Tra-2 Mediates Cross-Talk Between Sex Determination and Wing Polyphenism in Female *Nilaparvata lugens*

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ABSTRACT Sexual dimorphism and wing polyphenism are important and evolutionarily conserved features of many insect species. In this article, we found a cross-talk linking sexual differentiation with wing polyphenism in the brown planthopper (BPH) *Nilaparvata lugens* (order: Hemiptera). Knockdown of the sex determination gene *Transformer-2* in *N. lugens* (*NTra-2*) in nymph caused females to develop into infertile pseudomales containing undeveloped ovaries. Whereas males treated with ds*NTra-2* exhibited normal morphology, but lost fertility. Knockdown of *NTra-2* in adult females (maternal RNAi) resulted in long-winged female offspring, indicating that maternal RNAi changed the wing morphs in female offspring. In addition, silencing of *NTra-2* down-regulated the expression of the *forkhead* transcription factor FoxO (*NI*FoxO), and simultaneously up-regulated the expression of *phosphatidylinositol-3-OH kinase* (*PI(3)K*)-protein kinase B (*NI*Akt), the two critical genes in the insulin signaling pathway. Furthermore, the long-winged effect caused by maternal ds*NTra-2* RNAi could be reversed by silencing of *NlInR1* and *NI*Akt, leading to short-winged morphs. We propose that there is a cross-talk between the sexual differentiation and wing polyphenism pathways mediated by *NTra-2* during embryonic stages.

KEYWORDS sex determination; wing morphs; *Transformer-2*; BPH; cross-talk

GENES that commit an embryo to either a male or female developmental pathway are known as sex determination genes. Recent studies in representative species of holometabolous insects suggest that sex determination genes are well conserved. Many of the complex genetic regulatory hierarchies of Diptera have been well characterized, including the sex determination pathway of *Drosophila melanogaster*.

In *D. melanogaster*, X chromosome dose, or the ratio X:A, is the signal that determines female or male gender (Penalva and Sanchez 2003; Cmkovich *et al.* 2007). *Doublesex* (*Dm*dsx) is the final gene in the cascade and shows sex-specific splicing that promotes either female or male sexual development (Burtis and Baker 1989). In females, *Sex-lethal* (*Sxl*) acts as a molecular

memory device and is activated by an X:A ratio of 1, or XX. Functional *Sxl* can not only regulate its own pre-mRNA to be female specific, but it can also activate the feminizing switch gene *Transformer* (*Tra*) through female-specific splicing of its pre-mRNA (Cmkovich *et al.* 2007). Functional *Tra* protein induces the splicing of *Dm*dsx into the female form with the help of *Transformer-2* protein (*Tra-2*) (Amrein *et al.* 1988; Hoshijima *et al.* 1991). In males, there is no signal to turn on the switch. Therefore, without functional *Tra* protein, *Dm*dsx is spliced into the male form. From then on, sex-specific *Dm*dsx isoforms control many aspects of somatic sexual differentiation, including the development of genitalia, sex combs, abdominal tergites, the nervous system, and courtship behavior.

The *D. melanogaster* gene *Tra-2* (*DmTra-2*), which also shows sex-specific splicing, plays a critical role in both female and male development (Amrein *et al.* 1988; McGuffin *et al.* 1998). In females, the *DmTra-2* protein contains an RNA Recognition Motif (RRM) domain and SR (serine/arginine)-rich regions, which heterodimerize with *DmTra* and bind the *Dm*dsx pre-mRNA. In males, auto-regulation of *DmTra-2* splicing results in two distinct proteins, *DmTra-2*¹⁷⁹ and *DmTra-2*²²⁶. *DmTra-2*²²⁶ functions in the germ line, and mutant males

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lacking *DmTra-2* lose fertility due to sperm immotility (Belote and Baker 1983; Madigan *et al.* 1996; McGuffin *et al.* 1998). In other dipteran insects, such as *Musca domestica*, *Ceratitis capitata*, and *Anastrepha suspense*, knockdown of *Tra-2* influences female-specific splicing of the genes *tra* and *dsx* and causes females to transform into pseudomales (Salvemini *et al.* 2009; Sarno *et al.* 2010). In *Aedes aegypti*, knockdown of *Tra-2* causes segregation distortion at the level of gametic function, yielding up to 100% male offspring, rather than triggering female to male sex conversion (Hoang *et al.* 2016). The functions of *Tra-2* in other holometabolous insects have also been well documented. In *Apis mellifera*, knockdown of *Transformer-2*, which has six protein isoforms without sex-specific expression, affects embryonic viability and female splicing of the *fem* and *doublesex* transcripts (Nissen *et al.* 2012). In *Bombyx mori*, *Tra-2* does not regulate the sex-specific splicing of *doublesex*, although it is required for normal testis development (Suzuki *et al.* 2012).

The brown planthopper (BPH), *Nilaparvata lugens*, of the order Hemiptera, is an incomplete metamorphic insect in which males have an XY sex-determining mechanism and females have an XX sex-determining mechanism, and in the sexual dimorphism, the females had bigger wing area and body size than males (Den Hollander 1982; Kobayashi and Noda 2007). BPH is the most destructive rice pest around Asian countries. As a typical monophagous herbivore, it migrates over long distances every year to locate its only food source, rice sap (Xue *et al.* 2014). Migratory BPHs of both sexes are long-winged morphs with fully developed forewings and hindwings, whereas short-winged morphs with undeveloped wings are obligate flightless, but usually possess higher fecundity than the winged morphs, and no obvious sex biases in wing morphs have been identified in wild type (Xu *et al.* 2015). Environmental cues, such as population density and host quality, are thought to be relevant to wing morph switching (Iwanaga *et al.* 1985; Iwanaga and Tojo 1986; Syobu *et al.* 2002). Apart from environmental cues, wing dimorphism in the BPH might be genetically determined. Iwanaga *et al.* found that the short-winged morphs of female BPHs might be controlled by a single pair of dominant alleles, although male BPHs did have this type of regulation; therefore, they hypothesized that BPH wing morphism is regulated through sex-limited inheritance (Iwanaga *et al.* 1985). Recently, Xu *et al.* reported that a specific nutrient-sensing pathway, the insulin/insulin-like growth factor signaling pathway, determined alternative wing morphs; in particular, they showed that two insulin receptors (InR1 and InR2) play opposing roles by regulating the activity of forkhead transcription factor (FoxO) via the phosphatidylinositol-3-OH kinase (PI(3)K)-protein kinase B (Akt) signaling cascade (Xu *et al.* 2015).

In this paper, we report that *NTra-2* plays an important role in sex determination in BPH. The results showed that maternal RNA interference (RNAi) *NTra-2* caused female offspring to be strictly long-winged by influencing genes of the insulin signaling pathway. We found that there is a cross-talk between wing polyphenism and the sex differentiation pathway in BPH.

Materials and Methods

Insect rearing and embryo collection

The BPH populations used in this paper were collected in Hangzhou (30°16'N, 12°11'E), China, in 2008. BPHs were reared at $26 \pm 0.5^\circ$ on rice seedlings (Xiushui 128) under a 16 hr light: 8 hr dark photoperiod. Immature eggs were collected by dissecting virgin females 3 days after emergence. Eggs were collected by dissecting rice seedlings on which mated females laid.

Cloning of *NTra-2*

The *DmTra2* (CAA40722.1) sequence was downloaded from NCBI and used as a query to search for homologous genes in the BPH transcriptome (SRX023419). The full open reading frames (ORFs) from female and male complementary DNA (cDNA) libraries were amplified using the primers *NTra-2*-ORF-S (CGAGGAATCATCTTGTT) and *NTra-2*-ORF-AS (CGTCAGTCACGGTTTAG). The resulting PCR products were cloned into the PMD-19T vector (TaKaRa, Dalian, China) and then sequenced.

Sequence analysis and comparison

The Splign tool from NCBI (www.ncbi.nlm.nih.gov/sutils/splign/splign.cgi) was used to predict the exons and introns of *NTra-2*³⁰¹ and *NTra-2*²⁹⁸ by aligning the sequences of the RT-PCR products with the genomic sequence of BPH (PRJNA177647). The alignment of *NTra-2* and other *Tra-2* sequences was generated using Clustal X (JEANMOUGIN *et al.* 1998) and GENEDOC (Nicholas and Nicholas, 1997, <http://www.softpedia.com/get/Science-CAD/GeneDoc.shtml>).

RNA interference

The two common regions of the *NTra-2* isoforms were used as DNA templates for double-stranded RNA (dsRNA) synthesis, and the primers were ds*NTra-2*-S-1 (5'-TAATACGACTCAC TATAGGGAGACGAGGAGTCCCATGTCGA-3'), ds*NTra-2*-AS-1 (5'-TAATACGACTCACTATAGGGAGACGGTTTAGTAGCGTCT GG-3'), ds*NTra-2*-S-2 (5'-TAATACGACTCACTATAGGGAGA TCATGGAGGCAGCAAT-3'), and ds*NTra-2*-AS-2 (5'-TAATAC GACTCACTATAGGGAGACCCGTGTGTCGAGAGC-3'). The MEGAscript T7 High Yield Transcription Kit (catalog no. AM1334; Ambion) was used to synthesize the dsRNAs, according to the manufacturer's instructions. The concentration of the product was quantified using a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA). Afterward, we followed the BPH dsRNA treatment method described by Xue *et al.* (Xue *et al.* 2015, <http://dx.doi.org/10.1038/protex.2015.005>).

Absolute quantification in real-time qPCR

PCR products (Primer-S: GAGCACATTCTTCAGGTAA; Primer-AS: CGTATTTTCGAGAATATGTTGAG) were purified and quantified. The products were serially diluted eightfold, and the logarithm of their initial template copy numbers and the Ct values of the primers in every dilution were used to construct standard curves for both *NTra-2*³⁰¹ and *NTra-2*. The concentrations of the PCR products were measured using a NanoDrop

Figure 1 Genomic organization of *NITra-2* and alignment of *Tra-2* homologous sequences. (A) Genomic organization. Alternative splicing of the 5' splicing site (5'SS) in the *NITra-2* ORF. The black boxes denote exons, and the black lines denote introns. (B) Multiple alignment of *NITra-2* with homologs from insect species. *D. melanogaster* (NP_476764.1), *Anastrepha amita* (CBJ17290.1), *C. capitata* (NP_001266337.1), *Lucilia cuprina* (ACS34688.1), *Bactrocera tryoni* (AIK25409.1), *Tribolium castaneum* (AHF71088.1), *A. aegypti* (AGW27097.1), *Bombyx mori* (NP_001119705.1), and *A. mellifera* (NP_001252514.1). The alignment was made using Clustal X and GENEDOC. The arginine/serine-rich (R1 and R2) domains are indicated by single lines, the RNA recognition motif (RRM) is indicated by a broken line, the ribonucleoprotein-1 (RNP-1) and ribonucleoprotein-2 (RNP-2) regions are indicated by braces, and the linker region is indicated by a plus sign.

2000 (Thermo Fisher Scientific), and the copy numbers of the PCR products were calculated using the following equation:

$$\text{DNA(copy)} = \frac{6.02 \times 10^{23} (\text{copy/mol}) \times \text{DNA amount(g)}}{\text{DNA length(dp)} \times 660 (\text{g/mol/dp})}$$

Whelan *et al.* (2003), Lee *et al.* (2006). The Ct values of the dilutions were obtained by real-time qPCR with Primer1 and Primer2 (Primer1S: GAGCACATTCTTCAGGTAA, Primer1AS: TCCATGATCGCTAGTGTT; primer2S: GCGGACGGCTTTGAGT, primer2AS: CGTATTTGAGAATATGTTGAG). The PCR amplification efficiency (*E*) of each primer was obtained using the Bio-Rad CFX manager 3.1 (Bio-Rad Laboratories, Hercules, CA) (Supplemental Material, Figure S1). Ideally, the value of *E* should range from 90 to 110%, and in this study, *E* (Primer1) was 95.5% and *E*(Primer2) was 104.9%.

PCR of male-specific genomic DNA fragment

Genomic DNA for every PCR was purified from 5 to 10 treated planthoppers (5 females, 10 males) using the Wizard Genomic

DNA Purification Kit, according to the manufacturer's instructions (Promega, Madison, WI), starting with 600 µl Nuclei Lysis Solution, and the genomic DNA was concentrated and desalted by isopropanol precipitation. The DNA concentrations were measured and diluted to 50 ng/µl. Primers for a male-specific sequence (PM3nF: 5'-GAGCTGGAGTGTGTTGATGT-3' and PM3nR: 5'-CAAGTTGATTGAAACAGACT-3') and primers for a sequence common to both sexes (PM3femaleF: 5'-ATCATACCCGCATGTTGGAC-3' and 5'-GAATCTGAAGGGAGCTGTGC-3') were used as previously described (Kobayashi and Noda 2007). PCR was performed on 1 µl genomic DNA in a 25-µl reaction mixture (50 units/µl Taq DNA polymerase, W/Mg²⁺ buffer for DNA polymerase, Biocolors, Shanghai, China; 2 µmol dNTPs, TaKaRa). Thirty cycles of amplification were performed, each of which consisted of denaturation for 30 sec at 94°, annealing for 30 sec at 56°, and extension for 30 sec at 70°. Samples of the PCR reactions were analyzed on 2% agarose gels.

Quantitative real-time PCR (qRT-PCR) analysis

Total RNA was first isolated from the BPHs and tissues using RNAiso Plus (TaKaRa), and then each RNA sample (1 µg) was

Figure 2 Expression of *NITra-2*. (A) Expression of *NITra-2*³⁰¹ (blue) and *NITra-2*²⁹⁸ (red) in female and male BPHs. As there were only two splicing isoforms of the gene *NITra-2* in BPH, the absolute copy number of *NITra-2*²⁹⁸ (ACN) = ACN (*NITra-2*) – ACN (*NITra-2*³⁰¹). (B) Expression of *NITra-2* in different developmental stages and sexes. Primer2 was used to detect expression of *NITra-2* in the following different stages: unfertilized eggs, embryos, first, second, third, fourth, fifth-instar nymphs, female and male adults, and gene *N18s* rRNA was used as a positive PCR control.

reverse transcribed using the PrimeScript first strand cDNA synthesis kit (catalog no. 6110A; TaKaRa). The internal control for the qRT-PCR was the 18S rRNA gene of BPH (*N18s*-S: GTAACCGCTGAACCTCCT, *N18s*-AS: TCCGAAGACCTCAC TAAATC), and the SYBR Premix ExTaq Kit (TaKaRa) was used for qRT-PCR. The relative quantitative method ($\Delta\Delta CT$) was used to evaluate quantitative variations.

Semiquantitative RT-PCR

Total RNA was extracted from unfertilized eggs, embryos, first-, second-, third-, fourth-, fifth-instar nymphs, and female and male adults, respectively, and 1 μ g RNA was used to perform reverse

transcription in 20- μ l reactions using Quant reverse transcriptase (TIANGEN, Beijing, China) according to the manufacturer's instructions, diluted 10 times, and 1 μ l was used in the following PCR reaction. The PCR reactions were performed on 1 μ l of Primer 2 and Primer of *N18s* rRNA gene. The PCR conditions included 5 min of denaturation at 94°; 30 cycles of 30 sec at 95° and 30 sec at 60°; followed by final extension at 72° for 10 min.

Dissection and fertility analysis

Third-instar nymphs were injected with dsGFP or ds*NITra-2* and then dissected or used for fertility analysis 3 days after emergence. The treated BPH pair was reared on rice seedlings,

Figure 3 Phenotypes of dsGFP- and ds*NITra-2*-treated female and male adults. (A, a) Adult females treated with dsGFP at the third-instar stage. (B, b) Adult females treated with ds*NITra-2* at the third-instar stage. Abnormal tissues are indicated. (C, c) Adult males treated with dsGFP at the third-instar stage. (D, d) Adult males treated with ds*NITra-2* at the third-instar stage. All BPHs were collected on the third day after emergence. The external genitalia names of ds*NITra-2* males are labeled in B and b.

Figure 4 *NITra-2* regulates tissue development in a sex-dependent manner. (A) Adult body length in *dsGFP* females (*dsGFP*, $n = 20$), *dsGFP* males (*dsGFP*, $n = 19$), *dsNITra-2* females (*dsNITra-2*, $n = 21$), and *dsNITra-2* males (*dsNITra-2*, $n = 20$) treated at the second instar. Body length in *dsNITra-2* females (3.91 ± 0.035 mm) was shorter than in *dsGFP* females (4.26 ± 0.038 mm), although it was still longer than in *dsGFP* males (3.03 ± 0.030 mm). (B) Ovipositor length in *dsGFP* ($n = 21$) and *dsNITra-2* ($n = 21$). Ovipositor length in *dsNITra-2* females was shorter than in *dsGFP* females by $>35\%$. (C) Wing area in *dsGFP* and *dsNITra-2* females and males treated at the second-instar stage ($n = 20$). (D) Aedeagus length in *dsGFP* ($n = 18$) and *dsNITra-2* males ($n = 18$). Student's *t*-test was used (* $P < 0.01$, ** $P < 0.001$).

and the number of eggs was examined 10 days after deposition. Each pair of adults was fed on the same rice seedling. At least 10 pairs of adults per treatment were tested. Nymphs from the first, second, third, fourth, and fifth-instar stages were injected with *dsGFP* and *dsNITra-2*, and their phenotypes were recorded after emergence.

Image processing

dsRNAs targeting *GFP* and the common region of *NITra-2*³⁰¹ and *NITra-2*²⁹⁸ were injected into third-instar nymphs, and adult body length, wing area, aedeagus length, and ovipositor length were determined after emergence using a DFC320 digital camera attached to a LEICA S8APO stereomicroscope

and measured with ImageJ (National Institutes of Health, Bethesda, Maryland).

Sperm viability

The LIVE/DEAD Sperm Viability Kit (L-7011) (Invitrogen, Carlsbad, CA) was used to measure sperm activity, according to the manufacturer's protocol. The semen was released by cutting open the vas deferens with dissection scissors in 300 μ l buffer (10 mM HEPES, 150 mM NaCl, 10% BSA, pH 7.4). Then, 5 μ l diluted SYBR14 dye was added and incubated for 10 min at 36°. Next, 5 μ l propidium iodide was added to 300 μ l buffer and incubated for another 10 min. Finally, the number of green sperm (live) and red sperm (dead) in a 20- μ l

Figure 5 Influence of *NITra-2* on females in different instars. (A and G) The external genitalia of *dsGFP*-treated males and females, respectively. (B–F) The external genitalia of adult females treated at the first, second, third, fourth, and fifth-instar stages, respectively, with *dsNITra-2*. The external genitalia are labeled in A, D, and G.

Figure 6 Influence of *NITra-2* on the development of ovaries and testes. (A and B) Ovaries of *dsGFP*- and *dsNITra-2*-treated virgin females 3 days after emergence. (C and D) Testes of *dsGFP*- and *dsNITra-2*-treated males 3 days after emergence. (E and F) Sperm from *dsGFP* and *dsNITra-2* male adults 3 days after emergence; green indicates live sperm and red indicates dead sperm.

sample were counted using a Zeiss LSM 780 confocal microscope (Carl Zeiss MicroImaging, Göttingen, Germany). Each semen sample represented 10 individual insects, and each treatment was pooled from three independent experiments.

Maternal RNAi and egg counting

Virgin female adults collected <12 hr after emergence were injected with *dsGFP* and *dsNITra-2*, reared for 3 days, and then mated with wild-type males. Each pair was reared separately. The offspring from each day were reared separately, and the emergence time and number and wing morphs of the offspring were counted every day. When the offspring had grown to the 48th hr of the fifth instar, their wing buds were dissected, and the sex of the fifth-instar offspring was identified by the gonotome difference (Ding *et al.* 2012).

Detection of *NIFoxO* protein in wing buds

Virgin female adults collected <12 hr after emergence were treated with dsRNAs targeting *GFP* and *NITra-2* respectively, mated with males, and their offspring were reared. Wing buds ($n = 80$) were collected from fifth instars (48 hr after ecdysis), homogenized in 200 μ l RIPA (Thermo Fisher Scientific), quantified using the BCA method (catalog no. 23227; Thermo Fisher Scientific), and denatured in SDS-PAGE loading buffer. In total, 45 μ g protein was loaded per lane of the SDS-PAGE gel, and then transferred to a PVDF membrane. Western blotting was performed using antibodies against *NIFoxO* and β -Actin (1:5000), which were prepared by our laboratory (Xu *et al.* 2015). Blots were imaged using a Chemiluminescence Detection Kit (Bio-Rad) with Bio-Rad Molecular Imager ChemiDoc XRS+ System (Bio-Rad).

Table 1 Influence of *NItra-2* on female and male fertility (offspring number)

Treatment	#1	#2	#3	Average
Wild-type female × wild-type male	192	100	99	130 ± 38
ds <i>NItra-2</i> female × wild-type male	0	0	0	0
Wild-type female × ds <i>NItra-2</i> male	0	0	0	0

Data availability

The BPH strain generated in this study is available upon request. Sequences of *Nltra-2* are available at GenBank, with accession numbers: MF942728 and MF942729.

Results

NItra-2 produces two non-sex-specific splicing isoforms

To identify the homologous genes of *Tra-2* in BPH, the protein sequence of *D. melanogaster* *Tra-2* (Dm*Tra-2*) (NP_476764.1) was used as a query to blast the BPH transcriptome (SRX023419). Two transcripts homologous to *DmTra-2* were identified in *N. lugens* (*NItra-2*³⁰¹ and *NItra-2*²⁹⁸) and subsequently amplified by PCR. A comparison with genomic sequences (BioProject PRJNA177647) revealed that the two *NItra-2* transcripts were generated by alternative 5' splicing, but shared most amino acids (Figure 1A and Figure S1).

Multiple alignments of *NItra-2* protein with counterparts from other insects showed a high degree of sequence conservation in certain domains. These included domains that were common to *Tra-2* in different species, including the RRM, Ribonucleoprotein-1 (RNP-1) octapeptide, and Ribonucleoprotein-2 (RNP-2) hexapeptide. These domains confer the *Tra-2* to bind single-stranded nucleic acids (Dreyfuss *et al.* 1988; Niu *et al.* 2005) (Figure 1B).

Given two *Tra-2* isoforms were identified in *N. lugens*, we investigated the expression levels of *NItra-2*³⁰¹ and *NItra-2*²⁹⁸ by absolute quantification (Figure S1). Results showed that *NItra-2*³⁰¹ and *NItra-2*²⁹⁸ were present in both BPH females and males. However, the expression of *NItra-2*³⁰¹ was five times greater than *NItra-2*²⁹⁸ in both sexes (Figure 2A). In addition, *NItra-2* (both *NItra-2*²⁹⁸ and *NItra-2*³⁰¹) were expressed across developmental stages, including unfertilized eggs, laid eggs, first-, second-, third-, fourth-, fifth-instar nymphs, and female and male adults (Figure 2B), which suggests that *NItra-2* could play important roles during all BPH development stages.

NItra-2 influences somatic sexual development of females and males

RNA interference (RNAi)-mediated gene silencing was performed using dsRNAs targeting the common region of *NItra-2*³⁰¹ and *NItra-2*²⁹⁸. Third-instar BPH nymphs were

Figure 7 The influence of maternal RNAi knockdown of *NItra-2*. (A) The efficiency of maternal ds*NItra-2* on offspring development: eggs ($n = 200$), first ($n = 30$), second ($n = 20$), third ($n = 15$), fourth ($n = 10$), and fifth instar ($n = 10$). The cDNA copy number of *NItra-2* in different instars of offspring from dsGFP-treated females are shown in blue columns, and the cDNA copy number of *NItra-2* in different instars of offspring from ds*NItra-2*-treated females are shown in red columns. (B) The number of eggs from dsGFP- ($n = 9$) and ds*NItra-2*-treated females ($n = 8$). Pairwise statistical comparisons were performed using Student's *t*-test, and results are indicated with asterisks on top of the columns (** $P < 0.001$). (C) The female/male ratio of dsGFP female offspring (blue) and ds*NItra-2* female offspring (red).

Figure 8 Wing polyphenism and nymph duration of offspring of *NITra-2* maternal RNAi. (A) The percentage of short-winged females and males of offspring after maternal RNAi; the percentage of long-winged females and males of offspring after maternal RNAi. Red indicates short winged and blue indicates long winged. (B) The nymph duration of offspring after maternal RNAi; red indicates females and blue indicates males (Student's *t*-test, ***P* < 0.001).

injected with *dsNITra-2*, which led to over 80% down-regulation of its expression 3 days later (Figure S3A). After adult eclosion, we found that *dsNITra-2*-treated females were developed into pseudomales containing male external genitalia, such as claspers and intromittent organs (Figure 3B and b and Figure S2). To further confirm the sex of these insects, genomic DNA was isolated for PCR verification using the male-specific primers PM3n (Kobayashi and Noda 2007). The results verified their genomes are female. In addition, ovipositor length, wing size, and body length in *dsNITra-2*-treated females were smaller compared with *dsGFP*-treated females (Figure 4). For *dsNITra-2*-treated males, no obvious differences in wing, intromittent organ, or testes morphology were observed, except for increased body size. Moreover, the external genitalia showed no discernible change in *dsNITra-2*-treated males (Figure 3, C, D, c, d and Figure 4).

To better understand the influence of *NITra-2* on the BPH females, nymphs from different instar stages were injected with *dsGFP* and *dsNITra-2*. We found that the earlier female nymphs were injected with *dsNITra-2*, the more efficient the masculinization was (Figure 5). *dsNITra-2*-treated first- and second-instar female nymphs transformed into pseudomale adults and showed genital segments and external genitalia that were very similar to males in the ninth abdominal segment. However, these pseudomale adults remained shorter and had smaller ovipositors in the eighth abdominal segment (Figure 5, B and C). Females treated with *dsNITra-2* at the fourth instar had shorter ovipositors, compared to *dsGFP*-treated females, whereas no morphological difference was observed in females treated with *dsNITra-2* at the fifth instar.

Taken together, these findings demonstrate that the *NITra-2* gene is required for sex determination in BPH.

BPH females and males became infertile without *NITra-2*

To investigate whether *NITra-2* also influences fertility in BPH, adult females treated with *dsNITra-2* at the third instar were dissected 3 days after emergence. Results showed that the ovaries were poorly developed compared with *dsGFP* females (Figure 6, A and B), and all *dsNITra-2* females were infertile (Table 1). *dsNITra-2* and *dsGFP* males (3-day-old adult) treated at the third instar were also dissected, and there were no obvious differences in the appearance of the male reproductive system between the two groups (Figure 6, C and D). However, no offspring was produced when *dsNITra-2* males mated with wild-type virgin females (Table 1). The sperm viability test showed that over 80% of the sperm from *dsNITra-2* males treated at the third instar were dead (Figure 6, E and F).

These results indicate that *NITra-2* influences fertility in BPH both for females and males.

Maternal RNAi knockdown of *NITra-2* influences reproduction without altering the sex ratio

A previous study demonstrated that BPH was susceptible to maternal RNAi (Xu *et al.* 2013). To test whether maternal RNAi of *dsNITra-2* could affect their offspring, we used RNAi to knock down *NITra-2* in newly emerged females. qRT-PCR tests indicated that the RNAi effects of *dsNITra-2* injected into newly emerged females were effective in their offspring from the egg to the third-instar nymph stages, and the expression of *NITra-2* recovered by the fourth and fifth instars (Figure 7A). Moreover, the number of offspring from *dsNITra-2*-treated females was only 20% of that from *dsGFP*-treated females, although the sex ratio was the same between them (Figure 7, B and C).

Nearly all female offspring treated with *NITra-2* maternal are long-winged morphs

Interestingly, we found that nearly all female offspring from females treated with *dsNITra-2* developed into long-winged adults, whereas the wing morphs of male offspring were the same as those from females treated with *dsGFP* or wild-type females. By contrast, >90% of offspring from females treated with *dsGFP* or wild-type females were short-winged under

Figure 9 Expression of BPH insulin pathway genes in nymph wing buds. (A) Expression of *NInR1*, *NInR2*, *NIAkt*, and *NIFoxO* in the wing buds of fifth-instar female offspring (48 hr) treated with ds*NITra-2* or ds*GFP*. (B) Expression of *NInR1*, *NInR2*, *NIAkt*, and *NIFoxO* in the wing buds of fifth-instar male offspring (48 hr) treated with ds*NITra-2* or ds*GFP* maternal RNAi. (C) Expression of *NInR1*, *NInR2*, *NIAkt*, and *NIFoxO* in the wing buds of fifth-instar female offspring (48 hr) treated with ds*NITra-2* or ds*GFP* at the third instar. (D) Expression of *NInR1*, *NInR2*, *NIAkt*, and *NIFoxO* in the wing buds of fifth-instar male offspring (48 hr) treated with ds*NITra-2* or ds*GFP* at the third instar. (E) Expression of *NIFoxO* protein in fifth-instar offspring (48 hr) wing buds of ds*GFP* and ds*NITra-2* groups. β-Actin was used to show equal protein loading. Error bars represent the SEM of three biological replicates; every replicate had >120 wing buds.

laboratory rearing conditions (Figure 8A). Moreover, ds*NITra-2* maternal RNAi also influenced nymph developmental duration in a sex-specific manner, with the developmental duration for female nymphs being 3–4 days longer than for normal offspring (Figure 8B). By contrast, injection of ds*NITra-2* at different nymph stages had no obvious influence on wing polyphenism or nymph duration (data not shown), suggesting that *NITra-2* influences BPH wing polyphenism and nymph duration during the embryonic period.

Cross-talk between the insulin and sex determination pathways in BPH

Xu *et al.* demonstrated that two insulin receptors control wing morphs in BPH (Xu *et al.* 2015). To investigate whether ds*NITra-2* maternal RNAi influences wing morphs in offspring females through the insulin pathway, we measured the expression levels of related genes (*NInR1*, *NInR2*, *NIAkt*, and *NIFoxO*) in wing buds of fifth-instar nymphs 48 hr after molting. In female

offspring nymphs treated with *NITra-2* maternal RNAi, the expression levels of *NIFoxO* were down-regulated >50% compared with those treated with ds*GFP* maternal RNAi, whereas the expression levels of *NInR2* and *NIAkt* were up-regulated. In male offspring treated with *NITra-2* maternal RNAi, wing bud genes showed no significant changes (Figure 9). As third-instar nymphs treated with ds*NITra-2* were primarily molted into short-winged and did not show female-specific long-wing morphs, we also determined the expression level of *NInR2* and *NIFoxO* in these wing buds, but found no significant differences between the ds*GFP* and ds*NITra-2* groups (Figure 9, C and D). Moreover, Western blotting analysis of fifth instars (48 hr) using an antibody against C-terminal of the target protein revealed almost no *NIFoxO* proteins in female offspring treated with ds*NITra-2* maternal RNAi compared with three other samples. This result indicates that *NIFoxO* plays a critical role in cross-talk between the insulin and sex determination pathways.

Because of increased *NTra-2* expression in fifth-instar nymphs after maternal RNAi (Figure 7A), we investigated whether this led to female-specific long-winged morphs. To this purpose, third-instar nymph offspring of the *dsNTra-2* maternal RNAi were again injected with *dsNTra-2* and *dsGFP*. In both cases, females primarily developed into long-winged morphs, indicating that the late expression of *NTra-2* had no influence on wing polyphenism (Figure 10, A and B and Figure S3B). Xu *et al.* reported that double-gene knockdown with *dsNInR1* and *dsNIAkt* led to short-winged morphs in BPH, as silencing either *NInR1* or *NIAkt* could activate *NI*FoxO activity, leading to short-winged morphs (Xu *et al.* 2015). Therefore, we repeated this double-gene knockdown with *dsNInR1* and *dsNIAkt* in fourth-instar offspring treated with maternal RNAi of *dsNTra-2* (Figure S3, C and D). We found that *dsNInR1* females tended to yield short-winged morphs, with shorter forewings and hindwings; males were all short-winged (Figure 10C). Moreover, *dsNIAkt* females became short-winged (Figure 10D). These results show that although female-specific low-level expression of *NI*FoxO can lead to long-winged morphs, silencing upstream genes that activate *NI*FoxO proteins can inhibit the influence of *dsNTra-2* maternal RNAi, leading to wing morphs intermediate between long and short morphs.

In conclusion, maternal RNAi of the *NTra-2* gene could influence offspring wing morphs in a female-specific manner, and the *NIAkt* and *NI*FoxO genes, which are downstream of the insulin pathway, play an important role in this phenomenon.

Discussion

Sexual dimorphism is one of the most important factors influencing insect morphology. Genes that determine female and male phenotypes influence morphological development in different sexes in distinct ways (Herpin and Schartl 2015). In *Drosophila*, females are larger than males, and Rideout *et al.* reported that the *Tra* gene acts as a link between the sex determination and insulin signaling pathways that promote growth specifically in females (Rideout *et al.* 2015). Gotoh *et al.* reported a causal link between the sex determination and JH signaling pathways, as *doublesex* determines the developmental fates and final sizes of nutrition-dependent secondary-sexual characteristics in *Cyclommatus metallifer* (Gotoh *et al.* 2014). Insulin has been reported to control trehalosemia in adults as well as sex-specific differences in locomotor activity in adult *Drosophila*, and insulin signaling also mediated the sexual attractiveness in *Drosophila* (Belgacem and Martin 2006; Kuo *et al.* 2012). In this paper, we found that there is cross-talk between the wing determination pathway (insulin pathway) and the sex determination mediated by *NTra-2* and that knockdown of this gene produced female-specific long-winged morphs in BPHs.

First, we confirmed that *NTra-2* was involved in the sex determination pathway. *NTra-2* has two splice isoforms (*NTra-2³⁰¹* and *NTra-2²⁹⁸*) that are not sex-specific (Figure 2A), and *NTra-2³⁰¹* and *NTra-2²⁹⁸* possess nearly identical

Figure 10 Wing morphs of maternal *dsNTra-2*-treated offspring injected with dsRNAs against various insulin pathway genes. (A) Females and males injected with *dsGFP* at the third instar. Females were primarily long winged, and males were primarily short winged. (B) Females and males injected with *dsNTra-2* at the third instar. Females were long winged, and males were primarily short winged. (C) Females and males injected with *dsNInR1* at the third instar. Females were long-winged, although the forewings and hindwings were shorter than in wild-type males. Males were short winged. (D) Females and males injected with *dsNIAkt* at the third instar. Females were short winged, and the forewings of males were smaller than in wild-type males.

ORFs (Figure S1). Similarly, *Tribolium castaneum* has three *TcTra-2* isoforms in both females and males that share common start and stop codons (Shukla and Palli 2013). Our

results showed that knockdown of *Tra-2* in female nymphs resulted in the development of pseudomales. Similar phenotypes were also observed in *Anastrepha*, *Ceratitis*, *Musca*, *Lucilia*, and *Bactrocera dorsalis* (Burghardt *et al.* 2005; Salvemini *et al.* 2009; Sarno *et al.* 2010, 2011; Liu *et al.* 2015). The novel finding of this study is that injecting ds*NTra-2* into earlier instars of female nymphs leads to more efficient masculinization of females. These results suggest that the influence of *NTra-2* in this hemimetabolous insect is accumulatory, a phenomenon that appears distinct from that in holometabolous insects.

Second, we found that ds*NTra-2* maternal RNAi could influence wing morphs in offspring in a sex-specific manner. The wing morphs of female offspring treated with maternal ds*NTra-2* were nearly all long-winged, and the offspring of ds*GFP*-treated females were nearly all short-winged, whereas the male offspring of both ds*NTra-2*- and ds*GFP*-treated females were primarily short-winged. Recently, Xu *et al.* reported that Insulin receptor 1 (InR1) and Insulin receptor 2 (InR2) play opposing roles in controlling long-wing vs. short-wing development by regulating *NI*FoxO, the key downstream transcription regulator that determines wing morph in BPH females and males. Inactivation of *NI*FoxO led to long wings, and activation led to short wings. Moreover, BPH nymphs treated with ds*NI*FoxO developed into long-winged adults without a sex bias (Xu *et al.* 2015). In this study, we found that in the wing buds of fifth-instar females treated with ds*NTra-2* maternal RNAi, *NI*FoxO transcription was only half as strong as in controls and that *NI*FoxO protein was nearly undetectable by Western blotting; in addition, expression of *NI*Akt in the buds of offspring treated with ds*NTra-2* maternal RNAi was higher than in ds*GFP*-treated offspring (Figure 9, B and E). However, there were no differences in the expression of *NI*FoxO and *NI*Akt between buds in ds*NTra-2* or ds*GFP* maternal RNAi-treated male offspring (Figure 9C). We also found that ds*NI*InR1 and ds*NI*Akt could inhibit the influence of ds*NTra-2* maternal RNAi, as female-specific wings tended to be short-winged (Figure 10, C and D). Our results suggest that ds*NTra-2* influences wing form in female offspring via the insulin pathway. We found that ds*NTra-2* maternal RNAi did not influence the sex ratio of offspring, although the nymph stage of ds*NTra-2*-treated maternal RNAi female offspring was 4 days longer than in other offspring, suggesting that *NTra-2* can influence female BPHs in a sex-specific manner (Figure 8B).

In recent studies by our group, we showed that *N. lugens doublesex* is not involved in this cross-talk (data not shown) (Figure 11). Whether there are other genes involved in sex determination in BPH requires further study. In our model of the cross-talk, maternal knockdown of *NTra-2* causes the genes *NI*FoxO and *NI*Akt, which are involved in the BPH wing determination pathway, to be down- and up-regulated, respectively. As ds*NTra-2* maternal RNAi only influenced wing morphs in female offspring, we propose that there are female-specific embryonic genes that are influenced by ds*NTra-2* and act as a bridge between the sex determination and wing determination pathways (Figure 11).

Figure 11 Cross-talk between the sex determination and wing determination pathways in BPH females. In this model, ds*NTra-2* maternal RNAi causes the up- and down-regulation of *NI*Akt and *NI*FoxO respectively, through some unknown female-specific embryonic genes, leading female offspring to adopt long-winged morphs. Genes involved in the cross-talk are indicated in red, unknown genes are indicated with question marks, ds*NTra-2* maternal RNAi is indicated by red scissors, and genetic up- or down-regulation are indicated by the red up or down arrows.

In conclusion, we found that *NTra-2* mediates cross-talk between sexual dimorphism and wing polyphenism. This is the first time that one sex determination gene (*NTra-2*) has been found to influence wing morphs in a sex-specific manner, and it demonstrates that the downstream genes of the wing determination pathway *NI*FoxO and *NI*Akt are not controlled strictly by insulin receptors (*NI*InR1 and *NI*InR2) but are also influenced by other factors. We also found that this cross-talk is limited in time to the embryonic stage. This is an unusual mechanism, and we speculate that *NTra-2* acts through embryonic genes to link these two pathways. Future studies should focus on identifying these embryonic genes to better understand the functions of sex determination genes in determining wing morphs.

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