Chapter 3

Maternal provision of *transformer-2* is required for female development and embryo viability in *Nasonia vitripennis*

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ABSTRACT

Two molecular mechanisms of haplodiploid sex determination are currently known, Complementary Sex Determination (CSD) in *Apis mellifera* and Maternal Effect Genomic Imprinting Sex Determination (MEGISD) in the parasitoid *Nasonia vitripennis*. A primary signal starts the genetic cascade of sex determination which subsequently in most insect orders is transduced by a *transformer (tra)* ortholog. Only a female-specifically spliced *tra* mRNA yields a functional TRA-protein, that forms a complex with TRA2, encoded by *transformer-2* (*tra2*), to act as a sex specific splicing regulator of the downstream genes *doublesex* (*dsx*) and *fruitless* (*fru*). Here, we identify the *tra2* ortholog in *N. vitripennis* (*Nv-tra2*) and describe its function in *Nasonia* sex determination. Knock down of *Nv-tra2* by parental RNA interference results incomplete sex reversal of diploid offspring from female to male, indicating the requirement of *Nv-tra2* for female sex determination. As knockdown also led to lethality in early developmental stages *Nv-tra2* apparently served another, non sex determining, function during embryogenesis. Lethality of diploid *Nv-tra* pRNAi embryos is observed and lethality after *Nv-tra2* pRNAi appears more pronounced in diploid than in haploid offspring, indicating dosage effects. In contrast to haploid offspring, diploid embryos possess a paternal chromosome set, suggesting a disruption of development in diploids due to active paternal allele copies of unspecified genes. These results are discussed in the light of the MEGISD model of *Nasonia* sex determination.

INTRODUCTION

Insect sex determination is accomplished by a multistep process, involving a cascade of regulatory genes that is evolving bottom-up with *doublesex* (*dsx*), the interpreter for the sexual identity of the cell, as the most conserved downstream gene (Wilkins, 1995; Shukla & Nagaraju, 2010; Verhulst & van de Zande, 2015). The primary signal, that directs the cascade into the female or male developmental pathway, is highly diverse (Sánchez, 2008). In the hourglass model (Bopp *et al.*, 2014), the primary signal is considered as the instruction, and the action of *dsx* as the execution phase. In between is *transformer* which processes the primary signal towards an "on or off" state through sex-specific splicing. Only if the zygotic state of *tra* is ON, it will produce a functional TRA protein that will lead to female specific splicing of *dsx*. In addition, an autoregulatory loop in which *tra* maintains its own splicing mode functions as a "memory" to ensure proper sexual differentiation (Bopp *et al.*, 2014). The *tra-dsx* axis appears to be the conserved transduction mechanism in insect sex determination, yet *tra* orthologs are absent in several lineages (Shukla & Nagaraju, 2010; Bopp *et al.*, 2014; Geuverink & Beukeboom, 2014). This absence suggests that the conserved transduction mechanism of the pathway can change its executing elements.

In dipteran insects, two primary signals are known to instruct the zygotic state of *tra*: a masculinizing gene (M-factor) prevents the ON state (as for example in *Ceratitis capitata, Musca domestica*) or an insufficient dose of X-linked factors fails to timely activate the transcription of *sex-lethal* (*sxl*) to effectuate the ON state of *tra* (e.g. *Drosophila melanogaster*) (Dübendorfer *et al.*, 2002; Pane *et al.*, 2002; Erickson & Quintero, 2007). For Hymenoptera, that have a haplodiploid reproductive system, two primary signals have been described: complementation of alleles at the *csd* locus, in which heterozygosity at one or more *csd* loci leads to female development (Cook, 1993; van Wilgenburg *et al.*, 2006) and a genomic imprinting gene, called *womanizer* under Maternal Effect Genomic Imprinting Sex Determination (MEGISD) (Beukeboom *et al.*, 2007b; Beukeboom & van de Zande, 2010). The genetic basis of CSD has thus far only been elucidated in the honeybee (*Apis mellifera*), where the *csd* locus was identified as a duplication of *feminizer* (*fem*), the honeybee ortholog of *tra* (Beye *et al.*, 2003; Hasselmann *et al.*, 2008a). MEGISD has been proposed for the non-CSD parasitoid *Nasonia vitripennis* (Verhulst *et al.*, 2010a, 2013) and entails maternal imprinting of the putative *womanizer* (*wom*) gene to prevent activation of *Nv-tra* transcription in haploid zygotes. Upon fertilization, the non-silenced paternal *wom* allele will initiate early zygotic transcription of *Nv-tra*. Additionally, like in many insect species (e.g. *C. capitata*, *M. domestica* and *Tribolium castaneum*) (Pane *et al.*, 2002; Hediger *et al.*, 2010; Shukla & Palli, 2012a), the *Nasonia* female sex-determining mechanism is dependent on maternal provision of *tra* mRNA to the egg to help initiate the zygotic ON state of *tra* (Verhulst *et al.*, 2010a).

Transformer-2 (TRA2) is an essential co-factor in the sex determination of many, if not all, insect species (Amrein et al., 1990; Hedley & Maniatis, 1991; Inoue et al., 1992; Salvemini et al., 2009; Hediger et al., 2010; Schetelig et al., 2012). Knockdown studies of *tra2* in Diptera

revealed the involvement of TRA2 in the female-specific splicing of *tra* mRNAs (Burghardt *et al.*, 2005; Concha & Scott, 2009; Salvemini *et al.*, 2009; Sarno *et al.*, 2010; Martín *et al.*, 2011). In some insects species, such as *Sciara ocellaris*, *Bradysia coprophila*, *Bombyx mori*, *A. mellifera* and *T. castaneum* (Niu *et al.*, 2005; Martín *et al.*, 2011; Nissen *et al.*, 2012; Shukla & Palli, 2013) TRA2 has alternative isoforms which all code for a basic RNA-binding domain (RBD), but none of these identified isoforms are sex-specific at any life stage. In *M. domestica*, *C. capitata*, *Lucilia cuprina*, *Anastrepha obliqua*, *Anastrepha suspensa* and *Bactrocera dorsalis* only one *tra2* splice variant was detected (Burghardt *et al.*, 2005; Concha & Scott, 2009; Salvemini *et al.*, 2009; Sarno *et al.*, 2010; Schetelig *et al.*, 2012; Liu *et al.*, 2015). Overall, *tra2* is more conserved than *tra*, as even in species without a *tra* ortholog, like the lepidopteran *B. mori*, a *tra2* homolog was identified (Niu *et al.*, 2005; Kiuchi *et al.*, 2014).

The roles of *tra* and *dsx* in the *Nasonia* MEGISD model have been identified, but that of *tra2* is still lacking. Therefore, we investigated the function of *tra2* in *Nasonia* sex determination. We identified the structure and splicing of *Nv-tra2* and compared it to other known *tra2* orthologs. Using parental RNAi (pRNAi) knockdown we confirm that *Nv-tra2* is indeed involved in *Nasonia* sex determination. In addition, it became apparent that *Nv-tra2* must have other roles during development.

MATERIAL AND METHODS

N. vitripennis *strains and rearing*

The *N. vitripennis* lab strain AsymCX (Werren *et al.*, 2010) and the recessive red eye color mutant strain ST^{DR} were used throughout the experiments. ST^{DR} females mated with AsymCX males produce diploid female offspring with wild-type eyes and red-eyed haploid male offspring (Verhulst *et al.*, 2010a). This allows the detection of wild type diploid males resulting from knockdown of either *Nv-tra2* or *Nv-tra*. Wasps were reared on *Calliphora sp.* hosts and cultured at 25°C at a L16:D8 cycle.

RNA extraction and cDNA synthesis

Total RNA of individual wasps and embryo pools was extracted with TriZol (Invitrogen, Carlsbad, CA, USA) according to manufacturer's protocol. RNA was reverse-transcribed with oligo-dT and hexamer primers in a 1:6 ratio with the RevertAidTM H Minus First Strand cDNA Synthesis Kit (Fermentas, Hanover, MD, USA) and stored at -80°C. RNA samples for gene identification were individual male and female samples of the AsymCX strain. For 3'RACE (Rapid Amplification of cDNA Ends), RNA was reverse-transcribed with the RevertAidTM H Minus First Strand cDNA Synthesis Kit (Fermentas, Hanover, MD, USA) using 25µM 3'RACE adapter (5'-GCG AGC ACA GAA TTA ATA CGA CTC ACT ATA GGT 12VN-3') from FirstChoice RLM-RACE kit (Ambion, Austin, TX, USA). For 5'RACE, RNA was processed according to manufacturer's instructions (FirstChoice

RLM-RACE kit, Ambion, Austin, TX, USA) and reverse-transcribed using the RevertAidTM H Minus First Strand cDNA Synthesis Kit (Fermentas, Hanover, MD, USA).

Identification of Nv-tra2 *genomic structure*

Primers were designed based on the putative *Nv-tra2* transcript (LOC100116671) from the *N. vitripennis* genome (Werren *et al.*, 2010) (Table 3.1). 5'RACE-PCR was performed with outer primer Nvtra2_5Rout and inner primer Nvtra2_5Rin at 94°C for 3 minutes, 35 cycles of 94°C for 30 seconds, 58°C for 30 seconds and 72°C for 2 minutes, with a final extension of 10 minutes at 72°C. Outer primer Nvtra2_3Rout and inner primer Nvtra2_3Rin were used in 3'RACE-PCR with Phusion High-Fidelity DNA polymerase (Fermentas, Hanover, MD, USA). Cycling conditions were 98°C for 1 minute, 35 cycles of 98°C for 10 seconds, 57(out)/55(in)°C for 30 seconds and 72°C for 60 seconds, with a final extension of 10 minutes at 72°C. Resulting PCR fragments were run and visualized on ethidiumbromide-containing 1.5% agarose gel with 1×TAE buffer.

All RACE-PCR products were ligated into pGEM-T vector (Promega, Madison, WI, USA) after purification using GeneJET Gel Purification Kit (Fermentas, Hanover, MD, USA). Ligation reactions were used to transform competent JM-109 *Escherichia coli* (Promega, Madison, WI, USA). Colony-PCR was conducted by use of pGEM-T primers (5'-GTAAAACGACGGCCAGT-3') and 5'-GGAAACAGCTATGACCATG-3') at 94°C for 3 minutes, 40 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 2 minutes, with a final extension of 7 minutes at 72°C.

Additional Reverse Transcription-PCRs (RT-PCR) were used to confirm splice variation. These PCRs were performed with primers Nvtra2_exon1F/Nvtra2_exon4R and Nvtra2_exon2F/Nvtra2_exon6R at 94°C for 3 minutes, 40 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 2 minutes, with a final extension of 7 minutes at 72°C. PCR-fragments were sequenced on an ABI 3730XL capillary sequencer (Applied Biosystems) and reads were inspected in Chromas (Technelysium) and aligned in MEGA4 (Tamura *et al.*, 2007). Exon-intron structure of the genes was constructed by comparing the mRNA sequences to *N. vitripennis* genome and visualized with Exon-Intron Graphic Maker (http://wormweb.org/exonintron).

Phylogeny and alignment construction

Amino acid sequences of TRA2 orthologs in the following species (Genbank accession) were used in alignments and phylogenetic analyses: *Acromyrmex echinatior* (EGI70155), *A. mellifera* (AFJ15561), *B. mori* (NP_001119705), *C. capitata* (ACC68674), *Ceratosolen solmsi* (XP_011500657), *Daphnia pulex* (EFX90042), *D. melanogaster* (NP_476764), *M. domestica* (AAW34233) and *T. castaneum* (AHF71088). A neighbour-joining tree was constructed in MEGA4 (Tamura et al., 2007) and alignments were produced in Geneious8 (Biomatters Ltd).

Table 3.1. Overview of all primers used in this study. Noted between square brackets is the binding site sequence for T7 RNA polymerase.

Primername	Gene	Application	Primer sequence 5'-3'	Design
EF1α_F1	*EF1α*	qRT-PCR	CACTTGATCTACAAATGCGG	(Verhulst *et al.*, 2010a)
EF1α_R1	*EF1α*	qRT-PCR	GAAGTCTCGAATTTCCACAG	(Verhulst *et al.*, 2010a)
NvDsxU_F3	*dsx*	RT-PCR	AGCCACTGCCGAGTATACCA	(Verhulst *et al.*, 2010a)
NvDsxM_R1	*dsx*	RT-PCR	TCGGAGAAGATTGGCAGAAC	(Verhulst *et al.*, 2010a)
NvDsx_qPCR_F1	*dsx*	qRT-PCR	GGTGACATGCGTAGTTTGAG	(Verhulst *et al.*, 2010a)
NvDsx_qPCR_R1	*dsx*	qRT-PCR	CAAGTCGTGGATTTGGTTCG	(Verhulst *et al.*, 2010a)
NvTra_F2	*tra*	RT-PCR	GACCAAAAGAGGCACCAAAA	(Verhulst *et al.*, 2010a)
NvTra_R3	*tra*	RT-PCR	GGCGCTCTTCCACTTCAAT	(Verhulst *et al.*, 2010a)
NvTra_qPCR_F1	*tra*	qRT-PCR	CGCCGTTCTAAGTCATTGAG	(Verhulst *et al.*, 2010a)
NvTra_qPCR_R1	*tra*	qRT-PCR	ATCGGAATAATGCCTATCGT	(Verhulst *et al.*, 2010a)
NvTra_RNAi_F1	*tra*	RNAi	[TAATACGACTCACTATAGGG]CGAG ACATCAGTTAGAAGAT	(Verhulst *et al.*, 2010a)
NvTra_RNAi_R1	*tra*	RNAi	[TAATACGACTCACTATAGGG]GTCTT GTGGTCCTATGAAAC	(Verhulst *et al.*, 2010a)
Nvtra2_5Rout	*tra2*	RACE-PCR	GCCCGTTCTGTGATAGAATAATCC	This study
Nvtra2_5Rin	*tra2*	RACE-PCR	GTCCATCAATTGCCATTCCA	This study
Nvtra2_3Rout	*tra2*	RACE-PCR	GTGTACTTTGAATCACTGGA	This study
Nvtra2_3Rin	*tra2*	RACE-PCR	ATTGATGGACGACGAATCAG	This study
Nvtra2_exon1F	*tra2*	RT-PCR	CGCTTTGATTATTATGGACGAC	This study
Nvtra2_exon4R	*tra2*	RT-PCR	GACGGTAGTTTCCTCTGCTG	This study
Nvtra2_exon2F	*tra2*	RT-PCR	CAAGAAGATCAAGAGCCGAC	This study
Nvtra2_exon6R	*tra2*	RT-PCR	CTGAGGTTTGGAATGGTGGA	This study
Nvtra2_qF	*tra2*	qRT-PCR	ACAGAGATAATCCTACTCCAAGCC	This study
Nvtra2_qR	*tra2*	qRT-PCR	CGCTTTGTCTTTGCATCAATGAC	This study
Nvtra2_RNAiF	*tra2*	RNAi	[TAATACGACTCACTATAGGG]CCAAG AAGATCAAGAGCCGA	This study
Nvtra2_RNAiR	*tra2*	RNAi	[TAATACGACTCACTATAGGG]GTCCA TCAATTGCCATTCCA	This study

Parental RNAi and sample collection

Parental RNAi knockdown was induced in white female ST^{DR} pupae (Lynch & Desplan, 2006). Non-sex-specific regions were amplified using Nvtra2_RNAiF and Nvtra2_RNAiR primers for *Nv-tra2* dsRNA (564bp) and NvTra_RNAiF1 and NvTra_RNAiR1 primers for *Nv-tra* dsRNA (452bp) (Table 3.1). The 5' and 3' sides of those amplicons were flanked with a T7 promotor as a template for dsRNA production using the Megascript RNAi kit (Ambion, Austin, Texas, USA) according to the manufacturer's protocol.

Approximately 600 female ST^{DR} pupae were injected in the abdomen with 1.3µg/µl of *Nv-tra2* dsRNA, *Nv-tra* dsRNA or sterile water. *Nv-tra* was used as a positive control (see Verhulst *et al.* (2010a) for prior use) and sterilized milliQ as a negative control for the injection procedure. Injections were performed with Femtotips II (Eppendorf) needles connected to a Femtojet (Eppendorf, Hamburg, Germany) with continuous injection flow. Hundred and twenty adult females of each category were mated with AsymCX males and split over two sets for either embryo collections (60 wasps) or offspring production (60 wasps). Virgin females (60 wasps) were used to produce haploid male controls.

Embryonic levels of *Nv-tra2*, *Nv-tra* and *Nv-dsx* mRNAs and sex-specific splicing of *Nv-tra* and *Nv-dsx* were assessed in early embryos of less than three hours old (<3 h). Injected and control females were provided with *Calliphora sp.* hosts in individual egg laying chambers assembled from filter tips (1ml) capped with a cutoff 1.5ml vial as a host carrier. The anterior side of the hosts was exposed to the parasitizing females to facilitate localization of the embryos during collections. After two hours of oviposition in egg-laying chambers, hosts were gently opened to collect approximately 55 embryos in 100% ethanol per sample. Eight embryo samples per category were snap-frozen in liquid nitrogen and stored at -80°C until RNA extraction.

Levels and sex-specific splicing of *Nv-tra2*, *Nv-tra* and *Nv-dsx* mRNAs were determined in adult progenies that were injected with either sterile water, or *Nv-tra2* or *Nv-tra* dsRNA. Injected females were provided fresh hosts daily to oviposit for 24 hours at 25°C. Parasitized hosts were incubated for 14 days at 25°C after which offspring emerged. Offspring of *Nv-tra2* and *Nv-tra* injected females were phenotypically screened for sex and eye color and 10 diploid and 10 haploid males were collected per gene knockdown. Expected sex ratio and eye color of the offspring of control-group females were checked, and 10 diploid females and 10 haploid males were collected and stored at -80°C. Of all categories, another two adult offspring were collected and stored at -20°C for flow cytometry analysis to confirm ploidy (see below).

mRNA levels in embryos and adults

Quantitative real-time PCR (qPCR) analysis was performed using 5µl of a 50-fold cDNA dilution and 300nM PerfeCTaTM SYBR® Green mix (Quanta Biosciences, Gaithersburg, MD, USA) on an Applied Biosystems 7300 Real Time PCR System (Foster City, CA, USA). *Nv-tra2*, *Nv-tra* and *Nv-dsx* were amplified with non-sex-specific, exon-spanning primers at 250nM (Table 3.1) (Verhulst *et al.*, 2010a). The *elongation factor 1 alpha* (*EF1α*) was used as a reference gene using exon-

spanning primers (250nM) (Table 3.1) (Verhulst *et al.*, 2010a). qPCR profiles were 95°C for 3 minutes, 45 amplification cycles of 15 seconds at 95°C, 58°C/58°C/57°C/55°C for *Nv-tra2*/*Nv-tra*/*Nv-dsx*/*Nv-EF1α*, 72°C for 30 seconds and followed up by a standard ABI7300 dissociation curve. Raw fluorescence data generated by 7300 System SDS Software (Applied Biosystems, Foster City, CA, USA) were base-line corrected and the N0 value calculated with PCR efficiencies per amplicon using LinRegPCR 11.0 (Ramakers *et al.*, 2003). Relative levels were determined by dividing *Nv-tra2*, *Nv-tra* and *Nv-dsx* N0 values by *Nv-EF1α* N0. A one-way ANOVA was used for each life stage (embryo, adult offspring) to test relative differences between the *Nv-tra2*, *Nv-tra* dsRNA injected samples and the water-injected control samples.

Splice variant analysis of Nv-tra *and* Nv-dsx *following pRNAi*

Sex-specific fragments of *Nv-tra* and *Nv-dsx* in injected and control females and their embryonic and adult offspring were analyzed by RT-PCR. 5µl of a 50-fold cDNA dilution was used in a PCR. For sex-specific *Nv-tra* amplification primers NvTra_F2 and NvTra_R3 were used, located at exon 2 and 3, yielding a single 228 bp fragment in females and three fragments of 514, 460 and 282 bp in males depending on their age. Primers NvDsxU_F3 and NvDsxM_R1 were used for sex-specific *Nv-dsx* amplification, yielding 543 bp in females and 651 bp in males (Verhulst *et al.*, 2010a). For amplification and cDNA integrity control *Nv-EF1α* was amplified with NvEF1α_F1 and NvEF1α_R1 primers yielding a 174 bp fragment in both genders. The PCR profile was 45 cycles of 95°C for 15 seconds, 55°C for 30 seconds and 72°C for 30 seconds. Fragments were run and visualized on a 2% non-denaturing TAE gel and containing ethidiumbromide.

Flow cytometry of diploid males

Ploidy of *Nv-tra2* and *Nv-tra* diploid males was confirmed by flow cytometry analysis. Adult male wasp heads were homogenized in Galbraith buffer (21mM $MgCl_2$, 30mM tri-sodium citrate hydrate, 20mM MOPS, 0,1% Triton X-100, 1mg/l RNAse A) using Dounce homogenizers, filtered by 0.7µm cell strainer caps (BD Falcon Cell strainer #352235, BD Biosciences, San Jose, California, USA) and stained in propidium iodide (Sigma, St. Louis, Missouri, USA). Samples were loaded on a BD FACS Aria II and analyzed by BD FACSDiva software (BD Biosciences, San Jose, California, USA). References for ploidy were set by haploid and diploid males from a polyploid strain (Beukeboom & Kamping, 2006).

Viability and fertility of offspring after parental Nv-tra2 *and* Nv-tra *knockdown*

Brood sizes were analyzed to assess the survival of offspring from *Nv-tra2* dsRNA, *Nv-tra* dsRNA, sterile water injected and non-injected mothers. The number of embryos was compared to the number of emerging adult offspring. ST^{DR} female pupae were injected with dsRNA targeting *Nv-tra2* (N=200), *Nv-tra* (N=200) and sterile water (N=200). Of each category 100 adult females were mated with AsymCX males, while the other 100 remained virgin. Mated and virgin females

were individually cultured in egg laying chambers and split over 2 batches which were alternately used for embryo or adult offspring counts. This prevented biases caused by knockdown efficiency or fecundity of the wasps. Wasps were allowed to parasitize on the anterior side of the hosts for 2 hours at 25°C, which was repeated on four consecutive days at a similar time of day. Females were allowed to oviposit for two hours, after which they were removed and hosts were either opened for embryo counts or retained at 25°C for 14 days to count adult offspring. Numbers of embryos and adult offspring were compared in a general linear model and a post-hoc Tukey-test was used to examine sample differences in embryo and adult number. Differences in egg number laid by females in each category were compared with a Kruskal-Wallis test.

Adult male offspring from these experiments were used to assess fertility of haploid and diploid *Nv-tra2* and *Nv-tra* pRNAi males compared to control males (haploid males from water-injected mothers). Each male (N=20 per category) was mated to 7 virgin females very shortly after one another to induce sperm depletion (Ruther *et al.*, 2009). These females were hosted on 4 *Calliphora sp.* pupae every other day over a period of 10 days (5 data points). Offspring were scored after emergence and the fraction of data points (of 7 females x 5 rehostings) producing daughters was calculated for each male. This provided a measurement of (diploid) male fertility. Differences between categories were compared with a one-way ANOVA.

RESULTS

Structure and conservation of tra2 *in* N. vitripennis

To identify a *tra2* homolog in *N. vitripennis*, the reference genome of AsymCX (Werren *et al.*, 2010) was screened with an *A. mellifera* TRA2 query (Genbank accession AFJ15561) using tblastn. A putative homolog of *tra2* could be detected that was previously described (Genbank accession XP_001601106, predicted isoform X3) in an amino acid comparison in Nissen *et al.* (2012). This predicted sequence was used for RACE-PCR and RT-PCR primer design. Four splice variants of *Nv-tra2* were detected in *N. vitripennis* based on a combination of RACE-PCRs and additional RT-PCRs to verify the splice variation (Figure 3.1). An additional splice variant having 24 bp (5'-CATCATTTGCTACCTTACACACAG-3') joined to the 5' end of exon 2 was found in very low frequencies. It was not possible to detect whether this 24bp inclusion is present in one or all four splice variants. This 24 bp variation was also described in the predicted isoforms X1 (Genbank accession XP_008205153) and X2 (Genbank accession XP_008205154). Splice variant *Nv-tra2*^{A} containing exon 5 and the second part of exon 6 was most abundant (Figure 3.1). This splice variant matches exactly with the predicted isoform X3 (Genbank accession XP_001601106). Only splice variants *Nv-tra2*^{C} and *Nv-tra2*^{D} were not predicted by the automated computational analysis of the *tra2* locus (NCBI *Nasonia vitripennis* Annotation Release 101). No sex-specific splice variant differences were detected between females and males.

The structure of TRA2, consisting of a single RBD flanked by RS-rich regions, is conserved in *N. vitripennis*. Unlike in *A. mellifera* (Nissen *et al.*, 2012) no variation was found in the length of the first RS-rich domain. The 24 bp variation is just upstream of this region. The second RS-rich region is entirely absent in splice variants C and D and both splice variants are not abundant. Variant Nv-Tra2^{D} lacks a large part of the RBD region, which is fully present in the other splice variants (Figure 3.1).

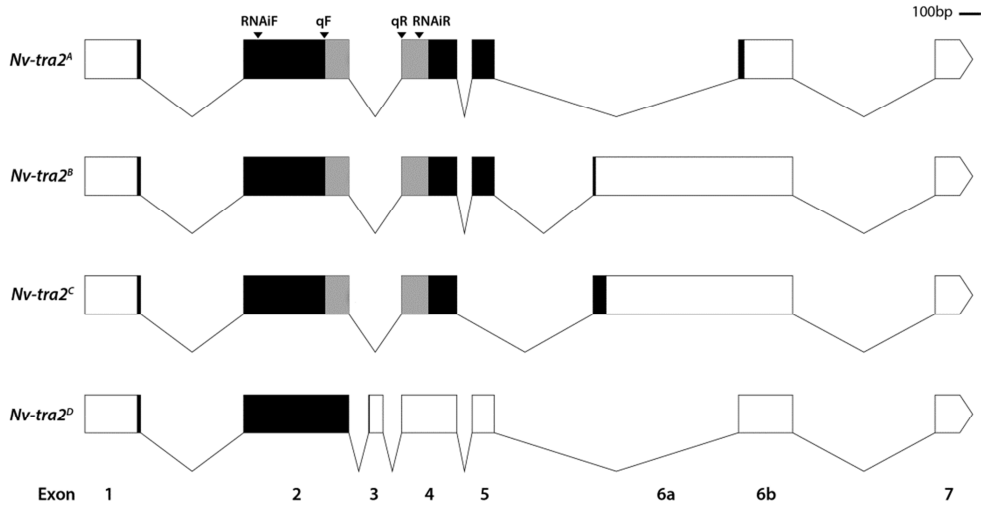

Figure 3.1. Genomic structure of *Nv-tra2*. Blocks represent exons, numbered at the bottom of the figure, and the lines represent the introns. The white regions depict the 5'UTR and 3'UTR, and the black exons depict the coding region. The RBD domain is plotted in grey on the exons, only when present in full. Positions of primers used to generate the dsRNA construct (RNAi) and to measure the *tra2* expression (q) are indicated at the top. The scale bar depicts 100 base pairs.

The amino acid sequence of NV-TRA2 was aligned to known TRA2 homologs, revealing strong conservation, in particular within the Hymenoptera (Figures 3.2 and 3.3). A notable feature, compared to other insect TRA2 peptides, is the presence of a glycine-rich region at the 3'end of the *Nv-tra2*^{A}, *Nv-tra2*^{B} and *Nv-tra2*^{C} splice variants (presence in *Nv-tra2*^{A} depicted in figure 3.2 between positions 266 to 288). This glycine-rich region is also detected in the chalcid *Ceratosolen solmsi* (XP_011500657 (Xiao *et al.*, 2013)) and *B. mori* (Niu *et al.*, 2005) (Figure 3.2).

Sex reversal effects of Nv-tra2 *parental RNAi knockdown*

In order to target all the different *Nv-tra2* splice variants, a shared sequence of 565 bp containing the RBD was used to synthesize dsRNA (primer positions shown in figure 3.1). This *Nv-tra2* dsRNA was injected into females in the pupal stage. RNAi off-target effects were predicted with WaspAtlas (Davies & Tauber, 2015) and no targets other than *Nv-tra2* were detected.

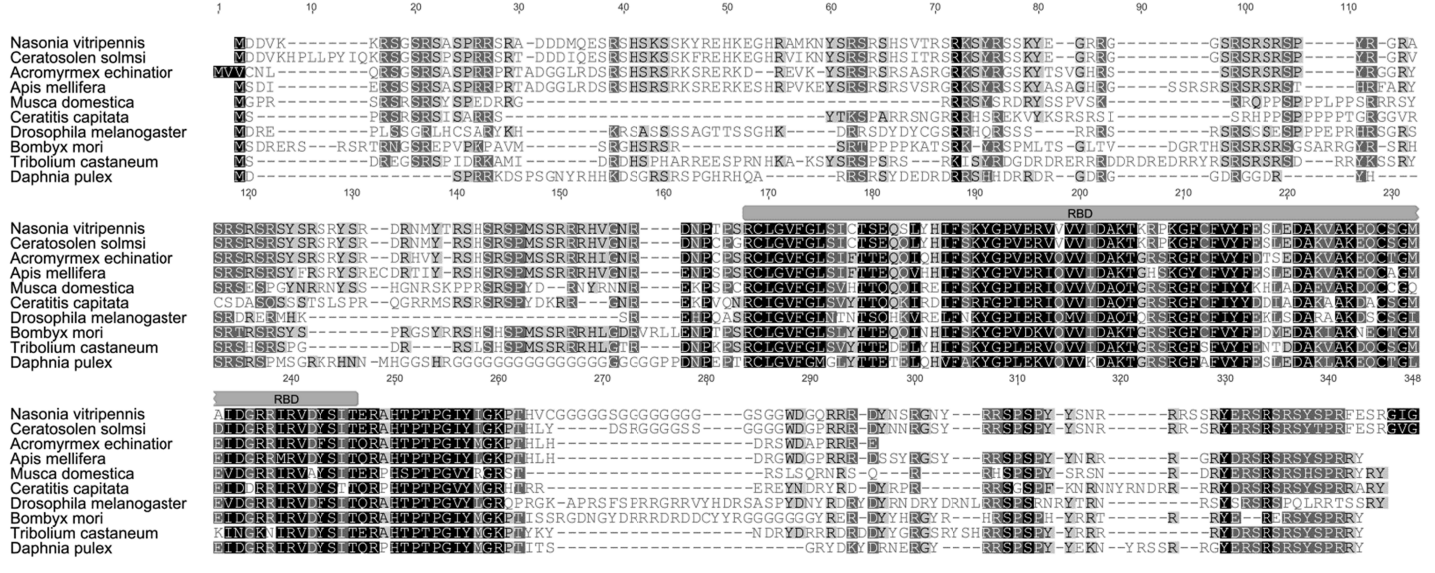

Figure 3.2. Alignment of TRA2 amino acid sequences. The RBD domain is marked in grey above the sequences. Conservation of sites is depicted in grey scale, with darker colours indicating higher conservation, and lighter colours indicating lower conservation.

Parental knockdown with *Nv-tra2* dsRNA led to a 10-fold reduction of *Nv-tra2* transcripts in the offspring compared to that of water injected females ($F_{(2,15)}$=27.1, P<0.001) (Figure 3.4a), but no effect on the level of *Nv-tra* mRNA (Tukey-test: P=0.22) (Figure 3.4b). Parental knockdown of *Nv-tra* led to a significant decrease in *Nv-tra* mRNA levels in the offspring compared to that of water-injected females ($F_{(2,15)}$=13.3, P<0.001) (Figure 3.4b), but no significant effect on *Nv-tra2* mRNA levels (Tukey-test: P=0.18) (Figure 3.4a). Hence, pRNAi of either gene does not interfere with the other.

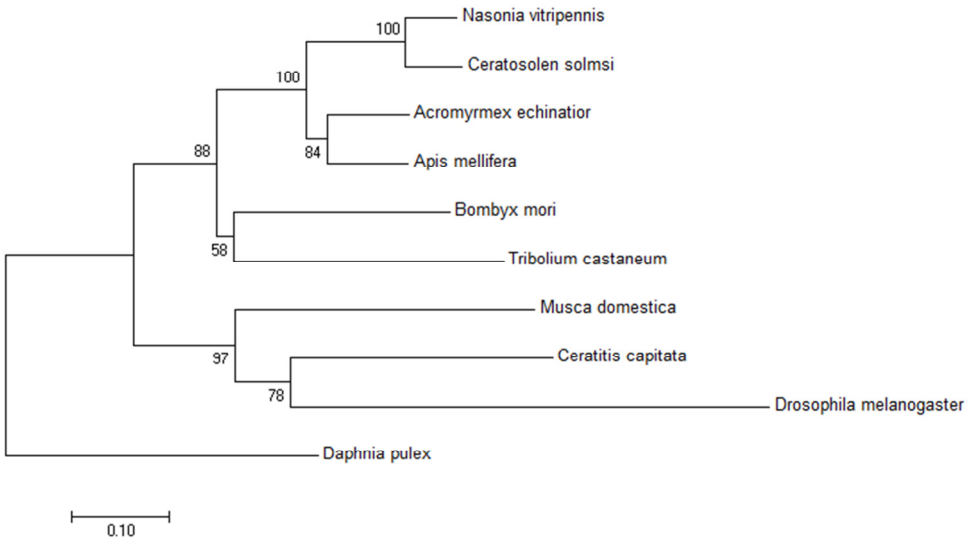

Figure 3.3. Gene tree of TRA2 based on amino acid sequence alignment and constructed with a pairwise deletion neighbour-joining method consisting of 1.000 bootstraps. The scale bar displays the number of substitutions per site.

The adult offspring of *Nv-tra2* knockdown females was scored after emergence, and consisted solely of males (Table 3.2). *Nv-tra* pRNAi resulted in male offspring, in agreement with earlier observations (Verhulst *et al.*, 2010a). Males with wild type and red eye color were confirmed with flow cytometry to be diploid and haploid, respectively. The water-injected females produced progeny with a low sex ratio (calculated as the number of males divided by the total number of offspring). In the adult offspring of injected mothers, relative levels of *Nv-tra2* and *Nv-tra* mRNA had recovered, indicating that the RNAi response is transient. The sex-specific splicing of sex determination genes *Nv-tra* and *Nv-dsx* was assessed in the adult offspring of *Nv-tra2* dsRNA-, *Nv-tra* dsRNA- and water-injected females. Diploid females and haploid males resulting from water-injected mothers have normal female- or male-specific splicing of *Nv-tra* and *Nv-dsx* as expected. Both *Nv-tra2* and *Nv-tra* dsRNA-injected females produced an all-male progeny, consisting of haploid and diploid males, that all showed splicing

of *Nv-tra* and *Nv-dsx* in the male-specific mode (Figure 3.5). This suggests that the role of *Nv-tra2* in the sex determination cascade is conserved, and that both *Nv-tra* and *Nv-tra2* mRNA is provided by the mother to the zygote and is required for female development of fertilized eggs.

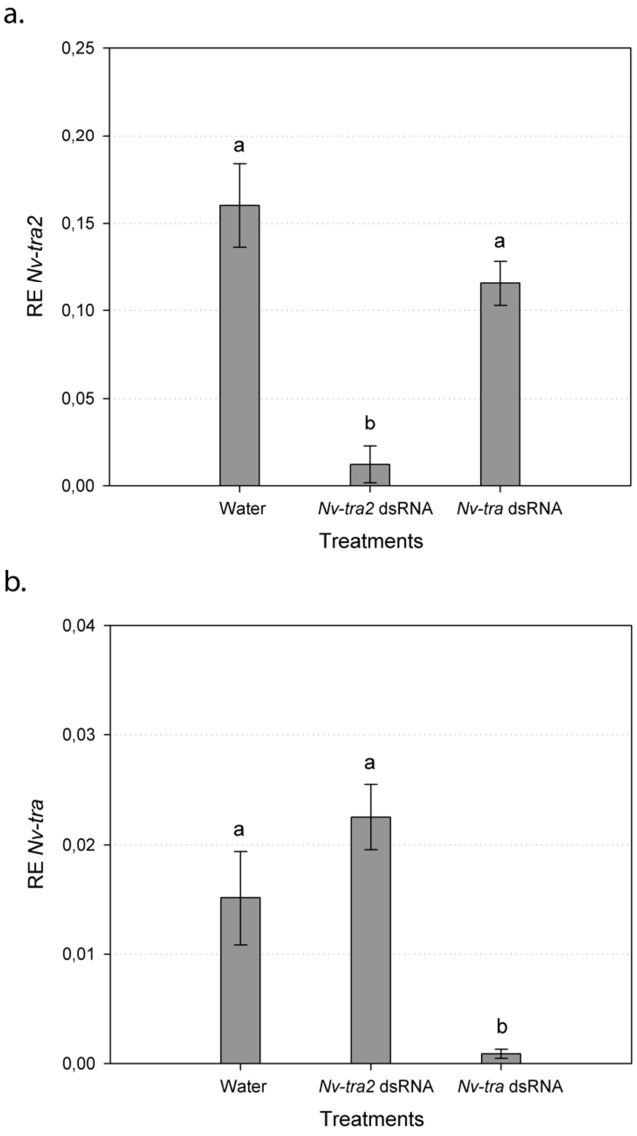

Figure 3.4. Relative expression (RE) in embryos. Relative levels of *Nv-tra2* mRNA(a) and *Nv-tra* (b) in control embryos (water), embryos of mothers injected with *Nv-tra2* dsRNA and embryos of mothers injected with *Nv-tra* dsRNA. Different letters above the bars indicate significant differences between treatment groups (Tukey test: $P<0.05$).

Table 3.2. *Nv-tra2* dsRNA, *Nv-tra* dsRNA and water-injected females and their offspring scored by sex and ploidy. Numbers of mated females (P: females (RNAi)) and counts of their offspring (F1: haploid males, F1: diploid females and F1: diploid males) are shown. Mean amounts of haploid and diploid offspring per female are calculated based on these counts.

Construct	P: ♀(RNAi)	F1: haploid ♂	F1: diploid ♀	F1: diploid ♂	Mean # haploid offspring per female	Mean # diploid offspring per female
Nv-tra2	61	251	0	46	4.11	0.75
Nv-tra	56	212	0	450	3.79	8.04
water	55	210	1195	0	3.82	21.73

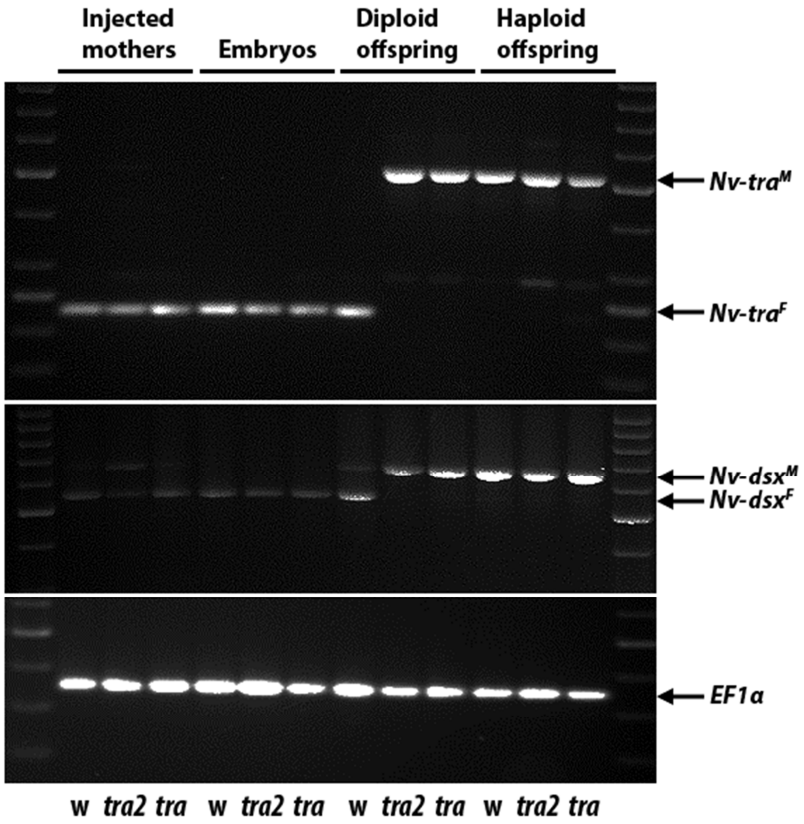

Figure 3.5. Sex-specific splicing patterns of *Nv-tra* and *Nv-dsx* in pRNAi mothers and offspring. Amplicons are produced by RT-PCRs on samples of mothers, embryos and diploid (2N) and haploid (N) adult offspring. The injected substance (water, *Nv-tra2* dsRNA or *Nv-tra* dsRNA) is depicted underneath each sample. Arrows mark female-specific splicing and male-specific splicing of *Nv-tra* and *Nv-dsx*. Control gene *EF1α* is displayed at the bottom.

Functionality of Nv-tra2*: viability and additional knockdown effects*

All diploids observed after *Nv-tra2* knockdown were males, but their numbers were very low. Additionally, a reduction in adult offspring numbers of both *Nv-tra* and *Nv-tra2* pRNAi females compared to those produced by water-injected mothers (Table 3.2) was noted. This reduction in offspring after pRNAi could result from a reduced oviposition or from lethality during development. To determine which was the case, the amount of oviposited embryos and adult offspring produced by mated and virgin females were scored. A large discrepancy between the number of oviposited eggs and emerging adults, in absence of larval or pupal remains, would indicate embryonic inviability, whereas lower numbers but equal for eggs and adults, would indicate reduced fertility.

In *Nv-tra2* pRNAi virgin females, a significant difference was detected between the number of oviposited eggs and the number of emerged offspring ($F_{(4,1014)}$=9.8, P<0.001, Tukey-test: P<0.001). The number of eggs deposited by *Nv-tra* pRNAi virgin females did not significantly differ from the number of emerged offspring (Tukey-test: P=0.74) (Figure 3.6a). Mated females injected with either *Nv-tra2* or *Nv-tra* dsRNA showed a discrepancy between number of oviposited eggs and adult emergence ($F_{(4,971)}$=34.9, P<0.001) (Figure 3.6b), indicating embryonic lethality in both categories. Overall, fewer embryos were found for both *Nv-tra* and *Nv-tra2* dsRNA-injected females ($H_{(3,507)}$=84.7, P<0.001) when compared to non-injected females, but this reduced fecundity was also present in the water-injected control category, suggesting an effect of handling and injection trauma more so than potential dsRNA side effects.

Since maternal *Nv-tra2* knockdown caused a dramatic mortality in the offspring of these wasps, the fertility of the surviving male offspring was tested. Haploid and diploid *Nv-tra* pRNAi males as well as haploid and diploid *Nv-tra2* pRNAi males had normal capabilities to mate and inseminate females compared to the control haploid males of water-injected mothers (Figure 3.7). A significant difference was only found between haploid and diploid *Nv-tra* pRNAi males ($F_{(4,94)}$=3.5916, P=0.009, Tukey: p=0.003). Neither of these categories differed in fertilization abilities from the haploid control males, suggesting that this may be a sample size (N=20 per category) or environmental effect.

DISCUSSION

Conservation of tra2 *in the* N. vitripennis *sex determination cascade*

We showed that an ortholog of *tra2* is present in the genome of *N. vitripennis,* but it is not sex-specifically spliced. This is also observed for *tra2* genes of other insects, although there are exceptions (Chapter 6). The *tra2* gene is an important part of the *Nasonia* sex-determining cascade, as prevention of *Nv-tra2* maternal provision by pRNAi leads to male-specific splicing of both *Nv-tra* and *Nv-dsx* pre-mRNA in diploid fertilized eggs and subsequent differentiation into functional males.

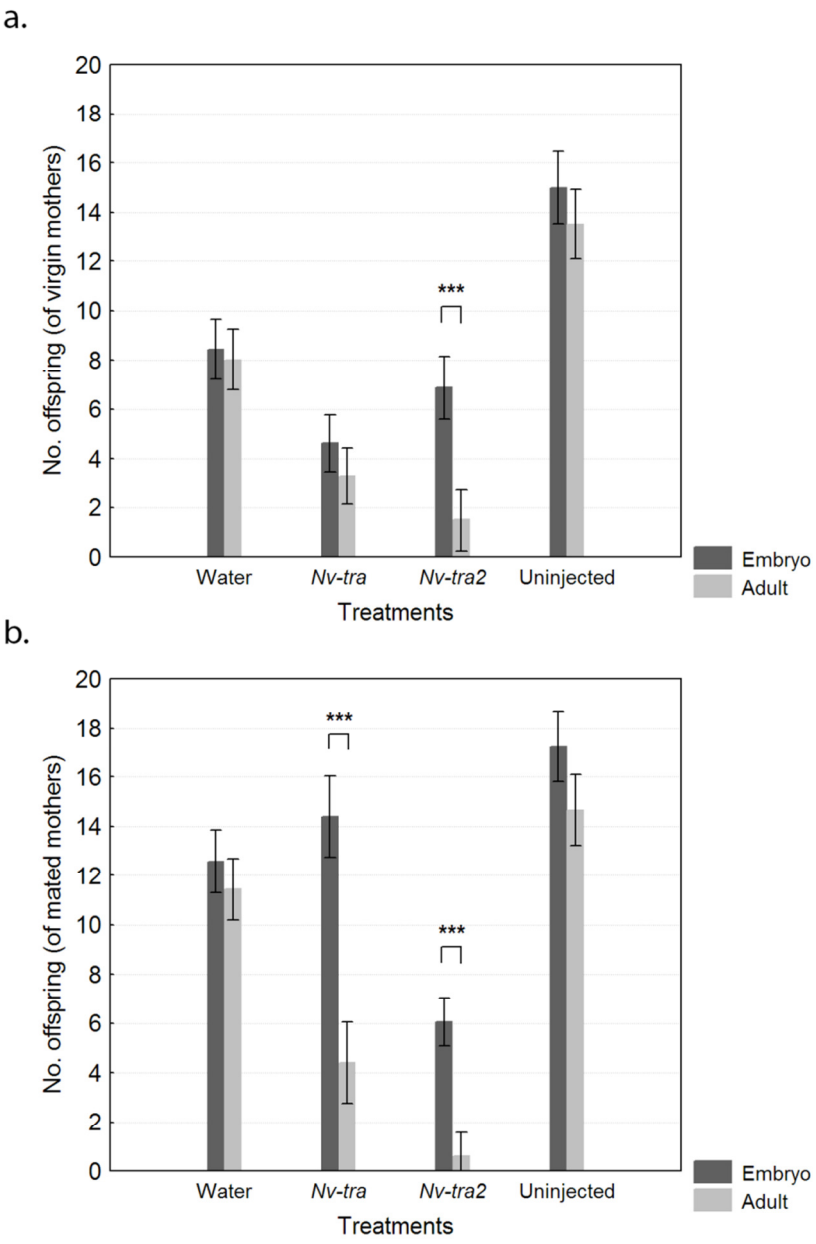

Figure 3.6. Offspring counts of (a) virgin and (b) mated females. Virgin females produced only haploid offspring and mated females produce a majority of diploid offspring when hosted individually. The number of eggs laid in a host is plotted in dark grey and the number of adult offspring which emerged from a host is plotted in light grey. The stars indicate the level of significance between the number of embryos and adults of the same treatment: *** P<0.001.

The change from female to male development of fertilized eggs upon parental knockdown of *Nv-tra2* expression indicates functional and positional conservation of *tra2* in the *Nasonia* sex determination cascade. Absence of maternal *Nv-tra2* mRNA provision fails to direct the sex determining pathway of diploid embryos towards female development, as only male-specific splice variants of *Nv-tra* and *Nv-dsx* are detected. Our results also suggest that NV-TRA2 is required for initiation of the female-specific *Nv-tra* autoregulatory loop. After all, if NV-TRA2 would only interact with *Nv-dsx*, maintenance of female-specific splicing of *Nv-tra* would be unaffected and only male-specific splicing of *Nv-ds*x would occur. Similar to *A. mellifera* (Nissen *et al.*, 2012), in *Nasonia* TRA2 acts on two levels in the sex determination cascade, regulating the splicing of both *tra* and *dsx*.

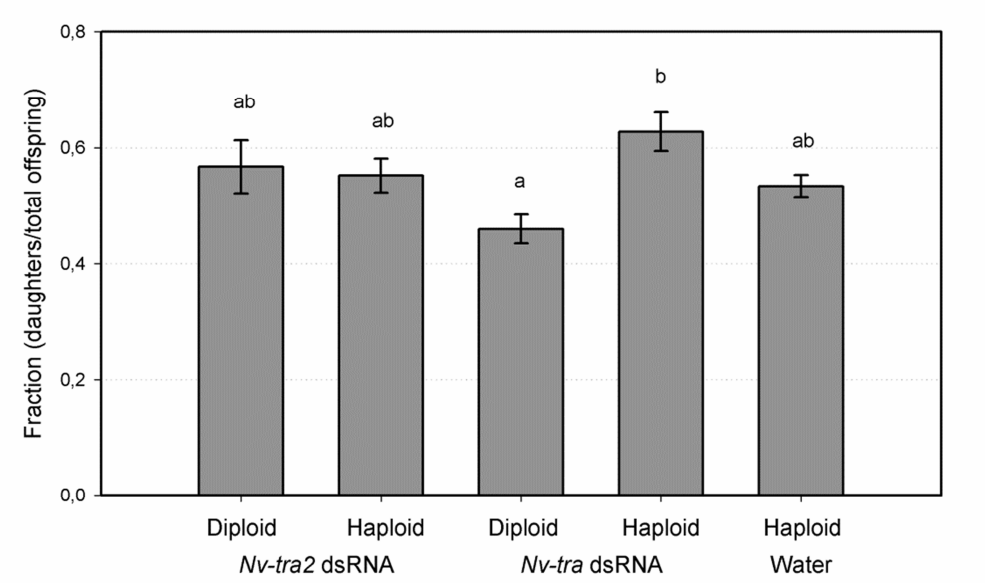

Figure 3.7. Fertility of knockdown male offspring**.** Mean fraction of daughters/total offspring per vial produced by haploid and diploid males of pRNAi *Nv-tra2*, *Nv-tra* and water-injected mothers.

Role of Nv-tra2 *in embryo viability*

Following pRNAi of *Nv-tra2,* a high lethality of both haploid and diploid offspring was observed, while *Nv-tra* pRNAi led to only high lethality in the diploid offspring. This suggests that (1) *Nv-tra2* likely has other functions in early development, as lowered maternal *Nv-tra2* provision leads to inviable haploid and diploid offspring and (2) the lack of *Nv-tra,* and possibly also *Nv-tra2*, maternal mRNA impairs the development of the diploid zygote (Figure 3.8). Unfortunately, we cannot distinguish between the causes of the observed inviable offspring and diploid lethality after *Nv-tra2* knockdown. Our first conclusion is corroborated by Nissen *et al.* (2012) who reported effects of *tra2* knockdown on embryogenesis in *A. mellifera* that were not sex-

specific and independent of *tra* regulation. This suggests a similar role of *Nv-tra2* in embryogenesis as in *A. mellifera*, but our experimental approach differs from Nissen *et al.* (2012). Our parental RNAi knockdown blocks maternal provision, whereas in *A. mellifera* dsRNA is injected directly in embryos to silence zygotic transcription. In dipterans and lepidopterans, RNAi studies have not shown any function for *tra2* in embryogenesis (Burghardt *et al.*, 2005; Salvemini *et al.*, 2009; Suzuki *et al.*, 2012). However, in *T. castaneum* (Shukla & Palli, 2013) RNAi with *tra2* dsRNA at the larval stage led to developmental arrest, suggesting that *tra2* could have acquired additional developmental functions in Hymenoptera and Coleoptera or has lost such functionality in Diptera and Lepidoptera.

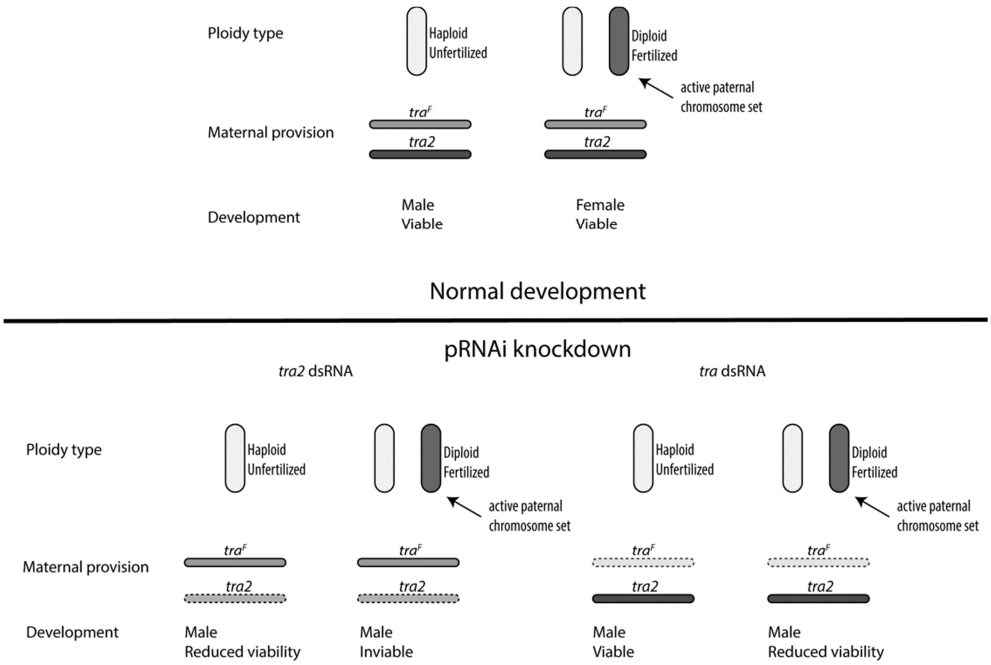

Figure 3.8. Overview of maternal provision and resulting offspring development in non-manipulated and pRNAi *N. vitripennis*. Depicted at the top is the normal embryonic development of *N. vitripennis;* at the bottom is the embryonic development following pRNAi with *Nv-tra* or *Nv-tra2* dsRNA. The ploidy of the embryo is represented by one or two chromosomes, with the maternal chromosome in light gray and the paternal chromosome in dark gray. Maternal provision is indicated by horizontal bars representing mRNA of *Nv-tra* (*tra*^{F}) and *Nv-tra2* (*tra2*). Knockdown of maternal provision by pRNAi is portrayed with a dotted outline, while the unaffected maternal provision is indicated with a solid outline. Underneath, the resulting sexual development is stated with the observed viability.

Our second conclusion may be caused by an additional mechanism. The decreased survival of diploids following *Nv-tra* pRNAi can also be seen in the data from Verhulst *et al.* (2010a) where mated mothers produced 44% haploid offspring. This deviates from a normal progeny sex ratio of a single foundress that typically contains about 15% haploid (male) offspring, as observed in the water injected females of this study. *Nv-tra* or *Nv-tra2* pRNA knockdown probably results in the inadequate formation of a TRA/TRA2 complex and/or autoregulatory loop of *Nv-tra. Nv-tra* pRNAi knockdown does not seem to impact haploid offspring, who under normal developmental conditions would not produce functional TRA protein, and therefore the absence of the TRA/TRA2 complex will only be detrimental to diploid offspring. Viable diploid males are observed in a polypoid mutant *N. vitripennis* strain (Whiting, 1960), however, these individuals emerge from unfertilized diploid eggs, and thus do not carry a paternal genome set (Beukeboom *et al.*, 2007b). In this study, diploid offspring in both pRNAi classes have a notable difference compared to their haploid brothers, as they obtain a paternal chromosome set upon fertilization. One possible explanation for the higher mortality of diploid embryos resulting from *Nv-tra* or *Nv-tra2* pRNAi is therefore that activated genes from the paternal genome are incompatible with male development and this would lead to an early developmental arrest.

Surviving diploid males do not differ from haploids in splicing and fertility

Despite the reduced viability of diploid *Nv-tra* pRNAi males and an almost complete inviability of diploid *Nv-tra2* pRNAi males, the surviving males do not appear to suffer subsequent effects. Their sex determination cascade is firmly fixed in the male mode with male-specific splicing of both *Nv-tra* and *Nv-dsx*. Furthermore, their ability to mate with females and fertilize their eggs does not appear to be impacted. This suggests that only very early processes, possibly at the start of zygotic transcription, result in the observed inviability. If the male developmental pathway is firmly established, these males appear not to encounter any further costs of their diploid status.

Activation of tra *under CSD and MEGISD*

The requirement of maternally provided *Nv-tra* and *Nv-tra2* for survival of diploid embryos highlights the importance of maternal effects in the *N. vitripennis* MEGISD sex determination (Verhulst *et al.*, 2010a). Maternal provision of *Nv-tra* alone is not sufficient to start the female-specific cascade; *Nv-tra2* mRNA is required as well in the early embryo to ensure both female development and proper embryonic development. In contrast, in the honeybee, RNAi knockdown of *fem* does not lead to noticeable inviability (Beye *et al.*, 2003; Hasselmann *et al.*, 2008a; Gempe *et al.*, 2009). Diploid males resulting from homozygous *csd* alleles can develop in *A. mellifera*, but are killed by workers before reaching adulthood (Woyke, 1963). Apparently, silencing this transducing stage of the sex determination cascade has different consequences for the CSD mechanism of *A. mellifera* than for the MEGISD mechanism of *N. vitripennis*. Fem^F in *A.*

mellifera is not maternally provided contrary to the tra^F mRNA provision in *N. vitripennis* (Gempe *et al.*, 2009; Verhulst *et al.*, 2010a). The female-specific cascade in *A. mellifera* is activated in the presence of two different *csd* alleles. *N. vitripennis* sex determination relies on a silencing mechanism in the mother/activating signal in the father and its differently imprinted chromosome sets may result in additional detrimental effects when present in an embryo developing into the opposite sex.

Acknowledgements

We thank Charlotte Capt for performing a pilot *Nv-tra2* pRNAi study and the entire Evolutionary Genetics group for their input during a paper discussion meeting.