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JNK signaling mediates wing form polymorphism in brown planthoppers (*Nilaparvata lugens*)

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ABSTRACT

Wing polyphenism is considered to be an adaptive trade-off between migration (long winged forms) and reproduction (short winged forms), determined by various environmental conditions. The c-Jun NH2-terminal kinase (JNK) is crucial for the regulation of the activity of a number of transcription factors, and is activated under stress and environmental fluctuations where it functions in maintaining cell viability and proliferation. We used RNA interference and a pharmacological inhibitor of JNK to test the role of JNK signaling in regulating the wing dimorphism of the brown planthopper, *Nilaparvata lugens*. Silencing *NlJNK* increased the proportion of short winged female adults, reminiscent of the effect of silencing inhibitory components of the insulin-signaling pathway, such as *NlAkt*. However, silencing of the *JNK*-activated transcription factors *NlJun* and *NlFos* did not change the wing form ratio significantly, indicating that *NlJNK* may not act through *NlJun* and *NlFos* in mediating this process. In summary, JNK signaling may play a role in determining wing polymorphism in *N. lugens* females.

1. Introduction

Wing polyphenism in insects represents an evolutionary trade-off between migration (long winged morphs) and reproduction (short winged morphs) (Zera and Brink, 2000; Zera and Denno, 1997). In the brown planthopper, *Nilaparvata lugens*, a serious pest of rice, morph development can be determined by various environmental conditions such as density, host plant condition, temperature, or exposure to pesticides (Zera and Brink, 2000; Zera and Denno, 1997). In *N. lugens*, developmental control of wing morph has recently been shown to be under the control of the transcriptional activator FOXO. RNA interference (RNAi) mediated knockdown of FOXO results in a developmental shift to the long winged morph (Lin et al., 2015a; Xu et al., 2015). FOXO activity in brown planthoppers is at least partly controlled by the insulin signaling pathway and its downstream effector kinase Akt, which acts as an inhibitor of FOXO (Xu et al., 2015). RNAi knockdowns of Akt and upstream members of the insulin-signaling pathway (including the insulin receptor and an insulin-like peptide ligand) have been shown to have an effect opposite to that of FOXO, i.e. they result in a developmental shift to the short winged morph (Lin et al., 2015a; Xu et al., 2015). The downstream effector status of FOXO is indicated by the fact that dual knockdowns of FOXO and insulin signaling genes result in the FOXO knockdown (long winged) phenotype (Lin et al., 2015a; Xu et al., 2015). Presumably, this system would in some way serve to regulate the development of individuals specialized for reproduction or dispersal (short or long winged) by determining the availability or quality of food through the insulin signaling pathway, although this link has not been demonstrated.

Of course nutritional status is not the only (and not necessarily even the primary) determinant of wing morph in naturally occurring populations of *N. lugens*. As mentioned, factors such as juvenile hormone, density, temperature, and pesticide exposure can alter proportions of long and short winged morphs, even in the presence of abundant nutritional resources (Bao et al., 2009; Xu et al., 2015). It has been recently shown that wounding increases expression of *NlFOXO*, and this leads to a developmental shift to the short winged morph (Lin et al., 2015b). It is not clear if this represents an adaptive shift, or is simply a consequence of a pleiotropic role for FOXO in wound healing. What is apparent is that the regulation of FOXO is complex, and that the effects of different signaling pathways can be manifested in changes in wing form.

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The c-Jun NH2-terminal kinases (JNK), part of the mitogen-activated protein kinase (MAPK) family, are members of a well-conserved signaling pathway that mediates responses to diverse environmental stressors, including UV irradiation and heat and osmotic stress (Gehart et al., 2010; Keshet and Seger, 2010; Kyriakis and Avruch, 2001; Sinha et al., 2011). Thus the JNK pathway is a potential candidate for mediating wing morph development in brown planthoppers. JNK proteins can phosphorylate a wide variety of targets, including of course the transcription factor Jun, a crucial mediator of cell division (Davis, 2000; Dhanasekaran and Reddy, 2008; Huang et al., 2003; Noselli and Agnes, 1999; Wang et al., 2003). In addition to Jun, JNK-mediated phosphorylation is known to activate effector molecules such as Fos, P53, Stat3, HSF1, and Elk1, and inhibit effectors such as STAT3, NFAT4 and NFATC1 (Davis, 2000; Dhanasekaran and Reddy, 2008; Huang et al., 2003; Noselli and Agnes, 1999; Wang et al., 2003). In addition, JNK can influence FOXO activity, although the response may be complex and vary by tissue, stimulus, and FOXO isoform (Ahn et al., 2012; Bode and Dong, 2007; Wang et al., 2003, 2005). For example, in human lung carcinoma cells, UV irradiation causes JNK-mediated activation of FOXO3a, through inhibitory phosphorylation of Akt by JNK (Wang et al., 2012). In contrast, in mice, JNK appears to act as a negative regulator of FOXO in neurons (Xu et al., 2011). Here we investigate a potential role for JNK signaling in mediating *N. lugens* wing polyphenism. We cloned and determined the expression patterns of the *N. lugens JNK*, *Jun* and *Fos* genes, and used RNAi-mediated knockdown and a chemical inhibitor to investigate the effect of the JNK, Jun and Fos proteins on wing dimorphism.

2. Materials and methods

2.1. Insect rearing

The brown planthopper (*N. lugens*) population was established from a culture provided by Z.R. Zhu (Zhejiang University, China) and maintained in the laboratory at China Jiliang University, Hangzhou, China. The long- and short-winged adults were categorized according to the length of the wings. Those with forewings extending past the posterior end of the abdomen were considered long-winged, while those with forewings shorter than the sixth abdominal segment and hind wings shorter than the first abdominal segment were considered short-winged. The insects were fed rice seedlings of IIyou-023 (*Oryza sativa* L. cv.) in the lab at 28 °C, under 14:10 h light:dark cycle at 70%–80% humidity. *N. lugens* were reared at densities of 6–10 animals per 100 cm^3 space. The ratio of planthopper wing forms of the insects reared under these conditions without treatment was used as a no treatment control (NC).

2.2. Cloning of NlJNK and NlJun

Total RNA was extracted from 1st–5th instar nymphs using the Trizol-based RNAiso Plus total RNA extraction kit (Takara, Dalian), and first strand cDNA was transcribed using the Roche Transcriptor First strand cDNA synthesis kit (Roche Applied Science, Shanghai).

The brown planthopper homologues of *NlJNK* and *NlJun* were identified from the transcriptome data and published genome sequence of *N. lugens* (Xue et al., 2014). The full-length JNK and Jun sequences used as templates for dsRNA synthesis were amplified by PCR using Ex-Taq polymerase (Takara, Dalian). The primers used were: NlJNKF: 5′ AATGATGAACTTGGCCTGCT 3′, NlJNKR: 5′ GAACCTCATGTGCGATTTGA 3′; NlJunF: 5′ AATGATGAACTTGGCCT GCT 3′, NlJunR: 5′ GAACCTCATGTGCGATTTGA 3′. NlFosF: 5′AAC GAATACCAGTGG CGATC3′, NlFosR:5′AGCTGATGGATTTCGCTCTG3′.

The PCR products were then purified using a gel purification kit (Omega bio-tek, USA), sub-cloned into the PMD18-T vector (Takara, Dalian), sequenced (Sunny Biotechnology, Shanghai), and then submitted to NCBI (*NlJNK*: KU363813, *NlJun*: KU363812, *NlFos*: KX023893). The sequences were translated and aligned with JNK homologues using Clustal W in DNASTAR lasergene 7. The NCBI Blast program (http://blast.ncbi.nlm.nih.gov) was used to identify homologues of *NlJNK* and *NlJun*.

2.3. dsRNA preparation and injection

The RiboMAX™ Large Scale RNA Production System-T7 (Promega, Beijing) was used to synthesize the double stranded RNA (dsRNA). The genes sub-cloned into the PMD18-T plasmid (Takara, Dalian) were used to re-amplify the DNA fragment by PCR as a template for dsRNA synthesis, and the PCR products were purified with a DNA gel purification kit (Omega bio-tek, USA). The dsRNA synthesis was carried out as described in Promega technical bulletin TB166. The primers used to amplify the dsRNA synthesis of *NlJNK*, *NlJun*, *NlFos*, *NlFOXO* and *Green Fluorescent Protein* (*GFP*, control) are listed in Table 1.

Fourth instar *N. lugens* nymphs were CO_2-anesthesized before injection and dsRNA was injected intra-thoracically. The injection was carried out using a Nikon microscope and Narishige injection system (MN-151, Narishige). 0.1 μg dsRNA was injected per nymph. The nymphs were allowed to recover for 2 h after injection and then reared on rice seedlings as above, and the insects were used for total RNA preparation 3 days after injection.

2.4. JNK inhibitor treatment

The Jun N-terminal kinase (JNK) Inhibitor CC-401 HCl [3-(3-(2-(Piperidin-1-yl)ethoxy)phenyl)-5-(1H-1,2,4-triazol-3-yl)-1H-indazole hydrochloride] (Selleck Chemicals, USA) (Bennett et al., 2001; Kanellis et al., 2010) was dissolved in DEPC water at 300 μM, and 0.2 μl of this solution was injected per 4th instar nymph.

Table 1
Primers for dsRNA synthesis.

Name	Sequence (5′–3′)
dsNlJNKF	TAATACGACTCACTATAGGGAGACCACTTCAGCCGGCATCATACATA
dsNlJNKR	TAATACGACTCACTATAGGGAGACCACAGCTGGAGCATTCACTTCGT
dsNlJunF	TAATACGACTCACTATAGGGAGACCACACTTCTACGAGGAGGCGTCA
dsNlJunR	TAATACGACTCACTATAGGGAGACCACTTCGTTGACGTTTCCTTTCC
dsNlJFosF	TAATACGACTCACTATAGGGAGACCACAACGAATACCAGTGGCGATC
dsNlFosR	TAATACGACTCACTATAGGGAGACCACAGCTGATGGATTTCGCTCTG
dsNlFOXOF	TAATACGACTCACTATAGGGAGACCACCTGTTCCCTGAATCGCCGCT
dsNlFOXOR	TAATACGACTCACTATAGGGAGACCACCGTTGCAGTCGAATCCGTCG
dsGFPF	TAATACGACTCACTATAGGGAGATTTGTATAGTTCATCCATGCCATGT
dsGFPR	TAATACGACTCACTATAGGGAGAATGAGTAAAGGAGAAGAACTTTTCA

Table 2
Primers for qPCR.

Name	Forward (5′–3′)	Reverse (5′–3′)
NlRPS15	TAAAAATGGCAGACGAAGAGCCCAA	TTCCACGGTTGAAACGTCTGCG
NlJNK	TTCAGTTGATGAGTGCGAAA	GATTTTGTCCCATTGATTGC
NlJun	TTATCTTCTCCCATGGTTCG	GTTTCAGCTGGTTGACGTTT
NlFos	TTTCGACTCCAAACAGCCTG	CAGGAAGGTCTGCTCAATGT
NlFOXO	ACCGGTTCATGCGCGTACAG	CTCGACGGCGAGCTGATTTG

2.5. Quantitative PCR

qPCR was carried out using Roche SYBR® Green PCR Master Mix and SYBR® Green RT-PCR Reagents kits (Roche Applied Science, Shanghai). The reverse transcription was carried out as described by the supplier (Roche Applied Science, Shanghai). In brief, a 25 μl reaction was used (Roche Applied Science, Shanghai), and 2 μl of diluted cDNA (20× dilution of the 1st strand cDNA synthesis) was used per qPCR reaction. The comparison of expression levels was carried out using the $2^{-\Delta\Delta Ct}$ relative expression method (Livak and Schmittgen, 2001). The reference genes were selected according to a previous study by Yuan et al.(Yuan et al., 2014). The primers used are shown in Table 2.

2.6. Imaging and statistical analysis

Images of adult brown planthoppers were taken using a Nikon stereomicroscope (SMZ745T) with NIS-Elements. Adobe Photoshop CS5 was used for image processing.

All statistical analyses were carried out using SPSS 20.0. For comparisons of gene expression levels between dsRNA treatments and controls, student's *t*-test was used. For comparisons of the ratios of wing forms between the control and dsRNA treatments, Chi-square test was used. Duncan's multiple comparison was used for comparing the expression level among different developmental stages and different tissues. Logistic multivariate regression analysis was carried out using SAS 9,1. The wing form was used as dependent variable, injection/no injection of *NlFOXO* dsRNA, *NlJNK* dsRNA and sex were used as independent variables.

3. Results

3.1. Cloning and developmental expression of NlJNK, NlJun and NlFos

We cloned and sequenced the full length JNK, Jun and Fos sequences from *N. lugens* (*NlJNK*). The translated *NlJNK*, *NlJun* and *NlFos* sequences are aligned with JNK, Jun or Fos sequences from other organisms in Figs. S1, S2 and S3, which show the high conservation of these sequences across diverse taxa.

QRT-PCR showed that *NlJNK*, *NlJun*, and *NlFos* are expressed throughout all developmental stages. In nymphs, expression of both genes peaks in the 4th instar, a period when wing fate is determined (Bertuso et al., 2002) and declines in the final (5th) nymphal stage, although the relative size of the peak was much greater for *NlJNK* (Fig. 1ABD). For comparison, we also measured the relative expression of *NlFOXO*, which has already been demonstrated to be critical in determining wing morph. *NlFOXO* has a similar expression pattern to *NlJNK*, *NlJun* and *NlFos*, including a significant increase in the 4th instar (Fig. 1C). However, expression of *NlFOXO* differs in that expression remains high in the 5th instar.

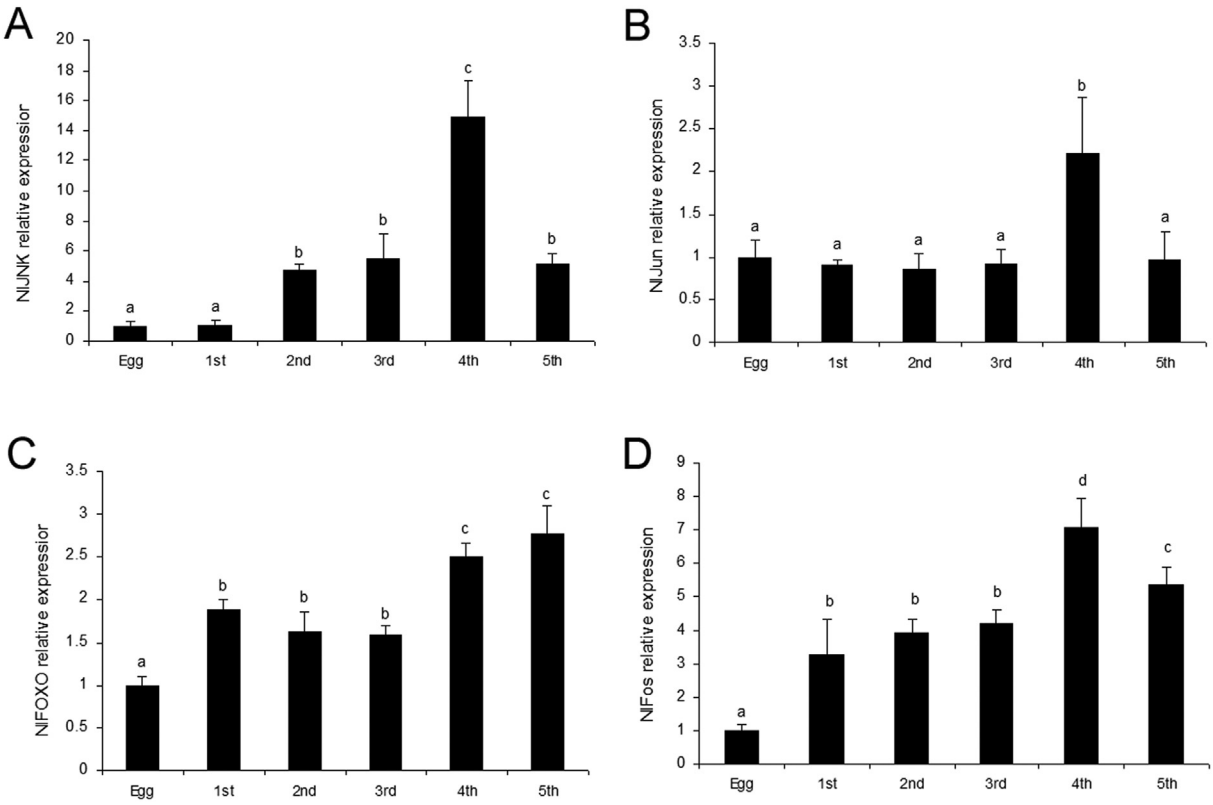

Fig. 1. Expression across *N. lugens* developmental stages of *NlJNK* (A); *NlJun* (B); *NlFOXO* (C); and *NlFos*(D). 1st through 5th indicates corresponding nymphal instar. Comparisons are by the $2^{-\Delta\Delta Ct}$ method, and all comparisons are relative to the amount of expression in egg for that gene. Unique letters indicate significance by Duncan's multiple comparison test, $P < 0.05$.

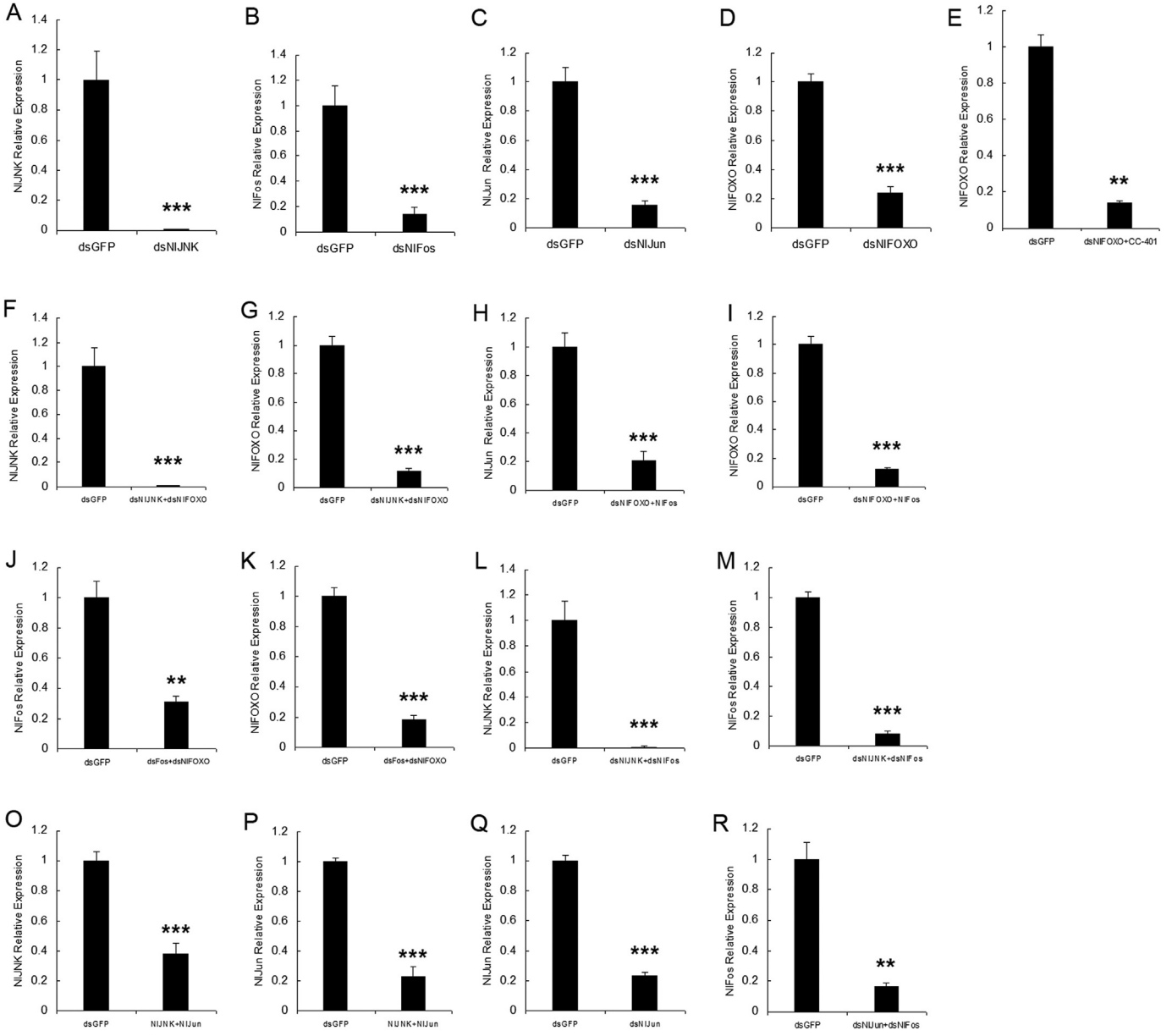

Fig. 2. QRT-PCR measurement of the expression of *NlJNK*, *NlFos*, *NlJun* or *NlFOXO* after dsRNA knockdown/double knockdown: A, *NlJNK*; B, *NlFos*; C: *NlJun*; D: *NlFOXO*; E, *NlJNK* + CC-401 (chemical inhibitor of JNK); F, G: *NlJNK* + *NlFOXO*; H, I: *NlJun* + *NlFOXO*; J, K: *NlFos* + *NlFOXO*; L, M: *NlJNK* + *NlFos*; O, P: *NlJNK* + *NlJun*; Q, R: *NlJun* + *NlFos*. Students *t*-test, **: $P < 0.01$; ***: $P < 0.001$.

3.2. Down regulation of JNK expression or inhibition of NlJNK activity increases the proportion of short winged females, but not males

To test for a role for *NlJNK* in mediating wing morph, we knocked down *NlJNK* in nymphs using RNAi. qRT-PCR measurement of the expression of *NlJNK* verified the dsRNA-mediated knockdown of *NlJNK* transcript (Fig. 2). *NlJNK* knockdown caused all females to develop into the short winged morph (Fig. 3A) [it should be noted that dsGFP injection does cause an increase in the proportion of short winged adults due to the effect of wounding (needle puncture) on *NlFOXO* upregulation (Lin et al., 2015a)]. However, knockdown of *NlJNK* did not cause a significant effect on male wing morph. In addition, injection of a pharmacological inhibitor of JNK, CC-401 (Han et al., 2001), into nymphs had a similar effect, significantly increasing the proportion of short-winged females to 98.1% compared to 87.7% for dsGFP controls (Chi-square test, $P < 0.05$, Fig. 3A), but having no significant effect on males (Fig. 3B).

3.3. Down-regulation of NlJun or NlFos does not change the wing morph ratio in males or females

Because of the effect of NlJNK on wing dimorphism, we also investigated its major targets, *NlJun* and *NlFos*. qRT-PCR measurement of the expression of *NlJun* verified the dsRNA-mediated knockdown of *NlJun* and *NlFos* transcripts (Fig. 2). However, down-regulation of either *NlJun* or *NlFos* by RNAi had no significant effect on the wing form ratio in males or females, and co-injection of *NlJNK* and *NlJun*/*NlFos* dsRNA together had no different effect than injection of dsNlJNK alone (Chi-square test, $P > 0.05$, Fig. 3). Thus we did not find any evidence that the effect of NlJNK on wing

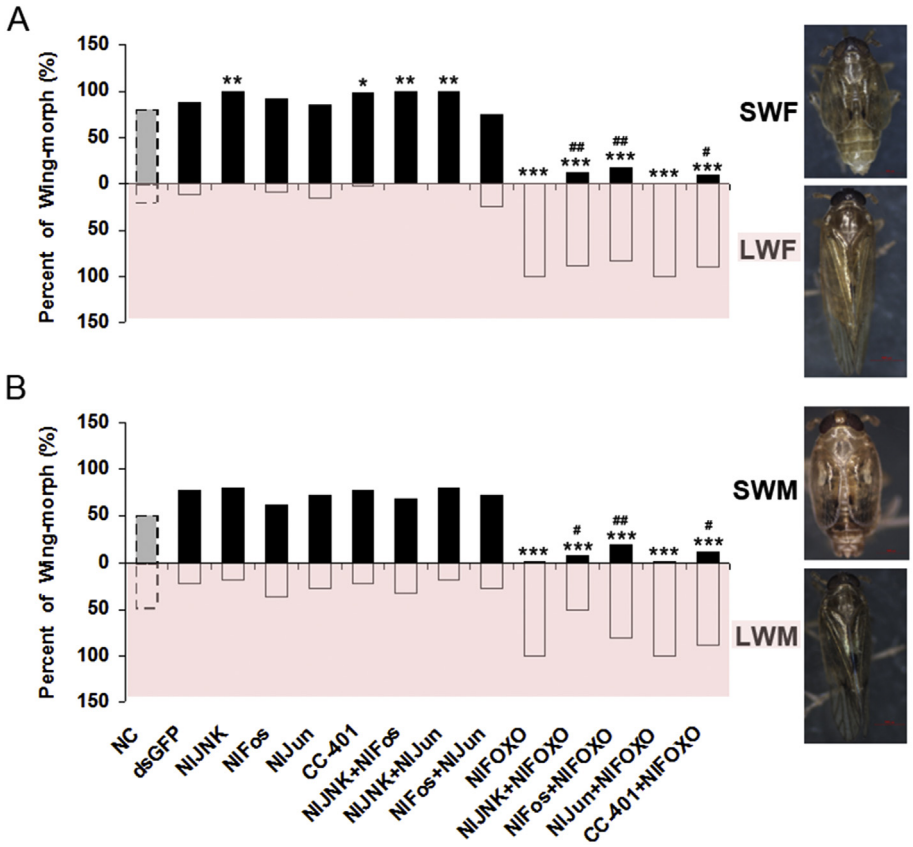

Fig. 3. Effect of injection of dsRNA or the JNK inhibitor CC-401 into 4th instar nymphs on adult wing morph ratios. A: female; B: male. NC (no treatment control) (n = 134 females, 103 males), *GFP* (n = 57 females, 52 males), *NlJNK* (n = 73 females, 71 males), *NlJun* (n = 134 females, 86 males), *NlFos*(n = 114 females, 80 males), CC-401 (n = 73 females, 35 males), *NlJNK* + *NlJun* (n = 98 females, 47 males), *NlFOXO* (n = 74 females, 50 males), *NlJNK* + *NlFOXO* (n = 53 females, 41 males), *NlJun* + *NlFOXO* (n = 68 females, 56 males), CC-401 + *NlFOXO* (n = 51 females, 38 males). Chi-square tests: */#: P < 0.05; **/##: P < 0.01; ***: P < 0.001; *: comparison is with dsGFP injected. #: comparison between *NlFOXO* dsRNA injected alone and other combinations that included *NlFOXO* dsRNA.

morph was mediated through its activation of NlJun or NlFos.

3.4. Co-knockdown of NlJNK and NlFOXO reduces the effect of NlFOXO knockdown alone

As mentioned above, the *N. lugens* homolog of the FOXO transcription factor has recently been found to regulate wing polyphenism, under negative regulation of the insulin-signaling pathway (Xu et al., 2015). To see if the action of *NlJNK* might also be due its downstream effect on *NlFOXO*, we did co-knockdown experiments. Consistent with previous reports (Lin et al., 2015a; Wang et al., 2003; Xu et al., 2015), treatment with ds*NlFOXO* alone resulted in 100% of the long winged form of brown planthopper adults, for both males and females (Fig. 3). However, co-injection of dsNlFOXO and dsNlJNK caused a significant increase in the proportion of adults of both sexes developing into the short winged form (12.2% females, 7.3% males), relative to dsNlFOXO injection alone (0% males and females) (Fig. 3). In addition, co-injection of the JNK inhibitor CC-401 with *NlFOXO* dsRNA had a similar effect to *NlJNK* dsRNA, with significant increases in the proportions of short winged adults for both females (9.8%) and males (10.5%) (Fig. 3), thus indicating that the effect of knockdown of *NlJNK* is not through a downstream effect of JNK on FOXO (in which case we would expect all long-winged individuals in the JNK/FOXO double knockdown). Co-injection of *NlJun* and *NlFOXO* dsRNA was identical to injection of *NlFOXO* dsRNA alone, with 100% of adults developing into the long winged morph (Fig. 3).

4. Discussion

Our results demonstrate an effect of the JNK signaling pathway on wing morph determination in *N. lugens*. Either down regulation of *NlJNK* expression by injection of dsRNA or inhibition of NlJNK activity by injection of the JNK inhibitor CC-401 caused 4th instar females to develop only into the short winged morph. Surprisingly however, this effect was sex-specific, as dsRNA or CC-401 did not affect the ratio of wing morphs in males. Clearly there are sex-specific effects on the *JNK* signaling pathway in *N. lugens*. More broadly, there appear to be general sex-specific differences in wing morph development. For instance, in our laboratory-reared population of *N. lugens*, there was a naturally greater frequency of the short winged morph in females (80.6%) than in males (50.5%) (Fig. 3). Also, this is not the first time we have observed sex-specific differences in wing morph due to gene knockdown: we found that knockdown of the FOXO inhibitor Akt in 4th instar nymphs caused a significant increase in the proportion of short winged females, but had no effect on males (Lin et al., 2015a), although knockdown of *NlFOXO* affected males and females identically (Fig. 3). We have not identified the mechanism for these sex-specific differences at this time, although differential regulation of JNK or other signaling pathways by the sex determination pathway is a likely candidate. For example in Drosophila, the sex determination gene *transformer* regulates differential size in males and females through its regulation of the insulin-signaling pathway (Rideout et al., 2015). In any case, sex seems to play a significant role in wing polymorphism in *N. lugens*, and is a topic worthy of further investigation.

Despite the effect of *NlJNK* knockdown on wing morph in females, knockdown of its targets *NlJun* and *NlFos* had no effect on wing morph in females (or males). Given the fact that RNAi only mediates a reduction (knockdown) of transcript, and not a full knockout (Fig. 2), we can not dismiss a potential role for *NlJun* or *NlFos* in mediating wing morph. However, our indication at this point is that this particular effect of *NlJNK* is not mediated through *NlJun* or *NlFos*. Of course JNK is known to activate or repress a wide variety of downstream effectors (Davis, 2000; Dhanasekaran and Reddy, 2008; Han et al., 2001; Huang et al., 2003; Kanellis et al., 2010; Noselli and Agnes, 1999; Wang et al., 2005, 2012), including in some cases FOXO (Wang et al., 2012). We also knocked down *NlFOXO* by RNAi in tandem with knockdowns of *NlJNK* in inhibit JNK activity, either through RNAi or CC-401. In these cases there was a wing morph ratio between *NlFOXO* knockdown and *NlJNK* knockdown: there were some short winged females in the double knockdowns (as opposed to none when knocking down *NlFOXO* alone), but not nearly to the levels seen in *NlJNK* knockdown alone, or even in control (GFP injected) treatments (Fig. 3A). Surprisingly, this effect also occurred in males: although *NlJNK* knockdown alone had no effect on male wing polymorphism, *NlJNK* knockdown in conjunction with *NlFOXO* knockdown significantly increased the ratio of males developing short wings relative to *NlFOXO* alone (Fig. 3B). Thus our results differ from what occurs with co-knockdowns of *NlFOXO* and insulin-signaling pathway genes (such as Akt): in these cases only the phenotype of the FOXO knockdown (all long winged adults) is expressed, indicating that the insulin signaling genes function in wing morph determination through their effect on FOXO (Lin et al., 2015a; Xu et al., 2015) Of course these results are further complicated by the fact that RNAi represents a knockdown, rather than a true knockout phenotype: (Fig. 3). Thus these results represent differentially reduced titers of the JNK and FOXO proteins, not their complete absence. However, at this point our evidence does not support the hypothesis that the effects of *NlJNK* on wing morph are due to its downstream effects on *NlFOXO*.

As well as investigating the function of *NlJNK NlJun* and *NlFos* in wing morph development, we examined their developmental transcriptional patterns (Fig. 1). The peak in transcript abundance of *NlJNK* and *NlJun* (as well as *NlFOXO*) in the 4th nymphal instar, during which wing fate is determined (Bertuso et al., 2002), temporally correlates abundance of *JNK*, *Jun*, *Fos* and *FOXO* with wing fate. Of course these nymphs represent a mixed population of what will develop into both short and long winged forms (with some bias toward the short winged forms, given the characteristics of our lab-reared population), so we are unable to draw any correlations between nymphal transcript abundance and adult wing morph.

Our evidence showing that *JNK* can affect wing dimorphism in *N. lugens* females expands and complicates our understanding of wing morph development in this insect. As previously mentioned, *JNK* responds to changes in environmental conditions, especially conditions causing stress. We surmise that activation of the *JNK* pathway might be a signal leading to an adaptive developmental shift to the long winged form, facilitating migration of the adults away from unfavorable environmental conditions. We hope to test this hypothesis more explicitly in the future. What is clear is that multiple signaling pathways, not only the insulin pathway but also *JNK* and perhaps other pathways, affect wing morph. These pathways may act not only through *FOXO*, but through other transcription factors as well. In addition, there appear to be sex-specific differences in how signaling pathways act on wing morph development. Clearly there still remains much to learn about this fascinating topic.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibmb.2016.04.005.

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