( A. mellifera \) (27) are located in the male-specific exon \( 2 m l \) (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.

![Fig. 2](https://www.sciencemag.org/content/328/5971/622/F2.large.jpg)

**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 (\( F_2 \): haploid males; \( F_1 \): diploid females; and \( F_2 \): diploid males).

<table>
<thead>
<tr>
<th></th>
<th>( P ): ( \varnothing ) (RNAi)</th>
<th>( P ): ( \varnothing ) (fertile)</th>
<th>( F_1 ): haploid ( \sigma )</th>
<th>( F_1 ): diploid ( \varnothing )</th>
<th>( F_2 ): diploid ( \sigma )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virgin</td>
<td>60</td>
<td>17</td>
<td>418</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mated</td>
<td>60</td>
<td>26</td>
<td>295</td>
<td>0</td>
<td>379</td>
</tr>
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</table>
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 thylotoky reproduction in haplodiploid insects.

References and Notes

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Supporting Online Material

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Materials and Methods

Figs. S1 and S2

Tables S1 and S2

References

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