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Genomic Imprinting and Maternal Effect Genes in Haplodiploid Sex Determination

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Key Words

doublesex · Genomic imprinting · Haplodiploidy · Maternal effect · *Nasonia* · Sex determination · *transformer*

Abstract

The research into the *Drosophila melanogaster* sex-determining system has been at the basis of all further research on insect sex determination. This further research has made it clear that, for most insect species, the presence of sufficient functional Transformer (TRA) protein in the early embryonic stage is essential for female sexual development. In Hymenoptera, functional analysis of sex determination by knockdown studies of sex-determining genes has only been performed for 2 species. The first is the social insect species *Apis mellifera*, the honeybee, which has single-locus complementary sex determination (CSD). The other species is the parasitoid *Nasonia vitripennis*, the jewel wasp. *Nasonia* has a non-CSD sex-determining system, described as the maternal effect genomic imprinting sex determination system (MEGISD). Here, we describe the arguments that eventually led to the formulation of MEGISD and the experimental data that supported and refined this model. We evaluate the possibility that DNA methylation lies at the basis of MEGISD and briefly address the role of genomic imprinting in non-CSD sex determination in other Hymenoptera.

For most insect species, the presence of sufficient functional Transformer (TRA) protein in the early embryonic stage is essential for female sexual development [McKeown et al., 1987; Pane et al., 2002; Ruiz et al., 2007; Concha and Scott, 2009; Gempe et al., 2009; Salvemini et al., 2009; Hediger et al., 2010; Verhulst et al., 2010a; Saccone et al., 2011]. The sex-determining function of TRA is to direct female-specific splicing of the *doublesex (dsx)* pre-mRNA, resulting in a female-specific DSX protein. Without TRA, *dsx* pre-mRNA is male-specifically spliced, leading to a male-specific DSX protein. Both male and female variants of DSX are DNA-binding transcription factors that direct downstream development of sex-specific characteristics like morphology, pheromone production and behavior [Kijimoto et al., 2012; Kopp, 2012; Matson and Zarkower, 2012; Wang and Yoder, 2012]. This central role of TRA has enabled, or perhaps even initiated, the evolution of a plethora of insect sex-determining mechanisms, all directed to either cause or prevent the production of a functional TRA protein [Verhulst et al., 2010b; Salz, 2011].

Gempe and Beye [2011] defined 2 zygotic sex-determining mechanisms, based on the prezygotic state of the *tra* gene: ON (producing a functional TRA protein) or OFF (not producing a functional TRA protein). If the prezygotic state of *tra* is ON, then female development is

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the default route and sex-determining mechanisms are masculinizing. If the prezygotic state of *tra* is OFF, then male development is the default route and sex-determining mechanisms are feminizing.

In Diptera, the best studied sex determination system, that of *Drosophila melanogaster,* is dependent on early activation of *tra* by Sex-lethal (SXL), which is only effectuated when a double dose of X chromosomes is present [Erickson and Quintero, 2007]. Hence, the prezygotic state of *tra* in *D. melanogaster* is OFF, and active feminizing activity is required for female development. ON systems have been identified in *Ceratitis capitata* and *Musca domestica*, where masculinizing M-factors that suppress TRA function are required for male development [Pane et al., 2002; Hediger et al., 2010]. Either way, in Diptera normally a paternally-derived genome provokes male development by inhibiting the ON state (M-factors) or (passively) maintaining the OFF state (transmitting only a single dose of X chromosomes) of prezygotic *tra*.

Hymenopteran insects have haplodiploid sex determination: males develop parthenogenetically from unfertilized eggs, and females develop from fertilized eggs. The fact that unfertilized eggs develop into males indicates that the haploid prezygotic state of *tra* in Hymenoptera is OFF and that, consequently, active feminizing activity is required for female development. In fertilized eggs, this is normally accomplished by the paternal genome set that activates the feminizing pathway in a variety of ways. This, in combination with the OFF state of haploid prezygotic *tra*, leads to the assumption that only the paternal genome is capable of inducing an active feminization process in Hymenoptera by switching zygotic *tra* in the ON state. Thus, in contrast to the diploid system, in Hymenoptera a paternal genome is required to provoke female development. The hymenopteran sex-determining system is disrupted if the OFF state of haploid pre-zygotic *tra* is incomplete or if the paternal genome is incapable of activating the feminization process (or both). Disruption of the sex-determining system has indeed been observed in different hymenopteran species, making it hard to unambiguously state the requirement of a paternally derived genome. This will be addressed later in this review.

Once zygotic *tra* has been switched into the ON state, it provides a continuous supply of female-specific *tra* mRNA via an autoregulatory splicing loop, thus acting as a cellular memory maintaining the female pathway. The presence of such a feminizing loop has been demonstrated for both dipteran and hymenopteran species [Pane et al., 2002; Concha and Scott, 2009; Gempe et al., 2009; Salvemini et al., 2009; Hediger et al., 2010; Saccone et al., 2011]. Only in *D. melanogaster* this function has been taken over by *Sxl* [Siera and Cline, 2008]. For a more detailed description, see the article of Bopp et al. in this issue.

Ploidy Itself Is Not the Sex-Determining Factor

Ever since the first observation that fertilization, and hence diploidy, leads to female production in the honeybee *(Apis mellifera)*, biologists have tried to elucidate the functional mechanism of haplodiploid sex determination. One of the first models, complementary sex determination (CSD), was based upon studies on the parasitoid wasp *Bracon hebetor.* Whiting [1933] proposed that the allelic state of a single locus (sl-CSD) determines the sex: heterozygosity leads to female development and hemizygosity or homozygosity leads to male development (table 1, sl-CSD). A variant on this theme is multilocus CSD (ml-CSD), in which heterozygosity for at least one of multiple sex-determining loci would lead to female development [Whiting, 1943; Crozier, 1977] (table 1, ml-CSD). The presence of CSD in a species can be easily determined by inbreeding experiments to increase the number of homozygous individuals in a population. If CSD is the sex-determining mechanism, inbreeding will therefore result in an increase in diploid male production. Evidence for both sl-CSD and ml-CSD has recently accumulated for many hymenopteran species [Cook, 1993; de Boer et al., 2007; reviewed in Heimpel and de Boer, 2008]. However, the only functional description of sl-CSD in hymenopteran sex determination comes from the work on *A. mellifera*. The *csd* locus was identified as a duplication of *feminizer (fem)*, the *A. mellifera* ortholog of *tra*. It was shown that heterozygosity at the *csd* locus is essential for activation of *fem*, to produce female-specific *fem* mRNA and to initiate the autoregulatory feminizing loop [Beye et al., 2003; Hasselmann et al., 2008; Gempe et al., 2009]. More details on CSD sex determination are presented in the contribution by C. Vorburger in this special issue.

The fact that inbreeding results in an increase in the proportion of diploid males may lead to a disadvantage of sl-CSD but also of ml-CSD as the sex-determining system. Diploid males, in many cases, have lower fitness, mostly due to reduced fertility [Zayed and Packer, 2005]. Therefore, natural selection will favor non-CSD sex-determining mechanisms in inbreeding-prone hymenopteran species like non-social insects such as parasitoid wasps. Inbreeding experiments have indeed shown absence of CSD in many non-social insects [van Wilgenburg et al., 2006; Heimpel and de Boer, 2008], including *Nasonia vitripennis* [Werren

Table 1. Previously proposed models for sex determination under haplodiploidy

Model	Based on	Prediction	Reason for rejection of model in *Nasonia*
sl-CSD	single-locus complementarity	inbreeding would quickly lead to diploid male production	no diploid male production after many generations of inbreeding [Skinner and Werren, 1980]
ml-CSD	multi-locus complementarity	inbreeding for many generations would eventually lead to diploid male production	
FSD	fertilization	fertilization in itself leads to female development	fertilization with sperm containing the PSR chromosome leads to male development [Dobson and Tanouye, 1998]
GBSD (ploidy)	dosage effect of non-cumulative male (M) loci and cumulative female (F) loci	in haploids: M > F resulting in male development in diploids: 2 F > M resulting in female development	diploid offspring of virgin triploid females develop as males even though they are 2 F > M [Whiting, 1960; Beukeboom and Kamping, 2006]
MESD	balance of cytoplasmic (M) (i.e. provided by mother) and nuclear components (F)	in haploids: cytoplasmic component is masculinizing (M) – M > F in diploids: cytoplasmic component is outweighed by nuclear genes (2 F) – 2 F > M	triploid female provides 3 M to diploid eggs containing only 2 nuclear genes – 3 M > 2 F; diploid offspring from triploid females should therefore always develop as males (but see table 2)
GISD	genomic imprinting	paternally inherited set of chromosomes is functionally different from a maternal one and required for female development	irradiated mated females produced haploid male offspring with only paternal genome [Friedler and Ray, 1951]; triploid females can produce diploid females parthenogenetically [Beukeboom and Kamping, 2006]

sl-CSD = Single-locus complementary sex determination; ml-CSD = multi-locus complementary sex determination; FSD = fertilization sex determination; GBSD = genic balance sex determination; MESD = maternal effect sex determination; GISD = genomic imprinting sex determination.

et al., 2010] and the complete genus of *Asobara* [Ma et al., 2013]. Many models for non-CSD sex determination under haplodiploidy have been proposed, primarily based upon ploidy level and the introduction of a paternal genome (table 1). However, all models proposed were challenged by the many occurrences of diploid males in the field and in laboratory experiments. The crucial problem appeared to be to define the exact role of a paternally-derived genome in provoking female development in non-CSD Hymenoptera. For the parasitoid *Nasonia*, eventually the maternal effect genomic imprinting sex determination (MEGISD) model was formulated. Apart from experimental evidence, this model was proposed based on previous models that, although refuted by empirical observations, were gradually becoming close to a satisfying description of non-CSD haplodiploid sex determination.

Initially, the models proposed were mainly based upon balancing, dose-dependent systems that focused on the developing zygote only. During haploid zygotic development, feminizing gene products (F) would be outweighed by the stronger male factors (M) when present in equal doses. A double dose of feminizing gene products, transcribed from the 2 chromosome sets in diploids, would lead to feminization [Cunha and Kerr, 1956] (table 1, GBSD). It should be noted that this model implies a form of dose compensation to account for the different doses of M and F factors in diploids, and thus already could hint at a non-equivalence of maternally and paternally inherited genomes, as Hymenoptera do not have male- and female-specific sex chromosomes. To circumvent this non-equivalence, the maternal effect theory [Crozier, 1977; Cook, 1993] invokes a maternal masculinizing cytoplasmic factor; the dose of this factor would be sufficient in haploids only, while in diploids double doses of feminizing nuclear factors cause female development (table 1, MESD). The basic idea of both models is that the number of chromosome sets essentially determines male or female development; the effect of having 2 chromosome sets is feminizing (diploidy). However, little experimental support was obtained for any of these models to completely explain non-CSD haplodiploid sex determination, and they were especially challenged by the many observations of diploid males in hymenopteran species.

MEGISD: A Non-CSD Sex Determination Model for *Nasonia* Based upon Genomic Imprinting and Maternal Effect

Studies of the parasitoid wasp *N. vitripennis* have proved to be very fruitful, owing to the occurrence of mutant strains (table 2), its low number of chromosomes,

Table 2. Overview of the ploidy of different types of mutant *Nasonia* strains and the ploidy of the offspring they produce

Strain	Parents		Offspring			Reference
	virgin female	female × male	male	gynandromorph	female	
Wild type	2n		1n	–	–	
Gynandromorphic strain	2n		1n	1n	1n[a]	Beukeboom et al., 2007b; Kamping et al., 2007
Polyploid strain (triploid)	3n		1n; 2n	1n; 2n[a]	2n[a]	Beukeboom and Kamping, 2006
Wild type		2n × 1n	1n		2n	
Gynandromorphic strain		2n × 1n	1n	1n	1n[a]; 2n	Beukeboom et al., 2007b; Kamping et al., 2007
Polyploid strain (triploid)		3n × 1n	1n; 2n	1n; 2n[b]	2n; 3n	Beukeboom and Kamping, 2006; Whiting, 1960

[a] This type of offspring is produced in very low numbers.
[b] This type of offspring has not been observed but is theoretically expected.

and, recently, the availability of a completed genome sequence [Werren et al., 2010]. In addition, the easy husbandry, short generation time (14 days at 25°C) and gregarious lifestyle make *Nasonia* an attractive model organism for biological studies.

As the number of chromosomes of *Nasonia* is only 5, polyploid mutant females may still produce appreciable offspring numbers as the formation of aneuploid eggs is relatively low. Indeed, a natural mutant line has been described in which high proportions of triploid females occur [Whiting, 1960]. Unmated triploid females from this line produce both haploid and diploid males, both of which are fertile (table 2). The fact that individuals from unfertilized diploid eggs develop as males was one of the arguments to reject both the idea that ploidy alone determines the sex of *Nasonia* and the theory that different doses and strengths of zygotically transcribed M and F factors from equivalent chromosomes determine sexual fate (table 1, GBSD).

Alternatively, if a maternal cytoplasmic M factor would be provided to the oocytes upon oogenesis by 3 genome complements (table 1, MESD), it is to be expected that both mated and unmated triploid females always produce male offspring from haploid and diploid eggs, as neither the haploid nor diploid zygote can outweigh the triple dose of M factors provided by a triploid female. This is because the nuclear F factor is zygotically transcribed from 2 genome complements at most, while the maternal M factor comes from 3 genome complements. This 3:2 imbalance will always lead to diploid male development [Beukeboom et al., 2007a]. This was not observed for the triploid *Nasonia* strain: offspring from mated triploid females consist of both haploid and diploid males (unfertilized eggs), but also of diploid and triploid females (fertilized eggs) (table 2).

Finally, the hypothesis that fertilization is required for the onset of female development does not hold given the effect of the *Nasonia* paternal sex ratio chromosome (PSR). Diploid females mated by haploid males that harbor this supernumerary PSR produce only haploid sons, since PSR causes its accompanied paternal genome to be destroyed in the zygote shortly after fertilization (table 1, FSD) [Nur et al., 1988].

Apparently, neither the process of fertilization nor diploidy alone trigger female development, and a paternally-derived genome is needed for female development. As stated above, in *Nasonia* this cannot be explained by the allelic states of sex-determining loci, as inbreeding does not lead to an increase in the proportion of diploid males [Skinner and Werren, 1980]. This implies that the paternal and maternal gametes contain genome sets that are somehow different and that only a genome in the 'paternal state' is capable of feminization. One attractive way to explain the different 'maternal and paternal states' of the gametes is parental epigenetic modification. These data led to the genomic imprinting sex determination model (GISD) [Poirié et al., 1992; Beukeboom, 1995], stating that differentially imprinted sex-determining loci lie at the basis of female versus male development in *Nasonia* (table 1, GISD).

The results of Trent et al. [2006] supply additional support for this model. Some X-ray mutagenized *Nasonia* males produced only diploid male offspring when mated to normal diploid females, indicating that the irradiation had destroyed the feminizing capacity of their genome. These diploid males were fully fertile and produced trip-

loid daughters when mated to diploid females. This suggests that the mutant phenotype was rescued in the diploid males by the inherited non-irradiated genome set from their mothers. Epigenetic modification of this maternal genome set into the paternal state, either in the germline or during gametogenesis, is an attractive explanation for the restoration of the feminizing capacity. It is interesting to note that the triploid females never produced haploid males that fathered only diploid sons, i.e. showing the mutant phenotype. This suggests that the mutagenized gene(s) are lethal during haploid male development. Taken together, female development in *Nasonia* seems to require at least 1 functional genome of paternal origin or in a 'paternal state'.

Despite these results, GISD cannot fully explain *Nasonia* sex determination. Friedler and Ray [1951] irradiated female *Nasonia*, which upon mating produced haploid male offspring, containing the (not mutagenized) paternal genome set only (table 1, GISD). Therefore, also a paternally-derived genome alone is not sufficient to induce female development; some maternal component still needs to be provided.

The experiments of Beukeboom and Kamping [2006] provided more evidence for a maternal component involved in sex determination of the offspring in addition to the paternal genome. They observed that females from the polyploid (triploid) mutant strain [Whiting, 1960] occasionally produced uniparental diploid females or gynandromorphs (GISD; tables 1, 2). In another mutant strain, unmated diploid females were observed to produce haploid gynandromorphic offspring that could be morphologically complete females (table 2) [Beukeboom et al., 2007b; Kamping et al., 2007]. Apparently, the maternal genome can be sufficient to provoke female development without the presence of the paternal genome. The explanation is that, as the result of aberrant epigenetic modification, the maternal gametes contain genomes that are both in the 'maternal state' and the 'paternal state'. It should be noted, however, that these mutant strains do not represent the 'default' haplodiploid sex-determining system, but their aberration can be used to study the *Nasonia* sex determination system.

These results led to the formulation of a model, in which a masculinizing maternal factor *(msd)* was inferred to be responsible for the silencing of a feminizing zygotic sex determiner gene *(zsd)* in the maternal genome. Through this silencing by genomic imprinting, *msd* transgenerationally prevents zygotic expression of *zsd*, leading to haploid male development. The non-imprinted and therefore active *zsd* on the paternal genome set would initiate female development of diploid (fertilized) eggs. Failure of imprinting *zsd* in the eggs would lead to haploid female development of unfertilized eggs, while incomplete imprinting would lead to production of gynandromorphs, which are both observed in the gynandromorph-producing strain. In addition, failure to imprint *zsd* could explain uniparental females observed in the triploid strain.

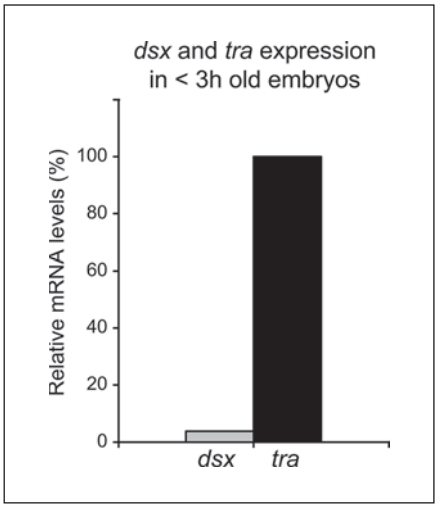

Fig. 1. Relative expression levels of *Nvtra* and *Nvdsx* in early embryos. Maternal provision of *Nvtra* and *Nvdsx* mRNA in <3-hour-old embryos of mated *N. vitripennis* females.

This model was termed maternal effect genomic imprinting sex determination (MEGISD) [Beukeboom et al., 2007a]. The maternal effect is not a cytoplasmic masculinizing factor to be directly transmitted to the zygote, but instead the masculinizing effect of indirect prevention of zygotic *zsd* production by silencing the maternal *zsd* copy by imprinting. The identity of *zsd*, however, remains a mystery.

MEGISD Revisited

In 2004, the *Nasonia* genome was proposed for sequencing [Werren et al., 2004], and its sequence was published in 2010 [Werren et al., 2010]. This led to the identification of the *Nasonia dsx* ortholog *(Nvdsx)* [Oliveira et al., 2009] and the *Nasonia tra* ortholog *(Nvtra)* [Werren et al., 2010] genes. In early (less than 3 h after egg laying) embryos of *N. vitripennis,* already appreciable levels of *Nvtra* mRNA were present in comparison to *Nvdsx* (fig. 1), leading to the hypothesis that maternal input of

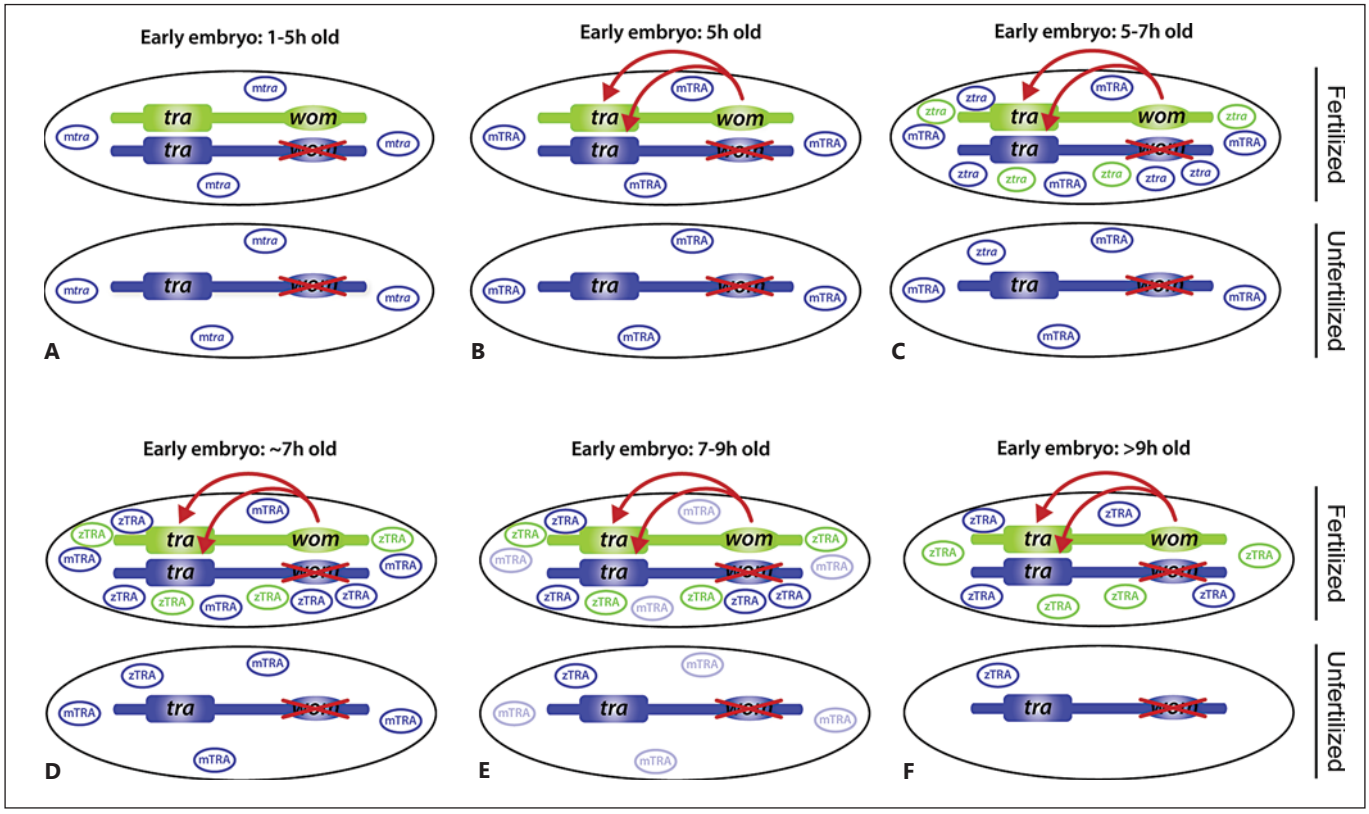

Fig. 2. *N. vitripennis* sex determination. **A** *Wom* is silenced on the maternal (blue) chromosome but active on the paternal (green) chromosome. Maternally provided *Nvtra* (m*tra* in blue circles) is present in both the haploid and diploid early zygotes. **B** In diploid zygotes, the active *wom* copy on the paternal chromosome initiates expression from both the maternal and paternal *Nvtra* allele (*wom* and *Nvtra* do not necessarily reside on the same chromosome). The maternally provided *Nvtra* transcripts are decaying. **C** Only in diploids, zygotic *Nvtra* is transcribed from both alleles (z*tra* in circle). **D** The amount of zygotic *Nvtra* transcripts is increasing in the diploid zygote only. The haploid zygote produces only trace amounts of *Nvtra* transcripts. Only in the diploid zygotes this production of NvTRA (zTRA) protein is sufficient to maintain autoregulation. **E** The maternally provided *Nvtra* transcripts are decaying, but only in diploid zygotes this decay is compensated by zygotic transcription of *Nvtra*. **F** The expression of *Nvtra* continues under the regulation of *wom* for the duration of embryonic development in the diploid zygote, while in haploid zygotes only trace amounts of *Nvtra* transcript are present. See text for details. m*tra* = Maternally provided *Nvtra* mRNA; mTRA = protein from m*tra*; z*tra* = zygotically transcribed *Nvtra* mRNA; zTRA = protein from z*tra*.

female-specific *Nvtra* mRNA is essential for female development. This maternal provision supplies a sufficiently high dose of NvTRA to direct female-specific splicing of early zygotically transcribed *Nvdsx* pre-mRNA in diploid embryos, ensuring enough female-specific DSX protein to activate downstream feminizing target genes. Indeed, Verhulst et al. [2010a] showed that preventing maternal input of *Nvtra* mRNA by RNAi treatment of the mated mothers led to a disruption of female-specific splicing of *Nvdsx* pre-mRNA and caused them to produce male offspring only, both diploid and haploid. Moreover, prevention of maternal *Nvtra* mRNA did not only lead to disrupted splicing of zygotic *Nvdsx* pre-mRNA, but also to a disruption of *Nvtra* pre-mRNA splicing itself. This indicates that *Nvtra* female-specific splicing also establishes an autoregulatory memory loop, as has been found in several other insect species [Pane et al., 2002; Ruiz et al., 2007; Concha and Scott, 2009; Gempe et al., 2009; Salvemini et al., 2009; Hediger et al., 2010; Saccone et al., 2011]. As blocking maternal provision of female-specific *Nvtra* mRNA led to a complete sex-reversal of diploid offspring, surprisingly, the mother provides feminizing in addition to masculinizing information to the zygote. However, the feminizing effect is dependent on timely zygotic expression of *Nvtra* in order to establish the autoregulatory memory loop that is essential for female development.

Verhulst et al. [2010a] found that in unfertilized eggs, *Nvtra* expression is not initiated in early embryonic development, while in fertilized eggs zygotic *Nvtra* expression starts around 7 h after egg-laying. Apparently the female *Nvtra* gene is maternally silenced in haploids, and a paternal genome is needed to elicit its expression in diploids, indicating that for female development at least a 'paternal state' genome is needed as long as the maternal provision of *Nvtra* mRNA is not disrupted. This means that the prezygotic state of *tra* in *Nasonia* is OFF, even though the mother also provides *Nvtra* mRNA as feminizing factor to the zygotes.

In a recent paper, Verhulst et al. [2013] showed, by cloning *Nvtra* expression products from 7-hour-old diploid embryos and nascent in situ hybridization, that zygotic expression of *Nvtra* originates from both the maternal and paternal allele. This implicates that it is not the *Nvtra* gene itself that is maternally imprinted, so *Nvtra* cannot be *zsd*. *Nasonia* female sex determination is dependent on zygotic activation of *Nvtra* expression by an as yet unknown factor, which the authors proposed to term *womanizer (wom)* to indicate its feminizing activity through activation of *Nvtra* (fig. 2). We suggest that this factor is *zsd*. The still unknown *msd* factor maternally silences *wom* upon oogenesis or during germline development to ensure male development of unfertilized eggs. The maternal silencing of *wom* explains why virgin triploid females produce diploid sons while mated triploid females produce diploid daughters (table 2). These results refine the MEGISD model [Beukeboom et al., 2007a], involving both maternal effects (provision of female-specific *Nvtra* mRNA) and genomic imprinting (prevention of zygotic transcription of *Nvtra* in haploid zygotes). The net effect of this genomic imprinting is maternal silencing and paternal activation of zygotic *Nvtra* expression. The identity of the *msd* gene, however, remains unknown.

DNA Methylation Is a *Nasonia* Imprinting Mechanism

The publication of the *Nasonia* genome [Werren et al., 2010] revealed that it contains all 3 DNA methyltransferase families: 3 maintenance *DNA methyltranferase1 (Dnmt1)* genes, 1 tRNA methylation *Dnmt2* and 1 *Dnmt3* gene for de novo methylation. In their supplementary online material, Werren et al. [2010] showed methylation patterns of 5 genes: XM_001607338.1 (vitellogenin), XM_001600728.1 (epithelial membrane protein), XM_001600593.1 (polypeptide of 976 amino acids), XM_001606530.1 (eIF 2a kinase), and XM_001601041.1 (eIF2B-gamma protein). Later, Park et al. [2011] suggested that the observed methylated CpG sites in exon 2 of *Nvtra* are markers to guide alternative splicing in sex determination. Zwier et al. [2012] showed maternal provision of *Dnmt1a, Dnmt1c* and *Dnmt3* mRNA to the zygote. Parental RNAi treatment of the mothers to prevent maternal provision of *Dnmt1c* and *Dnmt3* mRNAs showed (1) no effect on maternal phenotypes, (2) did not lead to the production of haploid females or diploid males and (3) also did not affect the sex-specific splicing of *Nvtra* in zygotes. Only inhibition of maternal *Dnmt1a* provision led to embryonic lethality and arrested development at the onset of gastrulation. The authors concluded that the activation and expression of zygotic *Nvtra* does not critically depend on maternally provided *Dnmts*. Moreover, if the unknown *msd* factor was a *Dnmt*, one would expect disruption of the maternal silencing of *zsd* after parental RNAi and, consequently, the production of haploid females. Therefore, it can be concluded that DNA methylation plays an important role in *Nasonia* biology, but its involvement in sex determination is unknown. Embryonic knockdown of *Dnmt* genes will reveal if they play a sex-determining role in the early zygotic stages.

Non-CSD in Non-*Nasonia*

Recently, Ma et al. [2013] showed absence of CSD in the complete genus of *Asobara*, and there are many other hymenopteran species without CSD [van Wilgenburg et al., 2006; Heimpel and de Boer, 2008]. It is completely unknown if the *Nasonia* system is underlying these non-CSD mechanisms of sex determination and, if not, how many alternative sex-determining systems have evolved. Genomic imprinting is an attractive system to explain the occurrence of thelytoky in Hymenoptera, as it would require only 2 steps: restoration of diploidy and the release of maternal *tra* silencing. Perhaps the induction of thelytoky in *Asobara japonica* by *Wolbachia* infection involves (bacterial) modification of imprinting to allow zygotic *tra* production without a paternal genome.

Schmieder et al. [2012] identified frequent duplications of *tra/fem* in many Hymenoptera. Although it is unknown if these duplications have a function in sex determination, the dynamics of the *tra/fem* genomic region in these Hymenoptera is conspicuous and indicates high evolutionary potential. After all, *csd* in the honeybee is such a duplication of *fem* [Hasselmann et al., 2008] that was shown to be recruited into the sex determination cas-

cade. The foreseen availability of many more insect genomes (i5K: http://arthropodgenomes.org/wiki/i5K; 1KITE: http://www.1kite.org) will enable researchers to investigate more (hymenopteran) sex determination systems and hopefully reveal the universality of parental imprinting in non-CSD hymenopteran species.

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