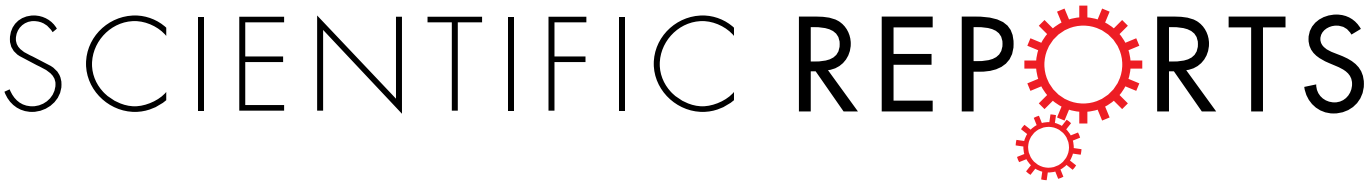

OPEN

Methoprene-tolerant (*Met*) and *Krüpple-homologue* 1 (*Kr-h1*) are required for ovariole development and egg maturation in the brown plant hopper

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The brown plant hopper is one of the most destructive known pests of rice. We studied the roles of the JH receptor Met and the downstream transcription factor Kr-h1 in ovariole development and egg maturation. The predicted Met protein in *N. lugens* (NlMet) contained 517 amino acids. qRT-PCR showed that *NlMet* was expressed in all tissues and that the highest expression occurred in the embryonic stage. In *NlMet*- or *NlKr-h1*-silenced female adults, ovarian development varied significantly, whereas the numbers of ovarioles were less variable in those injected with dsRNA targeting *NlMet*, *NlKrh-1* or both *NlMet* and *NlKr-h1*. In females injected with dsNlKr-h1 or with dsNlMet in combination with dsNlKr-h1 dsRNA, the preoviposition period was prolonged, whereas the females injected with *NlMet* dsRNA showed no significant changes. Moreover, we found no differences in the length of the preoviposition period between macropterous and brachypterous females. The disruption of *Nlmet* or *NlKr-h1* or the dual knockdown of *NlMet* and *NlKr-h1* significantly reduced the number of eggs laid. Moreover, significant differences were also found between the macropterous and the brachypterous brown plant hoppers. These results indicated that Met and Kr-h1 are required for ovariole development and egg maturation in the brown plant hopper.

The brown plant hopper *Nilaparvata lugens* Stål is dimorphic and is one of the most destructive known pests of rice. The yield losses caused by the brown plant hopper are severe in several countries, including China, India, Indonesia, Japan, South Korea, the Philippines, and Sri Lanka, and infestations are also observed in many other countries[1,2]. The brown plant hopper is a serious threat to rice production in Asia. The brown plant hopper feeds directly on rice tillers and causes the rice to turn yellow, which ultimately leads to "hopper burn" when the pest population is large. This pest also transmits viruses, such as the virus causing grassy stunt disease and the rice stripe virus (RSV), which increase yield loss[3,4].

The brown plant hopper is dimorphic, and typically, the wings develop such that there are long-winged macropterous forms and short-winged brachypterous forms. In the laboratory, the macropterous and brachypterous forms develop in similar proportions. However, in the field, an increase in the formation of macropterous forms typically indicates migration and leads to colonization of new fields. After colonization, the proportion of brachypterous forms increases. The brachypterous females are the primary reproductive form and typically lay more eggs than the macropterous females.

The signalling mediated by juvenile hormone (JH) is one of the key signalling pathways that controls insect development and normal adult physiological function[5]. The basic-helix-loop-helix/Per-Arnt-Sim (bHLH-PAS) domain protein *methoprene- tolerant* (*Met*) is the receptor for the JH ligand, and this domain contains the specific binding domain of the JH ligand-binding site (charles 2011; Miura 2005). *Met* forms a complex with another bHLH-PAS domain protein, *Tai/SRC/FISC* (*Taiman* (*Tai*), as well as with *steroid receptor coactivator* (*SRC*) and *Ftz-F1-interacting steroid receptor coactivator* (*FISC*))[6–9]. In mosquitoes, *Met* forms a complex with Cycle to regulate the circadian expression of JH-induced genes[10]. *Krüppel-homologue 1* (*Kr-h1*) is a zinc finger protein that

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acts downstream of the JH receptor and is expressed in response to JH signalling. *Kr-h1* is antimetamorphic and is required for the development of wings and external genitalia[11]. *Kr-h1* also participates in neural development and maturation[12,13], in addition to maintaining normal physiological function in adults.

The signalling mediated by JH is a key pathway in the regulation of insect metamorphosis[5], cellular immunity[14], and trehalose homeostasis[15]. In *Drosophila melanogaster*, JH regulates female mating and pheromone production[16] and male reproduction[17]. JH signalling is involved in vitellogenesis and oogenesis in female reproduction[18]. In *Tribolium*, the JH receptor *TcMet* is required for egg production and vitellogenin expression[19]. In *Aedes aegypti*, *Met* is required for ovarian development[20] and egg production[7]. More recently, in *Pyrrhocoris apterus*, the silencing of *Met* or *Tai* blocked the development of the ovaries, whereas the silencing of *Kr-h1* did not[21]. However, in *Locusta migratoria*, the silencing of *Kr-h1* blocked oocyte maturation and ovarian development[22]. In our previous study with *N. lugens*, treatment with JH III or the analogues methoprene or pyriproxifen induced the expression of the downstream transcription factor Kr-h1[11]. Similar induction was reported in *Pyrrhocoris apterus*[23], *Bombyx mori*[24,25], *Drosophila melanogaster*[26], *Tribolium castaneum*[27] and *Aedes aegypti*[10,28].

Understanding the development of the ovary is essential for making predictions about migratory insect pests. The development and maturation of eggs is also the basis of pest outbreaks. The development of the ovary of *N. lugens* has five stages: I. Undeveloped ovarioles. II. Developed ovarioles with immature eggs. III. A few ovarioles containing full-grown eggs. IV. More of the ovarioles containing full-grown eggs, and vacuity appears in some ovarioles. V. Ovarioles become wethered[29,30]. The stage of ovarian development of *N. lugens* is affected by various environmental factors and the developmental stage of the rice[29,31]. In *N. lugens*, JH is closely associated with migration, and the stage of ovarian development is significantly affected by JH and the antijuvenile hormone prococeneII[29,32]. However, the molecular mechanism underlying the effects remains unclear. In this study, we examined the roles of the JH receptor *Met* and the downstream transcription factor *Kr-h1* and their interaction during ovariole development and egg maturation in the hemimetabolous brown plant hopper.

Results

Phylogenetic analysis of the Nilaparvata lugens Methoprene-tolerant (Met) gene. We performed a BLAST search in the transcriptome database and then cloned the *N. lugens methoprene-tolerant* (*NlMet*) gene. The *N. lugens NlMet* contains 2040 bp in the coding region and encodes 680 amino acids. The nucleotide sequence and the predicted amino acid sequence were deposited in NCBI Genbank (accession number: KP797880). We aligned the predicted protein sequence of *N. lugens Met* (*NlMet*) with homologues from *Diploptera punctata, Drosophila melanogaster, Bombyx mori* and *Tribolium castaneum*. The *NlMet* protein had typical bHLH, PAS-A, PAS-B and PAC (PAS C terminal motif) domains throughout the protein (Fig. 1).

Based on the phylogenetic analysis, the NlMet protein was highly conserved and clustered with the DpunMet protein, and these two proteins clustered with RpMet and PaMet (Figure S1).

Spatial and temporal expression of *NlMet* during *N. lugens* development. The spatial and temporal expression of a gene effectively indicates the function of the gene. We measured the expression of *NlMet* at different developmental stages. The *NlMet* gene was expressed at all stages. The highest expression was in the embryonic stage (egg), whereas the expression was variable in the nymphal stage and was relatively constant in adults (Fig. 2). In the embryonic stage, the expression of *NlMet* was relatively high on the 2nd, 5th and 3rd days, but the expression was relatively low on the 4th day (Fig. 2). The levels of expression were similar on the other days (Fig. 2). The expression of *NlMet* was also determined in various tissues, including the brain (Br), thorax (Th), wing (Wi), leg, midgut (Mi), ovary (Ov) and testis (Ts). In the midgut, ovary and testis, the expression of *NlMet* was relatively low, whereas the expression was relatively high in the wing and brain (Fig. 2). We also measured the expression of *NlMet* during the development of the ovaries of brachypterous and macropterous *N. lugens*. *NlMet* was expressed from the 1st to the 4th stage of ovarian development, but the expression decreased significantly in the 5th stage of development in both the brachypterous and macropterous forms (Fig. 2D).

Silencing of *NlMet* and *NlKr-h1* through RNAi in the nymphal stage of *N. lugens* disrupted the development of the ovariole. To test whether the development of ovarioles and the maturation of eggs required components of JH signalling, 5th instar nymphs were injected with dsRNA or the control dsGFP. The ovaries were dissected, and the phenotypes were observed. For 7.69% of the ds*NlMet* adults and 6.9% of the adults that underwent dual knockdown of *NlMet* and *NlKr-h1*, the stage of ovary development could not be determined (Fig. 3F,G), because these ovaries either fit criteria for more than one stage of development or one of the two ovaries had fewer mature oocytes.

We then determined whether the "normal" remaining ovaries had equivalent numbers of ovarioles and similar developmental rates. The number of ovarioles was not significantly reduced in the *NlMet*-silenced macropterous or brachypterous females compared with that of the control female adults injected with dsGFP. However, in the *NlKr-h1*-silenced females and the *NlMet* and *NlKr-h1* double-silenced females, the numbers of ovarioles were reduced significantly compared with the control dsGFP brachypterous females (Fig. 4). In the macropterous females, the difference in the numbers of ovarioles between the females with dual knockdown of *NlMet* and *NlKr-h1* and the control was not significant, whereas the difference was significant when the dual knockdown females were compared with the *NlKr-h1*-silenced females ($P < 0.05$; Fig. 4).

To study the effect of gene silencing on the development of the ovariole, we recorded the stage of ovarian development in the adult females with dual knockdown of *NlMet* and *NlKr-h1*. Based on the grading criteria reported previously[29], we measured the level of ovarian development 2 days after adult emergence. All the dsRNA-injected females had delayed ovarian development, which resulted in the delayed maturation of eggs. In the *NlMet*- or *NlKr-h1*-silenced brachypterous females, the development of the ovaries was slowed significantly compared with that of the control ($P < 0.05$; Fig. 5); it was slowed further in the females with the dual knockdown of *NlMet* and

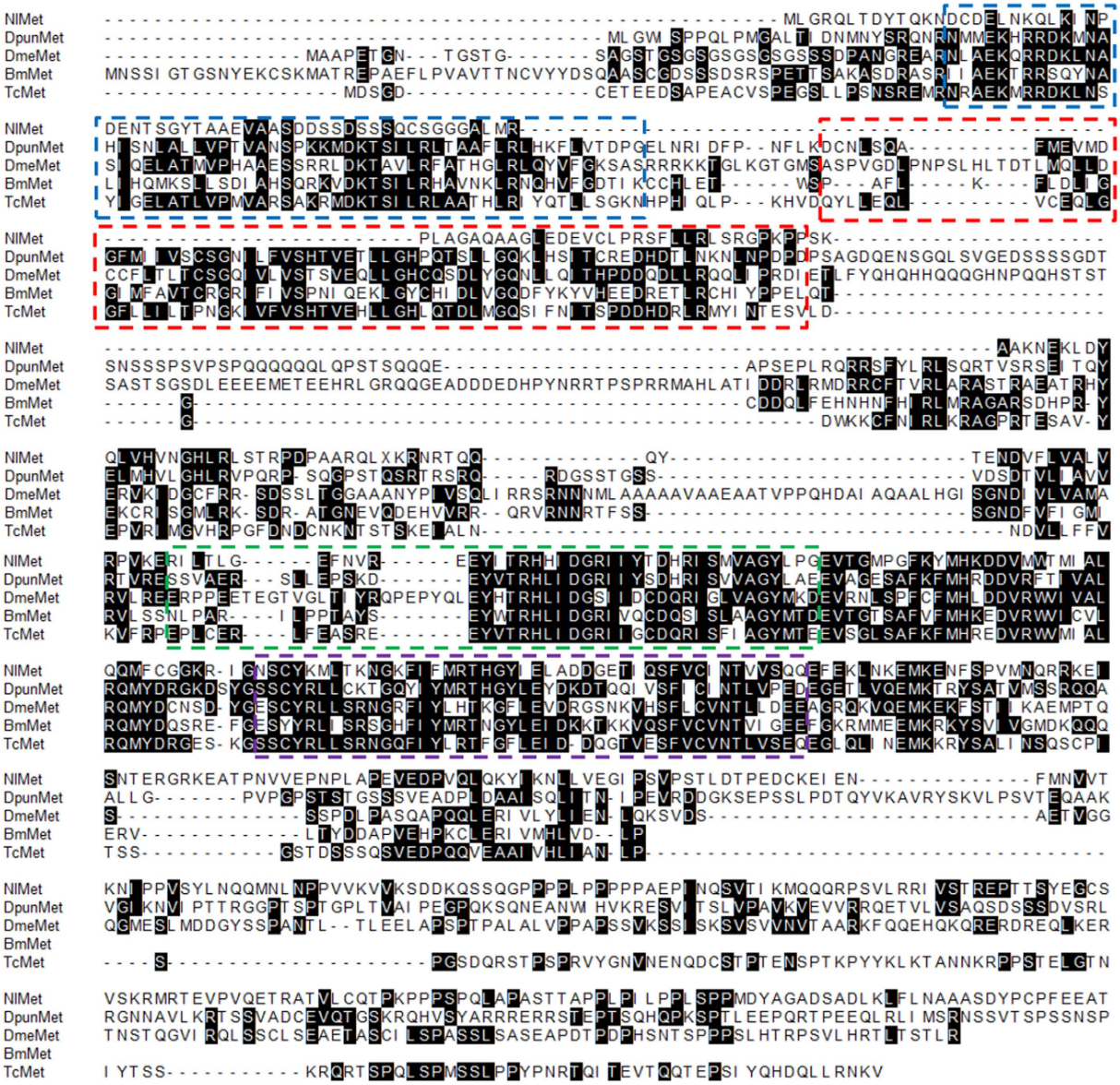

Figure 1. Alignment of the amino acid sequence of *N. lugens* Met with those of its homologues in *Bombyx mori*, *Diploptera punctata*, *Drosophila melanogaster*, and *Tribolium castaneum*. Domains—bHLH (Blue), PAS-A (Red), PAS-B (Green) and PAC (Purple)—are boxed. Bm, *Bombyx mori* (ACJ04052.1); Dpun, *Diploptera punctata* (AIM47235.1); Nl, *Nilaparvata lugens* (AIE12451.1); Tc, *Tribolium castaneum* (NP_001092812.1).

NlKr-h1 ($P < 0.01$; Fig. 5). In contrast to the brachypterous form, the rate of ovarian development in macropterous females was not significantly different between the *NlMet* or *NlKr-h1*-silenced females and the dsGFP control. Notably, the rate of ovarian development was significantly slowed upon dual knockdown of *NlMet* and *NlKr-h1* ($P < 0.01$; Fig. 5).

The roles of *NlMet* and *NlKr-h1* in maturation and the number of eggs. The preoviposition period occurs between emergence and the time the first eggs are deposited. To further investigate the roles of *Nlmet* and *NlKr-h1* in the development of ovaries and the maturation of eggs, we measured the preoviposition period of the *Nlmet*- and *NlKr-h1*-silenced females and the females with dual knockdown *NlMet* and *NlKr-h1*. The preoviposition period was prolonged in both the macropterous and the brachypterous females that were injected with *dsNlKr-h1* or *dsNlmet* and *dsNlKr-h1* dsRNA, whereas for the females injected with *NlMet* dsRNA, the preoviposition period was not different from that of the control (Fig. 6A). Moreover, the preoviposition period was not different between the macropterous and the brachypterous females upon any treatment or in the controls.

The number of eggs laid is an important indicator of an outbreak of an insect pest. We found that disruption of *Nlmet* and *NlKr-h1* and dual knockdown of *NlMet* and *NlKr-h1* significantly reduced the number of eggs laid ($P < 0.05$; Fig. 6B). Moreover, significant differences ($P < 0.01$) were found between the macropterous and the brachypterous brown plant hoppers in the numbers of eggs laid (Fig. 6B).

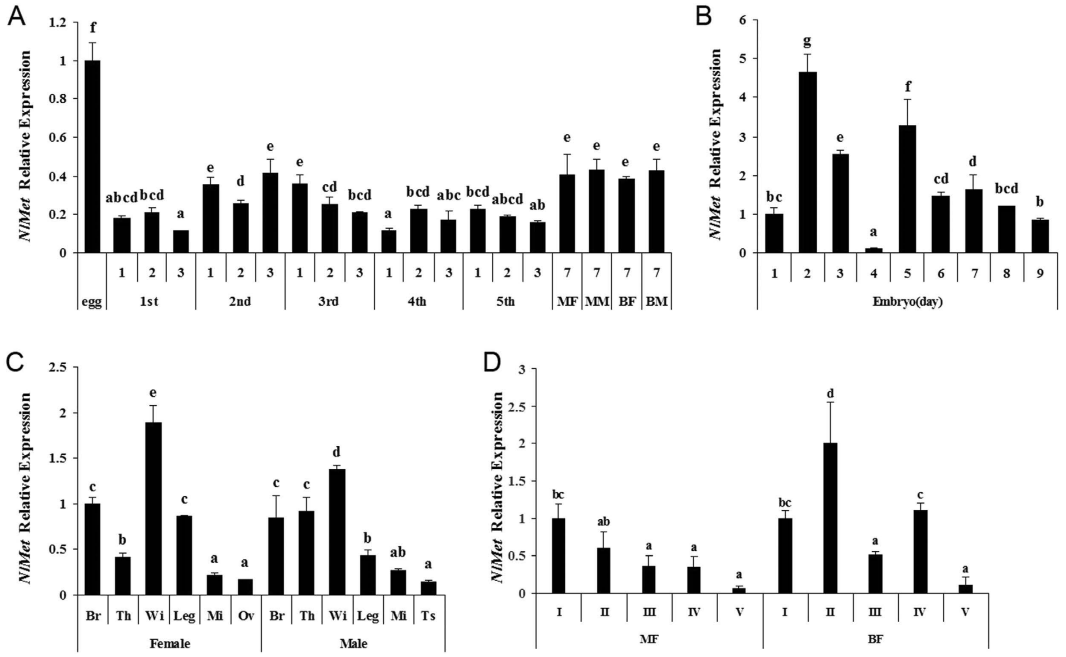

Figure 2. Spatial and temporal expression of NlMet. Expression during the embryonic development of the brown plant hopper (1st–9th days, **B**), at each of the nymph stages (1st to 5th, **A**) and in adults of the two wing forms (**A**). Also shown is the expression of *NlMet* in various tissues of the female and male (**C**). The relative expression of *NlMet* in the 1st to 5th stage ovaries of the brachypterous and macropterous females is shown in (**D**) The letters a–e indicate significant differences at a 0.05 level according to Duncan's multiple range tests Macropterous female (MF), Macropterous male (MM), Brachypterous female (BF), Brachypterous male (BM), Adults (**A**). Tissue-specific expression of NlMet in the brain (Br), thorax (Th), wing (Wi), leg (Leg), midgut (Mi), fat body (Fb), ovary (Ov) and testis (Ts) of adult *N. lugens* (**C**).

Based on the qRT-PCR results, the expression of *NlMet* declined significantly in the dsNlMet-injected brown planthoppers (17.8%; Fig. 7A) and in the dsNlKr-h1 injected brown plant hoppers (1.53%, Fig. 7B), whereas the expression of *NlKr-h1* was reduced to 33.4% and 23.1% compared with the dsGFP-injected controls, respectively (Fig. 7). The expression levels of *NlMet* and *NlKr-h1* were reduced by 33.5% and 26.6%, respectively, upon the dual knockdown of *NlMet* and *NlKr-h1* in brown plant hoppers (Fig. 7).

Discussion

JH signalling and ovarian development. *NlMet* was expressed in all developmental stages, which was consistent with previous studies in which *Met* played a role during development and had a physiological function in *N. lugens*. The development of the ovarioles of *N. lugens* is affected by a variety of factors, including temperature and food availability[33,34]. Additionally, JH affects ovariole development in *N. lugens*[32]. The retarded development of the ovaries of female brown plant hoppers is typically caused by a deficiency of JH, and when JH is added in the required amount, the speed of ovary development recovers, even under conditions consisting of a lack of food and unfavourable temperatures[29]. JH plays an important role in oogenesis[35,36], and JH is required for female reproductive maturation and vitellogenesis[35–37] . In a comparison with a study by Smykal *et al.*[38], *Met* or *Tai* was required for the ovarian development of the linden bug, *Pyrrhocoris apterus*, whereas *Kr-h1* was not. According to Song *et al.*, however, *Kr-h1* was required for oocyte maturation and ovarian development in the migratory locust *Locusta migratoria*[22].

In this study, in *N. lugens* females with disrupted JH signalling, the development of the ovarioles was affected, and abnormal phenotypes were observed (Fig. 3); moreover, we found that the progression through the stages of ovarian development declined significantly (Fig. 4), which indicated that JH signalling is required for the development of the ovary. Typically, the more highly evolved insects have longer preoviposition periods. In our experiment that measured the preoviposition period, the disruption of both *NlKr-h1* or *NlKr-h1* and *NlMet* significantly prolonged the preoviposition period, whereas the silencing of *NlMet* had no significant effect. This result indicated that *NlKr-h1* was required for the maturation of eggs in *N. lugens* but that *NlMet* was not required. In the *NlMet* knockdown or the *NlMet* and *NlKr-h1* dual knockdown brown plant hoppers, the ovaries developed unevenly (Fig. 3F,G). This result was similar to that in *D. menalogaster* in which a retinoic-like JH might be involved in genitalia rotation[39], indicating that a similar signalling pathway might control the looping of left-right asymmetric organ formation.

The interaction of *NlMet* and *NlKr-h1*. As previously mentioned, Met, along with its interaction partner Taiman, blocked ovarian development in the linden bug *Pyrrhocoris apterus*, although *Kr-h1* did not[21]. According to the current model of the JH signalling pathway, the transcription factor *Kr-h1* functions downstream of the JH

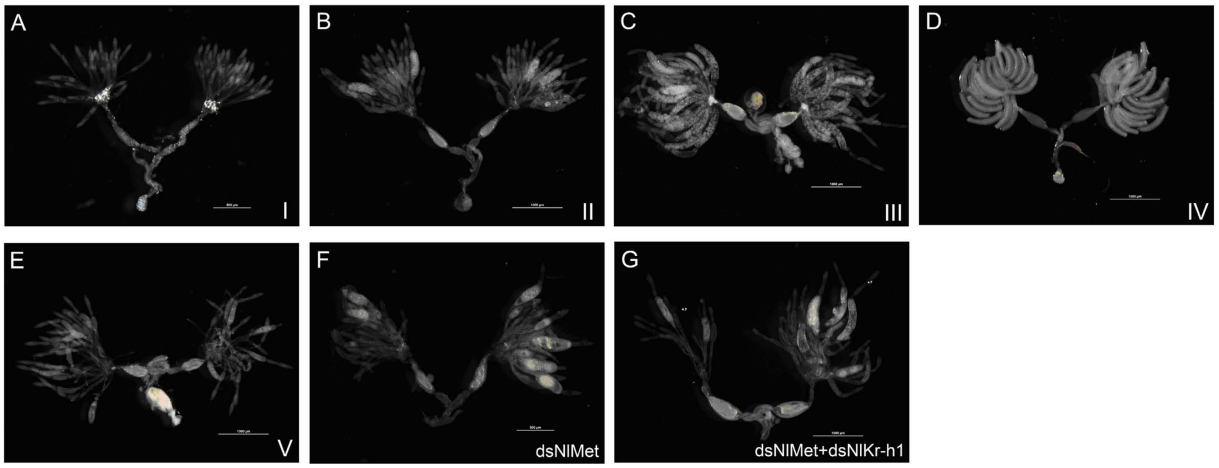

Figure 3. The stages I to V of the ovaries of *N. lugens* females (A–E) and the ovaries that did not fit the grading criteria (F,G) after silencing of *NlMet* alone or the dual knockdown of *NlMet* and *NlKr-h1*.

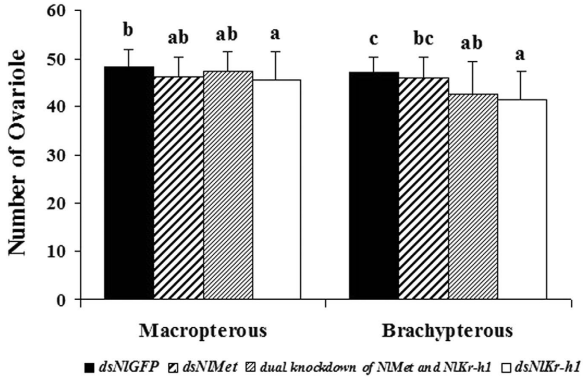

Figure 4. Effect of silencing NlMet and NlKr-h1 on the ovariole number of N. lugens females . The letters a–e indicate significant differences according to Duncan's multiple range tests at a 0.05 level. The numbers of female adults used for the experiment: dsGFP (n = 43 brachypterous, 42 macropterous), dsNlMet (n = 42 brachypterous, 44 macropterous), dual knockdown of *NlMet* and *NlKr-h1* (n = 46 brachypterous, 37 macropterous), and dsNlKr-h1 (n = 38 brachypterous, 41 macropterous).

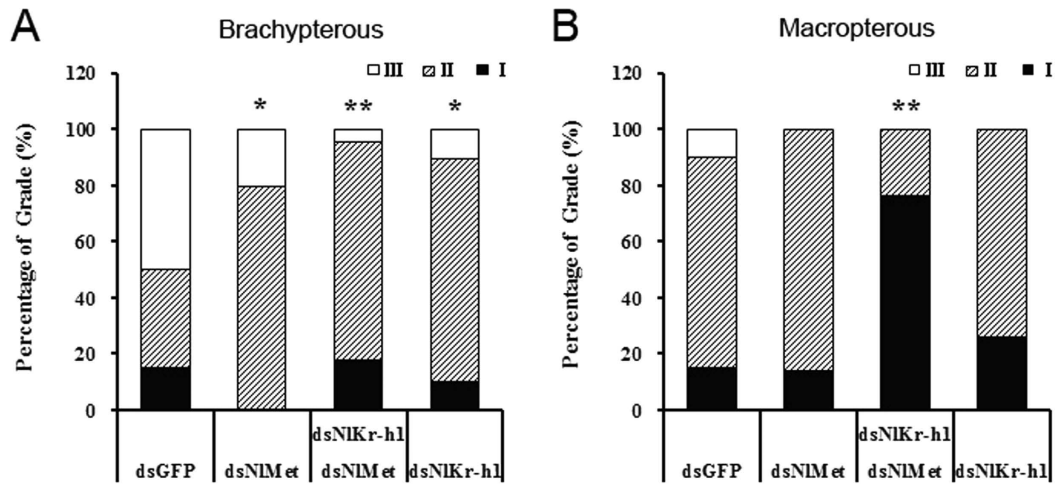

Figure 5. Effect of silencing NlMet and NlKr-h1 on ovariole development of N. lugens females. (**A**) Brachypterous; (**B**) Macropterous. Percentages of stages I, II, and III of ovariole development 2 days post-adult eclosion (PAE) are shown. For the Chi-square test, $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with injection with dsGFP as the control. See Fig. 4 for the numbers of females used in the experiment.

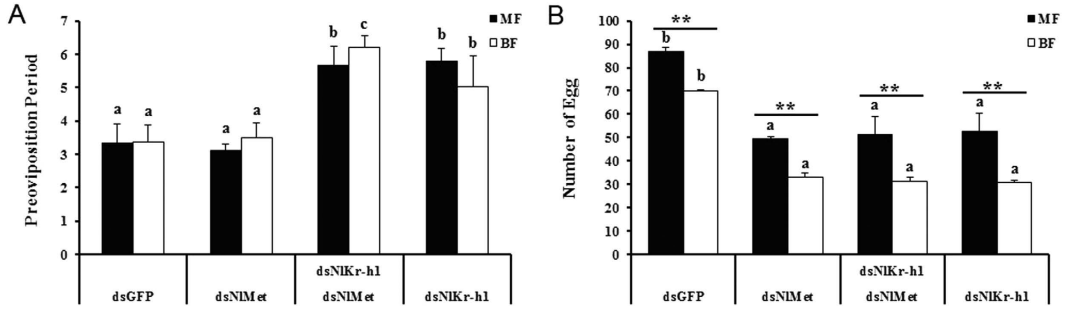

Figure 6. Effect of silencing NlMet and NlKr-h1 on the preoviposition period and the number of eggs of N. lugens females. (**A**) Preovipositon period; (**B**) Number of eggs laid. Comparison between macropterous and brachypterous adults for each dsRNA treatment, based on t-tests, $P < 0.01$. Comparison among different treatments, based on Duncan's multiple range tests, $P < 0.05$. The letters a–e indicate significant differences according to Duncan's multiple range tests at a 0.05 level. The numbers of females used for the experiment: dsGFP (n = 15 brachypterous and 17 macropterous), dsNlMet (n = 33 brachypterous and 14 macropterous), dual knockdown of NlMet and NlKr-h1 (n = 27 brachypterous and 6 macropterous), and dsNlKr-h1 (n = 22 brachypterous and 17 macropterous).

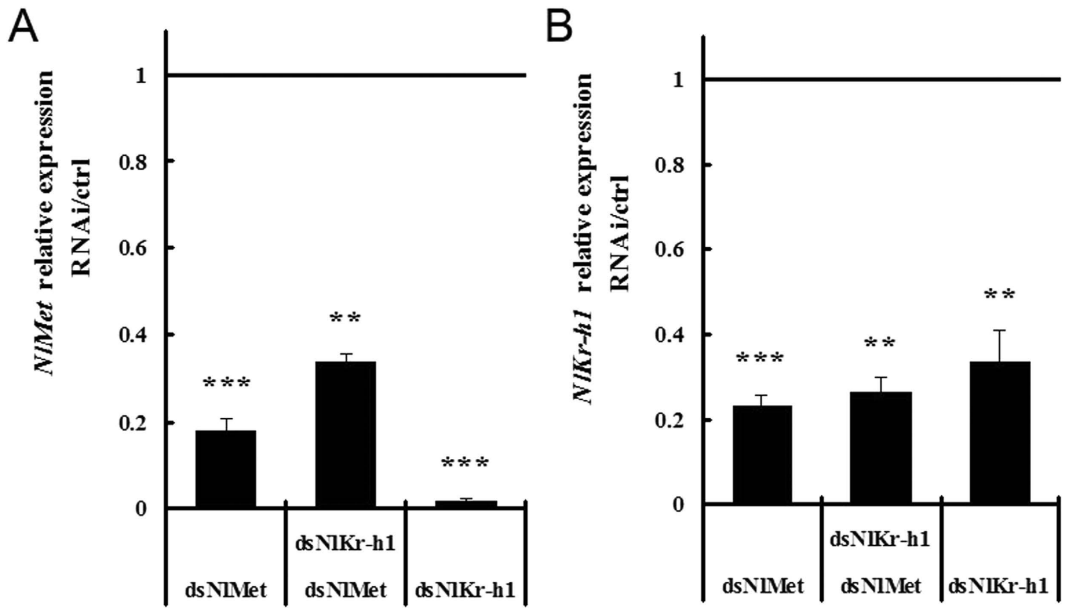

Figure 7. Relative expression levels of *NlMet* and *NlKr-h1* three days after gene silencing of *NlMet* or *NlKr-h1* or both in 5th instar larvae of *N. lugens*. (**A**) *NlMet*; (**B**) *NlKr-h1*. The level of significance is based on t-tests, $^{*}P < 0.01$, and error bars represent the standard error of the difference (SED).

receptor *Met*. The development of the ovarioles of dual knockdown *NlMet* and *NlKr-h1 N. lugens* brachypterous females was significantly slower than that in the adults treated separately with ds*NlMet* or ds*NlKr-h1*. This result suggests a possible interaction between *NlMet* and *NlKr-h1*. This interaction was more evident in the macropterous brown plant hoppers, i.e., silencing of neither *NlMet* nor *NlKr-h1* delayed ovariole development, whereas when both genes were silenced, ovariole development declined significantly ($P < 0.01$; Fig. 5B). Thus, when compared with the ovariole number and egg maturation, the development of the ovariole was more sensitive to the JH signalling.

The migration of *N. lugens* and ovary development. Insect migration is an adaptive behaviour in response to environmental change and physiological factors. JH plays an important role in the migration and the reproduction of insects. A low level of JH typically stimulates insects to migrate, whereas a high level of JH stimulates the development of the ovary and the maturation of eggs. The macropterous form is typically responsible for long-distance migration and colonization of rice fields, whereas the brachypterous forms lay many more eggs than the macropterous forms. The role of JH in insect migration is complicated because in a dimorphic insect such as the brown plant hopper, the wing form is not only regulated by the levels of JH but also by the window of time for JH action, i.e., the 3rd and the 4th instar nymph stages are critical for the formation of wings. In the laboratory, the treatment of 3rd and 4th instar nymphs with JH significantly increased the proportion of brachypterous plant

Name	Forward (5′–3′)	Reverse (5′–3′)
NlRPS11	CCGATCGTGTGGCGTTGAAGGG	ATGGCCGACATTCTTCCAGGTCC
NlMet	GGTGGTAAACGGATTGGAAA	CATCGTCAGCCAACTCGATA
NlKr-h1	TGATGAGGCACACGATGACT	ATGGAAGGCCACATCAAGAG

Table 1. Primers for qRT-PCR.

hoppers, whereas treatment with procene I increased the proportion of macropterous brown plant hoppers[32]. Notably, during migration, the ovary of *N. lugens* is typically underdeveloped, i.e., typically, before stage II[29], and this observation indicates that JH affects both the wing form of the brown plant hopper and the reproductive state of the females. The earlier stage of ovarian development is an adaption of ovary development to migration because of the clear benefits obtained from an underdeveloped ovary for long-distance migration. The development of the ovaries of *N. lugens* continues after migration and colonization. The slowing of ovariole development after the silencing of JH signalling by RNAi targeting the JH receptor *NlMet* or its downstream transcription factor *NlKr-h1* is similar to that observed after treatment with antijuvenile hormone[29,32]. Therefore, in *N. lugens*, the role of JH in the development of wings and ovaries in females is clear. These results also explain, in part, the molecular mechanism of the role of JH in the migration of the brown plant hopper. Based on the results, JH is most likely an important factor in the regulation of ovarian development in *N. lugens*. In the future, the regulation of brown plant hopper populations through the molecular regulation of female reproduction and wing formation by modification of the JH signalling pathway will be possible.

Materials and Methods

Brown plant hopper. The brown plant hopper *Nilaparvata lugens* (Stål) was kindly provided by Professor Zengrong Zhu of Zhejiang University and was maintained in the laboratory. Seedlings of *Oryza sativa* L. cv. IIyou-203 were used to feed the brown plant hoppers at 25–28°C with a light:dark photoperiod of 14:10 h and humidity of 70–80%. To obtain sufficient numbers of brown plant hoppers for quantitative real-time PCR (qRT-PCR) and the RNA interference (RNAi) experiment, the insects were cultured asynchronously to ensure that all developmental stages were available simultaneously.

RNA preparation, gene cloning and phylogenetic analysis. Total RNA was extracted from an equal mix of brown plant hopper nymphs and adults using the Trizol-based RNAiso Plus total RNA extraction kit (Takara, Dalian, China). First strand cDNA was synthesized using a Transcriptor First strand cDNA synthesis kit (Roche, Shanghai) and used as a template to amplify the gene of interest. The brown plant hopper homologue of *Met1* (*NlMet*) was searched in the transcriptome database. The full-length *NlMet* used for the synthesis of dsRNA was amplified by PCR using Ex-Taq polymerase (Takara, Dalian). The primers used were as follow: *NlMet*F: 5′CAACCAGCAGATGAACCTGA3′, and *NlMet*R: 5′GCAAAGCCTCGTACTCTTGG3′.

The PCR fragments were then purified with a gel purification kit (Omega, USA) and cloned into the PMD18-T vector (Takara, Dalian, China) for sequencing at Shanghai Sunny Biotechnology Co., Ltd. The sequences were aligned with ClustalW using Met homologues, and phylogenetic trees were constructed using MEGA6. For the sequence alignment and the phylogenetic tree, the search for homologues was performed using the Blast program at the NCBI (http://blast.ncbi.nlm.nih.gov).

RNA extraction and qPCR. Quantitative Reverse Transcriptase PCR(qRT-PCR) was conducted using the Roche SYBR® Green PCR Master Mix and SYBR® Green RT-PCR Reagents Kit (Roche Applied Science, Shanghai, China). The reverse transcription was conducted as described by the supplier. The primers used were listed in Table 1.

A 25 μl reaction was used (Roche Applied Science, Shanghai), and 2 μl of diluted cDNA (20× dilution of the first strand cDNA synthesis reaction) was used for each reaction. A No Template Control (NTC) was used to monitor contamination and primer dimers. The $2^{-\Delta\Delta Ct}$ method[40] was used to compare the relative levels of expression of the gene of interest at different developmental stages and in different tissues or before and after gene silencing. The reference genes used for qRT-PCR were previously reported[41]. Three biological replicates were used for the qRT-PCR experiment.

RNAi experiment. For the RNAi experiment, the double-stranded RNA (dsRNA) was synthesized using the synthesis kit RiboMAX™ Large-Scale RNA Production System-T7 (Promega, Beijing). The templates for the synthesis of the dsRNA were amplified by PCR using the PMD18-T plasmid (Takara, Dalian, China) with a DNA fragment of the gene of interest inserted, and then the PCR products were purified with a DNA gel purification kit (Omega bio-tek, USA). The dsRNAs were synthesized as described in the Promega technical bulletin TB166. The primers used for the dsRNA synthesis of *NlMet*, *NlKr-h1* and the control gene encoding Green Fluorescent Protein (GFP) are listed in Table 2.

The 5th instar nymphs were anaesthetized with CO_2 before the intra-thoracic dsRNA injection. The injection was conducted using a Nikon microscope and a Narishige injection system (MN-151; Narishige). In total, 0.1 μg of dsRNA was injected into each insect. The nymphs were allowed to recover for 1–2 h after the injection and then were reared on rice seedlings. The mortality, wing-morph and sex were recorded after injection, and then the females were used for ovary dissections or were prepared for total RNA extraction. Three independent biological replicates were used for the nymphs that were injected with dsNlMet, dsNlKr-h1, both dsN1Met and dsN1Kr-h1, or only dsGFP.

Name	Nucleotide sequence (5′–3′)
dsNlMetT7F	TAATACGACTCACTATAGGGAGACCACCAACCAGCAGATGAACCTGA
dsNlMetT7R	TAATACGACTCACTATAGGGAGACCACGCAAAGCCTCGTACTCTTGG
dsNlKrhT7F	TAATACGACTCACTATAGGGAGACCACGTGGGGTTCAGTCCTGAGGA
dsNlKrhT7R	TAATACGACTCACTATAGGGAGACCACCAGTCGAACACACACCGGAG
dsGFPT7F	GGATCCTAATACGACTCACTATAGGAAGGGCGAGGAGCTGTTCACCG
dsGFPT7R	GGATCCTAATACGACTCACTATAGGCAGCAGGACCATGTGATCGCGC

Table 2. Primers for dsRNA Synthesis.

Ovary dissection and slide preparation. The female brown plant hoppers were anaesthetized using CO_2 and were dissected in 1x PBS. The dissection was performed with a pair of tweezers, one to hold the thorax and the other to hold the lower part of the abdomen to gently tear the abdomen apart and expose the ovaries. The dissected ovaries were mounted in 70% glycerol on glass slides for further observation of the phenotypes or were prepared for total RNA extraction. The number of ovarioles was also recorded from counts under the microscope.

Imaging and statistical analyses. The images of adult *N. lugens* and the ovaries from the gene silencing experiment were taken under a Nikon stereomicroscope (SMZ745T) and a Nikon fluorescence microscope (Eclipse 80i, 4X) with NIS-Elements. The images were processed with Adobe Photoshop CS5.

The statistical analyses were conducted using the SPSS 20 statistical software package. For the spatial and temporal expression of *NlMet*, significant differences were determined by one-way ANOVA and then Duncan's multiple range tests. A Chi-square test was used for the comparison of the grading criteria for ovariole development.

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Author Contributions

X.L. conceived and designed the study, analysed and interpreted the data, and wrote the manuscript, Y.Y. performed the experiments and prepared Figures 4–7, and B.W. performed the experiments and prepared Figures 1–3 and supplementary figure 1. All authors have read and approved the final manuscript.

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