Sexually dimorphic traits and male-specific differentiation are actively regulated by Doublesex during specific developmental windows in *Nasonia vitripennis*

Yidong Wang [a], Anna Rensink [b], Ute Fricke [a,*], Megan C. Riddle [c,$], Carol Trent [c], Louis van de Zande [b], Eveline C. Verhulst [a,d]

a. Wageningen University, Laboratory of Entomology, Wageningen, The Netherlands
b. Evolutionary Genetics, Development and Behaviour, Groningen Institute for Evolutionary Life Sciences, University of Groningen, Groningen, The Netherlands
c. Biology Department, Western Washington University, Washington, United States of America
d. Wageningen University, Laboratory of Genetics, Wageningen, The Netherlands

* Current address: Departement of Animal Ecology and Tropical Biology, Biocenter, University of Würzburg, Würzburg, Germany
$ Current addresses: Department of Psychiatry and Behavioral Sciences, University of Washington, Seattle, WA, United States of America; Eating Recovery Center
1231 116th Ave NE Suite 800, Bellevue, WA 98004

Corresponding author: eveline.verhulst@wur.nl

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Abstract

Sexually dimorphic traits in insects are rapidly evolving due to sexual selection which can

ultimately lead to speciation. However, our knowledge of the underlying sex-specific

molecular mechanisms is still scarce. Here we show that the highly conserved gene,

Doublesex (Dsx), regulates rapidly diverging sexually dimorphic traits in the model parasitoid

wasp *Nasonia vitripennis* (Hymenoptera: Pteromalidae). We present here the revised full *Dsx*

gene structure with an alternative first exon, and two additional male *NvDsx* isoforms, which

gives important insights into the evolution of the sex-specific oligomerization domains and C-

termini. We show the 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, transient silencing of *NvDsx*

in early male larvae affects male genitalia tissue growth but not morphology. This indicates

that male *NvDsx* is actively required to suppress female-specific traits and to promote male-

specific traits during specific developmental windows. These results also strongly suggest that

in *N. vitripennis* most sex-specific tissues fully differentiate in the embryonic stage and only

need the input of *NvDsx* for growth afterwards. This provides a first insight into the regulatory

activity of *Dsx* in the Hymenoptera and will help to better understand the evolutionary and

molecular mechanisms involved in sex-specific development in this parasitoid wasp, which

can eventually lead to the development of new synthetic genetics-based tools for biological

pest control by parasitoid wasps.

Significance Statement

In insects, male and female differentiation is regulated by the highly conserved transcription

factor Doublesex (Dsx). The role of Dsx in regulating rapidly evolving sexually dimorphic traits

has received less attention, especially in wasps and bees. Here, we mainly focused on Dsx

regulation of two sexually dimorphic traits and male genitalia morphology in the parasitoid

wasp, *Nasonia vitripennis.* We demonstrate that *Dsx* actively regulates male-specific tissue

growth and morphology during specific developmental windows. These findings will help to

better understand the molecular mechanisms underlying the rapid evolution of sexual

differentiation and sexually dimorphic traits in insects, but may also be the starting point for

the development of new tools for biological control of pest insects by parasitoid wasps.

Introduction

Insects are well known for their distinct differences between male and female morphology.

The reproductive organs and sexually dimorphic traits such as wing shapes and colour

patterns are one of the fastest evolving traits in insects. Therefore, they are often used to

distinguish species, as these traits can differ dramatically between species. Development of

these fast changing sexually dimorphic traits usually depends on specific genes that are

expressed in a tissue and sex specific manner (1). Many of these genes are under control of

the transcription factor, Doublesex (Dsx), that initiates sexual differentiation in insects (2–4).

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 (5). 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. These C-terminal sex-specific differences

are assumed to be involved in the regulation of the development of sex-specific traits (6,7).

For instance, in *Drosophila*, DsxM represses and DsxF activates the expression of *bric-à-brac*

(bab) which results in male-specific abdominal pigmentation (8). A similar action of Dsx is

observed in the formation of sex combs, an important feature for performing mating rituals in

males of some *Drosophila* species (9). In *Bactrocera dorsalis, Dsx* knockdown in females

triggers the interruption of yolk protein gene expression, leading to ovipositor deformation and

a delay in ovary development (10). It confirms the essential role of DsxF to initiate and

maintain female development in Diptera. In Lepidoptera (butterflies and moths) and

Coleoptera (beetles), sex-specific *Dsx* expression in both sexes also affects many sex

specific traits. For example, transient expression of *DsxM* in female *Bombyx mori* silkmoths

distorts the development of female-specific genital organs by partially introducing male

characters in female genitalia (11), whereas introducing mutations in the male-specific *Dsx*

isoform of *B. mori* yields either malfunctioning males or intersexes (12). In *Papilio polytes*

butterflies, differential expression of *Dsx* isoforms results in sex-specific wing patterns (13).

Dsx also promotes the mandible growth in males but constrains it in females of *Cyclommatus*

metallifer beetles (14). In *Trypoxylus dichotomus* beetles, the formation of head horns is

antagonistically regulated by *DsxM* and *DsxF*, while the thoracic horn formation is *DsxM*

independent (15,16). Noteworthy, all these experiments were conducted in a specific

developmental stage and only few focused on sexually dimorphic traits. However, the

expression of *Dsx* starts in early embryogenesis and continues through maturity which implies

its ongoing action (10,17,18).

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*), Japanese ants (*Vollenhovia emeryi*) and fire ants (*Solenopsis invicta*) (19–23), but the role of *Dsx* in regulating reproductive organ development in males has only been shown in hymenopteran sawflies, *Athalia rosae* (24). To understand the rapid evolution of sexual dimorphism in insects and to compare the molecular mechanisms of *Dsx*-mediated regulation, it is paramount to gain more knowledge on how *Dsx* directs sex-specific and sexually dimorphic trait development in the Hymenoptera. Therefore, we set out to 1) identify the sex-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*.

Nasonia vitripennis belongs to the genus *Nasonia* (*Hymenoptera: Pteromalidae*), and has become the model system for parasitoids as it is highly suitable for evolutionary and developmental genetic studies. Multiple sexually dimorphic traits within the genus *Nasonia* make it a perfect system to study sex differentiation (25,26). For instance, the femur of the hind legs and the antennae of female *Nasonia* are pigmented with a dark-brown color, whereas in males these are pale yellow (26). Only in *N. vitripennis,* males have dramatically shorter forewings and are flightless (26), while the females can fly and have large forewings in all species. Although *Dsx* has been identified in *N. vitripennis* (27), its role in regulating these sexually dimorphic traits has not been shown before. Previous research identified one male *Dsx* (*NvDsxM*) and one female *Dsx* (*NvDsxF*) splice variant (27) and showed that the conserved splicing factor Transformer (Nvtra) is responsible for female-specific splicing of *Dsx* transcripts, while in males the *Dsx* transcript is spliced by default into the male isoform (28).

To assess the role of *Dsx* in regulating sexual dimorphism 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 a great start to study the molecular basis of sexual differentiation and rapidly evolving sexual dimorphism in insects in general and particularly in the Hymenoptera.

Results and discussion

Identification and characterization 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* (27). 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 retaining this 108bp intron, a form of alternative splicing which has not been observed for *Dsx* in other insects (27) (Fig. 1, *NvDsxF* and *NvDsxM1*). This splice form translates into a Dsx protein with a very short OD2 domain of only four amino acids (aa). The gene structure of this published *NvDsx* does not resemble any other published *Dsx* structures. To investigate the possibility of additional splice variants of *Dsx* pre-mRNA in *N. vitripennis,* we performed extensive 5' and 3' RACEs. The results reveal a novel alternative starting exon that we designated exon 1' (Fig. 1). Note that the start codon of protein translation is in exon 2. 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 (Fig. S1). Alternative splicing at the 5' end has not been shown for *Dsx* before. We assume that the alternative exon is used in all splice variants (Fig. 1), although long-read RNA sequencing would be required to confirm this. From here on we refer to the alternative variants collectively. The 3' RACEs identified two additional longer male splice variants (Fig. 1). We designated the previously published male-specific variant *NvDsxM1* (M1: NM_001162517; M1': MT043363); the newly found longer male splice forms were 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 which is 105 aa longer than the open reading frame of *NvDsxM1*. Compared to *NvDsxM2,* we find that exon 8 is spliced out in *NvDsxM3* which puts the stop codon in exon 9 and yields a protein of 330 aa. These two additional male specific splice variants skip the female-specific exon 6 completely which is a shared feature of male splice forms in many insect species including fruit flies (*D. melanogaster*) (29) (Fig. 1), wild silkmoths (*Antheraea assama*) (30), honeybees (*A. mellifera*) (21) and fire ants (*S. invicta*) (20). We did not find any non-sex specific splice variants in *N. vitripennis* as opposed to *A. mellifera* (Fig. 1) (21). NvDsxM2 and NvDsxM3 protein isoforms are almost completely identical and only differ at the C-terminus after the male-specific OD2 domain (Fig. S6A).

NvDsxM2 and NvDsxM3 are spliced by default

To determine whether the additionally identified splice variants are exclusively expressed in males, we first observed the overall expression of different *Nvdsx* splice forms in both males and females. Across development, *NvDsxF* is only expressed in females while *NvDsxM1, NvDsxM2* and *NvDsxM3* are highly expressed in males (Fig. S2), and expressed at a low

level in females. Only in males a slight increase of *NvDsxM1, -2 and -3* expression from 36h

toward the early pupal stage was observed. Afterwards, the expression remains relatively

constant until adult stage (Fig. S2). Overall, *NvDsxM2* is more abundant than *NvDsxM3* (Fig.

S2)*.* Females express *NvDsxM1*, *NvDsxM2* and *NvDsxM3* at a low level probably due to

leaky default splicing of male-specific transcripts (Fig. 2B and Fig. S2).

We have shown previously that parental *Nvtra* RNA interference (RNAi) in the female pupal

stage leads to a shift in splicing from *NvtraF* to *NvtraM* (28)*.* 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 (28). The reduced levels of NvtraF

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

NvTra independent splicing of *NvDsxM2* and *NvDsxM3*, we used *Nvtra* RNAi in the last larval

instar (4th instar) of females. RT-PCR was applied to amplify the different *NvDsx* and *Nvtra*

splice variants from 3 hours post injection (hpi) to 5 days post injection (dpi). After confirming

that *Nvtra* RNAi leads to *NvtraM* splicing in females (Fig. 2), we did not observe a clear

reduction of female *NvDsxF* expression, but a minor increase of *NvDsxM1* expression from

4dpi onwards was observed. *NvDsxM2* and *NvDsxM3* expression levels increase

substantially from 1dpi onward compared to *gfp* RNAi control (Fig. 2).

NvDsxM represses female-specific pigmentation

To assess the sex-specific function of Dsx, we studied the phenotypic effect of *NvDsx*

silencing at different developmental time points. We were unable to silence the male-specific

splice variants of *NvDsx* specifically (Fig. S3B), therefore we targeted the common region of

NvDsx (*NvDsxU*)*.* We observed that the developmental stage at which *NvdsxU* dsRNA was

injected greatly influenced the expression levels of *NvDsx* measured using qRT-PCR. We

found no significant decrease of *NvDsx* expression in the 2nd instar *NvDsx* knockdown

(*NvDsxU*-i) male larvae in comparison to non-treated (NT) and *gfp* mock (*gfp*-i) when

sampling after the white pupal stage (Fig. S3*A*). The strong increase in *NvDsx* expression

from 4th instar male larvae onwards (Fig. S2) may be the main cause as the limited amount of

siRNA present in the 2nd larval instar is not capable of silencing the sudden increase of

transcript numbers in later developmental stages. However, this allows us to transiently

suppress *NvDsx* during different developmental time windows. Injecting *NvDsxU* dsRNA in 4th

instar male larvae led to a significant reduction of *NvDsx* in newly emerged adults when

compared with both NT (Tukey's HSD, P<.001) and *gfp*-i males (Tukey's HSD, P<.001) (Fig.

S3*B*). It confirmed a successful *NvDsx* knockdown starting in 4th instar male larvae with an

effect lasting into adulthood. The 4th instar *gfp*-i males also showed significant reduction of

NvDsx expression compared to NT (Tukey's HSD, P=0.021) suggesting that in the 4th instar

larvae there might be a non-target effect of *gfp* dsRNA as was shown before in the honey bee

(31) (Fig. S3*B*). Clear reduction was observed at the adult stage of pupal stage *NvDsxU*-i

males when compared to NT (Tukey's HSD, P=0.003) and pupal stage *gfp*-i males (Tukey's

HSD, P=0.002) (Fig. S3*C*) which indicates that we also obtained a highly significant

knockdown of *NvDsx* from the pupal stage onwards.

Non-treated and *gfp*-i males showed unpigmented antennae and legs (Fig. 3*A*, *B*, *C*),

whereas females have pigmented antennae and legs (Fig. 3*D*). *NvDsx* knockdown (*NvDsx*-i)

in 2nd instar male larvae increased the amount of pigmentation in the adults slightly (Fig. 3*F*),

but depleting *NvDsx* from 4th instar male larvae onwards increased pigmentation in their adult

stage to the point that it resembled female-like pigmentation (Fig. 3*E*). No pigmentation was

observed in adult males when *NvDsx* was depleted from the early white pupal stage onwards

(Fig. S4).

We did not find changes in pigmentation intensity by *NvDsx* RNAi experiments in 4th instar

female larvae (Fig. S5), and silencing *NvDsx* led to female-like pigmentation in males.

Therefore, we conclude that female pigmentation is the default developmental state, and in

males NvDsxM actively interferes with the melanin synthesis pathway. In *D. melanogaster,*

expression of sex-specific abdominal pigmentation is regulated by interaction of the HOX

protein Abdominal-B (Abd-B) and Dsx. Combined input of Abd-B and DsxF leads to the

expression of *bric-a-brac* (*bab*) which suppresses the pigmentation in the posterior abdomen

segments while DsxM represses *bab* to maintain the posterior abdomen pigmentation (8). As

we found no true intersex phenotype, we hypothesize that NvDsxF plays no role in enhancing

expression of the genes required for pigmentation in females. The slight reduction in antenna

pigmentation of 4th instar *NvDsx*-i males (Fig. 3E) is likely due to 1) a structural difference in

the antennae with males having less plate organs on the sub-segments than females causing

a different color reflection (32), and/or 2) incomplete knockdown of *NvDsx*. The deposition of

the pigments in the cuticle often starts from late pupal to early adult stages in *Drosophila* (33).

Therefore, initiating the RNAi response from 4th instar larva onwards creates enough time to

deplete *NvDsx* mRNA and NvDsx protein production to activate the expression of

pigmentation genes in *N. vitripennis* males.

NvDsx silencing leads to increased forewing size of males

The second sex-specific character that we assessed was forewing size and shape in

N.vitripennis adult males. Silencing *NvDsx* in both 2nd and 4th instar larvae resulted in

increased forewing length and width when compared to *gfp*-i (Tukey's HSD, P<0.001) and NT

males (Tukey's HSD, P<0.001) (Fig. 4*B* and *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) (Fig. 4*A*) 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) (Fig. 4*A*, *B* and *C*). Interestingly, here we found an effect of

timing as the forewing length and width increased significantly between 2nd and 4th instar

larvae *NvDsx*-i treatments in which earlier stage *NvDsx* knockdown led 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) (Fig. 4*A*, *B* and *C*). Depleting

NvDsx from the white pupal stage onwards had no effect on forewing size or shape in males

(Fig. S4).

Previous research showed that a non-coding region of *Dsx* in *N. giraulti*, termed wing-size1

locus (*ws1*), is correlated with the development of long wings in males, whereas the *ws1*

variant in *N. vitripennis* is correlated with the severely reduced forewing size in males (34).

Although higher *NvDsxM1* expression levels were detected in individuals with the *N.

vitripennis ws1* variant (34), the mechanism of *NvDsx* regulation is unclear and the *NvDsxM2*

and *-3* splice variants were unknown at the time. Our research confirmed that the regulation

of the male forewing size depends on high *NvDsxM* levels and illustrates the role of *NvDsxM*

in repressing wing growth. Changes in spatial and temporal expression of *unpaired-like* (*upd-

like*) has been shown to affect wing width in *Nasonia* males which is caused by multiple

regulatory changes (35). *NvDsxM* regulation is likely responsible for differentially expressed

upd-like. In contrast to the observed complete female-specific pigmentation after *NvDsxM*

depletion in 4th instar males, the depletion of *NvDsxM* from 2nd instar onwards is inadequate

to restore female forewing size. All these results suggest that forewing size and shape

development might already take place during embryonal development and our chosen time

window for RNAi is not early enough to induce a full switch. We cannot rule out antagonistic

effects of NvDsxM and NvDsxF on wing size development in *Nasonia*, since in females *Aedes

aegypti, Dsx* knockdown leads to smaller wing development (36). However, we observed no

wing size change after *NvDsx* knockdown in females (Fig. S5).

Growth of male genitalia is controlled by *NvDsx*

Next, we assessed the role of *NvDsx* in male genitalia development and growth after embryogenesis is completed by depleting *NvDsx* at either 2nd or 4th instar larval stage. This is in contrast to many studies using genome editing in which *Dsx* is knocked out from early embryogenesis onwards (10,12). *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) (Fig. 5A). *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 phallomere structure (genital phallic lobes) was also severely reduced in size in both 2nd and 4th instar *NvDsx* depleted males (Fig. 5B-c and e) compared to *gfp*-i and NT males (Fig. 5B-a, b and d), whereas the structure of the paramere including the outer parameres, cuspis and digitus, was still intact (Fig. 5B). Compared to *gfp*-i males (Movie S1), the severe length reduction of the aedeagus rendered *NvDsx*-i males unable to copulate with females (Movie S2), showing that in this case size matters.

Dsx is shown to be involved in the differentiation of genitalia structures in male and female insects in many cases by interactions with *HOX* genes during early embryogenesis (38). However, whether *Dsx* is continuously required for genitalia development has not received much attention. Previous studies using *Dsx* RNAi experiments in other species conducted at late larval stages resulted in either deformed external genitalia or chimeras that contain the structures of both sexes (24,39). Here, even though *NvDsx* RNAi was done at a distinctly earlier stage (2nd larval instar) compared to other studied insect species, we did not observe any abnormalities in genital organs other than size. This shows that contrary to other studied insects, in *N. vitripennis* the differentiation of external genital organs is completed before the 2nd larval instar and only growth is required during later development. It also suggests that besides the sex-specific tissue differentiation, *NvDsx* is required for the direct sex-specific trait growth as it has been shown in stag beetles *Cyclommatus metallifer* where it was demonstrated that *Dsx* can manipulate the sensitivity of JH in a sex-specific way to regulate mandible growth in males and females (14).

Evolution of Dsx sex-specific OD2 domains and C-termini

Aligning the OD2 domains of NvDsxM1, NvDsxM2 and NvDsxM3 (Fig. S6A) or NvDsxF (Fig. S6B) with other DsxM or DsxF OD2 domains, respectively, showed that across insect orders the male-specific OD2 domain is far less conserved than the female-specific OD2 domain. Within insect orders, also a strong reduction of the sequence identity in male-specific OD2 domain alignment was seen. This observed rapid evolution of male-specific *Dsx* regions is

274 supported by other research and is indicative of its fast evolution likely due to increased

275 diversity of secondary sexual traits in male insects (40). In females, the sex-specific region of

276 OD2 is relatively conserved across different insect orders. However, within the Hymenoptera,

277 the female-specific OD2 domain showed a lower similarity. Only one out of eight conserved

278 amino acids is being shared with all other tested species in the hymenopteran sequences,

279 when compared to other insect orders that have half of the amino acids conserved (Fig. S6B).

280 In *D. melanogaster* the C-terminus including the OD2 domain of DsxF binds the co-factor

281 Intersex (41). The differences in female-specific OD2 amino acid composition in the

282 Hymenoptera could indicate that either no co-factors or different co-factors play a role in

283 regulating sexual development in this order compared to other insect orders. In addition, the

284 female specific OD2 domain in the Hymenoptera was found to evolve much faster than the

285 male-specific regions (40). This is contrary to Diptera, Lepidoptera and Coleoptera and has

286 been suggested to correlate with the evolution of female-dominated eusociality and female-

287 specific caste differentiation (40). However, *Nasonia* is solitary as are many other

288 hymenopteran species. In the Hymenoptera, females arise from fertilized eggs while male

289 development initiates by default from haploid, unfertilized eggs. We suggest that the rapid

290 evolution of the female-specific OD2 domain in the Hymenoptera could be linked to this

291 difference. All in all, the identification of the two additional male NvDsx isoforms opens the

292 door to better comparative phylogenetic studies, and to better understand the evolution of *Dsx*

293 in insects.

295 **Outlook**

296 In this research we identified two sexually dimorphic traits and one male-specific trait that are

297 actively regulated during specific developmental windows by Dsx in *N. vitripennis.* Our results

298 showed that male-specific lack of antennal and femur pigmentation is strictly regulated during

299 pupal development as silencing *NvDsx* from the larval stage onwards resulted in build-up of

300 pigmentation in the males, while silencing *NvDsx* from the pupal stage onwards did not have

301 any effect. In addition, our results suggest that sex-specific wing size and shape is regulated

302 by *NvDsx* during embryogenesis and early larval development. As silencing *NvDsx* from the

303 2nd larval instar onwards significantly increases wing size in males, while silencing from the

304 pupa stage onwards does not change wing size and shape. However, *NvDsx* RNAi in the 2nd

305 larval instar did not result in complete female-like wing size, indicating that the initial wing

306 development occurs during embryogenesis and possibly the 1st larval instar. We additionally

307 show that *NvDsx* regulates only male external reproductive organ growth and not morphology

from 2nd larval instar onwards, suggesting that the differentiation of external reproductive organ tissue takes place before the 2nd instar which has not been reported before. 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 differentation (19). Here, we expand this knowledge using a non-drosophilid species by showing that different morphological characteristics require their own specific timing and action of Dsx protein and we speculate that this timing and action is also 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.

Material and methods:

Insects rearing

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

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

For identification of the 3' ends of *NvDsx* mRNA, total RNA of 3 adult male- and female AsymCx wasps were 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 RevertAidTM H Minus First Strand cDNA Synthesis Kit (Fermentas, Hanover, MD, USA) and stored at -80°C. The cDNA was subjected to 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 (27) and sequences are listed in the Table S1.

For identification on the 5' end of *NvDsx*, total RNA of 3 female AsymCx wasps were 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 RevertAidTM 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 (27) and sequences are listed in the Table S1.

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 kit 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 (42). 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.

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 RevertAidTM H Minus First Strand cDNA Synthesis Kit (Fermentas, Hanover, MD, USA) with random hexamers according to 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 used to prepare the mastermix following manufacturer's instruction and 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.

Alignment and phylogenetic analysis of Dsx OD2 domain across insect orders

Dsx isoforms from 22 species across insect taxa were downloaded from NCBI protein

database with accession number attached in the end of each sequence name (Fig. S6A and

S6B). OD2 common domain and sex-specific region of OD2 domain selection is based on An

et al. 1996 (2). Both the male and female Dsx OD2 domain sequences were separately

aligned using Guidance2 Online Server with Guidance2 method, settings are MAFFT

algorithm, max iteration of 10, 100 alternative guide-trees. Geneious 2019.1 (created by

Biomatters. Available from https://www.geneious.com) was used to shade the amino acids

with Blosum62 score matrix applied to display the similarity between each sequence

(threshold at 3).

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.* (28). To conduct functional analysis of *NvDsx*,

same RNAi Kit was used to generate dsRNA based on either the common region of *NvDsx*

(exon 2-5) that presents in both male and female splice-variants or the specific region of

NvDsx (exon 7-9) that only presents 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 the Table S1. For male-

specific *NvDsx* RNAi, offspring of AsymCX strain was collected at 2nd and 4th larval instars

and white pupa stage which are described in Table S2. All dsRNAs were diluted with RNase-

free elution buffer (MEGAscript RNAi Kit (Thermo Fisher)) to 4000 ng/µl (NanoDrop™ 2000

Spectrophotometer, ThermoFisher) before proceeding to microinjection.

Microinjection of wasps was carried out with IM300 Microinjecctor (Narishige) and FemtoJet®

4i (eppendorf) and followed the protocols described by Werren *et al*. (43) and Lynch &

Desplan (44) 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 S3).

Second and 4th instar larvae were collected from parasitoid hosts and placed on a 1X PBS

agar plate before injection. Early pupal stage samples were fixed on the glass slides with

double-sided tape. After injection, 2nd instar larvae were transferred back to 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.

NvDsx splicing and expression throughout development

Male and female wasps were separated at black pupal stage by the 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 clogged with
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 extremely female-biased eggs, offspring from mated
females was considered female (45). 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 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 Nv_traF_ 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 S2. More than 50 embryo eggs were collected at 36h and five to 10
larvae, pupae or adults were collected at 2.5d and onwards. Collected samples were
subsequently frozen in liquid nitrogen and stored in -80°C. Sex-specific primers of _NvDsx_
were designed using Geneious 10.0.9. and are listed in the Table S1. Both _NvRP49_ and
NvEF-1a were assessed for their expression stability in this semi-quantitative RT-PCR setup,
and _NvEF-1a_ 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. On column
DNase treatment step was added to all samples. Subsequently, total RNA of each samples
was eluted in 16 µl of DNase/RNase free water. After verifying the purity and concentration by
NanoDrop™ 2000 Spectrophotometer (ThermoFisher), 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 manufacture'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.

Silencing efficiency of *NvDsx*

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[TM]

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 S1). *NvEF-1a* was used as

reference genes. 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 95,15 seconds of 55°C, 30

seconds of 72°C and a final standard dissociation curve step to check for non-specific

amplification.

Measurements of male forewing size, external reproduction organs

Forewing size was measured by wing length and width based on the standard methods

described by Loehlin *et al.* (46). Tibia length of the hind leg was used to standardize the body

size differences among individuals (47). Forewings and external reproduction organ of adult

males were dissected on clean slides with a drop of 1 X PBS buffer using dissection tweezers

and subsequently mounted in a drop of ethanol on slides which was allowed to evaporate.

External reproduction apparatuses were identified based on the description in Liu *et al.* (48)

and E. Snodgrass (37). Photos were taken by Dino-Lite Edge 5MP digital microscope and

measured by using DinoCapture 2.0 software.

Data analysis

qPCR data was first imported to LinRegPCR software (LinRegPCR, 2017.1.0.0, HFRC,

Amsterdam, The Netherlands) (49). 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.

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Figures

Figure 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 was modified from Oliveira *et al.* (27) 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. Light grey, black and dark grey blocks indicate coding region, DM domain and OD2 Dsx dimer region respectively. Scales are provided behind each species splice structures.

Figure 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).

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.

Figure 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.

Figure 4. Comparison of forewing size of adult after *NvDsx* or *gfp* dsRNA treatment in the 2nd

or 4th larval instar males and non-treated control. Y-axis represents: (A) Forewing length/width

ratio corrected with tibia length, (B) Length corrected with tibia length, (C) Width corrected

with tibia length. X-axis shows six different 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. 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.

Figure 5. Comparison of external reproduction organs in males of different treatments. (A) Comparison of genitalia length of adult males treated with dsRNA of *NvDsx* (*NvDsx*-i) or *gfp* (*gfp*-i) in the 2nd or 4th larval instar males and non-treated (NT) control males. Y-axis represents genitalia length corrected with 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. (B) Comparison of paramere length (without aedeagus) of adult males after *NvDsx* or *gfp* dsRNA treatment in the 2nd or 4th larval instar and NT control. (a) paramere structure (without aedeagus) in NT males, (b and d) 4th and 2nd larval instar *gfp*-i males, (c and e) 4th and 2nd larval instar *NvDsx*-i males. Each paramere component is described in *E. Snodgrass (1957)* (37) and labeled with the following abbreviations: outer paramere (opar), cuspis (cus) and digitus (dig).

NT♀   *NvDsx*-i♂   *gfp*-i♂   NT♂

NT♀   *NvDsx*-i♀   *gfp*-i♀   NT♂

A

common OD2 domain | male OD2

1. Apis_mellifera_M(NP_001104725)
2. Asobara_tabida_M(ATE86740)
3. Athalia_rosae_M(*)
4. Nasonia_vitripennis_M2(XP_008205424)
5. Nasonia_vitripennis_M3(XP_008205423)
6. Nasonia_vitripennis_M1(NP_001155989)
7. Trichomalopsis_dubius_M(ACJ65506)
8. Drosophila_melanogaster_M(NP_731197)
9. Musca_domestica_M(AAR23813)
10. Lucilia_cuprina_M(ADG37648)
11. Ceratitis_capitata_M(AAN63597)
12. Bactrocera_dorsalis_M(ACN73399)
13. Anastrepha_obliqua_M(AAY25167)
14. Anopheles_gambiae_M(AAZ78362)
15. Aedes_aegypti_M(ABD96573)
16. Culex_quinquefasciatus_M(AJB28477)
17. Antheraea_mylitta_M(ADL40855)
18. Bombyx_mori_M(NP_001104815)
19. Helicoverpa_armigera_M(AHF81649)
20. Ostrinia_furnacalis_M(AHF81635)
21. Tribolium_castaneum_M(AFQ62105)
22. Cyclommatus_metallifer_finae_A(BAO23808)
23. Cyclommatus_metallifer_finae_B(BAO23809)
24. Onthophagus_taurus_M(AEX92938)
25. Nilaparvata_lugens_M(AWJ25055)

B

common OD2 domain | female OD2

1. Apis_mellifera_F(NP_001128407)
2. Asobara_tabida_F(ATE86739)
3. Athalia_rosae_X1(XP_012262256)
4. Nasonia_vitripennis_F(NP_001155990)
5. Trichomalopsis_dubius_F(ACJ65505)
6. Drosophila_melanogaster_F(NP_731198)
7. Musca_domestica_F(AAR23812)
8. Lucilia_cuprina_F(ADG37649)
9. Ceratitis_capitata_F(AAN63598)
10. Bactrocera_dorsalis_F(AAV85891)
11. Anastrepha_obliqua_F(AAY25166)
12. Anopheles_gambiae_F(AAZ78363)
13. Aedes_aegypti_F1(ABD96571)
14. Aedes_aegypti_F2(ABD96572)
15. Culex_quinquefasciatus_F(AJB28478)
16. Antheraea_mylitta_F1(ADL40853)
17. Antheraea_mylitta_F2(ADL40854)
18. Bombyx_mori_F(NP_001036871)
19. Helicoverpa_armigera_F1(AHF81652)
20. Helicoverpa_armigera_F2_(AHF81653)
21. Ostrinia_furnacalis_F1(AHF81640)
22. Ostrinia_furnacalis_F2(AHF81641)
23. Tribolium_castaneum_F1(AFQ62106)
24. Tribolium_castaneum_F2(AFQ62107)
25. Tribolium_castaneum_F3(AFQ62108)
26. Cyclommatus_metallifer_finae_C(BAO23810)
27. Cyclommatus_metallifer_finae_D(BAO23811)
28. Onthophagus_taurus_F1(AEX92939)
29. Onthophagus_taurus_F2(AEX92940)
30. Onthophagus_taurus_F3(AEX92941)
31. Onthophagus_taurus_F4(AEX92942)
32. Onthophagus_taurus_F5(AEX92943)
33. Nilaparvata_lugens_F(AWJ25056)