Controlling sex - evolution of sex determination, sexual differentiation and endosymbiont-induced asexual reproduction Yidong Wang 2021

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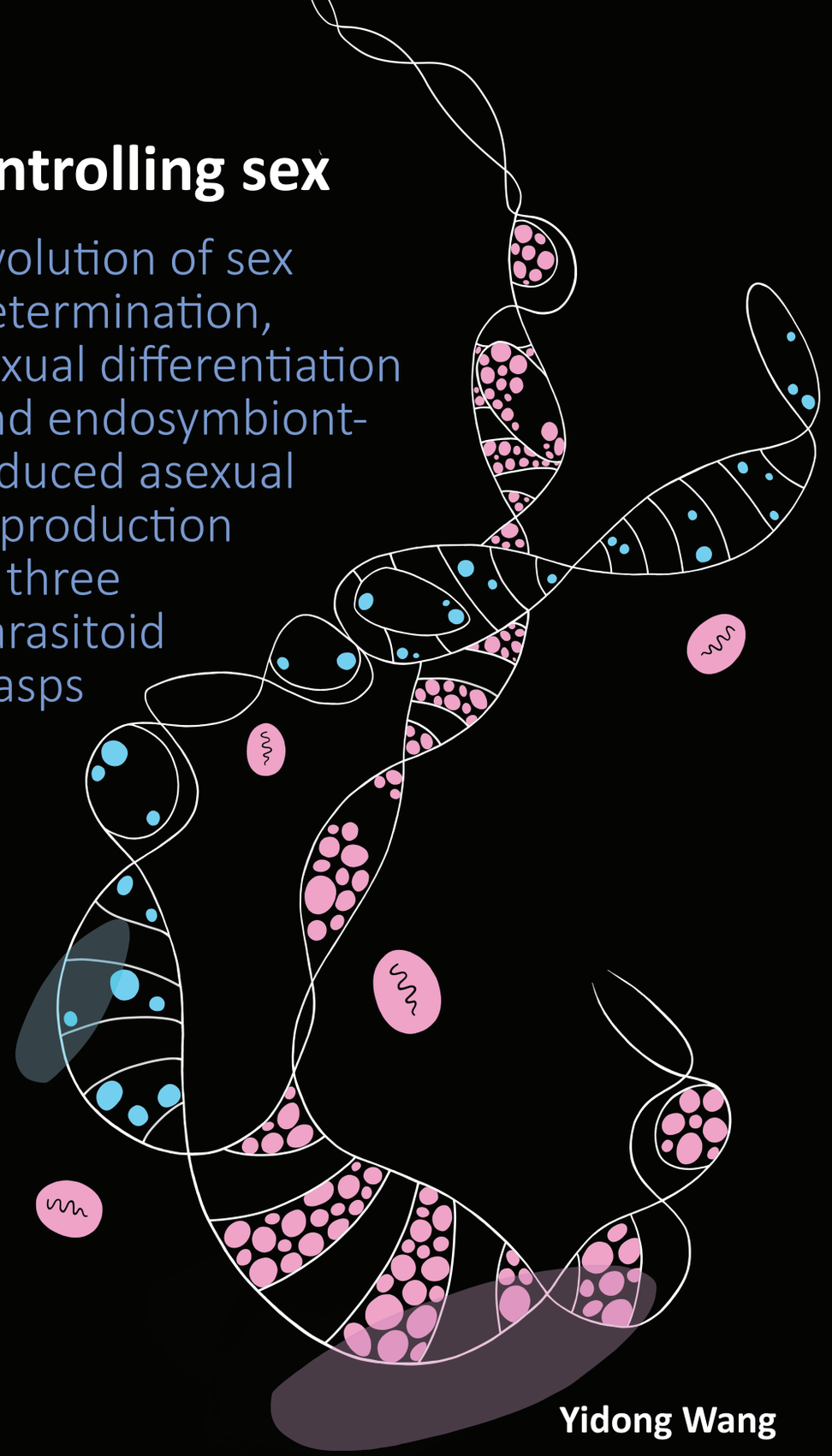
- evolution of sex determination, sexual differentiation and endosymbiont-induced asexual reproduction in three parasitoid wasps



Yidong Wang

Propositions

1. Bottom-to-top conservation of the sex-determination cascade does not apply when *Wolbachia* induces thelytoky.
(this thesis)

2. *Doublesex* is required at different developmental stages to establish sexual identity of *Nasonia vitripennis* males.
(this thesis)

3. The absence of ethical regulation leads to overkilling in insect studies.

4. Statistical significance is useless to determine the biological relevance of gene expression.

5. Protecting endangered species is a human intervention against natural selection.

6. A good cook knows how to flavour food but not how to give the food flavour.

Propositions belonging to the thesis, entitled

"Controlling sex - evolution of sex determination, sexual differentiation and endosymbiont-induced asexual reproduction in three parasitoid wasps"

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Controlling sex
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Thesis

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Chapter 1

General introduction

Sex — a universal feature of eukaryotic life

The origin of sex has always been a bewildering question in evolutionary biology. One of the biological definitions of sex is the presence of meiosis, which includes the pairing of homologous chromosomes originating from different parents, the recombination between the chromosomes, and the transfer to progeny (Bernstein and Bernstein, 2013). Bacterial transformation, with the integration of exogenous DNA into their genome, shares a striking similarity with eukaryote meiosis (Bernstein and Bernstein, 2013; Lin et al., 2006), and therefore is considered to be the precursor of meiosis. Bearing the extra energy cost and lower efficiency (Otto, 2009), sexual reproduction can hardly outcompete asexual reproduction; however, its widespread occurrence in different lineages of eukaryotes gives a strong indication that there must be sufficient evolutionary benefit to maintain it. Colnaghi et al. (2020) suggest that the dramatic eukaryote genome-size expansion was likely the trigger for early eukaryotes to evolve a more systematic system to maintain recombination, and is thus likely the starting point of the evolution of meiotic sexual reproduction.

Diversity in reproduction systems

While genome expansion and recombination are the driving force for sexual reproduction, sex determination and differentiation are required for sexual reproduction. Sex determination is a crucial developmental event which determines whether an embryo develops into a male or female. Although developing into a male or a female is a basal developmental process among eukaryotes, sex determination systems are highly diverse among different taxa and even among closely related species (Bachtrog et al., 2014).

Environmental (ESD) and genotypic sex determination (GSD) are two major mechanisms that control offspring sex differentiation, and can sometimes simultaneously occur in the same species (Radder et al., 2008; Yamamoto et al., 2014). Under ESD, sex is determined during embryonic development by non-genetic cues such as temperature. Since the first ESD discovery in reptiles, many more cases have been reported in nematodes, crustaceans, insects and vertebrates including fish, turtle, crocodiles and lizards (Janzen and Krenz, 2004; Korpelainen, 1990). However, very few studies demonstrate the underlying molecular mechanisms by which the external, physical signal is transduced to biological signals to initiated sexual differentiation (Deveson et al., 2017; Ge et al., 2017). In contrast, the GSD mechanisms are more thoroughly studied in various species (Bachtrog *et al*., 2014).

The GSD mechanism is mostly based on diploid sex chromosomes to produce either homogametic females (XX) and heterogametic males (XY), or heterogametic females (ZW) and homogametic males (ZZ) in all mammals and birds, a majority of beetles and flies, many reptiles, some fish, butterflies and moths (reviewed by Bachtrog et al., 2014; The Tree of Sex Consortium, 2014). In many cases, sex can be distinguished by XX/XO or ZZ/ZO as a variation of the XX/XY or ZZ/ZW system which likely arose as a consequence of Y- or W-chromosomal loss (Blackmon and Demuth, 2015).

Haplodiploidy, although not involving sex chromosomes, also belongs to GSD. This system is present in approximately 15% of all arthropods (De La Filia et al., 2015), and has at its basis a simple sex-determination mechanism: a female is diploid and a male is haploid (Heimpel and de Boer, 2008). Haplodiploidy can be further classified into the subgroups arrhenotoky, paternal genome elimination (PGE) and thelytoky. Arrhenotokous parthenogenesis describes a reproductive system where females emerge from fertilized, diploid eggs, while males parthenogenetically emerge from unfertilized, haploid eggs. Arrhenotoky has been predominantly identified in the Hymenoptera as a prevalent and ancestral mode of sex-determination (Heimpel and de Boer, 2008), but has also been found in scale insects, beetles, mites, thrips, and nematodes (Hansell et al., 1964; Luk and Spiridonov, 1990; Mcculloch and Owen, 2012; Nault et al., 2006; Normark et al., 1999; Ross et al., 2010). PGE refers to a system where both male and female emerge from fertilized eggs, but in males the paternal genome is destroyed during early development, rendering them effectively haploid (Gardner and Ross, 2014; Nur et al., 1988). Thelytokous parthenogenesis is a less common reproduction system in, but not limited to, the Hymenoptera. This system consists of diploid female offspring that parthenogenetically arise from unfertilized eggs produced by females. Genetically based thelytoky includes two ways to restore diploidy in unfertilized eggs: apomictic thelytoky and automictic thelytoky. Both have different consequences for the heterozygosity of the descendant (Leach et al., 2009; Suomalainen et al., 1987). Apomixis is based on mitotic offspring production, as there is complete absence of meiosis. It prevents any chromosomal rearrangements which leads to the conservation of the complete genetic make-up of the mother resulting in full clones (van Wilgenburg et al., 2006). Automixis on the other hand, includes the meiotic process in which either the genome is duplicated before meiosis (Asher, 1970), or fusion of division sister nuclei (terminal fusion) or non-sister nuclei (central fusion) during second meiotic division taking place (Gottlieb et al., 2002). In some of these cases, complete homozygosity is the result, but in other cases, crossing-over events are still possible allowing some recombination to take place which prevents offspring from

becoming fully homozygous (van Wilgenburg et al., 2006). Apomixis has been observed in several orders of insects, but only a few cases have been found in the Hymenoptera (Alavi et al., 2018; Schwander and Crespi, 2009), whereas automixis seems to be more prevalent in the Hymenoptera (Suomalainen et al., 1987; van Wilgenburg et al., 2006). Besides genetically based parthenogenesis, thelytoky can also be induced by endosymbiotic bacteria. Currently, *Wolbachia*, *Cardinium* and *Rickettsia* are the only bacterial genera that are known to induce thelytokous parthenogenesis (PI) in their hosts, which constitutes at least 15–31% of known species reproducing through parthenogenesis (reviewed by Ma and Schwander, 2017).

Wolbachia – a master of sex manipulation

Wolbachia pipientis (Order: Rickettsia), hereafter *Wolbachia*, was first reported in the mosquito *Culex pipiens* (Hertig and Wolbach, 1924). It is an obligate intracellular bacterium that is mostly found in the reproductive tissues of a wide range of hosts (Hilgenboecker et al., 2008; Werren, 1997). *Wolbachia* thrives in a variety of hosts and quickly establishes its population mainly through vertical transmission from female to offspring via the egg cytoplasm. Of all the confirmed cases of endosymbiont-induced parthenogenesis, more than half are caused by *Wolbachia* (reviewed by Ma and Schwander, 2017). The overrepresentation of *Wolbachia* and its host sex manipulation ability render it one of the most fascinating bacteria to study. The research directions include, for instance, its phylogenetic distribution, mechanisms of host sex manipulation, evolution and speciation in the hosts, and the application of biocontrol programs (Werren, 1997).

Currently, there are several distinguishable supergroups of *Wolbachia* (Gerth et al., 2014). Among these supergroups, the two major supergroups A and B that share a single origin contain a large range of taxonomically diverged hosts of arthropods (Gerth et al., 2014). Supergroup C and D are commonly found in nematodes. *Wolbachia* is well known for its ability to alter host reproduction (Werren et al., 2008). Host cytoplasmic incompatibility (CI) is the most frequent *Wolbachia*-induced phenotype. If a certain strain of CI-*Wolbachia* infects males, it will cause modification of the sperm. This modified sperm can only be rescued by the same *Wolbachia* strain in the egg (Werren, 1997). A cytological study showed that the presence of CI-*Wolbachia* will delay the male nuclear envelope breakdown in the embryo, causing asynchrony between the male and female pronuclei, eventually leading to the loss of paternal chromosomes at the first mitosis (Tram and Sullivan, 2002). In the diploid system, embryonic development will be disrupted. In haplodiploids, embryonic lethality only

occurs if the paternal chromosomes segregate improperly into the daughter nuclei (Tram et al., 2006), while the maternal chromosome set may continue normal development if only one daughter nucleus is affected, resulting in haploid males (Lassy and Karr, 1996; Tram and Sullivan, 2002). This toxin-antidote (TA) model of CI was found to be under the control of prophage WO genes *cifA* and *cifB*. These genes additively recapitulate host CI, and can be rescued by the same *cifA* in the embryo (Dylan Shropshire et al., 2018; Le Page et al., 2017).

Male killing (MK) induced by *Wolbachia* is another reproductive manipulation mechanism that has been described in several orders of arthropods and occurs mainly during embryo development (Fialho and Stevens, 2000; Hurst et al., 2000; Jiggins et al., 2001). This male-specific lethality during early embryogenesis in fruit flies is induced by the expression of a eukaryotic association module *WO-mediated killing* (*wmk*) by *Wolbachia* (Perlmutter et al., 2019). Transgenic expression of *wmk* in *Drosophila* causes male-specific cytological defects in embryos that are similar in MK, and is correlated with the accumulation of DNA damage in association with dosage compensation (Perlmutter et al., 2019), which was observed in MK-*Wolbachia* infected embryos (Harumoto et al., 2018). In *Ostrinia furnacalis*, *Wolbachia* induces MK by specifically targeting the homolog *Masculinizer* (*Masc*) gene (Fukui et al., 2015), which was first identified in the silkworm, *Bombyx mori,* as the initial signal for male development (Kiuchi et al., 2014). *Wolbachia* reduces the expression of *Masc*, which induces feminization but also causes dosage compensation failure, thereby killing male embryos (Fukui et al., 2015). This shows that *Wolbachia* can manipulate male-determination signals to destroy the dosage compensation system in its host leading to the MK phenotype.

Another *Wolbachia* reproductive manipulation mechanism is feminization which turns genetic males into females. *Wolbachia*-induced feminization is relatively scarce and is only found in a few species within the Hemiptera, Lepidoptera and Isopoda (review by Kageyama et al., 2012). Although the exact mechanism for inducing feminization has not yet been identified, it has been suggested that the presence of *Wolbachia* is essential during both embryo and larval development to accomplish complete feminization of the butterfly *Eurema hecabe* (Narita et al., 2007). Moreover, removing *Wolbachia* from *Eurema mandarina* larvae resumed the male-specific expression of the sex-determination gene *Doublesex* (*Dsx*) that is located at the bottom of the sex determination cascade and regulates the development of different sexual traits (Kageyama et al., 2017). Male differentiation in the early developmental stage of woodlouse *Armadillidium vulgare* requires high level of insulin-like androgenic gland hormone (IAG) expression, and recent research shows that

feminization is realized by *Wolbachia* through the suppression of IAG expression (Herran et al., 2020). These results indicate that different from CI and MK, feminization depends on the continuous action of *Wolbachia* either through direct regulation of the sex-determination genes or the hormones that promote sexual differentiation.

Compared to other sex-distortion manipulations, *Wolbachia*-induced PI is thus far found solely in haplodiploid species suggesting that the type of *Wolbachia* manipulation has a unique association with certain types of reproductive systems (Ma and Schwander, 2017). Under haplodiploidy, unfertilized eggs will develop into haploid males. Therefore, *Wolbachia* has to diploidise the haploid egg and start the host female-determination pathway to create thelytokous females. The cellular mechanism underlying parthenogenesis is through gamete duplication in all verified cases of *Wolbachia* triggered PI (Leach et al., 2009; Ma and Schwander, 2017). This is where after completion of meiosis, two sets of chromosomes fuse back together during or after the first mitosis, and the egg ends up with a diploid nucleus (Gottlieb et al., 2002; Pannebakker et al., 2004; Stouthamer and Kazmer, 1994). Although this cytological distortion has been observed and described a decade ago in several wasp species, few studies have elucidated the mechanisms and the requirements for *Wolbachia* to achieve thelytoky. Genome comparison between CI and PI *Wolbachia* yields little evidence for specific genes that could be involved in either of the two types of manipulations (Kampfraath et al., 2019; Newton et al., 2016). In addition, *Wolbachia* seems to induce diploidization and feminization separately in the wasps *Asobara japonica* and *Trichogramma kaykai* (Ma et al., 2015; Tulgetske, 2010). Clearly, this result points to the fact that, except for the cytological alteration (diploidization), PI-*Wolbachia* requires a separate regulation to induce sex change, which very likely acts directly on host sex determination system as has been shown in other systems with a feminizing or male-killing *Wolbachia* (Beukeboom, 2012). It is becoming clear that in many cases *Wolbachia* requires the host sex-determination system to manipulate host reproduction, and therefore it is imperative to understand its elements and processes.

Conserved genes in the sex-determination pathway

Despite the extreme diversity of sex-determination systems in insects, a remarkably well conserved sex-determination pathway was identified in many holometabolous insects (Bopp et al., 2014; Gempe and Beye, 2011; Geuverink and Beukeboom, 2014; Verhulst and van de Zande, 2015; Verhulst et al., 2010a). The core of the pathway includes several conserved

genes and is presumed to evolve from the bottom upwards by recruiting new elements to the top of the cascade (Wilkins, 1995). This is reflected by the fact that the gene at the bottom of the cascade, *Doublesex*, is present in all insects and possibly all arthropods, while the upstream genes are present in only a subset of the insect orders.

Transformer (or *feminizer*, *tra/fem*, *fem* is a *tra* homolog identified in honey bee *Apis mellifera* (Hasselmann et al., 2008). *Tra* encodes a splicing factor that is itself sex-specifically spliced to expresses male- or female-specific splice variants. This gene has been identified in several major insect orders including the Hymenoptera, Diptera and Coleoptera, but no *tra* homologues have yet been found in the Lepidoptera (Geuverink and Beukeboom, 2014; Mita et al., 2004), and *tra* seems absent from the basal dipteran mosquito lineage (Geuverink and Beukeboom, 2014). In female insects, the female-specific *tra* splice-variant encodes a full-length female-specific Tra protein (Tra^F), whereas in males *tra* yields a truncated male-specific Tra protein (Tra^M) that is assumed to have no function (Verhulst et al., 2010a). In all insect species with *tra* identified, except for the genus *Drosophila,* the female-specific

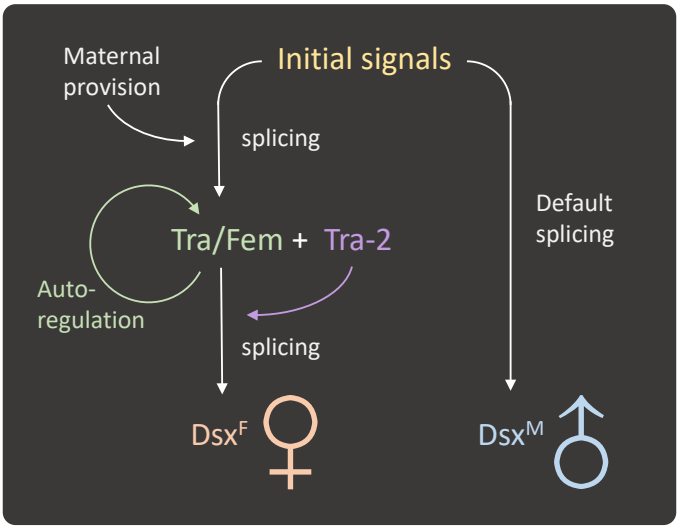


Figure 1.1 Common elements of the conserved sex determination cascade in insects. A huge variety of initial signals initiate the sex determination cascade. In many species, the initial signal initializes downstream female-specific *transformer/feminizer* (*tra/fem*) splicing in combination with maternal provision of *tra* and *transformer-2* (*tra-2*) or by initiating the female splicing of *fem*. The resulting Tra/Fem protein further maintains its own splicing in an auto-regulatory loop in females in combination with Tra-2. Tra/Fem and Tra-2 also splice the downstream sex differentiation gene *Doublesex* (*Dsx*) into the female-specific form (Dsx^F), which encodes Dsx^F to promote female development. In other species, the initial signal breaks the auto-regulatory loop and no functional Tra is produced leading to a default *Dsx* splicing and the production of male-specific Dsx (Dsx^M) and male development.

Tra/Fem protein is required for female-specific splicing of its own transcript forming an positive auto-regulatory loop and a cellular memory of the sex (Figure 1.1, Concha and Scott, 2009; Gempe et al., 2009; Geuverink et al., 2018b; Hediger et al., 2010; Lagos et al., 2007; Pane et al., 2002; Verhulst et al., 2010b). A *Ceratitis capitata, Apis mellifera, Musca domestica* (CAM, Hediger et al., 2010) domain is present in Tra^F that has been shown to be required for the auto-regulation of Tra/Fem female-specific splicing (Tanaka et al., 2018). This domain is absent in *Drosophila* Tra (Verhulst et al., 2010a) and the cellular memory is formed by the auto-regulation of the upstream regulator *Sex-lethal* (*Sxl*) that is part of the sex-determination cascade in *Drosophila* only (Bell et al., 1991). In addition to the CAM domain, Tra features an order-specific domain in the Hymenoptera (HYM) and Diptera (DIP), and commonly contains an arginine/serine-rich (RS) and a proline-rich region near the C-terminus (Geuverink and Beukeboom, 2014; Verhulst et al., 2010a). Embryonic silencing of *tra* expression in *C. capitata*, *M. domestica* and *A. mellifera* results in female to male sex reversal, proving its role in maintaining the female differentiation pathway (Hasselmann et al., 2008; Hediger et al., 2010; Pane et al., 2002). Parental RNA interference (pRNAi) of *tra* in the beetle *Tribolium castaneum* and the wasp *Nasonia vitripennis* leads to a female to male sex ratio shift of the offspring, further verifying its crucial function in some species for initiating the zygotic tra^F expression for offspring female development (Shukla and Palli, 2012a; Verhulst et al., 2010b).

Another splicing factor located at the same instruction level of *tra* is *transformer-2* (*tra-2,* Figure 1.1). It is non-sex-specifically expressed throughout all developmental stages and varies in the number of splicing variants (Burghardt et al., 2005; Geuverink et al., 2017; Geuverink et al., 2018b; Martín et al., 2011; Niu et al., 2005). These variants encode full-length proteins including an RNA-binding domain (RBD) flanked by two RS regions (Amrein et al., 1988). In some species it has been shown that Tra-2 is required for successful embryonic and larval development (Burghardt et al., 2005; Geuverink et al., 2017; Nissen et al., 2012). Because Tra^F contains no DNA binding domain, Tra-2 is required to facilitate Tra^F in the female-specific splicing of its own splice-variant and that of *Doublesex* (Geuverink et al., 2017; Geuverink et al., 2018b; Inoue et al., 1992; Nissen et al., 2012; Salvemini et al., 2009).

At the bottom of the sex-determination cascade is *Doublesex* (*Dsx*), which is conserved in all the investigated insect species across the insect orders (Baker and Wolfner, 1988; Cho et al., 2007; Concha et al., 2010; Hediger et al., 2004; Kijimoto et al., 2012; Ledón-Rettig et al., 2017; Mine et al., 2017; Miyakawa et al., 2018; Nguantad et al., 2020; Ohbayashi et al., 2001; Oliveira

et al., 2009; Permpoon et al., 2011; Ruiz et al., 2015; Saccone et al., 1996; Salvemini et al., 2011; Scali et al., 2005; Shukla and Nagaraju, 2010a; Shukla and Palli, 2012b; Sugimoto et al., 2010; Ugajin et al., 2016; Wexler et al., 2019; Zhuo et al., 2018). *Dsx* belongs to a structurally and functionally conserved *Dmrt* gene family (Erdman and Burtis, 1993; Wexler et al., 2014). Through alternative splicing, *Dsx* is subsequently translated into sex-specific Dsx proteins (Dsx^M and Dsx^F), leading to male and female differentiation (Gotoh et al., 2014; Gotoh et al., 2016; Ito et al., 2013; Ledón-Rettig et al., 2017; Mine et al., 2017; Prakash and Monteiro, 2020; Suzuki et al., 2005; Xu et al., 2017). Dsx and its orthologs share a zinc finger module (DM) containing a DNA-binding domain (DBD) and an oligomerization domain (OD1, Erdman and Burtis, 1993). Another oligomerization domain (OD2) located near the C-terminus of Dsx consists of a common non-sex-specific and a sex-specific region (Geuverink and Beukeboom, 2014; Price et al., 2015; Verhulst and van de Zande, 2015). The major differences between the sex-specific OD2 and non-sex-specific OD2 is speculated to account for the differentially regulated sexual dimorphic traits between sexes (Baral et al., 2019).

Dsx has mainly been studied in *Drosophila* species regarding its spatial and temporal expression, and its role in regulating different sexually dimorphic-trait-development in and among different *Drosophila* species (Rice et al., 2018; Tanaka et al., 2011; Williams et al., 2008). The role of Dsx in regulating the formation of sexually dimorphic horn and mandible structures in beetles (Gotoh et al., 2014; Ito et al., 2013; Kijimoto et al., 2012; Ledón-Rettig et al., 2017; Morita et al., 2018), the divergent sex-specific pheromone organs in butterflies (Prakash and Monteiro, 2020), or the sexual dimorphism of abdominal segments in silkworms (Xu et al., 2017) are also well characterized. Nevertheless, few studies have focused on the Dsx function in sexually dimorphic trait development in the Hymenoptera. Given the great diversity of sexual dimorphism in bees and wasps, this lack of knowledge hampers not only the understanding of Dsx within the Hymenoptera order, but also the overall evolution of Dsx regulation in insects in general.

Molecularly elucidated initial signals in insect species

X-chromosome dose – Compared to the downstream conserved genes in the sex determination cascade, the initial signals are highly variable among insect species. In *D. melanogaster*, which has a XY sex chromosome system, a double dose of instructive X chromosome signals activates the expression of *Sxl* in the embryo (Erickson and Quintero, 2007). Then, Sxl establishes a positive feedback loop to maintain its own production (Bell

et al., 1991) and controls the female-specific splicing of *tra*. Female development further proceeds by the action of Tra^F and Tra-2 to splice *Dsx* pre-mRNA into the female-specific *Dsx* variant, which encodes a Dsx^F protein to control downstream sexual differentiation target gene expression. In male development, X linked elements from only one X-chromosome are not sufficient to induce *Sxl* expression which results in default male development. However, *Sxl* orthologs characterized in other dipteran species, for instance *C. capitata* and *M. domestica*, show no sex-specific expression patterns and failed to take over the *Sxl* function in *D. melanogaster* (Meise et al., 1998; Saccone et al., 1998), suggesting *Sxl* is unique to the *Drosophila* sex-determination cascade.

M-factors – In *C. capitata,* the masculinizing factor (M-factor) is located on the long arm of the Y chromosome, (Willhoeft and Franz, 1996), and has recently been identified as *Maleness-on-the-Y* (*MoY*, Meccariello et al., 2019). It functions as a repressor to switch off the female functional *tra* splicing during early embryo development in order to promote male development (Meccariello et al., 2019; Pane et al., 2002). Another M-factor that has recently been identified in *M. domestica* is called *Musca domestica male determiner* (*Mdmd*, Sharma et al., 2017). *Mdmd* arose from a duplication of the spliceosomal factor gene CWC22 (*nucampholin*), and promotes male development by preventing the otherwise female-specific expression of *tra* and *Dsx* (Sharma et al., 2017). In mosquitos, which lack the conserved *tra*, male sex is also determined by Y-chromosome-linked dominant M-factors. In *Aedes aegypti* this factor is *Nix*, a distant homolog of *tra2* and it was shown that *Nix* null-mutants develop into feminized genetic males with female *Dsx* splicing (Hall et al., 2015). In *Anopheles gambiae* this M-factor is *Yob*, a 56 amino acid (aa) short protein possibly containing a helix-loop-helix motif, which causes lethality in genetic female embryos when ectopically expressed (Krzywinska et al., 2016). In both cases the exact mechanism by which *Nix* and *Yob* initiate the sex determination cascade leading to male-specific splicing of *Dsx* is unknown. Recently a new component was identified that controls female development in *A. gambiae* termed *femaleless* (*fle*) (Krzywinska et al., 2021). *Fle* shares a similar RNA recognition motif (RRM) with insect Tra-2 orthologs, but differs substantially from Tra-2 in protein length, lack of RS domains and the presence of two additional putative RRM regions (Krzywinska et al., 2021). Fle controls the female-specific splicing of *Dsx*, regulates the dosage compensation in females, and transient knockdown results in female embryo death (Krzywinska et al., 2021). Taken together, the major function of M-factor in Diptera is to supress the female sex determination gene function in order to allow male development.

piRNA – In the Lepidoptera WZ sex chromosome system, one of the W-chromosome-derived PIWI-interacting RNAs (piRNAs), called *Feminizer* (*Fem*), acts as the feminizing factor of the silk moth *Bombyx mori* (Kiuchi et al., 2014). PIWI proteins are involved in regulating differentiation of germ cells and stem cells and *Fem* piRNA induces feminization by disrupting the expression of *Masculinizer* (*Masc*). *Masc* is required for male development by directly regulating the expression of *insulin-like growth factor II mRNA-binding protein* (*Imp^{M}*, Sakai et al., 2015). Imp^{M} resembles Tra^{F} regulation in an opposite (sex-reversed) fashion: insufficient amount of Imp^{M} results in default female-specific *BmDsx^{F}* expression while unhampered expression of Imp^{M} forms an auto-regulatory loop to splice *BmDsx^{M}* and promote male development (Suzuki et al., 2014). This *Fem* piRNA regulation is seemingly conserved in Lepidoptera based on the fact that several *Masc* homologs have been identified in other Lepidoptera species (Lee et al., 2015; Visser et al., 2021).

These mentioned primary sex determination signals are present on or activated by sex chromosomes. As haplodiploid systems lack sex chromosomes, other mechanisms need to be in place to determine the sex. To date two sex-determination mechanisms have been molecularly uncovered and are now functionally studied: complementary sex determination (CSD) in *A. mellifera* and maternal effect genomic imprinting sex determination (MEGISD) in *N. vitripennis*.

Complementary Sex Determination (CSD) – Under CSD, allelic composition at a single locus (sl-CSD) or multiple loci (ml-CSD) determines an individual's sex. When an individual is heterozygous (diploid) in at least one of the loci it will develop as a female, but when an individual is hemizygous (haploid) or homozygous (diploid) in all the loci it will develop into a male (Whiting, 1939; Whiting, 1943). The CSD mechanism is easy to demonstrate in a given species: inbreeding results within a few (sl-CSD) or after a larger number of generations (ml-CSD) in complete male offspring of which a subset is diploid. In this way it has been shown that CSD is widely distributed in major hymenopteran subgroups (reviewed in van Wilgenburg et al., 2006). However, the exact locus remained elusive for a long time until the *csd* locus was molecularly characterized in *A. mellifera* (Beye et al., 2003). *Csd* displays structural similarity with *tra* and is derived from its downstream target *fem* (Gempe et al., 2009) by gene duplication (Hasselmann et al., 2008), and contains a hypervariable region that accounts for its CSD function (Beye et al., 2003). The sl-CSD system is inherently disadvantageous in mass rearing and low genetic variation in the population because mother/son mating, easily results in homozygous diploid males that are in most cases infertile or are viable but sire

few daughters (Cook, 1993; Harpur et al., 2013). Therefore, especially social insects with an sl-CSD system evolved several traits to escape from sib mating, such as different eclosion time between sex, or actively removing diploid males at early stage (van Wilgenburg et al., 2006). Most species that commonly inbreed appear not to have CSD (Wu et al., 2003), illustrating an evolutionary avoidance. MEGISD on the other hand, provides an alternative for sex determination in haplodiploid insects.

Maternal Effect Genomic Imprinting Sex Determination (MEGISD) – The MEGISD system has so far only been molecularly demonstrated in *Nasonia* (Beukeboom and van de Zande, 2010; Beukeboom et al., 2007; Dobson and Tanouye, 1998; van de Zande and Verhulst, 2014). The MEGISD system relies on a *wasp overruler of masculinization* (*wom*) which contains a P53-like domain coding region and arose from gene duplication (Zou et al., 2020). Haploid, unfertilized eggs inherit a silenced *wom* copy from the mother. This maternally imprinted *wom* is not expressed during early embryogenesis and, therefore, cannot initiate zygotic *tra* expression which is necessary for the *tra* autoregulatory loop, leading to default male development (Zou et al., 2020). Diploid, fertilized eggs receive the paternal chromosome set that contains the active paternal *wom* allele. In this case, *wom* is activated in embryos and initiates zygotic *tra* expression. Maternal provision of tra^F and *tra2* together with *wom* activates the expression of zygotic tra^F which maintains its own auto-regulatory loop. Sufficient tra^F in zygotic eggs directs the female splicing of *Dsx* that further encodes Dsx^F protein to ensure female development (Verhulst *et* al., 2010; Verhulst *et al*., 2013).

Genera of *Nasonia* and *Muscidifurax*

Nasonia vitripennis (Hymenoptera: Pteromalidae) is a cosmopolitan ectoparasitic wasp that parasitizes a variety of dipteran pupae (Whiting, 1967). They are minute in size (2–3 mm) and show clear sexual dimorphism. The forewings in females are proportionally larger and both antennae and femur of rear legs have a dark pigmentation. Males on the other hand, possess smaller forewings with unpigmented antennae and rear leg femurs (Darling and Werren, 1990). The *Nasonia* genus contains four species that diverged in a short period of time, and they all display clear differences in their forewing size, mating preference and pheromone profiles (Buellesbach et al., 2013; Leonard and Boake, 2006; Loehlin et al., 2010b; Lynch, 2015; Raychoudhury et al., 2010). *Nasonia vitripennis* is the parasitoid wasp model species in the Hymenoptera to study ecology and genetic evolution due to the molecularly elucidated sex-determination mechanism, thoroughly sequenced and

annotated genome and transcriptome data, clearly documented stereotypic behaviours and well characterized abdominal sex pheromones and contact pheromones (Lynch, 2015; Mair and Ruther, 2019; Pultz and Leaf, 2003; van den Assem et al., 1980; Werren and Loehlin, 2009). *Nasonia vitripennis* was found to harbour CI-*Wolbachia* in its natural populations (Bordenstein et al., 2001). Interestingly, the parasitoid family Chalcidoidea to which *N. vitripennis* belongs, is one of the several super families where most PI-*Wolbachia* are found, and the family completely lacks the CSD mechanism (van Wilgenburg et al., 2006; Wu et al., 2003). Therefore, the genetic basis of sex determination in *N. vitripennis* may also shed light on other related haplodiploid insect species without CSD, providing not only a great opportunity to analyse how these crucial genes control the morphology and behaviour, but also a foundation to understand the interaction between PI-*Wolbachia* and its hosts.

The genus *Muscidifurax* is closely-related to *Nasonia* and consists of five described species (Marcos & Legner, 1970). Serving as potential bio-control agents of synanthropic fly species (Geden and Hogsette, 2006; Petersen and Cawthra, 1995), the genus *Muscidifurax* continuously receives attention from researchers. Within the genus, *Muscidifurax raptorellus* is a gregarious species that is first found in Uruguay and Chile. It reproduces sexually and shows a male-biased sex ratio in the commercial mass-rearing conditions (Heimpel and Lundgren, 2000). Its solitary sister species, *M. uniraptor,* originates from Puerto Rico, and has a thelytokous reproduction controlled by PI-*Wolbachia*. Feeding antibiotics to adult females transiently removes *Wolbachia*, but sexual reproduction cannot be resumed (Gottlieb and Zchori-Fein, 2001; Zchori-Fein et al., 2000). Artificial transformation of *PI-Wolbachia* from *M. uniraptor* to other species only lasts a few generations with no induced PI phenotype in the new hosts (van Meer and Stouthamer, 1999). All these results suggest that the manipulation of *PI-Wolbachia* is complex and could exert tremendous modification in its host during long-term co-evolution.

Scope of the research

In this dissertation I set out to study three major topics: (1) the evolutionary conservation of sex determination mechanisms, (2) the PI-*Wolbachia* induced reproductive manipulation of the host sex determination system, and (3) the role of Doublesex in regulating the sexual differentiation in parasitoid wasps. To investigate the mechanism of PI-*Wolbachia* manipulation, *M. uniraptor* was chosen as study species, as it has a sexually reproducing sister-species, *M. raptorellus*, and it is a close relative to *N. vitripennis.* This means that

well-established research methods in *N. vitripennis* can be applied to *Muscidifurax* to facilitate the research. I first tried to identify the core sex determination genes and to understand their functions in a comparison of *M. uniraptor* and *M. raptorellus* (**Chapter 2**), expecting to find some differences that could provide an explanation for the loss of sexual reproduction in *M. uniraptor* following PI-*Wolbachia* infection. In **Chapter 3**, I further investigated the required steps for *Wolbachia* to achieve thelytoky. I primarily focused on the sex determination pathway, aiming to understand whether *Wolbachia* takes direct control of the sex determination system during early embryogenesis. Then the research was directed into another aspect of sex determination by researching how sexually dimorphic traits are promoted and regulated by the sexual differentiation gene *Dsx* in haplodiploid species. I used *N. vitripennis* as a research subject because of its clear sexually dimorphic phenotypes, the extensive knowledge of its behaviour and its ease by which it can be manipulated. In **Chapter 4**, the effect of Dsx was first evaluated on the differentiation of major dimorphic traits in early developmental stages. In addition, a new sex determination gene homolog from *A. mellifera* was evaluated for its effect on dimorphic trait development in *N. vitripennis* (**Chapter 5**). Subsequently, I explored the potential of Dsx to regulate the courting and mating behaviour of adult *N. vitripennis* (**Chapter 6**). In **Chapter 7**, I discuss the results of this thesis in a wider context, critically reflecting on limitations and contributions of my results to the overall understanding of sex determination in the Hymenoptera, and highlighting the directions for future research.

Chapter 2

Comparison of sex determination mechanisms of a *Wolbachia*-infected thelytokous parasitoid wasp *Muscidifurax uniraptor* and its sexually reproducing sister species, *Muscidifurax raptorellus*

Yidong Wang and Eveline C. Verhulst

Abstract

Insects possess diverse sex-determination mechanisms to regulate their sexual development. The common feature of different sex determination systems is a relatively conserved sex-determination cascade that evolved from the bottom upwards with genes at the bottom being more conserved than genes at the top. The endosymbiont *Wolbachia*, however, can manipulate its host sex determination, resulting in the host being completely dependent on *Wolbachia* for reproduction. To understand the molecular mechanism of this type of *Wolbachia* manipulation, knowledge of the sex determination cascades in these infected insect species needs to be acquired first. In this research, we studied the sex-determination system of the thelytoky-inducing *Wolbachia*-infected *Muscidifurax uniraptor* wasp species and compared it with the system from its sexually reproducing sister species *M. raptorellus* which has no *Wolbachia* infection. We identified transcripts of three conserved sex-determination genes *transformer* (*tra*), *transformer-2* (*tra2*) and *Doublesex* (*Dsx*) in the two species. Similar structures of sex-specific and non-sex-specific *tra* and *tra2* splice variants were found in both species. Using RNA interference, we depleted the expression of both *tra* and *tra2* in females and confirmed that the maternal provision of *tra* and *tra2* is essential for female offspring development. We also found that the knockdown of maternally provided *tra2* has a detrimental effect on embryo development. Although no differences were observed in the upstream sex-determination genes, we found a clear divergent female-specific splicing in the downstream gene *Dsx* in the two species. In *M. uniraptor*, two female-specific *Dsx* splice variants are found while in *M. raptorellus* only one female-specific and one non-sex-specific *Dsx* splice variant are present, in addition to the two male-specific splice variants in both species. Moreover, an alternatively spliced 5'UTR is uniquely present in *M. uniraptor.* We hypothesize that these differences in *Dsx* are related to the co-evolution with *Wolbachia*.

Introduction

The insect sex-determining cascade is a complex pathway of interacting genes that evolves and diversifies rapidly among species. Based on Wilkins (1995) hypothesis, the genetic pathway of sex determination initiated from most downstream signal and evolved by adding genes upstream. Especially at the top of the cascade, insects possess a vast variety of diversified initial signals that induce the downstream genes in the sex-determination cascade to promote sexual differentiation (Bachtrog et al., 2014). In most insect orders, sex is determined by the sex chromosomes. In Hymenoptera, sex is determined by the ploidy state of the individual using a wide array of genetic and cytoplasmic mechanisms leading to diploid female development from fertilized eggs, and haploid male development from unfertilized eggs (Heimpel and de Boer, 2008). Only two initial signals directing sex determination have been molecularly elucidated in Hymenoptera: complementary sex determination (CSD) in the honeybee, *Apis mellifera* (Beye et al., 2003) and the maternal-effect genomic imprinting sex determination (MEGISD) in the parasitoid wasp, *Nasonia vitripennis* (Verhulst et al., 2013; Zou et al., 2020). The actual gene underlying CSD, *csd*, is only characterized in bees, with heterozygous *csd* activating female development, and homozygous or hemizygous *csd* leading to male development (Beye et al., 2003; Gempe et al., 2009). MEGISD has recently been verified with the identification of the *wasp overruler of masculinization* (*wom*) gene only being transcribed from the paternal chromosome set to activate the early zygotic sex-determination pathway for female development (Zou et al., 2020). Although diverse in the initial signals, in all studied insects, *Doublesex* (*Dsx*), the switch gene that initiates male or female differentiation, is at the bottom of the sex-determination cascade and is conserved in all insects studied to date (Geuverink and Beukeboom, 2014; Price et al., 2015; Verhulst and van de Zande, 2015). Then, in most, but not all insect species the sex-determination pathways contain a conserved set of upstream genes called *transformer/feminizer* (*tra/fem, fem* is a *tra* homolog identified in *Apis mellifera* (Hasselmann et al., 2008)) and *transformer-2* (*tra2*). *Transformer* is a splice factor that expresses sex-specific splice variants, encoding either a full-length female-specific Tra (Tra^F) protein or a truncated male-specific Tra (Tra^M) (reviewed in Verhulst et al., 2010a). Common features of functional Tra include a putative autoregulatory CAM domain (Bopp et al., 2014; Tanaka et al., 2018) which is found in all *tra* genes except for *Drosophila*; an order-specific domain (Verhulst et al., 2010a), and arginine/serine-rich (RS) and proline-rich regions that are located near the C-terminus (reviewed by Geuverink and Beukeboom, 2014). *Transformer-2* (*tra2*) is another splice factor that expresses non-sex-specific variants that encode full-length proteins containing an RNA

binding domain (RBD) flanked by RS regions (Amrein et al., 1988). Tra^F and Tra2 interact to facilitate female-specific splicing of the most downstream gene *Dsx* (Geuverink et al., 2017; Inoue et al., 1992; Nissen et al., 2012; Salvemini et al., 2009). Male-specific splicing of *Dsx* occurs by default without facilitation of Tra and Tra2 (Inoue et al., 1992). The different sex-specific Dsx proteins control the development of sexual differentiation and dimorphism. The omnipresence of Dsx is confirmed in diverse insect species across insect orders (Shukla and Nagaraju, 2010b). Dsx contains a DM domain which includes a DNA binding domain (DBD) and an oligomerization domain (OD1) to facilitate binding downstream targets, and a dimerization domain (OD2), which consists of a common OD2 sequence and a sex-specific OD2 sequence (An et al., 1996). These sex-specific OD2 sequences are suggested to account for the regulation of sexual trait development in males and females (Price et al., 2015; Verhulst and van de Zande, 2015).

Besides the inherent sex-determination mechanisms controlling the sex in different insect species, intracellular endosymbiotic bacteria such as *Wolbachia*, *Rickettsia* and *Cardinium* can build up an intimate relationship with infected insect hosts and take over the control of their reproduction to benefit their own propagation (Chigira and Miura, 2005; Hagimori et al., 2006; Stouthamer et al., 1990). As one of the most prevalent endosymbionts, *Wolbachia* evolved four distinct sex-manipulation mechanisms to control the reproduction of its hosts (Werren et al., 2008). One of these, thelytokous parthenogenesis inducing (PI) *Wolbachia* are found exclusively in haplodiploid species (Ma and Schwander, 2017). In this case, asexually reproduced unfertilized eggs will develop into diploid females but not haploid males, indicating that *Wolbachia* achieves diploidy-restoration of the haploid eggs coupled with feminization (Gottlieb et al., 2002; Ma et al., 2015; Pannebakker et al., 2004; Stouthamer and Kazmer, 1994). Once this vertical transmission of *Wolbachia* from female to offspring is established, sexual reproduction becomes unnecessary for maintaining female development.

For the past several decades, many parasitoid wasps have been selected as biocontrol agents to reduce pest species pressure. Undoubtedly, better understanding of the mechanism by which *Wolbachia* can induce thelytokous parthenogenesis can provide a great benefit to the mass rearing of parasitoid wasps as needed for biocontrol. *Muscidifurax uniraptor* belongs to a wasp genus that is commercially used to reduce filth flies present in stables and dairy farms (Legner, 1985). Compared to its gregarious, sexually reproducing sister species *Muscidifurax raptorellus*, *M. uniraptor* is a solitary species infected with PI-*Wolbachia*

(Stouthamer et al., 1993). Several attempts have been made to transfer PI-*Wolbachia* from native hosts to closely related species, however, none of these led to the maintenance of a stable infection in descendants or induced thelytokous parthenogenesis in the recipient hosts (Huigens et al., 2004; van Meer and Stouthamer, 1999; Watanabe et al., 2013).

In order to understand the molecular regulation mechanism used by *Wolbachia* to induce thelytokous parthenogenesis in *M. uniraptor*, the sex-determination cascade of *M. uniraptor* has to be identified first. In this research, we set out to characterize the sex-determination genes *tra*, *tra2* and *Dsx* in *M. uniraptor* and *M. raptorellus*. We then determined their expression levels during development, and verified their function using RNA interference (RNAi). Comparative analysis of the sex determination cascade of both species will highlight differences between the two species that could explain the mechanism of sex-manipulation induced by *Wolbachia*.

Materials and methods

1. Insect rearing

Muscidifurax uniraptor and *Muscidifurax raptorellus* used in the experiments were kindly provided to us by Jack Werren and Koppert Biological Systems respectively. The two cultures are continuously reared on *Calliphora spp.* pupae under laboratory conditions of light/dark 16h/8h at 25°C.

2. Rapid amplification of cDNA ends (RACE) PCR to identify the splice variants of sex determination genes

To identify full transcripts of the sex-determination genes *Dsx*, *tra* and *tra2* in *M. uniraptor*, we performed 3' and 5' RACE PCR. Based on a draft genome of *M. raptorellus* (Jack Werren, personal communication) we designed nested RACE PCR primers in Geneious 10.0.9 (Table S2.1).

For 3' RACE PCR, RNA from three individual males and females of *M. uniraptor* adults was extracted separately using ZR Tissue & Insect RNA MicroPrep™ (Zymo Research) following the manufacturer's instructions with the on-column DNase treatment step applied to all samples. Subsequently, the 3' RACE adapter provided by the FirstChoice RLM-RACE Kit (Invitrogen™) was annealed to the purified RNA during reverse-transcription using RevertAid™ H Minus First Strand cDNA Synthesis Kit (Thermo Fisher). Nested touch-down

PCR was first performed using GoTaq® G2 Flexi DNA Polymerase (Promega) following manufacturer's instruction with the outer primer sets (Table S2.1) with the PCR profile: 95°C for 3 minutes, 5 cycles of 95°C for 30 seconds, 60°C (1°C decrease after each cycle) for 30 seconds, and 72°C for 1.5 minutes, and 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1.5 minutes with a final extension of 10 minutes at 72°C in a thermal cycler (T100TM Thermal Cycler, Bio-Rad). Subsequently, the inner primer set was used (Table S2.1) with the PCR profile: 95°C for 3 minutes, 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1.5 minutes with a final extension of 10 minutes at 72°C.

For 5' RACE PCR, total RNA pooled from 30 adult males and from 30 adult females was extracted separately using ZR Tissue & Insect RNA MicroPrep™ (Zymo Research) following the standard protocol. The 5' RACE was carried out using the FirstChoice RLM-RACE Kit (Invitrogen™) with manufacturer's instructions. Nested touch-down PCR using GoTaq® G2 Flexi DNA Polymerase (Promega) with the 5' RACE inner and outer primers was conducted with the same PCR profile used for amplifying the 3' ends.

All PCR amplified products were separated by gel electrophoresis using a 1.5% TAE agarose gel stained with Midori Green (NIPPON Genetics). Bands were excised from gel and purified using QIAquick Gel Extraction Kit (QIAGEN). All purified fragments were cloned into pGEM®-T Easy Vector (Promega) for sequencing. Sequencing results were aligned to the draft *M. raptorellus* genome in Geneious 10.0.9 to acquire the structure of the transcripts.

3. Phylogenetic analysis of the conserved domain

Representative insect Tra, Tra2 and Dsx protein sequences were acquired from the NCBI database. DM and OD2 domains in Dsx, and complete protein sequences of Tra2 and Tra were used to construct the multi-sequence alignments in Clustal Omega 1.2.2 (Sievers et al., 2011). Phylogenetic trees were inferred with Maximum Likelihood method and the confidence of the branches was estimated by 1000 bootstraps (Kumar et al., 2016). The Genbank accession numbers of the Tra, Tra2 and Dsx proteins of the different species are provided in the Figures 2.2, S2.2 and S2.3.

4. Sex-determination gene expression analysis at different developmental stages by reverse transcriptase PCR (RT-PCR)

Muscidifurax uniraptor females were cured from *Wolbachia* infection to obtain male offspring, by feeding them 0.5% tetracycline solution (dissolved tetracycline hydrochloride powder (Sigma-Aldrich) in 10% sucrose (Sigma-Aldrich) solution) using strip-cut filter paper. Female offspring was collected directly from *Wolbachia*-infected mothers. For the sexually reproducing *M. raptorellus*, male and female offspring samples were obtained from mated and virgin females, respectively. Samples were collected at 2 days post oviposition (2d po) for *M. raptorellus* and 3d po for *M. uniraptor,* fourth larval instar (6d po), white pupal stage (WP), red-eye white pupal stage (RWP), black and white pupal stage (BWP), black pupal stage (BP) and Adult stage (A). Collected samples were fast frozen in liquid nitrogen and total RNA was extracted using ZR Tissue & Insect RNA MicroPrep™ (Zymo Research) including on column DNase treatment step. Subsequently, one µl of total RNA from each sample was synthesized into cDNA with a standard reaction mix (SensiFAST™ cDNA Synthesis Kit, Bioline) in a thermal cycler (Bio-Rad T100TM Thermal Cycler, Bio-Rad). The PCR program consisted of 5 minutes priming at 25°C, 30 minutes reverse transcription at 46°C and 5 minutes reverse transcriptase inactivation at 85°C. Afterwards, RT-PCR was performed using GoTaq® G2 Flexi DNA Polymerase (Promega) following manufacturer's instruction with the following PCR profile: 3 minutes at 95°C, 31 amplification cycles of 30 seconds at 95°C, 30 seconds at 55°C, 50 seconds at 72°C and a final extension of 5 minutes at 72 °C in a thermal cycler (T100TM Thermal Cycler, Bio-Rad) for all genes. Primers of the internal reference gene *RP-49* and the target genes are provided in Table S2.1. All PCR products (including gDNA as control) were separated and visualized on a 1.5% agarose gel with 1×TAE buffer stained with Midori Green (NIPPON Genetics).

5. RNAi and silencing efficiency analysis of *tra* and *tra2* in *M. uniraptor* and *M. raptorellus*

Parental RNAi (pRNAi) was used to silence maternal provision of *tra* and *tra2* mRNA to the eggs of *M. uniraptor* and *M. raptorellus* females as described by Wang et al. (2020). Primers that were used for generating the dsRNA and conducting the quantitative RT-PCR (qPCR) were designed in Geneious 10.0.9 (Table S2.1). Silencing efficiency was determined through qPCR by comparing the expression of sex determination genes of the target gene dsRNA injected and mock control *gfp* dsRNA injected female wasps. Five to six replicates

of individual wasps from control and treatment groups were fast frozen in liquid nitrogen. Total RNA extraction and cDNA synthesis followed the procedure described in the RT-PCR section. Subsequently, the cDNA template was diluted 50 times and SensiFAST™ SYBR® No-ROX Kit (Bioline) was used to conduct the qPCR following manufacturer's instruction. qPCR was carried out using the CFX96TM Real-Time System (Bio-Rad) with Bio-Rad CFX Manager 3.1 Software (Bio-Rad). The standard qPCR profile consisted of 95°C for 3 minutes, 45 amplification cycles of 15 seconds at 95°C, 15 seconds of 55°C, 30 seconds of 72°C and a final standard dissociation curve step to check for non-specific amplification.

6. Verification of the sex ratio and ploidy level of the offspring from pRNAi treated mothers

Injected female *M. uniraptor* wasps were kept individually in small glass tubes, sealed with a cotton plug and kept at rearing conditions prior to eclosion. Twenty-four hours after eclosion, each of the female wasps was provided with two host pupae for food, which was exchanged for ten host pupae per day in the following two days for offspring production. All the pupae that were collected from the same mother were kept together under rearing conditions until offspring emergence. For *M. raptorellus*, newly emerged injected females were first mated with virgin males for 24 hours. One host per female was given for 24h for food which was exchanged for two consecutive days for offspring production. Only the hosts that had been successfully parasitized (no fly emergence) were counted and used in the final calculation. The percentage of male offspring was calculated and average number of offspring per host per female was determined by dividing the total offspring numbers of single female by the successfully parasitized host numbers.

Flowcytometry was used to determine the ploidy level of offspring from different treatments following the protocol adapted from Ma et al. (2015). One frozen wasp head was put into an Eppendorf tube with 0.3 ml ice-cold Galbraith buffer. A plastic pestle was used to crack open the head and homogenize the samples. The homogenate was then poured through a 5 ml Cell Strainer Cap (Stemcell Technologies) to filter out the debris and collected in 96-well plates. Samples were stained with 5 µl propidium iodide (1.0 mg/ml, Sigma-Aldrich). The total DNA content of each sample was measured by a MACSQuant Analyzer (Miltenyi Biotec) and visualized by a log scale generated fluorescence intensity plot using MACSQuantify™ Software 2.11. Representative figures were generated by gating the raw data in flowjo v10.6.2 program.

7. Fecundity bioassay

To assess the developmental potential of the produced eggs after pRNAi, offspring eggs from *tra2*-i and *gfp*-i *M. raptorellus* females were collected. Females were restricted in host access using an egg-laying chamber as described in Wang et al. (2020) to facilitate egg collection. Prior to the experiment, individual females were trained in the laying chamber for 24h with single host. Afterwards, one hour was given to female wasps for oviposition. Subsequently, host pupae were opened and eggs from the same mother were collected and carefully transferred to a 1XPBS agar plate. Plates were incubated under rearing conditions for 48 hours until all surviving embryos had hatched as larvae. Egg mortality was determined by counting the number of hatched and dead eggs of *tra2*-i and *gfp*-i treated mothers to determine egg mortality.

Due to the difficulty of collecting enough eggs from individual solitary *M. uniraptor* females, fecundity of *tra2* RNAi treated females was determined by comparing the number of matured eggs in *M. uniraptor* female ovaries after *tra2*-i or *gfp*-i treatment. The abdomen of freshly eclosed females was immersed in 1XPBS buffer in a dissection tray and dissected. Afterwards, ovaries were carefully mounted in a drop of 1XPBS buffer on a glass slide. Photos of the ovaries were captured by a sCMEX-20 camera (Euromex Scientific) coupled with a Leitz Dialux 20 EB light microscope. The number of mature eggs in the ovaries was counted per female.

Results

Characterization of the *M. uniraptor* sex determination genes

To understand the mechanism of sex determination in both *Muscidifurax* species, we first characterized the genes in the sex-determination cascade that are conserved in other insect species. We identified the gene structure by 3' and 5' RACE PCR using regions conserved in other insect sex-determination genes to design primers. *M. uniraptor transformer* (*Mutra*) contains nine exons that are present in all *Mutra* transcripts, and is alternatively spliced in exon 2 (Figure 2.1A). Female *Mutra* (*Mutra*^{F}) includes only exon 2a and translates into a 400-amino-acid (aa) protein. By skipping exon 2b and 2c, *Mutra*^{F} encodes the complete CAM domain, HYM domain, SR-domain and proline-rich region which are conserved features in most if not all identified female-specific Tra proteins so far (Geuverink and Beukeboom, 2014; Verhulst et al., 2010a). The male *Mutra* (*Mutra*^{M}) consists of the complete exon 2

which shifts the open reading frame (ORF) to include stop-codons, resulting in a 172 aa truncated protein. A non-sex-specific (NSS) splice variant was characterized in *M. uniraptor* that contains exon 2a and 2c and encodes a 190 aa protein. Both *Mutra*^{M} and *Mutra*^{NSS} contain the HYM domain (Figure 2.1A).

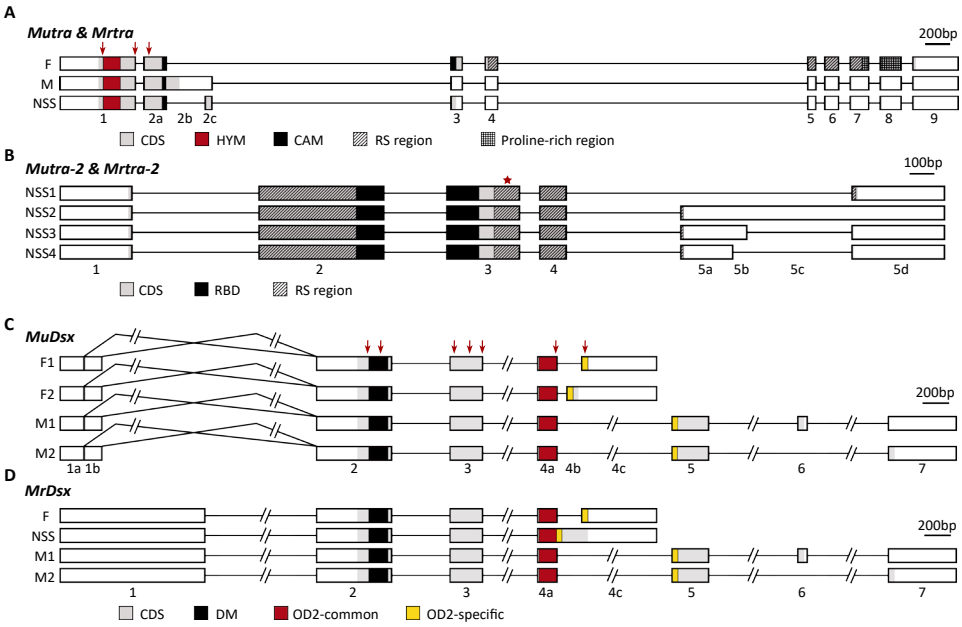


Figure 2.1 Transcript structures of *transformer*, *transformer-2* and *Doublesex* in *Muscidifurax*. Similar structures of transcripts are present between **(A)** *Mutra* and *Mrtra*, **(B)** *Mutra2* and *Mrtra2*, but different structures of **(C)** *MuDsx* and **(D)** *MrDsx* are observed in this study. Blocks and horizontal lines in the structures represent the exons and introns respectively. Exons are marked by numbers below each gene. Female (F), male (M) sex-specific and none-sex-specific (NSS) splice forms are indicated in front of each transcript and scales are provided behind the gene structures. CDS, HYM, CAM, RBD, DM and OD2 domains are shaded with colours, RS region and Proline-rich region are shaded with patterns, and empty regions refer to the non-coding sequence of 5' and 3' UTRs. Positions of nonsynonymous substitutions of nucleotides between the two species are indicated with red arrows and the deletion is indicated with a red star. The size of the introns is reduced with double slash lines for visualization purpose.

Next, we identified four *M. uniraptor transformer-2* (*Mutra2*) transcripts in both males and females, indicating there is no sex-specific splicing (Figure 2.1B). These four splice variants differ in the last exon 5, of which *Mutra2*^{NSS1} contains exon 5d, *Mutra2*^{NSS2} retains exon 5 completely, *Mutra2*^{NSS3} excludes exon 5c, and *Mutra2*^{NSS4} retains exon 5a and 5d. As a result, *Mutra2*^{NSS1} is translated into a 307 aa protein and the other splice variants all encode the same protein isoform of 302 aa. The conserved RBD domain is present in all identified *Mutra2* isoforms (Figure 2.1B).

Finally, we characterized four *Dsx* sex-specific splice variants in *M. uniraptor*, of which two are female-specific and two are male-specific splice variants (Figure 2.1C). An alternatively spliced 5'-UTR was detected by 5'RACE PCR and confirmed by RT-PCR in both sexes in exon 1, containing a 181 bp or 318 bp long sequence respectively (Figure S2.1C), albeit that further identification is required to confirm their presence in all splice variants. *MuDsx*^{F} and *MuDsx*^{M} splice forms share exon 1 to 4a but differ in their C-terminus (Figure 2.1C). Based on the draft *M. raptorellus* genome, in female-specific splice variants *MuDsx*^{F1} the last part of exon 4 (4c) is included and a 181 bp part of exon 4 is spliced out, encoding a protein of 233 aa. Female-specific splice variant *MuDsx*^{F2} contains a larger part of exon 4 (4b and 4c) and a 75 bp part of exon 4 is spliced out, encoding a protein of 245 aa. In both male-specific splice variants only exon 4a is retained and downstream exons 5, 6 and 7 are incorporated. The difference between the two male-specific variants is the inclusion of exon 6 in *MuDsx*^{M1} which is skipped in *MuDsx*$^{M2.}$ This results in slightly different coding sequences (CDS) in the 3' end, resulting in MuDsx^{M1} protein of 324 aa and a MuDsx^{M2} protein of 326 aa (Figure 2.1C).

Characterization of the *M. raptorellus* sex determination genes

In *M. raptorellus,* we identified *Mrtra* and *Mrtra2* and observed that they share their structures with *Mutra* and *Mutra2* completely (Figure 2.1A,B) and we found the same number of female-, male- and non-sex specific variants. There are three amino acids that differ between MrTra^{F} and MuTra^{F}, however, none of them are located inside the known putative domains (Figure 2.1A). Compared to *Mutra2*, *Mrtra2* has six additional base pairs in exon 3 leading to a two aa longer total protein size (Figure 2.1B). The biggest difference between the two species is in the splicing of *Dsx*, as *MrDsx* has different splice-variants compared to *MuDsx*. Only one female-specific *MrDsx* variant is present, which resembles *MuDsx*^{F1} (Figure 2.1C,D). Instead of a second female-specific splice variant*, M. raptorellus* contains the non-sex-specific *MrDsx*^{NSS} splice variant that incorporates the entire exon 4 but this does not shift the ORF and yields a 293 aa protein, 60 aa longer than *MrDsx*^{F}. *MrDsx*^{M1} and *MrDsx*^{M2} splice variants both have similar structures as the respective ones in *M. uniraptor*. Furthermore, in *M. raptorellus* exon 1 shows no alternative splicing compared to *MuDsx* (Figure S2.1)*,* but in all *MrDsx* transcripts exon 1 contains an additional 1093 bp. This *MrDsx* exon 1 initiates at the same position as it does in *MuDsx* but extends further downstream resulting in a longer 5'-UTR (Figure 2.1C,D and S2.1). A comparison of the coding sequence of Dsx in both species shows in total seven nonsynonymous substitutions of which three are located in the DM and OD2 functioning domains (Figure 2.1C).

Phylogenetic analysis with Tra^{F} (Figure S2.2) and Tra2 (Figure S2.3) protein sequences indicates that their evolution is closely reflected in the divergence of insect orders. A similar phylogenetic analysis with DM and OD2 domains shows that *MuDsx*^{F1} and *MrDsx*^{F} follow species divergence and cluster with Dsx proteins from their close relative *N. vitripennis* and *Trichomalopsis dubius*, while *MuDsx*^{F2} does not exhibit species divergence (Figure 2.2). Still, despite their variation, all female Dsx isoforms in *Muscidifurax* belong to the monophyletic subgroup within the Hymenoptera (Figure 2.2).

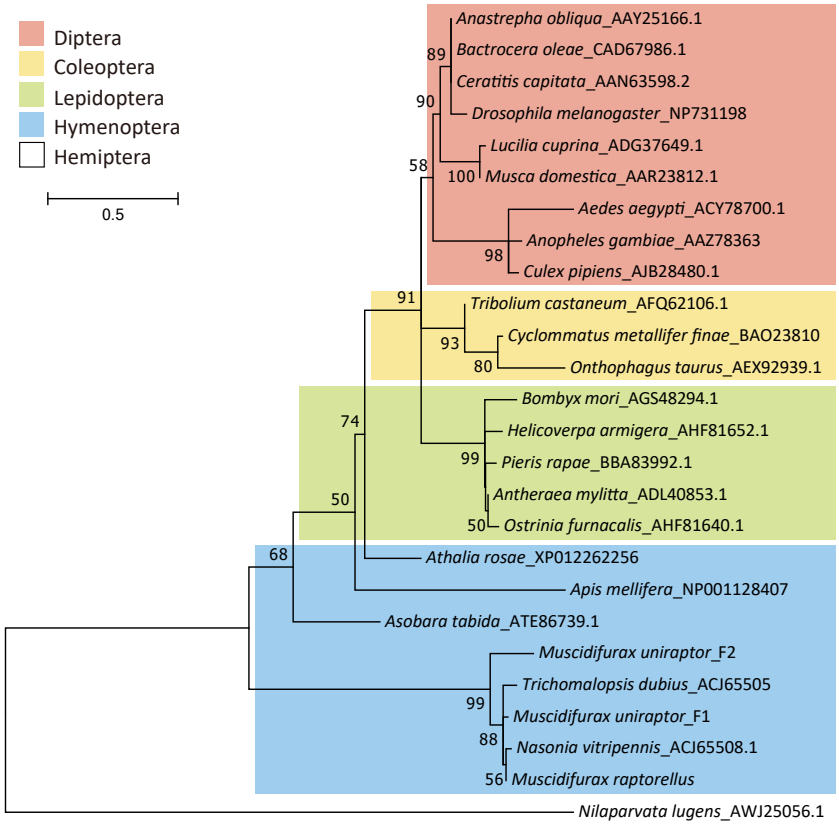


Figure 2.2 Molecular phylogenetic analysis of Doublesex in different insect species. Based on the annotation of *Drosophila melanogaster*_NP731198, the conserved DM and OD2 domains of different Dsx isoforms from different species were used to construct the tree. The evolutionary history is inferred by using the Maximum Likelihood method based on the JTT matrix-based model (Jones et al., 1992). A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 1.7563)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 8.02% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. 1000 replicates of bootstraps were applied and the percentage of replicate trees that above 50% are shown at nodes. Accession numbers (if applicable) are provided next to the species names. Colour blocks indicate the insect order of the species.

Sex-specific expression of *tra, tra2* and *Dsx* in different developmental stages of *M. uniraptor* and *M. raptorellus*

We collected male and female samples at different developmental stages to investigate sex-specific expression of the different sex-specific splice variants of *tra*, *tra2* and *Dsx* in *M. uniraptor* and *M. raptorellus* using RT-PCR analysis. We found that $Mutra^{F}$ is only present in females, while $Mutra^{M}$ is highly expressed in males at all assessed time points from early larval (3 days post oviposition (po)) to adult stage (Figure 2.3A) and is expressed at a very low level in females. In both sexes $Mutra^{NSS}$ is expressed at a low level throughout development (Figure 2.3A). A slight increase of $Mutra^{NSS}$ expression towards pupal and adult stages is observed in females (Figure 2.3A). In females the additional band that is slightly larger than $Mutra^{NSS}$ could not be identified. Since primers amplifying *Mutra* cover large

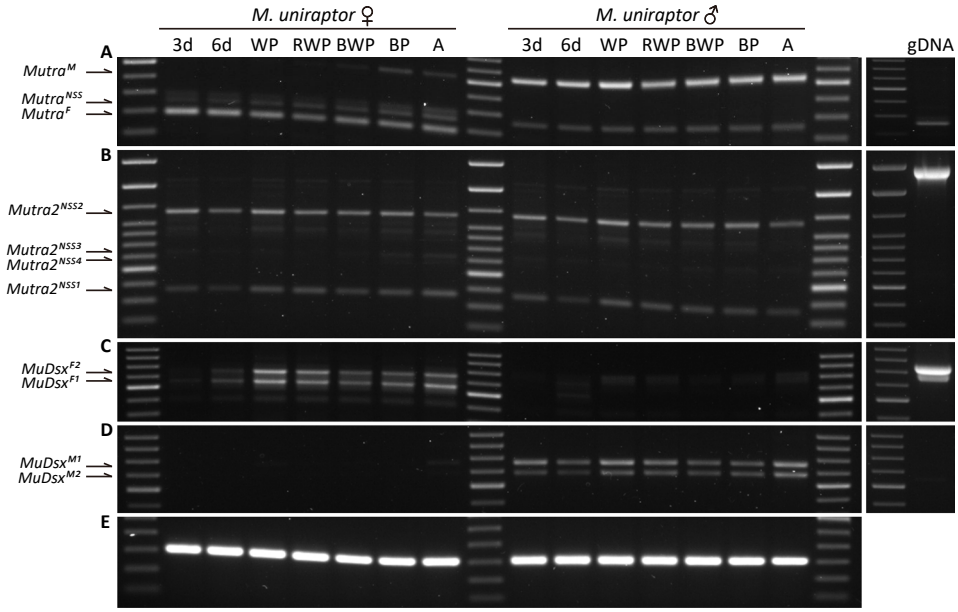


Figure 2.3 Expression of sex-determination genes (A) *Mutra*, (B) *Mutra2*, (C) $MuDsx^{F}$, (D) $MuDsx^{M}$ and the internal control gene (E) *MuRP49* at different developmental stages of *M. uniraptor* males and females. Developmental stages indicated above the figure are: 3 days post oviposition (po) (3d), 6 days po (6d), white pupal stage (WP), red eye white pupal stage (RWP), black and white pupal stage (BWP), black pupal stage (BP) and adult stage (A). Male (M) and female (F) sex-specific and non-sex-specific (NSS) splice variants were indicated next to the figure. *MuRP49* serves as internal control and 100bp ladder (Thermo fisher) indicates fragment size. Fragments are visualized on 1.5% TAE agarose gel stained with Midori Green (NIPPON Genetics).

introns, the weak band amplified from gDNA that has a similar size as *Mutra*^{NSS} comes from the non-specific amplification. *Mutra2* expression was also observed in all developmental stages in both sexes, with *Mutra2*^{NSS1} and *Mutra2*^{NSS2} being predominantly expressed while minor expression of *Mutra2*^{NSS3} and *Mutra2*^{NSS4} becomes visible from the black pupal stage onwards (Figure 2.3B). Additional bands might indicate additional splice variants but these could not be identified (Figure 2.3B). Clear *MuDsx* sex-specific expression was observed as *MuDsx*^{F1} and *MuDsx*^{F2} were expressed at a similar level in females only, and *MuDsx*^{M1} and *MuDsx*^{M2} were expressed in males only (Figure 2.3C,D). An increase in expression of *MuDsx*^{F} from early larval to pupal stage was observed in females while *MuDsx*^{M} displayed a relatively constant expression level throughout male development (Figure 2.3C,D).

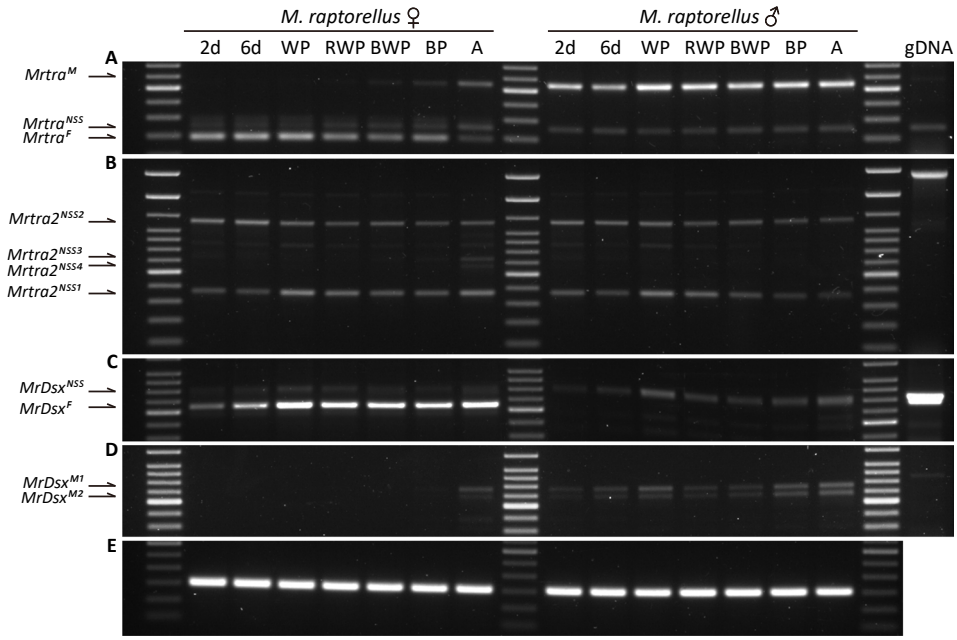


Figure 2.4 Expression of sex-determination genes (A) *Mrtra*, (B) *Mrtra2*, (C) *MrDsx*^{F}, (D) *MrDsx*^{M} and the internal control gene (E) *MrRP49* at different developmental stages of *M. raptorellus* males and females. Developmental stages indicated above the figure are: 2 days post oviposition (po) (2d), 6 days po (6d), white pupal stage (WP), red eye white pupal stage (RWP), black and white pupal stage (BWP), black pupal stage (BP) and adult stage (A). Male (M) and female (F) sex-specific and non-sex-specific (NSS) splice variants were indicated next to the figure. *MrRP49* serves as internal control and 100bp ladder (Thermo fisher) indicates fragment size. Fragments are visualized on 1.5% TAE agarose gel stained with Midori Green (NIPPON Genetics).

Similar expression patterns of *Mrtra* and *Mrtra2* were observed in *M. raptorellus* (Figure 2.4A,B) when compared to *M. uniraptor*. The expression of female-specific *MrDsx*^{F} is increasing from 2 days po to the white pupal stage and remains at this level until adulthood

(Figure 2.4C). *MrDsx*^{M}, on the other hand, maintains its expression level throughout the different developmental stages (Figure 2.4D). In addition, *MrDsx*^{NSS}, albeit at a low expression level, is observed at all sampled time points in both sexes (Figure 2.4C).

Maternal provision of *tra* and *tra2* determines female development in *Muscidifurax*

In the studied hymenopteran non-CSD sex determination systems, maternal input of *tra* and *tra2* is required for female development (Geuverink et al., 2017; Verhulst et al., 2010b). To unravel the sex determination mechanism in *Muscidifurax*, we performed parental RNAi (pRNAi) targeting *tra* and *tra2* in females of both species. In *M. raptorellus*, silencing of *Mrtra* or *Mrtra2* (Figure 2.5A,B) in females that were subsequently mated, resulted in 100% male offspring (Figure 2.5C), which is significantly different from the *gfp* RNAi (*gfp*-i) treated

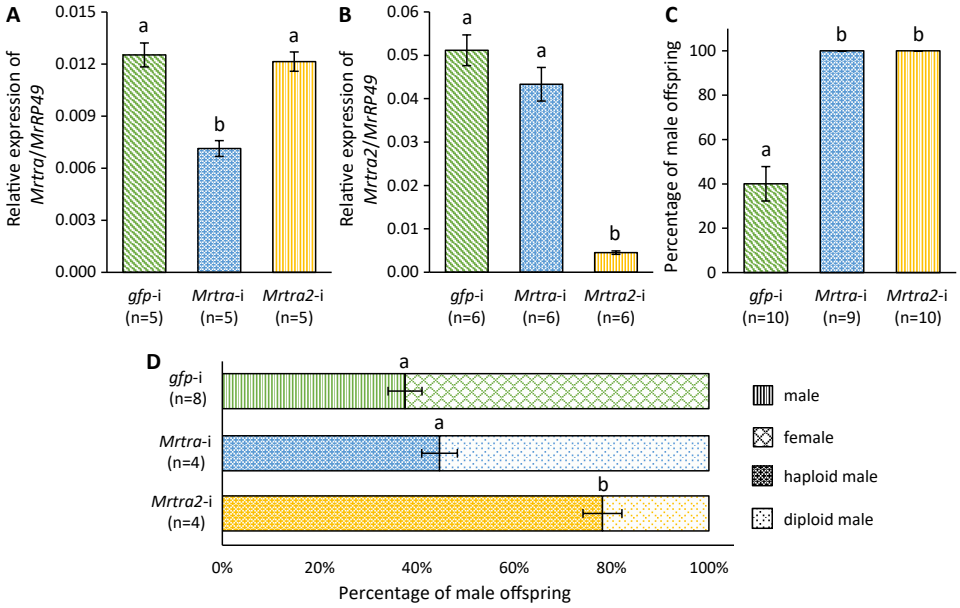


Figure 2.5 Silencing efficiency of *Mrtra* and *Mrtra2* knockdown in *M. raptorellus* females and the different sex ratio of the offspring produced by *gfp*-i, *Mrtra*-i and *Mrtra2*-i *M. raptorellus* females. (A) Relative expression of *Mrtra/MrRP49* in *gfp*-i, *Mrtra*-i and *Mrtra2*-i females. **(B)** Relative expression of *Mrtra2/MrRP49* in *gfp*-i, *Mrtra*-i and *Mrtra2*-i females. **(C)** Percentage of male offspring of *gfp*, *Mrtra* and *Mrtra2* dsRNA injected females. **(D)** Haploid and diploid percentage of offspring in *gfp*-i, *Mrtra*-i and *Mrtra2*-i females. Different colours represent different treatments in bar graphs. Numbers of females that used to produce offspring in each treatment are provided below or next to the bar graphs and error bars represent standard error. Statistics was performed with ANOVA/Tukey HSD for A,D and with Kruskal-Wallis multiple comparison Dunn-test for B,C. Letters refer to statistically significant differences with $p<0.05$.

and subsequently mated females that produced about 40% male offspring (Figure 2.5C). *Gfp*-i had no effect on *Mrtra* or *Mrtra2* expression levels, and no cross-silencing effects were found in either *Mrtra*-i or *Mrtra2*-i females (Figure 2.5A,B). Flowcytometry analysis of all males shows that both *Mrtra*-i and *Mrtra2*-i mothers produced haploid and diploid male offspring (Figure S2.4). In addition, the ratio of haploid and diploid offspring from *Mrtra*-i mated mothers is similar to the offspring sex-ratio produced by *gfp*-i mated mothers (Figure 2.5D), indicating that the ratio of fertilized and unfertilized eggs is similar for these two treatments. However, *Mrtra2*-i mated mothers produced a significantly higher percentage of haploid male offspring (Figure 2.5D).

Silencing *Mutra* and *Mutra2* by pRNAi in *M. uniraptor* renders a similar effect as in *M. raptorellus*. *Muscidifurax uniraptor* normally asexually produces only female offspring due to the thelytoky-inducing *Wolbachia* infection. However, a significant reduction of maternally provided *Mutra* or *Mutra2* (Figure 2.6A,C) leads to male development of the offspring (Table 2.1). Flowcytometry analysis showed that all tested male offspring produced by both *Mutra*-i and *Mutra2*-i mated females are diploid (Table 2.1, Figure S2.5). In addition, we observed no reduction of *Wolbachia* density in *Mutra*-i and *Mutra2*-i females compared to *gfp*-i females (Figure 2.6B,D).

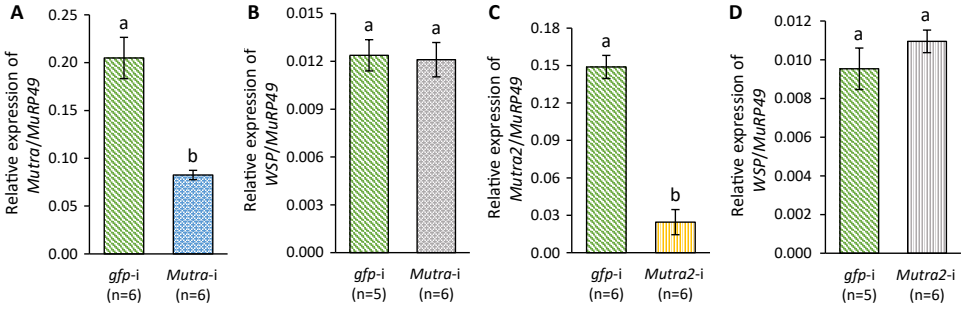


Figure 2.6 Silencing efficiency of *Mutra* and *Mutra2* pRNAi and relative expression of *WSP* in *M. uniraptor* females indicating *Wolbachia* titre. (A) Relative expression of *Mutra/MuRP49* in *gfp*-i and *Mutra*-i females. **(B)** Relative expression of *WSP/MuRP49* in *gfp*-i and *Mutra*-i females. **(C)** Relative expression of *Mutra2/MuRP49* in *gfp*-i and *Mutra2*-i females. **(D)** Relative expression of *WSP/MuRP49* in *gfp*-i and *Mutra2*-i females. Numbers of females that were used in each treatment are provided below the bar graphs and error bars represent standard error. Statistical analysis was performed with Wilcoxon rank sum test for A,C and with *t*-test for B,D. Letters refer to statistically significant differences with $p<0.05$.

Table 2.1 Total number and ploidy level of male and female offspring from *M. uniraptor gfp*-i, *Mutra*-i and *Mutra2*-i mated females. **Mutra*-i: Five male offspring from each treated female were checked by flowcytometry. *Gfp*-i: All males (if applicable) and two female offspring from each control female were checked by flowcytometry. *Mutra2*-i: All fully developed offspring from each treated female were checked by flowcytometry.

Treatment*	Male offspring			Female offspring		
	Haploid	Confirmed diploid	Total number	Haploid	Diploid	Total number
gfp-i (n=8)	1	0	1	0	16	119
Mutra-i (n=14)	-	70	150	0	0	0
Mutra2-i (n=6)	-	23	26	0	0	0

Tra2 silencing affects embryo viability

Parental RNAi of *tra2* in *M. uniraptor* and mated *M. raptorellus* females resulted in severely decreased offspring numbers when compared to the offspring of corresponding *tra*-i and *gfp*-i females (Figure 2.7A, 2.8A). We determined whether this was due to offspring mortality using *M. raptorellus*, by collecting freshly laid eggs from seven *Mrtra2*-i and *gfp*-i mated females and placing these on 1XPBS agar plates for an embryo development test. Only

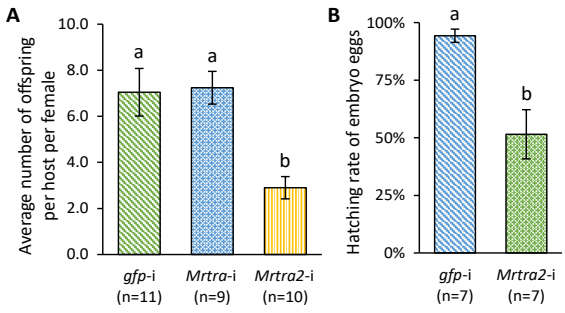


Figure 2.7 Average number of *M. raptorellus* offspring per host per female and the embryo hatching rates after different RNAi treatments. (A) Average number of survived offspring per host per female laid by *gfp*-i, *Mrtra*-i and *Mrtra2*-i females. **(B)** Hatching rate of the embryo eggs from *gfp*-i and *Mrtra2*-i females. Numbers of individually treated females used are provided below the bar graphs and error bars represent standard error. Statistical analysis was performed with ANOVA/Tukey HSD for A and with Wilcoxon rank sum test for B. Letters refer to statistically significant differences with $p<0.05$.

half of the embryos from *Mrtra2*-i females developed successfully and hatched as larvae compared to offspring developmental success from *gfp*-i females indicating a significant embryo mortality after *Mrtra2*-i (Figure 2.7B). We then checked whether egg production was limited by counting the number of mature eggs in the ovaries using *M. uniraptor.* No reduction in fecundity and no observable aberration in the ovary structure were found in *Mutra2*-i females when compared to *gfp*-i females (Figure 2.8B,C). Ploidy analysis of

surviving *Mrtra2*-i offspring suggests that the reduced average amount of offspring per *Mrtra2*-i female is caused by a high mortality of specifically diploid embryos while the average number of haploid offspring per female remains constant among treatments and control (Table 2.2).

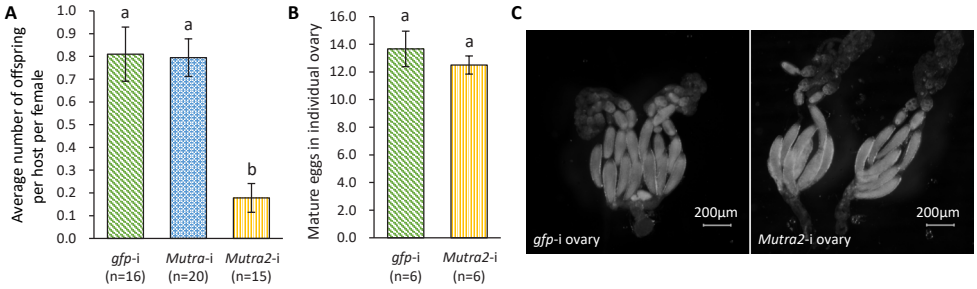


Figure 2.8 Average number of *M. uniraptor* offspring per host per female and ovary structure with the number of mature eggs after different RNAi treatments. (A) Average number of surviving offspring per host per female that laid by *gfp*-i, *Mutra*-i and *Mutra2*-i females. **(B)** Numbers of mature eggs in the ovaries of *gfp*-i and *Mutra2*-i females. Numbers of treated females that had been tested are provided below the bar graphs and error bars represent standard error. Statistical analysis was performed with Wilcoxon rank sum test for A and with *t*-Test for B. **(C)** comparison of the ovary structure of *gfp*-i and *Mutra2*-i females. Letters refer to statistically significant difference with $p<0.05$. Scales are provided in the photos.

Table 2.2 Average number of haploid and diploid male offspring from *Mrtra*-i and *Mrtra2*-i mated females, and haploid male and diploid female offspring from *gfp*-i females as determined by flowcytometry analysis. Statistical analysis of average haploid and diploid offspring number were performed with $: ANOVA/Tukey HSD and #: GLM/Tukey HSD. Letters in different cells refer to statistically significant difference with $p<0.05$. "n" refers to the number of replicate females that is used to produce offspring in each treatment. Standard errors are provided.

Treatment	$Male	#Male	#Female
	haploid	diploid	diploid
gfp-i (n=8)	6.75±0.77 **a**		11±0.64 **a**
Mrtra-i (n=4)	6±0.61 **a**	7.75±1.14 **a**	
Mrtra2-i (n=4)	5.5±0.56 **a**	1.5±0.25 **b**	

Discussion

In this research, we showed that sex determination gene orthologs *tra*, *tra2* and *Dsx* are present in *M. uniraptor* and *M. raptorellus*. In both species, *tra* and *Dsx* are sex-specifically spliced, whereas *tra2* is not, as confirmed by observations for all three genes in many other insect species (reviewed in Bopp et al., 2014; reviewed in Geuverink and Beukeboom, 2014; Geuverink

et al., 2017; Nissen et al., 2012; reviewed in Price et al., 2015; reviewed in Verhulst and van de Zande, 2015; reviewed in Verhulst et al., 2010b). Both *tra* and *tra2* are important components of the *Muscidifurax* sex-determination cascade, as reduction of *tra* or *tra2* maternal transcript provision leads to male differentiation of diploid fertilized eggs in *M. raptorellus* and of diploid asexually produced eggs in *M. uniraptor* despite a PI-*Wolbachia* infection.

Conservation of the *tra* and *tra2* genes in *Muscidifurax*

The splicing structures of *tra* and *tra2* are highly similar in both *Muscidifurax* species, but also show high resemblance to the splicing structure of *tra* and *tra2* in *N. vitripennis*, and *fem* and *tra2* in *A. mellifera* (Geuverink et al., 2017; Hasselmann et al., 2008; Nissen et al., 2012; Werren et al., 2010). Protein sequence analysis showed that both *Muscidifurax tra* and *tra2* cluster tightly together with their closely related species *N. vitripennis* and *T. dubius*. The presence of the specific Hymenoptera (HYM) motif encoded in all *tra* transcripts of *M. uniraptor* and *M. raptorellus* solidifies its conservation in the Hymenoptera (Verhulst et al., 2010a). Yet, only the female-specific *tra* splice variants encode a complete CAM domain in both species, suggesting the preservation of the auto-regulatory loop for continuous tra^{F} splicing in *Muscidifurax* species as shown for many other insect species (reviewed in Geuverink and Beukeboom, 2014; Verhulst et al., 2010a). *Tra2* is not sex-specifically spliced in both species, which is in line with the identified *tra2* transcripts in *N. vitripennis* and *A. mellifera* (Geuverink et al., 2017; Nissen et al., 2012). All *tra2* splice variants encode the RNA binding domain (RBD) indicating that the RNA binding property is present in all Tra2 isoforms (Inoue et al., 1992). The two variants $tra2^{NSS3}$ and $tra2^{NSS4}$ have low expression levels and are difficult to detect by RT-PCR. In addition, unidentified fragments present in *tra2* RT-PCR analysis could yield alternative *tra2* splice variants. However, these transcripts would be expected to encode the same protein as the first in-frame stop codon is encoded at the beginning of exon 5.

Variation in the *Dsx* gene between *Muscidifurax* species

Dsx alternative splicing differs between both *Muscidifurax* species in three regions. First, the first *MrDsx* exon is not alternatively spliced which differs from *MuDsx* that has a non-sex-specific alternatively spliced first exon. This difference in splicing does not affect the CDS. Alternative splicing of the 5' UTR is a relatively common feature, at least in the mammalian transcriptome (Araujo et al., 2012), and the alternative usage of first exon has been shown to control tissue-specific expression (Tsukamoto et al., 2007; Zhang et al., 2004). Alternatively,

non-sex-specific splicing of the first *Dsx* exon is also observed in other Hymenoptera (Mine et al., 2017; Wang et al., 2020, Chapter 4), but more research is needed to understand the regulatory function of the *Dsx* 5' UTR region in general. Effect of 5' UTR alternative splicing on *Dsx* expression patterns and the regulation of downstream genes in both *Muscidifurax* species also requires further investigation. Second, intron retention in the fourth exon of *Dsx* in *M. raptorellus* leads to the splicing of a non-sex-specific *Dsx* transcript. A similar type of intron retention was found in *A. rosae* and *N. vitripennis Dsx* splicing, but it results in a male-specific splice variant (Mine et al., 2017; Wang et al., 2020, Chapter 4). In *A. mellifera*, the non-sex-specific *Dsx* is assumed not to have a specific function in sex determination due to the absence of the OD2 domain (Cho et al., 2007). *MrDsx*^{NSS}, however, contains all the protein binding properties, including DM/OD1, and the common OD2. The exact function of *MrDsx*^{NSS} in sexual differentiation requires further investigation. Third, the additional female-specific *MuDsx* results also from intron retention in the fourth exon, and the equal expression level of both *MuDsx*^{F} splice variants throughout female developmental suggests both function in female sexual differentiation.

A comparison of both *Muscidifurax* Dsx protein sequences revealed one nonsynonymous substitution in the DM domain and two in the OD2 domain. As these conserved domains are crucial for Dsx to bind *cis*-regulatory sites to regulate downstream targets, the alteration in the amino acid sequence may affect its function. Sexual traits needed for reproduction are degenerated in *M. uniraptor* females and other insect species infected by PI-*Wolbachia* (Gottlieb and Zchori-Fein, 2001; Ma et al., 2014; Pannebakker et al., 2005) likely due to selection against the expressed female reproductive traits that could be costly in asexually reproducing females (Pijls et al. 1996). Further research is needed to determine whether the differences observed in *Dsx* in these two sister species is the result from *Wolbachia* infection and its induced thelytokous parthenogenesis.

Role of *tra* and *tra2* in *Muscidifurax* embryo viability and sex determination

Tra2 pRNAi in both *Muscidifurax* species reduces offspring numbers significantly compared to *tra*-i and *gfp*-i treatments. Based on the reduced hatching rate of embryos observed in *M. raptorellus* in combination with the presence of normal numbers of mature eggs in *M. uniraptor*, we suggest that this offspring reduction is due to embryo mortality. We find that mostly haploid offspring of mated *M. raptorellus* females develop into adults, suggesting that diploid embryos die during development. This is corroborated by the

observation that in *M. uniraptor* the offspring number of *tra2* pRNAi females is sharply reduced, which consists of diploid males only. However, further confirmation on diploid embryo mortality in *M. uniraptor* will be needed to verify this *tra2* pRNAi effect. This is in contrast to *N. vitripennis* and *A. mellifera* in which reduced *tra2* input affects both haploid and diploid embryo development (Geuverink et al., 2017; Nissen et al., 2012). Embryo mortality following *tra2* silencing in mothers was observed not only in insects (Geuverink et al., 2017; Nissen et al., 2012) but also in the predatory mite *Phytoseiulus persimilis* (Arachnida) (Sijia et al., 2019), suggesting that its role in early embryonic development is conserved in arthropods.

Maternal provision of both *tra* and *tra2* is a requirement for female development in *M. raptorellus* and *M. uniraptor* as depletion of either one of them in the mother results in male offspring development. The same effect has been observed in *N. vitripennis* and *A. mellifera* (Geuverink et al., 2017; Nissen et al., 2012; Verhulst et al., 2010b). This adds *Muscidifurax* to the list of Hymenoptera where *tra* and *tra2* are core elements of the sex-determination cascade needed for female development. Further research will show whether the female-specific *tra* splice variant is maternally provided to both *Muscidifurax* species as in *N. vitripennis* (Verhulst et al., 2010b) or the tra^{NSS} splice variant as in the parasitoid *Asobara tabida* (Geuverink et al., 2018a).

Conclusion

The sex-determination cascade evolves from the bottom upwards indicating that downstream sex-determination genes are normally more conserved than upstream genes (Wilkins, 1995). However, our comparison of the sex-determination genes in *M. raptorellus* and *M. uniraptor* shows that in this case the more upstream genes, *tra* and *tra2*, are more conserved than the most downstream gene, *Dsx*. The structure, expression pattern and function of *tra* and *tra2* in both species are highly similar, whereas the structure of *Dsx* shows clear differences. This discrepancy with current theory may result from the long-term co-infection of *M. uniraptor* with its endosymbiont *Wolbachia* that induces parthenogenesis. The known decay of costly sexual traits in insects infected with thelytoky-inducing endosymbionts (Gottlieb and Zchori-Fein, 2001; Ma et al., 2014; Pannebakker et al., 2005) may be taking place at the level of the central transcription factor for sexual differentiation, *Dsx*. Ultimately, understanding the mechanism by which unfertilized eggs develop into males in *M. raptorellus* but develop into females in the *Wolbachia*-induced thelytokous wasp *M. uniraptor*, requires more in-depth research into the sex determination mechanism of both *Muscidifurax* species.

Acknowledgements

We would like to thank Marcel Dicke for his insightful suggestions and valuable comments, Marlieke Dekker for her pioneering work in the identification of *M. uniraptor Dsx*. This work is part of the Dutch Research Council (NWO) research programme Innovational Research Incentives Scheme Veni with project number 863.13.014 granted to ECV.

Supplements

Table S2.1 Primers used in this research. "[]" T7 promoter sequence that is recommended by the manufacturer.

	Name	Sequence
Reference	RP49_F	5'-GTGTACAGGCCGAAAATCGT-3'
Reference	RP49_R	5'-CGCTTCTTGCTGCTAACTCC-3'
RNAi	U&R_TRA_RNAi_F2	5'-[TAATACGACTCACTATAG]CGTTCTGTGTCACTGAGTCC-3'
RNAi	U&R_TRA_RNAi_R2	5'-[TAATACGACTCACTATAG]AGTATACGGGGAATGGCACT-3'
RNAi	U&R_TRA2_RNAi_F1	5'-[TAATACGACTCACTATAG]CGTTCACATTCAAAGTCAAGCA-3'
RNAi	U&R_TRA2_RNAi_R1	5'-[TAATACGACTCACTATAG]GCCTATGTAAATTCCTGGGGTT-3'
RNAi	GFP_RNAi_F	5'-[TAATACGACTCACTATAG]GTGACCACCTTGACCTACG
RNAi	GFP_RNAi_R	5'-[TAATACGACTCACTATAG]TCTCGTTGGGGTCTTTGCT
qPCR	U&R_TRA_qpcr_F1	5'-AAGCACAGAAGTAGCAGATCTC-3'
qPCR	U&R_TRA_qpcr_R1	5'-CACTAGTTTCAGTTGCAGATGC-3'
qPCR	U&R_TRA2_qpcr_F1	5'-GCTTAGGCGTCTTTGGTTTATC-3'
qPCR	U&R_TRA2_qpcr_R1	5'-CTGCACTGTTCTTTAGCTACCT-3'
qPCR	WSP_F	5'-GCAAAATTTACGCCAGATGCTA-3'
qPCR	WSP_R	5'-ACCATTTTGACTACTCACAGCA-3'
3RACE	U&R_TRA_outer_3RACE	5'-GAACCGCAAGCGAAGATCAC-3'
3RACE	U&R_TRA_inner_3RACE	5'-CGTAGCAGGTCCGAAGAGTCGCA-3'
3RACE	U&R_TRA_outer_3RACE end	5'-AGAAGACGTCGGTCACCC-3'
3RACE	U&R_TRA_inner_3RACE end	5'-CGCACTCTAGAGAACGCGACTACCA-3'
3RACE	U&R_DSX_outer_3RACE	5'-AATTTGCCACCCCCTCTACC-3'
3RACE	U&R_DSX_inner_3RACE	5'-CACACAACTTCGCCCGCTGTCA-3'
3RACE	U&R_TRA2_outer_3RACE	5'-ACACAGAAGCGGAGTGGAAG-3'
3RACE	U&R_TRA2_inner_3RACE	5'-GCCGTAGCCGCTCACCCTAC-3'
5RACE	U&R_TRA_outer_5RACE	5'-TTCACAACACGTCGAATTTCAC-3'
5RACE	U&R_TRA_inner_5RACE	5'-AGTTTTGGAACCTCTTTTGGTCCAT-3'
5RACE	U&R_DSX_outer_5RACE	5'-ATTTGCTGCCTCTTGGTCGTGA-3'
5RACE	U&R_DSX_inner_5RACE	5'-TGACCTGGACCTTCTTGCCGTG-3'
5RACE	U&R_TRA2_outer_5RACE	5'-GAAAAGCAATCCAGAGGCGGCG-3'
5RACE	U&R_TRA2_inner_5RACE	5'-TCTCTGTTGGTGTCGCTAGCGC-3'
RT-PCR	U&R_DSX_5UTR_F	5'-ACCGACTGCATACTGGTGAG-3'
RT-PCR	U&R_DSX_5UTR_R	5'-GGACCTTCTTGCCGTGATTC-3'

RT-PCR	Rap_DSX_5UTR_F	5’-GCATGCTCGTACGCTGTATA -3’
RT-PCR	Rap_DSX_5UTR_R	5’-ACGCTTGTGAAACTTGACCT -3’
RT-PCR	U&R_TRA2_5UTR_F	5’-CGGAGCAGCTTTTGTTTCTG-3’
RT-PCR	U&R_TRA2_5UTR_R	5’-ACTTCTCGAGTGCGATCTAGT-3’
RT-PCR	U&R_TRAF&M_F	5’-TGGATGAGAAGGAACTGCGA-3’
RT-PCR	U&R_TRAF&M_R	5’-TGTGGACTAGGACTGCGATG-3’
RT-PCR	U&R_DSXF_F	5’-TGGCTACCATTGGCAATCGT-3’
RT-PCR	U&R_DSXF_R	5’-ACCGTTTCATGAACCCAGCT-3’
RT-PCR	U&R_DSXM_F	5’-GAGAAAGAAAACGGCCAAGTCC-3’
RT-PCR	U&R_DSXM_R	5’-TTCTATCTTGAAGCGATGGC-3’
RT-PCR	U&R_TRA2F&M_F	5’-GCGTGTGGTTGTCGTTATTG-3’
RT-PCR	U&R_TRA2F&M_R	5’-TGAAACTTGCAAACTTCCCTC-3’

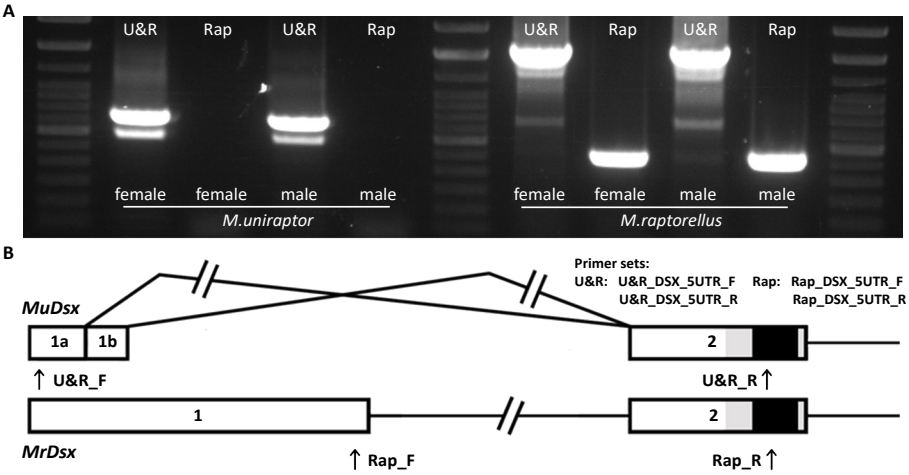


Figure S2.1 Confirmation of the alternative splicing of 5’ UTR in *MuDsx*. (A) Alternative splicing of exon 1 in *M. uniraptor* and the expression of longer exon 1 in *M. raptorellus*. **(B)** 5’ UTR structure of *MuDsx* and *MrDsx* and the primer binding sites. Targets (top) and name and the sex of the species (bottom) are indicated in the gel. Fragment size is indicated by 100bp ladder (Thermo Fisher). Visualized on 1.5% TAE agarose gel stained with Midori Green (NIPPON Genetics).

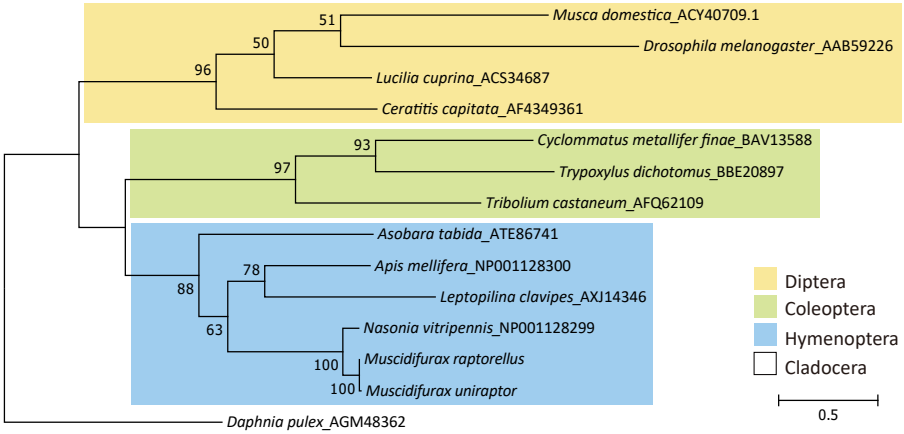


Figure S2.2 Molecular phylogenetic analysis of Transformer (Tra) in different insect species. Full sequences of female-specific Tra were used to construct the tree. The evolutionary history inferred by using the Maximum Likelihood method based on the Whelan And Goldman + Freq. model (Whelan and Goldman, 2001). A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 5.3071)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. 1000 bootstraps were applied and the percentage of replicate trees that are above 50% are shown at the nodes. Accession numbers (if applicable) were provided next to the species names. Colour blocks indicate the insect order of the species.

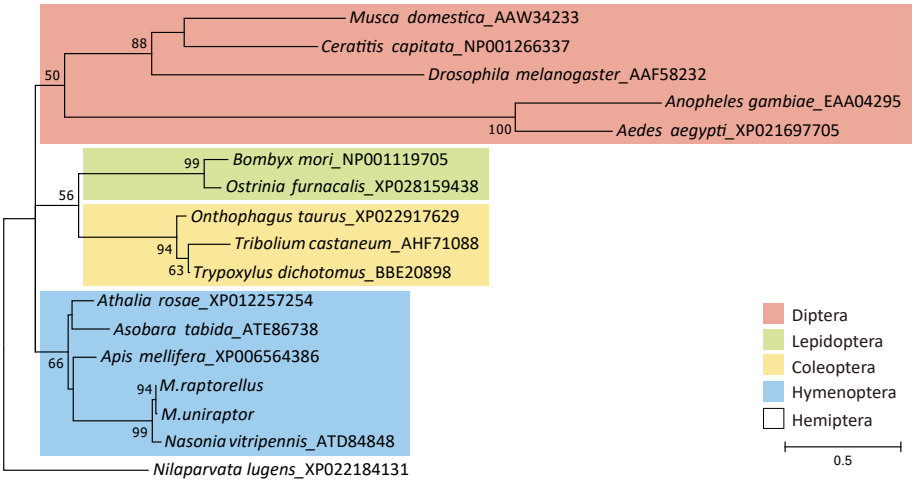


Figure S2.3 Molecular phylogenetic analysis of Transformer-2 (Tra2) in different insect species. Full sequences of Tra2 were used to construct the tree. The evolutionary history inferred by using the Maximum Likelihood method based on the Whelan And Goldman + Freq. model (Whelan and Goldman, 2001). A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 1.8333)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. 1000 bootstraps were applied and the percentage of replicate trees that are above 50% are shown at the nodes. Accession numbers (if applicable) were provided next to the species names. Colour blocks indicate the insect order of the species.

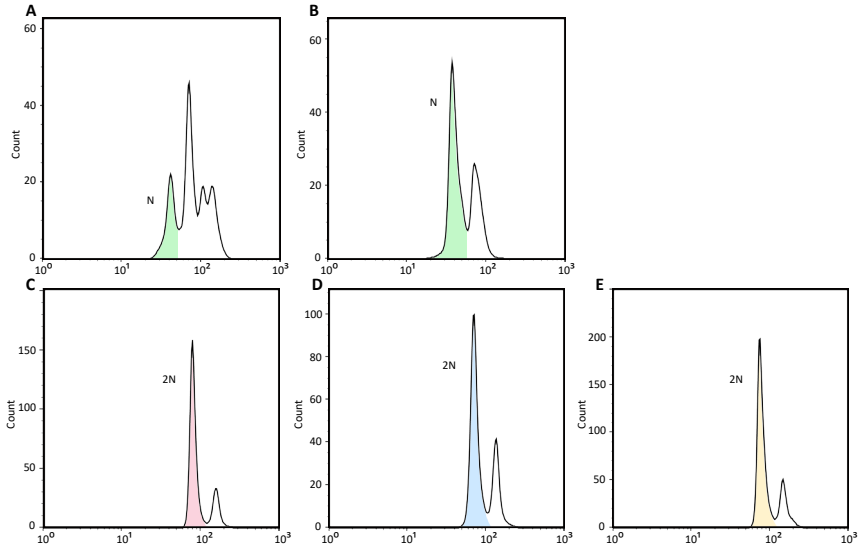


Figure S2.4 Representative plots depict the ploidy level of *gfp*-i, *Mrtra*-i, *Mrtra2*-i offspring and wild-type (WT) male and female of *M. raptorellus*. Peaks marked with colour in the figures represent the accumulation of the nucleus. **(A)** Ploidy plot of WT or *gfp*-i female produced male offspring (bad nuclei separation), **(B)** Ploidy plot of WT or *gfp*-i female produced male offspring (good nuclei separation), **(C)** Ploidy plot of WT female, **(D)** Ploidy plot of male offspring from *Mrtra*-i mother, and **(E)** Ploidy plot of male offspring from *Mrtra2*-i mother. Y axis refers to the number of the nuclei counts. 'N' indicates haploid sample and '2N' indicates diploid sample.

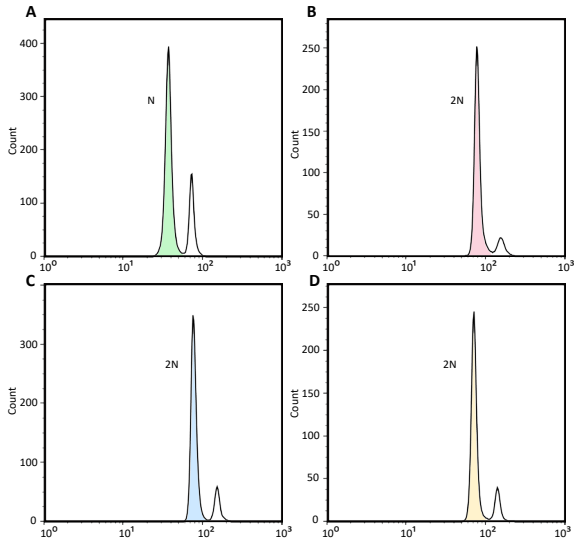


Figure S2.5 Representative plots depict the ploidy level of *gfp*-i, *Mutra*-i, *Mutra2*-i offspring and WT male and female of *M. uniraptor*. Peaks that marked with colour in the figures represent the accumulation of the nucleus in **(A)** Ploidy plot of male offspring from WT female or *gfp*-i female, **(B)** Ploidy plot of female offspring from WT female or *gfp*-i female, **(C)** Ploidy plot of male offspring from *Mutra*-i female and **(D)** Ploidy plot of male offspring from *Mutra2*-i female. Y axis refers to the number of the nuclei counts. 'N' indicates haploid sample and '2N' indicates diploid sample.

Chapter 3

Evidence for a one-step mechanism of endosymbiont-induced thelytoky in the parasitoid wasp, *Muscidifurax uniraptor*

Yidong Wang and Eveline C. Verhulst

Photo by Jennifer Wolve

Abstract

Wolbachia manipulates host reproduction in various haplodiploid insect species, in which fertilized eggs normally develop into diploid females while unfertilized eggs develop into haploid males. Females infected with a thelytoky-inducing *Wolbachia* produce diploid daughters from unfertilized eggs (thelytoky), but in some infected species diploid males spontaneously occur in low numbers. This suggests that diploidization and feminization are induced separately. In the *Wolbachia*-infected thelytokous parasitoid wasp, *Muscidifurax uniraptor,* occasional males have been found but with unknown ploidy. Therefore, we studied the mechanism of *Wolbachia*-induced thelytoky in *M. uniraptor* in the context of sex determination. We started by feeding different concentrations of tetracycline (antibiotic) to *M. uniraptor* females to gradually reduce the *Wolbachia* titre. We found that a decreased *Wolbachia* titre leads to an increased proportion of haploid male offspring, but we found no diploid males. Next, we studied the effect of *Wolbachia* infection on the expression and splicing of the sex determination genes *transformer* (*Mutra*) and *transformer-2* (*Mutra2*) in female ovaries and conclude that *Wolbachia* does neither affect the expression nor the splicing of *Mutra* and *Mutra2*. We then measured *Mutra* and *Mutra2* expression levels in developing zygotes at different time points and found a two-fold *Mutra* expression increase in *Wol+* compared to *Wol-*. Finally, we used the sexually reproducing sister species, *Muscidifurax raptorellus* and artificially created triploid females to determine whether diploidization is sufficient for feminization. These triploid females, when virgin, produced haploid sons and diploid daughters, showing that in *Muscidifurax* feminization solely depends on ploidy level. This strongly suggests that *Wolbachia* only needs to induce diploidization and that bi-allelic *Mutra* expression is sufficient for female development.

Introduction

All insects belonging to the order Hymenoptera have a haplodiploid mode of reproduction, whereby fertilized eggs develop into diploid females and unfertilized eggs develop into haploid males (Cook, 1993). However, in a small subset of haplodiploid species, unfertilized eggs develop into females in a process termed thelytoky. This type of parthenogenesis can be induced by nuclear genes (Beukeboom and Pijnacker, 2000; Lattorff et al., 2005; Rössler and DeBach, 1973; Schilder et al., 1999; Vavre et al., 2004; Verma and Ruttner, 1983) or by intracellular endosymbionts, such as *Wolbachia*, *Rickettsia* and *Cardinium* (Chigira and Miura, 2005; Furihata et al., 2015; Hagimori et al., 2006; Stouthamer et al., 1990; Zchori-Fein et al., 2004). These endosymbionts are vertically transmitted to the offspring only through the egg cytoplasm, as sperm cells do not contain enough cytoplasm (Ma and Schwander, 2017). By increasing the ratio of infected females in the population through endosymbiont-inducted thelytoky, they obtain a selective advantage. It is assumed that endosymbiont-induced thelytoky is only present in host species with haplodiploid sex determination as egg development can commence without fertilization (Leach et al., 2009).

Realizing diploidization of haploid eggs seems a prerequisite for endosymbionts to induce thelytoky in a haplodiploid reproduction system, as feminization would follow automatically from the host's haplodiploid mode of reproduction (Kageyama et al., 2012; Werren et al., 2008). However, in some endosymbiont-induced thelytokous species it was found that lowering the endosymbiont titre by antibiotic curing or heat treatment resulted in diploid male progeny, and only complete endosymbiont removal leads to haploid male progeny (Ma et al., 2015; Tulgetske, 2010). This suggests that a low endosymbiont titre is sufficient for diploidization and a higher endosymbiont titre is required for feminization, essentially decoupling diploidization and feminization from each other in a two-step mechanism (Ma et al. 2015).

The mechanisms by which endosymbionts would induce feminization in its host are unknown, and it is further complicated by the extraordinary diversity of hymenopteran sex determination mechanisms (Heimpel and de Boer, 2008). In all Hymenoptera studied to date, the splicing of terminal sexual differentiator, *Doublesex (Dsx)*, is controlled by the upstream splicing regulators Transformer (Tra) or its ortholog Feminizer (Fem), and Transformer-2 (Tra2) (Gempe et al., 2009; Geuverink et al., 2017; Hasselmann et al., 2008; Inoue et al., 1992; Nissen et al., 2012; Verhulst et al., 2010b). To guarantee female offspring

development in Hymenoptera, it is essential to maintain the female-specific splicing of *tra* (or *fem*). In the parasitoid wasp *Nasonia vitripennis*, *tra* and *tra2* are maternally provided to the eggs to initiate female-specific splicing of early zygotic *tra* messenger RNA (mRNA) (Geuverink et al., 2017; Verhulst et al., 2010b). The continuous splicing of female-specific *tra* (or *fem*) mRNA is regulated by its own protein, leading to female development (Geuverink et al., 2017; Verhulst et al., 2010b). Recently, it has been shown in *N. vitripennis* that an active copy of *wasp overruler of masculinization* (*wom*) from the paternal genome is also required for zygotic *tra* expression in order to start its autoregulation and female development (Beukeboom and van de Zande, 2010; Verhulst et al., 2010b; Verhulst et al., 2013; Zou et al., 2020). An inactive, imprinted *wom* copy from only the maternal allele does not lead to sufficient zygotic *tra* expression, resulting in male development (Zou et al., 2020). In the honey bee, *Apis mellifera*, heterozygosity at the *complementary sex determiner* (*csd*) locus and *tra2* expression result in initial female-specific *fem* splicing (Beye et al., 2003; Hasselmann et al., 2008; Nissen et al., 2012). Then, a positive feedback loop of female-specific *fem* splicing is mediated by its own Fem protein (Gempe et al., 2009). Hemi- or homozygosity at the *csd* locus results in male development (Beye et al., 2003). In *Asobara tabida*, although the exact initial signal leading to female-specific *tra* autoregulation is still unknown, maternal provision of *tra* and *tra2* are presumed to be important for female development (Geuverink et al., 2018a). The ubiquity of *tra2* and female-specific *tra*/*fem* and their crucial function in the female sex determination cascades, offers a potential for endosymbionts to manipulate their early expression in order to induce feminization.

In the *Wolbachia*-infected thelytokous wasp, *Muscidifurax uniraptor,* spontaneous males are occasionally produced by *M. uniraptor* females in laboratory rearing. It was shown previously that curing females from *Wolbachia* infection using antibiotics results in male offspring production, and an increasing dose of antibiotics results in a higher frequency of male occurrence (Zchori-Fein et al., 2000). However, diploid males have not been recorded in literature, and ploidy testing of the spontaneous males in our mass-rearing indicated they were haploid, suggesting that the two-step mechanism of endosymbiont-induced thelytoky is not applicable in this wasp.

Therefore, we investigated the mechanism by which *Wolbachia* induces thelytoky in *M. uniraptor* and propose a model for the sex determining cascade to understand the mechanism by which feminization could be achieved. First, we assessed the frequency of haploid and diploid male offspring after selective removal of *Wolbachia* from *M. uniraptor*

females using an increasing concentration of tetracycline. The sex determination genes in *M. uniraptor* share features with those of other hymenopterans, and *N. vitripennis* in particular, in terms of splicing structures and function (see Chapter 2). Based on this knowledge, we next analysed the association of *Wolbachia* presence with both maternal and zygotic expression and splicing of sex determination genes. As several sexual reproduction barriers prevent the establishment of *M. uniraptor* sexual lines, including a reduced ability to store sperm, female mating reluctancy and male infertility (Gottlieb and Zchori-Fein, 2001), we used the sister species *M. raptorellus* to generate triploid females that would asexually produce haploid and diploid offspring, to investigate whether diploidization is sufficient for feminization in this genus.

Our results suggest that in the genus *Muscidifurax*, being diploid is sufficient for female development. We prove that endosymbiont-induced thelytoky can be a one-step process, in which *Wolbachia* only has to induce diploidization which is automatically followed by feminization. Our analysis of the *M. uniraptor* sex determination mechanism is discussed in the light of the different mechanisms that *Wolbachia* can employ to induce thelytoky in haplodiploid insects.

Material and method

1. Insect rearing

Muscidifurax uniraptor and *M. raptorellus* were kindly provided to us by Jack Werren and Koppert Biological Systems respectively. The two cultures were continuously reared on *Calliphora spp.* pupae under laboratory conditions of light/dark 16h/8h at 25°C.

2. Partial *Wolbachia* removal from *M. uniraptor* female

To reduce the *Wolbachia* titre in *M. uniraptor* females, one 10% sucrose control solution and seven different concentrations of tetracycline solutions: $5x10^{-2}$%, $2.5x10^{-2}$%, $1.25x10^{-2}$%, $5x10^{-3}$%, $2.5x10^{-3}$%, $1.25x10^{-3}$% and $5x10^{-4}$% were prepared by diluting the tetracycline hydrochloride power (Sigma-Aldrich) into 10% sucrose solution. All prepared solutions were filtered by 0.22 µl Millex-GP Syringe Filter (Sigma-Aldrich) and stored at -20°C.

Newly emerged *M. uniraptor* females were collected and transferred to separate glass vials and closed with a cotton plug. A strip of filter paper was placed in each vial with 25 µl of

prepared tetracycline solution for 24 hours. Each treatment included 40 individual females. Ten females of each treatment were subsequently offered five *Calliphora* hosts to lay eggs, every 24 hours, for three consecutive days. Parasitized hosts were collected separately and incubated at rearing conditions until offspring emerged. For each mother, the offspring numbers and their sex was recorded. Since offspring emerging from the host presented on the first day were almost all females across all treatments, the offspring that emerged from the second- and third-day parasitized hosts was used for further ploidy analysis. Experiments conducted by Ma et al. (2015) suggest that diploid male offspring often has an intermediate *Wolbachia* titre, which we assumed to have in samples from our low concentration tetracycline treatments. Therefore, the heads from male wasps emerging from the four lowest tetracycline concentration treatments $5x10^{-3}$%, $2.5x10^{-3}$%, $1.25x10^{-3}$% and $5x10^{-4}$% were collected and stored at -20°C for ploidy check. The remaining 30 mothers from each treatment were dissected in a drop of 1 X PBS solution on glass slides to collect ovaries. Five ovaries were pooled together as one replicate, and six replicates per treatment were collected in 1.5 ml Eppendorf tubes and were fast frozen in liquid nitrogen before storage in -80°C.

3. Offspring ploidy level determination

Flowcytometry was used to identify the ploidy level of offspring from different tetracycline treatments following the protocol adapted from Ma et al. (2015). One frozen wasp head was put into an Eppendorf tube with 0.3 ml ice-cold Galbraith buffer. A plastic pestle was used to crack open the head and homogenize the sample. The homogenate was then poured through a 5 ml cell strainer cap (Stemcell Technologies) to filter out the debris, and was subsequently collected in 96-well plates. Samples were stained with 5 µl Propidium iodide (1.0 mg/ml, Biotium). The total DNA content of each sample was measured by a MACSQuant Analyzer (Miltenyi Biotec) and visualized by a log scale generated fluorescence intensity plot using MACSQuantify™ Software 2.11. Representative figures were generated by gating the raw data in Flowjo v10.6.2 (Becton, Dickinson & Company).

4. Collecting embryo stage samples

Newly emerged females were collected in separate glass vials, closed with a cotton plug and fed with either 25 µl of 10% sucrose solution for *Wolbachia*-positive control (*Wol+*) or 25 µl of 0.5% tetracycline in 10% sucrose solution for *Wolbachia*-negative treatment (*Wol-*). Solutions were first provided on strip-type filter papers to females for 12 hours. Then, the filter papers

were removed and two hosts were provided per single female to lay eggs within 12 hours. These procedures were applied for two consecutive days prior to embryo egg collection. In order to confirm complete *Wolbachia* removal, the parasitized hosts from these two days were collected and incubated at rearing conditions for offspring sex check. To facilitate the embryo egg collection, an egg-laying chamber was used as described in Wang et al. (2020). Fed females were transferred into the assembled vials in which they could only access the head portion of the fly host. After 24 hours training in the egg-laying chamber with a single host, females were given two hours to lay eggs in the hosts and 30 mins was used for egg collection. For later time point samples, parasitized hosts were first collected after a two-hour egg-laying period and incubated in the rearing condition before the embryo eggs were collected. Eight time windows of 0-2.5h, 2.5-5h, 5-7.5h, 7.5-10h, 10-12.5h, 12.5-15h, 15-17.5h and 17.5-20h were set for embryo collection. A needle with a hook was used for embryo collection to open the shell of the fly pupa and transfer the embryos into a 1.5 ml Eppendorf tube filled with 10 µl of absolute ethanol. More than 40 embryo were pooled as one sample and three replicates were collected per time point of both *Wol+* and *Wol-* treatments. The embryo samples for the *gfp* spike-in experiment were prepared as described before and were collected at 7.5-10h for both *Wol+* and *Wol-* treatment. Each sample contained exactly 35 embryos.

5. Maternal provision and zygotic expression of *Mutra* and *Mutra2*

To analyse the maternal provision and zygotic expression of *Mutra* and *Mutra2* alternative splice forms, collected ovaries or embryos were homogenized by plastic pestles and total RNA was extracted using ZR Tissue & Insect RNA MicroPrep™(Zymo Research) following manufacturer's instructions. For embryo samples, an on-column DNase treatment step (Zymo Research) was added during RNA extraction and 16 µl of DNase/RNase free water was added to each sample to recover the total RNA from the extraction columns. RNA from ovary samples was recovered from the extraction columns by adding 13 µl DNase/RNase free water to each sample. One µl of RNA was used to verify the RNA concentration on a spectrophotometer (DS-11-DeNovix). DNase treatment was carried out on ovary samples using RQ1 RNase-Free DNase (Promega) following manufacture's protocol. One µg RNA of each ovary sample or 0.5 µg RNA of each embryo sample was synthesized into cDNA by reverse transcriptase PCR (RT-PCR) with standard reaction mix (SensiFAST™ cDNA Synthesis Kit, Bioline) in a thermal cycler (Bio-Rad T100TM Thermal Cycler, Bio-Rad) with 5 minutes priming at 25°C, 15 minutes reverse transcription at 46°C and 15 minutes reverse transcription at 42°C followed by 5 minutes reverse transcriptase inactivation at 85°C.

GoTaq® G2 Flexi DNA Polymerase (Promega) was used according to manufacturer's instructions to prepare the mastermix for PCR (primer concentration: 0.4 µM, $MgCl^2$ solution: 1.5 mM). Target genes *Mutra* and *Mutra2* and internal control gene *MuRP49* were amplified with a standard PCR profile: 3 minutes at 95°C, 32 amplification cycles of 30 seconds at 95°C, 30 seconds at 55°C, 50 seconds at 72°C and a final extension of 5 minutes at 72 °C in a thermal cycler (T100TM Thermal Cycler, Bio-Rad). Primer sequences are listed in Table S3.1.

Ovary and embryo samples were further analysed with quantitative reverse transcriptase PCR (qPCR) to acquire the relative expression of *Mutra* and *Mutra2.* cDNA that was generated in the RT-PCR was diluted 50 times to be used as qPCR templates and qPCR mastermix was prepared according to SensiFAST™ SYBR® No-ROX Kit manual (primer concentration: 0.4µM, Bioline). *MuRP49* was used as the internal control for ovary samples and *MuEF-1α* was selected as the internal control for embryo samples. *Wolbachia surface protein* (*WSP*) was used for *Wolbachia* quantification. Primer sequences used in qPCR are listed in Table S3.1. qPCR was carried out using the CFX96TM Real-Time System (Bio-Rad) with Bio-Rad CFX Manager 3.1 Software (Bio-Rad). The standard qPCR profiles were used for all target genes consisting of 95°C for 3 minutes, 40 amplification cycles of 15 seconds at 95°C,15 seconds of 55°C, 30 seconds of 72°C followed by a melting curve check.

6. Quantification of *Mutra* and *Mutra2* in embryos using *gfp* spike-in

To account for the influence of ploidy levels in gene expression difference, we performed the quantification of *Mutra* and *Mutra2* in embryo at 7.5-10h between *Wol+* and *Wol-* treatments using *gfp* spike-in. *Gfp* template was first amplified from the vector pOPINEneo-3C-GFP which was a gift from Ray Owens (Addgene plasmid # 53534; http://n2t.net/addgene: 53534; RRID: Addgene_53534). Subsequently, *gfp* RNA was reverse synthesized using MEGAscript™ T7 Transcription Kit (Thermo Fisher) and purified using MEGAclear™ Transcription Clean-Up Kit (Thermo Fisher) following manufacturer's instructions. Purified *gfp* RNA was further quantified using Qubit RNA BR Assay Kit (Thermo Fisher) with Qubit 2.0 Fluorometer (Thermo Fisher). Prior to the embryo RNA extraction, 0.03ng *gfp* RNA was added to each sample. Subsequent embryo RNA extraction, cDNA synthesis and qPCR procedures were done as described in the former section. Primers details are provided in Table S3.1.

7. Generating triploid *M. raptorellus* females

To investigate whether female development relies solely on ploidy level in *Muscidifurax* species, triploid females of *M. raptorellus* were created. We used the same parental RNA interference (pRNAi) protocol described in Chapter 2 to maternally silence the *Mrtra* in pupal stage *M. raptorellus* females to first produce diploid males. These diploid males were mated with virgin females to create triploid female offspring. The rearing scheme is illustrated in Figure S3.1.

Initially, 40 F0 female pupae of *M. raptorellus* were injected with *Mrtra* dsRNA. Once these injected F0 females emerged, we kept them in separate glass vials sealed with cotton plugs. A single male was provided to each F0 female for 24 hours for mating. Thereafter, one fly pupa host was offered to each female and another 24 hours were given for oviposition. Subsequently, the F0 females were freeze-killed and stored in -80°C, and parasitized pupa hosts were incubated in rearing condition until F1 adult emerged. Eight F0 females that produced only male offspring were chosen and their offspring was used for the next step. All F1 males (being either haploid or diploid) from the eight selected F0 females were mated with a virgin female for 24 hours and subsequently freeze-killed and stored in -80°C. Each female that mated with a F1 male was given a single fly pupa host for oviposition for 24 hours, resulting in F2 female offspring that was subsequently collected before eclosion and separated in individual glass vials. These F2 females (containing both triploid and diploid individuals) were further provided with five fly hosts per day for four consecutive days to boost their offspring production. When we observed females in F3, these F3 females were used for further analysis and their male siblings (if present) were freeze-killed and stored in -80°C. To assess the reproductive ability of females uniparentally produced by triploid mothers, we set them up to produce offspring as virgins and after mating. We collected five F3 virgin female offspring of triploid mothers and provided them with two fly pupa as hosts per female to check their asexual reproductive ability and offspring sex. The remaining F3 females were given 24 hours to mate with males, and two hosts per mated female were offered for 24 hours to check their sexual reproductive ability (including mating) and offspring sex. After oviposition, tested F3 females were freeze-killed and stored in -80°C. All the offspring in each generation that was descendent from same F0 female, F1 male and F2 female was noted and the numbers of male and female offspring of each generation from each mother were counted. After collecting all samples, the heads of the wasps were used to verify the ploidy level in flowcytometry following the same procedures described in former section.

8. Data analysis

qPCR data was first imported to LinRegPCR software (LinRegPCR, 2017.1.0.0, HFRC, Amsterdam, The Netherlands) (Ramakers et al., 2003). After baseline correction, the initial number of templates (N0) were calculated based on the average PCR efficiency of each amplicon. Relative expression levels of *NvDsx* in each sample was obtained by dividing the N0 value of *NvDsx* by N0 value of internal or external reference genes. The absolute mass of each transcript in the *gfp* spike experiment is based on the ratio of its N0 and *gfp* N0 and multiplied by 0.03. Then, the exact transcript number is calculated using the formula:

Transcript number=(Mass*6.022×10^{23})/(Length*10^{9}×321.47)

One mole is 6.022x10^{23} molecules; Length – transcript length (*Mutra*: 172bp, *Mutra2*:184bp, *WSP*: 174bp); Mass - Average weight of an RNA base pair (bp) is 321.47 Daltons.

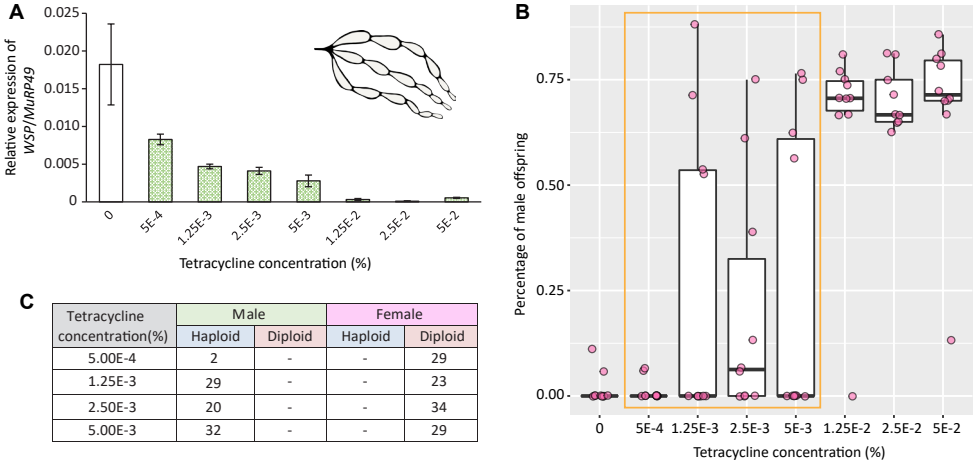
Statistical analysis was performed in R (R core Team, 2018). Ovary qPCR data was analysed using One-way ANOVA and Tukey's honest significant difference (HSD) as post hoc test or Kruskal-Wallis multiple comparison Dunn-test; embryo different developmental stage and *gfp* spike-in qPCR data were analysed by *t*-test and corrected with false discovery rate (FDR), and Kruskal-Wallis rank sum test. Triploid female offspring data was analysed using Kruskal-Wallis rank sum test and Dunn-test.

Results

Reduction of *Wolbachia* titre in *M. uniraptor* female ovaries leads to haploid male offspring production

To investigate thelytokous reproduction induced by *Wolbachia* in our study species *M. uniraptor,* we first asked if diploidization and feminization is decoupled by reducing the amount of *Wolbachia* in females. We expected to find diploid male offspring if *M. uniraptor* follows a two-step feminization model as described in Ma et al. (2015). We reduced the *Wolbachia* titre in a step-wise fashion by feeding increasing concentrations of tetracycline to different groups of freshly emerged female wasps. The results show that we managed to partially remove *Wolbachia* in different treatments and, importantly, that *Wolbachia* titre in female ovaries is inversely correlated with increasing tetracycline concentrations (Figure 3.1A). A decreased *Wolbachia* titre directly affects the percentage of male offspring which

decreased from around 70% in the treatment with the highest tetracycline concentration to almost 0% in the treatment with lowest concentration (Figure 3.1B). We observed that female offspring always emerged from the fly hosts provided on the first day, regardless of the tetracycline concentration treatment. Therefore, we could not obtain a complete sex reversal even in the treatment with the highest antibiotic concentration. We assume that the penetration of antibiotic was incomplete in the first set of matured eggs, and penetration increased with egg maturation over the next two consecutive days as was shown by Zchori-Fein et al. (2000). In four treatments with lower tetracycline concentrations, *Wolbachia* titre was reduced to intermediate levels in the female ovaries, and after examining offspring ploidy in these treatments, we found no diploid males (Figure 3.1C, S3.2C,D).



C

Tetracycline concentration(%)	Male		Female	
	Haploid	Diploid	Haploid	Diploid
5.00E-4	2	-	-	29
1.25E-3	29	-	-	23
2.50E-3	20	-	-	34
5.00E-3	32	-	-	29

Figure 3.1 Lower *Wolbachia* titre is inversely correlated with increasing percentage of haploid male offspring in tetracycline treated females. (A) Relative expression of *Wolbachia* gene, *WSP/MuRP49,* in different tetracycline treated female ovaries. The concentration of tetracycline solution of each treatment is indicated on the X-axis. Mean plus standard error is given for each treatment (n=6). **(B)** Percentage of male offspring from females treated with increasing concentrations of tetracycline. The box-and-whisker plots show median (horizontal line), 25-75 % quartiles (box) and maximum/minimum range (whiskers). The pink data points represent the individual sample points (n=10). The yellow box indicates the treatments that are used for ploidy level confirmation. The concentration of tetracycline solution of each treatment is indicated on the X-axis. **(C)** Ploidy level of the offspring produced by female treated with different concentrations of tetracycline.

Mutra and Mutra2 expression is not affected by *Wolbachia* density in the ovaries

Previous data (Chapter 2) confirmed that the maternal provision of both *Mutra* and *Mutra2* plays an essential role in female development. Silencing *Mutra* or *Mutra2* in female *M. uniraptor*, results in 100% male offspring regardless of the presence of *Wolbachia*. We

hypothesized that *Wolbachia* is involved in the regulation of maternal provision of *Mutra* and *Mutra2* transcripts to the eggs. Therefore, we performed both RT-PCR and qPCR to study their expression pattern and expression level in tetracycline-treated female ovaries. The expression level of *Mutra* varied among treatments with increasing dose of tetracycline (Figure 3.2A). We conclude that there is no correlation between *Mutra* expression and

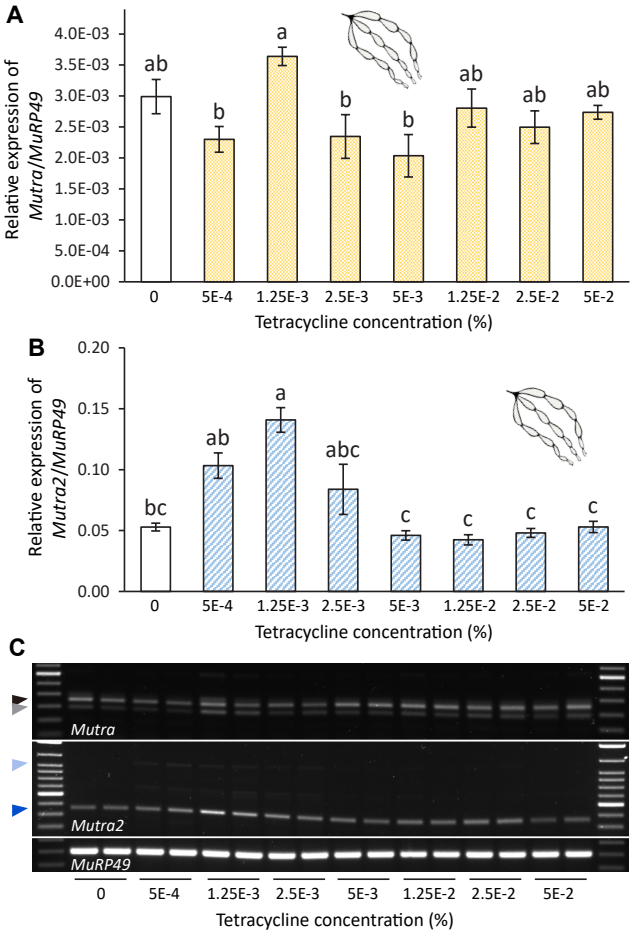


Figure 3.2 *M. uniraptor transformer* (*Mutra*) and *M. uniraptor transformer-2* (*Mutra2*) expression and splicing patterns in the ovaries of females treated with different concentrations of tetracycline. Relative expression of **(A)** *Mutra/MuRP49* and **(B)** *Mutra2/MuRP49* in ovaries of *M. uniraptor* females treated with increasing dose of tetracycline concentration (x-axis represents the percentage of tetracycline solution). Error bars represent standard error (SE) (n=6). Statistics was performed with One-way ANOVA and Tukey's HSD for A, with Kruskal-Wallis multiple comparison Dunn-test for B. Letters refer to statistically significant differences with $P<0.05$. **(C)** Gel electrophoresis of RT-PCR products of *Mutra* and *Mutra2* splice variants and *MuRP49* fragments in ovaries of *M. uniraptor* females treated with increasing tetracycline concentration indicated under the gels. Black and grey arrows refer to non-sex-specific (NSS) and female-sex specific (F) *Mutra*, dark blue and light blue arrows refer to more abundant non-sex specific splicing *Mutra2*^{NSS1} and *Mutra2*^{NSS2}. *MuRP49* serves as internal control and 100bp ladder (Thermo Fisher) indicates fragment size. Fragments are visualized on 1.5% TAE agarose gel stained with Midori Green (NIPPON Genetics).

Wolbachia titre (Figure 3.2A). *Mutra2* expression on the other hand, significantly increased in the 1.25x10^{-3}% tetracycline-treated group compared to the non-treated group (P=0.028, Figure 3.2B). However, in the treatment groups with increasing dose of tetracycline, *Mutra2*

expression decreased to levels comparable to the non-treated group (Figure 3.2B). We cannot explain the variable levels of *Mutra2* in response to tetracycline treatment of the females and suggest that observed differences in *Mutra2* expression are due to biological variation and are not related to *Wolbachia* titre (Figure 3.2B).

Next, we hypothesized that *Wolbachia* is involved in the sex-specific splicing of *Mutra* and *Mutra2* transcripts that are maternally provide to the eggs which would result in a shift from female-specific splicing to male-specific splicing. Currently, three *Mutra* splice variants have been identified in *M. uniraptor* (see Chapter 2), including a male-specific $Mutra^{M}$, a female-specific $Mutra^{F}$, and a non-sex-specific $Mutra^{NSS}$. Both $Mutra^{F}$ and $Mutra^{NSS}$ were found to be expressed in the female ovary (Figure 3.2C) while only $Mutra^{F}$ was present in the rest of the female body (Figure S3.3). The splice variant $Mutra^{F}$ and $Mutra^{NSS}$ are present in all treatment groups (Figure 3.2C), and a relatively higher expression level of $Mutra^{NSS}$ compared to $Mutra^{F}$ was observed. However, expression of both splice variants is similar across different treatment groups (Figure 3.2C). The non-sex specific $Mutra2^{NSS1}$ is predominantly expressed in all treatment groups (Figure 3.2C). $Mutra2^{NSS2}$ splice form was found to be very abundant in whole body tissues in different developmental stages of female (see Chapter 2, Figure 2.3) but is barely expressed in ovaries (Figure 3.2C). Concludingly, we observed no shift in *Mutra* or *Mutra2* splicing patterns among treatments with increasing dose of tetracycline (Figure 3.2C), and we determine that *Wolbachia* is not involved in sex-specific splicing of *Mutra* or *Mutra2* in female ovaries.

Wolbachia affects zygotic expression of *Mutra* but not *Mutra2* during early embryogenesis

Wolbachia infection does not affect expression levels or splicing patterns of maternal provision of *Mutra* and *Mutra2*, still, a reduction in *Wolbachia* titre results in a shift of offspring sex to males. Therefore, we hypothesized that *Wolbachia* manipulates sex determination during early zygotic development by changing zygotic expression of sex determination genes. We analysed the zygotic expression profiles of *Mutra* and *Mutra2* in embryo samples from both tetracycline-treated (*Wol*-) and non-treated (*Wol*+) females that were collected in eight time windows from zero to 20 hours post oviposition (hpo). We first confirmed that *Wol*- zygotes have significantly reduced *Wolbachia*-infection throughout zygotic development, showing the success of antibiotic treatment (Figure 3.3A). *Mutra* expression increased from zero to 10 hpo and showed peak expression at 7.5-10 hpo (Figure 3.3B). No significant difference of

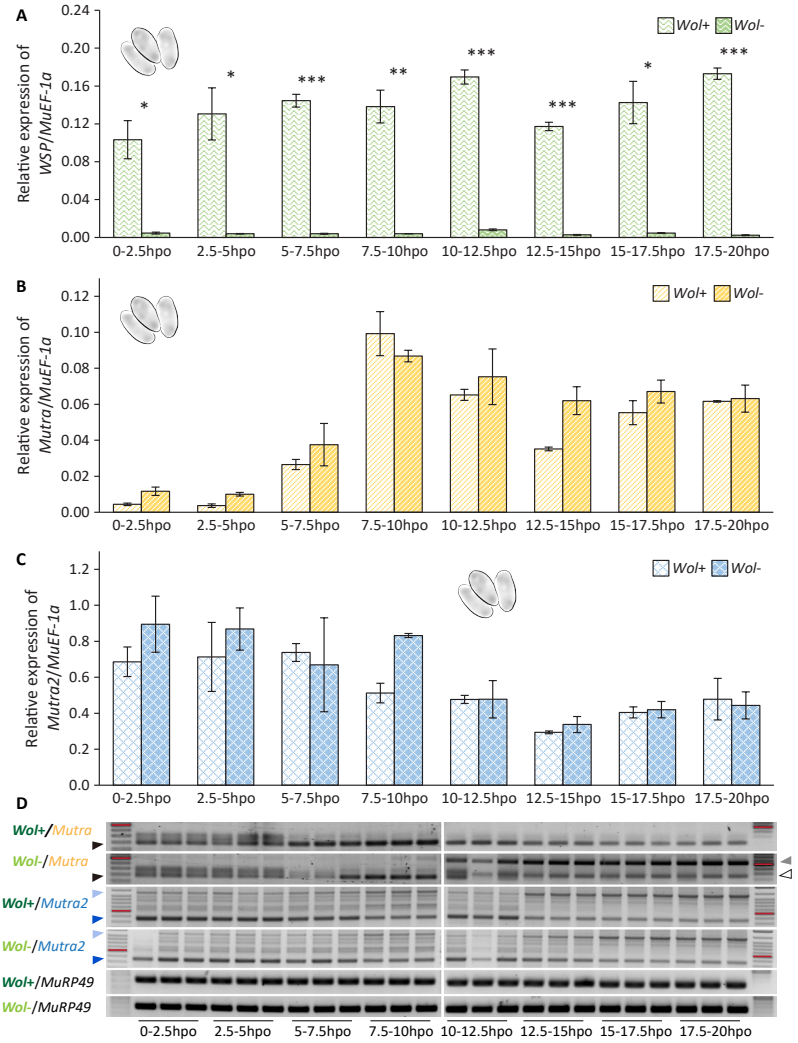


Figure 3.3 Expression of *M. uniraptor transformer (Mutra)* and *M. uniraptor transformer-2* (*Mutra2*) in early embryogenesis of *Wolbachia* positive (*Wol+*) and *Wolbachia* negative (*Wol-*) embryos. Figures show the relative expression of **(A)** *Wolbachia* gene, *WSP/MuEF-1a,* **(B)** *Mutra/MuEF-1a* and **(C)** *Mutra2/MuEF-1a* in embryo samples from both tetracycline-treated (*Wol-*) and non-treated (*Wol+*) females at eight developmental time windows from zero to 20 hours post oviposition (hpo). Error bars are standard errors with n=3. Statistics was performed with *t*-test and corrected with false discovery rate (FDR) for A, B and C. Asterisks were assigned for significant differences: * = $P<0.05$, ** = $P<0.01$ and *** = $P<0.001$. **(D)** Gel electrophoresis of RT-PCR products of *Mutra* and *Mutra2* splice variants and *MuRP49* fragments in *Wol-* embryos and *Wol+* embryos at eight developmental time windows from zero to 20 hours post oviposition (hpo). Black, grey, white, light blue and dark blue arrows indicate $Mutra^{F}$, $Mutra^{M}$, $Mutra^{NSS}$, $Mutra2^{NSS2}$ and $Mutra2^{NSS1}$ respectively. MuRP49 serves as internal control and 100bp ladder (Thermo Fisher) indicates fragment size (600bp fragments in ladders are marked with red lines). Fragments are visualized on 1.5% TAE agarose gel stained with Midori Green (NIPPON Genetics).

Mutra expression between *Wol*- and *Wol*+ treatments was found in 0-2.5 hpo, corroborating that *Wolbachia* does not affect maternal provision of *Mutra*. From 2.5-7.5 hpo onwards there is no difference in *Mutra* expression between *Wol*+ and *Wol*+. After the peak, *Mutra* expression remains consistent from 10 hpo onward with no significant differences in both treatments, although overall a slightly higher expression of *Mutra* was observed in *Wol*- treatment (Figure 3.3B). Next, we determined *Mutra* sex-specific splicing during zygotic development in *Wol*- and *Wol*+ embryos (Figure 3.3D). From 0-5 hpo, the maternal provision of $Mutra^{NSS}$ *and* $Mutra^{F}$ is visible in both *Wol*- and *Wol*+ treatments (Figure 3.3D). The zygotic expression of $Mutra^{F}$ increases visibly from 5-10 hpo in *Wol*+ embryos and only from 7.5-10 hpo in *Wol*- embryos except for one sample that increases visibly from 5-10 hpo in *Wol*- embryos. Overall, *Mutra* expression starts earlier and appears stronger in *Wol*+ embryos compared to *Wol*- embryos in the 5-10 hpo developmental window. From 10 hpo onwards a clear shift in sex-specific splicing from $Mutra^{F}$ to $Mutra^{M}$ and $Mutra^{NSS}$ can be observed in *Wol*- embryos, whereas *Wol*+ embryos maintain $Mutra^{F}$ splicing (Figure 3.3D). Therefore, we conclude that in *Wol*- embryos male development initiates from 10 hpo, but the effect of *Wolbachia* infection on the zygotic *Mutra* expression levels are not conclusive from this experiment.

Mutra2 is also maternally provided to the eggs (0-2.5 hpo) and shows no difference in expression in both *Wol*+ and *Wol*- embryos from all time windows. *Mutra2* expression is approximately 10-fold higher than *Mutra* expression at 7.5-10 hpo, and remains relatively high until 10 hpo after which it gradually decreases (Figure 3.3C). Next, we determined *Mutra2* sex-specific splicing during early embryogenesis and observed that in both *Wol*+ and *Wol*- treatments splice variants remain unchanged (Figure 3.3D). Most abundant splice variants are $Mutra2^{NSS1}$ and $Mutra2^{NSS2}$, but also the less abundant splice variants $Mutra2^{NSS3}$ and $Mutra2^{NSS4}$ are visible (Figure 3.3D). We observed that $Mutra2^{NSS1}$ is expressed predominantly from 0-12.5 hpo in both treatments and decreases in expression from 12.5 hpo onwards, while $Mutra2^{NSS2}$ expression increases from 12.5 hpo onwards in both treatments (Figure 3.3D). We conclude that *Wolbachia* does not affect *Mutra2* splicing and expression.

We could not definitively conclude whether or not *Wolbachia* affects *Mutra* expression using relative quantification, but we asked if a diploid individual would have a double dose of *Mutra*, caused by bi-allelic expression, when compared to *Mutra2* expression. Therefore we performed an absolute quantification of *Mutra* and *Mutra2* expression in *Wol*- and *Wol*+ embryos. We collected an equal number of embryos per sample for both treatments, and

added a predefined amount of an exogenous synthesized *gfp* RNA (spike RNA) as external standard in every sample before RNA extraction. In this way we could calculate the exact initial RNA input of different samples and correct this against the amount of *gfp* spike RNA for technical variation. As relative *Mutra* expression increases rapidly from 7.5 hpo and the shift of female-specific splicing to male-specific splicing occurs from 10 hpo, we chose to sample at the 7.5-10 hpo time window.

We first confirmed that also in this experiment *Wol*- embryos have a significant reduction of *Wolbachia* (*P*=0.006, Figure 3.4A). Next, we found that the *Mutra* expression in *Wol*+ embryos is more than two-fold compared to the *Mutra* expression in *Wol*- embryos (*P*=0.003, Figure 3.4B). As expected, we did not find a significant difference in *Mutra2* expression between *Wol*- and *Wol*+ treatments (*P*=0.535, Figure 3.4C). These results show that a *Wolbachia*-infected diploid individual has a two-fold higher *Mutra* expression than an uninfected haploid individual, suggesting that either *Wolbachia* regulates *Mutra* expression or *Mutra* has bi-allelic expression compared to *Mutra2* and possibly other zygotic genes. This hints towards a sex-determination model in which diploidization results in enough *Mutra* expression to maintain the positive autoregulatory loop of female-specific *Mutra* splicing as shown for other Hymenoptera.

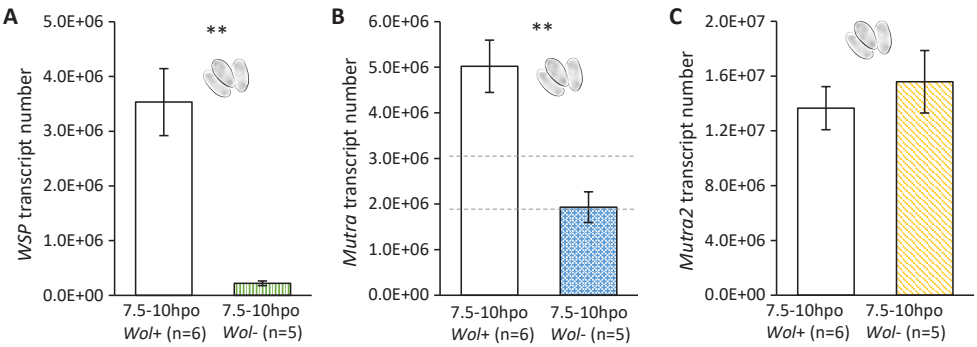
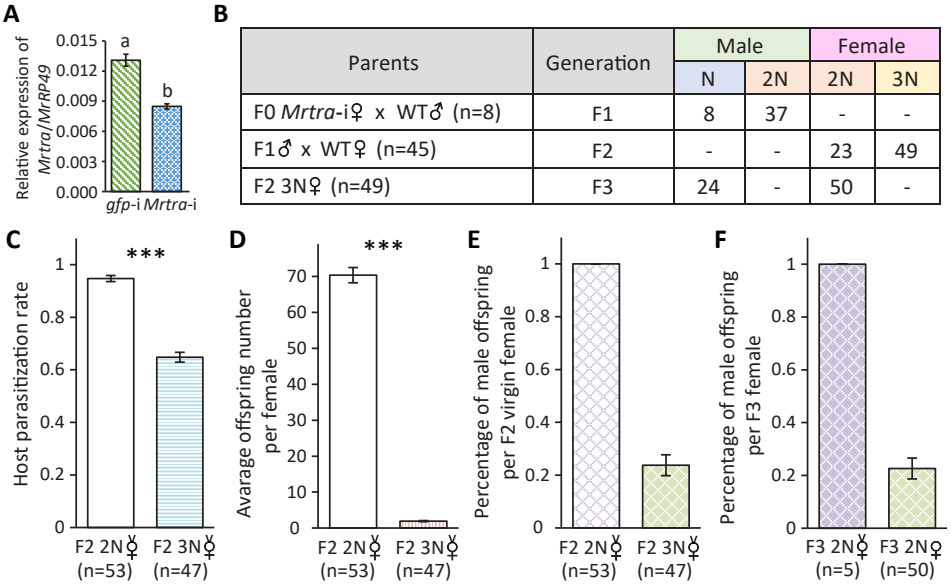


Figure 3.4 Expression of *WSP*, *Mutra* and *Mutra2* in *Wolbachia* positive (*Wol+*) and *Wolbachia* negative (*Wol-*) embryos at 7.5-10 hours post oviposition (hpo). Figures show **(A)** *WSP*, **(B)** *Mutra* and **(C)** *Mutra2* transcript number in embryo samples from both tetracycline-treated (*Wol-*) and non-treated (*Wol+*) females. RNA spike is *gfp*. Error bars represent standard errors. Dashed lines in (B) represent the upper and lower range of mono-allelic *Mutra* expression in *Wol+* control embryos. Statistics was performed with Kruskal-Wallis rank sum test for A, with One-way ANOVA for B and C. Asterisks represent significant difference: ** = P<0.01.

Ploidy level determines the sex in *M. raptorellus* and possibly *M. uniraptor*

In *M. uniraptor*, *Wolbachia* induces diploidy restoration of unfertilized eggs after completion of the first mitosis directly after egg laying (Gottlieb et al., 2002). We hypothesized that in *M. uniraptor*, *Wolbachia*-induced diploidization is sufficient for feminization and *Wolbachia*-induced thelytoky is a one-step mechanism. In *N. vitripennis* it has been shown that triploid females produce diploid and haploid offspring when virgin (Beukeboom and Kamping, 2006), and triploid females can be artificially created by mating a diploid female to a diploid male (Leung et al., 2019). In Chapter 2, we show that parental RNAi of *Mutra* or *Mrtra* results in a shift of female development into male development, thereby creating functional diploid males. In order to prove our hypothesis, we used *M. raptorellus*, a sexually reproducing sister species of *M. uniraptor* to first generate diploid males by maternally silencing *Mrtra* in females and then mate these diploid males with normal diploid *M. raptorellus* females to obtain triploid females (Figure S3.1). After successfully silencing *Mrtra* in F0 females (Figure 3.5A), these females were mated to normal haploid males to produce diploid male F1 offspring (Figure S3.1). These diploid males were subsequently mated to normal diploid females to produce triploid female offspring and haploid male offspring in the F2 (Figure S3.1).

Flowcytometry analysis confirms that we obtained 37 diploid males in the F1 offspring and 49 triploid females in the F2 offspring (Figure 3.5B, Figure S3.2H,J). These F2 triploid females were set up as virgin and parthenogenetically produced male F3 offspring and female F3 offspring (Figure 3.5B, Figure S3.1), which were confirmed to be haploid and diploid respectively (Figure S3.2K,L). Compared to the F2 diploid females, these F2 triploid females parasitized significantly fewer fly hosts, and offspring likely suffered from aneuploidy, resulting in extremely low average offspring numbers (Figure 3.5C,D). However, despite the low fecundity of F2 triploid females, they produced a high proportion of impaternate (uniparental reproduction by mother) female offspring by thelytokous reproduction (Figure 3.5E). The impaternate F3 diploid females were tested for asexual reproduction, and were mated to normal haploid males to confirm their ability to mate and produce offspring. Result shows that impaternate F3 diploid females parthenogenetically produced only F4 male offspring and after mating produced F4 male and female offspring in a ratio similar to F3 triploid females (Figure 3.5F). We conclude that in *M. raptorellus* and, by extension, in *M. uniraptor*, being diploid is sufficient for female development.



Parents	Generation	Male		Female	
		N	2N	2N	3N
F0 *Mrtra*-i♀ x WT♂ (n=8)	F1	8	37	-	-
F1♂ x WT♀ (n=45)	F2	-	-	23	49
F2 3N♀ (n=49)	F3	24	-	50	-

Figure 3.5 Triploid *M. raptorellus* females produce impaternate (uniparental reproduction by mother) diploid females but have low fecundity. (A) Relative expression of *Mrtra/MuRP49* in *Mrtra* silenced (*Mrtra*-i) females and *gfp/MuRP49* in *gfp* silenced (*gfp*-i) females. **(B)** Table showing the number of parental crosses to generate polyploid offspring, the generation number, and the amount of haploid, diploid and triploid offspring and their sexes. Number of parental crosses in each generation is indicated in the column "Parents" with "n". "N", "2N" and "3N" refers to haploid, diploid and triploid male or female offspring. **(C)** Host parasitization rate of F2 diploid and triploid females. **(D)** Average offspring number of F2 diploid and triploid females. **(E)** Proportion of male offspring produced by F2 diploid and triploid virgin females. **(F)** Proportion of male offspring produced by F3 diploid virgin and mated females. "2N" and "3N" in C, D, E and F refers to diploid and triploid females. "v" on top of the female symbols represents virgin females. Error bars are standard error of A (n=4), C, D, E and F. Statistics was performed with *t*-test for A, with Kruskal-Wallis rank sum test for C, D. Asterisks represent significant difference: *** = $P<0.001$.

Discussion

In this research, we investigated the possible regulatory mechanism for *Wolbachia* to induce thelytoky in *M. uniraptor*. As spontaneous diploid males are reported in several wasp species infected with PI-*Wolbachia*, it was assumed that *Wolbachia*-induced thelytoky would take two steps: diploidy restoration and feminization. We asked if *Wolbachia*-induced thelytoky in *M. uniraptor* also requires two steps. We gradually lowered the *Wolbachia* titre in *M. uniraptor* female ovaries by oral feeding increasing concentrations of tetracycline solution, and observed a proportional increase in the male offspring from *Wolbachia*-reduced females. This result confirms the observation by Zchori-Fein et al. (2000) that female development requires a sufficient amount of *Wolbachia* in the ovaries. Using flowcytometry,

we verified that even the lower concentrations of tetracycline treatments led to haploid male offspring development, indicating that in *M. uniraptor* separate diploidization and feminization steps are not needed for thelytokous reproduction. Our result is different from *Asobara japonica* and *Trichogramma kaykai* studies that show that *Wolbachia* uses a two-step mechanism to achieve thelytoky (Ma et al., 2015; Tulgetske, 2010). In addition, this sex determination mechanism supports a long-standing model in Hymenoptera in which ploidy itself determines sex.

Further studies on the sex determination genes *tra* and *tra2* in *Wol*- and *Wol*+ female ovaries suggests that neither the expression levels nor the expression patterns of these genes are correlated with the *Wolbachia* titre, indicating that *Wolbachia* does not manipulate the level of *Mutra* and *Mutra2* maternal provision. By comparing the relative expression of *Mutra* and *Mutra2* between *Wol*- and *Wol*+ treatments during early embryo development, we observed comparable expression level of *Mutra* and *Mutra2* from both *Wol*- and *Wol*+ embryos during eight developmental time windows. Strikingly, in both *Wol*- and *Wol*+ the expression of *Mutra* peaked at 7.5-10 hpo, but the splicing patterns of *Mutra* displayed a clear shift from $Mutra^{F}$ to $Mutra^{NSS}$ and $Mutra^{M}$ from 10 hpo onwards only in *Wol*- treatment and not in *Wol*+ treatment. This suggests that the expression of $Mutra^{F}$ cannot be maintained from 10 hpo onwards without *Wolbachia*. Absolute quantification of transcript numbers of *Mutra* and *Mutra2* at 7.5-10 hpo showed that *Mutra* expression increased significantly only in *Wol*+ embryos, while no significant changes in the *Mutra2* expression were detected in *Wol*+ embryos compared to *Wol*- embryos. Intriguingly, the reduction of *Mutra* in *Wol*- treatment is within the range of half the *Mutra* expression in *Wol*+ treatment, indicating it could due to the bi-allelic expression of *Mutra* in diploid embryos. This sudden increase of *Mutra* expression at 7.5-10 hpo resembles the more than ten-fold higher expression during peak expression in fertilized embryo of *N. vitripennis* compared to unfertilized embryos (Verhulst et al., 2010b; Zou et al., 2020). Assuming the *tra* auto-regulatory loop is well conserved in *Muscidifurax* as has been shown in almost all identified insect species (Verhulst et al., 2010a), the threshold for the number of *tra* transcripts needed to initiate and maintain the auto regulatory loop is unclear and could vary among species.

By silencing *Mrtra* in *M. raptorellus* females with subsequent mating, we artificially created diploid males in *M. raptorellus*. Mating these diploid males to normal diploid females generated triploid females and these triploid females in our experiments could parthenogenetically reproduce diploid female and haploid male offspring. This further

strengthens the idea that diploidization is the only requirement for female development in *Muscidifurax*. *Wolbachia* would then achieve diploidy restoration in *M. uniraptor* by the end of the first mitosis as shown by Gottlieb et al. (2002), and it would not need to take control of the sex determination system.

One remaining question for our proposed *Muscidifurax* sex determination model is whether there is dosage compensation. Dosage compensation has first been observed in *Drosophila*, in which X-chromosome-linked genes, although having a double copy in females (XX) and one copy in males (XY), show equal expression levels in both sexes. Further research discovered that the number of X chromosomes controls the X-chromosome-located *Sex lethal* (*Sxl*) expression in females (Erickson and Quintero, 2007), which encodes a female-specific RNA-binding protein that not only initiates the female sex determination cascade, but also suppresses the dosage compensation machinery (Kelley et al., 1997). This transcriptionally regulated dosage compensation is commonly found in species with sex chromosomes but receives less attention in haplodiploid species without sex chromosomes. In Hymenoptera, dosage compensation was suggested by Rasch et al. (1977) to account for the ploidy difference between sexes. Interestingly, they found that in *N. vitripennis* haploid embryos have indeed half the amount of DNA in the nuclei compared to diploid embryos, but in adult somatic cells an equivalent amount of DNA per cell is found in both males and females. This dosage compensation therefore is thought to be realized by an endoreplication cycle, in which DNA content is increased without cell division (Edgar and Orr-Weaver, 2001). Rasch et al. (1977) postulated that dosage compensation by endoreplication possibly takes place several hours before the first larval instar. If this is the case in *Muscidifurax*, the observed two-fold higher expression of *tra* at 7.5-10 hpo is only possible in diploid but not haploid embryo, which is in accordance with our one-step-diploidization-causes-feminization model. Furthermore, if increased zygotic *tra* expression is only exhibited through doubled genomic DNA content, we would expect to see that the expression of *tra* relative to reference gene expression will remain unchanged in later developmental time windows, which seems the case based on our embryo qPCR and RT-PCR results. However, a more precise quantification method would be required for further justification. Given the fact that we only observed an increase of *tra* expression but not *tra2* expression in *gfp*-spiked *Wol*+ embryos, it means that there must be a transcriptional level dosage compensation mechanism to suppress *tra2* expression and activate (or release) *tra* expression in diploid embryos. *Sxl* in *Drosophila* and *femaleless* in *Anopheles gambiae* are X-linked genes that are both involved in sex determination and the transcriptional dosage compensation (Kelley et al., 1997; Krzywinska et al., 2021). If

there is an upstream regulator in *Muscidifurax* controlling both sex determination and the transcriptional dosage compensation process, it should be able to specifically increase the zygotic *tra* expression in relation to the ploidy level of the embryo. Another possibility is the involvement of *tra* itself in transcriptional dosage compensation. At the moment we severely lack knowledge in this regard and we have no enough evidence to support either theory, more research is required to fully understand the initial sex determination signal in *Muscidifurax.*

Even if the exact sex determination mechanism remains elusive, our result at least suggests that the sex determination mechanism in *M. raptorellus* and possibly in *M. uniraptor* are different from the only two currently known sex determination mechanisms in Hymenoptera: maternal effect genomic imprinting sex determination (MEGISD) in *N. vitripennis* (Verhulst et al., 2010b; Verhulst et al., 2013; Zou et al., 2020) and the complementary sex determination (CSD) in *Apis mellifera* (Beye et al., 2003; Gempe et al., 2009; Hasselmann et al., 2008). MEGISD requires an active copy of *wom* provided by the male sperm during fertilization to direct zygotic *tra* expression resulting in female development (Zou et al., 2020). Our result rejects the MEGISD system for *Muscidifurax* by showing that female development in *M. raptorellus* can be independent of paternal input. On the other hand, CSD, in which a homozygous *csd* locus leads to male development (Beye et al., 2003; Gempe et al., 2009) is also not compatible with homozygous parthenogenesis production of *Muscidifurax* females (Heimpel and de Boer, 2008). This again demonstrates the diversity of sex determination mechanisms in insects that is awaited our exploration.

Acknowledgements

We would like to thank Marcel Dicke for his insightful suggestions and valuable comments, Julia Ruiz Capella, Lina Ojeda Prieto, Roxane Snijders and Sander Koene for their pioneering work in determining the proper concentrations of tetracycline used for oral feeding *M. uniraptor*, and Min Xu for helping collect and prepare different wasp samples.

Supplement

Table S3.1 Primers used in this research. "[]" T7 promoter sequence that is recommended by the manufacturer.

	Name	Sequence
Reference	RP49_F	5'-GTGTACAGGCCGAAAATCGT-3'
Reference	RP49_R	5'-CGCTTCTTGCTGCTAACTCC-3'
Reference	Nv_EF-1a_qPCR_F	5'-CACTTGATCTACAAATGCGG-3'
Reference	Nv_EF-1a_qPCR_R	5'-GAAGTCTCGAATTTCCACAG-3'
gfp spike-in	GFP_RNAi_F	5'-[TAATACGACTCACTATAG]GTGACCACCTTGACCTACG
gfp spike-in	GFP_RNAi_R	5'-[TAATACGACTCACTATAG]TCTCGTTGGGGTCTTTGCT
RNAi	U&R_TRA_RNAi_F2	5'-[TAATACGACTCACTATAG]CGTTCTGTGTCACTGAGTCC-3'
RNAi	U&R_TRA_RNAi_R2	5'-[TAATACGACTCACTATAG]AGTATACGGGGAATGGCACT-3'
qPCR	U&R_TRA_qpcr_F1	5'-AAGCACAGAAGTAGCAGATCTC-3'
qPCR	U&R_TRA_qpcr_R1	5'-CACTAGTTTCAGTTGCAGATGC-3'
qPCR	U&R_TRA2_qpcr_F1	5'-GCTTAGGCGTCTTTGGTTTATC-3'
qPCR	U&R_TRA2_qpcr_R1	5'-CTGCACTGTTCTTTAGCTACCT-3'
qPCR	WSP_F	5'-GCAAAATTTACGCCAGATGCTA-3'
qPCR	WSP_R	5'-ACCATTTTGACTACTCACAGCA-3'
qPCR	GFP_qPCR_F	5'-TCGTGACCACCTTGACCTAC-3'
qPCR	GFP_qPCR_R	5'-GTCTTGTAGTTGCCGTCGTC-3'
RT-PCR	U&R_TRAF&M_F	5'-TGGATGAGAAGGAACTGCGA-3'
RT-PCR	U&R_TRAF&M_R	5'-TGTGGACTAGGACTGCGATG-3'
RT-PCR	U&R_TRA2F&M_F	5'-GCGTGTGGTTGTCGTTATTG-3'
RT-PCR	U&R_TRA2F&M_R	5'-TGAAACTTGCAAACTTCCCTC-3'

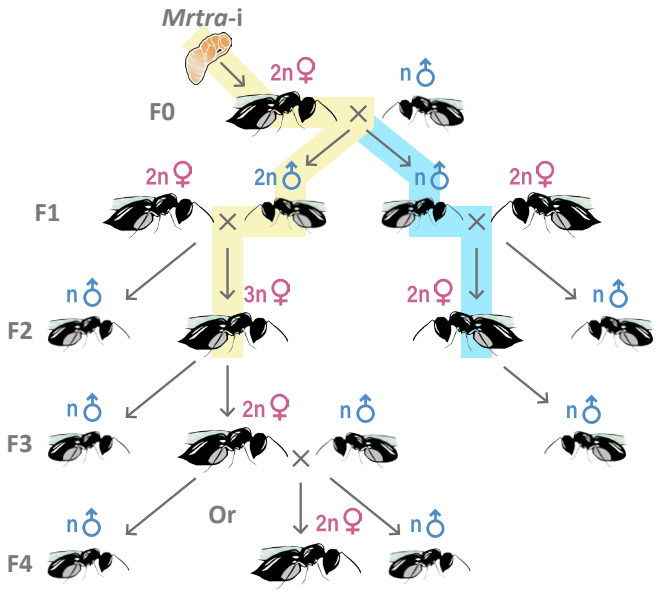


Figure S3.1 Schematic of the triploid *M. raptorellus* females creation and the resulting offspring sex and ploidy. The generation F0 as indicated on the left of the figure refers to the females treated with *Mrtra* dsRNA (*Mrtra*-i) in the white pupal stage. After emergence they were mated to haploid (n) males and produced haploid and diploid (2n) males (F1). These F1 males were mated to normal diploid females, resulting in the production of F2 triploid (3n) females and haploid males (yellow line, diploid father), or the production of diploid females and haploid males (blue line, haploid father). The F2 triploid females were setup as virgin and produced male and female F3 offspring (yellow line); the F2

diploid female was setup as virgin and produced only male F3 offspring. The impaternate (uniparental reproduction by mother) diploid F3 females were mated to normal haploid males and produced male and female (F4) offspring.

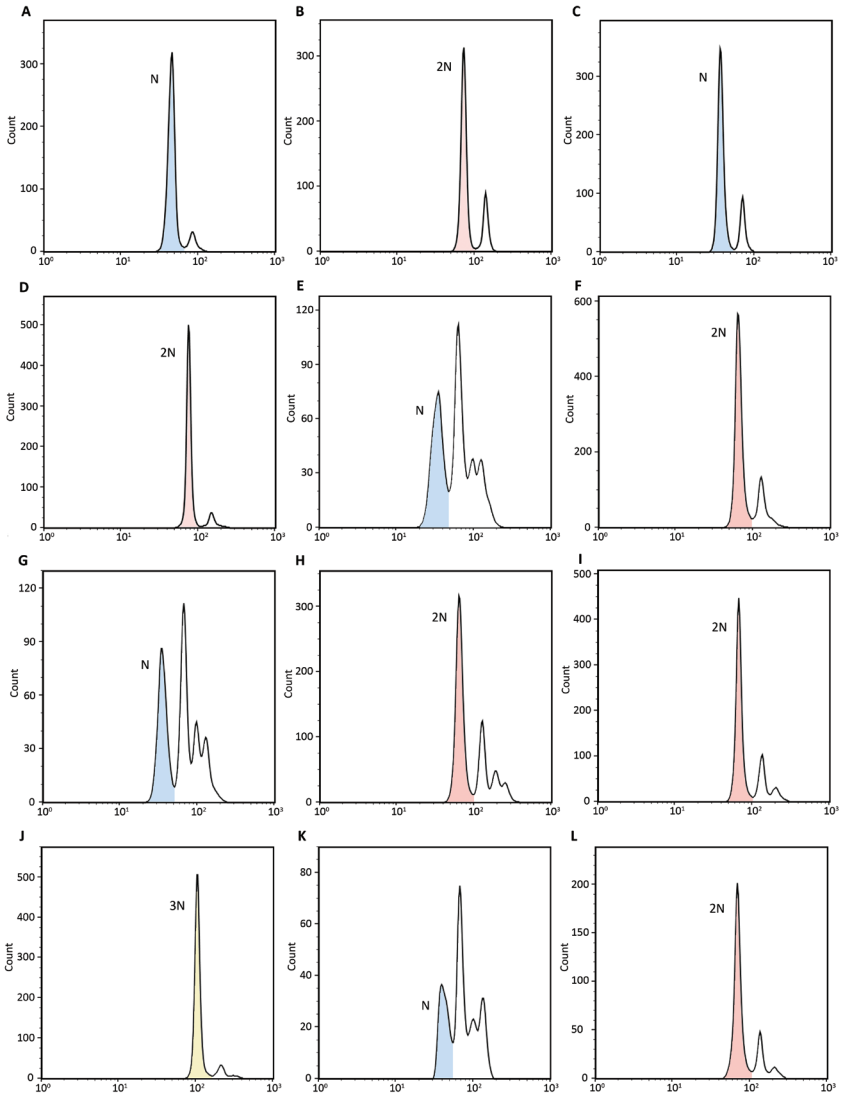


Figure S3.2 Representative plots depict the ploidy level of wild-type (WT) and offspring male and female from different treatments or generations of *M. uniraptor* and *M. raptorellus*. Peaks that are marked with colour in the figures represent the accumulation of the nucleus in ploidy plots of **(A)** WT *M. uniraptor* male, **(B)** WT *M. uniraptor* female, **(C)** male offspring of tetracycline treated *M. uniraptor* female, **(D)** female offspring of tetracycline treated *M. uniraptor* female, **(E)** WT *M. raptorellus* male, **(F)** WT *M. raptorellus* female, **(G)** *M. raptorellus* haploid F1 male, **(H)** *M. raptorellus* diploid F1 male, **(I)** *M. raptorellus* diploid F2 female, **(J)** *M. raptorellus* triploid F2 female, **(K)** *M. raptorellus* haploid F3 male and **(L)** *M. raptorellus* diploid F3 female. Y axis refers to the number of the nuclei counts. 'N', '2N' and '3N' indicate haploid, diploid and triploid represented peaks.

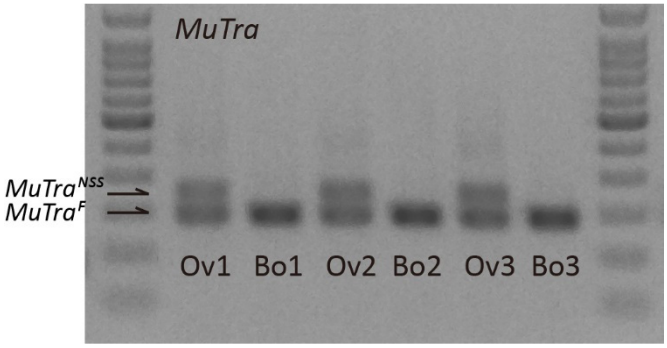


Figure S3.3 Expression of *Mutra* splicing variants in ovary and body samples of *M. uniraptor*. Female sex-specific (F) and non-sex-specific (NSS) *Mutra* splice variants are indicated with arrows. 'Ov' refers to ovary samples and 'Bo' refers to body samples without ovaries. 100bp ladder (Thermo Fisher) indicates fragment size. Fragments were visualized on 1.5% TAE agarose gel stained with Midori Green (NIPPON Genetics).

Chapter 4

Doublesex actively regulates male-specific differentiation during distinct developmental time windows in the parasitoid wasp, *Nasonia vitripennis*

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Abstract

Sexually dimorphic traits in insects are rapidly evolving due to sexual selection. Yet, our knowledge of the underlying sex-specific molecular mechanisms is still scarce. Here we show that the highly conserved gene, *Doublesex (Dsx),* regulates sexual dimorphism in the model parasitoid wasp *Nasonia vitripennis* (Hymenoptera: Pteromalidae). We present the revised *Dsx* gene structure including a novel alternative first exon, and two additional male *NvDsx* isoforms. We show sex-specific *NvDsx* expression throughout development, and demonstrate that transient *NvDsx* silencing in different male developmental stages dramatically shifts the morphology of two sexually dimorphic traits from male to female, with the effect being dependent on the timing of silencing. In addition, we also determined the effect of *NvDsx* on reproductive organs. Transient silencing of *NvDsx* in early male larvae affects the growth and differentiation of the internal and external reproductive tissues. Our results indicate that male *NvDsx* is constantly required to suppress female-specific traits and to promote male-specific traits during distinct developmental windows. This provides a first insight into the regulatory activity of *Dsx* during wasp development in the Hymenoptera.

Introduction

Insects are well known for their distinct differences in male and female morphology. Reproductive organs and sexually dimorphic traits such as wing shapes and colour patterns are one of the fastest evolving traits in insects. As these traits can differ dramatically among insects, they are often used to distinguish species. Development of these rapidly changing sexually dimorphic traits usually depends on specific genes that are expressed in a tissue and sex-specific manner (Barmina and Kopp, 2007). Many of these genes are under control of the Doublesex (Dsx) transcription factor that initiates sexual differentiation in insects (Clough et al., 2014; Shukla and Palli, 2012b). In general, *Dsx* pre-mRNA is alternatively spliced to yield male-specific *Dsx* (*DsxM*) or female-specific *Dsx* (*DsxF*) transcripts, resulting in distinct sex-specific protein isoforms (Shukla and Nagaraju, 2010b; Verhulst and van de Zande, 2015). In most insects, the conserved splicing factor *transformer* (*tra*) is responsible for female-specific splicing of *Dsx* transcripts, while in males the *Dsx* transcript is spliced by default into the male isoform (Bopp et al., 2014; Geuverink and Beukeboom, 2014; Verhulst et al., 2010a). Two functional domains are present in all insect Dsx homologs: 1) the DM domain which consists of a DNA binding domain (DBD) and an oligomerization domain (OD1) with a zinc module and binding sites to facilitate binding downstream targets, 2) a dimerization domain (OD2) with a common N-terminal part and a sex-specific C-terminal part (An et al., 1996; Ohbayashi et al., 2001). These C-terminal sex-specific differences are assumed to be involved in the regulation of the development of sex-specific traits (Price et al., 2015; Verhulst and van de Zande, 2015). For instance, in *Drosophila,* DsxM represses and DsxF activates the expression of *bric-à-brac (bab)* which results in male-specific abdominal pigmentation (Williams et al., 2008). A similar action of Dsx is observed in the formation of sex combs, an important feature for male mating success of some *Drosophila* species (Kopp, 2011). In *Bactrocera dorsalis, Dsx* knockdown in females triggers ovipositor deformation and the interruption of yolk protein gene expression, leading to a delay in ovary development (Chen et al., 2008). These studies confirm the essential role of DsxF in initiating and maintaining female development in Diptera. Also, in Lepidoptera (butterflies and moths) and Coleoptera (beetles), sex-specific *Dsx* isoforms regulate many sex-specific traits. For example, transient expression of *DsxM* in female *Bombyx mori* silk moths distorts the development of female-specific genital organs by partially introducing male-specific characteristics in female genitalia (Suzuki et al., 2005), whereas introducing mutations in the male-specific *Dsx* isoform of *B. mori* yields either malfunctioning males or intersexes (Xu et al., 2017). In *Papilio polytes* butterflies, differential expression of *Dsx* isoforms results in

sex-specific wing patterns (Kunte et al., 2014). *Dsx* also promotes the mandible growth in males but constrains it in females of *Cyclommatus metallifer* beetles (Gotoh et al., 2014). In *Trypoxylus dichotomus* and *Onthophagus taurus* beetles, the formation of head horns is antagonistically regulated by *DsxM* and *DsxF*, while in *T. dichotomus* the thoracic horn formation is *DsxM* independent (Ito et al., 2013; Kijimoto et al., 2012). Noteworthy, all these experiments were conducted in a very specific developmental stage focusing on sexual differentiation; only few focused on sexually dimorphic traits. However, the expression of *Dsx* starts in early embryogenesis and continues through maturity, implying an ongoing action in regulating both sexual differentiation and the development of sexually dimorphic traits (Chen et al., 2019; Morrow et al., 2014; Robinett et al., 2010).

Thus far, the specific role of *Dsx* in regulating sexually dimorphic traits has not been studied in the Hymenoptera. *Dsx* sex-specific splice variants and expression differences have been identified in honeybees (*Apis mellifera*), bumble bees (*Bombus ignites),* ants (*Cardiocondyla obscurior*), Japanese ants (*Vollenhovia emeryi*) and fire ants (*Solenopsis invicta*) (Cho et al., 2007; Klein et al., 2016; Miyakawa et al., 2018; Nipitwattanaphon et al., 2014; Ugajin et al., 2016). The role of *Dsx* in regulating reproductive organ development in males has only been shown in hymenopteran sawflies, *Athalia rosae* (Mine et al., 2017). As the species in the order Hymenoptera often possess the most distinctive dimorphic characters between sexes, it is paramount to gain more knowledge on how *Dsx* directs sex-specific and sexually dimorphic trait development in the Hymenoptera.

N. vitripennis belongs to the genus *Nasonia* (Hymenoptera: Pteromalidae), and has become the model system for parasitoid as it is highly suitable for evolutionary and developmental genetic studies (Lynch, 2015; Werren et al., 2010). Multiple sexually dimorphic traits within the genus *Nasonia* make it a perfect system to study sex differentiation (Darling and Werren, 1990; Raychoudhury et al., 2010). For instance, the femur and tibia of the hind legs, and the antennae of female *Nasonia* are pigmented with a dark-brown colour, whereas in males these are pale yellow (Darling and Werren, 1990). *Nasonia vitripennis* males have dramatically shorter forewings and are flightless (Darling and Werren, 1990), while the females can fly and have large forewings in all species. Although *Dsx* has been identified in *Nasonia* (Oliveira et al., 2009), its role in regulating these sexually dimorphic traits has not been shown before. Previous research identified one female *Dsx* (*NvDsxF*) and one male *Dsx* (*NvDsxM*) isoform, but the identified *NvDsxM* isoform encodes only four amino acids in its sex-specific OD2 domain and its function in regulating male development is unclear (Oliveira et al., 2009).

Therefore, we set out to 1) identify male-specific traits that are regulated by *Dsx,* and 2) study the timing by which *Dsx* regulates these traits during development in the parasitoid wasp *Nasonia vitripennis*. To assess the role of *NvDsxM* in regulating male sexual development in *Nasonia,* we first revisited the *NvDsx* genetic structure. We confirmed existing splice-variants and identified an alternative starting exon and two additional male *NvDsx* splice-variants. We then used RNA interference to temporally silence *Dsx* in different male developmental stages to study its function in sexual differentiation and dimorphism. Ultimately, our results provide an important starting point to study the molecular basis of sexual differentiation and rapidly evolving sexual dimorphism in insects in general and particularly in the Hymenoptera.

Material and methods

1. Insects rearing

The *Nasonia vitripennis* lab strain AsymCx was reared on *Calliphora sp.* hosts at 16h/8h light/dark and 25°C. AsymCx strain has been maintained in the laboratory for over 10 years and is cured from *Wolbachia* infection.

2. 5' and 3' RACE-PCR to identify additional *NvDsx* splice variants

For identification of the 3' ends of *NvDsx* mRNA*,* total RNA of three adult male- and female AsymCx wasps was extracted individually using TriZol (Invitrogen, Carlsbad, CA, USA) and cleared from genomic DNA by using the DNA-*free* DNA removal kit according to manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Total RNA was annealed with the 3' adapter from the FirstChoice RLM-RACE kit (Ambion, Austin, TX, USA) during the reverse-transcription using RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, Hanover, MD, USA) and stored at -80°C. The cDNA was subjected to a series of (semi-) nested PCRs using primers complementary to the adapter and gene-specific primers targeting *NvDsxU*, and NvDsx_X7, NvDsx_X8 and NvDsx_X9 at 94°C for 3 minutes, 35 cycles of 94°C for 30 seconds, 55-62°C for 30 seconds and 72°C for 2 minutes, with a final extension of 10 minutes at 72°C. Primers were designed based on the initial *NvDsx* gene structure and sequences are listed in Table S4.1.

For identification of the 5' end of *NvDsx*, total RNA of three female AsymCx wasps was extracted using TriZol (Invitrogen, Carlsbad, CA, USA). After decapping, a 45-base RNA adapter

was ligated to the RNA population using the FirstChoice RLM-RACE kit (Ambion, Austin, TX, USA) and reverse transcribed using the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, Hanover, MD, USA). The cDNA was subjected to series of (semi-) nested PCRs using primers complementary to the adapter and gene-specific primers targeting *NvDsxU* at 94°C for 3 minutes, 35 cycles of 94°C for 30 seconds, 55-60°C for 30 seconds and 72°C for 2 minutes, with a final extension of 10 minutes at 72°C. Primers were designed based on the initial *NvDsx* gene structure and sequences are listed in Table S4.1.

5' and 3' RACE-PCR fragments were run and visualized on ethidium bromide-containing 1.5% agarose gel with 1×TAE buffer. All RACE-PCR products were purified using GeneJET Gel Purification Kit (Fermentas, Hanover, MD, USA) and ligated into pGEM®-T vector (Promega, Madison, WI, USA) and transformed into competent JM-109 *Escherichia coli* cells (Promega, Madison, WI, USA). Colony-PCRs were conducted by using primers recommended by the manufacturer 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. All isoforms were sequenced on an ABI 3730XL capillary sequencer (Applied Biosystems) and reads were visualized and aligned in MEGA4 (Tamura et al., 2007)which expands on the existing facilities for editing DNA sequence data from autosequencers, mining Web-databases, performing automatic and manual sequence alignment, analyzing sequence alignments to estimate evolutionary distances, inferring phylogenetic trees, and testing evolutionary hypotheses. Version 4 includes a unique facility to generate captions, written in figure legend format, in order to provide natural language descriptions of the models and methods used in the analyses. This facility aims to promote a better understanding of the underlying assumptions used in analyses, and of the results generated. Another new feature is the Maximum Composite Likelihood (MCL. Exon-intron structure of the genes was constructed by aligning the mRNA sequences to *N. vitripennis* genome and visualizing in Geneious Prime 2019.1.3 and CLC Workbench 6.

3. Reverse transcriptase PCR (RT-PCR) to confirm usage of alternative 1' exon

Total RNA was extracted from four female samples and three male samples containing three adults each using NucleoSpin XS (Brand) with final elution done in 15 µl nuclease-free water. Eleven µl of eluted total RNA was converted to cDNA using RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, Hanover, MD, USA) with random hexamers according to the manufacturer's protocol and stored at -80°C. One female sample was not converted to cDNA but used as gDNA contamination control in RT-PCR. The primer sets for

the RT-PCR reactions are: NvEF-1a and Nv_DsxU_F6 or Nv_DsxU_F7 with Nv_Dsx_qPCR_R (Table S1). GoTaq® G2 Flexi DNA Polymerase (Promega) was added to the mastermix which was prepared following manufacturer's instruction, and the reaction was amplified with a standard PCR profile: 3 minutes at 95°C, 35 amplification cycles of 30 seconds at 95°C, 30 seconds at 55°C, 30 second at 72°C and a final extension of 7 minutes at 72 °C in a thermal cycler (T100TM Thermal Cycler, Bio-Rad). Five µl of PCR product was visualized on a non-denaturing 1% agarose gel with a 100bp ladder. PCR fragments were Sanger sequenced at Eurofins Scientific to confirm the correct amplification.

4. RNA interference (RNAi) of *NvtraF* and *NvDsx* gene

For the *Nvtra* RNAi knockdowns, MEGAscript RNAi Kit (Thermo Fisher) was used to generate dsRNA to target exon 5-8 of *Nvtra* mRNA in 4th larval instar females which resulted in a male splice form of *NvDsx* as shown in Verhulst et al. (2010b). To conduct functional analysis of *NvDsx*, the same RNAi kit was used to generate dsRNA based on either the common region of *NvDsx* (exon 2-5) that is present in both male and female splice-variants or the specific region of *NvDsx* (exon 7-9) that is only present in two longer male splice-variants. *Gfp* dsRNA was generated from the vector pOPINEneo-3C-GFP which was a gift from Ray Owens (Addgene plasmid # 53534; http://n2t.net/addgene: 53534; RRID: Addgene_53534). This *gfp* dsRNA was used as control in all experiments as it has no target in *Nasonia*. Primers designed using Geneious 10.0.9 to construct the dsRNA T7 template are listed in Table S4.1. For male-specific *NvDsx* RNAi, offspring of AsymCx strain was collected at 2nd and 4th larval instars and the white pupa stage which are described in Table S4.2. All dsRNAs were diluted with RNase-free elution buffer (MEGAscript RNAi Kit (Thermo Fisher)) to 4000 ng/µl (NanoDrop™ 2000 Spectrophotometer, Thermo Fisher) before proceeding to microinjection.

Microinjection of wasps was carried out with IM300 Microinjector (Narishige) and FemtoJet® 4i (Eppendorf) and followed the protocols described by Werren et al. (2009) and Lynch and Desplan (2006) with minor changes. Red colour food dye was pre-mixed with the dsRNAs in a ratio of 1:9. Experiments were performed using AsymCx strain at different developmental stages for different purposes (Table S4.3).

Second and 4th instar parasitoid larvae were collected from their hosts and placed on a 1X PBS agar plate before injection. Early pupal stage samples were fixed on glass slides with double-sided tape. After injection, the 2nd instar larvae were transferred back to a host (6-8

per host) and sealed with the host shell. Afterward, sealed hosts were kept in 8 tube-PCR strips with open lid. Subsequently, all injected samples either in 8 tube-PCR strip or on slides were placed on 1X PBS plates to prevent dehydration and incubated at standard rearing conditions until adult emergence.

5. *NvDsx* splicing and expression throughout development

Male and female wasps were separated at the black pupal stage by sex-specific traits such as forewing size and presence/absence of the ovipositor. To generate female offspring, one selected male and one selected female pupa were collected in a single glass vial closed with a cotton plug until eclosion and kept for one more day to make sure they had mated. After fertilization, two hosts per day for the first two days were provided to mated females to initiate oviposition. Afterwards, one-hour host presentation was repeated twice in a row per day. Since mated *N. vitripennis* tend to lay eggs with an extremely female-biased sex ratio, offspring from mated females was considered female (Hamilton, 1967). Male offspring was collected by using virgin females with the same setup. To facilitate early stage (eggs) sample collection, egg-laying chambers (1.5 ml Eppendorf tubes with cut-off tip presenting the head of a host pupa plugged in 1000 µl filter pipet tips) were made to allow the females only access to the head portion of the fly hosts.

To confirm that male-specific splice variants are not under the regulation of NvTraF but splice in default mode independently, female samples were collected from 3h, 1d, 2d, 3d, 4d, 5d and 6d after *NvtraF* knockdown. To study the expression pattern of *NvDsx* sex-specific splice variants throughout whole developmental stages, samples were collected at specific time points listed in Table S4.2. More than 50 embryo eggs were collected at 36h and five to ten larvae, pupae or adults were collected at 2.5d and onwards. Collected samples were subsequently frozen in liquid nitrogen and stored at -80°C. Sex-specific primers of *NvDsx* were designed using Geneious 10.0.9. and are listed in the Table S4.1. Both *NvRP49* and *NvEF-1α* were assessed for their expression stability in a semi-quantitative RT-PCR setup, and *NvEF-1α* was finally chosen as the reference gene.

Frozen samples were homogenized by pestles which were designed for 1.5 ml microcentrifuge tubes (Biosphere SafeSeal Tube 1.5ml). Total RNA was extracted using ZR Tissue & Insect RNA MicroPrep™(Zymo) following manufacturer's instructions. An on-column DNase treatment step was added to all samples. Subsequently, total RNA of each sample was eluted in 16 µl of

DNase/RNase free water. After verifying the purity and concentration by NanoDrop™ 2000 Spectrophotometer (Thermo Fisher), one µg of each template was synthesized into cDNA with a standard reaction mix (SensiFAST™ cDNA Synthesis Kit, Bioline) in a thermal cycler (Bio-Rad T100TM Thermal Cycler, Bio-Rad) with 5 minutes priming at 25°C, 30 minutes reverse transcription at 46°C and 5 minutes reverse transcriptase inactivation at 85°C.

For all RT-PCR reactions, GoTaq® G2 Flexi DNA Polymerase (Promega) was used to prepare the mastermix following the manufacturer's instruction and amplified with a standard PCR profile: 3 minutes at 95°C, 34 (male-specific splicing verification) / 29 (expression pattern of *NvDsx*) amplification cycles of 30 seconds at 95°C, 30 seconds at 55°C, 50 seconds at 72°C and a final extension of 5 minutes at 72 °C in a thermal cycler (T100TM Thermal Cycler, Bio-Rad). In order to standardize the expression pattern of *NvDsx*, the number of PCR cycles needed was determined by comparison of the brightness of the bands in gels from different PCR reactions with cycles ranging from 25 to 34 to achieve non-oversaturated brightness. In the end 29 cycles were used for both reference genes and target genes.

6. Silencing efficiency of *NvDsx*

Quantitative RT-PCR (qPCR) was conducted to verify the silencing efficiency of *Dsx* knockdown. After dsRNA injection, samples were collected at either adult stages (injected at the 4th larval instar and the white pupa stage) or the white pupa stage (injected at the 2nd larval instar). Four to five pupae or adults were pooled to produce one biological replicate and six biological replicates per treatment were prepared. All testing samples were used in qPCR according to SensiFAST™ SYBR® No-ROX Kit manual (Bioline). *NvDsx* qPCR primers were designed on the common region (exon 2-5) of male and female splice variants (Table S4.1). *NvEF-1a* was used as reference gene. qPCR was carried out using the CFX96TM Real-Time System (Bio-Rad) with Bio-Rad CFX Manager 3.1 Software (Bio-Rad). The standard qPCR profile consists of 95°C for 3 minutes, 45 amplification cycles of 15 seconds at 95°C ,15 seconds of 55°C, 30 seconds of 72°C and a final standard dissociation curve step to check for non-specific amplification.

7. Measuring and visualizing leg and antenna pigmentation, forewings and reproductive organs

Legs, antennae, forewings and external reproductive organs of adult males were dissected on clean slides with a drop of 1 X PBS buffer using dissection tweezers, and subsequently

mounted on slides in a drop of ethanol which was allowed to evaporate. Internal reproductive organs were dissected in dissection trays that were filled with 1 X PBS buffer using dissection tweezers, and were subsequently mounted in a drop of 1 X PBS on a slide and covered with a coverslip. Photos of leg, antenna, forewing and external reproductive organs were taken by Dino-Lite Edge 5MP digital microscope and measured by using DinoCapture 2.0 software. Photos of internal reproductive organs were captured by the sCMEX-20 camera (Euromex Scientific) coupled with a Leitz Dialux 20 EB light microscope and generated in ImageFocusAlpha software. Zerene Stacker was used for photo stacking. Forewing size was measured by quantifying wing length and width based on the standard methods described by Loehlin et al., (2010b). Tibia length of the hind leg was used to standardize the body size differences among individuals (Godfray, 1994). Reproduction structures were identified based on the description in Liu et al. (2017) and Snodgrass (1957).

8. Data analysis

qPCR data was first imported to LinRegPCR software (LinRegPCR, 2017.1.0.0, HFRC, Amsterdam, The Netherlands) (Ramakers et al., 2003). After baseline correction, the initial number of templates (N0) were calculated based on the average PCR efficiency of each amplicon. Relative expression levels of *NvDsx* in each sample was obtained by dividing the N0 value of *NvDsx* by N0 value of *NvEF-1a*. Statistical analysis of qPCR and measurement data was performed in R using One-way ANOVA and Tukey's Honest Significant Difference (HSD) for post hoc test.

Results

Identification of two additional male-specific *Dsx* splice variants

Previously, only one female (*NvDsxF*) and one male (*NvDsxM*) *Dsx* splice form were identified in *N. vitripennis* (Oliveira et al., 2009). The *NvDsxF* splice variant shares the same first four exons with the *NvDsxM* splice variant, but 108 bases are spliced out from exon 5 which leads to female-specific exons 5F and 6F. In *NvDsxM,* exon 5M results from retention of this 108bp intron, and the entire transcript encodes a Dsx protein with a very short OD2 domain of only four amino acids (aa). This intron retention does not resemble any *Dsx* splice variant in other insect orders (Oliveira et al., 2009) (Figure 4.1, *NvDsxF* and *NvDsxM1*). To investigate the possibility of additional splice variants of *Dsx* pre-mRNA in *N. vitripennis,* we

performed extensive 5' and 3' Rapid Amplification of cDNA Ends (RACE). The results reveal a novel alternative starting exon that we designated exon 1' (Figure 4.1). Using RT-PCR to target exon 1' to exon 5 we verified its presence in transcripts of both sexes but with a higher frequency in female transcripts (Figure S4.1). Alternative splicing at the 5' end has not been shown for *Dsx* before, but does not change the start codon of Dsx translation. From here on we refer to the alternative variants collectively. 3' RACE yielded two additional male splice variants (Figure 4.1). We designated the previously published male-specific variant *NvDsxM1* (M1: NM_001162517; M1': MT043363); the longer male splice forms we present here are designated *NvDsxM2* (M2: MT043359; M2': MT043360) and *NvDsxM3* ((M3: MT043361; M3': MT043362). All male isoforms share the same exons 1 (or 1') to 5 with the female isoform *NvDsxF* (F: NM_001162518; F': MT043364). The *NvDsxM2* open reading frame ends with a stop codon in exon 8 and translates into a protein of 328 aa that is 105 aa longer than the open reading frame of *NvDsxM1*. In *NvDsxM3,* compared to *NvDsxM2,* exon 8 is spliced out and the stop codon is encoded in exon 9, yielding a protein of 330 aa. NvDsxM2 and NvDsxM3 protein isoforms are almost completely identical and only differ at the C-terminus downstream of the male-specific OD2 domain.

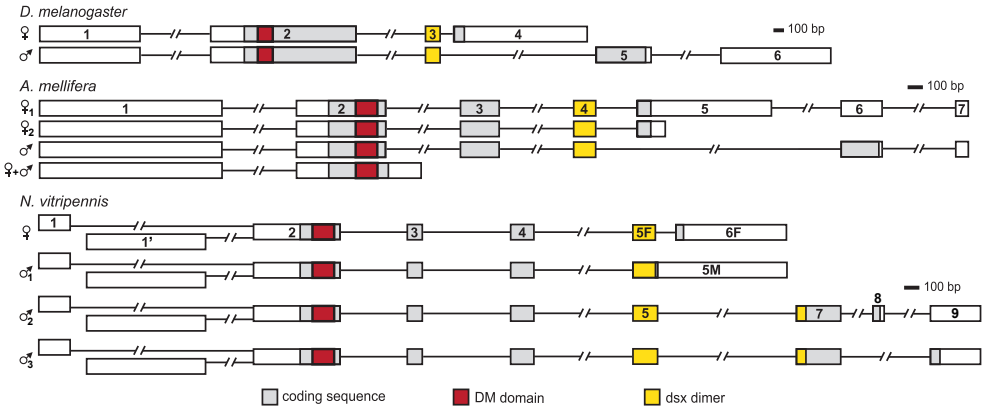


Figure 4.1 Comparison of insect *Doublesex* (*Dsx*) gene models. The eight *N. vitripennis* male and female *Dsx* splice variants are compared to *Dsx* splice variants of *D. melanogaster* and *A. mellifera*. Figure modified from Oliveira et al. (2009) to include the alternative 1st exon (1') and the additional *NvDsx* male splice variants. Male and female specific splice forms are indicated by ♂ and ♀. Exon numbers are noted on each exon. Grey, red and yellow blocks indicate coding regions, DM domain and OD2 Dsx dimer region respectively. Scales are provided behind each species splice structures.

To determine whether the newly identified splice variants are exclusively expressed in males, we observed the overall expression of different *NvDsx* splice forms in both males

and females. Across development, *NvDsxF* is only expressed in females whereas *NvDsxM1, NvDsxM2* and *NvDsxM3* are highly expressed in males (Figure S4.2), while being expressed at a very low level in females. In males an increase of *NvDsxM1, -2 and -3* expression from 36h toward the 4th instar larvae was observed (Figure S4.2). Afterwards, the expression remains relatively constant until the adult stage (Figure S4.2). Overall, *NvDsxM2* transcripts are more abundant than *NvDsxM3* (Figure S4.2). Females express *NvDsxM1*, *NvDsxM2* and *NvDsxM3* at a very low level probably due to leaky default splicing of male-specific transcripts (Figure 4.2B and Figure S4.2).

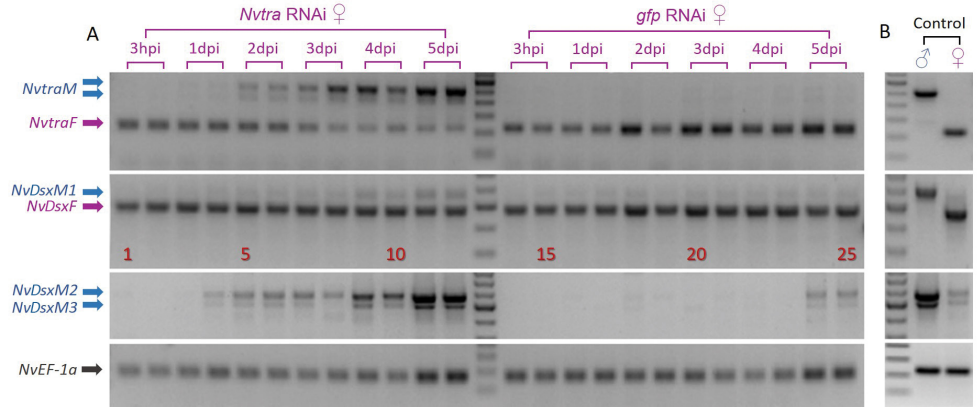


Figure 4.2 Confirmation of sex-specific *Dsx* splicing after *Nvtra* knockdown in *N. vitripennis*. (A) Sex-specific splice variants of *Nvtra* and *NvDsx* in *N. vitripennis* females that are injected in the 4th instar larval stage with *Nvtra* dsRNA or *gfp* dsRNA. **(B)** Male and female non-treated (NT) control samples for each primer sets. Arrows indicate male (in blue) and female (in purple) splice forms of the different genes. Lanes 1-12: Female *Nvtra* knockdown samples from 3-hour post injection (hpi) to 5-day post injection (dpi). Lane 13: 100bp ladder (Thermo Fisher). Lanes 14-25: Female *gfp* knockdown samples from 3dpi to 5dpi. For each stage two samples are used. *NvEF-1a* is used as expression and loading control.

We have shown previously that parental *Nvtra* RNAi in the female pupal stage leads to a shift in splicing from *NvtraF* to *NvtraM* (Verhulst et al., 2010b). This subsequently induces a shift from *NvDsxF* to *NvDsxM* (now *NvDsxM1*) splicing in both the treated adult females and the offspring, without consequences for adult female fertility (Verhulst et al., 2010b). The reduced levels of *NvtraF* transcripts are no longer sufficient to regulate the splicing of all *NvDsx* transcripts into the female-specific form, leading to default expression of *NvDsxM1* splice variants. To verify the default and NvTraF independent splicing of *NvDsxM2* and *NvDsxM3*, we silenced *Nvtra* in 4th instar female larvae. We assessed the different *NvDsx* and *Nvtra* splice variants from 3 hours post injection (hpi) to 5 days post injection (dpi) using RT-PCR. We confirmed that

Nvtra RNAi leads to *NvtraM* splicing in females (Figure 4.2A) and observed a minor increase in *NvDsxM1* expression from 4dpi onwards, while *NvDsxM2* and *NvDsxM3* expression levels increase substantially from 1dpi onward compared to mock *gfp* RNAi treatment (Figure 4.2A). We did not observe a clear reduction in female *NvDsxF* splicing (Figure 4.2A).

Effects of *NvDsx* RNAi in males depend on the timing of injection

To assess the sex-specific function of Dsx, we started by silencing *NvDsx* using RNAi in different developmental stages (Figure S4.3A,B,C) and determined *NvDsx* silencing by quantitative RT-PCR in later stages. We were unable to silence the male-specific splice variants of *NvDsx* specifically (*NvDsxM*-i, Figure S4.3B), so we targeted the common region of *NvDsx* by injecting *NvDsxU* dsRNA (from here on indicated as *NvDsx*-i). We observed that the developmental stage at which *NvDsx*-i was done, greatly influenced the expression levels of *NvDsx* we measured using qPCR five to seven days later. First, we injected 2nd instar male larvae with dsRNA against *NvDsx* or *gfp* (mock) and sampled these males five days later when they were in the white pupal stage to assess *NvDsx* expression levels. We found no significant decrease of *NvDsx* expression in comparison to non-treated (NT) and *gfp* mock (*gfp*-i) (Figure S4.3A) in these males. It could be that the strong increase in male *NvDsx* expression from 4th instar larvae onwards (Figure S4.2) is the main cause as the limited amount of siRNA present in the 2nd larval instar is not capable of silencing the increased transcript numbers in later developmental stages. Next, we performed *NvDsx*-i in 4th instar male larvae, which is one day prior to pupation, and sampled these males seven days later as newly emerged adults to assess *NvDsx* expression. In this case we observed a significant reduction of *NvDsx* when compared with both NT (Tukey's HSD, P<0.001) and *gfp*-i males (Tukey's HSD, P<0.001) (Figure S4.3B). This confirms a successful *NvDsx* knockdown starting in 4th instar male larvae with an effect lasting until adulthood. Finally, we treated white pupal stage males with *NvDsx*-i and sampled at the adult stage (six days after injections). We observed a significant reduction of *NvDsx* expression when comparing *NvDsx*-i to NT (Tukey's HSD, P=0.003) and pupal stage *gfp*-i males (Tukey's HSD, P=0.002, Figure S4.3C) which indicates that we also obtained a highly significant knockdown of *NvDsx* from the pupal stage onwards. The transient nature of RNAi in *Nasonia* allows us to suppress *NvDsx* during specific developmental time windows to assess the effects on morphological development. Our experimental setup is therefore different from many genome editing studies in which *Dsx* is knocked out from early embryogenesis onwards (Chen et al., 2019; Xu et al., 2017).

Silencing *NvDsx* in males results in female-like morphological trait development

After determining the silencing of *NvDsx* through RNAi at three different developmental stages, we continued by observing the morphological changes after *NvDsx*-i in males of the different developmental stages. We first observed changes in antenna pigmentation, and femur and tibia pigmentation of the hind legs (hereafter legs). NT and *gfp*-i males showed unpigmented antennae and legs (Figure 4.3A,B,C), whereas NT females have pigmented antennae and legs (Figure 4.3D). *NvDsx*-i in 2nd instar male larvae increased the amount of pigmentation in the adults slightly (Figure 4.3F), even though we did not observe a significant reduction in *NvDsx* expression in these samples at the white pupal stage (Figure S4.3A). Silencing *NvDsx* from 4th instar male larvae onwards resulted in female-like pigmentation in the adult stage (Figure 4.3E). When we silenced *NvDsx* from the white pupal stage onwards we did not see an increase in pigmentation (Figure S4.4B), suggesting that upon pupal metamorphosis the developmental window of NvDsx regulation on sex-specific pigmentation is closed.

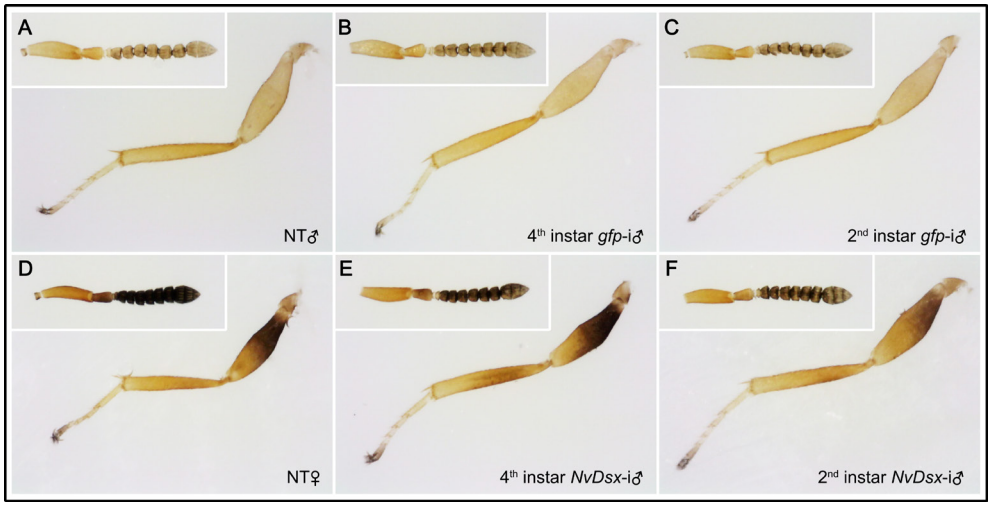


Figure 4.3 Pigmentation of antennae and hind legs of adult wasps after *NvDsx* or *gfp* dsRNA treatment in different developmental stages. (A) non-treated (NT) male **(B)** 4th instar *gfp* RNAi (*gfp*-i) male **(C)** 2nd instar *gfp*-i male **(D)** NT female **(E)** 4th instar *NvDsx* RNAi (*NvDsx*-i) male and **(F)** 2nd instar *NvDsx*-i male. Light source and exposure time are equal for all pictures.

Then we observed changes in forewing size and shape in N. vitripennis adult males following NvDsx-i in different developmental stages. Silencing NvDsx in both 2nd and 4th instar

larvae resulted in increased forewing length and width of adult males when compared to gfp-i (Tukey's HSD, P<0.001) and NT males (Tukey's HSD, P<0.001) (Figure 4.4B,C). Length/width (L/W) ratio of NvDsx-i treatments also showed severe reduction compared to gfp-i (Tukey's HSD, P<0.001) and NT males (Tukey's HSD, P<0.001) (Figure 4.4A) indicating that the forewing became wider and more female-like. However, the forewings of the NvDsx-i males were still smaller and narrower than those of the NT females (Length: Tukey's HSD, P<0.001; Width: Tukey's HSD, P<0.001; L/W ratio: Tukey's HSD, P<0.001) (Figure 4.4A,B,C).

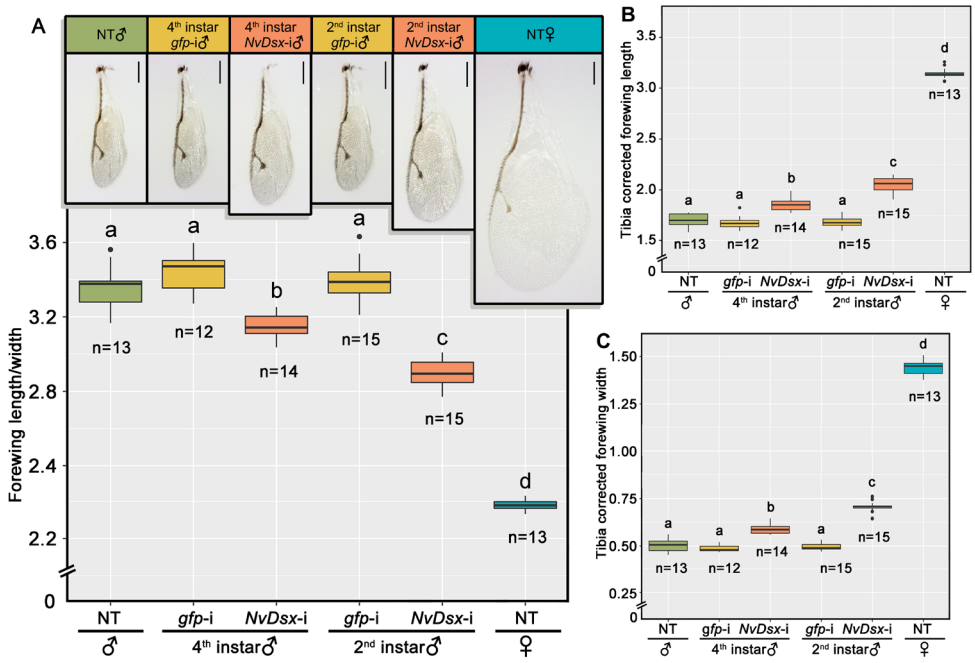


Figure 4.4 Comparison of forewing size of adult after *NvDsx* or *gfp* dsRNA treatment in 2nd or 4th larval instar male larvae and non-treated control. Y-axis represents: **(A)** Depiction of forewings of wasps of different RNAi treatments and the forewing length/width ratio corrected for tibia length, **(B)** Wing length corrected for tibia length, **(C)** Wing width corrected for tibia length. X-axis shows six groups: males treated with dsRNA of *NvDsx* (*NvDsx*-i) and *gfp* (*gfp*-i) in the 2nd and 4th larval instar, non-treated (NT) males and NT females. Wing sizes were corrected for tibia length and scales indicate 200µm. Boxplots were generated in R and display minimum, first quartile (Q1), median, third quartile (Q3), and maximum. Dots outside the boxplots represent outliers. Replicates are provided in the figures of each treatment. One-way ANOVA and post-hoc Tukey's HSD were used for data analysis. Letters in the figures denote statistical significance (*P*<0.05) among treatments.

Interestingly, also here we found an effect of NvDsx-i timing as the forewing length and width increased significantly in adults that developed from larvae treated in the 2nd and 4th instar, with earlier stage NvDsx knockdown leading to more severe changes (Tukey's HSD,

P<0.001), steering the L/W ratio towards NT females dramatically (Tukey's HSD, P<0.001). No significant differences were observed between 2nd and 4th instar *gfp*-i males (Tukey's HSD, *P*=0.062), 2nd instar *gfp*-i and NT males (Tukey's HSD, *P*=0.070) and 4th instar *gfp*-i and NT males (Tukey's HSD, *P*=0.999) (Figure 4.4A,B,C). Silencing *NvDsx* in the white pupal stage had no effect on forewing size or shape in adult males (Figure S4.4F), suggesting that the developmental window of *NvDsx* regulation influencing sex-specific wing size is also closed upon pupal metamorphosis.

Silencing *NvDsx* affects growth and development of male genitalia

Next, we assessed the role of *NvDsx* in male genitalia development by silencing *NvDsx* in the 2nd and 4th instar larval stage. *NvDsx*-i in both developmental stages reduced the aedeagus length significantly when compared to *gfp*-i in both developmental stages and NT males (Tukey's HSD, *P*<0.001) (Figure 4.5A,B). *NvDsx*-i males from both developmental stages had the same extent of aedeagus length reduction (Tukey's HSD, *P*=0.998). Aside from the shortened aedeagus, the paramere (genital organ structure without aedeagus) was also severely reduced in size in both 2nd and 4th instar *NvDsx*-i males compared to *gfp*-i and NT males (Figure 4.5C), whereas the structure of the paramere including the outer parameres, cuspis and digitus, was still intact (Figure 4.5C). Interestingly, unlike the aedeagus differences, the 2nd instar *NvDsx*-i males displayed more dramatic reduction in size of the outer paramere than the 4th instar *NvDsx*-i samples (Figure 4.5C). We recorded the mating ability of the *gfp*-i (Movie S4.1) and *NvDsx*-i (Movie S4.2) males and observed that the severe length reduction of the genitalia rendered *NvDsx*-i males unable to copulate with females, showing that in this case size matters. Taken together, these results suggest that the NvDsx regulatory window of aedeagus and paramere growth in *N. vitripennis* males is mostly during larval development.

Finally, we assessed the effect of *NvDsx* silencing at different developmental stages on the internal reproductive tissue development in the adult stage. Compared to the reproductive tissue development from normal female (Figure 4.6A), normal male (Figure 4.6B) and *gfp*-i males (Figure 4.6D,F), it was clear that the most pronounced effect was in the 2nd larval instar *NvDsx*-i males. In these males a severely deformed testis resembling more a female-like ovary structure is observed (Figure 4.6E). Seminal vesicles are also malformed and resemble a female-like lateral oviduct (Figure 4.6E). In comparison, 4th larval instar *NvDsx*-i males develop a mostly normal formed testis structure (Figure 4.6C),

however the seminal vesicles in 4th larval instar *NvDsx*-i males are smaller in size and do not contain any observable mature sperm (Figure 4.6H) when compared to *gfp*-i males (Figure 4.6D) and NT males (Figure 4.6G). These results suggest that the NvDsx regulation of sex-specific reproductive tissue development ends around pupal metamorphosis, but NvDsx continues regulating sperm maturation most likely into adulthood.

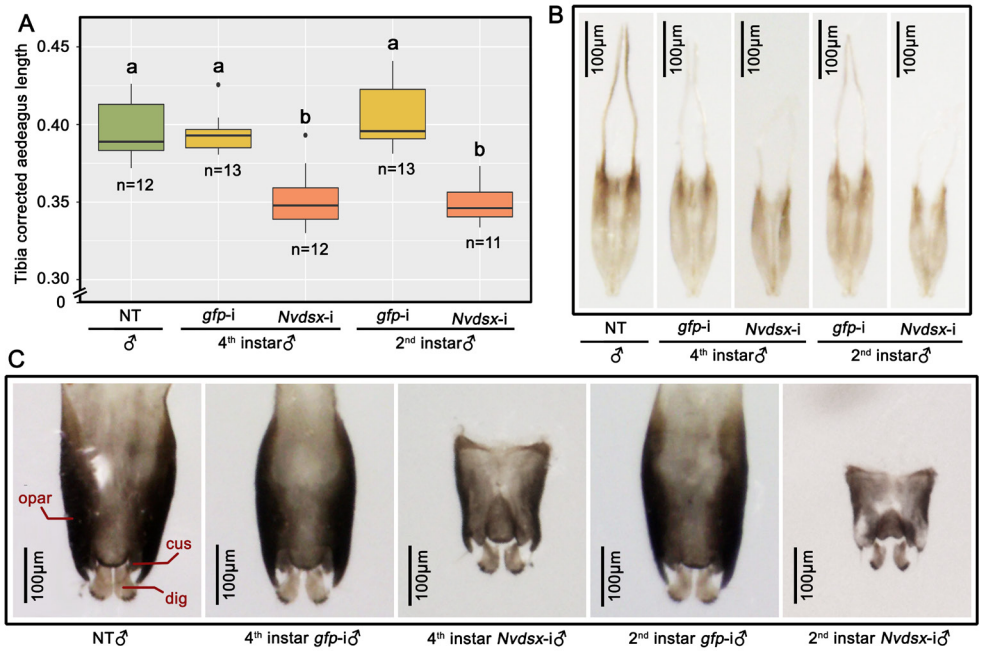


Figure 4.5 Comparison of external reproductive organs in males of different treatments. (A) Comparison of aedeagus length of adult males treated with *NvDsx* dsRNA (*NvDsx*-i) or *gfp* (*gfp*-i) in 2nd or 4th larval instar males and non-treated (NT) control males. Y-axis represents aedeagus length corrected by tibia length. Boxplots were generated in R and display minimum, first quartile (Q1), median, third quartile (Q3), and maximum. Dots outside the boxplot represent outliers. One-way ANOVA and post hoc Tukey's HSD were used for data analysis. Letters in the figures denote statistical significance ($P<0.05$) among treatments. Phenotypes of **(B)** aedeagus and **(C)** paramere (without aedeagus) of adult males after *NvDsx* or *gfp* dsRNA treatment in the 2nd or 4th larval instar and NT control. Each paramere component is described in Snodgrass, (1957) and labelled with the following abbreviations: outer paramere (opar), cuspis (cus) and digitus (dig).

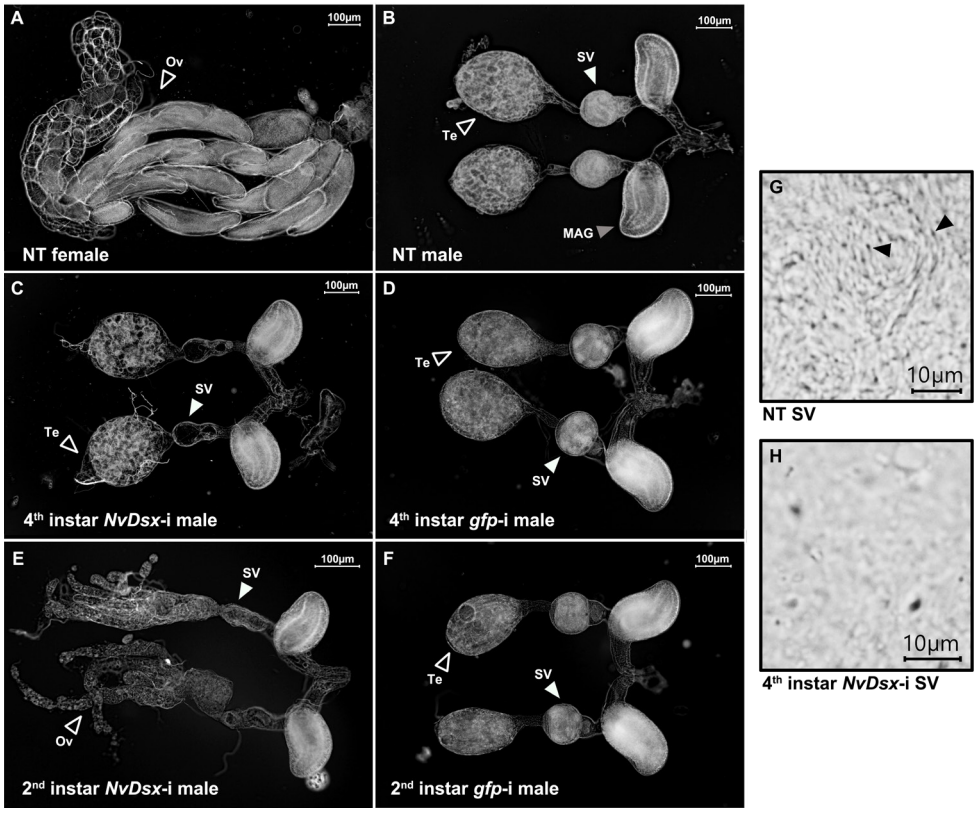


Figure 4.6 Structure of the internal reproductive organs shown for (A) non-treated (NT) female, (B) NT male, (C) male treated with *NvDsx* dsRNA (*NvDsx*-i) and (D) *gfp* (*gfp*-i) in the 4th larval instar, (E) *NvDsx*-i male and (F) *gfp*-i male in the 2nd larval instar and the comparison between (G) NT male and (H) 4th instar *NvDsx*-i male seminal vesicle. MAG, SV and Te refers to male accessory gland, seminal vesicle and testis. Active spermatozoa in the NT male seminal vesicle are indicated with black arrows (G). Scales are provided in the photos.

Discussion

In this study we set out to assess the role of male-specific Dsx isoforms in regulating male sexual dimorphism and sexual differentiation in *N. vitripennis*. In addition to the already known very short male-specific isoform, we identified two additional male-specific NvDsx isoforms with full-length OD2 domains. Within insects, *Dsx* splice variants often differ in terms of structure and number. *N. vitripennis* expresses three male and one female sex-specific splice form of *Dsx,* but contrastingly, *D. melanogaster* has only one male-specific and one female-specific splice form described (Burtis and Baker, 1989) (Figure 4.1). The two newly

identified male-specific *NvDsx* splice variants skip the female-specific exon 6 completely, yet this is a shared feature of male-specific splicing in many insect species including fruit flies (*D. melanogaster*) (Burtis and Baker, 1989) and honeybees (*A. mellifera*) (Cho et al., 2007) (Figure 4.1), but also wild silkmoths (*Antheraea assama*) (Shukla and Nagaraju, 2010a) and fire ants (*S. invicta*) (Nipitwattanaphon et al., 2014). We did not find any non-sex-specific splice variants in *N. vitripennis* as opposed to data for *A. mellifera* (Figure 4.1) (Cho et al., 2007). Then, the *NvdsxM1* isoform retains a 108bp intron that is spliced out from *NvDsxF*, and a similar splicing mechanism was also observed in *A. rosae* in which exon 5 (119bp) is retained in males but spliced out in female-specific *Dsx* splice variants (Mine et al., 2017). In both cases this intron retention creates early in-frame stop codons resulting in a very short male-specific OD2 domain. The mechanism by which this short male-specific isoform would perform its sex-specific regulatory function in *N. vitripennis* is unclear, and therefore initially we planned to silence all three male NvDsx isoforms individually to understand the specific functions of the different NvDsx isoforms during development. However, we were unable to silence the individual *NvDsx* splice variants using RNAi. For future studies we plan to apply different techniques, such as CRISPR/Cas9 in *N. vitripennis*, to further investigate the roles of the different NvDsx isoforms in *N. vitripennis* sexual development.

Pigmentation

The observation that silencing *NvDsx* induced female-like pigmentation in males leads us to conclude that female pigmentation is actively suppressed by NvDsx in male *N. vitripennis*, most likely by interfering with the melanin synthesis pathway. Pigmentation is a highly variable trait among diverse insect species. It provides physical protection through e.g. thermoregulation, UV protection and desiccation resistance (True, 2003). In *D. melanogaster,* sex-specific pigmentation of the abdominal segments is regulated by interaction of the HOX protein Abdominal-B (Abd-B) and Dsx (Kopp et al., 2000). However, in this case DsxM activates the pigmentation pathway leading to abdomen pigmentation in males only (Kopp et al., 2000; Williams et al., 2008). Although the pigmentation intensity of the legs is similar in appearance between NT females and 4th instar *NvDsx*-i males, we observed a slight reduction in antenna pigmentation of 4th instar *NvDsx*-i males (Figure 4.3E). This can be caused by 1) a structural difference in the antennae with males having less plate organs on the sub-segments than females, causing a different colour reflection (Slifer, 1969); or 2) incomplete penetration of *NvDsx*-i in head tissue. We suggest that the less intense pigmentation in the legs of 2nd instar *NvDsx*-i males is due to the transient nature of RNAi, in which the

NvDsx silencing effect was already diminished but the still reduced levels of NvDsx protein could mildly repress the pigmentation pathway. No pigmentation was observed in adults developed from early pupal stage *NvDsx*-i males indicating that NvDsx fully repressed the pigmentation pathway before the RNAi silencing could take effect. Although the deposition of the pigments in the cuticle often starts from late pupal to early adult stages (Massey and Wittkopp, 2016), our results suggest that *NvDsx* represses the pigmentation pathway in male larvae before pupal metamorphosis.

Forewing size

Previous research showed that backcrossing the *N. giraulti wing-size1* locus (*ws1*), a non-coding region of *Dsx,* into the *N. vitripennis* genetic background is correlated with the development of larger wings in *N. vitripennis males* (Loehlin et al., 2010a). Although higher *NvDsxM1* expression levels were detected in individuals with the *N. vitripennis ws1* variant (Loehlin et al., 2010a), the mechanism of wing size regulation by NvDsx is unclear and the *NvDsxM2* and *-3* splice variants were unknown at the time. Our research confirmed that in males high *NvDsx* levels are required for the regulation of the male forewing size by repressing wing growth. In addition, the sex-specific *widerwing* (*wdw*) locus has been backcrossed from *N. giraulti* into *N. vitripennis* thereby increasing male but not female forewing width (Loehlin et al., 2010b), with *wdw* encoding a *upd-like* growth gene (Loehlin and Werren, 2012). The wing size increase of the introgressed *wdw* or *ws1* from *N. giraulti* to the *N. vitripennis* background is due to the growth of both the cell size and the cell number (Loehlin et al., 2010a; Loehlin et al., 2010b). We observed that 2nd instar *NvDsx*-i males developed larger, more female-like wings than 4th instar *NvDsx*-i males, but an increase in wing size and width was observed for both treatment groups, suggesting that NvDsx continuously regulates imaginal wing disc growth throughout larval development. On one hand, the wing imaginal disc cells are assumed to experience an exponential growth from the beginning of larval development until pupal metamorphosis, as has been shown for *D. melanogaster* (Garcia-Bellido and Merriam, 1971). Therefore, we could not obtain a complete female-specific wing phenotype as male-specific wing disc development started well before the applied 2nd instar *NvDsx*-i window. On the other hand, antagonistic effects of NvDsxM and NvDsxF can also play a role in wing size development in *Nasonia*, since in *Aedes aegypti Dsx* knockdown leads to smaller wing development in females (Mysore et al., 2015). Moreover, further research is required to identify the downstream target genes that are regulated by NvDsx to repress wing growth in males.

External genitalia

Dsx is known to be involved in the differentiation of external genitalia structures in male and female insects (Christiansen et al., 2002); however, whether *Dsx* is continuously required for genitalia development has not received much attention. Previous studies using *Dsx* RNAi experiments in other species were conducted at late larval stages and resulted in either deformed external genitalia or chimeras that contain the structures of both sexes (Mine et al., 2017; Zhuo et al., 2018). In contrast, in *N. vitripennis*, both 2nd instar and 4th instar *NvDsx*-i treatments result in only a size reduction of the external genitalia, but no visible effect on morphology was recorded. In the first larval instar of *D. melanogaster*, *Dsx* expression is already detected in the few cells of the genital disc (Robinett et al., 2010), which later directs the differentiation of genital primordia (Keisman et al., 2001). This Dsx-induced regulation starts relatively early to determine the sex-specific fate of genitalia development (Keisman and Baker, 2001). This likely explains the absence of external reproductive tissue deformation in our study. We suggest that in *N. vitripennis* the sexual differentiation of male external genital organs is determined well before 2nd instar larval stage, but sex-specific input from NvDsx for male external genitalia growth is constantly required during development. In addition, our results show that there is a slight difference in the exact NvDsx regulatory window of male genitalia growth. The window in which *NvDsx*-i can affect paramere growth is larger than that for aedeagus growth, indicating the delicate regulation of NvDsx on the growth of sex-specific traits.

Internal genitalia

Contrary to its regulation of growth of external genitalia, NvDsx regulates the morphology of the internal genitalia during larval development. Silencing the *NvDsx* in 2nd instar *N. vitripennis* males resulted in a significant effect on internal genitalia with a female-like ovarian structure and malformed seminal vesicles. Silencing *NvDsx* in 4th instar larvae has a less pronounced effect, although testis shape malformation and sperm production impairment can still be observed. In both treatments, the accessory glands remain intact.

In *Drosophila*, gonads develop from a combination of germ cells and somatic gonadal precursors (SGPs) (Whitworth et al., 2012). Sexual differences in gonad size can already be seen during early larval stage (Kerkis, 1931). This size difference is due to male-specific somatic gonadal precursors (msSGPs), and although initially present in both males and

females, they are only recruited for testes formation (DeFalco et al., 2003). In females, *Dsx* regulates the removal of msGFPs through programmed cell death (DeFalco et al., 2003). Although this sexual dimorphism is established in early development, the cells in the gonad remain highly plastic in their sexual development until late larval stage (Camara et al., 2019). Furthermore, seminal vesicles in *Drosophila* also show a potential for later sexual differentiation as a recruitment of mesodermal cells into genital disc happens in the last larval stage of *Drosophila* and these recruited cells eventually contribute to major parts of the internal genitalia (Ahmad and Baker, 2002). This might explain the difference in development of external and internal genitalia that are regulated by NvDsx. Further research will be needed to fully uncover the Dsx-related mechanism in *Nasonia* that achieves this refined regulation in developing the male genitalia at specific time points.

Visualizing *Dsx*-expressing cells in *D. melanogaster* has demonstrated that *Dsx* gene expression is under elaborate temporal and spatial control to regulate tissue-specific sexual differentiation (Robinett et al., 2010). Here, we build on this knowledge by using a non-drosophilid species to show that different morphological characteristics require their own specific *Dsx* timing and action, and our data suggest that this timing and action is species-specific. Our findings solidify the view that in insects, sexual development is not only a set and forget mechanism that only operates during embryogenesis, but one that requires continuous input from Dsx on a species-specific basis.

We include some extra results of this Chapter in the following box section.

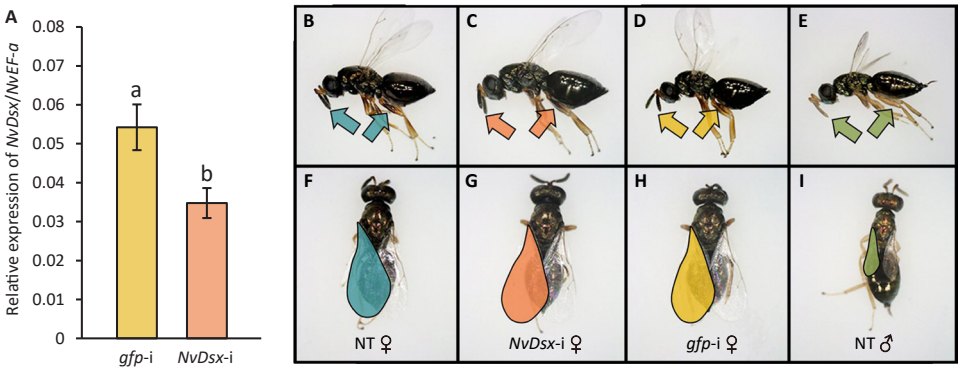
Box section of Chapter 4

Yidong Wang and Eveline C. Verhulst

Silencing *NvDsx* in female larvae shows no altered adult phenotype

In this box, the results of *NvDsx* RNAi silencing (*NvDsx*-i) in females are shown. We hypothesized that *NvDsx* in females also regulates the development of dimorphic traits and that *NvDsx*-i in females would result in male-like phenotypic trait development, such as loss of pigmentation or reduction of wing size. Therefore, we silenced *NvDsx* in females at the 4th instar larval stage. After reducing the *NvDsx* expression in female larvae (Box 4.1A), we observed no differences in pigmentation in antennae and rear leg femur (Box

4.1B-E) and forewing size (Box 4.1F-I) when comparing *NvDsx*-i to *gfp*-i females at adult stage (Box 4.1B-E). It should be noted that the reduction of *NvDsx*, although significantly different from *gfp*-i females (Tukey's HSD, *P*=0.02), might not be sufficient to induce a phenotypic change in females, and the *NvDsx* RNAi efficiency needs to be improved.

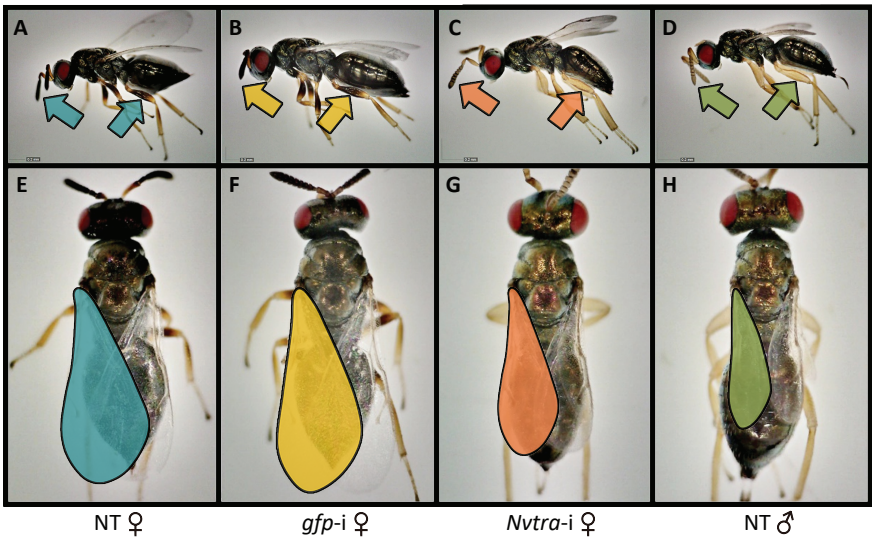


Box 4.1 *NvDsx* silencing efficiency and adult phenotype comparison of 4th instar larval stage *NvDsx* knockdown females with *gfp* control injected females. (A) Relative expression levels of *NvDsx* in adult female after 4th instar RNAi in *NvDsx* knockdown (*NvDsx*-i) and in 4th instar mock *gfp* knockdown (*gfp*-i) females. *NvEF-1a* was used to standardize the expression levels between replicates. Error bars show standard error (n=6). ANOVA and post hoc Tukey's HSD were used for data analysis. Letters in the figures show statistical significance (*P*<0.05) between treatments. Comparison of 4th instar larval stage **(C and G)** *NvDsx*-i female with **(D and H)** *gfp*-i female, non-treated (NT) female **(B and F)** and NT male **(E and I)**. Top row shows the lateral side of tested *N. vitripennis*. Two arrows in each photo indicate the position of pigmentation in the antennae and hind legs. Bottom row shows the dorsal side of tested *N. vitripennis*. Coloured areas mark forewing area of different treatments.

Silencing *Nvtra* in female larvae shows intermediate phenotype

In order to verify the role of NvDsx in female differentiation we then turned to the *NvDsx* upstream regulator *Nvtra* in 2nd instar females. After silencing *Nvtra* using RNAi (*Nvtra*-i), we observed an intermediate phenotype in adult females, including reduced forewing size, less pigmented antennae and completely unpigmented rear leg femur (Box 4.2). Reduction of *Nvtra* not only impairs the female-specific splicing of *NvDsxF*, but also increased the default expression of *NvDsxM* as shown in Figure 4.2. Therefore, the male-like intermediate phenotype observed may be explained by the initiation of male-specific morphological trait development by *NvDsxM* as consequence of *Nvtra*-i, or the reduced levels of NvDsxF lead to improper maintenance of the normal female morphological differentiation. Future research involves optimizing *NvDsx* RNAi efficiency in females, but also genetic modification techniques such as CRISPR-Cas9 (Chaverra-Rodriguez et al., 2020; Li et al.,

2017) need to be optimized in *N. vitripennis* to facilitate gene functional analysis, and further confirm the role of *NvDsx* in females.



Box 4.2 Adult phenotype comparison of 2nd instar larval stage *Nvtra* knockdown females with *gfp* control injected females. Comparison of 2nd instar larval stage *gfp*-i female **(B and F)** with *Nvtra*-i female **(C and G)**, non-treated (NT) female **(A and E)** and NT male **(D and H)**. Top row shows the lateral side of tested *N. vitripennis*. Two arrows in each photo indicate the position of pigmentation in the antennae and hind legs. Bottom row shows the dorsal side of tested *N. vitripennis*. Coloured areas mark forewing area of different treatments.

Acknowledgments

We thank Marcel Dicke for his insightful suggestions and valuable comments; we thank Dr. Hans Smid for assisting with phenotype recording; Rutger Diepeveen for his contribution to the *NvDsx* RNAi work; Joan Diaz Calafat, Age Muller, Simon Pleiter and Romy Gielings for their contribution to the *NvtraF* RNAi work; Min Xu for supporting the sample preparation, collection and measuring; and Weizhao Sun for recording the mating behaviour of RNAi treated males.

Supplementary Information

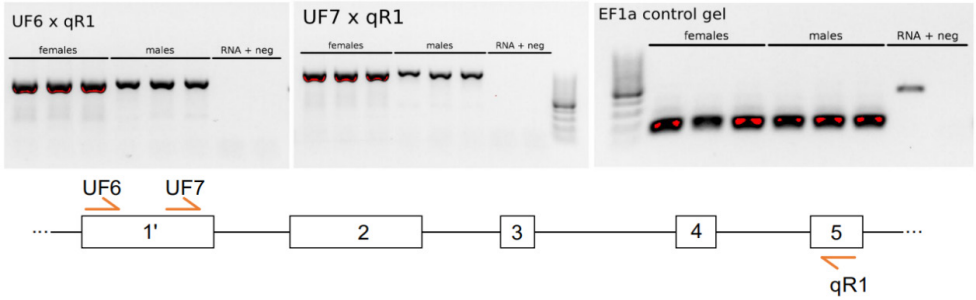


Figure S4.1 Schematic overview of putative *NvDsx* exons with the correlated RT-PCR results. RT-PCR shows the joining of exon 1' with exon 2 of the *NvDsx* transcript. A higher usage of exon 1' in females compared to males is observed. *NvEF-1α* was used as expression control. RNA indicates sample that has not been converted to cDNA and includes any remaining gDNA in the sample. Neg. is negative template RT-PCR control. Primer qR1 is Nv_Dsx_qPCR_R (Table S4.1). All samples were loaded on 1% agarose gel with 100bp ladder (Thermo Fisher).

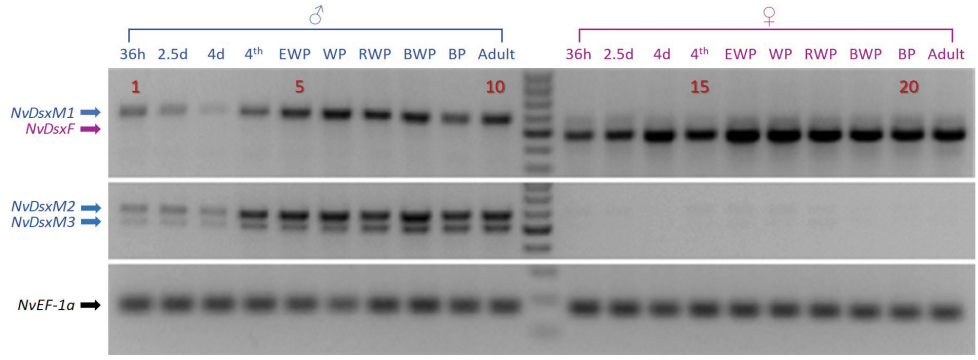


Figure S4.2 Confirmation of sex-specific *NvDsx* transcript expression throughout different developmental stages in *N. vitripennis* males and females. The overall expression profile of each splice variant was determined in ten developmental stages ranging from embryo to adult (Table S4.2). Arrows indicate male (in blue) and female (in purple) splice forms. Lanes 1-10: *N. vitripennis* male samples from 36h to adult stage (2.5d: 2.5 day, 4d: 4 day, 4th: 4th larval instar, EWP: early white pupa stage, WP: white pupa stage, RWP: red eye white pupa stage, BWP: black and white pupa stage, BP: black pupa stage, Adult: Adult stage). Lanes 11: 100 bp ladder. Lanes 12-21: *N. vitripennis* female samples from 36h to adult stage. All samples were loaded on 1.5% agarose gel with 100bp ladder (Thermo Fisher) indication. *NvEF-1α* was used as an expression control.

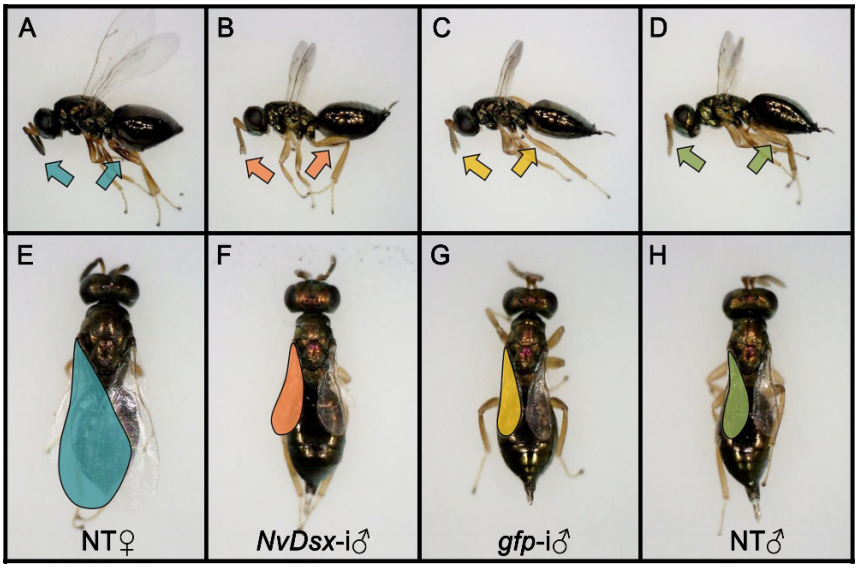


Figure S4.3 Relative expression levels of *NvDsx* after RNAi in different developmental stages. To verify the function of *NvDsxM* in general and *NvDsxM2* and *NvDsxM3* specifically, dsRNAs *NvDsxU* was designed against the common region of *NvDsx* (exon 2-5) and *NvDsxM* was designed against the common region of *NvDsxM2* and *NvDsxM3* (exon 7-9) respectively. A *gfp* dsRNA was used as a mock. Relative expression of *NvDsx* / *NvEF-1α* was determined in non-treated and RNAi treated males at the 2nd instar **(A)**, 4th instar **(B)**, and white pupal stage **(C)**. *NvEF-1α* was used to standardize the variance between samples. X-axis shows different treatments: *gfp*-i, *NvDsxU*-i and *NvDsxM*-i refers to *gfp* knockdown, *NvDsxU* RNAi (*NvDsxU*-i) and *NvDsxM* RNAi (*NvDsxM*-i). Y-axis shows percentage of *NvDsx* / *NvEF-1α* expression of treatments relative to NT control. Error bars show standard error (n=6). ANOVA and post hoc Tukey's HSD were used for data analysis. Letters in the figures show statistical significance (P<0.05) among treatments.

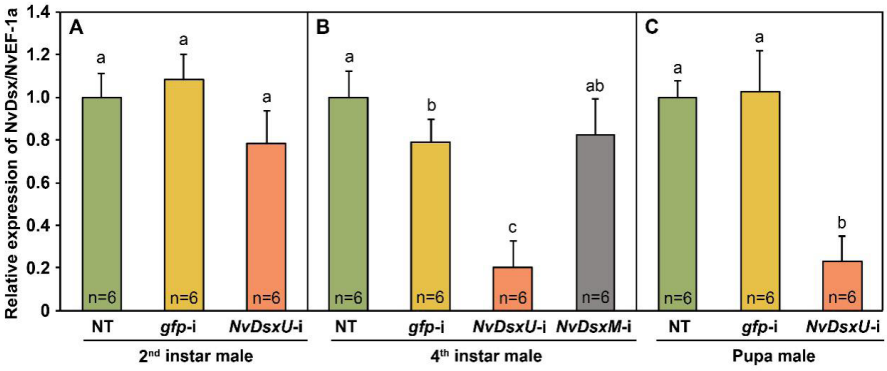


Figure S4.4 Adult phenotype comparison of early white pupal stage *NvDsx* knockdown males with *gfp* control injected males. Comparison of NT female **(A and E)** with early white pupal stage *NvDsx* knockdown (*NvDsx*-i) male **(B and F)** with mock *gfp* knockdown (*gfp*-i) male **(C and G)**, and non-treated (NT) male **(D and H)**. Samples were collected at adult stage and killed by freezing in liquid nitrogen before being placed on glass slides for photo shooting. Top row shows the lateral side of tested *N. vitripennis*. Two arrows in each photo indicate the position of

pigmentation in the antennae and hind legs. Compared to NT males (D), none of the *NvDsx*-i males (B) and *gfp*-i males (C) have pigmentation in antennae and hind legs, while NT females have dark pigmentation in the antennae and hind legs (A). Bottom row shows the dorsal side of tested *N. vitripennis*. Colours mark forewing area of different treatments. Compared among NT males (H), *NvDsx*-i males (F) and *gfp*-i males (G) have relatively smaller forewing and there is no observable difference among them, while NT females have large forewings (E).

Table S4.1 Primers used in this research. 'Region' indicates which *NvDsx* or *Nvtra* exons are targeted from the given primer sets. * '[]' represent the T7 adaptor sequence that is provided with the MEGAscript RNAi Kit (Thermo Fisher). a. (Verhulst et al., 2010b)

Target	Region	Primer	Sequence
		3' race adapter	5'-GCGAGCACAGAATTAATACGACTCACTATAGGT12VN-3'
		3' race outer primer	5'-GCGAGCACAGAATTAATACGACT-3'
		3' race inner primer	5'-CGCGGATCCGAATTAATACGACTCACTATAGG-3'
		5' race outer primer	5'-GCTGATGGCGATGAATGAACACTG-3'
		5' race inner primer	5'-CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATG-3'
NvDsxM2 & NvDsxM3	Exon 5-9	A14_Nvit_DsxU_F1	5'-ACGGTTTGGCTACCATTGGC-3'
		Nv_DsxM_RNAi_R	5'-TAGTAGACGCTCTCTTTGGG-3'
NvDsxF & NvDsxM1	Exon 4-6	A14_Nvit_DsxU_F2	5'-TGAGCAATAACAACAGCAACAG-3'
		Nv_DsxM_R1[a]	5'-TCGGAGAAGATTGGCAGAAC-3'
NvDsx common region	Exon 2	A14_Nv_DsxU_R2	5'-GTATGCGCTGACTTGGCTTG-3'
		A14_Nv_DsxU_R3	5'-GACTTGGCTTGGGCTTTTTCGTC-3'
		A14_Nv_DsxU_R4	5'-ACACTTTTGCACTGCAGACCCT-3'
	Exon 3-5	Nv_Dsx_qPCR_F[a]	5'-CAGCAACACGAGATGCTGATGG-3'
		Nv_Dsx_qPCR_R[a]	5'-TGTCATTACTGCCATTCATGCTTGG-3'
	Exon 1'	Nv_DsxU_F6	5'-CAAGCAGAGAGCAGCTCAGG-3'
		Nv_DsxU_F7	5'-ACAACGGAGCAAGAGGGAAG-3'
	Exon 2-5	Nv_DsxU_RNAi_F	5'-*[TAATACGACTCACTATAGGG]CCAAGAGGCAGCAAATTATG-3'
		Nv_DsxU_RNAi_R	5'-*[TAATACGACTCACTATAGGG]GTTATACGCCGCATGGCTAC-3'
NvDsx male-specific region	Exon 7-9	Nv_DsxM_RNAi_F	5'-* [TAATACGACTCACTATAGGG]CGGCTACTATCCACCCTCG-3'
		Nv_DsxM_RNAi_R	5'-* [TAATACGACTCACTATAGGG]TAGTAGACGCTCTCTTTGGG-3'
	Exon 7	A14_Nvit_Dsx_X7_R1	5'-CAAAGGCGTAGGGCAGGAG-3'
	Exon 8	A14_Nvit_Dsx_X8_R1	5'-GACGCTTGCTTAATCCGTGG-3'
	Exon 9	A14_Nvit_Dsx_X9_R1	5'-AATCCTCGGCCGGAAATAGC-3'
Nvtra common region	Exon 5-8	Nv_Tra_RNAi_F1[a]	5'-*[TAATACGACTCACTATAGGG]CGAGACATCAGTTAGAAGAT-3'
		Nv_Tra_RNAi_R1[a]	5'-*[TAATACGACTCACTATAGGG]GTCTTGTGGTCCTATGAAAC-3'
NvtraF & NvtraM	Exon 2-3	Nv_Tra_F2[a]	5'-GACCAAAAGAGGCACCAAAA-3'
		Nv_Tra_R3[a]	5'-GGCGCTCTTCCACTTCAAT-3'
NvEF-1a		Nv_EF-1a_qPCR_F[a]	5'-CACTTGATCTACAAATGCGG-3'
		Nv_EF-1a_qPCR_R[a]	5'-GAAGTCTCGAATTTCCACAG-3'
GFP		GFP_RNAi_F	5'-*[TAATACGACTCACTATAGGG]GTGACCACCTTGACCTACG-3'
		GFP_RNAi_R	5'-*[TAATACGACTCACTATAGGG]TCTCGTTGGGGTCTTTGCT-3'

Table S4.2 Developmental stages and time points in the development of *Nasonia vitripennis*. Overview of different developmental stages and time points used in this study with their abbreviations.

Abbreviation	Description	Development stage	Duration of development at 25 °C, LD 16:8
36h	1st instar larva	Embryo/larva	36 h
2.5d	2nd instar larva	Larva	2.5~3 d
4d	3rd instar larva	Larva	4 d
4th	4th instar larva	Larva	~ 6 d
EWP	Early white pupa	Pupa	~ 7 d
WP	White pupa	Pupa	~ 8 d
RWP	White pupa, red eyes	Pupa	~ 9 d
BWP	Black pupa, white abdomen	Pupa	~ 10 d
BP	Black pupa	Pupa	~ 12 d
Adult	Adult	Imago	~ 14 d

Table S4.3 Target genes and developmental stages of *Nasonia vitripennis* for microinjection for different purposes.

Purpose	Target genes	Targeted Development stages
NvDsxM splicing variants verification	*gfp & NvtraF*	4th larval instar
NvDsx functional analysis	*gfp, NvDsx & NvtraF*	2nd larval instar, 4th larval instar & early white pupa

Movie S4.1 (separate file). A naive adult male injected with *gfp* dsRNA at the 4th instar larval stage courts with an untreated naive female adult. Male is on top and performs a series of head nods in which pheromones are exchanged with the female. At 8 seconds the female becomes receptive and the male slides down to copulate. At 20 seconds, the male climbs back up and performs post-copulatory head nods to end the copulation.

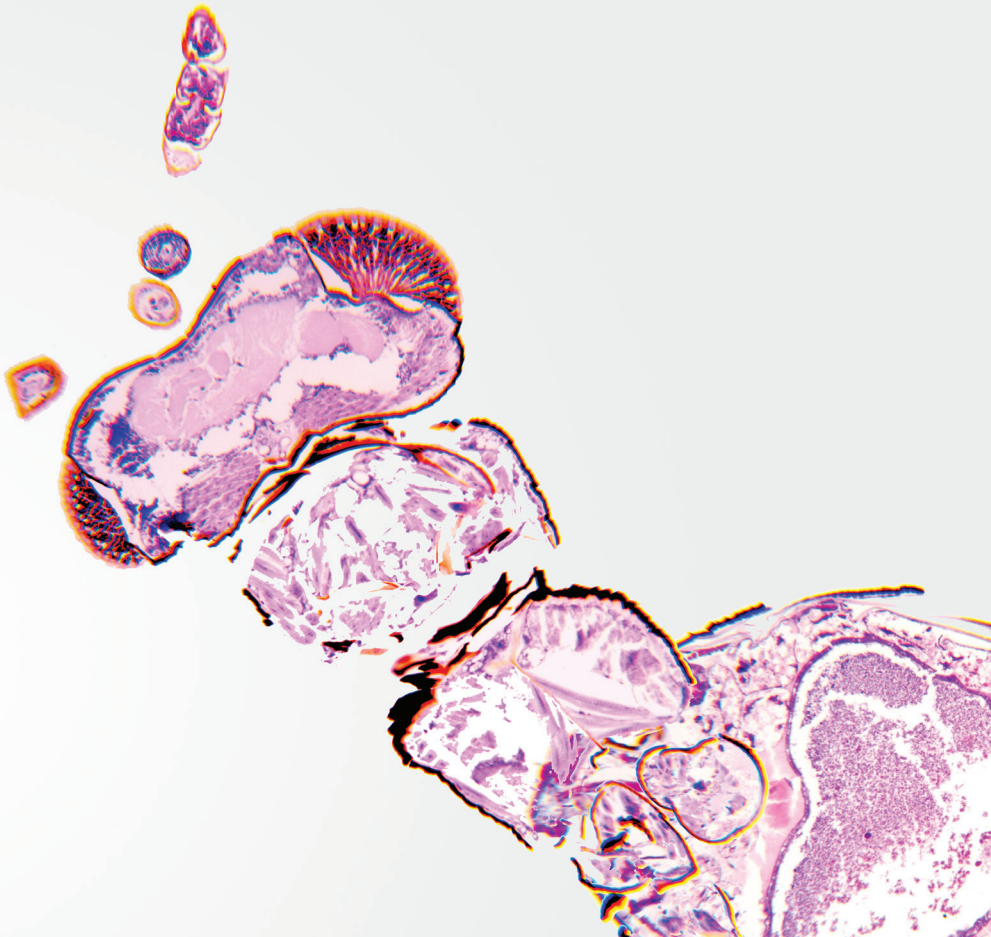
Movie S4.2 (separate file). A naive adult male injected with Dsx dsRNA at the 4th instar larval stage courts an untreated naive female adult. Male is on top and performs a series of head nods in which pheromones are exchanged with the female. At 8 seconds the female becomes receptive and the male slides down in an attempt to copulate. The male tries to copulate for 21 seconds until the female walks away at 30 seconds, and no copulation took place.

Supplementary movies are available on https://www.dx.doi.org/10.6084/m9.figshare.12152322

Chapter 5

The homolog of *Apis mellifera glubschauge* (*glu*) gene is sex-specifically spliced and affects male eye width in *Nasonia vitripennis*

Yidong Wang and Eveline C. Verhulst



Abstract

Sex-specific eye morphology is commonly present in insects. Recently a new sex-determination element, *glubschauge* (*glu*), was identified in *Apis mellifera* that controls sexually dimorphic eye development. *Glu* is seemingly directly regulated by the sex-determination gene *feminizer*, which suggests that *glu* sits at the same instruction level in the sex-determining cascade as the highly conserved sexual differentiation gene *Doublesex*. In this research, we characterized the homolog *glu* (*Nvglu*) in the parasitoid wasp, *Nasonia vitripennis,* and provide a preliminary functional analysis. We confirmed that *Nvglu* is sex-specifically spliced in males and females, and this alternative splicing is regulated by the *Nasonia feminizer* ortholog, *transformer*. We functionally analysed *Nvglu* by RNA interference in second larval instar males and females and found that the relative eye width is decreased in *Nvglu* silenced (*Nvglu*-i) males. In *Nvglu*-i females the relative eye width shows a rising trend, however this observation is not conclusive for *Nvglu* function in females. These results provide a first attempt to investigate the possible utilization of other sex differentiation genes, besides *Dsx,* in regulating sexual dimorphism in *N. vitripennis*.

Introduction

Sexually dimorphic traits commonly exist in insects and have essential roles in insect survival and reproduction (Bear and Monteiro, 2013). Among the variety of sex-specific traits in insects, compound eyes often display sexually dimorphic features in size, eye span and dorsal-ventral regions between sexes (Baker and Wilkinson, 2001; Lau and Meyer-Rochow, 2006; Ziemba and Rutowski, 2000). For instance, in the firefly *Rhagophthalmus ohbaia*, only male eyes have a distinguishable smaller dorsal region and a larger ventral region, which regional difference affects spectral sensitivity (Lau et al., 2007). The ventral region is suggested to have evolved sex-specifically in males to search for conspecific females that emit a yellow light in the evening (Lau et al., 2007). Because the sexual differences of insect morphology are often regulated by the highly conserved gene *Doublesex* (*Dsx*) that is located at the bottom of the sex determination cascade, it is logical to assume that Dsx would also control the sexual differentiation of eye morphology.

In the honeybee, *Apis mellifera*, compound eye size is a sexually dimorphic trait. The eyes of queens and workers (females) cover less than a quarter of the head surface, and are dorsally separated, while in drones (males) two large oval-shaped eyes are attached in the dorsal region and cover one third of the head surface (Menzel et al., 1991; Winston, 1987). The largely increased dorsal region of the eyes in males enhances spatial resolution and facilitates approaching the queen during the mating flight (Menzel et al., 1991; Praagh et al., 1980). However, *A. mellifera Dsx* mutants only differ in their reproductive organs compared to wild type bees, which would suggest that *Dsx* is not involved in the regulation of sexually dimorphic eyes (Roth et al., 2019).

Recently, transcriptome studies of *A. mellifera* early-embryo development revealed a sex-specifically spliced gene, which was termed *glubschauge* (*glu*), that encodes a C2H2 zinc finger motif (ZnF) only in females and determines female-specific eye development in females (Netschitailo et al., unpublished data). It was shown that *glu* is the first gene aside from *Dsx* and *Fruitless* (*Fru*) to be spliced by the sex determination splicing factor Feminizer (Fem) (Netschitailo et al., unpublished data).

The *glu* homologs are also found in related honeybee species, the parasitic wasp *Nasonia vitripennis* and the fruit fly *Drosophila melanogaster*, but no alternative splicing forms are present in *D. melanogaster* (Netschitailo et al., unpublished data). Although a sexually

dimorphic difference in compound eyes is observable in *N. vitripennis*, with larger eyes in males and smaller eyes in females in general, no research has been carried out to study this dimorphic feature in detail. Therefore, in this research we investigated the possible involvement of a *glu* homolog in the sexually dimorphic eye differentiation in *N. vitripennis*.

Material and methods

1. Insects rearing

The *Nasonia vitripennis* lab strain AsymCx was reared on *Calliphora spp.* hosts at 16h/8h light/dark and 25˚C. The AsymCx strain has been maintained in the laboratory for over 10 years and is cured from *Wolbachia* infection.

2. RNA interference (RNAi) of *Nvtra* and *Nvglu* genes

Based on *Nasonia* genome annotation (*Nasonia vitripennis* annotation release 104, Nvit_psr_1.1), and *Nasonia* transcriptome data of female heads (Accession: SAMN07837303, SAMN02687583) and male testes (Accession: SAMN02258478, SAMN02258476) from the National Centre for Biotechnology Information search (NCBI) database, we identified the potential sex-specific alternative splicing of *glu* homolog (Accession: *LOC100678462*) and designed primers for RNA interference (RNAi), quantitative reverse-transcriptase polymerase chain reaction (qPCR) and reverse transcriptase-PCR (RT-PCR) using Geneious 10.0.9.

MEGAscript RNAi Kit (Thermo Fisher) was used to produce dsRNAs to target *Nvtra* and *Nvglu* mRNA. *Gfp* dsRNA was used as a control (Rougeot et al., 2021), which was generated from the vector pOPINEneo-3C-GFP, a gift from Ray Owens (Addgene plasmid # 53534; http://n2t.net/addgene: 53534; RRID: Addgene_53534). Primers to generate *Nvglu* and *Nvtra* dsRNA are provided in Table S5.1.

For *Nvtra* RNAi, dsRNA injection was performed on 4th instar female larvae. RNAi on *Nvglu* was performed by injecting *Nvglu* dsRNA in 2nd instar male and female larvae. Both 2nd and 4th instar larvae were collected from parasitoid hosts and placed on 1X PBS agar plates before injection. Microinjection in *N. vitripennis* larvae was carried out with a FemtoJet® 4i (Eppendorf) and followed the protocol described by Wang et al. (2020). After *Nvglu* dsRNA injection, 2nd instar larvae resumed feeding from the foster fly hosts under rearing conditions. Five to seven replicates (each replicate contains 4-5 individual wasps) per

treatment were collected at the white pupal stage to check for *Nvglu* expression, and the remainder of the injected males and females, once emerged, were collected in separate 1.5 ml Eppendorf tubes. After 4th instar female larva *Nvtra* knockdown, three biological replicate samples were collected at 3-hour-post-injection (h), 1-day-post-injection (d), 2d, 3d, 4d and 5d, and were fast frozen in liquid nitrogen and stored at -80°C.

3. Alternative splicing of *Nvglu* after *Nvtra* knockdown

To confirm sex-specific splicing of *Nvglu,* total RNA of three individual males and females was extracted using ZR Tissue & Insect RNA MicroPrep™(Zymo) following manufacturer's instructions. To test if the splicing of *Nvglu* is under control of *Nvtra*, RNA of different time point samples (described in former section) was extracted using the same kit. An on-column DNase treatment step was added to all samples. Subsequently, one µg of each template was synthesized into cDNA with a standard reaction mix (SensiFAST™ cDNA Synthesis Kit, Bioline) in a thermal cycler (Bio-Rad T100TM Thermal Cycler, Bio-Rad) with 5 minutes priming at 25°C, 30 minutes reverse transcription at 46°C and 5 minutes reverse transcriptase inactivation at 85°C. Primers were designed on the possible alternatively spliced region (Figure 5.2A) and *NvEF-1α* was used as internal reference (Table S5.1). For all RT-PCR reactions, GoTaq® G2 Flexi DNA Polymerase (Promega) was used to prepare the master mix (primer concentration: 0.4µM, MgCl2 solution: 1.5mM) following the manufacturer's instruction and amplified with a standard PCR profile: 3 minutes at 95°C, 35 (adult samples for sex-specific splicing confirmation) / 32 (*Nvtra* knockdown samples) amplification cycles of 30 seconds at 95°C, 30 seconds at 55°C, 50 seconds at 72°C and a final extension of 5 minutes at 72 °C in a thermal cycler (T100TM Thermal Cycler, Bio-Rad).

4. Silencing efficiency of *Nvglu*

To test silencing efficiency of *Nvglu* dsRNA injection, total RNA extraction and cDNA synthesis were done according to the same protocols described above. SensiFAST™ SYBR® No-ROX Kit manual (Bioline) was used to conduct the qPCR (primer concentration: 0.4µM). *NvEF-1α* transcripts were used as reference. qPCR was carried out using the CFX96TM Real-Time System (Bio-Rad) with CFX Manager 3.1 Software (Bio-Rad). The standard qPCR profile consists of 95°C for 3 minutes, 40 amplification cycles of 15 seconds at 95°C, 15 seconds of 55°C, 30 seconds of 72°C and a final standard dissociation curve step to check for non-specific amplification.

5. Head measurement

Individual male and female heads were separated from the body on a glass slide using a scalpel. Thereafter, heads were mounted on clean slides and photos were taken from dorsal view using a Dino-Lite Edge 5MP digital microscope. Head measurements (Figure 5.1), including head length from top of head to bottom of head (HL), maximum head width (MHW) and interocular distance at the maximum head width (MIO) were based on the description from Werren et al. (2016) and measured by using DinoCapture 2.0 software.

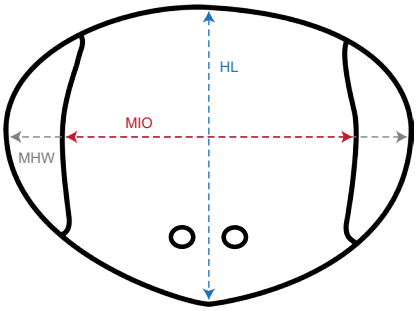


Figure 5.1 Head measurement of *N. vitripennis*. (HL) Length from top of head to bottom of head, (MHW) maximum head width and (MIO) interocular distance at maximum head width are indicated.

6. Data analysis

qPCR data was first imported to LinRegPCR software (LinRegPCR, 2017.1.0.0, HFRC, Amsterdam, The Netherlands (Ramakers et al., 2003). After baseline correction, the initial number of templates (N0) were calculated based on the average PCR efficiency of each amplicon. Relative expression levels of *Nvglu* in each sample were obtained by dividing the N0 value of *Nvglu* by N0 value of *NvEF-1a*. Statistical analysis was performed in R (R core Team, 2018). qPCR data was analysed using One-way ANOVA. Head measurement data was analysed using Kruskal-Wallis rank sum test and Dunn-test.

Results

Sex-biased expression of *Nvglu* in *N. vitripennis* adults is under the control of *Nvtra*

In *A. mellifera,* transcriptome studies in female and male embryos revealed the gene *glubschauge* (*glu*, *LOC552468*) to be sex-specifically spliced and to encode a ZnF motif in females only (Netschitailo et al., unpublished data). An NCBI Blast search using *glu* as query

against the *N. vitripennis* genome resulted in the identification of an uncharacterized gene *LOC100678462* (Netschitailo et al., unpublished data), that we termed *Nvglu.* In the available *N. vitripennis* RNAseq data in NCBI, we observed a low expression of exon 7 in male testes tissue (the only male RNAseq data available in NCBI), while this exon is highly expressed in female heads, suggesting possible sex-specific splicing of this homolog (Figure 5.2A, Figure S5.1). To confirm this, we designed primers to target the region where potential sex-specific splicing occurs including exon 7 (Figure 5.2A), and conducted RT-PCR on whole body adult males and females. The results show that indeed *Nvglu* is sex-specifically spliced, with exon 7 being retained in the females only. This female-specific *Nvglu* (*Nvglu*^{F}) is, therefore, only expressed in female samples and is absent in male samples, which show only faint genomic fragments (gDNA, Figure 5.2B). The male-specific *Nvglu* (*Nvglu*^{M}) is present in higher amounts in males than in females (Figure 5.2B). The ZnF motif, however, is not encoded in either sex-specific *Nvglu* splice variant.

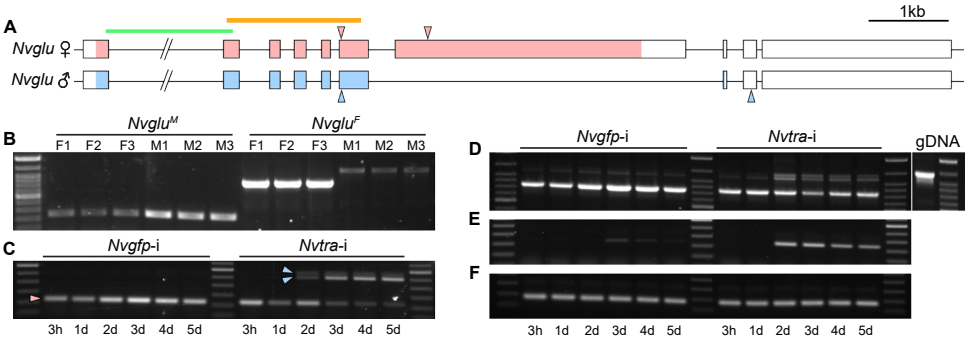


Figure 5.2 *Nvglu* gene structure, sex-specific expression and splicing patterns after *Nvtra* knockdown in *N. vitripennis*. (A) Sex-specific splicing structure of *Nvglu* based on NCBI RNAseq data and partial RT-PCR confirmation. One kb scale is provided. Male- and female-specific splice forms are indicated by ♂ and ♀. Blocks indicate exons and coding regions are marked with colours. Pink and blue arrows refers to the sex-specific primers to amplify the sex-specific region of *Nvglu*. Double slash lines were used to reduce the intronic region for visualization purpose. Yellow lines indicate the dsRNA target region and green lines indicate the qPCR primer target. **(B)** Sex-specific splicing of *Nvglu* in male (M) and female (F) adults. Expression of **(C)** *Nvtra*, **(D)** *Nvglu*^{F}, **(E)** *Nvglu*^{M} and **(F)** internal reference *NvEF-1a* in *N. vitripennis* females that are injected in the 4th instar larval stage with *Nvtra* dsRNA or *gfp* dsRNA. Male and female *Nvtra* splice variants are indicated by blue and pink arrows accordingly. Target genes are indicated on gels and hours (h) and days (d) after injection are indicated under the gels. All samples were loaded on 1.5% agar gel with 100bp ladder (Thermo Fisher) indication.

We hypothesized that *Nvglu*^{F} splicing is under the control of NvTra^{F} as *glu* is under control of the Tra ortholog, Fem, in *A. mellifera* (Netschitailo et al., unpublished data). Therefore we transiently silenced *Nvtra* (*Nvtra*-i) in 4th instar females and compared the splicing patterns of *Nvglu* to the mock control *gfp* knockdown (*gfp*-i). *Nvtra*^{F} splicing is regulated by its own

protein in an autoregulatory loop, and it has been shown that *Nvtra* RNAi results in a shift of female-specific *Nvtra* splicing to male-specific *Nvtra* splicing (Verhulst et al., 2010b). If NvTra^F controls *Nvglu* splicing, in addition to its own splicing, the shift in splicing from female- to male-specific *Nvtra* and *Nvglu* should occur simultaneously. After two-days (d) following *Nvtra* dsRNA injection, we observed a reduction of *Nvtra*^{F} expression and an elevation of the default *Nvtra*^{M} expression (Figure 5.2C), proving we successfully knocked down *Nvtra.* Then, a slight reduction of *Nvglu*^{F} and an increase of unspecific gDNA amplification in *Nvtra*-i treatment from 2d onwards was observed (Figure 5.2D), in addition to a clear increase of *Nvglu*^{M} splicing, which was not observed in *gfp*-i control samples (Figure 5.2E). This strongly suggests that NvTra^F not only splices its own transcripts but also splices *Nvglu* into the female-specific splice variant.

Nvglu has a small effect on male head shape

We next accessed the effect of reducing *Nvglu* expression in males and females on head morphology. After knocking-down *Nvglu* in 2nd instar male and female larvae, we detected a significant reduction of *Nvglu* in both *Nvglu*-i males (P=0.006) and females (P<0.001) at the pupal stage compared to the respective *gfp*-i samples (Figure 5.3A,B).

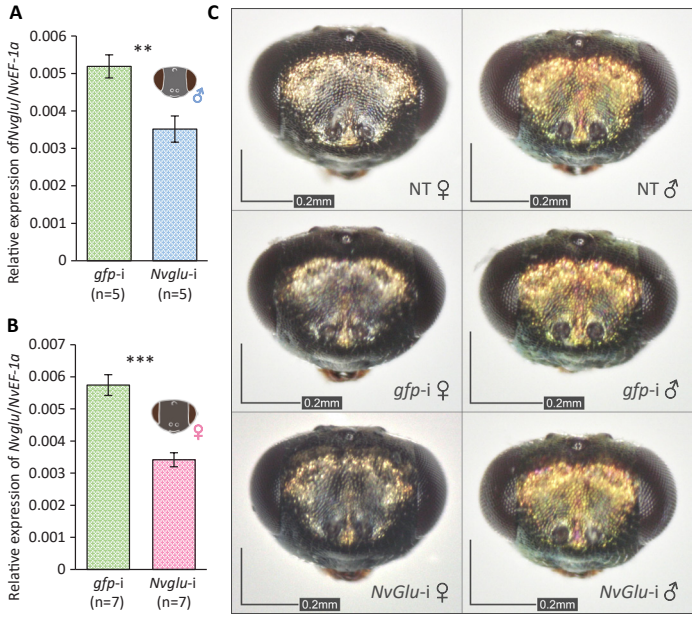


Figure 5.3 Relative expression levels of *Nvglu* in males and females after *Nvglu* RNAi (*Nvglu*-i) and phenotypes of control and treated male and female heads. Relative expression of *Nvglu*/*NvEF-1a* in *Nvglu*-i and *gfp* RNAi (*gfp*-i) **(A)** males and **(B)** females at white pupal stage. Error bars denote standard error and numbers of replicates are indicated below the bar graphs. Asterisks show significant differences: ** = P<0.01 and *** = P<0.001. **(C)** Front view of the representative head morphology of non-treated (NT), *gfp*-i and *Nvglu*-i male and female. Male and female are indicated by ♂ and ♀. Scales are provided in each photo.

However, no observable difference in head morphology can be observed between *gfp*-i and *Nvglu*-i in either sex (Figure 5.3C).

Further detailed measurements on male heads show that the maximum head width (MHW) corrected by head length (HL) is significantly reduced in *Nvglu*-i compared to *gfp*-i (P=0.003) and non-treated (NT) (P<0.001) (Figure 5.4A), while interocular distance at maximum head width (MIO) corrected by HL does not change with different treatments (P=0.97, Figure 5.4B). In females, on the other hand, MHW/HL shows an increasing trend in *Nvglu*-i compared to NT (P=0.076), and it is significantly different between *gfp*-i and *Nvglu*-i (P=0.016, Figure 5.4C). The effect of *Nvglu*-i on MIO/HL in females is inconsistent, as *gfp*-i has a highly significant effect on female MIO/HL compared to NT treatments (P=0.004 Figure 5.4D), while there is no significant difference between *Nvglu*-i and NT treatments (P=0.353, Figure 5.4D).

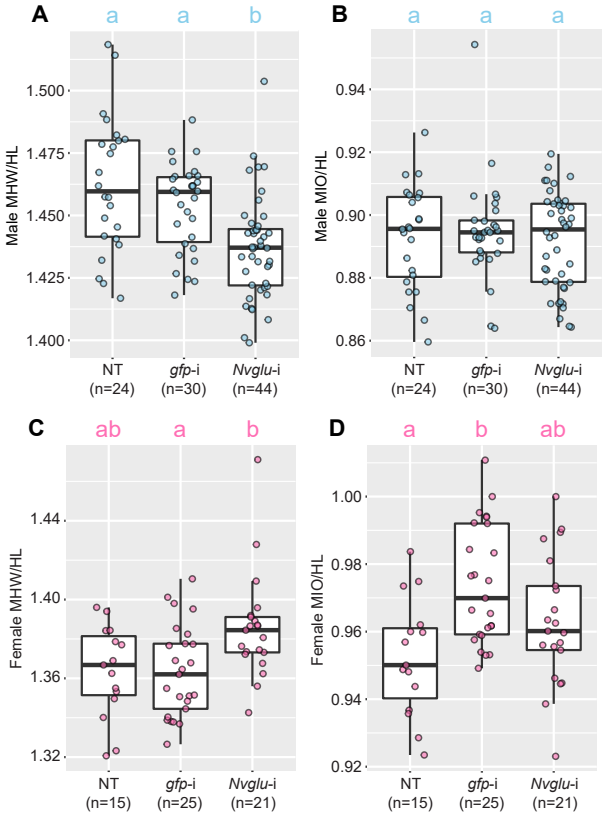


Figure 5.4 Adult head and eye measurements of 2nd instar *Nvglu* RNAi (*Nvglu*-i) and *gfp* RNAi (*gfp*-i) treated males and females. Box-and-whisker plots represent **(A)** the maximum width of the male head (MHW) corrected by head length (HL), **(B)** the interocular distance at maximum male head width (MIO) corrected by HL, **(C)** female MHW/HL and **(D)** female MIO/HL. Numbers of replicates are provided under the box-and-whisker plots. Each plot shows median (horizontal line), 25-75% quartiles (box), maximum/minimum range (whiskers) and sample points. Letters represent significant differences at P<0.05.

Discussion

In this experiment, we identified an *A. mellifera glu* homolog in *N. vitripennis* and show that it is a potential regulatory gene for sexual dimorphism of head morphology, as has been shown in *A. mellifera* (Netschiltailo et al., unpublished data). In *A. mellifera*, *glu* is sex-specifically spliced in exon 8 (Netschitailo et al., unpublished data), shifting the open reading frame (ORF) in males, resulting in an early stop codon thereby encoding only 16 amino acids (aa). In females, the inclusion of exon 8 creates an ORF that encodes a 1256 aa long peptide which harbours a ZnF motif (Netschitailo et al., unpublished data). We show that also in *N. vitripennis*, *Nvglu* is sex-specifically spliced, with exon 7 retention in females only. However, *Nvglu* in *N. vitripennis* does not appear to be a transcription factor as no C2H2 ZnF motif was identified in *Nvglu* (Netschitailo et al., unpublished data). Analysing the likelihood of the ancestral states of the amino acid positions in 49 homologous sequences revealed this core C2H2 motif was gained in the stinging wasps (Aculeata) after the divergence of the stinging wasps and parasitoid wasps. The sex-specific splicing of *glu* is not a conserved feature in its homologs in *Drosophila melanogaster* (Diptera) and *Cimex lectularius* (Hemiptera) which are outside the Hymenoptera (Netschitailo et al., unpublished data).

We strongly suggest that the sex-specific splicing of *Nvglu* is under the control of NvTra. After silencing *Nvtra* in female pupae, we observed an increased expression of male-specific *Nvglu* variants and a reduction of female-specific *Nvglu* variants in *Nvtra*-i compared to *gfp*-i control simultaneously with the shift in $Nvtra^{F}$ to $Nvtra^{M}$ splicing. The upstream regulatory gene, *feminizer* (*tra* homolog in *A. mellifera*) has been shown to direct the sex-specific splicing of *glu* in *A. mellifera* (Netschitailo et al., unpublished data). In addition, in *A. mellifera,* sex-specific expression of *glu* in embryo development happens before *Dsx* splicing, suggesting it is not under Dsx regulation (Netschitailo et al., unpublished data). We have no *N. vitripennis* transcriptomic data of the early embryo developmental stage right after peak expression of *Nvtra* in *N. vitripennis*, to corroborate the timing of *Nvglu* splicing is before *NvDsx* splicing. However, the fact that the observed shift of *Nvglu* splicing after *Nvtra*-i coincides precisely with the shift of *Nvtra* splicing, suggests that also in *N. vitripennis Nvglu* splicing is not under control of NvDsx. It is notable that in both *N. vitripennis* and *A. mellifera*, *glu* is sex-specifically spliced by Tra/Fem, showing that the regulatory pathway seems conserved between *A. mellifera* and *N. vitripennis.*

Sexual dimorphism in head shapes has been observed in *N. vitripennis* before (Darling and Werren, 1990), but with our detailed measurements, we quantified the differences between *N. vitripennis* males and females. In general males have a bigger head width to head length ratio than females, but females have a bigger interocular eye distance to head length ratio than males. In our *Nvglu* knockdown males, the MHW/HL decreased and became more female-like, while the MIO/HL remained unchanged, suggesting that the alteration is specifically reducing eye width. The MHW/HL shows an increasing trend in *Nvglu*-i females, and became more male-like. However, no consistent difference was observed in MIO/HL when comparing to *gfp*-i and NT females. This could be due to the relatively low silencing efficiency of *Nvglu* in general. In *A. mellifera,* deletion of *glu* exons 2-8 by CRISPR/Cas9 increased female eye width and length and reduced interocular distance (Netschitailo et al., unpublished data). A targeted mutagenesis of the C2H2 Zn finger resulted in unchanged female eye width only while length and interocular distance showed a similar phenotype as *glu* exon 2-8 deletion (Netschitailo et al., unpublished data). We observed a very modest change in the ratio of head width to head length in both males and females after *Nvglu* RNAi but not in the interocular distance in relation to head length, indicating that *Nvglu* could be involved in regulating this part of the eye morphology. The effects in *N. vitripennis* are less pronounced than in *A. mellifera* which could be due to the difference in technique, as we used transient RNAi, whereas CRISPR/Cas9 was used in *A. mellifera* to establish a full gene knockout (Netschitailo et al., unpublished data), or because of the lacking of the ZnF motif in *Nvglu*. More research is required to fully understand the function of *Nvglu* and its evolution in Hymenoptera.

Acknowledgements

We would like to thank Marcel Dicke for his insightful suggestions and valuable comments; Martin Beye and Oksana Netschitailo for their suggestions on the experimental design and for providing the information that helped construct the introduction and discussion.

Supplementary

Table S5.1 Primers used in this research. * T7 promoter sequence that is recommended by the manufacturer.

Target	Primer	Sequence
Nvtra dsRNA	Nv_Tra_RNAi_F1[a]	5'-*[TAATACGACTCACTATAGGG]CGAGACATCAGTTAGAAGAT-3'
	Nv_Tra_RNAi_R1[a]	5'-*[TAATACGACTCACTATAGGG]GTCTTGTGGTCCTATGAAAC-3'
Nvglu dsRNA	glu RNAi_F3	5'-*[TAATACGACTCACTATAG]AAACACGAACAATTAGGCGC-3'
	glu RNAi_R3	5'-*[TAATACGACTCACTATAG]ATCTGTACAAACTTGGCGCA-3'
Nvglu RT-PCR for male splicing	glu_M2_Forward	5'-AAGTTTTACCGTCTGCTGCA-3'
	glu_M2_Reverse	5'-CGCGTACCGAATTGTTTTGT-3'
Nvglu RT-PCR for female splicing	glu_M&F_Forward	5'-TACGACTGATCCCGGCTAAT-3'
	glu_F_Reverse	5'-ACGACTTTGATTCGAGCTCC-3'
Nvglu qPCR	glu_qpcr_Forward	5'-AGTCGAGAGTTTTCCGCCT-3'
	glu_qpcr_Reverse	5'-GGGAAACTGTATGTCAACGTTC-3'
Reference gene for qPCR and RT-PCR	Nvit_EF1a_qPCR_F1[a]	5'-CACTTGATCTACAAATGCGG-3'
	Nvit_EF1a_qPCR_R1[a]	5'-GAAGTCTCGAATTTCCACAG-3'
RT-PCR for *Nvtra*^{F} & *Nvtra*^{M} splicing	Nv_Tra_F2[a]	5'-GACCAAAAGAGGCACCAAAA-3'
	Nv_Tra_R3[a]	5'-GGCGCTCTTCCACTTCAAT-3'

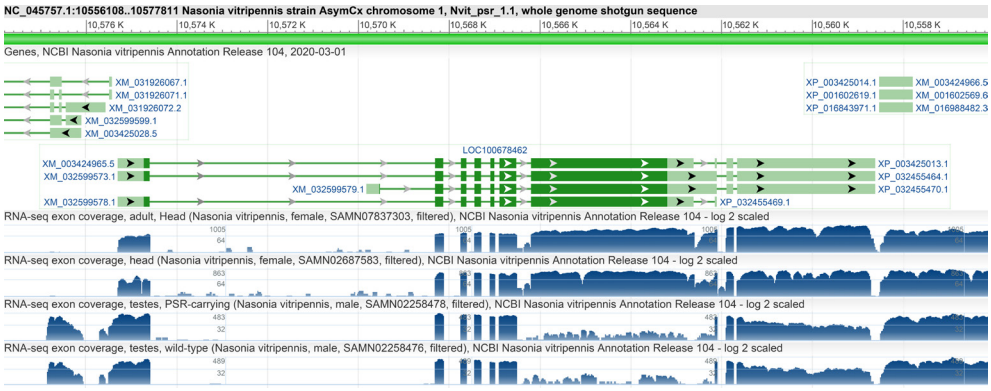


Figure S5.1 Screen shot of *Nasonia* genome annotation in national centre for biotechnology information search (NCBI) (*Nasonia vitripennis* annotation release 104, Nvit_psr_1.1) and *Nasonia* transcriptome data of female heads (Accession: SAMN07837303, SAMN02687583) and male testes (Accession: SAMN02258478, SAMN02258476) of the *Nasonia glu* homolog (*LOC100678462*).

Chapter 6

Silencing *Doublesex* expression triggers three-level pheromonal feminization in *Nasonia* males

Yidong Wang, Weizhao Sun, Sonja Fleischmann, Jocelyn G. Millar, Joachim Ruther and Eveline C. Verhulst

Photo by Hans Smid

Abstract

The transcription factor Doublesex (Dsx) has a conserved function in controlling sexual morphological differences in insects, but our knowledge on its role in regulating sexual behaviour is widely limited to *Drosophila*. Here, we show in the parasitoid wasp *Nasonia vitripennis* that males whose Dsx gene had been silenced by RNA interference (*NvDsx*-i) underwent a three-level pheromonal feminization: (1) *NvDsx*-i males were no longer able to attract females from a distance, owing to drastically reduced titres of the abdominal long-range sex pheromone. (2) *NvDsx*-i males were courted by wild-type males like females which correlated with a lower abundance of alkenes in their cuticular hydrocarbon (CHC) profiles. Supplementation of *NvDsx*-i male CHC profiles with realistic amounts of synthetic (*Z*)-9-hentriacontene (*Z*9C31), the most significantly reduced alkene in *NvDsx*-i males, interrupted courtship by wild-type conspecific males. Supplementation of female CHC profiles with *Z*9C31 reduced courtship and mating attempts by wild-type males. These results prove that *Z*9C31 is crucial for sex discrimination in *Nasonia.* (3) *NvDsx*-i males were hampered in eliciting female receptivity during courtship and thus experienced severely reduced mating success, suggesting that they are unable to produce the hitherto unidentified oral aphrodisiac pheromone reported in *N. vitripennis* males. We conclude that Dsx is a multi-level key regulator of pheromone-mediated sexual communication in *N. vitripennis*. Silencing *Dsx* by RNA interference provides a new avenue for unravelling the molecular mechanisms underlying the pheromone-mediated sexual communication in insects.

Introduction

Insect sex pheromones play a major role in initiating and guiding the mating process, and in allowing individuals to reliably recognize conspecific mates. In many insect species one sex releases a blend of volatile sex pheromones that conveys species-specific information leading to attraction of conspecifics of the opposite sex (Chuman et al., 1987; Lebreton et al., 2017; Roelofs and Rooney, 2003). The attractiveness of the blend is often based on the quantity and ratio of the pheromone components, which may be correlated with nutritional condition, age, or gamete quality of the sender (Chemnitz et al., 2015; Ruther et al., 2009). Over shorter distances, sex-specific mixtures of low-volatility, fatty-acid-derived hydrocarbons on the insect cuticle (cuticular hydrocarbons, CHC) play a role in the recognition and mating processes of many insect species (Buellesbach et al., 2013; Geiselhardt et al., 2009; Ginzel, 2010; Jungwirth et al., 2021; Silk et al., 2009), and species can often be identified by their unique CHC profiles (Buellesbach et al., 2013; Page et al., 1990; Ruther, 2013). In many insects, the same CHC compounds are shared by both sexes but vary in their relative abundance (Ginzel et al., 2003; Steiner et al., 2006; Uebel et al., 1975), while others have evolved subsets of sex-specific CHC to either attract potential mates or to repel competitors (Carlson et al., 1971; Ferveur and Sureau, 1996).

Because sex pheromone production is sexually dimorphic in nature, it is generally assumed that the sex determination pathway is involved in regulating sex pheromone production. In insects, sexual development is initiated by diverse species-specific primary signals, but downstream, the pathway contains several transcription factors that have relatively conserved functions (Bopp et al., 2014; Wilkins, 1995). One major transcription factor, Doublesex (Dsx), is located at the bottom of the cascade, and regulates sexual differentiation in various species across insect orders (Chen et al., 2019; Ito et al., 2013; Mine et al., 2017; Rice et al., 2018; Shukla and Palli, 2012b; Williams et al., 2008; Xu et al., 2017). At first, Dsx was considered to be exclusively a regulator of sex-specific morphological development, while the transcription factor Fruitless (Fru), as identified in *Drosophila melanogaster,* was thought to mediate sex-specific behaviours, including mating behaviour. In *Drosophila*, the male Fru protein (Fru^M) is a key regulator of crucial mating rituals in both the central nervous system (CNS) and the peripheral nervous system (Demir and Dickson, 2005; Ryner et al., 1996). Accumulating evidence has been shown that Dsx coordinates the development of the CNS, and together with Fru^M, shapes courtship behaviour in *D. melanogaster* males (Pan et al., 2011; Rideout et al., 2007; Rideout et al., 2010). Nevertheless, Dsx knockdown

experiments in other insect species, for example in *Onthophagus taurus* beetles, changed aggressiveness, but did not yield solid evidence for changes in mating behaviours (Beckers et al., 2017). Our knowledge of the role of Dsx in directly regulating the mating behaviour of insects is therefore primarily limited to *Drosophila* flies.

Mating is a complex process in insects, which is influenced by morphological characteristics, behaviour, and chemical communication, making it a challenge to study the role of Dsx in regulating these interacting factors. The jewel wasp *Nasonia vitripennis* (Hymenoptera: Pteromalidae), as an emerging model species, offers great opportunities for this kind of research. *Nasonia vitripennis* is a gregarious parasitoid of several pest fly species (Whiting, 1967). The sex determination mechanism in *N. vitripennis* has been completely elucidated (van de Zande and Verhulst, 2014; Verhulst et al., 2010b; Verhulst et al., 2013; Zou et al., 2020), and recently we illustrated the role of Dsx in regulating sexually dimorphic traits (Wang et al., 2020). In parallel, the chemical communication system of *N. vitripennis* has been intensively studied (Mair and Ruther, 2019). The male-derived long-range sex attractant pheromone of *N. vitripennis* consists of the major components (4*R*,5*R*)- and (4*R*,5*S*)-5-hydroxy-4-decanolide (HDL-*RR* and HDL-*RS*) with a synergistic minor compound, 4-methylquinazoline (4MQ) (Ruther et al., 2007; Ruther et al., 2008). The pheromone is synthesized by males in the rectal vesicle (Abdel-Latief et al., 2008) and is deposited onto the natal host and other substrates through a typical "abdominal dipping" behaviour (Mair and Ruther, 2018; Steiner and Ruther, 2009). Pheromone marks are highly attractive to conspecific virgin females, whereas mated females are no longer attracted (Lenschow et al., 2018). In addition to the male-derived sex attractant, sex- and species-specific CHC profiles have been identified in wasps of the genus *Nasonia* (Buellesbach et al., 2013; Buellesbach et al., 2018; Niehuis et al., 2011; Steiner et al., 2006). CHC are used for mate recognition, and elicit stereotyped courtship behaviour in *N. vitripennis* males which has been well-characterized and described in detail (Barrass, 1960; van den Assem and Werren, 1994; van den Assem et al., 1980). After encountering a female, the male immediately mounts, places his fore tarsi on her head, and starts to perform the mating ritual, which includes stereotypical wing movements, a series of head nods, and antennal sweeping (Barrass, 1960). During head nodding, the male moves his mouthparts close to the proximal region of the female's antennae and synchronizes the extrusion and retraction of his mouthparts with the upward and downward nodding process (Barrass, 1960; van den Assem and Werren, 1994). During this crucial step, males are believed to secrete an aphrodisiac pheromone from the mouthparts onto the female antennae to elicit female receptivity (van den Assem et al., 1980). The female signals receptivity by lowering her antennae while raising her abdomen

to expose her genital orifice, after which mating usually occurs. Subsequently, another short round of courting, termed post-copulatory courtship, is repeated by the male, during which the female often again exhibits a receptive posture. The sequence terminates with the male dismounting from the female. The blend of oral pheromones apparently not only serves as an aphrodisiac, but also inhibits the response of mated females to the male sex pheromone, thus lowering the chance of re-mating (Mair and Ruther, 2019). Taken together, many aspects of the *Nasonia* mating behaviour and chemical signalling are known, but the possible role of *Dsx* in regulating these sex- and species-specific traits has not yet been investigated.

In this research, we explored the role of *N. vitripennis Dsx* (*NvDsx*) in regulating male mating behaviour and sex pheromone production. We silenced *NvDsx* in male pupae by RNA interference (*NvDsx*-i) and observed each step of the resulting adults' mating behaviour after emergence. Using coupled gas chromatography-mass spectrometry (GC-MS), we then investigated the effects of *NvDsx* on the pheromone chemistry by comparing long-range sex pheromone titres and CHC profiles of *NvDsx*-i males with those of *green fluorescent protein* RNA interference mock control males (*gfp*-i). Finally, we performed Y-tube olfactometer bioassays and bioassays with treated dummies to determine the attractiveness of *NvDsx*-i males to virgin females with which we could identify key components of the male CHC that inhibit male-male mating attempts.

Material and methods

1. Insect rearing

The *Wolbachia*-free AsymCx laboratory strain of *N. vitripennis* was used in all experiments. The laboratory cultures were continuously reared on the host *Calliphora sp.* and *Lucilia caesar* (only the wasps for the dead wasp bioassay) at 25°C with a 16h/8h light/dark cycle.

2. *NvDsx* dsRNA preparation

NvDsx knockdown was conducted by using RNA interference (RNAi) as described in Wang et al. (2020). MEGAscript RNAi Kit (Invitrogen™, Waltham, Massachusetts, USA) was used to produce the dsRNA that targets the common region (exons 2-5) of *NvDsx* in all male splice-variants. *Gfp* dsRNA was used as a control for all experiments and was generated from the vector pOPINEneo-3C-GFP (Addgene plasmid # 53534; http://n2t.net/addgene:

53534; RRID: Addgene_53534). Primers that were used to construct the dsRNA T7 template were designed in Geneious 10.0.9 (Table S6.1). Both *NvDsx* and *gfp* dsRNAs were diluted in RNase-free water to a final concentration of 4000 ng/ul (determined using a NanoDrop™ 2000 Spectrophotometer, Thermo Scientific™) and stored at -20°C.

3. Microinjection

DsRNAs were pre-mixed with a prepared red food dye at a 9:1 ratio before microinjection. Commercially available red food dye was prepared by diluting it 2X in DNase/RNase free water. Because asexual reproduction leads to all-male broods in the haplodiploid sex determination system of Hymenoptera, virgin females were used to produce the male offspring that were used at the pupal stage for microinjection. Thirty female pupae were collected in the black pupal stage (~12 days after egg laying), transferred to a single glass vial (7.5 cm length, one cm diameter), plugged with a cotton plug, and kept under rearing conditions as described above. Twenty-four hours after eclosion, each female was provided with two *Calliphora sp.* host pupae per day. Host pupae were continuously incubated under rearing conditions until parasitoid offspring reached the white pupal stage (~7 days after egg laying). Pupae of male *N. vitripennis* were subsequently collected from hosts and fixed on glass slides with double-sided adhesive tape (3M). Microinjection was performed with a FemtoJet® 4i microinjector (Eppendorf, Hamburg, Germany) following the protocol described by Lynch and Desplan (2006). Phosphate Buffered Saline (PBS) plates were prepared by adding 1.5 g BD Bacto™ Agar (Fisher Scientific, Sparks, Nevada, USA) and one Oxoid™ phosphate buffered saline tablet (Thermo Scientific™, USA) into 100 ml distilled water and autoclaving at 115°C for 15 minutes before pouring them into plastic Petri dishes. After microinjection, slides with injected pupae were placed on PBS plates to prevent dehydration and incubated under rearing conditions until adult emergence.

4. Quantification of *NvDsx* expression by quantitative reverse transcriptase PCR (qPCR)

NvDsx-i males were collected once they emerged. Six individuals were pooled as one biological replicate and six biological replicates per treatment were collected in 1.5 ml microcentrifuge tubes and flash frozen in liquid nitrogen. ZR Tissue & Insect RNA MicroPrep™ (Zymo Research Corp., Irvine, California, USA) kit was used under the manufacturer's instructions to extract the total RNA with an on-column DNase treatment step. Sixteen µl of DNase/

RNase free water was applied to elute each sample. RNA concentration was determined with a NanoDrop™ 2000 Spectrophotometer (Thermo Scientific™) and one µg of total RNA from each sample was converted into cDNA with SensiFAST™ cDNA Synthesis Kit (Bioline, London, UK) using a T100™ Thermal Cycler (Bio-Rad) with an incubation program consisting of five minutes priming at 25°C, 30 minutes at 46°C and five minutes at 85°C.

Following the manufacturer's instruction, a SensiFAST™ SYBR® No-ROX Kit (Bioline, London, UK) was used to perform the qPCR. cDNA templates were further diluted 1:100 and five µl aliquots of each diluted template were used in qPCR reactions to verify gene expression. *Elongation factor 1α* of *N. vitripennis* (*NvEF-1α*) was used as a reference gene. Primers that were used to amplify the common region of *NvDsx* in all male splice-variants were designed in Geneious 10.0.9. Detailed primer sequences are listed in Table S6.1.

5. Mating trials

In the mating trials, non-injected (Non-i) females were presented with Non-i males (n=40), *gfp*-i males (n=40), or *NvDsx*-i males (n=60). One- to two-day-old virgin males and females for the mating trials were separated at the black pupal stage by sex-specific traits such as forewing size and presence/absence of the ovipositor, and were collected in separate glass vials (7.5 cm length, one cm diameter) plugged with cotton until eclosion. Each wasp was used only once. A virgin female was transferred into a glass vial (7.5 cm length, one cm diameter) that contained a male, and the wasps were brought in contact at the bottom by quickly flicking the vial. Observations started immediately after transferring the female. The durations of four distinctive elements of the mating process were recorded: (1) searching time, which is the time from the start of the observation until the male mounts the female; (2) mounting time, the duration of the male mounting the female; (3) copulation time, the duration of the copulation; (4) post-copulation time, the duration of the male remounting the female after copulation until the male dismounts. Recordings were excluded if mounting did not occur within five minutes. Mating success was pronounced positive if all four mating steps were finalized in a single mating trial and was used to calculate the mating success rate.

6. Y-tube olfactometer bioassay

The responses of virgin females to the odours of differently treated conspecifics were tested in a glass still-air Y-tube olfactometer (0.9 cm diameter), consisting of an 8.5 cm stem and two

5 cm arms. Prior to the tests, ten wasps of either treatment were kept in five ml transparent pipette tips that were sealed at both ends with parafilm. Wasp odours were allowed to accumulate in the pipette tips by keeping the wasps inside the pipette tips for two days under rearing conditions. Afterwards, the bottom parafilm was replaced by a piece of fibre mesh, and pipette tips were mounted to the arms of the Y-tube olfactometer. Prior to each experiment, odours were allowed to diffuse for five minutes from the pipette tips into the arms of the Y-tube olfactometer. Tests started once a glass vial containing a one- to two-day-old virgin female was connected to the stem of the Y-tube olfactometer. Tested females were given three minutes to enter the stem of the Y-tube olfactometer and another three minutes to make a decision. Replicates were excluded when females did not enter the stem of the Y-olfactometer within three minutes. A decision was recorded, if a female entered one arm of the olfactometer and stayed less than one cm away from the respective mesh for one minute. Any other cases were recorded as "no choice". After each test, the Y-tube olfactometer was turned by 180° to compensate for any unforeseen asymmetry bias. The Y-tube olfactometer was replaced by a clean one after five tests and pipette tips with wasps were replaced by new ones after 10 tests. Each responding female was tested only once and after the test the female was gently brushed out of the Y-tube. Tested combinations of two choices were between: 10 Non-i females and 10 Non-i males (n=20), 10 Non-i females and 10 *gfp*-i males (n=25), 10 Non-i females and 10 *NvDsx*-i males (n=25), 10 Non-i males and 10 *gfp*-i males (n=40), and 10 Non-i males and 10 *NvDsx*-i males (n=40).

7. Behavioural experiments with dead wasps

Experiments were performed in a round bioassay chamber (10 mm diameter x 3 mm high) made from acrylic glass and covered by a cover slip (Ruther et al., 2000). Behaviours were observed by using a stereo microscope with illumination from a microscope light, and recorded using The Observer XT software (Noldus Information Technology, Wageningen, The Netherlands). Non-i males were exposed to differently treated, freeze-killed one- to two-day-old cadavers (dummies) for five minutes. For each test, the time was recorded that the males spent mounting the dummy, whether these males tried to copulate with the dummy and, if so, the time that these copulation attempts lasted. In the first experiment, we tested the responses of Non-i males (n=25) to untreated male *gfp*-i and *NvDsx*-i dummies as well as to male *NvDsx*-i dummies treated with one µl dichloromethane (DCM, control) or one µl of a solution of *Z*9C31 in DCM (30 ng/µl). In the second experiment, we tested the responses of Non-i males (n=20) to untreated Non-i male and female dummies as well as to

Non-i female dummies treated with one µl DCM (control) or one µl of a solution of *Z*9C31 in DCM (30 ng/µl). *Z*9C31 was synthesized based on Saul-Gershenz and Millar (2006). The dose of *Z*9C31 was based on the amount that was found in the CHC extracts of *gfp*-i males (see results). In all treatments that used DCM, the solvent was allowed to evaporate for one minute before the start of the bioassays.

8. Chemical analysis

For CHC analysis, individual freeze-killed two-day-old *gfp*-i and *NvDsx*-i males (n = 10) were extracted for 30 minutes with 15 µl hexane containing 10 ng/µl tetracosane as an internal standard. For analysis of the volatile sex attractant pheromone (HDL), dissected abdomens of individual freeze-killed two-day-old *gfp*-i and *NvDsx*-i males (n = 10) were extracted with 30 µl of DCM containing 10 ng/µl methyl undecanoate as an internal standard. Aliquots of two µl of extracts (HDL analyses: one µl) were analysed on a Shimadzu QP2010 Plus GC/MS system equipped with a non-polar BPX5 capillary column (60 m x 0.25 mm inner diameter, 0.25 µm film thickness; SGE Analytical Science Europe, Milton Keynes, UK). Samples were injected at 300°C in splitless mode using an AOC 20i auto sampler. Helium was used as carrier gas at a linear velocity of 30 cm s^{-1} (HDL: 37.8 cm s^{-1}). The initial oven temperature of 150°C (HDL: 80°C) was increased at 2°C min^{-1} (HDL: 5°C min^{-1}) to 300°C (HDL: 280°C) and held for 30 minutes. The mass spectrometer was operated in the electron ionization mode at 70 eV and the mass range was *m/z* 35-600. Identifications of compounds were done by comparing mass spectra and linear retention indices with those of authentic reference chemicals (HDL-*RR*, HDL-*RS*, 4MQ, *Z*9C31) or previously published identifications of specific CHCs in this species (Steiner et al., 2006) (*Z*7C31, *Z*9C33, *Z*7C33). Quantification of alkenes and HDL was done by comparing the peak areas of the analytes to those of the respective internal standards.

9. Data analysis

qPCR data was first imported to LinRegPCR software (LinRegPCR, 2017.1.0.0, HFRC, Amsterdam, The Netherlands). After baseline correction, the initial number of templates (N0) was calculated based on averaging PCR efficiency in each amplicon. Relative expression levels of target genes were obtained by dividing the N0 value of target genes by the N0 value of the reference gene. Statistical analysis of qPCR and mating behaviour data was performed in R (R core Team, 2018) using general linear models (GLM) with gamma distribution and

Tukey's Honestly Significant Difference (HSD) test for post-hoc comparisons. Mating success rates were compared by Bonferroni-corrected multiple 2x2 Chi² tests. Y-tube olfactometer data were analysed in R using GLM with binominal distribution with cloglog link function and HSD for post-hoc comparisons. The amounts of alkenes in the CHC extracts and HDL and 4MQ in the abdomen extracts of *gfp*-i and *NvDsx*-i males were compared by Mann-Whitney *U*-tests. In tests with dummies, the total mounting times and the durations of copulation attempts were compared by a Kruskal-Wallis H-test followed by Bonferroni-corrected multiple Mann-Whitney *U*-tests for pairwise comparisons. The percentages of males showing copulation attempts were compared by Bonferroni-corrected multiple 2x2 Chi² tests.

Results

Courting *NvDsx*-i males are less successful in rendering conspecific females receptive

We first asked if the silencing of *NvDsx* expression affects the mating behaviour of males. We microinjected *Dsx* dsRNA into white male pupae and measured a significant suppression of *NvDsx* in these *NvDsx*-i males after emergence when compared to the *gfp*-i control males. (GLM, $P<0.001$), or to Non-injected males (Non-i, GLM, $P<0.001$) (Figure 6.1A). In mating trials, we exposed virgin females to *NvDsx*-i, *gfp*-i, or Non-i males and recorded the duration of the behavioural elements searching, mounting including head nodding, copulation, and post-copulatory courtship, as well as male mating success rate. Only 10.9% of *NvDsx*-i males elicited receptivity and subsequently mated, which was significantly less than Non-i (97.6%) and *gfp*-i males (100%, Figure 6.1B). *NvDsx*-i males did not differ from Non-i (GLM, $P=0.940$) and *gfp*-i (GLM, $P=0.951$) males with respect to searching times, but the high fraction of unreceptive females led to a significantly longer mounting time for *NvDsx*-i males (Non-i: GLM, $P<0.001$; *gfp*-i: GLM, $P<0.001$, Figure 6.1C). The few *NvDsx*-i males that elicit female receptivity after mounting showed no significant difference in copulation time (Non-i: GLM, $P=0.540$; *gfp*-i: GLM, $P=0.266$), but displayed significantly shorter post-copulatory courtship than Non-i males (GLM, $P<0.001$) and *gfp*-i males (GLM, $P=0.002$). There was an effect of *gfp* RNAi on the courtship behaviour, as *gfp*-i males showed longer mounting time (GLM, $P<0.001$) and shorter post-copulatory courtship (GLM, $P=0.014$) than Non-i males. However, the 100% mating success rate of *gfp*-i males indicates overall mating performance was unaffected.

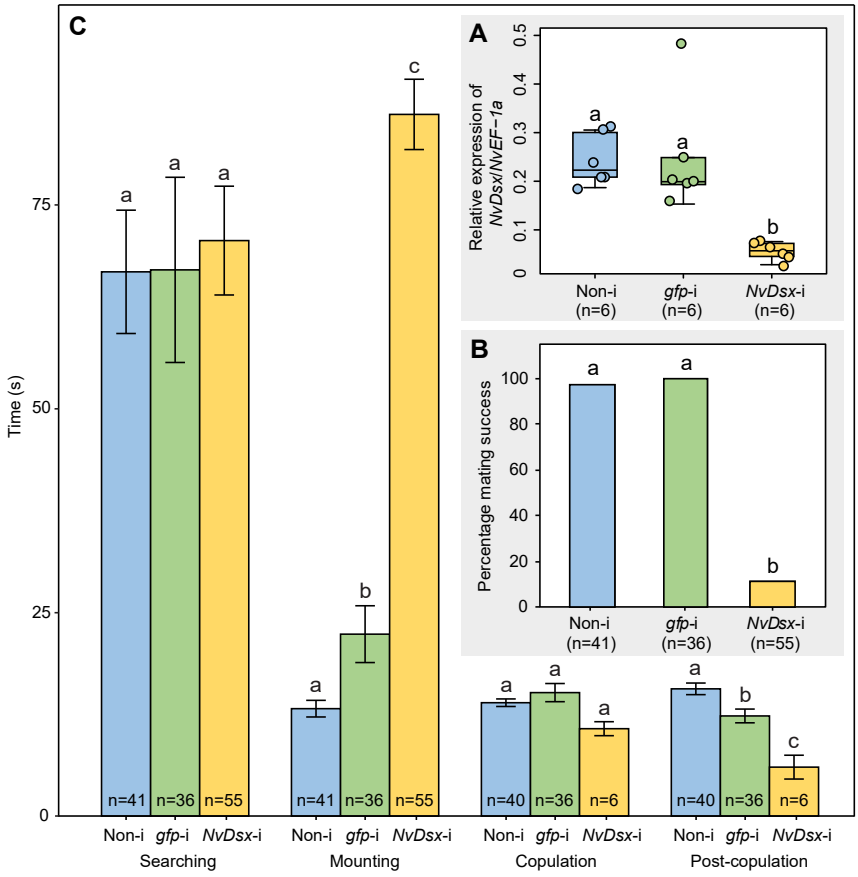


Figure 6.1 Effects of *NvDsx* knockdown on *NvDsx* expression and mating behaviour of *N. vitripennis* males. (A) Relative expression of *NvDsx/NvEF-1α* in Non-i, *gfp*-i and *NvDsx*-i males. Box-and-whisker plot shows median values (horizontal line), 25-75% quartiles (box), maximum/minimum range (whiskers) and individual data points. **(B)** Percentage mating success of Non-i, *gfp*-i and *NvDsx*-i males in mating trials with virgin females. **(C)** Duration of selected behavioural elements (means ± SE) of the *N. vitripennis* mating sequence of Non-i, *gfp*-i, and *NvDsx*-i males in mating trials with virgin females. Different lowercase letters indicate significant differences at $P<0.05$ (A, C: GLM, B: Bonferroni-corrected multiple Chi² tests).

NvDsx-i males are no longer attractive to virgin females due to decreased abdominal sex attractant pheromone production

To evaluate whether *NvDsx*-i males were less successful in attracting virgin females from a distance, we gave virgin females the choice between the odours of two groups of ten live conspecifics in a Y-tube olfactometer. As expected, virgin females preferred the odour of

Non-i males (GLM, $P<0.01$, Figure 6.2A) or *gfp*-i males (GLM, $P<0.001$) over that of Non-i females.

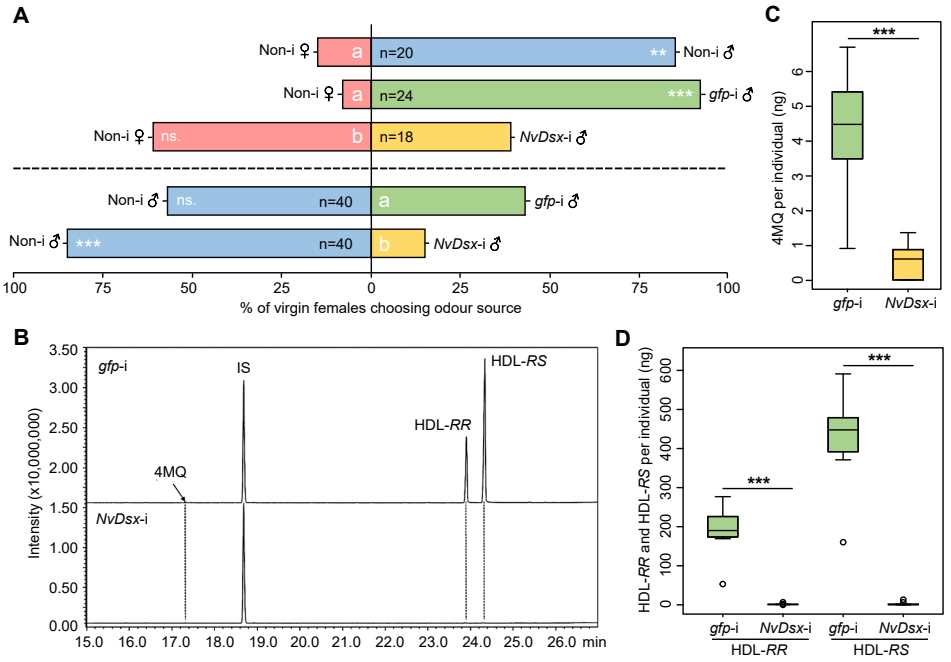


Figure 6.2 Response of virgin females to the odours of differently treated conspecifics in a Y-tube olfactometer, and chemical analysis of the abdominal sex attractant pheromone in *gfp*-i and *NvDsx*-i males. (A) Percentage of virgin females choosing between the odour of ten live Non-i females, Non-i males, *gfp*-i, or *NvDsx*-i males. The treatments tested in each experiment are given on each side of the bars. Statistical analyses were conducted in two groups separated by the dashed line. Letters indicate significant differences at $P<0.05$ (GLM) and deviations from equality are indicated by asterisks, ns. refers to not significant ($P>0.05$), ** refers to $P<0.01$, *** refers to $P<0.001$ (GLM). **(B)** Total ion current chromatograms of abdominal extracts from *gfp*-i and *NvDsx*-i males (IS = internal standard, 10 ng methyl undecanoate, HDL-*RR* and HDL-*RS* = (4*R*,5*R*)- and (4*R*,5*S*)-5-hydroxy-4-decanolide, 4MQ = 4-methylquinazoline). Quantification of **(C)** 4MQ, and **(D)** HDL-*RR* and HDL-*RS* in abdominal extracts from *gfp*-i and *NvDsx*-i males. Box-and-whisker plots show median (horizontal line), 25-75 % quartiles (box), maximum/minimum range (whiskers) and outliers (° >1.5 x interquartile range outside the first or the third quartile). Asterisks indicate significant differences between treatments at $P<0.001$ (Mann-Whitney *U*-test, n=10).

The attractiveness of Non-i and *gfp*-i males did not differ in these trials (GLM, $P=0.904$). In contrast, virgin females were not attracted by the odours of *NvDsx*-i males, showing no preference between those odours and the odours of Non-i females (GLM, $P=0.856$). When given the choice, virgin females did not discriminate between the odours of Non-i males and *gfp*-i males but they preferred Non-i males over *NvDsx*-i males (GLM, $P<0.001$, Figure 6.2A). The reduced attractiveness of *NvDsx*-i males in comparison to *gfp*-i males correlated with

significantly reduced titres of the abdominal sex attractant pheromones, as determined by GC-MS (Figure 6.2B). All three abdominal sex attractant pheromone components, HDL-*RR*, HDL-*RS*, and 4MQ, were detected only in trace amounts in extracts of the abdomens of *NvDsx*-i males, while the total amount and relative proportions of abdominal sex attractant pheromone components in *gfp*-i males were within the range reported previously for Non-i males (Blaul and Ruther, 2011; Ruther et al., 2008; Ruther et al., 2014) (Figure 6.2C,D).

NvDsx-i males elicit courtship in conspecific males due to decreased alkene abundance in their CHC profiles

Earlier observations of *NvDsx*-i mating behaviour by our team suggested that *NvDsx*-i males are mistaken for females by normal males. Previous research also showed that males use CHC signalling to discriminate between the sexes, and that female-derived CHC arrests males and elicits courtship behaviour (Steiner et al., 2006). Hence, we hypothesized that the unusual courting of *NvDsx*-i males by Non-i males is elicited by changes in their CHC profiles. One characteristic feature of *N. vitripennis* CHC profiles is the male-biased relative abundance of alkenes (Steiner et al., 2006). Analysis of the CHC profiles of *NvDsx*-i and *gfp*-i males indeed revealed a significant reduction in alkenes (Figure 6.3A,B). The amounts of (*Z*)-9-hentriacontene (*Z*9C31) in CHC extracts from *NvDsx*-i males were less than one-fourth of those found in *gfp*-i males (mean ± SE *gfp*-i: 31.8 ± 5.7 ng; *NvDsx*-i: 6.6 ± 1.1 ng). Similar reductions, albeit to a lesser extent, were found for (*Z*)-7-hentriacontene (*Z*7C31), (*Z*)-9-tritriacontene (*Z*9C33), and (*Z*)-7-tritriacontene (*Z*7C33).

These results suggest that the decrease in alkenes explains the same-sex courtship of *NvDsx*-i males. Given that *NvDsx*-i treatment affected the amounts of *Z*9C31 the most, we tested whether the application of natural amounts of this compound to *NvDsx*-i males was sufficient to interrupt this male-male courtship. *Nasonia* males are known to respond to attractive dead conspecifics by mounting and copulation attempts (van den Assem et al., 1980). Therefore, we conducted a series of behavioural experiments with differently treated dead wasps (dummies). We presented Non-i males with untreated *gfp*-i and *NvDsx*-i dummies as well as with those treated with 30 ng of synthetic *Z*9C31 dissolved in dichloromethane (DCM) or the pure DCM solvent (control). In general, a higher percentage of *N. vitripennis* Non-i males tried to copulate with *NvDsx*-i male dummies than with *gfp*-i male dummies (X^2(1, *N*=50)=8, *P*=0.028, Figure 6.4A), spent significantly more time on *NvDsx*-i male dummies than on *gfp*-i male dummies (Mann-Whitney *U*=149, n_1=n_2=25,

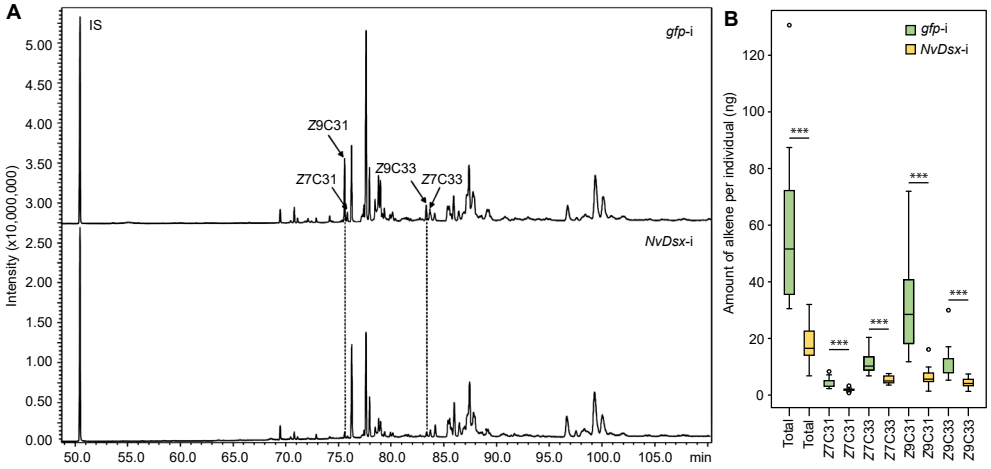


Figure 6.3 Chemical analyses of cuticular hydrocarbons in *NvDsx*-i and *gfp*-i males. (A) Total ion chromatograms of whole-body hexane extracts from *gfp*-i and *NvDsx*-i males. Arrows indicate peaks of the alkenes (*Z*)-9- and (*Z*)-7-hentriacontene (*Z*9C31 and *Z*7C31) as well as (*Z*)-9- and (*Z*)-7-tritriacontene (*Z*9C33 and *Z*7C33). IS = internal standard (10 ng tetracosane). **(B)** Quantification of alkenes in dichloromethane extracts of abdomens of *gfp*-i and *NvDsx*-i males. The box-and-whisker plots show median (horizontal line), 25-75 % quartiles (box), maximum/minimum range (whiskers) and outliers (° >1.5 x interquartile range outside the first or third quartile). Asterisks indicate significant differences between treatments at $P<0.001$ (Mann-Whitney *U*-test, n = 10).

$P<0.01$, Figure 6.4B), and tried to copulate with *NvDsx*-i male dummies longer than with *gfp*-i male dummies (Mann-Whitney $U=209$, $n_1=n_2=25$, $P=0.012$, Figure 6.4C). The application of pure DCM to *NvDsx*-i male dummies did not significantly alter the courtship behaviour of Non-i males (Figure 6.4A-C). However, compared to the pure DCM solvent treated *NvDsx*-i male dummies, the application of *Z*9C31 to *NvDsx*-i male dummies significantly reduced the proportion of Non-i males attempting to copulate with those dummies ($X^2(1, N=50)=20.053$, $P<0.001$), the duration of mounting (Mann-Whitney $U=46.5$, $n_1=n_2=25$, $P<0.001$) and copulation attempts (Mann-Whitney $U=130$, $n_1=n_2=25$, $P<0.01$), to the levels of *gfp*-i dummies (Figure 6.4A-C). These results support our hypothesis that males use the increased abundance of alkenes in the CHC profiles of males to discriminate between sexes. Therefore, in the next step we investigated whether treatment with *Z*9C31 would make female dummies less attractive to responding males. To this end, we performed a similar dummy experiment as described above, using Non-i male (negative control) and differently treated Non-i female dummies. A significantly higher proportion of *N. vitripennis* Non-i males tried to copulate with Non-i female dummies than with Non-i male dummies ($X^2(1, N=40)=28.972$, $P<0.001$), and Non-i males mounted

(Mann-Whitney U=22, n_1=n_2=20, P<0.001) and tried to copulate (Mann-Whitney U=21, n_1=n_2=20, P<0.001) significantly longer with Non-i female dummies than with Non-i male dummies (Figure 6.4D-F). Application of pure DCM solvent to Non-i female dummies did not influence the response of Non-i males. However, comparing to the pure DCM solvent treated Non-i female dummies, application of 30 ng of *Z*9C31 to Non-i female dummies reduced the proportion of mating attempts by Non-i males (X^2(1, N=40)=13.333, P<0.01) as well as the total time Non-i males spent trying to copulate with the Non-i female dummies to intermediate levels (Mann-Whitney U=59, n_1=n_2=20, P<0.001, Figure 6.4D,F). The duration of mounting was also reduced and was not significantly different than that of Non-i male dummies (Mann-Whitney U=121, n_1=n_2=20, P=0.202, Figure 6.4E).

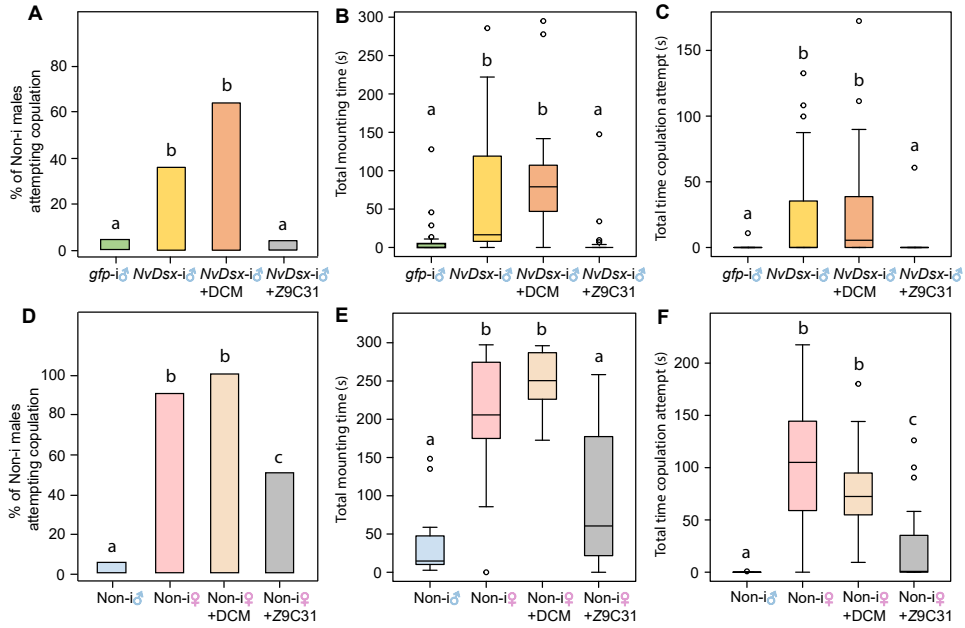


Figure 6.4 Effect of synthetic *Z*9C31 on the courting behaviour of Non-i *N. vitripennis* males. Responding Non-i males were presented with dead conspecifics (dummies) for a five minutes observation period. Dummies were either differently treated *gfp*-i or *NvDsx*-i males (a-c, n=25) or Non-i wasps of either sex (d-f, n=20). Tested dummies were either untreated, treated with 30 ng of (*Z*)-9-hentriacontene (*Z*9C31) dissolved in dichloromethane (DCM), or with pure DCM. Shown is **(A)**, **(D)** the percentage of Non-i males trying to copulate with the dummy, **(B)**, **(E)** the total mounting time, and **(C)**, **(F)** the duration of copulation attempts. Box-and-whisker plots (B, C, E, and F) show median (horizontal line), 25-75 % quartiles (box), maximum/minimum range (whiskers) and outliers (° >1.5 x interquartile range outside the first or third quartile). Letters indicate significant differences at P<0.05 (data analysis by Kruskal-Wallis H-test followed by Bonferroni-corrected multiple Mann-Whitney U-tests (B, C, E, and F) or by Bonferroni-corrected multiple 2x2 Chi²-tests (A and D)).

Discussion

In this study we set out to identify the role of Dsx in regulating *N. vitripennis* male mating behaviour and pheromone production, and we found a major effect of NvDsx in the regulation of the production of both volatile and non-volatile sex pheromones. Using Y-olfactometer two-choice assays and GC-MS analyses, we observed a reduced attractiveness of *NvDsx*-i males to virgin females accompanied by the almost complete disappearance of the abdominal volatile sex attractant pheromone components HDL-*RR*, HDL-*RS*, and 4MQ. HDL-*RR* and HDL-*RS* are synthesized by *N. vitripennis* males in the rectal vesicle from fatty acids, and are released via the anal orifice (Abdel-Latief et al., 2008). Our results suggest that NvDsx directly regulates the biosynthetic pathway, but which of the genes involved in the biosynthesis of HDL (Ruther et al., 2021) are targeted by NvDsx, remains unknown. Although it has been suggested that the production of the CHC pheromones of females is controlled by Dsx in *D. melanogaster* (Jallon et al., 1988; Waterbury et al., 1999), hardly any research has focused on this topic in other insects. Hence, the present study is, to our knowledge, the first to demonstrate a regulatory effect of Dsx on the biosynthesis of pheromones other than CHC in insects.

Despite their lack of abdominal sex attractant pheromones for long-range attraction of females, all tested *NvDsx*-i males were still able to mount and court females, and displayed stereotypical mating behaviours such as head nodding and antennal sweeping. Apparently, the substantial reduction in the titres of the sex attractant pheromone did not affect the acceptance of courtship by females, initiated by the males through mounting. We also found no significant differences in the searching times for the different treatments in the mating experiment, which may have been due to the experimental set-up, in which the male and female were enclosed in a small space. At this level of the mate finding process, the abdominal sex attractant pheromone of *N. vitripennis* males no longer plays a role, and it is up to the male to recognize and court the female via the female-specific CHC (Steiner et al., 2006). Intriguingly, *NvDsx*-i males were courted by Non-i males in a similar manner to females, suggesting a kind of "feminization" of the *NvDsx*-i CHC profiles. *NvDsx*-i males apparently became sexually attractive to Non-i males because of a substantial reduction of alkenes, in particular *Z*9C31, in their CHC profile. Supplementation of the profile by applying synthetic *Z*9C31 to *NvDsx*-i male dummies eliminated the courting behaviour of Non-i males, suggesting that *Z*9C31 is a key compound for sex discrimination by *N. vitripennis* males. Just as the cuticular pheromone (*Z*)-7-tricosene inhibits male-male courtship in *D. melanogaster*

(Ferveur and Sureau, 1996; Lacaille et al., 2007), *Z*9C31 helps to prevent male-male courtship in *N. vitripennis*. The low abundance of *Z*9C31 in the CHC profile of females (Steiner et al., 2006) might even underlie males' recognition of potential mates. This idea is supported by our finding that applying *Z*9C31 to female dummies reduced Non-i male courting attempts and duration, although not to the low levels shown towards Non-i males. In this context, it is important to note that the other three alkenes, and many other CHC components that are found in different relative amounts in *N. vitripennis* males and females might also contribute to this effect (Carlson et al., 1999; Steiner et al., 2006). Hence, the addition of *Z*9C31 alone might not be sufficient to entirely disrupt a female's pheromone signal.

We analysed the presence and amounts of alkenes that we found to be directly or indirectly regulated by NvDsx in the previously published, species-specific CHC profiles of *Nasonia* (Buellesbach et al., 2013). We found a male bias in the expression of these alkenes in *N. vitripennis* but not in the other three *Nasonia* species (Buellesbach et al., 2013; Niehuis et al., 2011). In *D. melanogaster,* alkenes are a major fraction of the CHC profiles (Jallon and David, 1987), and their biosynthesis requires different types of desaturases (Chertemps et al., 2006). Niehuis et al. (2011) found that two of the *Drosophila* desaturase gene homologs map to regions in the *Nasonia* genome that contain the QTL clusters for these alkenes. NvDsx is a prime candidate for regulation of the expression of the desaturase genes that controls alkene biosynthesis. The gain and loss of the binding-site of Dsx in these desaturase genes may account for the species-specific differences in alkene synthesis, as has been shown for *D. melanogaster* (Shirangi et al., 2009). This difference might be related to specific life-history traits of different *Nasonia* species. Females mating inside the host puparium (within-host mating, WHM) is rare in parasitoids, but it does happen in *Nasonia* (Drapeau and Werren, 1999). Compared to *N. vitripennis,* the sibling species *N. giraulti* and *N. longicornis* show a significantly higher WHM rate (Drapeau and Werren, 1999). Because *N. vitripennis* is ancestral to the other species in the genus (Werren et al., 2010), this sex-specific difference in alkene expression might have been lost in *N. giraulti* and *N. longicornis* as a result of decreased mate competition due to their higher WHM rates. Further research will be required to verify this. In addition, behavioural assays to determine mate discrimination among *Muscidifurax uniraptor, N. vitripennis*, and *Trichomalopsis sarcophagae* showed that *N. vitripennis* males do not discriminate against *T. sarcophagae* females, displaying courtship behaviour and copulation attempts, but reject *M. uniraptor* females (Buellesbach et al., 2018). Despite the general similarity of the CHC profiles of females of both species, both *Z*9C31 and *Z*9C33 are produced by *N. vitripennis* and *T. sarcophagae* females at low levels,

whereas *M. uniraptor* females have higher amounts of these alkenes in their CHC profiles (Buellesbach et al., 2018). Hence, *Z*9C31 and the other alkenes might also be involved in species discrimination by *N. vitripennis* males. It would be interesting to further investigate the consequences and benefits of maintaining this alkene-dependent olfactory recognition system.

By comparing the courtship and copulation behaviours of *NvDsx*-i, *gfp*-i, and Non-i males when presented with live virgin females, we did not find obvious differences in male courtship performance between *NvDsx*-i and Non-i males. Male courtship behaviour in insects is thought to be governed primarily by Fruitless (Fru, Ryner et al., 1996). However, in *Drosophila*, Dsx was shown to facilitate the formation of the CNS, and these neuronal networks develop during pupation (Shirangi et al., 2016). This suggests that silencing *NvDsx* from the pupal stage onwards does not affect the development of the regions of the CNS that control mating behaviour in *N. vitripennis* males. Hence, our results add to the current theory that Fru is the main regulator of courtship behaviour in insects, but it should be noted that silencing *NvDsx* earlier in development might produce different outcomes.

We observed significantly prolonged mounting time and a strong reduction in mating success of *NvDsx*-i males when they courted normal females, indicating that an important component inducing receptivity of females might be missing from the *NvDsx*-i males. It has been shown that males with sealed mouthparts cannot induce receptivity in females, which indicates the existence of an aphrodisiac produced by the mouthparts (Ruther et al., 2010; van den Assem et al., 1980). The identity of this aphrodisiac is still unknown despite extensive efforts to unravel its chemical structure (Mair and Ruther, 2019). So far, three fatty acid ethyl esters have been identified in the oral secretion of *N. vitripennis* males, but these compounds were unable to elicit female receptivity (Ruther and Hammerl, 2014). The inability of *NvDsx*-i males to elicit receptivity in conspecific females suggests that the production of the oral aphrodisiac is affected by Dsx, possibly in combination with, or due to malformed mandibular glands, which are known to be present in *N. vitripennis* (Mikó and Deans, 2014). Therefore, comparative chemical analyses of the heads of *NvDsx*-i and Non-i males will be a promising approach to unravel the identity of the oral aphrodisiac, as a next step towards a comprehensive understanding of pheromone communication in *N. vitripennis*.

We include some extra results of this Chapter in the following box section.

Box section of Chapter 6

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Background

In this box we present the results of silencing *NvDsx* in male 4th instar larvae, and female 4th instar larvae and pupae by RNA interference (*NvDsx*-i) and the effects on adult mating behaviour. We hypothesized that silencing *NvDsx* earlier in development causes a more drastic disruption of male sexual traits which would affect behaviour in addition to the changes in sex pheromone production. First identified in *Drosophila,* the transcription factor *Fruitless* (*Fru*) was shown to regulate male mating behaviours (Ryner et al., 1996) and male-specific neural circuit development (Kimura et al., 2005). As *Dsx* was also shown to coordinate the generation of male-specific neurons for courtship behaviours in *Drosophila* (Kimura et al., 2008; Sanders and Arbeitman, 2008), we hypothesized that *Dsx* expression could affect *Fru* expression and vice versa. Therefore, we additionally investigated whether *NvDsx* knockdown would have an effect on *Fru* expression in *N. vitripennis*.

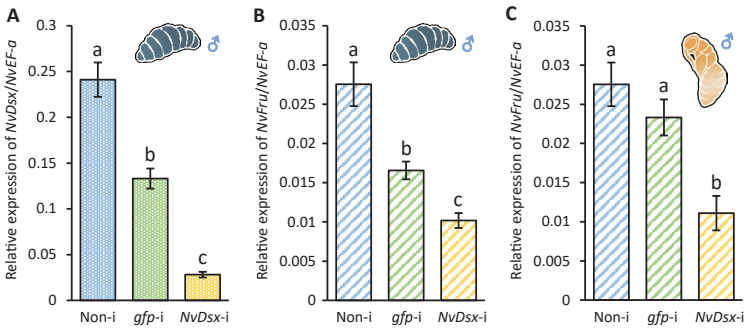
Results

Silencing *NvDsx* in male 4th instar larval stage affects mating and male specific *Fru* expression

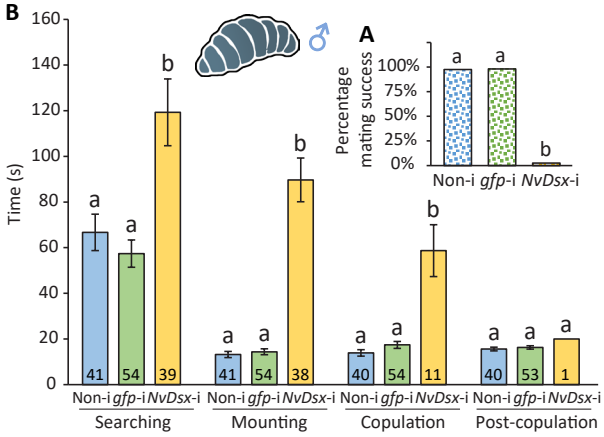
We injected *NvDsx* dsRNA in 4th instar larval stage of *N. vitripennis* males, and obtained a substantial reduction of *NvDsx* expression in the adult stage of *NvDsx*-i males when comparing to Non-i (GLM, $P<0.001$) and *gfp*-i (GLM, $P<0.001$) males (Box 6.1A).

The suppression of *NvDsx* in 4th larval instar males has a significant effect on adult mating behaviour. Overall, only one out of 39 *NvDsx*-i males (2.56%) elicited receptivity of non-treated (NT) females, mated with NT females and subsequently finished post-copulatory behaviour, which was significantly less than Non-i (97.56%) and *gfp*-i males (98.15%, Box 6.2A). We then analysed the separate courtship elements (Box 6.2B). *NvDsx*-i males spent significantly more time in searching for females compared to Non-i males (GLM, $P=0.0036$) and *gfp*-i males (GLM, $P<0.001$). Significantly more time was also spent by *NvDsx*-i males in mounting and copulation with NT females when comparing to Non-i (GLM, $P<0.001$)

and *gfp*-i (GLM, *P*<0.001) males. In the mating trails, only one *NvDsx*-i male successfully finalized the mating and performed the standard post-copulation behaviour with a similar duration as Non-i and *gfp*-i males (Box 6.2B).



Box 6.1 Effects of *NvDsx* knockdown on *NvDsx* and *NvFru^M* expression in *N. vitripennis* males. (A) Relative expression of *NvDsx*/*NvEF-1a* in adults of 4th instar Non-injected (Non-i), *gfp,* and *NvDsx* dsRNA injected males (means ± SE). **(B)** Relative expression of *NvFru^M*/*NvEF-1a* in adults of 4th instar Non-i, *gfp*-i, and *NvDsx*-i males. **(C)** Relative expression of *NvFru^M*/*NvEF-1a* in adults of pupal stage Non-i, *gfp*-i, and *NvDsx*-i males. Letters indicate significant differences at *P* < 0.05 (A, B: GLM, C: ANOVA/Tukey HSD, n=6).



Box 6.2 Effects of *NvDsx* knockdown on mating behaviour of *N. vitripennis* males. (A) Percentage mating success of Non-injected (Non-i), 4th larval instar *gfp* and *NvDsx* dsRNA injected males in mating trials with virgin females. **(B)** Duration of selected elements (means ± SE) of the *N. vitripennis* mating sequence of Non-i and 4th instar *gfp*-i and *NvDsx*-i males. Numbers of replicates are provided in the bars. Letters indicate significant differences at *P* < 0.05 (A: Bonferroni-corrected multiple Chi^2 tests, B: GLM).

In *Drosophila melanogaster*, Dsx establishes sexual dimorphism in the central nervous system by controlling the cell numbers (Sanders and Arbeitman, 2008), and the majority of brain and thoracic ganglion neurons in *D. melanogaster* are suggested to co-express

Dsx and *Fru* (Pan et al., 2011). We further examined if depletion of *NvDsx* affects *NvFru* expression in *N. vitripennis*. For this, we conducted qPCR by using sex-specific primers to target $NvFru^{M}$ transcript (P1-m transcript in Bertossa et al., 2009) in *NvDsx*-i and control adult males (Table S6.1). Reducing the *NvDsx* expression from 4th instar male larval development onwards corresponds to a significant down-regulation of $NvFru^{M}$ in *NvDsx*-i adults compared to Non-i males (GLM, $P<0.001$) and *gfp*-i males (GLM, $P=0.002$, Box 6.1B). For completeness, we also analysed the pupal *NvDsx*-i males and found a similar correlation between Non-i males (GLM, $P=0.002$) and *gfp*-i males (GLM, $P=0.015$) (Box 6.1C). In addition, we also observed a significant reduction of *NvDsx* and $NvFru^{M}$ in 4th instar *gfp*-i males compared to Non-i males (*NvDsx*: GLM, $P<0.001$, Box 6.1A; $NvFru^{M}$: GLM, $P=0.0012$, Box 6.1B) but this did not have any effect on their general mating performances.

Intriguingly, prolonged searching time was observed only in the adult of 4th instar *NvDsx*-i males and not in the adult of pupa *NvDsx*-i males (Box 6.2B and Figure 6.1C). Because the adult of 4th instar larval *NvDsx*-i males displayed several morphological changes (Chapter 4) that are not observed in the adult of pupal *NvDsx*-i males, it is likely that there are also developmental changes in the brain and head morphology which may decrease the mate searching efficiency. For instance, changes in eye morphology could directly affect the visual perception of *NvDsx*-i adult males, which seems an important aspect of males chasing females during courtship (Barrass, 1960). In addition, ten out of 11 adults of 4th instar *NvDsx*-i males could not copulate, even though the female became receptive. Because the silencing of *NvDsx* affects *NvFru* expression in both developmental stages, we cannot rule out the possibility that the reduction of $NvFru^{M}$ in the adult of 4th instar *NvDsx*-i males contributes to the extended or unsuccessful copulation, as was shown for *D. melanogaster* (Jois et al., 2018). However, we did not observe obvious changes in mating rituals in different treatments. Moreover, during mating observation (Chapter 4, Movie S4.2) the adult of 4th instar *NvDsx*-i males had notable difficulty in making genital contact with the females. We consider this the prime reason for the observed copulation failures, and this is corroborated by the reduced size of external genitalia in the adult of 4th instar *NvDsx*-i males that were observed earlier (Chapter 4).

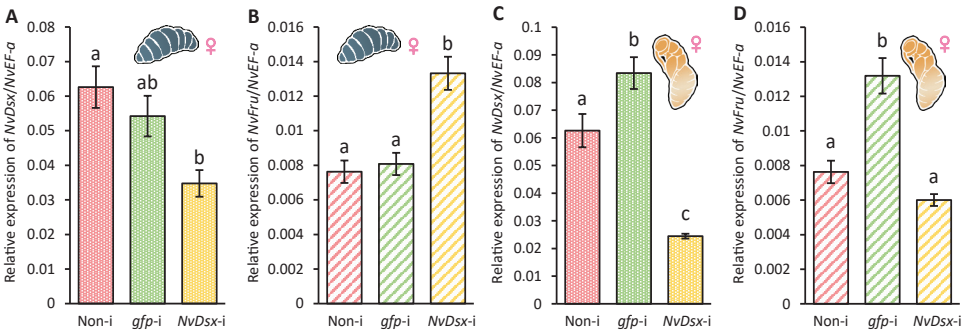
A synergistic effect between *NvDsx* and $NvFru^{M}$ expression may suggest a feedback loop between these two genes. However, it is also possible that $NvDsx^{M}$ like Dsx^{M} in *Drosophila*, regulates Dsx-expressing cell numbers in a dose-dependent manner (Sanders

and Arbeitman, 2008), and NvDsx^{M} may indirectly affect NvFru^{M} expression by controlling the number of NvDsx^{M} and NvFru^{M} co-expressing cells.

In conclusion, we did not observe obvious aberrant mating behaviours in the adult of 4th instar *NvDsx*-i males despite observing a significant decrease in both *NvDsx* and *NvFru^{M}* expression. The reduction of *NvFru^{M}* expression following *NvDsx* silencing in males is striking and requires further research. The involvement of *NvFru* in regulating male mating behaviour is still illusive and *NvFru* silencing studies are required to improve our understanding of this gene.

Silencing *NvDsx* in females at different developmental stages does not impair mating behaviour and *NvFru* expression

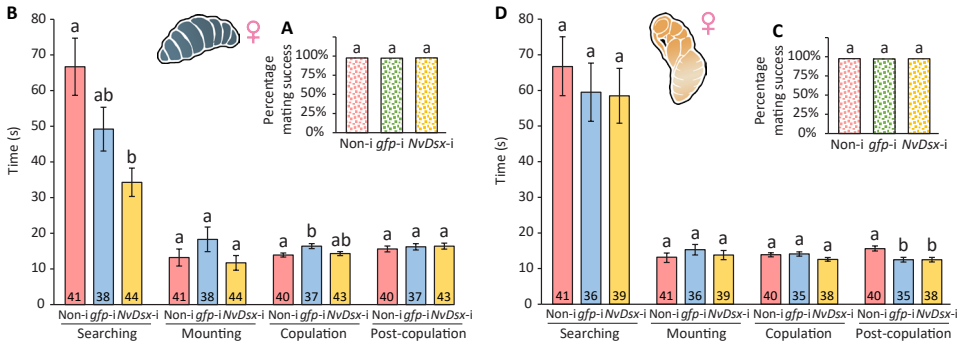
We also performed *NvDsx* silencing in 4th instar and pupal females and evaluated their mating performance with NT males. A significant reduction of *NvDsx* expression at adult stage was observed in 4th instar *NvDsx*-i when compared to Non-i females (Tukey's HSD, *P*=0.0057) but not when compared to *gfp*-i females (GLM, *P*=0.052, Box 6.3A).



Box 6.3 Effects of *NvDsx* knockdown on *NvDsx* and *NvFru^{F}* expression in *N. vitripennis* females. (A) Relative expression of *NvDsx*/*NvEF-1α* in adults of 4th instar *gfp*, *NvDsx* dsRNA injected females and non-injected (Non-i) females. **(B)** Relative expression of *NvFru^{F}*/*NvEF-1α* in adults of 4th instar *gfp*-i, *NvDsx*-i and Non-i females. **(C)** Relative expression of *NvDsx*/*NvEF-1α* in adults of pupa stage *gfp*-i, *NvDsx*-i and Non-i females. **(D)** Relative expression of *NvFru^{F}*/*NvEF-1α* in adults of pupa stage *gfp*-i, *NvDsx*-i and Non-i females. Letters indicate significant differences at *P* < 0.05 (A, B, C: GLM, D: ANOVA/Tukey HSD, n=6, means ± SE).

In the pupal *NvDsx*-i females, a significant reduction of *NvDsx* expression at adult stage was observed when compared to both Non-i females (GLM, *P*<0.001, Box 6.3C) and *gfp*-i females (GLM, *P*<0.001, Box 6.3C). Strangely, adult of pupal *gfp*-i females showed an increase in *NvDsx* expression compared to the adult of Non-i females (GLM, *P*=0.013,

Box 6.3C). Despite the significant reduction of *NvDsx* in both developmental stages, no significant deviation was found in the time spent on the mating components mounting and copulation, nor in the mating success rates observed between *NvDsx*-i and control females (Box 6.4). Significantly reduced searching time was observed in the adult of 4th instar *NvDsx*-i females compared to Non-i females (GLM, $P<0.001$, Box 6.4B), where it took the NT males less time to find and mount the females, but this effect was not observed when comparing *NvDsx*-i and *gfp*-i (GLM, $P=0.091$, Box 6.4B) or Non-i and *gfp*-i females (GLM, $P=0.194$, Box 6.4B). We also observed a significant reduction of post-copulation duration in the adult of pupal *NvDsx*-i females compared to Non-i females (GLM, $P=0.005$), while no clear difference was found between *NvDsx*-i and *gfp*-i (GLM, $P=0.963$, Box 6.4D).



Box 6.4 Effects of *NvDsx* knockdown on mating behaviour of *N. vitripennis* females. (A) Percentage mating success of Non-i, 4th instar *gfp*-i and *NvDsx*-i females in mating trials with virgin males. **(B)** Duration of selected elements (means ± SE) of the *N. vitripennis* mating sequence of NT and 4th instar *gfp*-i and *NvDsx*-i females in mating trials with virgin males. **(C)** Percentage mating success of Non-i, pupal *gfp*-i and *NvDsx*-i females in mating trials with virgin males. **(D)** Duration of selected elements (means ± SE) of the *N. vitripennis* mating sequence of Non-i and pupal *gfp*-i and *NvDsx*-i females in mating trials with virgin males. Number of replicates are referred in bar graphs. Letters indicate significant differences at $P < 0.05$ (A, C: Bonferroni-corrected multiple Chi^2 tests, B, D: GLM).

Strikingly, the female-specific $NvFru^F$ (P1-f transcript in Bertossa et al., 2009) expression is significantly increased in the adult of 4th instar *NvDsx*-i females compared to Non-i (GLM, $P<0.001$) and *gfp*-i (GLM, $P<0.001$) females (Box 6.3B). However, in the adult of pupal *NvDsx*-i females, no significant change in $NvFru^F$ expression was detected when comparing to NT females (Tukey's HSD, $P=0.288$, Box 6.3D). In addition, adult of pupa *gfp*-i females displayed a significantly increased expression of $NvFru^F$ compared to Non-i females (Tukey's HSD, $P<0.001$, Box 6.3D) and *NvDsx*-i females (Tukey's HSD, $P<0.001$, Box 6.3D), as was also observed for *NvDsx* expression in the adult of pupal *gfp*-i females (Box

6.3C). At the moment, we cannot explain the increase of *NvDsx* and *NvFru*^{F} expression in the adult of pupal *gfp*-i females. But we noticed that the non-target effect of *gfp*-i treatments are more prominent in 4th instar injected males and pupal injected females regardless of which genes. Furthermore, we were not able to amplify another highly expressed female-specific *NvFru*^{F} transcript (referred as P0-fru in Bertossa et al., 2009) in *N. vitripennis* females, therefore the equal or increased expression of *NvFru*^{F} in *NvDsx*-i females merely represents partial *NvFru*^{F} expression. In *Drosophila* female development, cell death is triggered by the Dsx^{F} in a neuronal cluster that expresses Fru (Kimura et al., 2008). Repression of *NvDsx*^{F} in *N. vitripennis* females during pupation may help preserve these cells and eventually increase the *NvFru*^{F} expression, albeit that further research will be required to confirm this.

In *D. melanogaster,* research suggests that female mating behaviour is controlled by Dsx^{F} and Fru^{F} at different levels. For example, in female fruit flies, female-specific receptivity-promoting neurons do not express P1 *Fru* transcripts, but rely on the expression of *Dsx* to maintain their function (Zhou et al., 2014). Furthermore, non-sex-specific *Fru* was recently implicated in *D. melanogaster* to affect female rejection behaviour (Chowdhury et al., 2020). Here we find no decreased receptivity or increased mating failures in both 4th instar and pupal stage *NvDsx*-i females. Since we only obtained efficient *NvDsx* silencing in pupal stage females, our result suggests that Dsx expression after pupal stage in female *N. vitripennis* does not regulate their mating behaviours. Our limited knowledge of the function of *NvFru* in *N. vitripennis* mating behaviour (Bertossa et al., 2009) hampers our further interpretation of the regulatory mechanism. More in-depth research is needed to understand the role of *NvDsx* and *NvFru* in regulating the mechanism governing female mating behaviour in *N. vitripennis.*

Supplementary

Table S6.1 Primers used in the experiment.

Primer	Sequence
Nv_Dsx_qPCR_F[a]	CAGCAACACGAGATGCTGATGG
Nv_Dsx_qPCR_R[a]	TGTCATTACTGCCATTCATGCTTGG
Nv_EF-1a_qPCR_F[a]	CACTTGATCTACAAATGCGG
Nv_EF-1a_qPCR_R[a]	GAAGTCTCGAATTTCCACAG
NvFru_P1-m_qPCR_F[b]	GTGTGCAACAGCAGAGCATC
NvFru_P1-m_qPCR_R[b]	AGGTTGGCCGGATGATTGTTC
NvFru_P1-f_qPCR _F[b]	CCGCACACAATGACCCGAT
NvFru_P1-f_qPCR_R[b]	AGGTTGGCCGGATGATTGTTC
Nv_Dsx_RNAi_F	*[TAATACGACTCACTATAGGG]CCAAGAGGCAGCAAATTATG
Nv_Dsx_RNAi_R	*[TAATACGACTCACTATAGGG]GTTATACGCCGCATGGCTAC
GFP_RNAi_F	*[TAATACGACTCACTATAGGG]GTGACCACCTTGACCTACG
GFP_RNAi_R	*[TAATACGACTCACTATAGGG]TCTCGTTGGGGTCTTTGCT

a. Used in Verhulst et al. (2010b)

b. Used in Bertossa et al. (2009)

* T7 promoter sequence that is recommended by the manufacturer.

Chapter 7

General discussion

Photo by Hans Smid

In this dissertation, I focused on two major aspects of sex determination in insects: the conservation of the core elements of the sex-determination cascade and the role of the final conserved sexual differentiator, *Doublesex.* In addition, I specifically focused on a unique aspect of hymenopteran sex determination, i.e. thelytokous reproduction induced by *Wolbachia.* I studied the sex-determination system in two species of the wasp genus *Muscidifurax* and explored the function of the master sex-differentiation gene *Dsx* in regulating sexually dimorphic traits throughout development in the emerging model species *Nasonia vitripennis* to expand our knowledge on the rapid evolution of sexually dimorphic traits in the Hymenoptera. Then I used the knowledge gained on *Muscidifurax uniraptor* sex determination to investigate the exact mechanism that thelytokous parthenogenesis inducing (PI) *Wolbachia* employs to induce thelytoky in this wasp species. The major results and their contributions to our overall understanding are briefly discussed here together with their limitations and the remaining knowledge gaps that require future investigation.

Alternatively spliced Dsx may serve different functions

Dsx was first identified in *Drosophila* as a regulatory gene that controls the somatic sexual differentiation (Baker and Wolfner, 1988; Hildreth, 1965). Until now, the ubiquitous presence of *Dsx* has been shown in diverse insect orders (Price et al., 2015). The hallmark of *Dsx* in insects is the presence of alternative splice forms. In the closest hexapod outgroup species *Daphnia magna* (Crustacea), two copies of *Dsx*-like genes were identified, showing no alternative splicing but sex-specific differences in expression (Kato et al., 2011). Price et al. (2015) speculate that the eventual loss of one *Dsx* copy is replaced by alternative sex-specific splicing and neo-functionalisation in insects. In addition, splice forms of *Dsx* differ in terms of structures and numbers in different insect orders. In the dipteran fruit fly *Drosophila*, only one male- and one female-specific splicing form have been described (Baker and Ridge, 1980), yet there are three male- and one female-specific splice forms of *Dsx* in the hymenopteran *N. vitripennis* (Chapter 4). Multiple splicing variants from the same sex are identified in other species as well, including the hymenopteran *Apis mellifera* (Cho et al., 2007), *Bombyx mori* and other Lepidopteran species (Shukla and Nagaraju, 2010a; Shukla et al., 2011; Wang et al., 2014) and *Tribolium castaneum* and other Coleoptera species (Gotoh et al., 2014; Kijimoto et al., 2012; Shukla and Palli, 2012b).

Sex-specific splicing of *Dsx* within a species results in differences in the sex-specific OD2 regions which are presumed to account for the differentiation of sexually dimorphic characteristics.

NvDsx in male *N. vitripennis* is confirmed to actively promote the development of male-specific traits (Chapter 4). The short NvDsxM1 isoform encodes a much shorter and different male-specific OD2 region compared to the two longer NvDsxM isoforms. Together with the fact that all *NvDsxM* isoforms rapid increase in expression from early larval until adult stage in males (Chapter 4), I expect that different male-specific NvDsx variants have distinctive functions. Evidence supporting this idea comes from a study in the beetle *T. castaneum* (Shukla and Palli, 2012b). Research conducted in *T. castaneum* suggests that different female Dsx isoforms could have specific regulation of downstream gene expression and only one of the Dsx isoforms appears responsible for oocyte development and maturation (Shukla and Palli, 2012). In other cases, the results are hard to interpret; often, a sex-specific trait is regulated by some but not all of the sex-specific Dsx isoforms, suggesting either non-functional Dsx isoforms are present or some Dsx isoforms regulate other dimorphic traits (Gotoh et al., 2016; Ito et al., 2013). Unfortunately, few studies have been carried out to investigate this.

Dsx evolution in Hymenoptera and its function in females

Within the animal kingdom, DM domain genes often play a central role in sexual differentiation (Kopp, 2012; Picard et al., 2015). Aligning the sex-specific *Dsx* region across insect orders, Baral et al. (2019) showed that the male-specific exons in general evolve much faster while the female-specific exons remain relatively conserved. Within the insect orders Diptera, Coleoptera and Lepidoptera, also a higher nonsynonymous substitution rate was observed in male-specific *Dsx* regions compared to female-specific regions (Hughes, 2011). This rapid evolution of male-specific *Dsx* regions is likely due to higher secondary sexual trait diversity in male insects (Baral et al., 2019). However, within the Hymenoptera, the situation is reversed. Female-specific *Dsx* regions were found to be highly variable (Baral et al., 2019) which is suggested to correlate with the evolution of female-dominated eusociality and female-specific caste differentiation (Baral et al., 2019). Moreover, this episodic selection in the female-specific *Dsx* region was not detected in chalcid wasps but was shown in other Hymenoptera species suggesting an evolutionary divergence of Dsx in the Hymenoptera based on sociality (Baral et al., 2019). Intriguingly, our phylogenetic analysis of the female-specific *Dsx* region in Chalcidoidea such as *N. vitripennis*, *M. uniraptor*, *M. raptorellus* and *Trichomalopsis dubius*, showed a very high sequence similarity (Chapter 2). The limited number of functionally identified *Dsx* genes in the Hymenoptera hampers our understanding of the evolution of the female-specific *Dsx* region, and identification of Dsx in more Hymenoptera species will help

to resolve the question whether female-specific *Dsx* regions face different selection forces depending on whether they are eusocial or non-eusocial insects.

On top of this, the exact function of female Dsx in regulating morphological differentiation in the Hymenoptera is far from being fully understood. Although sex-specific Dsx plays antagonistic roles in Diptera, Lepidoptera and Coleoptera (Hildreth, 1965; Kijimoto et al., 2012; Xu et al., 2017) in our experiments in *N. vitripennis*, we did not find any obvious phenotypic-variations in female forewing size, pigmentation or mating behaviours after silencing *NvDsx* in females (Box in Chapter 4 and Chapter 6). One possibility is that this antagonistic effect of sex-specific Dsx is not always occurring to modulate dimorphic sexual traits. For instance, the formation of the thoracic horn is only male-Dsx-dependent in the rhinoceros beetle *Trypoxylus dichotomus*, which is different from the head horn that is under the regulation of both male and female Dsx (Ito et al., 2013). Therefore, some other sexual traits that we did not investigate could be under the regulation of NvDsxF in *N. vitripennis.* However, there is also evidence to support another hypothesis. Across the animal phyla, the evolutionarily conserved function of the *Dmrt* gene family (including *Dsx*) is to promote testis formation and repress ovary development (Kopp, 2012). In the cockroach *Blattella germanica* (Blattodea) and the brown planthopper *Nilaparvata lugens* (Hemiptera) that belong to the basal insect orders, Dsx is required in males but is dispensable in females for directing sexually dimorphic trait development (Wexler et al., 2019; Zhuo et al., 2018). In the hymenopteran basal sawfly, *Athalia rosae*, interrupting *Dsx* expression in males induced female genitalia structure development, causing severe malformations of the male external genitalia (Mine et al., 2017). The female-specific signature gene, *Vitellogenin* (*Vg*, yolk proteins which provide essential nutrients for embryo growth), that is activated by DsxF but repressed by DsxM in *D. melanogaster* (Coschigano and Wensink, 1993), has also a different mode of Dsx regulation in *N. lugens*; only DsxM supresses the *Vg* expression in *N. lugens* males while in females, *Vg* is expressed by default (Zhuo et al., 2018). This result is consistent with our proteomics data collected from the abdomens of *NvDsx* RNAi males (*NvDsx*-i) and *gfp* RNAi males (*gfp*-i) (personal communication Joachim Ruther), in which *NvDsx*-i males contained a significantly higher amount of Vg protein than *gfp*-i males. If in the evolutionary history, Dsx used to be required for male-specific differentiation only and was incorporated for female-specific differentiation more recently, hymenopterans, which are present at the relatively basal lineages of insects, may represent the transition phase of Dsx evolution. As a result, I suggest the regulatory role of Dsx in *N. vitripennis* will look like this: the female phenotype is the default state, and the function of NvDsxF is merely to

prevent NvDsxM expression, which will actively repress the female default phenotype and promote the development of male-specific characteristics.

The possible Dsx regulation network in *N. vitripennis*

For a long time, sex determination and differentiation mechanisms were thought to be divergent between vertebrates and invertebrates. In vertebrates, genetic or environmental sex determination signals first determine the gonadal sex (Bear and Monteiro, 2013). Upon gonad differentiation, sex-specific hormones are secreted as signals to establish the sexual identity in other cells and tissues that will further develop into sexually dimorphic traits. In contrast, insects were thought to use cell-autonomous regulation, by which each single cell determines its own sexual fate directly by the genetic elements (Baker and Ridge, 1980). However, accumulating evidence from *Drosophila* studies suggests that non-cell-autonomous regulation also plays an important role in directing sexual-trait development in invertebrates (Camara et al., 2008; DeFalco et al., 2008; Prakash and Monteiro, 2016).

Firstly, short-distance (cell-to-cell) signalling pathways can be utilized by Dsx. In *Drosophila* gonads, Dsx is only expressed in subsets of the somatic gonadal cells (Hempel and Oliver, 2007) but it controls the surrounding male germ cell and the pigment cell identity through different types of male-specific cell-to-cell signals (DeFalco et al., 2008). Dsx also instructs fibroblast growth factors (FGF) to recruit the mesodermal cells which migrate into the genital imaginal disc to form part of the internal genitalia in later male developmental stages (Ahmad and Baker, 2002). These observations reflect the fact that some cells and tissues remain in a non-sex-specific state and await sex-specific differentiation triggers from other cells and tissues. The Dsx silencing results we found in the *Nasonia* male gonad (Chapter 4) seem to resemble this non-cell autonomous regulation by showing the alteration of male testes to female ovary-like structures when supressing the male-specific Dsx expression in the early larval stage.

Another aspect of Dsx-regulated sexual-trait development in insects involves the modulation of the long-distance hormonal pathways. The ecdysteroid hormones (ecdysone (E) and 20-hydroxyecdysone (20E)) and juvenile hormones (JH) that are commonly used by insect for growth and moulting, can also exert an influence on secondary sexual traits development. Sex-biased expression of ecdysone was found to control cellular sexual identity in *Drosophila* (Fagegaltier et al., 2014). The fact that 20E expression regulates dimorphic wing shapes in the

tussock moth *Orgyia recens* in a sex- and tissue-specific manner (Lobbia, et al., 2003) again suggests the involvement of Dsx. In houseflies, female-specifically expressed yolk protein is also regulated by Dsx-modulated ecdysone (Siegenthaler et al., 2009). In stag beetles, JH directly promotes the sex-specific mandible growth (Gotoh et al., 2014). The difference in JH sensitivity between sexes is suggested to be modulated by sex-specific Dsx (Gotoh et al., 2014). These results are in line with our observations of reduced forewing size and external genitalia in early larval stage *Dsx*-i *N. vitripennis* males. It suggests that hormones may also play a role in *N. vitripennis* sex-specific development.

Temporal expression of Dsx is required to determine sexual fate (Sánchez et al., 2001). In the somatic gonad, Dsx initiates the male stem cell niche development prior to the 2nd larval instar by which a group decision has been made and will be largely executed even without further Dsx function (Camara et al., 2019). Because the potential Dsx targets include the ones that encode transcriptional regulators, activating or repressing these transcription factor by Dsx at a specific time may be sufficient to guarantee the development of their regulated sexual traits (Clough et al., 2014). The spatial-specific expression of Dsx is also crucial for sex-specific trait development (Robinett et al., 2010). This delicate control of sexual traits requires Dsx to incorporate the HOX genes that are responsible for spatial patterning and differentiation (Barmina and Kopp, 2007). The best known examples are the sex-specific regulation of dimorphic abdominal pigmentation and the sex combs in *Drosophila* (Tanaka et al., 2011; Williams et al., 2008). The sex-specific pigmentation in legs and antennae of *N. vitripennis* may also rely on the collaboration of patterning signals and the sex determination genes. As Camara et al. (2008) pointed out that, because not all cells have their sexual "identity", the limitation of specific Dsx expression to dimorphic tissues should have its evolutionary advantages. On the other hand, because Dsx regulates some of the core signalling pathways (Christiansen et al., 2002) that are also required to promote the development of non-sex specific tissue, systematic expression will cause severe problems such as death in the developing insects (Jursnich and Burtis, 1993). Therefore, pinpointing the timing and place of Dsx action on sex-specific tissue development is essential for identifying direct Dsx targets and helping to understand the regulation of dimorphic traits.

In *Drosophila* species, both changes in the spatial expression patterns of Dsx (Rice et al., 2018; Tanaka et al., 2011), and changes in the Dsx *cis*-regulatory elements (CRE) of the genes that directly control the sexual traits (Shirangi et al., 2009; Williams et al., 2008) contributes to the evolution of species- and sex-specific morphology. Given the fact that sexually dimorphic

leg and antennae pigmentation is present in *N. vitripennis,* whereas closely-related species such as *Muscidifurax uniraptor* and *M. raptorellus* display only monomorphic pigmentation patterns, comparing the Dsx regulation pathways between these species will provide more knowledge on sexual-trait evolution in parasitoid wasps.

In our research, we investigated the role of Dsx in promoting sexual characters from early larval to the adult stage of *N. vitripennis* (Figure 7.1), as this is the first step towards fully uncovering Dsx function in *N. vitripennis*. Based on our obtained results and the knowledge from other studied species, we propose a possible Dsx regulatory network in *N. vitripennis*.

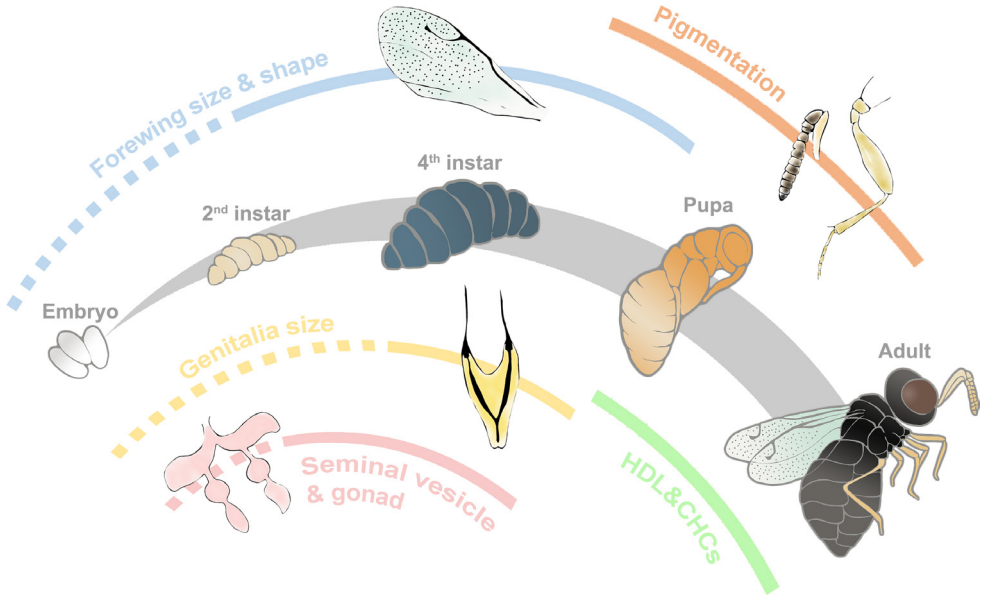


Figure 7.1 Summary of Dsx regulation of sexually dimorphic trait development in male *N. vitripennis*. Each line indicates the predicted regulation period for specific trait development. Dashed lines indicate the possible starting point of the regulation of different traits. HDL refers to male abdominal sex-attractant pheromone (4*R*,5*R*)- and (4*R*,5*S*)-5-hydroxy-4-decanolide and CHCs refers to male cuticular hydrocarbons.

Sexual traits such as wing size and genitalia morphology are initially formed in specific imaginal discs, which may require Dsx to determine the sexual identity during early embryogenesis. During early larval development, Dsx is likely required to constantly maintain its expression to modulate the long-term and short-term signalling pathways for continuous growth and differentiation. The non-cell-autonomous regulation might be also important for the tissues without sexual "identity" in early development, to differentiate

sex-specifically at later stages, likely the internal reproduction organs. Spatial and temporal expression of *N. vitripennis* Dsx is likely realized by interacting with the HOX genes (currently uncharacterized) in order to achieve direct cell-autonomous regulation at the site of sexual traits development, for example the production of pigmentation in the female legs. After the pupal stage, Dsx is still required to control sex-pheromone production to shape the adult behaviours. Dsx regulates male abdominal sex-attractant pheromone production necessary to attract females. In addition, Dsx is also responsible for the production of male-biased alkenes (cuticular hydrocarbons), especially (*Z*)-9-hentriacontene *Z*9C31, which helps to avoid male-male mating behaviours. In addition, the oral aphrodisiac that is crucial to elicit female receptivity during courtship is also likely controlled by Dsx. Further research will be needed to fill in all the knowledge gaps before we can generate a more complete Dsx regulation network in *N. vitripennis*.

Evolution of sexual manipulation strategies

Because of the high infection rate of *Wolbachia* in arthropods (Hilgenboecker et al., 2008; Weinert et al., 2015; Werren and Windsor, 2000), it is difficult to summarize a clear infection pattern of *Wolbachia* in insects. A comparative study in beetles did not manage to generalize a reliable relationship between the occurrence of *Wolbachia* and the ecological or biological traits of their host species (Kajtoch et al., 2019). On the other hand, although different *Wolbachia*-induced reproductive manipulation phenotypes seem clearly distinctive, the transition from one to another sometimes is less constrained. For example, *Wolbachia* induces cytoplasmic incompatibility (CI) in its natural host *Drosophila recens*, but it instantaneously induces complete male killing (MK) after being transferred into *D. subquinaria* by introgressive hybridization (Jaenike, 2007). In another case, *Wolbachia* causing feminization in the original host *Ostrinia scapulalis* altered the reproduction manipulation to a MK phenotype in a newly transfected host *Ephestia kuehniella* (Fujii et al., 2001). The change from MK to CI phenotype is also observed in *Hypolimnas bolina* (Hornett et al., 2008).

Recently, the molecular mechanisms underlying *Wolbachia*-induced CI (Beckmann et al., 2017; Le Page et al., 2017) and MK (Perlmutter et al., 2019) have been elucidated with the discovery of several functional genes (*cifA* and *cifB* for CI and *wmk* for MK) that originate from bacteriophages. These genes are located in the eukaryotic association module (EAM) of the prophage WO (named after *Wolbachia*), which is a mobile element similar to a transposable element in eukaryotes, and is inserted into the genome of *Wolbachia* (Kaur et al., 2021). This

EAM contains protein domains with putative eukaryotic functions, including host-microbe interaction, toxins and host-cell suicide etc. (Bordenstein and Bordenstein, 2016). These findings provide a possible explanation to the shift in reproductive manipulation between CI and MK after transferring *Wolbachia* into new hosts. If the operons that induce different sex distortions are present in the same *Wolbachia*, the reproductive manipulation phenotypes may be determined species-specifically. All these results indicate that *Wolbachia* may possess multiple reproductive manipulation tools.

However, transitions from other *Wolbachia* induced phenotypes to PI seem to have never happened. Intriguingly, frequently degradation and loss of the CI inducing gene, *cif,* was found in strains that do not trigger CI in their hosts. For example, phage WO is completely lost in *Wolbachia* supergroups C and D, which have obligate mutualistic relationships with their nematode hosts and do not induce reproductive manipulations (Gerth et al., 2014). It is also missing from most PI-*Wolbachia* in *Trichogramma* (Gavotte et al., 2007). In the parasitoid species studied in this thesis, *M. uniraptor*, although the *cif* allele is present in the *Wolbachia* (*w*Uni) genome, only one *cif* module was recovered and most of the modules are either missing or highly divergent from the functional *cif* in the *D. melanogaster Wolbachia* (Lindsey et al., 2018). In most of the cases, the reduction in genome size of endosymbionts depends on the mutual dependency on their hosts (McCutcheon and Moran, 2012). Therefore, the loss of prophage WO indicates a long-term co-adaptation of *Wolbachia* and its host. Based on this idea, host-*Wolbachia* association seems to be happened more recently in *M. uniraptor* than in *Trichogramma* and nematodes.

The initial signal to trigger thelytokous reproduction is unclear. However, growing evidence suggests that DNA methylation is involved in PI-*Wolbachia* reproductive manipulation (Negri et al., 2009). Recently, it was found that PI-*Wolbachia* (*w*Tpre) infection in *Trichogramma pretiosum* is associated with higher levels of DNA methylation in the host, including in the genes involved in chromosome segregation and oocyte development (Wu et al., 2020). As methylated genes tend to have higher expression level than non-methylated genes (Wang et al., 2013), and loss of DNA methylation often occurs in invertebrate genes that function in cellular signalling and reproductive processes, the changes in DNA methylation levels by *Wolbachia* may point to a *Wolbachia*-induced parthenogenesis mechanism in *Trichogramma.* Due to the divergent cytological manipulation induced by PI-*Wolbachia* in *M. uniraptor* and *T. pretiosum* (Gottlieb et al., 2002; Stouthamer and Kazmer, 1994), and the different steps for *Wolbachia* to achieve thelytoky in these two species (Tulgetske, 2010,

Chapter 3), it is unlikely that a similar DNA methylation regulation system governs female development in *M. uniraptor*.

PI-*Wolbachia* causes decay of sexual characteristics

A change from sexual to asexual reproduction has evolved repeatedly across insect species. Many traits required for sexual reproduction are expected to be lost during the transition to parthenogenesis reproduction. This is also the case for *Wolbachia*-infected thelytokous females, that often show a decay of sexual traits including sexual attractiveness, mating, plasticity in adjusting offspring sex ratio and, in most of the cases, the ability of fertilizing eggs (Kremer et al., 2009; Ma et al., 2014; Zchori-Fein et al., 1995). However, studies on *Apoanagyrus diversicornis* and *Telenomus nawai* for instance, found that males derived from *Wolbachia*-induced parthenogenesis reproducing populations (males are generated through asexual reproduction by females that are cured of *Wolbachia* infection) are capable of inseminating uninfected females (Arakaki et al., 2000; Pijls et al., 1996). Males of *Leptopilina clavipes* can also perform largely normal courtship behaviour towards females (Pannebakker et al., 2005). This evidence suggests that males suffer less from the switch to thelytokous reproduction and can largely preserve their sexual reproduction abilities.

On the other hand, females with an enhanced male production, i.e. lower fertilization rate, have an advantage in a female-biased population, where a thelytoky-inducing *Wolbachia* infection has not reached fixation, assuming these males can mate with the asexually produced females (Stouthamer et al., 2010). This so-called 'functional virginity' was proposed by Jeong and Stouthamer (2005), and suggests that traits underlying this advantage will become fixed in the population (Stouthamer et al., 2010). Simulation models further support that under PI-*Wolbachia* spread, selection will favour nuclear-gene mutations that reduce female fertilization rate, which may explain why female mating and egg fertilization are lost rapidly (Stouthamer et al., 2010). On the other hand, as males are normally absent in an asexual reproduction system, male sexual traits evolve under neutral selection and accumulate mutations, leading to a slow erosion of these traits. This would account for the loss of male-specific traits in PI-*Wolbachia* infected species.

Indeed, in *M. uniraptor*, females lost their willingness to mate, and both the spermatheca in females and sperm production in males lost their function during co-evolution with PI-*Wolbachia* (Gottlieb and Zchori-Fein, 2001). However, a comparison of the sex determination

cascades between *M. uniraptor* and its sexually reproducing sister species *M. raptorellus*, shows that in both species, the cascades are similar (Chapter 2). Maternal provision of *tra* and *tra2* are essential for female development in both species (Chapter 2 and 3). Interestingly, however, the conserved sexual differentiation gene *Dsx* shows extra female splicing variants in *M. uniraptor* and contains three mutations in the two crucial binding domains of Dsx when compared to *M. raptorellus*. It is very unlikely that random mutations get fixed in functional domains, because they may directly hamper the binding property of Dsx (Coschigano and Wensink, 1993), and this may have profound influence on a wide range of downstream targets (Clough et al., 2014). As these amino acid changes are not present in the most conserved positions, the consequences of these changes are difficult to evaluate currently. However, it does not exclude the possibility that the female functional virginity trait could be at the level of Dsx.

Recently, a single QTL has been found in *Asobara japonica* that underlies the female functional virginity trait, which adds to the idea that an irreversible transition to asexuality happens rapidly during evolution (Ma et al., 2014; Ma et al., 2021). Although the *Dsx* splicing machinery and sex-specific Dsx functions in *Muscidifurax* have not been verified, it can be expected that the extra female Dsx variant is likely involved in the loss of female sexual traits in *M. uniraptor*. Once mating rejection and/or fertilization failure has spread in the population, it cannot be reversed due to a lack of recombination during gamete duplication. Nevertheless, as a closely related species to *N. vitripennis*, female-specific Dsx in *M. uniraptor* may also be less important in promoting female sexual traits (assuming female Dsx has less regulative function in female dimorphic traits as I discussed before). Therefore the observed mutations and changes of the splicing patterns could only reflect the slow erosion of the gene. In general, it is still too early to make a conclusion and more research will be required to validate these ideas.

Wolbachia and the host sex-determination system

Chapter 3 addressed the possible regulatory mechanism that *Wolbachia* employs to achieve thelytokous parthenogenesis in *M. uniraptor*. In *A. japonica* and *Trichogramma kaykai, Wolbachia* induces thelytokous parthenogenesis by a separate diploidization step and feminization step (Ma et al., 2015; Tulgetske, 2010). This also suggests that *Wolbachia* manipulates the host sex-determination systems, which is indeed observed in two systems with *Wolbachia*-induced thelytoky where *Wolbachia* manipulated maternal provision of

tra (Geuverink, 2017; Geuverink et al., 2018a). We did not observe a direct manipulation of *tra* and *tra2* maternal provision in terms of changes in expression or splicing patterns (Geuverink et al., 2017). We observed normal patterns of maternal provision and subsequent tra^F expression in both infected and uninfected *M. uniraptor* offspring. However, we did observe a two-fold zygotic expression increase of *tra* when *Wolbachia* is present. We propose that this correlates with the diploidization process that is induced by *Wolbachia* (Gottlieb et al., 2002). Because *M. uniraptor* does not mate, we used *M. raptorellus* to artificially generate triploid females and test our hypothesis. These virgin triploid females produced uniparental diploid females, proving that in *Muscidifurax* female development requires no paternal input and is instructed directly by ploidy level. Therefore, *Wolbachia* can achieve thelytokous parthenogenesis in *M. uniraptor* without manipulating the host sex determination system.

To this date, two sex-determination mechanisms have been molecularly described in Hymenoptera. The first is the Complementary Sex Determination system (CSD) in which both parents provide a different *csd* allele to their offspring as only heterozygosity at the *csd* locus initiates female sex determination. Hemi- or homozygous *csd* leads to default male development (Figure 7.2). As it is easy to experimentally confirm --inbreeding results in diploid male development--, in many Hymenoptera species, this system has been identified (Heimpel and de Boer, 2008). However, only in the honeybee the *csd* locus has been identified (Beye et al., 2003). CSD seems not compatible with *Wolbachia*-induced parthenogenesis, because gamete duplication triggered by PI-*Wolbachia* renders the *csd* locus homozygous, resulting in only diploid males (van Wilgenburg et al., 2006). Only if a species has diploidy-restoration through central or terminal fusion, which retains some degree of heterozygosity, CSD and parthenogenesis can be present simultaneously (Leach et al., 2009). In general, it is assumed that *Wolbachia* can only induce thelytokous reproduction in species with a different sex-determination mechanism.

The other mechanism is the maternal effect genomic imprinting sex-determination system (MEGISD) that has been identified in *N. vitripennis*, in which the maternal copy of *wasp-overruler-of-masculinization* (*wom*) is silenced and will result in default male development, unless an active copy of *wom* is provided by the father through fertilization which will activate the female developmental pathway together with maternally provided *tra* and *tra2* (Figure 7.2). Also here, the system relies on the combined input from both parents to guarantee female production. However, unless *Wolbachia* evolves a way to release the

maternal imprinting or mimic the signal provided by male to direct female development, the MEGISD is not easily taken over by PI-*Wolbachia*.

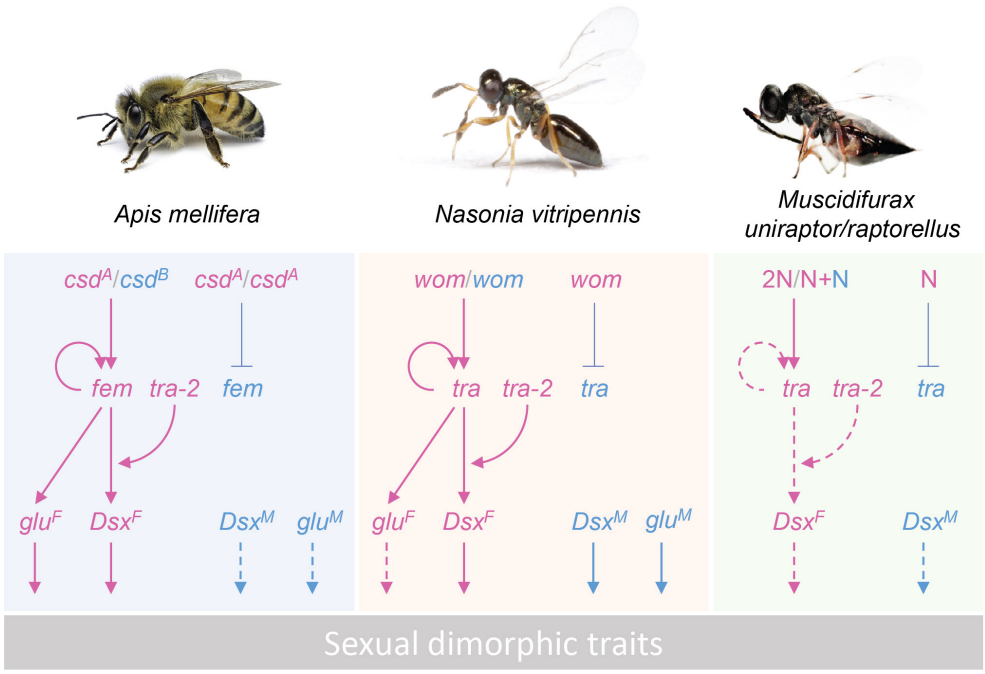


Figure 7.2 Sex determination cascades of *Apis mellifera*, *Nasonia vitripennis*, *Muscidifurax uniraptor* and *Muscidifurax raptorellus*. Blue and pink colours indicate the input and pathway of male and female development respectively. Lines indicate molecularly confirmed regulations and dashed lines refer to possible regulations.

Wom is an evolutionarily new gene, that contains a P53-like domain region and a partial *LOC100678853* homologous region (Zou et al., 2020). *Wom* and *LOC100678853* homologs are only found in *Nasonia* and the closely-related *Trichomalopsis.* In *Muscidifurax*, we searched for the *wom* homolog in the *M. raptorellus* genome but recovered only the sequence that represents the P53 element and not the *LOC100678853* homologous region (unpublished data). Therefore, we assume that the *M. raptorellus* does not contain a *wom* homolog. Moreover, *Muscidifurax* does not require paternal input for female development (Chapter 3). All evidence points to the fact that we have (partially) uncovered a third sex-determination system in the Hymenoptera.

In this sex-determination model, maternal provision of *tra* and *tra2* is required for female-specific splicing, and a bi-allelic *tra* is required to express enough zygotic *tra* to initiate and

maintain the auto-regulatory loop of tra^F splicing and Dsx^F splicing. Either diploidization of the haploid embryo by PI-*Wolbachia*-induced gamete duplication (Gottlieb et al., 2002), or the normal fertilization process (Figure 7.2) brings together two active *tra* alleles which is sufficient to surpass the threshold needed for auto-regulation. Without fertilization or diploidization, only one *tra* allele is present and mono-allelic *tra* expression is insufficient to surpass the threshold for auto-regulation, thus, the default male splicing of *tra* and *Dsx* commences.

Future perspective

This thesis provides the starting point towards an overall understanding of Dsx-regulated sex-differentiation in hymenopterans and *Wolbachia*-mediated thelytokous parthenogenesis in wasps. Many more questions remain for further investigation. In *Nasonia*, dimorphic traits are also present in the head and antennae. The development of a sex-specific neuronal network and specialized odour receptors are all important to guide their behaviours and communication. The role of *Dsx* in the regulation of these traits requires more study.

RNA interference has its merit to transiently compromise gene function, which facilitates functional analysis in specific developmental stages, as we have shown in our *Dsx* knockdown experiments. However, its efficiency may be greatly affected by the target gene size, the RNA silencing machinery of the investigated subjects, tissue types and developmental stages (Bansal and Michel, 2013; Mulot et al., 2016; Tomoyasu et al., 2008; Yoshiyama et al., 2013). Therefore, applying RNAi needs an cautionary approach. On the other hand, advanced gene editing techniques such as CRISPR/Cas9 are successfully applied in several insect orders including Diptera (Bassett *et al*., 2013; Hall *et al*., 2015) and Lepidoptera (Chen et al., 2018; Xu et al., 2017a). Also in *N. vitripennis,* CRISPR/Cas9 can be used to generate heritable germline mutations (Chaverra-Rodriguez et al., 2020; Li et al., 2017). This is especially useful for future functional studies of the *Dsx* sex-specific variants as they are difficult to individually target with RNAi knockdown.

In addition, we started to uncover the role of Dsx in *Nasonia* adult life, especially in the regulation of sex pheromones (Chapter 4 and 6). Mating behaviour in insects is mainly guided by long-range volatile and contact sex pheromones (Buellesbach et al., 2013; Chuman et al., 1987; Geiselhardt et al., 2009; Ginzel, 2010; Jungwirth et al., 2021; Lebreton et al., 2017; Roelofs and Rooney, 2003; Silk et al., 2009). Species-specific pheromone blends are crucial

to regulate intraspecific recognition (Weiss et al., 2015). Further exploration of the Dsx-mediated synthetic pathway of sex pheromone production and its evolution between species of *Nasonia* will shed more light on the overall understanding of Dsx function. Moreover, we advanced the identification of the elusive oral-aphrodisiac that triggers female receptivity in *Nasonia* (Chapter 6). With our *Dsx* RNAi experiment, we observed that the majority of females did not show receptivity during courtship with *Dsx* silenced males. These males can serve as null-mutants (negative controls) for chemical identification of the *Nasonia* oral-aphrodisiac compound in future.

The identification of the host reproductive manipulation mechanism by PI-*Wolbachia* helps to accumulate more knowledge in this research field. By combining molecular methods and insect "breeding", we convincingly identified the one-step feminization by diploidization mechanism in *Wolbachia*-induced parthenogenesis in *M. uniraptor*. Diploid males are observed in at least two other PI-*Wolbachia* infected hymenopterans, indicating a separate diploidization and feminization process (Ma et al., 2015; Tulgetske, 2010). Investigating the steps for PI-*Wolbachia* to trigger feminization in other haplodiploid species along with their sex-determination mechanism will greatly improve our understanding of the genetic basis and evolution of *Wolbachia*-induced parthenogenesis.

Moreover, it remains elusive how PI-*Wolbachia* got co-adapted with their host. One way to study the evolution of *Wolbachia*-induced parthenogenesis is to trans-infect new hosts with PI-*Wolbachia*. This has been done before, (Legner, 1987; van Meer and Stouthamer, 1999; Watanabe et al., 2013; Yamashita and Takahashi, 2018), however, a successful infection of PI-*Wolbachia* in a new host is hard to achieve (reviewed by Hughes and Rasgon, 2014). Even in closely-related species or even different strains of same species, a stable infection is difficult to maintain in additional generations and results in no or very mild PI phenotypes (Watanabe et al., 2013; Yamashita and Takahashi, 2018). Many studies observed that the trans-infection of *Wolbachia* into new hosts is often associated with a strong activation of the insect host's innate immune response (Da Silva Gonçalves et al., 2019; Herbert and McGraw, 2018), which explains the low trans-infection success. Studying how *Wolbachia* surpasses these host defence barriers would be the first step to understand its evolution and transmission.

On the other hand, reducing the host immune response by gene knockdown and pre-adapting *Wolbachia* in the cell lines of novel hosts before trans-infection (McMeniman et

al., 2008) would be alternatives to improve the trans-infection success. These optimization and pre-adaptation methods also have their advantages in biological control or gene-drive application (McMeniman et al., 2009; Xi et al., 2005). The development of asexually reproducing female lines will greatly contribute to the optimization of mass rearing of biological control agents.

Wolbachia is predominant in reproductive organs and germlines, however over the last decade, *Wolbachia* appears to be prevalent in many somatic tissues including central brains, muscles, midgut, wings and salivary gland as well (reviewed in Pietri et al., 2016). The presence of *Wolbachia* in these somatic tissues can increase the olfactory response to odours (Peng and Wang, 2009), shape female mating preference (Miller et al., 2010) and even regulate male pheromone profile, that is crucial for female acceptance (Schneider et al., 2019). These are also essential factors of *Wolbachia* host-reproduction manipulation, and understanding them will provide a more complete picture of the overall *Wolbachia*-host interactions.

The ability of *Wolbachia* to survive in cell-free medium (Krafsur et al., 2020; Rasgon et al., 2006) and the recent discovery of a *Wolbachia* plasmid (Reveillaud et al., 2019), opens the door to its genetic manipulation, and may provide more research tools for *Wolbachia* research in the near future.

Concluding remarks

The sex-determination gene *Dsx* is conserved in sequence and function in insects. We observed that in male *N. vitripennis Dsx* actively regulates sexually dimorphic trait development during different developmental stages. *Dsx* is also involved in the sex-specific and sex-biased pheromone production during adult stage to shape the conspecific recognition. We found that the core elements of the sex-determination cascade in *Muscidifurax* are very similar to those of its close relative, *Nasonia*. However, the sex-determination pathway is not manipulated by *Wolbachia* to achieve parthenogenesis in its host *M. uniraptor*. Instead, female development is determined by the diploid state of its chromosomes regardless of how this diploidization is achieved. This dissertation provides a starting point towards the comprehensive understanding of the evolution of sex-determination cascades, the role of *Dsx* in sexual differentiation and *Wolbachia*-induced parthenogenesis in wasps, which still requires many more research efforts in the future.

Reference list

A

Abdel-Latief, M., Garbe, L. A., Koch, M. and Ruther, J. (2008). An epoxide hydrolase involved in the biosynthesis of an insect sex attractant and its use to localize the production site. *Proc. Natl. Acad. Sci.* **105**, 8914–8919.

Ahmad, S. M. and Baker, B. S. (2002). Sex-specific deployment of FGF signaling in *Drosophila* recruits mesodermal cells into the male genital imaginal disc. *Cell* **109**, 651–661.

Alavi, Y., van Rooyen, A., Elgar, M. A., Jones, T. M. and Weeks, A. R. (2018). Novel microsatellite markers suggest the mechanism of parthenogenesis in *Extatosoma tiaratum* is automixis with terminal fusion. *Insect Sci.* **25**, 24–32.

Amrein, H., Gorman, M. and Nöthiger, R. (1988). The sex-determining gene *tra-2* of *Drosophila* encodes a putative RNA binding protein. *Cell* **55**, 1025–1035.

An, W., Cho, S., Ishii, H. and Wensink, P. C. (1996). Sex-specific and non-sex-specific oligomerization domains in both of the *doublesex* transcription factors from *Drosophila melanogaster*. *Mol. Cell. Biol.* **16**, 3106–3111.

Arakaki, N., Noda, H. and Yamagishi, K. (2000). *Wolbachia*-induced parthenogenesis in the egg parasitoid *Telenomus nawai*. *Entomol. Exp. Appl.* **96**, 177–184.

Araujo, P. R., Yoon, K., Ko, D., Smith, A. D., Qiao, M., Suresh, U., Burns, S. C. and Penalva, L. O. F. (2012). Before it gets started: regulating translation at the 5′ UTR. *Comp. Funct. Genomics* **2012**, 1–8.

Asher, J. H. (1970). Parthenogenesis and genetic variability. II. One-locus models for various diploid populations. *Genetics* **66**, 369–391.

B

Bachtrog, D., Mank, J. E., Peichel, C. L., Kirkpatrick, M., Otto, S. P., Ashman, T.-L., Hahn, M. W., Kitano, J., Mayrose, I., Ming, R., et al. (2014). Sex determination: why so many ways of doing it? *PLoS Biol.* **12**, e1001899.

Baker, B. S. and Ridge, K. A. (1980). Sex and the single cell. I. On the action of major loci affecting sex determination in *Drosophila melanogaster*. *Genetics* **94**, 383–423.

Baker, R. H. and Wilkinson, G. S. (2001). Phylogenetic analysis of sexual dimorphism and eye-span allometry in stalk-eyed flies (Diopsidae). *Evolution* **55**, 1373–1385.

Baker, B. S. and Wolfner, M. F. (1988). A molecular analysis of *doublesex*, a bifunctional gene that controls both male and female sexual differentiation in *Drosophila melanogaster*. *Genes Dev.* **2**, 477–489.

Bansal, R. and Michel, A. P. (2013). Core RNAi machinery and Sid1, a component for systemic RNAi, in the hemipteran insect, *Aphis glycines*. *Int. J. Mol. Sci.* **14**, 3786–3801.

Baral, S., Arumugam, G., Deshmukh, R. and Kunte, K. (2019). Genetic architecture and sex-specific selection govern modular, male-biased evolution of *doublesex*. *Sci. Adv.* **5**, eaau3753.

Barmina, O. and Kopp, A. (2007). Sex-specific expression of a HOX gene associated with rapid morphological evolution. *Dev. Biol.* **311**, 277–286.

Barrass, R. (1960). The Courtship Behaviour of *Mormoniella vitripennis* Walk. (Hymenoptera, Pteromalidae). *Behaviour* **15**, 185–209.

Bassett, A. R., Tibbit, C., Ponting, C. P. and Liu, J. L. (2013). Highly efficient targeted mutagenesis of *Drosophila* with the CRISPR/Cas9 system. *Cell Rep.* **4**, 220–228.

Bear, A. and Monteiro, A. (2013). Both cell-autonomous mechanisms and hormones contribute to sexual development in vertebrates and insects. *Bioessays* **35**, 725–732.

Beckers, O. M., Kijimoto, T. and Moczek, A. P. (2017). *doublesex* alters aggressiveness as a function of social context and sex in the polyphenic beetle *Onthophagus taurus*. *Anim. Behav.* **132**, 261–269.

Beckmann, J. F., Ronau, J. A. and Hochstrasser, M. (2017). A *Wolbachia* deubiquitylating enzyme induces cytoplasmic incompatibility. *Nat. Microbiol.* **2**, 17007.

Bell, L. R., Horabin, J. I., Schedl, P. and Cline, T. W. (1991). Positive autoregulation of Sex-lethal by alternative splicing maintains the female determined state in *Drosophila*. *Cell* **65**, 229–239.

Bernstein, H. and Bernstein, C. (2013). Evolutionary origin and adaptive function of meiosis. In *Meiosis*, pp. 41–75.

Bertossa, R. C., van de Zande, L. and Beukeboom, L. W. (2009). The *fruitless* gene in *Nasonia* displays complex sex-specific splicing and contains new zinc finger domains. *Mol. Biol. Evol.* **26**, 1557–1569.

Beukeboom, L. W. (2012). Microbial manipulation of host sex determination: endosymbiotic bacteria can directly manipulate their host's sex determination towards the production of female offspring. *Bioessays* **34**, 484–488.

Beukeboom, L. W. and Kamping, A. (2006). No patrigenes required for femaleness in the haplodiploid wasp *Nasonia vitripennis*. *Genetics* **172**, 981–989.

Beukeboom, L. W. and Pijnacker, L. P. (2000). Automictic parthenogenesis in the parasitoid *Venturia canescens* (Hymenoptera: Ichneumonidae) revisited. *Genome* **43**, 939–944.

Beukeboom, L. W. and van de Zande, L. (2010). Genetics of sex determination in the haplodiploid wasp *Nasonia vitripennis* (Hymenoptera: Chalcidoidea). *J. Genet.* **89**, 333–339.

Beukeboom, L. W., Kamping, A. and van de Zande, L. (2007). Sex determination in the haplodiploid wasp *Nasonia vitripennis* (Hymenoptera: Chalcidoidea): a critical consideration of models and evidence. *Semin. Cell Dev. Biol.* **18**, 371–378.

Beye, M., Hasselmann, M., Fondrk, M. K., Page, R. E. and Omholt, S. W. (2003). The gene *csd* is the primary signal for sexual development in the honeybee and encodes an SR-type protein. *Cell* **114**, 419–429.

Blackmon, H. and Demuth, J. P. (2015). Genomic origins of insect sex chromosomes. *Curr. Opin. Insect Sci.* **7**, 45–50.

Blaul, B. and Ruther, J. (2011). How parasitoid females produce sexy sons: a causal link between oviposition preference, dietary lipids and mate choice in *Nasonia*. *Proc. R. Soc. B Biol. Sci.* **278**, 3286–3293.

Bopp, D., Saccone, G. and Beye, M. (2014). Sex determination in insects: variations on a common theme. *Sex. Dev.* **8**, 20–28.

Bordenstein, S. R. and Bordenstein, S. R. (2016). Eukaryotic association module in phage WO genomes from *Wolbachia*. *Nat. Commun.* **7**, 13155.

Bordenstein, S. R., O'hara, F. P. and Werren, J. H. (2001). *Wolbachia*-induced incompatibility precedes other hybrid incompatibilities in *Nasonia*. *Nature* **409**, 707–710.

Buellesbach, J., Gadau, J., Beukeboom, L. W., Echinger, F., Raychoudhury, R., Werren, J. H. and Schmitt, T. (2013). Cuticular hydrocarbon divergence in the jewel wasp *Nasonia* : evolutionary shifts in chemical communication channels? *J. Evol. Biol.* **26**, 2467–2478.

Buellesbach, J., Vetter, S. G. and Schmitt, T. (2018). Differences in the reliance on cuticular hydrocarbons as sexual signaling and species discrimination cues in parasitoid wasps. *Front. Zool.* **15**, 22.

Burghardt, G., Hediger, M., Siegenthaler, C., Moser, M., Dübendorfer, A. and Bopp, D. (2005). The *transformer2* gene in *Musca domestica* is required for selecting and maintaining the female pathway of development. *Dev. Genes Evol.* **215**, 165–176.

Burtis, K. C. and Baker, B. S. (1989). *Drosophila doublesex* gene controls somatic sexual differentiation by producing alternatively spliced mRNAs encoding related sex-specific polypeptides. *Cell* **56**, 997–1010.

C

Camara, N., Whitworth, C. and Van Doren, M. (2008). Chapter 3: The creation of sexual dimorphism in the *Drosophila* soma. *Curr. Top. Dev. Biol.* **83**, 65–107.

Camara, N., Whitworth, C., Dove, A. and van Doren, M. (2019). Doublesex controls specification and maintenance of the gonad stem cell niches in *Drosophila*. *Development* **146**, dev170001.

Carlson, D. A., Mayer, M. S., Silhacek, D. L., James, J. D., Beroza, M. and Bierl, B. A. (1971). Sex attractant pheromone of the house fly: isolation, identification and synthesis. *Science* **174**, 76–78.

Carlson, D. A., Geden, C. J. and Bernier, U. R. (1999). Identification of pupal exuviae of *Nasonia vitripennis* and *Muscidifurax raptorellus* parasitoids using cuticular hydrocarbons. *Biol. Control* **15**, 97–106.

Chaverra-Rodriguez, D., Dalla Benetta, E., Heu, C. C., Rasgon, J. L., Ferree, P. M. and Akbari, O. S. (2020). Germline mutagenesis of *Nasonia vitripennis* through ovarian delivery of CRISPR-Cas9 ribonucleoprotein. *Insect Mol. Biol.* **29**, 569–577.

Chemnitz, J., Jentschke, P. C., Ayasse, M. and Steiger, S. (2015). Beyond species recognition: somatic state affects long-distance sex pheromone communication. *Proc. R. Soc. B Biol. Sci.* **282**, 20150832.

Chen, S.-L., Dai, S.-M., Lu, K.-H. and Chang, C. (2008). Female-specific *doublesex* dsRNA interrupts yolk protein gene expression and reproductive ability in oriental fruit fly, *Bactrocera dorsalis* (Hendel). *Insect Biochem. Mol. Biol.* **38**, 155–165.

Chen, X., Cao, Y., Zhan, S., Tan, A., Palli, S. R. and Huang, Y. (2019). Disruption of sex-specific *doublesex* exons results in male- and female-specific defects in the black cutworm, *Agrotis ipsilon*. *Pest Manag. Sci.* **75**, 1697–1706.

Chertemps, T., Duportets, L., Labeur, C., Ueyama, M. and Wicker-Thomas, C. (2006). A female-specific desaturase gene responsible for diene hydrocarbon biosynthesis and courtship behaviour in *Drosophila melanogaster*. *Insect Mol. Biol.* **15**, 465–473.

Chigira, A. and Miura, K. (2005). Detection of "*Candidatus* Cardinium" bacteria from the haploid host *Brevipalpus californicus* (Acari: Tenuipalpidae) and effect on the host. *Exp. Appl. Acarol.* **37**, 107–116.

Cho, S., Huang, Z. Y. and Zhang, J. (2007). Sex-specific splicing of the honeybee *doublesex* gene reveals 300 million years of evolution at the bottom of the insect sex-determination pathway. *Genetics* **177**, 1733–1741.

Chowdhury, T., Calhoun, R. M., Bruch, K. and Moehring, A. J. (2020). The *fruitless* gene affects female receptivity and species isolation. *Proc. R. Soc. B Biol. Sci.* **287**, 20192765.

Christiansen, A. E., Keisman, E. L., Ahmad, S. M. and Baker, B. S. (2002). Sex comes in from the cold: the integration of sex and pattern. *Trends Genet.* **18**, 510–516.

Chuman, T., Landolt, P. J., Heath, R. R. and Tumlinson, J. H. (1987). Isolation, identification, and synthesis of male-produced sex pheromone of papaya fruit fly, *Toxotrypana curvicauda* Gerstaecker (Diptera: Tephritidae). *J. Chem. Ecol.* **13**, 1979–1992.

Clough, E., Jimenez, E., Kim, Y. A., Whitworth, C., Neville, M. C., Hempel, L. U., Pavlou, H. J., Chen, Z. X., Sturgill, D., Dale, R. K., et al. (2014). Sex- and tissue-specific functions of *Drosophila doublesex* transcription factor target genes. *Dev. Cell* **31**, 761–773.

Colnaghi, M., Lane, N. and Pomiankowski, A. (2020). Genome expansion in early eukaryotes drove the transition from lateral gene transfer to meiotic sex. *Elife* **9**, 1–16.

Concha, C. and Scott, M. J. (2009). Sexual development in *Lucilia cuprina* (Diptera, Calliphoridae) is controlled by the *transformer* gene. *Genetics* **182**, 785–798.

Concha, C., Li, F. and Scott, M. J. (2010). Conservation and sex-specific splicing of the *doublesex* gene in the economically important pest species *Lucilia cuprina*. *J. Genet.* **89**, 279–285.

Cook, J. M. (1993). Sex determination in the Hymenoptera: a review of models and evidence. *Heredity* **71**, 421–435.

Coschigano, K. T. and Wensink, P. C. (1993). Sex-specific transcriptional regulation by the male and female doublesex proteins of *Drosophila*. *Genes Dev.* **7**, 42–54.

D

Da Silva Gonçalves, D., Iturbe-Ormaetxe, I., Martins-Da-Silva, A., Telleria, E. L., Rocha, M. N., Traub-Csekö, Y. M., O'Neill, S. L., Sant'Anna, M. R. V. and Moreira, L. A. (2019). *Wolbachia* introduction into *Lutzomyia longipalpis* (Diptera: Psychodidae) cell lines and its effects on immune-related gene expression and interaction with *Leishmania infantum*. *Parasit. Vectors* **12**, 1–13.

Darling, D. C. and Werren, J. H. (1990). Biosystematics of *Nasonia* (Hymenoptera: Pteromalidae): two new species reared from birds' nests in North America. *Ann. Entomol. Soc. Am.* **83**, 352–370.

De La Filia, A. G., Bain, S. A. and Ross, L. (2015). Haplodiploidy and the reproductive ecology of Arthropods. *Curr. Opin. Insect Sci.* **9**, 36–43.

DeFalco, T. J., Verney, G., Jenkins, A. B., McCaffery, J. M., Russell, S. and Van Doren, M. (2003). Sex-specific apoptosis regulates sexual dimorphism in the *Drosophila* embryonic gonad. *Dev. Cell* **5**, 205–216.

DeFalco, T., Camara, N., Le Bras, S. and Van Doren, M. (2008). Nonautonomous sex determination controls sexually dimorphic development of the *Drosophila* gonad. *Dev. Cell* **14**, 275–286.

Demir, E. and Dickson, B. J. (2005). *fruitless* splicing specifies male courtship behavior in *Drosophila*. *Cell* **121**, 785–794.

Deveson, I. W., Holleley, C. E., Blackburn, J., Marshall Graves, J. A., Mattick, J. S., Waters, P. D. and Georges, A. (2017). Differential intron retention in *Jumonji* chromatin modifier genes is implicated in reptile temperature-dependent sex determination. *Sci. Adv.* **3**, e1700731.

Dobson, S. L. and Tanouye, M. A. (1998). Evidence for a genomic imprinting sex determination mechanism in *Nasonia vitripennis* (Hymenoptera; Chalcidoidea). *Genetics* **149**, 233–242.

Drapeau, M. D. and Werren, J. H. (1999). Differences in mating behaviour and sex ratio between three sibling species of *Nasonia*. *Evol. Ecol. Res.* **1**, 223–224.

Dylan Shropshire, J., On, J., Layton, E. M., Zhou, H. and Bordenstein, S. R. (2018). One prophage WO gene rescues cytoplasmic incompatibility in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci.* **115**, 4987–4991.

E

Edgar, B. A. and Orr-Weaver, T. L. (2001). Endoreplication cell cycles: more for less. *Cell* **105**, 297–306.

Erdman, S. E. and Burtis, K. C. (1993). The *Drosophila* doublesex proteins share a novel zinc finger related DNA binding domain. *EMBO J.* **12**, 527–535.

Erickson, J. W. and Quintero, J. J. (2007). Indirect effects of ploidy suggest X chromosome dose, not the X:A ratio, signals sex in *Drosophila*. *PLoS Biol.* **5**, e332.

F

Fagegaltier, D., Fagegaltier, D., Gordon, A., Lai, E. C., Gingeras, T. R., Hannon, G. J. and Shcherbata, H. R. (2014). A genome-wide survey of sexually dimorphic expression of *Drosophila* miRNAs identifies the steroid hormone-induced miRNA let-7 as a regulator of sexual identity. *Genetics* **198**, 647–668.

Ferveur, J. F. and Sureau, G. (1996). Simultaneous influence on male courtship of stimulatory and inhibitory pheromones produced by live sex-mosaic *Drosophila melanogaster*. *Proc. R. Soc. B Biol. Sci.* **263**, 967–973.

Fialho, R. F. and Stevens, L. (2000). Male-killing *Wolbachia* in a flour beetle. *Proc. R. Soc. B Biol. Sci.* **267**, 1469–1474.

Fujii, Y., Kageyama, D., Hoshizaki, S., Ishikawa, H. and Sasaki, T. (2001). Transfection of *Wolbachia* in lepidoptera: The feminizer of the adzuki bean borer *Ostrinia scapulalis* causes male killing in the Mediterranean flour moth *Ephestia kuehniella*. *Proc. R. Soc. B Biol. Sci.* **268**, 855–859.

Fukui, T., Kawamoto, M., Shoji, K., Kiuchi, T., Sugano, S., Shimada, T., Suzuki, Y. and Katsuma, S. (2015). The endosymbiotic bacterium *Wolbachia* selectively kills male hosts by targeting the *Masculinizing* gene. *PLoS Pathog.* **11**, e1005048.

Furihata, S., Hirata, M., Matsumoto, H. and Hayakawa, Y. (2015). Bacteria endosymbiont, *Wolbachia*, promotes parasitism of parasitoid wasp *Asobara japonica*. *PLoS One* **10**, e0140914.

G

Garcia-Bellido, A. and Merriam, J. R. (1971). Parameters of the wing imaginal disc development of *Drosophila melanogaster*. *Dev. Biol.* **24**, 61–87.

Gardner, A. and Ross, L. (2014). Mating ecology explains patterns of genome elimination. *Ecol. Lett.* **17**, 1602–1612.

Gavotte, L., Henri, H., Stouthamer, R., Charif, D., Charlat, S., Boulétreau, M. and Vavre, F. (2007). A survey of the bacteriophage WO in the endosymbiotic bacteria *Wolbachia*. *Mol. Biol. Evol.* **24**, 427–435.

Ge, C., Ye, J., Zhang, H., Zhang, Y., Sun, W., Sang, Y., Capel, B. and Qian, G. (2017). *Dmrt1* induces the male pathway in a turtle species with temperature-dependent sex determination. *Development* **144**, 2222–2233.

Geden, C. J. and Hogsette, J. A. (2006). Suppression of house flies (Diptera: Muscidae) in Florida poultry houses by sustained releases of *Muscidifurax raptorellus* and *Spalangia cameroni* (Hymenoptera: Pteromalidae). *Environ. Entomol.* **35**, 75–82.

Geiselhardt, S., Otte, T. and Hilker, M. (2009). The role of cuticular hydrocarbons in male mating behavior of the mustard leaf beetle, *Phaedon cochleariae* (F.). *J. Chem. Ecol.* **35**, 1162–1171.

Gempe, T. and Beye, M. (2011). Function and evolution of sex determination mechanisms, genes and pathways in insects. *Bioessays* **33**, 52–60.

Gempe, T., Hasselmann, M., Schiøtt, M., Hause, G., Otte, M. and Beye, M. (2009). Sex determination in honeybees: two separate mechanisms induce and maintain the female pathway. *PLoS Biol.* **7**, e1000222.

Gerth, M., Gansauge, M.-T., Weigert, A. and Bleidorn, C. (2014). Phylogenomic analyses uncover origin and spread of the *Wolbachia* pandemic. *Nat. Commun.* **5**, 5117.

Geuverink, E. (2017). Parental and endosymbiont effects on sex determination in haplodiploid wasps: Who is in control? University of Groningen.

Geuverink, E. and Beukeboom, L. W. (2014). Phylogenetic distribution and evolutionary dynamics of the sex determination genes *doublesex* and *transformer* in insects. *Sex. Dev.* **8**, 38–49.

Geuverink, E., Rensink, A. H., Rondeel, I., Beukeboom, L. W., van de Zande, L. and Verhulst, E. C. (2017). Maternal provision of *transformer-2* is required for female development and embryo viability in the wasp *Nasonia vitripennis*. *Insect Biochem. Mol. Biol.* **90**, 23–33.

Geuverink, E., Verhulst, E. C., van Leussen, M., van de Zande, L. and Beukeboom, L. W. (2018a). Maternal provision of non-sex-specific *transformer* messenger RNA in sex determination of the wasp *Asobara tabida*. *Insect Mol. Biol.* **27**, 99–109.

Geuverink, E., Kraaijeveld, K., van Leussen, M., Chen, F., Pijpe, J., Linskens, M. H. K., Beukeboom, L. W. and van de Zande, L. (2018b). Evidence for involvement of a *transformer* paralogue in sex determination of the wasp *Leptopilina clavipes*. *Insect Mol. Biol.* **27**, 780–795.

Ginzel, M. D. (2010). Hydrocarbons as contact pheromones of longhorned beetles (Coleoptera: Cerambycidae). In *Insect hydrocarbons: biology, biochemistry, and chemical ecology*, pp. 375–389. Cambridge University Press, Cambridge, UK.

Ginzel, M. D., Blomquist, G. J., Millar, J. G. and Hanks, L. M. (2003). Role of contact pheromones in mate recognition in *Xylotrechus colonus*. *J. Chem. Ecol.* **29**, 533–545.

Godfray, H. C. J. (1994). *Parasitoids: behavioral and evolutionary ecology*. Princeton University Press.

Gotoh, H., Miyakawa, H., Ishikawa, A., Ishikawa, Y., Sugime, Y., Emlen, D. J., Lavine, L. C. and Miura, T. (2014). Developmental link between sex and nutrition; *doublesex* regulates sex-specific mandible growth via juvenile hormone signaling in stag beetles. *PLoS Genet.* **10**, e1004098.

Gotoh, H., Ishiguro, M., Nishikawa, H., Morita, S., Okada, K., Miyatake, T., Yaginuma, T. and Niimi, T. (2016). Molecular cloning and functional characterization of the sex-determination gene *doublesex* in the sexually dimorphic broad-horned beetle *Gnatocerus cornutus* (Coleoptera, Tenebrionidae). *Sci. Rep.* **6**, 1–10.

Gottlieb, Y. and Zchori-Fein, E. (2001). Irreversible thelytokous reproduction in *Muscidifurax uniraptor*. *Entomol. Exp. Appl.* **100**, 271–278.

Gottlieb, Y., Zchori-Fein, E., Werren, J. H. and Karr, T. L. (2002). Diploidy restoration in *Wolbachia*-infected *Muscidifurax uniraptor* (Hymenoptera: Pteromalidae). *J. Invertebr. Pathol.* **81**, 166–174.

H

Hagimori, T., Abe, Y., Date, S. and Miura, K. (2006). The first finding of a Rickettsia bacterium associated with parthenogenesis induction among insects. *Curr. Microbiol.* **52**, 97–101.

Hall, A. B., Basu, S., Jiang, X., Qi, Y., Timoshevskiy, V. A., Biedler, J. K., Sharakhova, M. V., Elahi, R., Anderson, M. A. E., Chen, X. G., et al. (2015). A male-determining factor in the mosquito *Aedes aegypti*. *Science* **348**, 1268–1270.

Hamilton, W. D. (1967). Extraordinary sex ratios. *Science* **156**, 477–488.

Hansell, R. I. C., Mollison, M. M. and Putman, W. L. (1964). A cytological demonstration of arrhenotoky in three mites of the family Phytoseiidae. *Chromosoma* **15**, 562–567.

Harpur, B. A., Sobhani, M. and Zayed, A. (2013). A review of the consequences of complementary sex determination and diploid male production on mating failures in the Hymenoptera. *Entomol. Exp. Appl.* **146**, 156–164.

Harumoto, T., Fukatsu, T. and Lemaitre, B. (2018). Common and unique strategies of male killing evolved in two distinct *Drosophila* symbionts. *Proc. R. Soc. B Biol. Sci.* **285**, 20172167.

Hasselmann, M., Gempe, T., Schiøtt, M., Nunes-Silva, C. G., Otte, M. and Beye, M. (2008). Evidence for the evolutionary nascence of a novel sex determination pathway in honeybees. *Nature* **454**, 519–522.

Hediger, M., Burghardt, G., Siegenthaler, C., Buser, N., Hilfiker-Kleiner, D., Dubendorfer, A. and Bopp, D. (2004). Sex determination in *Drosophila melanogaster* and *Musca domestica* converges at the level of the terminal regulator *doublesex*. *Dev. Genes Evol.* **214**, 29–42.

Hediger, M., Henggeler, C., Meier, N., Perez, R., Saccone, G. and Bopp, D. (2010). Molecular characterization of the key switch F provides a basis for understanding the rapid divergence of the sex-determining pathway in the housefly. *Genetics* **184**, 155–170.

Heimpel, G. E. and de Boer, J. G. (2008). Sex determination in the Hymenoptera. *Annu. Rev. Entomol* **53**, 209–230.

Heimpel, G. E. and Lundgren, J. G. (2000). Sex ratios of commercially reared biological control agents. *Biol. Control* **19**, 77–93.

Hempel, L. U. and Oliver, B. (2007). Sex-specific DoublesexM expression in subsets of *Drosophila* somatic gonad cells. *BMC Dev. Biol.* **7**, 1–15.

Herbert, R. I. and McGraw, E. A. (2018). The nature of the immune response in novel *Wolbachia*-host associations. *Symbiosis* **74**, 225–236.

Herran, B., Geniez, S., Delaunay, C., Raimond, M., Lesobre, J., Bertaux, J., Slatko, B. and Grève, P. (2020). The shutting down of the insulin pathway: a developmental window for *Wolbachia* load and feminization. *Sci. Rep.* **10**, 1–9.

Hertig, M. and Wolbach, S. B. (1924). Studies on *Rickettsia*-like micro-organisms in insects. *J. Med. Res.* **44**, 329–374.

Hildreth, P. E. (1965). *Doublesex*, recessive gene that transforms both males and females of *Drosophila* into intersexes. *Genetics* **51**, 659–678.

Hilgenboecker, K., Hammerstein, P., Schlattmann, P., Telschow, A. and Werren, J. H. (2008). How many species are infected with *Wolbachia*? - a statistical analysis of current data. *FEMS Microbiol. Lett.* **281**, 215–220.

Hornett, E. A., Duplouy, A. M. R., Davies, N., Roderick, G. K., Wedell, N., Hurst, G. D. D. and Charlat, S. (2008). You can't keep a good parasite down: evolution of a male-killer suppressor uncovers cytoplasmic incompatibility. *Evolution* **62**, 1258–1263.

Hughes, A. L. (2011). Runaway evolution of the male-specific exon of the *doublesex* gene in *Diptera*. *Gene* **472**, 1–6.

Hughes, G. L. and Rasgon, J. L. (2014). Transinfection: a method to investigate *Wolbachia*-host interactions and control arthropod-borne disease. *Insect Mol. Biol.* **23**, 141–151.

Huigens, M. E., de Almeida, R. P., Boons, P. A. H., Luck, R. F. and Stouthamer, R. (2004). Natural interspecific and intraspecific horizontal transfer of parthenogenesis-inducing *Wolbachia* in *Trichogramma* wasps. *Proc. R. Soc. B Biol. Sci.* **271**, 509–515.

Hurst, G. D. D., Johnson, A. P., Schulenburg, J. H. G. V. D. and Fuyama, Y. (2000). Male-killing *Wolbachia* in *Drosophila*: a temperature-sensitive trait with a threshold bacterial density. *Genetics* **156**, 699–709.

I

Inoue, K., Hoshijima, K., Higuchi, I., Sakamoto, H. and Shimura, Y. (1992). Binding of the *Drosophila* transformer and transformer-2 proteins to the regulatory elements of doublesex primary transcript for sex-specific RNA processing. *Proc. Natl. Acad. Sci.* **89**, 8092–8096.

Ito, Y., Harigai, A., Nakata, M., Hosoya, T., Araya, K., Oba, Y., Ito, A., Ohde, T., Yaginuma, T. and Niimi, T. (2013). The role of *doublesex* in the evolution of exaggerated horns in the Japanese rhinoceros beetle. *EMBO Rep.* **14**, 561–567.

J

Jaenike, J. (2007). Spontaneous emergence of a new *Wolbachia* phenotype. *Evolution* **61**, 2244–2252.

Jallon, J.-M. and David, J. R. (1987). Variations in cuticular hydrocarbons among the eight species of the *Drosophila melanogaster* subgroup. *Evolution* **41**, 294–302.

Jallon, J.-M., Lauge, G., Orssaud, L. and Antony, C. (1988). Female pheromones in *Drosophila melanogaster* are controlled by the doublesex locus. *Genet. Res.* **51**, 17–22.

Janzen, F. and Krenz, J. G. (2004). *Temperature dependent sex determination in vertebrates*.

Jeong, G. and Stouthamer, R. (2005). Genetics of female functional virginity in the parthenogenesis-*Wolbachia* infected parasitoid wasp *Telenomus nawai* (Hymenoptera: Scelionidae). *Heredity* **94**, 402–407.

Jiggins, F. M., Hurst, G. D. D., Schulenburg, J. H. G. V. D. and Majerus, M. E. N. (2001). Two male-killing *Wolbachia* strains coexist within a population of the butterfly *Acraea encedon*. *Heredity* **86**, 161–166.

Jois, S., Chan, Y. B., Fernandez, M. P. and Leung, A. K. W. (2018). Characterization of the sexually dimorphic fruitless neurons that regulate copulation duration. *Front. Physiol.* **9**, 780.

Jones, D. T., Taylor, W. R. and Thornton, J. M. (1992). The rapid generation of mutation data matrices from protein sequences. *Bioinformatics* **8**, 275–282.

Jungwirth, S., Ruther, J. and Pokorny, T. (2021). Similar is not the same – mate recognition in a parasitoid wasp. *Front. Ecol. Evol.* **9**, 174.

Jursnich, V. A. and Burtis, K. C. (1993). A positive role in differentiation for the male doublesex protein of *Drosophila*. *Dev. Biol.* **155**, 235–249.

K

Kageyama, D., Narita, S. and Watanabe, M. (2012). Insect sex determination manipulated by their endosymbionts: incidences, mechanisms and implications. *Insects* **3**, 161–199.

Kageyama, D., Ohno, M., Sasaki, T., Yoshido, A., Konagaya, T., Jouraku, A., Kuwazaki, S., Kanamori, H., Katayose, Y., Narita, S., et al. (2017). Feminizing *Wolbachia* endosymbiont disrupts maternal sex chromosome inheritance in a butterfly species. *Evol. Lett.* **1**, 232–244.

Kajtoch, Ł., Kolasa, M., Kubisz, D., Gutowski, J. M., Ścibior, R., Mazur, M. A. and Holecová, M. (2019). Using host species traits to understand the *Wolbachia* infection distribution across terrestrial beetles. *Sci. Rep.* **9**, 1–15.

Kampfraath, A. A., Klasson, L., Anvar, S. Y., Vossen, R. H. A. M., Roelofs, D., Kraaijeveld, K. and Ellers, J. (2019). Genome expansion of an obligate parthenogenesis-associated *Wolbachia* poses an exception to the symbiont reduction model. *BMC Genomics* **20**, 1–14.

Kato, Y., Kobayashi, K., Watanabe, H. and Iguchi, T. (2011). Environmental sex determination in the branchiopod crustacean *Daphnia magna*: deep conservation of a *Doublesex* gene in the sex-determining pathway. *PLoS Genet.* **7**, e1001345.

Kaur, R., Shropshire, J. D., Cross, K. L., Leigh, B., Mansueto, A. J., Stewart, V., Bordenstein, S. R. and Bordenstein, S. R. (2021). Living in the endosymbiotic world of *Wolbachia*: a centennial review. *Cell Host Microbe* **29**, 879–893.

Keisman, E. L. and Baker, B. S. (2001). The *Drosophila* sex determination hierarchy modulates *wingless* and *decapentaplegic* signaling to deploy dachshund sex-specifically in the genital imaginal disc. *Development* **128**, 1643–1656.

Keisman, E. L., Christiansen, A. E. and Baker, B. S. (2001). The sex determination gene *doublesex* regulates the A/P organizer to direct sex-specific patterns of growth in the *Drosophila* genital imaginai disc. *Dev. Cell* **1**, 215–225.

Kelley, R. L., Wang, J., Bell, L. and Kuroda, M. I. (1997). *Sex lethal* controls dosage compensation in *Drosophila* by a non-splicing mechanism. *Nature* **387**, 195–199.

Kerkis, J. (1931). The growth of the gonads in *Drosophila melanogaster*. *Genetics* **16**, 212–224.

Kijimoto, T., Moczek, A. P. and Andrews, J. (2012). Diversification of *doublesex* function underlies morph-, sex-, and species-specific development of beetle horns. *Proc. Natl. Acad. Sci.* **109**, 20526–20531.

Kimura, K. I., Ote, M., Tazawa, T. and Yamamoto, D. (2005). Fruitless specifies sexually dimorphic neural circuitry in the *Drosophila* brain. *Nature* **438**, 229–233.

Kimura, K., Hachiya, T., Koganezawa, M., Tazawa, T. and Yamamoto, D. (2008). Fruitless and Doublesex coordinate to generate male-specific neurons that can initiate courtship. *Neuron* **59**, 759–769.

Kiuchi, T., Koga, H., Kawamoto, M., Shoji, K., Sakai, H., Arai, Y., Ishihara, G., Kawaoka, S., Sugano, S., Shimada, T., et al. (2014). A single female-specific piRNA is the primary determiner of sex in the silkworm. *Nature* **509**, 633–636.

Klein, A., Schultner, E., Lowak, H., Schrader, L., Heinze, J., Holman, L. and Oettler, J. (2016). Evolution of social insect polyphenism facilitated by the sex differentiation cascade. *PLoS Genet.* **12**, e1005952.

Kopp, A. (2011). *Drosophila* sex combs as a model of evolutionary innovations. *Evol. Dev.* **13**, 504–522.

Kopp, A. (2012). *Dmrt* genes in the development and evolution of sexual dimorphism. *Trends Genet.* **28**, 175–184.

Kopp, A., Duncan, I. and Carroll, S. B. (2000). Genetic control and evolution of sexually dimorphic characters in *Drosophila*. *Nature* **408**, 553–559.

Korpelainen, H. (1990). Sex ratios and conditions required fro environmental sex determination in animals. *Biol. Rev.* **65**, 147–184.

Krafsur, A. M., Ghosh, A. and Brelsfoard, C. L. (2020). Phenotypic response of *Wolbachia pipientis* in a cell-free medium. *Microorganisms* **8**, 1060.

Kremer, N., Charif, D., Henri, H., Bataille, M., Prévost, G., Kraaijeveld, K. and Vavre, F. (2009). A new case of *Wolbachia* dependence in the genus Asobara: evidence for parthenogenesis induction in *Asobara japonica*. *Heredity* **103**, 248–256.

Krzywinska, E., Dennison, N. J., Lycett, G. J. and Krzywinski, J. (2016). A maleness gene in the malaria mosquito *Anopheles gambiae*. *Science* **353**, 67–69.

Krzywinska, E., Ferretti, L., Li, J., Li, J. C., Chen, C. H. and Krzywinski, J. (2021). *femaleless* controls sex determination and dosage compensation pathways in females of *Anopheles* mosquitoes. *Curr. Biol.* **31**, 1084–1091.

Kumar, S., Stecher, G. and Tamura, K. (2016). MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* **33**, 1870–1874.

Kunte, K., Zhang, W., Tenger-Trolander, A., Palmer, D. H., Martin, A., Reed, R. D., Mullen, S. P. and Kronforst, M. R. (2014). *Doublesex* is a mimicry supergene. *Nature* **507**, 229–232.

L

Lacaille, F., Hiroi, M., Twele, R., Inoshita, T., Umemoto, D., Manière, G., Marion-Poll, F., Ozaki, M., Francke, W., Cobb, M., et al. (2007). An inhibitory sex pheromone tastes bitter for *Drosophila* males. *PLoS One* **2**, e661.

Lagos, D., Koukidou, M., Savakis, C. and Komitopoulou, K. (2007). The *transformer* gene in *Bactrocera oleae*: The genetic switch that determines its sex fate. *Insect Mol. Biol.* **16**, 221–230.

Lassy, C. W. and Karr, T. L. (1996). Cytological analysis of fertilization and early embryonic development in incompatible crosses of *Drosophila simulans*. *Mech. Dev.* **57**, 47–58.

Lattorff, H. M. G., Moritz, R. F. A. and Fuchs, S. (2005). A single locus determines thelytokous parthenogenesis of laying honeybee workers (*Apis mellifera* capensis). *Heredity* **94**, 533–537.

Lau, T. F. (Stanley) and Meyer-Rochow, V. B. (2006). Sexual dimorphism in the compound eye of *Rhagophthalmus ohbai* (Coleoptera: Rhagophthalmidae): I. Morphology and ultrastructure. *J. Asia. Pac. Entomol.* **9**, 19–30.

Lau, T. F. S., Ohba, N., Arikawa, K. and Meyer-Rochow, V. B. (2007). Sexual dimorphism in the compound eye of *Rhagophthalmus ohbai* (Coleoptera: Rhagophthalmidae): II. Physiology and function of the eye of the male. *J. Asia. Pac. Entomol.* **10**, 27–31.

Le Page, D. P., Metcalf, J. A., Bordenstein, S. R., On, J., Perlmutter, J. I., Shropshire, J. D., Layton, E. M., Funkhouser-Jones, L. J., Beckmann, J. F. and Bordenstein, S. R. (2017). Prophage WO genes recapitulate and enhance *Wolbachia*-induced cytoplasmic incompatibility. *Nature* **543**, 243–247.

Leach, I. M., Pannebakker, B. A., Schneider, M. V., Driessen, G., van de Zande, L. and Beukeboom, L. W. (2009). Thelytoky in Hymenoptera with *Venturia canescens* and *Leptopilina clavipes* as case studies. In *Lost Sex*, pp. 347–375. Dordrecht: Springer Netherlands.

Lebreton, S., Borrero-Echeverry, F., Gonzalez, F., Solum, M., Wallin, E. A., Hedenström, E., Hansson, B. S., Gustavsson, A.-L., Bengtsson, M., Birgersson, G., et al. (2017). A *Drosophila* female pheromone elicits species-specific long-range attraction via an olfactory channel with dual specificity for sex and food. *BMC Biol.* **15**, 88.

Ledón-Rettig, C. C., Zattara, E. E. and Moczek, A. P. (2017). Asymmetric interactions between *doublesex* and tissue- and sex-specific target genes mediate sexual dimorphism in beetles. *Nat. Commun.* **8**, 14593.

Lee, J., Kiuchi, T., Kawamoto, M., Shimada, T. and Katsuma, S. (2015). Identification and functional analysis of a *Masculinizer* orthologue in *Trilocha varians* (Lepidoptera: Bombycidae). *Insect Mol. Biol.* **24**, 561–569.

Legner, E. F. (1985). Natural and induced sex ratio changes in populations of thelytokous *Muscidifurax uniraptor* (Hymenoptera: Pteromalidae). *Ann. Entomol. Soc. Am.* **78**, 398–402.

Legner, E. F. (1987). Transfer of thelytoky to arrhenotokous *Muscidifurax raptor* Girault and Sanders (Hymenoptera: Pteromalidae). *Can. Entomol.* **119**, 265–271.

Lenschow, M., Cordel, M., Pokorny, T., Mair, M. M., Hofferberth, J. and Ruther, J. (2018). The post-mating switch in the pheromone response of *Nasonia* females is mediated by dopamine and can be reversed by appetitive learning. *Front. Behav. Neurosci.* **12**, 14.

Leonard, J. E. and Boake, C. R. B. (2006). Site-dependent aggression and mating behaviour in three species of *Nasonia* (Hymenoptera: Pteromalidae). *Anim. Behav.* **71**, 641–647.

Leung, K., Zande, L. and Beukeboom, L. W. (2019). Life-history traits of the Whiting polyploid line of the parasitoid *Nasonia vitripennis*. *Entomol. Exp. Appl.* **167**, 655–669.

Li, M., Au, L. Y. C., Douglah, D., Chong, A., White, B. J., Ferree, P. M. and Akbari, O. S. (2017). Generation of heritable germline mutations in the jewel wasp *Nasonia vitripennis* using CRISPR/Cas9. *Sci. Rep.* **7**, 1–7.

Lin, Z., Kong, H., Nei, M. and Ma, H. (2006). Origins and evolution of the *recA/RAD51* gene family: Evidence for ancient gene duplication and endosymbiotic gene transfer. *Proc. Natl. Acad. Sci.* **103**, 10328–10333.

Lindsey, A. R. I. I., Werren, J. H., Richards, S. and Stouthamer, R. (2016). Comparative genomics of a parthenogenesis-inducing *Wolbachia* symbiont. *G3: Genes, Genomes, Genetics.* **6**, 2113–2123.

Lindsey, A. R. I., Rice, D. W., Bordenstein, S. R., Brooks, A. W., Bordenstein, S. R. and Newton, I. L. G. (2018). Evolutionary genetics of cytoplasmic incompatibility genes *cifA* and *cifB* in prophage WO of *Wolbachia*. *Genome Biol. Evol.* **10**, 434–451.

Liu, N.-Y., Wu, G.-X., Ze, S.-Z., Yang, B. and Zhu, J.-Y. (2017). Morphology and ultrastructure of the male reproductive system of the jewel wasp, *Nasonia vitripennis* (Walker) (Hymenoptera: Pteromalidae). *J. Asia. Pac. Entomol.* **20**, 577–582.

Lobbia, S., Niitsu, S. and Fujiwara, H. (2003). Female-specific wing degeneration caused by ecdysteroid in the Tussock Moth, *Orgyia recens*: Hormonal and developmental regulation of sexual dimorphism. *J. Insect Sci.* **3**, 11.

Loehlin, D. W. and Werren, J. H. (2012). Evolution of shape by multiple regulatory changes to a growth gene. *Science* **335**, 943–947.

Loehlin, D. W., Oliveira, D. C. S. G., Edwards, R., Giebel, J. D., Clark, M. E., Cattani, M. V., van de Zande, L., Verhulst, E. C., Beukeboom, L. W., Muñoz-Torres, M., et al. (2010a). Non-coding changes cause sex-specific wing size differences between closely related species of *Nasonia*. *PLoS Genet.* **6**, e1000821.

Loehlin, D. W., Enders, L. S. and Werren, J. H. (2010b). Evolution of sex-specific wing shape at the *widerwing* locus in four species of *Nasonia*. *Heredity* **104**, 260–269.

Luk, P. Van and Spiridonov, S. Ė. (1990). Experimental evidence of arrhenotoky in the nematode *Blatticola blattae* (Oxyurida: Thelastomatidae). *Helminthologia* **27**, 67–70.

Lynch, J. A. (2015). The expanding genetic toolbox of the wasp *Nasonia vitripennis* and its relatives. *Genetics* **199**, 897–904.

Lynch, J. A. and Desplan, C. (2006). A method for parental RNA interference in the wasp *Nasonia vitripennis*. *Nat. Protoc.* **1**, 486–494.

M

Ma, W.-J. and Schwander, T. (2017). Patterns and mechanisms in instances of endosymbiont-induced parthenogenesis. *J. Evol. Biol.* **30**, 868–888.

Ma, W.-J., Pannebakker, B. A., Beukeboom, L. W., Schwander, T. and van de Zande, L. (2014). Genetics of decayed sexual traits in a parasitoid wasp with endosymbiont-induced asexuality. *Heredity* **113**, 424–431.

Ma, W.-J., Pannebakker, B. A., van de Zande, L., Schwander, T., Wertheim, B. and Beukeboom, L. W. (2015). Diploid males support a two-step mechanism of endosymbiont-induced thelytoky in a parasitoid wasp. *BMC Evol. Biol.* **15**, 1–12.

Ma, W. J., Pannebakker, B. A., Li, X., Geuverink, E., Anvar, S. Y., Veltsos, P., Schwander, T., van de Zande, L. and Beukeboom, L. W. (2021). A single QTL with large effect is associated with female functional virginity in an asexual parasitoid wasp. *Mol. Ecol.* **30**, 1979–1992.

Mair, M. M. and Ruther, J. (2018). Territoriality and behavioural strategies at the natal host patch differ in two microsympatric *Nasonia species*. *Anim. Behav.* **143**, 113–129.

Mair, M. M. and Ruther, J. (2019). Chemical ecology of the parasitoid wasp genus *Nasonia* (Hymenoptera, Pteromalidae). *Front. Ecol. Evol.* **7**, 184.

Marcos, K. and E.F.Legner (1970). A biosystematic revision of the genus *Muscidifurax* (Hymenoptera: Pteromalidae) with descriptions of four new species. *Can. Entomol.* **102**, 1268–1299.

Martín, I., Ruiz, M. F. and Sánchez, L. (2011). The gene *transformer-2* of *Sciara* (Diptera, Nematocera) and its effect on *Drosophila* sexual development. *BMC Dev. Biol.* **11**, 1–13.

Massey, J. H. and Wittkopp, P. J. (2016). The genetic basis of pigmentation differences within and between *Drosophila species*. *Curr. Top. Dev. Biol.* **119**, 27–61.

Mcculloch, J. B. and Owen, J. P. (2012). Arrhenotoky and oedipal mating in the northern fowl mite (*Ornithonyssus sylviarum*) (Acari: Gamasida: Macronyssidae). *Parasit. Vectors* **5**, 1–5.

McCutcheon, J. P. and Moran, N. A. (2012). Extreme genome reduction in symbiotic bacteria. *Nat. Rev. Microbiol.* **10**, 13–26.

McMeniman, C. J., Lane, A. M., Fong, A. W. C., Voronin, D. A., Iturbe-Ormaetxe, I., Yamada, R., McGraw, E. A. and O'Neill, S. L. (2008). Host adaptation of a *Wolbachia* strain after long-term serial passage in mosquito cell lines. *Appl. Environ. Microbiol.* **74**, 6963–6969.

McMeniman, C. J., Lane, R. V., Cass, B. N., Fong, A. W. C., Sidhu, M., Wang, Y. F. and O'Neill, S. L. (2009). Stable introduction of a life-shortening *Wolbachia* infection into the mosquito *Aedes aegypti*. *Science* **323**, 141–144.

Meccariello, A., Salvemini, M., Primo, P., Hall, B., Koskinioti, P., Dalíková, M., Gravina, A., Gucciardino, M. A., Forlenza, F., Gregoriou, M. E., et al. (2019). *Maleness-on-the-Y* (*MoY*) orchestrates male sex determination in major agricultural fruit fly pests. *Science* **365**, 1457–1460.

Meise, M., Hilfiker-Kleiner, D., Dubendorfer, A., Brunner, C., Nothiger, R. and Bopp, D. (1998). *Sex-lethal*, the master sex-determining gene in *Drosophila*, is not sex-specifically regulated in *Musca domestica*. *Development* **125**, 1487–1494.

Menzel, J. G., Wunderer, H. and Stavenga, D. G. (1991). Functional morphology of the divided compound eye of the honeybee drone (*Apis mellifera*). *Tissue Cell* **23**, 525–535.

Mikó, I. and Deans, A. (2014). The mandibular gland in *Nasonia vitripennis* (Hymenoptera: Pteromalidae). *bioRxiv* 006569.

Miller, W. J., Ehrman, L. and Schneider, D. (2010). Infectious speciation revisited: impact of symbiont-depletion on female fitness and mating behavior of *Drosophila paulistorum*. *PLoS Pathog.* **6**, e1001214.

Mine, S., Sumitani, M., Aoki, F., Hatakeyama, M. and Suzuki, M. G. (2017). Identification and functional characterization of the sex-determining gene *doublesex* in the sawfly, *Athalia rosae* (Hymenoptera: Tenthredinidae). *Appl. Entomol. Zool.* **52**, 497–509.

Mita, K., Kasahara, M., Sasaki, S., Nagayasu, Y., Yamada, T., Hiroyuki Kanamori, N. N., Kitagawa, M., Yamashita, H., Yasukochi, Y., Kadono-Okuda, K., et al. (2004). The genome sequence of silkworm, *Bombyx mori*. *DNA Res.* **11**, 27–35.

Miyakawa, M. O., Tsuchida, K. and Miyakawa, H. (2018). The *doublesex* gene integrates multi-locus complementary sex determination signals in the Japanese ant, *Vollenhovia emeryi*. *Insect Biochem. Mol. Biol.* **94**, 42–49.

Morita, S., Ando, T., Maeno, A., Mizutani, T., Mase, M., Shigenobu, S. and Niimi, T. (2018). *doublesex* regulates sexually dimorphic beetle horn formation by integrating spatial and temporal developmental contexts in the Japanese rhinoceros beetle *Trypoxylus dichotomus*. *bioRxiv* https://doi.org/10.1101/328120.

Morrow, J. L., Riegler, M., Frommer, M. and Shearman, D. C. A. (2014). Expression patterns of sex-determination genes in single male and female embryos of two *Bactrocera* fruit fly species during early development. *Insect Mol. Biol.* **23**, 754–767.

Mulot, M., Boissinot, S., Monsion, B., Rastegar, M., Clavijo, G., Halter, D., Bochet, N., Erdinger, M. and Brault, V. (2016). Comparative analysis of RNAi-based methods to down-regulate expression of two genes expressed at different levels in *Myzus persicae*. *Viruses* **8**, 316.

Mysore, K., Sun, L., Tomchaney, M., Sullivan, G., Adams, H., Piscoya, A. S., Severson, D. W., Syed, Z. and Duman-Scheel, M. (2015). siRNA-mediated silencing of *doublesex* during female development of the dengue vector mosquito *Aedes aegypti*. *PLoS Negl. Trop. Dis.* **9**, e0004213.

N

Narita, S., Kageyama, D., Nomura, M. and Fukatsu, T. (2007). Unexpected mechanism of symbiont-induced reversal of insect sex: feminizing *Wolbachia* continuously acts on the butterfly *Eurema hecabe* during larval development. *Appl. Environ. Microbiol.* **73**, 4332–4341.

Nault, B. A., Shelton, A. M., Gangloff-kaufmann, J. L., Clark, M. E., Werren, J. L., Cabrera-la Rosa, J. C. and Kennedy, G. G. (2006). Reproductive modes in onion thrips (Thysanoptera: Thripidae) populations from New York onion fields. *Environ. Entomol.* **35**, 1264–1271.

Negri, I., Franchini, A., Gonella, E., Daffonchio, D., Mazzoglio, P. J., Mandrioli, M. and Alma, A. (2009). Unravelling the *Wolbachia* evolutionary role: the reprogramming of the host genomic imprinting. *Proc. R. Soc. B Biol. Sci.* **276**, 2485–2491.

Newton, I. L. G., Clark, M. E., Kent, B. N., Bordenstein, S. R., Qu, J., Richards, S., Kelkar, Y. D. and Werren, J. H. (2016). Comparative genomics of two closely related *Wolbachia* with different reproductive effects on hosts. *Genome Biol. Evol.* **8**, 1526–1542.

Nguantad, S., Chumnanpuen, P., Thancharoen, A., Vongsangnak, W. and Sriboonlert, A. (2020). Identification of potential candidate genes involved in the sex determination cascade in an aquatic firefly, *Sclerotia aquatilis* (Coleoptera, Lampyridae). *Genomics* **112**, 2590–2602.

Niehuis, O., Büllesbach, J., Judson, A. K., Schmitt, T. and Gadau, J. (2011). Genetics of cuticular hydrocarbon differences between males of the parasitoid wasps *Nasonia giraulti* and *Nasonia vitripennis*. *Heredity* **107**, 61–70.

Nipitwattanaphon, M., Wang, J., Ross, K. G., Riba-Grognuz, O., Wurm, Y., Khurewathanakul, C. and Keller, L. (2014). Effects of ploidy and sex-locus genotype on gene expression patterns in the fire ant *Solenopsis invicta*. *Proc. R. Soc. B Biol. Sci.* **281**, 20141776.

Nissen, I., Müller, M., Beye, M., Dauwalder, B. and Mattox, W. (2012). The *Am-tra2* gene is an essential regulator of female splice regulation at two levels of the sex determination hierarchy of the honeybee. *Genetics* **192**, 1015–1026.

Niu, B. L., Meng, Z. Q., Tao, Y. Z., Lu, S. L., Weng, H. B., He, L. H. and Shen, W. F. (2005). Cloning and alternative splicing analysis of *Bombyx mori transformer-2* gene using silkworm EST database. *Acta Biochim. Biophys. Sin. (Shanghai).* **37**, 728–736.

Normark, B. B., Jordal, B. H. and Farrell, B. D. (1999). Origin of a haplodiploid beetle lineage. *Proc. R. Soc. B Biol. Sci.* **266**, 2253–2259.

Nur, U., Werren, J. H., Eickbush, D. G., Burke, W. D. and Eickbush, T. H. (1988). A "selfish" B chromosome that enhances its transmission by eliminating the paternal genome. *Science* **240**, 512–514.

O

Ohbayashi, F., Suzuki, M. G., Mita, K., Okano, K. and Shimada, T. (2001). A homologue of the *Drosophila doublesex* gene is transcribed into sex-specific mRNA isoforms in the silkworm, *Bombyx mori*. *Comp. Biochem. Physiol. - B Biochem. Mol. Biol.* **128**, 145–158.

Oliveira, D. C. S. G., Werren, J. H., Verhulst, E. C., Giebel, J. D., Kamping, A., Beukeboom, L. W. and van de Zande, L. (2009). Identification and characterization of the *doublesex* gene of *Nasonia*. *Insect Mol. Biol.* **18**, 315–324.

Otto, S. P. (2009). The evolutionary enigma of sex. *Am. Nat.* **174**, S1–S14.

P

Page, M., Nelson, L. J., Haverty, M. I. and Blomquist, G. J. (1990). Cuticular hydrocarbons of eight species of north american cone beetles, *Conophthorus hopkins*. *J. Chem. Ecol.* **16**, 1173–1198.

Pan, Y., Robinett, C. C. and Baker, B. S. (2011). Turning males on: activation of male courtship behavior in *Drosophila melanogaster*. *PLoS One* **6**, e21144.

Pane, A., Salvemini, M., Bovi, P. D., Polito, C. and Saccone, G. (2002). The *transformer* gene in *Ceratitis capitata* provides a genetic basis for selecting and remembering the sexual fate. *Development* **129**, 3715–3725.

Pannebakker, B. A., Pijnacker, L. P., Zwaan, B. J. and Beukeboom, L. W. (2004). Cytology of *Wolbachia*-induced parthenogenesis in *Leptopilina clavipes* (Hymenoptera: Figitidae). *Genome* **47**, 299–303.

Pannebakker, B. A., Schidlo, N. S., Boskamp, G. J. F., Dekker, L., van Dooren, T. J. M., Beukeboom, L. W., Zwaan, B. J., Brakefield, P. M. and van Alphen, J. J. M. (2005). Sexual functionality of *Leptopilina clavipes* (Hymenoptera: Figitidae) after reversing *Wolbachia*-induced parthenogenesis. *J. Evol. Biol.* **18**, 1019–1028.

Peng, Y. and Wang, Y. (2009). Infection of *Wolbachia* may improve the olfactory response of *Drosophila*. *Chinese Sci. Bull.* **54**, 1369–1375.

Perlmutter, J. I., Bordenstein, S. R., Unckless, R. L., LePage, D. P., Metcalf, J. A., Hill, T., Martinez, J., Jiggins, F. M. and Bordenstein, S. R. (2019). The phage gene *wmk* is a candidate for male killing by a bacterial endosymbiont. *PLoS Pathog.* **15**, e1007936.

Permpoon, R., Aketarawong, N. and Thanaphum, S. (2011). Isolation and characterization of *Doublesex* homologues in the *Bactrocera* species: *B. dorsalis* (Hendel) and *B. correcta* (Bezzi) and their putative promoter regulatory regions. *Genetica* **139**, 113–127.

Petersen, J. J. and Cawthra, J. K. (1995). Release of a gregarious *Muscidifurax* species (Hymenoptera: Pteromalidae) for the control of filth flies associated with confined beef cattle. *Biol. Control* **5**, 279–284.

Picard, M. A.-L., Cosseau, C., Mouahid, G., Duval, D., Grunau, C., Toulza, È., Allienne, J.-F. and Boissier, J. (2015). The roles of *Dmrt* (Double sex/Male-abnormal-3 Related Transcription factor) genes in sex determination and differentiation mechanisms: Ubiquity and diversity across the animal kingdom. *C. R. Biol.* **338**, 451–462.

Pietri, J. E., DeBruhl, H. and Sullivan, W. (2016). The rich somatic life of *Wolbachia*. *Microbiologyopen* **5**, 923–936.

Pijls, J. W. A. M., van Steenbergen, H. J. and van Alphen, J. J. M. (1996). Asexuality cured: the relations and differences between sexual and asexual *Apoanagyrus diversicornis*. *Heredity* **76**, 506–513.

Praagh, J. P. van, Ribi, W., Wehrhahn, C. and Wittmann, D. (1980). Drone bees fixate the queen with the dorsal frontal part of their compound eyes. *J. Comp. Physiol. A* **136**, 263–266.

Prakash, A. and Monteiro, A. (2016). Molecular mechanisms of secondary sexual trait development in insects. *Curr. Opin. Insect Sci.* **17**, 40–48.

Prakash, A. and Monteiro, A. (2020). *Doublesex* mediates the development of sex-specific pheromone organs in *Bicyclus* butterflies via multiple mechanisms. *Mol. Biol. Evol.* **37**, 1694–1707.

Price, D. C., Egizi, A. and Fonseca, D. M. (2015). The ubiquity and ancestry of insect *doublesex*. *Sci. Rep.* **5**, 1–9.

Pultz, M. A. and Leaf, D. S. (2003). The jewel wasp *Nasonia*: querying the genome with haplo-diploid genetics. *Genesis* **35**, 185–191.

R

R core Team (2018). R: A Language and Environment for Statistical Computing. *R Foundation for Statistical Computing, Vienna, Austria*.

Radder, R. S., Quinn, A. E., Georges, A., Sarre, S. D. and Shine, R. (2008). Genetic evidence for co-occurrence of chromosomal and thermal sex-determining systems in a lizard. *Biol. Lett.* **4**, 176–178.

Ramakers, C., Ruijter, J. M., Deprez, R. H. L. and Moorman, A. F. . (2003). Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neurosci. Lett.* **339**, 62–66.

Rasch, E. M., Cassidy, J. D. and King, R. C. (1977). Evidence for dosage compensation in parthenogenetic Hymenoptera. *Chromosoma* **59**, 323–340.

Rasgon, J. L., Gamston, C. E. and Ren, X. (2006). Survival of *Wolbachia pipientis* in cell-free medium. *Appl. Environ. Microbiol.* **72**, 6934–6937.

Raychoudhury, R., Desjardins, C. A., Buellesbach, J., Loehlin, D. W., Grillenberger, B. K., Beukeboom, L., Schmitt, T. and Werren, J. H. (2010). Behavioral and genetic characteristics of a new species of *Nasonia*. *Heredity* **104**, 278–288.

Reveillaud, J., Bordenstein, S. R., Cruaud, C., Shaiber, A., Esen, Ö. C., Weill, M., Makoundou, P., Lolans, K., Watson, A. R., Rakotoarivony, I., et al. (2019). The *Wolbachia* mobilome in Culex pipiens includes a putative plasmid. *Nat. Commun.* **10**, 1–11.

Rice, G., Barmina, O., Hu, K. and Kopp, A. (2018). Evolving *doublesex* expression correlates with the origin and diversification of male sexual ornaments in the *Drosophila immigrans* species group. *Evol. Dev.* **20**, 78–88.

Rideout, E. J., Billeter, J.-C. and Goodwin, S. F. (2007). The sex-determination genes *fruitless* and *doublesex* specify a neural substrate required for courtship song. *Curr. Biol.* **17**, 1473–1478.

Rideout, E. J., Dornan, A. J., Neville, M. C., Eadie, S. and Goodwin, S. F. (2010). Control of sexual differentiation and behavior by the *doublesex* gene in *Drosophila melanogaster*. *Nat. Neurosci.* **13**, 458–466.

Robinett, C. C., Vaughan, A. G., Knapp, J.-M. and Baker, B. S. (2010). Sex and the single cell. II. There is a time and place for sex. *PLoS Biol.* **8**, e1000365.

Roelofs, W. L. and Rooney, A. P. (2003). Molecular genetics and evolution of pheromone biosynthesis in Lepidoptera. *Proc. Natl. Acad. Sci.* **100**, 9179–84.

Ross, L., Pen, I. and Shuker, D. M. (2010). Genomic conflict in scale insects: the causes and consequences of bizarre genetic systems. *Biol. Rev.* **85**, 807–828.

Rössler, Y. and DeBach, P. (1973). Genetic variability in a thelytokous form of *Aphytis mytilaspidis* (Le Baron) (Hymenoptera: Aphelinidae). *Hilgardia* **42**, 149–175.

Roth, A., Vleurinck, C., Netschitailo, O., Bauer, V., Otte, M., Kaftanoglu, O., Page, R. E. and Beye, M. (2019). A genetic switch for worker nutrition-mediated traits in honeybees. *PLoS Biol.* **17**, e3000171.

Rougeot, J., Wang, Y. and Verhulst, E. C. (2021). Effect of using green fluorescent protein double-stranded RNA as non-target negative control in *Nasonia vitripennis* RNA interference assays. *Exp. Results* **2**, e11.

Ruiz, M. F., Alvarez, M., Eirín-López, J. M., Sarno, F., Kremer, L., Barbero, J. L. and Sánchez, L. (2015). An unusual role for *doublesex* in sex determination in the Dipteran *Sciara*. *Genetics* **200**, 1181–1199.

Ruther, J. (2013). Novel insights into pheromone-mediated communication in parasitic hymenopterans. In *Chemical Ecology of Insect Parasitoids* (ed. Wajnberg, E. and Colazza, S., pp. 112–144. Chichester, UK: John Wiley & Sons, Ltd.

Ruther, J. and Hammerl, T. (2014). An oral male courtship pheromone terminates the response of *Nasonia vitripennis* females to the male-produced sex attractant. *J. Chem. Ecol.* **40**, 56–62.

Ruther, J., Homann, M. and Steidle, J. L. M. (2000). Female-derived sex pheromone mediates courtship behaviour in the parasitoid *Lariophagus distinguendus*. *Entomol. Exp. Appl.* **96**, 265–274.

Ruther, J., Stahl, L. M., Steiner, S., Garbe, L. A. and Tolasch, T. (2007). A male sex pheromone in a parasitic wasp and control of the behavioral response by the female's mating status. *J. Exp. Biol.* **210**, 2163–2169.

Ruther, J., Steiner, S. and Garbe, L.-A. (2008). 4-methylquinazoline is a minor component of the male sex pheromone in *Nasonia vitripennis*. *J. Chem. Ecol.* **34**, 99–102.

Ruther, J., Matschke, M., Garbe, L.-A. and Steiner, S. (2009). Quantity matters: male sex pheromone signals mate quality in the parasitic wasp *Nasonia vitripennis*. *Proceedings. Biol. Sci.* **276**, 3303–3310.

Ruther, J., Thal, K., Blaul, B. and Steiner, S. (2010). Behavioural switch in the sex pheromone response of *Nasonia vitripennis* females is linked to receptivity signalling. *Anim. Behav.* **80**, 1035–1040.

Ruther, J., McCaw, J., Böcher, L., Pothmann, D. and Putz, I. (2014). Pheromone diversification and age-dependent behavioural plasticity decrease interspecific mating costs in *Nasonia*. *PLoS One* **9**, e89214.

Ruther, J., Bruckmann, A. and Hofferberth, J. (2021). Pheromone biosynthesis in *Nasonia*. In *Insect Pheromone Biochemistry and Molecular Biology*, pp. 237–267. Elsevier Scientific.

Ryner, L. C., Goodwin, S. F., Castrillon, D. H., Anand, A., Villella, A., Baker, B. S., Hall, J. C., Taylor, B. J. and Wasserman, S. A. (1996). Control of male sexual behavior and sexual orientation in *Drosophila* by the *fruitless* gene. *Cell* **87**, 1079–1089.

S

Saccone, G., Peluso, I., Testa, G., Di Paola, F., Pane, A. and Polito, L. (1996). Drosophila *sex-lethal* and *doublesex* homologous genes in *Ceratitis capitata*: searching for sex-specific genes to develop a medfly transgenic sexing strain. *Vienna: IAEA/FAO*.

Saccone, G., Peluso, I., Artiaco, D., Giordano, E., Bopp, D. and Polito, L. C. (1998). The *Ceratitis capitata* homologue of the *Drosophila* sex-determining gene *sex-lethal* is structurally conserved, but not sex-specifically regulated. *Development* **125**, 1495–1500.

Sakai, H., Sakaguchi, H., Aoki, F. and Suzuki, M. G. (2015). Functional analysis of sex-determination genes by gene silencing with LNA-DNA gapmers in the silkworm, *Bombyx mori*. *Mech. Dev.* **137**, 45–52.

Salvemini, M., Robertson, M., Aronson, B., Atkinson, P., Polito, L. C. and Saccone, G. (2009). *Ceratitis capitata transformer-2* gene is required to establish and maintain the autoregulation of *Cctra*, the master gene for female sex determination. *Int. J. Dev. Biol.* **53**, 109–120.

Salvemini, M., Mauro, U., Lombardo, F., Milano, A., Zazzaro, V., Arcà, B., Polito, L. C. and Saccone, G. (2011). Genomic organization and splicing evolution of the *doublesex* gene, a *Drosophila* regulator of sexual differentiation, in the dengue and yellow fever mosquito *Aedes aegypti*. *BMC Evol. Biol.* **11**, 1–19.

Sánchez, L., Gorfinkiel, N. and Guerrero, I. (2001). Sex determination genes control the development of the *Drosophila* genital disc, modulating the response to Hedgehog, Wingles and decapentaplegic signals. *Development* **128**, 1033–1043.

Sanders, L. E. and Arbeitman, M. N. (2008). Doublesex establishes sexual dimorphism in the *Drosophila* central nervous system in an isoform-dependent manner by directing cell number. *Dev. Biol.* **320**, 378–390.

Saul-Gershenz, L. S. and Millar, J. G. (2006). Phoretic nest parasites use sexual deception to obtain transport to their host's nest. *Proc. Natl. Acad. Sci.* **103**, 14039–14044.

Scali, C., Catteruccia, F., Li, Q. and Crisanti, A. (2005). Identification of sex-specific transcripts of the *Anopheles gambiae doublesex* gene. *J. Exp. Biol.* **208**, 3701–3709.

Schilder, K., Heinze, J., Gross, R. and Hölldobler, B. (1999). Microsatellites reveal clonal structure of populations of the thelytokous ant *Platythyrea punctata* (F. Smith) (Hymenoptera; Formicidae). *Mol. Ecol.* **8**, 1497–1507.

Schneider, D. I., Ehrman, L., Engl, T., Kaltenpoth, M., Hua-Van, A., Le Rouzic, A. and Miller, W. J. (2019). Symbiont-driven male mating success in the neotropical *Drosophila paulistorum* superspecies. *Behav. Genet.* **49**, 83–98.

Schwander, T. and Crespi, B. J. (2009). Multiple direct transitions from sexual reproduction to apomictic parthenogenesis in *Timema* stick insects. *Evolution* **63**, 84–103.

Sharma, A., Heinze, S. D., Wu, Y., Kohlbrenner, T., Morilla, I., Brunner, C., Wimmer, E. A., Van De Zande, L., Robinson, M. D., Beukeboom, L. W., et al. (2017). Male sex in houseflies is determined by *Mdmd*, a paralog of the generic splice factor gene *CWC22*. *Science* **356**, 642–645.

Shirangi, T. R., Dufour, H. D., Williams, T. M. and Carroll, S. B. (2009). Rapid evolution of sex pheromone-producing enzyme expression in *Drosophila*. *PLoS Biol.* **7**, e1000168.

Shirangi, T. R. R., Wong, A. M. M., Truman, J. W. W. and Stern, D. L. L. (2016). Doublesex regulates the connectivity of a neural circuit controlling *Drosophila* male courtship song. *Dev. Cell* **37**, 533–544.

Shukla, J. N. and Nagaraju, J. (2010a). Two female-specific DSX proteins are encoded by the sex-specific transcripts of *dsx*, and are required for female sexual differentiation in two wild silkmoth species, *Antheraea assama* and *Antheraea mylitta* (Lepidoptera, Saturniidae). *Insect Biochem. Mol. Biol.* **40**, 672–682.

Shukla, J. N. and Nagaraju, J. (2010b). *Doublesex*: a conserved downstream gene controlled by diverse upstream regulators. *J. Genet.* **89**, 341–356.

Shukla, J. N. and Palli, S. R. (2012a). Sex determination in beetles: production of all male progeny by parental RNAi knockdown of *transformer*. *Sci. Rep.* **2**, 602.

Shukla, J. N. and Palli, S. R. (2012b). Doublesex target genes in the red flour beetle, *Tribolium castaneum*. *Sci. Rep.* **2**, 948.

Shukla, J. N., Jadhav, S. and Nagaraju, J. (2011). Novel female-specific splice form of *dsx* in the silkworm, *Bombyx mori*. *Genetica* **139**, 23–31.

Siegenthaler, C., Maroy, P., Hediger, M., Dübendorfer, A. and Bopp, D. (2009). Hormones and sex-specific transcription factors jointly control yolk protein synthesis in *Musca domestica*. *Int. J. Evol. Biol.* **2009**, 1–9.

Sievers, F., Wilm, A., Dineen, D., Gibson, T. J., Karplus, K., Li, W., Lopez, R., McWilliam, H., Remmert, M., Söding, J., et al. (2011). Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol. Syst. Biol.* **7**, 539.

Sijia, B., Jiale, L., Juan, X., Dianyi, S., Endong, W., Guiting, L. and Xuenong, X. (2019). RNAi mediated knockdown of *RpL11*, *RpS2*, and *tra-2* led to reduced reproduction of *Phytoseiulus persimilis*. *Exp. Appl. Acarol.* **78**, 505–520.

Silk, P. J., Ryall, K., Barry Lyons, D., Sweeney, J. and Wu, J. (2009). A contact sex pheromone component of the emerald ash borer *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae). *Naturwissenschaften* **96**, 601–608.

Slifer, E. H. (1969). Sense organs on the antenna of a parasitic wasp, *Nasonia vitripennis* (Hymenoptera, pteromalidae). *Biol. Bull.* **136**, 253–263.

Snodgrass, R. E. (1957). A revised interpretation of the external reproductive organs of male insects. *Smithson. Misc. Collect.* **135**, 1–60.

Steiner, S. and Ruther, J. (2009). Mechanism and behavioral context of male sex pheromone release in *Nasonia vitripennis*. *J. Chem. Ecol.* **35**, 416–421.

Steiner, S., Hermann, N. and Ruther, J. (2006). Characterization of a female-produced courtship pheromone in the parasitoid *Nasonia vitripennis*. *J. Chem. Ecol.* **32**, 1687–1702.

Stouthamer, R. and Kazmer, D. J. (1994). Cytogenetics of microbe-associated parthenogenesis and its consequences for gene flow in *Trichogramma* wasps. *Heredity* **73**, 317–327.

Stouthamer, R., Luck, R. F. and Hamilton, W. D. (1990). Antibiotics cause parthenogenetic *Trichogramma* (Hymenoptera/Trichogrammatidae) to revert to sex. *Proc. Natl. Acad. Sci.* **87**, 2424–2427.

Stouthamer, R., Breeuwer, J. A. J., Luck, R. F. and Werren, J. H. (1993). Molecular identification of microorganisms associated with parthenogenesis. *Nature* **361**, 66–68.

Stouthamer, R., Russell, J. E., Vavre, F. and Nunney, L. (2010). Intragenomic conflict in populations infected by parthenogenesis inducing *Wolbachia* ends with irreversible loss of sexual reproduction. *BMC Evol. Biol.* **10**, 1–12.

Sugimoto, T. N., Fujii, T., Kayukawa, T., Sakamoto, H. and Ishikawa, Y. (2010). Expression of a *doublesex* homologue is altered in sexual mosaics of *Ostrinia scapulalis* moths infected with *Wolbachia*. *Insect Biochem. Mol. Biol.* **40**, 847–854.

Suomalainen, E., Saura, A. and Lokki, J. (1987). *Cytology and evolution in parthenogenesis*. CRC Press.

Suzuki, M. G., Funaguma, S., Kanda, T., Tamura, T. and Shimada, T. (2005). Role of the male BmDSX protein in the sexual differentiation of *Bombyx mori*. *Evol. Dev.* **7**, 58–68.

Suzuki, M. G., Kobayashi, S. and Aoki, F. (2014). Male-specific splicing of the silkworm *Imp* gene is maintained by an autoregulatory mechanism. *Mech. Dev.* **131**, 47–56.

T

Tamura, K., Dudley, J., Nei, M. and Kumar, S. (2007). MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* **24**, 1596–1599.

Tanaka, K., Barmina, O., Sanders, L. E., Arbeitman, M. N. and Kopp, A. (2011). Evolution of sex-specific traits through changes in HOX-dependent *doublesex* expression. *PLoS Biol.* **9**, e1001131.

Tanaka, A., Aoki, F. and Suzuki, M. G. (2018). Conserved domains in the Transformer protein act complementary to regulate sex-specific splicing of its own pre-mRNA. *Sex. Dev.* **12**, 180–190.

The Tree of Sex Consortium (2014). Tree of sex: a database of sexual systems. *Sci. Data* **1: 140015**.

Tomoyasu, Y., Miller, S. C., Tomita, S., Schoppmeier, M., Grossmann, D. and Bucher, G. (2008). Exploring systemic RNA interference in insects: A genome-wide survey for RNAi genes in *Tribolium*. *Genome Biol.* **9**, 10.

Tram, U. and Sullivan, W. (2002). Role of delayed nuclear envelope breakdown and mitosis in *Wolbachia*-induced cytoplasmic incompatibility. *Science* **296**, 1124–1126.

Tram, U., Fredrick, K., Werren, J. H. and Sullivan, W. (2006). Paternal chromosome segregation during the first mitotic division determines *Wolbachia*-induced cytoplasmic incompatibility phenotype. *J. Cell Sci.* **119**, 3655–3663.

True, J. R. (2003). Insect melanism: the molecules matter. *Trends Ecol. Evol.* **18**, 640–647.

Tsukamoto, H., Takizawa, T., Takamori, K., Ogawa, H. and Araki, Y. (2007). Genomic organization and structure of the 5′-flanking region of the *TEX101* gene: alternative promoter usage and splicing generate transcript variants with distinct 5′-untranslated region. *Mol. Reprod. Dev.* **74**, 154–162.

Tulgetske, G. M. (2010). Investigations into the mechanisms of Wolbachia-induced parthenogenesis and sex determination in the parasitoid wasp, *Trichogramma*. University of california riverside.

U

Uebel, E. G., Sonnet, P. E., Miller, R. W. and Beroza, M. (1975). Sex pheromone of the face fly, *Musca autumnalis* De Geer (Diptera: Muscidae). *J. Chem. Ecol.* **1**, 195–202.

Ugajin, A., Matsuo, K., Kubo, R., Sasaki, T. and Ono, M. (2016). Expression profile of the sex determination gene *doublesex* in a gynandromorph of bumblebee, *Bombus ignitus*. *Sci. Nat.* **103**, 17.

V

van de Zande, L. and Verhulst, E. C. (2014). Genomic imprinting and maternal effect genes in haplodiploid sex determination. *Sex. Dev.* **8**, 74–82.

van den Assem, J. and Werren, J. H. (1994). A comparison of the courtship and mating behavior of three species of *Nasonia* (Hymenoptera: Pteromalidae). *J. Insect Behav.* **7**, 53–66.

van den Assem, J., Simbolotti, P. and Jachmann, F. (1980). Courtship behaviour of *Nasonia vitripennis* (Hymenoptera, Pteromalidae): Some qualitative, experimental evidence for the role of pheromones. *Behaviour* **75**, 301–307.

van Meer, M. M. M. and Stouthamer, R. (1999). Cross-order transfer of *Wolbachia* from *Muscidifurax uniraptor* (Hymenoptera: Pteromalidae) to *Drosophila simulans* (Diptera: Drosophilidae). *Heredity* **82**, 163–169.

van Wilgenburg, E., Driessen, G. and Beukeboom, L. W. (2006). Single locus complementary sex determination in Hymenoptera: an “unintelligent” design? *Front. Zool.* **3**, 1.

Vavre, F., De Jong, J. H. and Stouthamer, R. (2004). Cytogenetic mechanism and genetic consequences of thelytoky in the wasp *Trichogramma cacoeciae*. *Heredity* **93**, 592–596.

Verhulst, E. C. and van de Zande, L. (2015). Double nexus-*Doublesex* is the connecting element in sex determination. *Brief. Funct. Genomics* **14**, 396–406.

Verhulst, E. C., van de Zande, L. and Beukeboom, L. W. (2010a). Insect sex determination: it all evolves around *transformer*. *Curr. Opin. Genet. Dev.* **20**, 376–383.

Verhulst, E. C., Beukeboom, L. W. and van de Zande, L. (2010b). Maternal control of haplodiploid sex determination in the wasp *Nasonia*. *Science* **328**, 620–623.

Verhulst, E. C., Lynch, J. A., Bopp, D., Beukeboom, L. W., van de Zande, L., Sánchez, L., Sinclair, A., Berta, P., Palmer, M., Hawkins, J., et al. (2013). A new component of the *Nasonia* sex determining cascade is maternally silenced and regulates *transformer* expression. *PLoS One* **8**, e63618.

Verma, S. and Ruttner, F. (1983). Cytological analysis of the thelytokous parthenogenesis in the cape honeybee (*Apis mellifera capensis* Escholtz). *Apidologie* **14**, 41–57.

Visser, S., Voleníková, A., Nguyen, P., Verhulst, E. C. and Marec, F. (2021). A conserved role of the duplicated *Masculinizer* gene in sex determination of the Mediterranean flour moth, *Ephestia kuehniella*. *bioRxiv* 2021.02.15.431251.

W

Wang, X., Wheeler, D., Avery, A., Rago, A., Choi, J. H., Colbourne, J. K., Clark, A. G. and Werren, J. H. (2013). Function and evolution of DNA methylation in *Nasonia vitripennis*. *PLoS Genet.* **9**, 1003872.

Wang, X.-Y., Zheng, Z.-Z., Song, H.-S. and Xu, Y.-Z. (2014). Conserved RNA cis-elements regulate alternative splicing of Lepidopteran *doublesex*. *Insect Biochem. Mol. Biol.* **44**, 1–11.

Wang, Y., Rensink, A., Fricke, U., Riddle, M. C., Trent, C., van de Zande, L. and Verhulst, E. C. (2020). Sexually dimorphic traits and male-specific differentiation are actively regulated by *Doublesex* during specific developmental windows in *Nasonia vitripennis*. *bioRxiv* https://doi.org/10.1101/2020.04.19.048553.

Watanabe, M., Kageyama, D. and Miura, K. (2013). Transfer of a parthenogenesis-inducing *Wolbachia* endosymbiont derived from *Trichogramma dendrolimi* into *Trichogramma evanescens*. *J. Invertebr. Pathol.* **112**, 83–87.

Waterbury, J. A., Jackson, L. L. and Schedl, P. (1999). Analysis of the Doublesex female protein in *Drosophila melanogaster*: role in sexual differentiation and behavior and dependence on intersex. *Genetics* **152**, 1653–1667.

Weinert, L. A., Araujo-Jnr, E. V., Ahmed, M. Z. and Welch, J. J. (2015). The incidence of bacterial endosymbionts in terrestrial arthropods. *Proc. R. Soc. B Biol. Sci.* **282**, 20150249.

Weiss, I., Hofferberth, J., Ruther, J. and Stökl, J. (2015). Varying importance of cuticular hydrocarbons and iridoids in the species-specific mate recognition pheromones of three closely related *Leptopilina* species. *Front. Ecol. Evol.* **3**, 19.

Werren, J. H. (1997). Biology of *Wolbachia*. *Annu. Rev. Entomol.* **42**, 587–609.

Werren, J. H. and Loehlin, D. W. (2009). The parasitoid wasp *Nasonia*: an emerging model system with haploid male genetics. *Cold Spring Harb. Protoc.* pdb.emo134.

Werren, J. H. and Windsor, D. M. (2000). *Wolbachia* infection frequencies in insects: Evidence of a global equilibrium? *Proc. R. Soc. B Biol. Sci.* **267**, 1277–1285.

Werren, J. H., Baldo, L. and Clark, M. E. (2008). *Wolbachia*: master manipulators of invertebrate biology. *Nat. Rev Microbiol* **6**, 741–751.

Werren, J. H., Loehlin, D. W. and Giebel, J. D. (2009). Larval RNAi in *Nasonia* (parasitoid wasp). *Cold Spring Harb. Protoc.* **2009**, pdb.prot5311.

Werren, J. H., Richards, S., Desjardins, C. A., Niehuis, O., Gadau, J., Colbourne, J. K. and Nasonia Genome Working Group, A. (2010). Functional and evolutionary insights from the genomes of three parastoid *Nasonia* species. *Science* **327**, 343–348.

Werren, J. H., Cohen, L. B., Gadau, J., Ponce, R., Baudry, E. and Lynch, J. A. (2016). Dissection of the complex genetic basis of craniofacial anomalies using haploid genetics and interspecies hybrids in *Nasonia* wasps. *Dev. Biol.* **415**, 391–405.

Wexler, J. R., Plachetzki, D. C. and Kopp, A. (2014). Pan-metazoan phylogeny of the DMRT gene family: A framework for functional studies. *Dev. Genes Evol.* **224**, 175–181.

Wexler, J., Delaney, E. K., Belles, X., Schal, C., Wada-Katsumata, A., Amicucci, M. J. and Kopp, A. (2019). Hemimetabolous insects elucidate the origin of sexual development via alternative splicing. *Elife* **8**, e47490.

Whelan, S. and Goldman, N. (2001). A general empirical model of protein evolution derived from multiple protein families using a maximum-likelihood approach. *Mol. Biol. Evol.* **18**, 691–699.

Whiting, P. W. (1939). Sex determination and reproductive economy in *Habrobracon*. *Genetics* **24**, 110–111.

Whiting, P. W. (1943). Multiple alleles in complementary sex determination of *Habrobracon*. *Genetics* **28**, 365–382.

Whiting, A. R. (1967). The biology of the parasitic wasp *Mormoniella vitripennis* = *Nasonia brevicornis* (Walker). *Q. Rev. Biol.* **42**, 333–406.

Whitworth, C., Jimenez, E. and van Doren, M. (2012). Development of sexual dimorphism in the *Drosophila* testis. *Spermatogenesis* **2**, 129–136.

Wilkins, A. S. (1995). Moving up the hierarchy: a hypothesis on the evolution of a genetic sex determination pathway. *Bioessays* **17**, 71–77.

Willhoeft, U. and Franz, G. (1996). Identification of the sex-determining region of the *Ceratitis capitata* Y chromosome by deletion mapping. *Genetics* **144**, 737–745.

Williams, T. M., Selegue, J. E., Werner, T., Gompel, N., Kopp, A. and Carroll, S. B. (2008). The regulation and evolution of a genetic switch controlling sexually dimorphic traits in *Drosophila*. *Cell* **134**, 610–623.

Winston, M. L. (1987). *The biology of the honey bee*. Harvard University Press.

Wu, Z., Hopper, K. R., Ode, P. J., Fuester, R. W., Chen, J. and Heimpel, G. E. (2003). Complementary sex determination in Hymenopteran parasitoids and its implications for biological control. *Insect Sci.* **10**, 81–93.

Wu, X., Lindsey, A. R. I., Chatterjee, P., Werren, J. H., Stouthamer, R. and Yi, S. V. (2020). Distinct epigenomic and transcriptomic modifications associated with *Wolbachia*-mediated asexuality. *PLoS Pathog.* **16**, e1008397.

X

Xi, Z., Dean, J. L., Khoo, C. and Dobson, S. L. (2005). Generation of a novel *Wolbachia* infection in *Aedes albopictus* (Asian tiger mosquito) via embryonic microinjection. *Insect Biochem. Mol. Biol.* **35**, 903–910.

Xu, J., Zhan, S., Chen, S., Zeng, B., Li, Z., James, A. A., Tan, A. and Huang, Y. (2017). Sexually dimorphic traits in the silkworm, *Bombyx mori*, are regulated by *doublesex*. *Insect Biochem. Mol. Biol.* **80**, 42–51.

Y

Yamamoto, Y., Zhang, Y., Sarida, M., Hattori, R. S. and Strüssmann, C. A. (2014). Coexistence of genotypic and temperature-dependent sex determination in pejerrey *Odontesthes bonariensis*. *PLoS One* **9**, e102574.

Yamashita, S. and Takahashi, K. H. (2018). Artificial transfer of a thelytoky-inducing *Wolbachia* endosymbiont between strains of the endoparasitoid wasp *Asobara japonica* (Hymenoptera: Braconidae). *Appl. Entomol. Zool.* **53**, 403–409.

Yoshiyama, N., Tojo, K. and Hatakeyama, M. (2013). A survey of the effectiveness of non-cell autonomous RNAi throughout development in the sawfly, *Athalia rosae* (Hymenoptera). *J. Insect Physiol.* **59**, 400–407.

Z

Zchori-Fein, E., Faktor, O., Zeidan, M., Gottlieb, Y., Czosnek, H. and Rosen, D. (1995). Parthenogenesis-inducing microorganisms in *Aphytis* (Hymenoptera: Aphelinidae). *Insect Mol. Biol.* **4**, 173–178.

Zchori-Fein, E., Gottlieb, Y. and Coll, M. (2000). *Wolbachia* density and host fitness components in *Muscidifurax uniraptor* (Hymenoptera: Pteromalidae). *J. Invertebr. Pathol.* **75**, 267–272.

Zchori-Fein, E., Perlman, S. J., Kelly, S. E., Katzir, N. and Hunter, M. S. (2004). Characterization of a "*Bacteroidetes*" symbiont in *Encarsia* wasps (Hymenoptera: Aphelinidae): Proposal of "*Candidatus* Cardinium hertigii." *Int. J. Syst. Evol. Microbiol.* **54**, 961–968.

Zhang, T., Haws, P. and Wu, Q. (2004). Multiple variable first exons: a mechanism for cell- and tissue-specific gene regulation. *Genome Res.* **14**, 79–89.

Zhou, C., Pan, Y., Robinett, C. C., Meissner, G. W. and Baker, B. S. (2014). Central brain neurons expressing doublesex regulate female receptivity in Drosophila. *Neuron* **83**, 149–163.

Zhuo, J.-C., Hu, Q.-L., Zhang, H.-H., Zhang, M.-Q., Jo, S. B. and Zhang, C.-X. (2018). Identification and functional analysis of the *doublesex* gene in the sexual development of a hemimetabolous insect, the brown planthopper. *Insect Biochem. Mol. Biol.* **102**, 31–42.

Ziemba, K. S. and Rutowski, R. L. (2000). Sexual dimorphism in eye morphology in a butterfly (*Asterocampa leilia*; Lepidoptera, Nymphalidae). *Psyche A J. Entomol.* **103**, 25–36.

Zou, Y., Geuverink, E., Beukeboom, L. W., Verhulst, E. C. and van de Zande, L. (2020). A chimeric gene paternally instructs female sex determination in the haplodiploid wasp *Nasonia*. *Science* **370**, 1115–1118.

Summary

Sex-determination systems are highly diverse among different taxa and even among closely related species. Especially, insects evolved a great diversity of sex-determination mechanisms to facilitate reproduction by promoting sexual differentiation and development of sexual behaviour. However, sex is not always under the control of insects themselves. Endosymbiotic bacteria, such as *Wolbachia pipientis,* have different mechanisms to manipulate the reproduction of their insect hosts. Understanding this insect-endosymbiont interaction requires comprehensive knowledge on insect sex-determination mechanisms. In this thesis, we used three parasitoid wasp species as study models and set out to investigate the sex-determination system in the Hymenoptera by focussing on three major topics: (1) the evolutionary conservation of sex-determination mechanisms, (2) the manipulation of the host sex-determination mechanism by thelytokous parthenogenesis-inducing (PI)-*Wolbachia*, and (3) the role of *Doublesex* (*Dsx*) in regulating sexual differentiation in parasitoid wasps.

In Chapter 2, we used *Muscidifurax uniraptor* wasps that were infected by PI-*Wolbachia* to study its sex-determination mechanism and to compare it with its sexually reproducing sister species *M. raptorellus* which has no *Wolbachia* infection. We identified transcripts of three conserved sex-determination genes, *transformer* (*tra*), *transformer-2* (*tra2*) and *Dsx*, of which *tra* and *tra2* share similar structures and expression patterns in both species. We confirmed that in both species the maternally provided *tra* and *tra2* are essential for female offspring development and a sufficient amount of *tra2* is needed for embryo viability. The only divergent expression is observed in the downstream gene *Dsx* in the two species. *M. uniraptor* possesses an extra female-specific *Dsx* splice variant and alternatively spliced 5'UTR in all its *Dsx* transcripts. We hypothesize that these alterations in *Dsx* are related to the co-evolution with *Wolbachia*. Although PI-*Wolbachia* altered reproduction in *M. uniraptor*, we did not find substantial changes in host sex-determination mechanisms.

Based on the knowledge we gained from studying the sex-determination system of *Muscidifurax*, we next investigated the mechanism of *Wolbachia*-induced thelytoky in *M. uniraptor* in Chapter 3. Females infected with PI-*Wolbachia* produce diploid daughters from unfertilized eggs, but in some infected species, diploid males may occur with lower *Wolbachia* titre. This suggests that diploidization and feminization are two separate processes. We started our experiments by feeding different concentrations of tetracycline (antibiotics) to *M. uniraptor* females in order to gradually reduce the *Wolbachia* titre.

However, we did not find any diploid males to support the two-step feminization model. We further studied the effect of *Wolbachia* infection on the expression and splicing of the sex-determination genes *transformer* (*Mutra*) and *transformer-2* (*Mutra2*) in female ovaries and conclude that *Wolbachia* does not affect expression or splicing of *Mutra* and *Mutra2*. In the early development of *Wolbachia*-infected embryos, we observed a two-fold increase of *Mutra* expression. To determine whether diploidization is sufficient for feminization, we used the sexually-reproducing sister species, *Muscidifurax raptorellus* to artificially create triploid females. These triploid females, when virgin, produced haploid sons and diploid daughters, showing that in *Muscidifurax* feminization solely depends on ploidy level. This strongly suggests that *Wolbachia* only needs to induce diploidization and that bi-allelic *Mutra* expression is sufficient for female development in *M. uniraptor* species.

Sexually dimorphic traits in insects are rapidly evolving due to sexual selection. Yet, our knowledge of the underlying sex-specific molecular mechanisms is still scarce, especially in the Hymenoptera. Therefore, in Chapter 4, we studied how sexual dimorphisms are promoted with a special focus on the highly conserved gene *Dsx* in the model parasitoid wasp *Nasonia vitripennis* (Hymenoptera: Pteromalidae). We show sex-specific *Dsx* expression throughout development, and demonstrate that transient *Dsx* silencing in different male developmental stages dramatically shifts the morphology of two sexually dimorphic traits, forewing size and leg and antenna pigmentation, from male to female, with the effect being dependent on the timing of silencing. We also found that transient silencing of *NvDsx* in early male larvae affects the growth and differentiation of the internal and external reproductive tissues. On the other hand, we did not observe phenotypic alterations in females developing from early Dsx silenced larvae. Our results indicate that male NvDsx is constantly required to suppress female-specific traits and to promote male-specific traits during distinct developmental windows. This provides a first insight into the regulatory activity of Dsx during wasp development in the Hymenoptera.

In addition to *Dsx*, we investigated in Chapter 5 the function of a potential new sex differentiation gene, the *glubschauge* (*glu*) homolog in *N. vitripennis,* which controls the sex-specific development of compound eyes in *Apis mellifera*. We confirmed that *glu* is sex-specifically spliced in males and females in *N. vitripennis*, and the alternative splicing is regulated by the upstream splice regulator Tra. We additionally found that *glu* affects the sexually dimorphic eye width in *N. vitripennis*.

Besides the conserved function of Dsx in controlling sexually morphological traits, in Chapter 6, we expanded the knowledge of Dsx to adult behaviour. We show in the parasitoid wasp *N. vitripennis* that males whose *Dsx* gene had been silenced underwent a three-level pheromonal feminization: First of all, *Dsx*-silenced males had drastically reduced titres of the abdominal long-range sex pheromone, and therefore, can no longer attract females from a distance. Secondly, *Dsx*-silenced males were courted as female by wild-type males due to the reduction of their cuticular hydrocarbon (CHC) profiles, specifically the alkene (*Z*)-9-hentriacontene (*Z*9C31). This *Z*9C31 is further shown in our experiment to be a crucial CHC compound for sex discrimination in *N. vitripennis*. Last but not least, *Dsx*-silenced males were hampered in eliciting female receptivity during courtship, suggesting that they are unable to produce the hitherto unidentified oral aphrodisiac pheromone reported in *N. vitripennis* males.

Taken together, we elucidated a new sex determination cascade in *Muscidifurax* that takes ploidy as its instruction signal and we provide evidence that PI-*Wolbachia* requires no manipulation of the host sex determination to induce the parthenogenesis. We confirmed the conserved Dsx function in regulating morphological dimorphic traits in wasps, and we also shed light on Dsx functions in the control of sex-specific pheromone production that affects the mating behaviours of parasitoid wasps. This thesis contributes to the overall fundamental knowledge of sex-determination mechanisms in parasitoid wasps, but more research will be needed before we obtain a comprehensive understanding of this topic.

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Publication list

Wang, Y., **Wang, Y.**, Wang, Z., Bravo, A., Soberón, M. and He, K. **(2016)**. Genetic basis of Cry1F-resistance in a laboratory selected asian corn borer strain and its cross-resistance to other *Bacillus thuringiensis* toxins. *PLoS One* 11, e0161189.

Zhang, T., Coates, B. S., Wang, Y. Q., **Wang, Y.**, Bai, S., Wang, Z. Y. and He, K. **(2017)**. Down-regulation of aminopeptidase N and ABC transporter subfamily G transcripts in Cry1Ab and Cry1Ac resistant asian corn borer, *ostrinia furnacalis* (Lepidoptera: Crambidae). *Int. J. Biol. Sci.* 13, 835–851.

Song, Y., Liu, L., **Wang, Y.**, Valkenburg, D.-J., Zhang, X., Zhu, L. and Thomma, B. P. H. J. **(2018)**. Transfer of tomato immune receptor Ve1 confers Ave1-dependent Verticillium resistance in tobacco and cotton. *Plant Biotechnol. J.* 16, 638–648.

Wang, Y., Rensink, A., Fricke, U., Riddle, M. C., Trent, C., van de Zande, L. and Verhulst, E. C. (2020). Sexually dimorphic traits and male-specific differentiation are actively regulated by Doublesex during specific developmental windows in *Nasonia vitripennis*. *bioRxiv https://doi.org/10.1101/2020.04.19.048553.* **(in submission)**

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Wang, Y., Sun, W., Fleischmann, S., Millar, J. G., Ruther, J. and Verhulst, E. C. (2021). Silencing Doublesex expression triggers three-level pheromonal feminization in *Nasonia* males. *bioRxiv 2021.05.21.445141* **(in submission)**

Netschitailo, O., **Wang, Y.**, Wagner, A., Verhulst, E. C. and Beye, M. A novel regulator for sexual eye development evolved by gain of female-specific sequences in the Glubschauge protein. **(in preparation)**

Xiong, X., Kelkar, Y. D., Geden, C. J., Zhang, C., **Wang, Y**., Jongepier, E., Verhulst, E. C., Gadau, J., Werren, J. H. and Wang, X. Long-read assembly and annotation of the parasitoid wasp *Muscidifurax raptorellus*, a biological control agent for filth flies. **(in submission)**

Wang, Y. and Verhulst, E. C. (2021). Comparison of sex determination mechanisms of a *Wolbachia*-infected thelytokous parasitoid wasp *Muscidifurax uniraptor* and its sexually reproducing sister species, *Muscidifurax raptorellus*. **(in preparation)**

Wang, Y. and Verhulst, E. C. (2021). Evidence for an one-step mechanism of endosymbiont-induced thelytoky in the parasitoid wasp, *Muscidifurax uniraptor*. **(in preparation)**

About the author

Photo shot by dr. Hans Smid at Brain lab of Wageningen University & Research Entomology group. 22 March, 2018.

Yidong Wang was born the 11th of November 1990 in Beijing. He went to Shi-jia-hu-tong primary school and afterwards attended the No.5 middle school and high school. In 2009, he started his study in plant protection at China Agricultural University. During that time, Yidong gained his passion for plants and insects and after graduation he decided to pursue a professional career as a research assistant in the Maize Group that belongs to the Chinese Academy of Agricultural Sciences (CAAS), Plant Protection Institution. In this group, he was involved in different lines of research including the GMO maize safety evaluation, Bt-protein toxicity and insect diapause research. He was intrigued by the fascinating world of science and decided to gain more knowledge by enrol in the Master of Plant Science program at Wageningen University & Research. During his Master, Yidong did a major project at the Laboratory of Phytopathology under the supervision of prof. Bart P.H.J. Thomma and PhD student Yin Song. He studied the broad host resistance of the homolog tomato Ve1 immune receptor against *Verticillium wilt* in a variety of plants. His work contributed to the publication in the international journal Molecular Plant Pathology. Later, he switched to an aphid study in his second project at the Laboratory of Entomology with a special focus on the identification of the aphid effectors which can suppress the immune response of a resistant cabbage cultivar. He enjoyed his time and the research in the Laboratory of Entomology and immediately after his Master, Yidong started his PhD project under the supervision of prof. Marcel Dicke and dr. Eveline C. Verhulst. During his PhD, he investigated the function of a sex determination gene *Doublesex* in promoting sexually dimorphic trait development and pheromone-based mating behaviours in the parasitoid wasp *Nasonia vitripennis*. In addition, he identified the sex-determination genes in two other parasitoid wasp species, *Muscidifurax uniraptor* and *M. raptorellus,* and studied the mechanisms of the obligate endosymbiont *Wolbachia*-induced asexual reproduction in *M. uniraptor*. His passion for insect studies will never fade away and will keep stimulating him in his future career.

Education statement

PE&RC Training and Education Statement

With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of literature (4.5 ECTS)

- Genetic basis of sex determination in an asexually reproducing parasitic wasp

Post-graduate courses (5.2 ECTS)

- Nöthiger meeting of sex determination system; Nöthiger meeting committee (2017)
- Bayesian statistics; PE&RC (2017)
- Introduction to R for statistical analysis; PE&RC (2017)
- Electron microscopy course the basics: from A to W; PE&RC (2019)
- Nöthiger meeting of sex determination system; Nöthiger meeting committee (2019)
- Generalized linear models; PE&RC (2019)

Laboratory training and working visits (0.6 ECTS)

- Discussion of the experiment on Dsx regulating sex pheromone in *Nasonia vitripennis*; University of Regensburg, Institute for Zoology and Chemical Ecology (2019)

Invited review of journal manuscripts (1 ECTS)

- Frontiers in Zoology: phylogenetic analysis and embryonic expression of panarthropod *Dmrt* genes (2019)

Competence strengthening / skills courses (2.25 ECTS)

- Research data management; WGS (2019)
- Scientific writing; Wageningen into language (2020)

Scientific integrity/ethics in science activities (0.6 ECTS)

- Scientific integrity; WGS (2018)

PE&RC Annual meetings, seminars and the PE&RC weekend (1.5 ECTS)

- PE&RC First years weekend (2017)
- PE&RC Last years weekend (2020)

National scientific meetings, local seminars, and discussion groups (4.2 ECTS)

- National entomology day (2017)
- Netherlands society of evolutionary biology (2018)
- National Entomology day (2018)
- Wageningen evolution and ecology seminars (2018-2020)
- National entomology day (2019)

International symposia, workshops and conferences (6.3 ECTS)

- International symposium on Insect Physiology, Biochemistry and Molecular Biology (IPBMB) and International Conference of Insect Genomics (ICIG); Chongqing, China (2019)
- Virtual EMBO| EMBL Symposium: the molecular basis and evolution of sexual dimorphism; online (2020)
- New approaches and concepts in microbiology; online (2021)

Lecturing/Supervision of practicals/tutorials (10.5)

- Molecular aspects of bio-interactions (2017, 2018, 2019)
- Molecular and evolutionary ecology (2019)

BSc/MSc thesis supervision (5.25 ECTS)

- Timing of *Doublesex* splicing in male and female development of *Nasonia vitripennis*
- The effect of *Doublesex* in pheromone production and mating behaviour in *Nasonia vitripennis*
- Identification of potential parthenogenesis inducing genes in *Wolbachia* through comparative genomic approach

The research presented in this thesis was performed at the Laboratory of Entomology of Wageningen University & Research (WUR).

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