Maternal input and silencing of transformer regulates haplodiploid sex determination in the wasp Nasonia vitripennis

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Abstract
All Hymenoptera have haplodiploid sex determination where males develop from haploid unfertilized eggs while females are diploid. Although the molecular basis of sex determination has been described in the honeybee, where it relies on the allelic state of the complementary sex determination (csd) gene, little is known about other Hymenoptera. Here, the sex determination system of the parasitic wasp Nasonia, that has no csd locus, is presented. It is shown that maternal input of transformer (Nutra) mRNA is essential for female development since a decrease of Nutra in mothers by RNA interference results in the production of diploid male offspring. Splice form analysis indicates that Nutra auto regulates its female specific splicing as has been shown for other insect species. With these results the proposed maternal effect genomic imprinting sex determination (MEGISD) model can be further refined. This model implies that maternal imprinting prevents zygotic transcription of Nutra in embryos from unfertilized eggs, leading to male development. Upon fertilization zygotic Nutra transcription is initiated, auto regulates the female specific transcript, and leads to female development. This mechanism of haplodiploid sex determination constitutes a novel mode of sex determination in insects.

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Maternal control of haplodiploid sex determination in the wasp Nasonia.
Chapter 4

Introduction

In the previous Chapters the sex determining genes *Nasonia vitripennis double- sex (Nvdsx)* (Chapter 2) and *N. vitripennis transformer (Nvtra)* (Chapter 3) were identified. Transcripts of both genes are spliced in a sex specific manner. Both female and male Nvdsx mRNA result in a sex specific functional protein, while for Nvtra only the female specific mRNA yields a functional protein. Male Nvtra is spliced to produce transcripts with premature in-frame stop codons. Splicing analysis of a mutant strain that produces gynandromorphs revealed that the splice pattern of both genes follows the male or female morphology (Chapter 3).

While the identification of Nvtra was in progress, not only the identification of a tra ortholog in *Apis mellifera* (termed feminizer, see Chapter 5) was published (Hasselmann et al., 2008), but also three studies in which tra orthologs were identified in the dipteran species *Bactrocera oleae*, *Anastrepha sp.* and *Lucilia cuprina* (Lagos et al., 2007; Ruiz et al., 2007; Concha and Scott, 2009). In *Ceratitis capitata* tra had already been identified in 2002 (Pane et al., 2002), while in *Musca* it was established that the F factor, which is required for female development, was a tra ortholog (Hediger et al., 2010). In most studies the function of tra (or fem) was established by tra knockdown in early embryos using RNA interference which resulted in male specific dsx and tra splicing (Pane et al., 2002; Lagos et al., 2007; Hasselmann et al., 2008; Concha and Scott, 2009; Hediger et al., 2010). The subsequent transformation of otherwise female offspring was not always complete and resulted in intersexes with various stages of masculinization, while male development remained unaffected. In *C. capitata* and *M. domestica* it was shown that knockdown of transformer-2 (tra-2) by RNAi also resulted in (incomplete) transformation of female offspring into various stages of masculinization (Burghardt et al., 2005; Salvemini et al., 2009; Hediger et al., 2010). This confirmed the collaboration of tra and tra-2 in the splicing of dsx as was already demonstrated in *Drosophila* (Inoue et al., 1992). Moreover, it was shown that RNAi knockdown of tra-2 in *C. capitata* and *M. domestica* resulted in male specific tra splicing, indicating that tra-2 is necessary for maintaining the female autoregulation of tra (Burghardt et al., 2005; Salvemini et al., 2009; Hediger et al., 2010).

To ascertain the function of Nvtra in the sex determining cascade of *Nasonia* an attempt was made to knockdown tra in early embryos and this led to comparable results: various stages of masculinization of diploid individuals. As it was shown in Chapter 3 that Nvtra expression is already present in one hour old embryos as a result of maternal provision during oogenesis and it was shown in other studies that female specific tra was a maternal factor that was deposited in the eggs (Dübendorfer and Hediger, 1998; Pane et al., 2002; Lagos et al., 2007; Hediger et al., 2010), a functional RNA interference (RNAi) analysis of Nvtra was performed in female pupae using the protocol for parental RNAi in *Nasonia* from...
Lynch and Desplan (2006) to prevent deposition of *Nutra* in the eggs. If provision of maternal *Nutra* indeed is essential for female development, either the occurrence of intersexes or transformation of diploid, normally female, offspring into males is expected to result from this experiment.

**Results**

**Female specific Nvtra is maternally provided to all eggs**

Reverse Transcriptase PCR of one, three and five hours old embryos showed that the maternal provision of *Nutra* in eggs is the female specific splice variant for both fertilized and unfertilized eggs (Fig. 4.1).

![Unfertilized and Fertilized embryos](image)

**Figure 4.1:** Maternal input in early embryos. RT-PCR analysis of maternal input of female specific Nvtra mRNA in early embryos from unfertilized (top) and fertilized (bottom) eggs; one, three and five hours after egg laying. Open arrows indicate female specific Nvtra splice form. M is 100 bp size marker ranging from 200-600 bp.

**Screen for diploid males**

To distinguish diploid males from haploid males, a recessive red eye color mutant, st\(^{DR}\), was used. Crossing st\(^{DR}/st^{DR}\) females with AsymC (st\(^{+}\)) males, yields offspring that is either haploid st\(^{DR}\) with red eye color or diploid st\(^{DR}/st^{+}\) with wild type eye color (dark purple) (Fig. 4.2). Hence, diploid males can be distinguished from haploid males based on their wild type eye color.

**Parental RNA interference with Nvtra**

*Nutra* expression was knocked down by injecting dsRNA against a non sex specific part of *Nutra* in one to two day old female st\(^{DR}/st^{DR}\) pupae (Lynch and Desplan, 2006). After emergence, neither phenotypic nor behavioral changes were observed compared to control uninjected females. *Nutra* dsRNA injected females were capable of mating and ovipositing and were fully fertile. To control for
Maternal input of \textit{Nvtra}

**Figure 4.2:** Eye color cross used to identify diploid males. Crossing scheme of injected \(st^{DR}\) females (gray box) with AsymC males with wild type eye color (\(st^{+}\)). F1 offspring in black box are diploid males. F2 female offspring are triploid females and wild type, sired by diploid males.

Successful knockdown, the levels of \textit{Nvtra} mRNA five days after dsRNA injection were checked, when the females were in the late pupal stage, as well as in non-treated females of the same stage. A 2.8-fold decrease in \textit{Nutra} expression \((t_{(16)} = 3.86, P = 0.0007, \text{Fig. 4.3})\) was observed in the injected females. Because the females suffered no ill effect after RNAi, it can be concluded that either \textit{Nutra} is not essential for the development and maintenance of the female phenotype from the pupal stage onward or that the lower level of female specific \textit{Nvtra} is still sufficient to elicit female development.

**Splicing analysis of Nvdsx and Nvtra after RNAi**

Next, the effect of \textit{Nutra} knockdown on the splicing of \textit{Nutra} and \textit{Nvdsx} transcripts was examined by RT-PCR. In control females, only the female specific \textit{Nutra} splice form was present. In \textit{Nutra} dsRNA injected females, however, in addition to a decreased female specific splice form, all three male specific \textit{Nutra} splice forms were produced (Fig.4.4). Apparently, repression of \textit{Nutra} had also disrupted the female specific splicing of \textit{Nutra} pre-mRNA itself. For \textit{dsx}, the previously described (Chapter 2) female specific \textit{Nvdsx} splice pattern was observed in control females including very low quantities of a male specific \textit{dsx} splice form.
In Nutra dsRNA injected females, however, expression of the predominant female splice form of Nvdsx had decreased while the expression of the male specific splice form was markedly increased (as shown by semi-quantitative RT-PCR in Fig. 4.4). This indicates that, in Nasonia, an active NvTRA, is necessary for female specific splicing of Nvdsx mRNA. Nevertheless, the lower levels of female specific Nvdsx and the production of male specific Nvdsx transcripts did not disrupt development into fully functional females of pupae that were injected with Nutra RNAi. These results show that Nutra is part of the Nasonia sex determining cascade and responsible for the sex specific splicing of Nvdsx. In addition, sufficient levels of female specific Nutra transcripts are necessary to maintain the female specific splicing pattern of Nutra itself. This has also been described for the dipterans C. capitata, B. oleae and L. cuprina (Pane et al., 2002; Lagos et al., 2007; Concha and Scott, 2009), and for fem in A. mellifera (Hasselmann et al., 2008) in which female specific TRA protein splices tra mRNA into a female specific form. Without this female specific TRA protein, tra mRNA is spliced by default into a male specific form. This mode of female specific tra splicing is termed an autoregulatory loop, and resembles the regulation of Sxl in D. melanogaster (Bell et al., 1991).

**Diploid male production after RNAi**

As expected, virgin Nutra dsRNA injected st^DR/st^DR females produced only st^DR males (Fig. 4.2). When injected st^DR/st^DR 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 4.1). Both haploid and diploid adult males had only the male specific splice forms of both Nutra and Nvdsx (Fig. 4.4). As neither intersex nor female offspring were observed, Nutra dsRNA injected females exhibit a complete sex reversal in their offspring. Flow cytometry confirmed the diploidy of the st^DR/st^+ males (Fig. 4.5). A subset of these diploid st^DR/st^+ males was mated to st^DR/st^DR females. The female offspring of this cross all had wild type eyes indicating that these males had transmitted their complete diploid genome to generate triploid st^DR/st^DR/st^+ daughters (Fig. 4.2).
Figure 4.4: Sex specific differential splicing of $Nvtra$ and the functional relationship of $Nvtra$ and $Nvdsx$. RT-PCR analysis of sex specific splicing of $Nvtra$ (top), $Nvdsx$ (middle) and Ribosomal protein 49 (bottom) mRNA. Lanes 1–4: control females; lanes 5–10: $Nvtra$ dsRNA injected females; lanes 11–12: haploid male offspring from injected females; lanes 13–16: diploid male offspring from injected females; lanes 15–16: haploid male offspring from control females; M is 100bp molecular size marker. Black arrows indicate male specific splice forms, gray arrow indicates unknown splice form and white arrows female specific splice forms. A control for amplification from residual genomic DNA (gDNA) is present in the right most panel.

Figure 4.5: Flow cytometry on heads of adult $Nasonia$. (A) Normal haploid male. (B) Normal diploid female. (C) Diploid male. Peak P1 corresponds to haploid and P2 to diploid cells and P4 to tetraploid cells. Some endopolyploidization is evident in haploid males (P2) and females (P4) (Tarone et al., 2005; Beukeboom et al., 2007a).
Table 4.1: *Nutra* dsRNA injected females and their offspring numbers. Number of *Nutra* dsRNA injected females (P: Females (RNAi)) that produced offspring (P: Females (fertile)) as virgin or as mated to AsymC males and the offspring they produced (F1: haploid males; F1: diploid females and F1: diploid males).

<table>
<thead>
<tr>
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<th>P: (RNAi)</th>
<th>P: (fertile)</th>
<th>F1: haploid♂</th>
<th>F1: diploid♀</th>
<th>F1: diploid♂</th>
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<tr>
<td>Virgin</td>
<td>60 17</td>
<td>418 0</td>
<td>0</td>
<td>0</td>
<td></td>
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<tr>
<td>Mated</td>
<td>60 26</td>
<td>295 0</td>
<td>379</td>
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To assess if *Nutra* dsRNA injected mothers had indeed provided lower amounts of *Nutra* to the eggs, the relative levels of *Nutra* were measured in the offspring of *Nutra* dsRNA injected and non-injected females. It appeared that very early embryos (0–3 hours old), in which zygotic gene expression has not yet started, resulting from both virgin and mated *Nutra* dsRNA injected females, contained significantly lower levels of *Nutra* mRNA than early embryos from control non-injected females ($t_{35} = -3.92$, $p = 0.0002$, Fig. 4.6).

![< 3h old embryos](https://example.com/figure.png)

**Figure 4.6:** Maternal input in early embryos. Relative *Nutra* mRNA levels in embryos from control (gray bar) or *Nutra* dsRNA injected (black bar) females. Error bars represent s.e. *P < 0.001.

**Discussion**

In this chapter evidence is provided for a crucial role of *Nutra* as a maternal factor in *Nasonia* sex determination. Parental RNAi knockdown of *Nutra* in females was used to study the knockout effect on the offspring. The results show that a threshold level of maternally provided female specific *Nutra* mRNA is essential for female development of the fertilized egg, as knockdown of *Nutra* in mothers leads to the production of diploid male offspring. They also indicate that maternal NvTRA protein is necessary for female specific splicing of zygotic *Nutra*. First, knockdown of *Nutra* in the mother leads to disruption of their own female specific splicing of both *Nutra* and *Nudsx*. Second, the diploid male offspring from *Nutra*
dsRNA injected mothers had only male specific spliced \textit{Nvtra} transcripts, indicating the dependence of a functional \textit{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 \textit{Nvtra} resulting in a full sex reversal indicates that sufficient \textit{NvTRA} is needed for female specific splicing. In \textit{M. domestica} and \textit{A. mellifera} transgenic expression of female specific \textit{tra} in \textit{M} factor bearing individuals (\textit{M. domestica}) or female specific \textit{fem} in haploid individuals (\textit{A. mellifera}) changed the splicing from male specific to female specific (Gempe \textit{et al.}, 2009; Hediger \textit{et al.}, 2010), which indicates that a threshold level of female specific \textit{tra/fem} is necessary for female development. Fourth, eight putative TRA/TRA2 binding motifs \textit{(U/A)GAAGAU(U/A)} in the \textit{tra/fem}-regulated \textit{dsx} and \textit{fruitless (fru)} genes of \textit{N. vitripennis} and \textit{A. mellifera} (Bertossa \textit{et al.}, 2009) are located in the male specific exon 2m1 (Chapter 2) and in the intronic region between exon two and three of the \textit{Nvtra} gene. Based upon similar arguments, a \textit{tra} autoregulatory loop has been proposed for the dipteran \textit{C. capitata} (Pane \textit{et al.}, 2002).

Still, these \textit{Nvtra} dsRNA injected females showed no phenotypic aberration, even though male specific splice variants were detected in these females as well as a clear reduction of \textit{tra} expression in general. This indicates that the sexual development is defined earlier during development. Also, no effect on fecundity and fertilization were seen since both haploid and diploid offspring was produced by these treated females. \textit{Nvtra} dsRNA injected virgin females produced only haploid males, injected mated females produced both haploid and diploid males and no females. This shows that when the expression level of \textit{tra} is diminished in the \textit{Nvtra} dsRNA injected females, it prevents the input of sufficient \textit{Nvtra} in the eggs, leading to an interruption in the development of fertilized eggs as female offspring. Since \textit{Nudsx} is spliced into the male-specific form in the absence of female-specific \textit{Nvtra}, the knockdown of \textit{Nvtra} resulted in diploid males and had no effect on haploid males. Similar results were found after RNAi experiments with embryos from \textit{B. oleae}, \textit{C. capitata}, \textit{L. cuprina}, \textit{M. domestica} and \textit{A. mellifera} in which transient RNAi knockdown of \textit{tra/fem} resulted in fertile males (Pane \textit{et al.}, 2002; Lagos \textit{et al.}, 2007; Hasselmann \textit{et al.}, 2008; Concha and Scott, 2009; Hediger \textit{et al.}, 2010)

Notably, \textit{Nvtra} is maternally provided to all eggs, not only the ones that get fertilized but also the ones that remain unfertilized and develop as males, even though a feminizing factor is present in these eggs. Apparently some mechanism prevents the autoregulatory loop from initiating despite the maternally provided \textit{Nvtra}. In the dipterans, \textit{Ceratitis}, \textit{Musca} and \textit{Lucilia}, it is suggested that the \textit{M} factor blocks the transcription or translation of female \textit{tra} or by interfering with \textit{tra} splicing (Pane \textit{et al.}, 2002; Concha and Scott, 2009; Salvemini \textit{et al.}, 2009; Hediger \textit{et al.}, 2010). In \textit{Musca}, this \textit{M} factor can be located on the \textit{Y} chromosome but also on one of the autosomes (Schmidt \textit{et al.}, 1997). In most Diptera the presence of an \textit{M} factor leads to male development (Marín and Baker, 1998). Only males can provide the \textit{M} factor for the next generation. In Hymenoptera no \textit{M} factor can be present since females produce males uniparentally and they do not possess this \textit{M} factor.
Figure 4.7: The adjusted maternal effect genomic imprinting sex determination (MEGISD) model. A maternal effect gene (msd, two doses in diploid females) actively imprints transformer (tra) into a masculinizing state. The msd gene does not imprint in males and the paternally inherited tra allele is feminizing. Unfertilized haploid eggs with only a maternal tra develop into males and fertilized diploid eggs with a maternal and paternal tra develop into females (Figure modified from Beukeboom et al., 2007b).

The absence of an M factor to block the startup of the autoregulatory loop means that a different mechanism has to be responsible for the development of males despite the presence of maternal Nvtra mRNA. Here, an adjustment of the MEGISD imprinting model proposed by Beukeboom et al. (2007b) (see Chapter 1) is suggested that may further describe the sex determining process in Naso-nia. With the current knowledge we propose that the zygotic sex determiner (zsd) gene in the MEGISD model is Nvtra (Fig. 4.7). In an unfertilized embryo, where only the maternal genome set is present, the Nvtra gene is imprinted by the yet unknown maternal sex determiner (msd) gene rendering it inactive resulting in Nvtra messenger transcript that cannot be transcribed from the maternal gene. The maternal provision of Nvtra mRNA into the eggs is translated into a protein, but since no new (zygotic) Nvtra transcript is produced, the autoregulatory loop is unable to start and eventually the provided maternal TRA protein will break down. To account for the presence of male-specific transcript later on in development, it is suggested that the inactivation is lost at a certain moment during development. By then the TRA protein is already broken down and Nvtra is spliced in the default male specific form. In a fertilized embryo, both the maternal and the paternal genome set are present. The paternal genome provides an unimprinted, active Nvtra gene that will be transcribed into pre-mRNA enabling the autoregulation of Nvtra to start and eventually resulting in female development.

This theory is supported by results obtained by Trent et al. (2006). In their research they X-ray mutagenized wild type haploid males and crossed them to homozygous recessive eye-color mutant females. They obtained rare diploid male offspring that were of biparental origin rather than previously found uni-
Maternal input of \textit{Nvtra} parental diploid males from triploid virgin females (Beukeboom and Kamping, 2006). These biparental diploid males, when mated with a diploid female, produced triploid female offspring. The biparental diploid males were explained by an imprinting defect in the irradiated paternal germ line generating an epigenetic lesion. The exact nature of the mutation that generated the biparental males was unknown but it was hypothesized that a paternally inherited gene required for female sexual development was mutated. With the adjusted MEGISD model described above it can be assumed that the X-ray mutagenized males obtained a genetic mutation rendering \textit{Nvtra} dysfunctional. The biparental diploid male offspring can be explained by assuming that the maternal \textit{Nvtra} gene in the offspring is imprinted during early development and that the paternal \textit{Nvtra} gene is mutated. This means that no \textit{Nvtra} mRNA is produced in the early zygote preventing the autoregulatory loop from starting, which results in diploid male development. Assuming that upon spermatogenesis the imprint is erased, they transmit a mutated \textit{Nvtra} and a normal active \textit{Nvtra} while females transmit an imprinted \textit{Nvtra}. When these biparental diploid males were crossed with a female the active paternal \textit{Nvtra} yielded enough \textit{Nvtra} mRNA to start the autoregulatory loop leading to triploid female development.

Other more recent studies also provide additional information about sex determination in \textit{N. vitripennis}. Two natural strains of \textit{N. vitripennis} produce gynandromorphs and/or females from haploid unfertilized eggs (Beukeboom and Kamping, 2006; Beukeboom et al., 2007a; Kamping et al., 2007) (see Chapter 1). Under the proposed imprinting model it is suggested that in these particular strains a mutation occurred which caused incomplete imprinting in the maternal germ line, generating a (partial) active \textit{Nvtra} gene. When these females produce haploid offspring they would transmit this active \textit{Nvtra} gene to the next generation. The maternal input of \textit{Nvtra} together with the zygotic expression from the active \textit{Nvtra} gene would initiate the \textit{tra} autoregulatory loop, leading to female development. A partial imprinting may give a reduced transcription rate of \textit{Nvtra} in the haploid offspring which would cause a partial start up of the autoregulatory loop. This would then lead to gynandromorph development. These observations show that females can develop from eggs that do not receive a paternal chromosome complement.

In this Chapter, a \textit{Nasonia} \textit{tra} homolog was described and a hypothesis is proposed in which \textit{Nvtra} is the most likely candidate for the primary signal in \textit{Nasonia}. It is shown that \textit{Nvtra} is responsible for splicing \textit{Nudsx}. This suggests that an active copy of this gene is necessary for female development. Maternal \textit{tra} input seems conserved in insects: in \textit{Ceratitis}, \textit{Bactrocera}, \textit{Lucilia} and \textit{Musca} maternal \textit{tra} input is demonstrated and even in \textit{Drosophila} where \textit{tra} is regulated by \textit{Sxl} it is likely that the ancestral mode of sex determination is \textit{tra} autoregulation (Siera and Cline, 2008). In Hymenoptera the mode of sex determination with \textit{csd} has always been assumed to be ancestral, however Hasselmann et al. (2008) already provided evidence for the contrary. Our results substantiate the claim that \textit{csd} is a derived system. It is most likely that also in Hymenoptera the ancestral mode of sex determination is the maternal input of \textit{tra} and consequently
the maternal imprinting of tra. Whereas in this Chapter the focus was on the effect of Nutra dsRNA injection in adult females and offspring, in the next Chapter the expression levels of Nutra during embryonic development in fertilized and unfertilized eggs will be investigated to acquire more evidence for the maternal imprinting model.

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