Maternal control of haplodiploid sex determination in the wasp Nasonia

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Supporting Online Material for

Maternal Control of Haplodiploid Sex Determination in the Wasp *Nasonia*

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Insect strains

The *N. vitripennis* strains AsymC , the recessive red eye colour mutant strain STDR and a Russia Bait strain originally collected near Moscow, Russia were used throughout the experiments. The Russia Bait strain harbours an 18 bp deletion in exon one, 237 bp from the ATG startcodon of *Nvtra*. This deletion has no apparent effect on sex determination.

Primers

See Table S2 for all primer sequences.

Statistical analysis

All qPCR data was analyzed in Statistix 1.0 for Windows.

Parental RNAi with Nvtra

RNAi knockdown was induced in early female pupae. A non-sex specific *Nvtra* dsRNA fragment of 452 bp was used. At either the 5' or 3' end of the fragment a T7 promoter was placed using designed primers NvTra_RNAi_F1 and NvTra_RNAi_R1.

This fragment was transcribed in both directions using the Megascript RNAi kit (Ambion, Austin, Texas, USA) according to protocol to generate dsRNA. Approximately 200 white st^{DR} / st^{DR} female pupae were injected in the abdomen according to a protocol described by Lynch *et. al.* (*S1*) with two µg/µl of *tra* dsRNA mixed with red dye. Injections were done with Femtotips II (Eppendorf, Hamburg, Germany) needles under continuous injection flow. The female was injected until the abdomen of the female turned clearly pink. After emergence, females were kept virgin or were mated to AsymC (st^{+}) males, fed sugar water for 24h followed by four times hosting with one *Calliphora* pupa for 24h. Diploid males were identified by flowcytometry and by their wild-type eye color, since biparental males are st^{DR} / st^{+}. Haploid males are only st^{+} and have red eyes. Fertility and mating behavior of presumed diploid males was tested by crossing them to an st^{DR} / st^{DR} female. This also allowed us to determine the ability

of the diploid males to transmit their full genome set in each case. All diploid males fathered only daughters with wild type eye color indicating them as triploid st^{DR} / st^{DR} / st^{+} females (Fig. S1). If the males were unable to transmit their full genome set, they would have fathered diploid females with a mix of st^{DR} / st^{DR} (red) and st^{DR} / st^{+} (wild type) genotypes.

Flowcytometry analysis of diploid males

For flow cytometry, heads of adult wasps were homogenized in Galbraith buffer (21mM MgCl2, 30 mM tri-Sodium citrate dihydrate, 20mM MOPS, 0.1% Triton X-100, 1mg/l RNase A) using a Dounce homogenizer, filtered (40µm), stained in propidium iodide (Sigma, St. Louis, Missouri, USA) and loaded on a LFRII flow cytometer (BD BioSciences, Franklin Lakes, New Jersey, USA).

Splice form analysis following RNAi

Sex specific fragments of *Nvtra* and *Nvdsx* in normal and RNAi injected female as well as their offspring were analyzed by RT-PCR. Each sample consists of total RNA isolated from one adult wasp with TriZol (Invitrogen, Carlsbad, California, USA). 200 ng of this RNA was reverse transcribed with random hexamer provided with the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, Hanover, MD, USA). One microgram of cDNA was used in a PCR with *Nvtra* primers (*S2*) NvTra_F2 and NvTra_R3 that are located in exon two and three respectively and give a fragment of 228 bp in females and three fragments of of 514 bp, 460 bp and 282 bp in males. *Nvdsx* primers (*S3*) NvDsxU_F3 and NvDsxM_R1 that are located in exon 4 and 6 respectively and give a product of 543 bp in males and 435 bp in females.

For amplification and cDNA integrity control Ribosomal protein 49 (*Rp49*) was amplified with primer set Rp49_F1and Rp49_R1 (*S3*) yielding a product of 305 bp in both males and females. The PCR profile used was 35 cycles of 95°C for 15s, 53°C (Rp49 primers) or 55°C (*Nvdsx* and *Nvtra* primers) for 30s and 72°C for 45s. PCR fragments were visualized on a 1.5% non-denaturing agarose gel with ethidiumbromide.

Expression analyis of *Nvtra* after RNAi in females and offspring

Expression levels of *Nvtra* after RNAi were assessed in female pupae 5 days after RNAi injection (n=9). Those females were in their late pupal stage one day prior to emergence. They were compared to control females that were not injected in the same developmental stage (n=9). To assess maternal input after RNAi, embryos from an equal ratio of mated and virgin dsRNA injected females (n=19) and an equal ratio of mated and virgin not injected females (n=18) were each individually used for isolating total RNA with TriZol (Invitrogen). The entire isolated RNA sample was subsequently reverse transcribed with a mix of one part oligo-dT and six parts random hexamer both provided with the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas). The subsequent qPCR analysis was done with one micro liter cDNA mix and ABsolute™ QPCR SYBR Green ROX (500nM) Mix (Abgene, Hamburg, Germany) on an Applied Biosystems 7300 Real Time PCR System with 300 nM Nvtra qPCR primers NvTra_qPCR_F1 and NvTra_qPCR_R1.

The elongation factor 1 alpha (*EF1a*) was used as reference gene, with 300 nM of primers NvEF1a_F1 and NvEF1a_R1. *Nvtra* primer set was developed on non-sex specific parts of the transcript. All primers sets were developed using PerlPrimer (*S4*).

qPCR profile was as follows: 95°C for 15 min, 40 cycles of 95°C for 15s, 55°C for 30s and 72°C for 30s. A standard ABI7300 dissociation curve was applied to control for nonspecific amplification. LinregPCR (S5) was used for calculating starting concentrations of both genes. Relative expression levels of *Nvtra* in control and *Nvtra* dsRNA injected females and their offspring was calculated by comparing *Nvtra* starting concentrations to starting concentrations of the reference gene *EF1α*. An one-tailed unpaired *t-test* was used to calculate significant *Nvtra* expression differences between the *Nvtra* dsRNA injected samples and the control samples.

Expression of *Nvtra* and analysis of origin and splicing of *Nvtra* expression in early embryonic development

Thirty females from the Russian strain were individually mated to an AsymC male and given one *Calliphora* pupa every other day for a period of one week. Thereafter, these females were

allowed to oviposit for one hour at 25 °C in an egg laying chamber to facilitate embryo collection. The parasitized pupae were incubated at 25 °C. Therefore, the sample “five hours after egg laying” includes one hours of egg laying and four hours of incubation. Five replicates of 30 embryos per time point were collected in 100% ethanol at 2 °C in approximately 30 minutes and stored at -80 °C until RNA extraction.

The RNA extraction, reverse transcription and qPCR were performed in the same way as mentioned above. To amplify *Nvdsx* in the qPCR 300 nM dsx specific primers NvDsx_qPCR_F1 and NvDsx_qPCR_R1 were used. Relative expression levels of each of the five replicates per time point for *Nvtra* or *Nvdsx* were calculated by comparing *Nvtra* or *Nvdsx* starting concentrations to starting concentrations of the reference gene *EF1α*. The average of these normalized values plus their standard errors were calculated to construct the graph in fig. 3A, as well as the ratio of *Nvtra* expression over *Nvdsx* expression (Table S1). A two-tailed unpaired *t-test* was used to calculate significance gene expression level differences between the samples from unfertilized eggs and the samples from fertilized eggs. An One-Way ANOVA was used to calculate significant *Nvtra* expression level differences between samples from fertilized eggs and unfertilized eggs, 9-23 h after egg laying.

This set of five replicates for 12 time points was also used to analyze the source of *Nvtra* expression in embryos from fertilized eggs during embryonic development. For this all five replicates were mixed in an equal ratio and one micro liter was used in a RTPCR with 400 nM primers NvTra_poly_F1 and NvTra_poly_R1 with 95°C for 3 min, 35 cycles of 95°C for 15s, 57°C for 30s and 72°C for 30s. Another RT-PCR was done to analyze the sex specific splicing in this sample set. Again one micro liter of the mixed set was used in a RT-PCR with primers NvTra_F2 (exon one) and NvTra_R3 (exon two) as mentioned in the ‘Splice form analysis following RNAi’. These primers give a fragment of 328 bp in samples without the deletion, 310 bp in samples with the deletion and 416 bp on genomic DNA. The PCR fragments were analyzed on a 2% non-denaturing agaraose gel stained with Ethidiumbromide.

Supplemental References

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Figure S1. Eye colour cross used to identify diploid males. Crossing scheme of injected st^{DR} / st^{DR} females (grey box) with AsymC males (st^{+}) with wild type eye color. F1 offspring in black box are diploid st^{DR} / st^{+} males. F2 female offspring are triploid st^{DR} / st^{DR} / st^{+} females with wild type eye color, sired by diploid males.

Figure S2. Flow cytometry on heads of adult *Nasonia.* (a) Normal haploid male. (b) Normal diploid female. (c) Diploid male. Peak P1 corresponds to haploid, P2 to diploid cells and P4 to tetraploid cells. Some endopolyploidization is evident in haploid males (P2) and diploid females (P4) (*S6*, *S7*).

Table S1. Mean normalized expression levels of *Nvtra* and *Nvdsx* during embryonic development of fertilized and unfertilized eggs in *N. vitripennis*. Ratio *tra* / *dsx* indicates relative expression (± S.E..) of *Nvtra* in embryos from fertilized versus unfertilized eggs divided by the relative expression (±S.E.) of *Nvdsx* in embryos from fertilized versus unfertilized eggs. Total indicates ratio *tra* / *dsx* (±S.E.) in embryos from fertilized and unfertilized eggs pooled together. Time indicates hours after egg laying.

File: 1185805s3.ai

Table S2. Overview of primers.

File: 1185805s4.ai

Mated

P

RNAi treated

♀ $\frac{st^{DR}}{st^{DR}}$ x ♂ $\frac{st^{+}}{}$

F1

♂ $\frac{st^{DR}}{}$ ♂ $\frac{st^{DR}}{st^{+}}$ x ♀ $\frac{st^{DR}}{st^{DR}}$

F2

♀ $\frac{st^{DR}}{st^{DR}}$ $\frac{}{st^{+}}$ ♂ $\frac{st^{DR}}{}$

Virgin

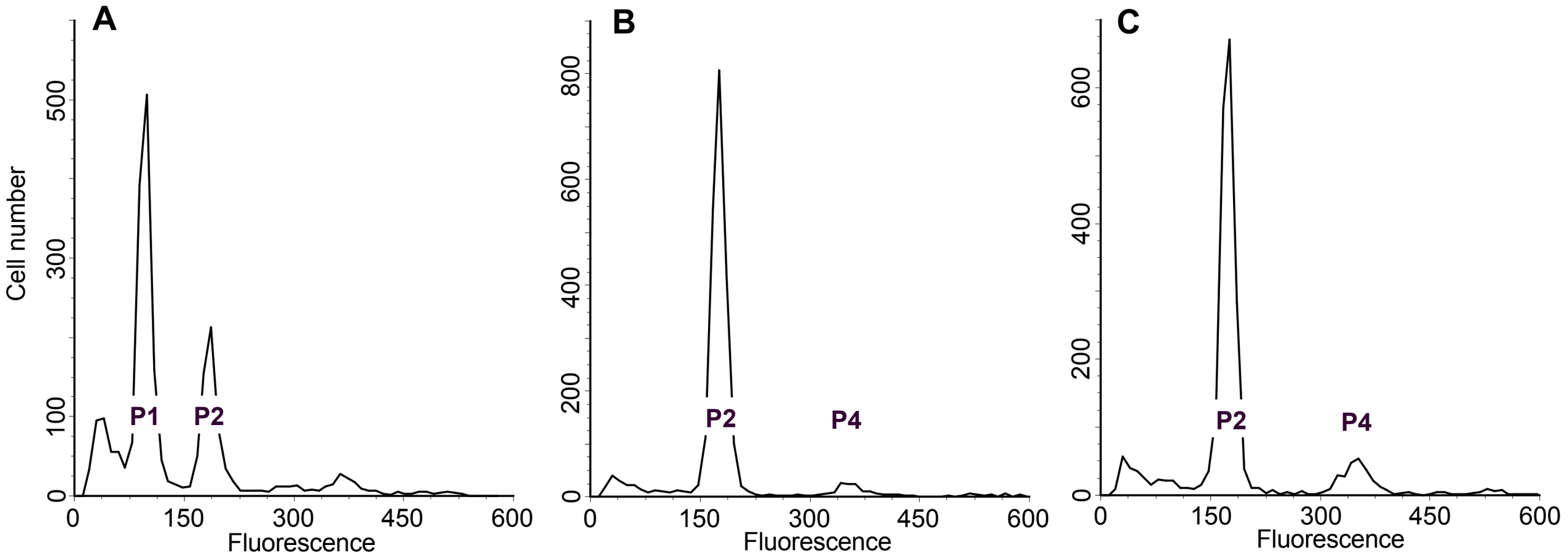
P

RNAi treated

♀ $\frac{st^{DR}}{st^{DR}}$

F1

♂ $\frac{st^{DR}}{}$


A
B
C
Cell number
0
100
300
500
200
400
600
800
P1
P2
P4
0
150
300
450
600
Fluorescence

	transformer		*doublesex*		Ratio *tra* / *dsx*		
Time	**unfertilized**	**fertilized**	**unfertilized**	**fertilized**	**unfertilized**	**fertilized**	**Total**
1	0.03450±0.01328	0.02266±0.00453	0.00370±0.00123	0.00331±0.00030	8.75424±0.85014	6.60760±0.76384	7.68092±0.64673
3	0.02351±0.01057	0.01519±0.00239	0.00268±0.00040	0.00287±0.00062	9.78664±4.98401	5.69537±0.82332	7.74101±2.47703
5	0.01104±0.00269	0.01112±0.00414	0.00264±0.00032	0.00204±0.00076	4.10580±0.63131	5.54875±0.70008	4.82727±0.50529
7	0.00673±0.00116	0.09919±0.02211	0.00210±0.00033	0.00108±0.00015	3.35696±0.50272	91.58505±14.77694	47.47101±16.27292
9	0.01164±0.00372	0.04961±0.01486	0.01168±0.00261	0.00485±0.00055	1.04364±0.23129	10.85896±2.57367	5.40601±2.02294
11	0.00767±0.00046	0.06718±0.02540	0.01111±0.00239	0.00519±0.00100	0.77298±0.10578	11.66565±2.51771	6.21931±2.16955
13	0.00497±0.00076	0.03346±0.00234	0.00610±0.00093	0.00336±0.00039	0.84935±0.10523	10.49024±1.25798	5.66979±1.71347
15	0.00596±0.00139	0.03395±0.00930	0.00761±0.00196	0.00538±0.00084	0.87902±0.25140	6.22116±1.24914	3.55009±1.07402
17	0.00490±0.00145	0.02457±0.00808	0.00642±0.00114	0.00475±0.00155	0.72356±0.15847	4.97909±0.68887	2.85133±0.78363
19	0.00198±0.00031	0.01347±0.00225	0.00340±0.00034	0.00276±0.00030	0.62082±0.12788	4.79588±0.68237	2.70835±0.76896
21	0.00310±0.00117	0.01640±0.00255	0.00326±0.00049	0.00285±0.00040	1.05517±0.41619	5.58703±0.78758	3.06933±0.88621
23	0.00578±0.00318	0.01736±0.00555	0.00245±0.00042	0.00269±0.00027	2.02779±0.75889	6.72179±2.22992	4.37479±1.35832

Gene	Application	Primer name	Primer sequence 5’ – 3’	Exon
transformer	RT-PCR	NvTra_poly_F1	GGATTGCTTGGATGGTACAG	1
transformer	RT-PCR	NvTra_poly_R1	TGGATGTTCACTACAACTTGTC	2
transformer	RT-PCR	NvTra_F2	GACCAAAAGAGGCACCAAAA	2
transformer	RT-PCR	NvTra_R3	GGCGCTCTTCCACTTCAAT	3
transformer	qPCR	NvTra_qPCR_F1	CGCCGTTCTAAGTCATTGAG	3
transformer	qPCR	NvTra_qPCR_R1	ATCGGAATAATGCCTATCGT	4/5
doublesex	RT-PCR	NvDsxU_F3	AGCCACTGCCGAGTATACCA	5
doublesex	RT-PCR	NvDsxM_R1	TCGGAGAAGATTGGCAGAAC	6
doublesex	qPCR	NvDsx_qPCR_F1	GGTGACATGCGTAGTTTGAG	3/4
doublesex	qPCR	NvDsx_qPCR_R1	CAAGTCGTGGATTTGGTTCG	4/5
transformer	RNAi	NvTra_RNAi_F1	[TAATACGACTCACTATAGGG]CGAGACATCAGTTAGAAGAT	5
transformer	RNAi	NvTra_RNAi_R1	[TAATACGACTCACTATAGGG]GTCTTGTGGTCCTATGAAAC	8
Rp49	RT-PCR	NvRp49_F1	GTGTACAGGCCGAAAATCGT	
Rp49	RT-PCR	NvRp49_R1	CGCTTCTTGCTGCTAACTCC	
EF1α	qPCR	NvEF1α_F1	CACTTGATCTACAAATGCGG	
EF1α	qPCR	NvEF1α_R1	GAAGTCTCGAATTTCCACAG	