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ORIGINAL ARTICLE

Expression patterns and functional analysis of the short neuropeptide F and NPF receptor genes in *Rhopalosiphum padi*

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Abstract The short neuropeptide F (*sNPF*) and NPF receptor (*NPFR*) genes play important roles in many physiological processes. However, information on the survival-related functions of *sNPF* and *NPFR* under different stress conditions is lacking in aphids. In this study, we cloned *sNPF* and *NPFR*, and investigated the expression levels of these genes in different developmental stages, wing morphs, and stress conditions of the bird cherry-oat aphid (*Rhopalosiphum padi* L.), an important agricultural pest. The *sNPF* and *NPFR* transcript levels varied among developmental stages, and their expression levels in alate females were significantly higher than those in apterous females. In addition, starvation resulted in significantly increased s*NPF* expression, which then recovered after refeeding. Heat stress and insecticides significantly affected transcription of both genes. *sNPF* and *NPFR* knockdown in *R. padi* using RNA interference revealed optimal interference efficiency at 48 h post-injection. *sNPF* knockdown significantly decreased adult longevity, 15-d fecundity, and food intake. Additionally, mortality under starvation, insecticides, and heat stress conditions was significantly higher after injection with double-stranded *sNPF* in *R. padi*. *NPFR* knockdown significantly affected food intake and starvation resistance in *R. padi*. These results strongly indicate that *sNPF* plays vital roles in food intake, longevity, and reproduction in *R. padi*, and it can significantly affect the pest's response to stress conditions.

Key words fecundity; food intake; heat stress; insecticides; starvation; survival rate

Introduction

Neuropeptides, a group of chemical signaling molecules, regulate a broad range of physiological and behavioral activities (Altstein & Nässel, 2010; Urbanski *et al*., 2019). Short neuropeptide F (sNPF) is one type of insect neuropeptide. In invertebrates, *sNPF* was first discovered in *Leptinotarsa decemlineata* (Spittaels *et al*., 1996). *sNPF* is evolutionarily conserved and characterized by the xPxLRLRFamide sequence at the carboxy terminus (Nässel & Wegener, 2011). NPF receptors (*NPFRs*) that belong to the G protein-coupled receptor superfamily were first cloned from the brain of *Lymnaea stagnalis* (Tensen *et al*., 1998). *sNPF* exerts its effects by specifically combining with *NPFR*.

Recently, the functions of *sNPF* and *NPFR* have drawn extensive attention in a wide variety of insect species, and research has shown that *sNPF* could serve as a neuroregulator with vital roles in many physiological processes, such as food intake in *Drosophila melanogaster* (Carlsson *et al*., 2013), *Schistocerca gregaria* (Dillen *et al*., 2014), *Acyrthosiphon pisum* (Li *et al*., 2018), and *Locusta migratoria* (Tan *et al*., 2019); metabolic stress

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responses and modulation of locomotor behavior in *D. melanogaster* (Kahsai *et al*., 2010a,b); sleep homeostasis in *D. melanogaster* (Chen *et al*., 2013); reproduction in *S. gregaria* (Dillen *et al*., 2013); host-seeking behavior in *Aedes aegypti* (Christ *et al*., 2017); olfactory sensitivity in *Bactrocera dorsalis* (Jiang *et al*., 2017); and regulation of hormone production or release in *D. melanogaster* (Nässel *et al*., 2008) and *Bombyx mori* (Kaneko & Hiruma, 2014). Unfortunately, there has been less research on the functions of *sNPF* and *NPFR* in aphids.

The bird cherry-oat aphid *Rhopalosiphum padi* is a globally distributed agricultural pest that causes severe economic losses through direct feeding and transmitting the barley yellow dwarf virus in Gramineae crops (Schliephake *et al*., 2013; Leybourne *et al*., 2020). Because of global warming conditions and an increase in the annual frequency of extreme high-temperature events, the proportional abundance of *R. padi* has increased; therefore, *R. padi* may become a more serious cereal pest in the future (Ma *et al*., 2015; Peng *et al*., 2020). Although the role of *sNPF* in regulating feeding behavior has been investigated in *A. pisum*, information on the functions of *sNPF* and *NPFR* in survival under different stress conditions is lacking in aphids.

In this study, *sNPF* and *NPFR* expression profiles were determined in different developmental stages, in alate and apterous wing forms, and at different starvation states. Additionally, the efficiency of RNA interference (RNAi) and the effects of *sNPF* and *NPFR* on aphid reproduction, longevity, feeding, and survival rate under starvation, insecticide exposure, and heat stress conditions were investigated in *R. padi*. The results indicate that longevity, fecundity, and survival rate under different stress conditions are critical for population growth and sustainability. Our study provides information that supports the improvement of eco-friendly pest control strategies for this serious pest.

Materials and methods

Aphid rearing and insecticide bioassays

The *R. padi* clone was obtained from a laboratory colony originally collected from *Triticum aestivum* (cultivar "Xiaoyan 22") in Yangling, Shaanxi Province, China. The aphids were reared in a fine mesh gauze cage (38 × 38 × 38 cm) in an artificial climate chamber (ICH750, Memmert Co., Ltd., Schönaich, Germany) under long photoperiod (16 : 8 L : D) conditions at 24 ± 1 °C and 70% relative humidity. All aphids were maintained on wheat seedlings of the same *T. aestivum* cultivar. For the RNAi experiment in each treatment, aphids from different generations were used to represent biological viability.

The two insecticides used for toxicity bioassays in this study were imidacloprid (95% purity; Jiangsu Changlong Chemical Co., Ltd., Nanjing, China) and beta-cypermethrin (96% purity; Yancheng Nongbo Biotechnology Co., Ltd., Yancheng, China). The leaf-dipping bioassay was used to assess insecticide toxicity to *R. padi* (Zuo *et al*., 2016). Five concentrations of beta-cypermethrin (0.5, 1, 2, 4, and 8 mg/L) and imidacloprid (0.5, 1, 2, 4, and 8 mg/L) were used to conduct the bioassays. Newly emerged apterous adult aphids treated with distilled water containing 0.01% (v/v) Triton X-100 and 0.01% acetone were used as a control, and three replicates of 30 apterous adult aphids were used for each concentration. After 24 h, the number of surviving aphids was recorded (Zuo *et al*., 2016).

Identification and phylogenetic analysis of sNPF and NPFR in R. padi

The *sNPF* and *NPFR* DNA sequences in *R. padi* were obtained from the *R. padi* genome published by Thorpe *et al*. (2018). The full-length *sNPF* and *NPFR* sequences were verified by reverse transcriptase polymerase chain reaction (PCR) using gene-specific primers (Table S1) designed by Primer Premier 6 (PREMIER Biosoft International, Palo Alto, CA, USA). The products were sequenced by Sangon Biotech (Shanghai, China). National Center for Biotechnology Information (NCBI) BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to identify nucleotide sequence similarities, and open reading frame (ORF) Finder (https://www.ncbi.nlm.nih.gov/orffinder/) was used to predict each ORF. The signal peptide was predicted using the SignalP 5.1 Server (http://www.cbs.dtu.dk/services/SignalP/). Transmembrane domains were predicted using MHMM Server 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/). Multiple alignments of the amino acid sequences of *sNPF* and *NPFR* from different aphids were performed by ClustalX (Larkin *et al*., 2007). The phylogenetic analysis was constructed using the neighbor-joining algorithm in MEGA 7 (Kumar *et al*., 2016) with 1000 bootstrap replications.

sNPF and NPFR expression patterns in different developmental stages and wing morphs of R. padi

To investigate the *sNPF* and *NPFR* expression patterns in *R. padi* at five developmental stages, total RNA was

extracted from 20 1st-, 2nd-, 3rd-, and 4th-instar nymphs and apterous adults from the clone described above using Invitrogen TRIzol Reagent (Life Technologies, Carlsbad, CA, USA). The aphids in all experimental groups were simultaneously collected to exclude the possible influence of circadian rhythm on the *sNPF* and *NPFR* transcript expression levels.

The DNA-free Kit (Applied Biosystems, Foster City, CA, USA) was used to eliminate DNA contamination. Two micrograms of total RNA from each sample were reverse transcribed into first-strand complementary DNA (cDNA) using the reverse transcriptase (Promega, Madison, WI, USA) protocol. Quantitative primers (Table S1) were designed by Primer Premier 6, and the specificity of these primers was determined before quantitative PCR (qPCR). The qPCR efficiency of these primers was analyzed using serially diluted cDNA. qPCR (95 °C for 3 min, 40 cycles of 95 °C for 10 s, 58 °C for 20 s, and 72 °C for 20 s; and one cycle at 72 °C for 10 min) was carried out on a Rotor Q thermocycler (Qiagen, Hilden, Germany). Each reaction system contained 10.0 μL of SYBR mix (Roche, Basel, Switzerland), 0.8 μL of forward and reverse primers (10 μmol/L), 2.0 μL of cDNA, and 6.4 μL of ddH_2O. A melting curve was used to further confirm qPCR primer specificity. All treatments were performed with three technical and biological replicates. To eliminate the possibility of reagent contamination, a blank (no-template) control was included for each run and gene. The β-actin gene was used as an internal control (Kang *et al*., 2016; Wang *et al*., 2016; Zuo *et al*., 2016). The relative expression patterns of *sNPF* and *NPFR* were calculated by the relative quantitative method ($2^{-\Delta\Delta Ct}$) (Livak & Schmittgen, 2001).

The transcriptional expression patterns of *sNPF* and *NPFR* in alate and apterous parthenogenetic females were compared. Each treatment consisted of 10 alate or 10 apterous adults, and three biological replicates were used for each treatment. The samples were frozen in liquid nitrogen for RNA extraction, and qPCR was performed according to the method described earlier.

Effects of three different stress conditions on sNPF and NPFR expression patterns

Our previous results showed that alate aphids are more resistant to starvation than apterous aphids, and short-term starvation has less of an impact on their survival (unpublished data). Therefore, newly emerged apterous adults were used in these experiments. The aphids underwent 1 d starvation (S1), 1 d starvation and refeeding for 1 d (S1F1), 2 d starvation (S2), or 2 d starvation and 1 d refeeding (S2F1). In the starvation-treated groups, filter paper filled with water was placed at the bottoms of the plastic dishes (diameter, 9 cm; height, 2 cm), and every aphid was placed on a plastic dish. The plastic dishes were checked daily, and newborn nymphs were removed. In the refeeding groups, the aphids that survived the starvation treatment were separately kept on the wheat seedlings. The apterous adults reared on the wheat seedlings were used as controls. The 10 surviving aphids per treatment were frozen in liquid nitrogen for RNA extraction, and three biological replicates were used for each treatment. qPCR was performed according to the method described earlier. The *sNPF* and *NPFR* expression levels were investigated among the five treatments.

For the heat stress treatment, 160 newly emerged adult aphids were heated at temperatures of 36, 37, 38, and 39 °C for 2 h. Newly emerged adult aphids reared at 24 °C were used as a control. After treatment, 10 surviving aphids per treatment were used to extract total RNA. The *sNPF* and *NPFR* expression levels were determined as described earlier. Three biological replicates were used for each treatment.

Three different sublethal concentrations of beta-cypermethrin (1, 2, and 4 mg/L) and imidacloprid (1, 2 and 4 mg/L) were used to conduct the bioassays. Thirty newly emerged adult aphids treated with distilled water that contained 0.01% (v/v) Triton X-100 and 0.01% acetone were used as a control. After 24 h, 10 surviving aphids per treatment were used to investigate the expression levels of *sNPF* and *NPFR* in different treatments according to the method described earlier. Three independent biological replicates were performed for each treatment.

RNAi targeting sNPF and NPFR

RNAi primers for *sNPF* and *NPFR* were designed using Primer Premier 6 (Table S1). To prevent off-target effects, we chose specific target fragments to avoid any overlap with other genes in the aphid genome that exceeded 19 bp, and the sequence specificity of target fragments was tested via NCBI BLAST. The double-stranded RNA (dsRNA) of the two genes was synthesized using the T7 RiboMAX^{TM} Express RNAi System (Promega, Madison, WI, USA) according to the manufacturer's instructions. We employed ds*GFP* as a control, and the purified dsRNA concentrations were examined using a biophotometer (Eppendorf BioPhotometer Plus, Eppendorf, Germany). To evaluate the RNAi efficiency, 480 newly emerged adult aphids were chosen for the RNAi assay. Four different concentrations of dsRNA solution (3, 6, 9, and 12 μg/μL) (55.2 nL) were injected into the suture

joining the ventral mesothorax and metathorax using an automatic nanoliter injector (Märzhäuser, Wetzlar, Germany) equipped with a microglass needle prepared using a P-97 Micropipette Puller (Sutter Instrument Co., Novato, CA, USA). Ten surviving adults were arbitrarily sampled at 1, 2, 3, and 4 d in each treatment after injection of ds*GFP*, ds*sNPF*, or ds*NPFR*. qPCR was used to measure the RNAi efficiency of the target genes, and three replicates were carried out per treatment.

Effects of RNAi targeting sNPF and NPFR on adult longevity and fecundity

To avoid the influence of individual size and weight on the experimental results, newly emerged adult aphids with similar body weights (~420 μg) and sizes (~1.60 mm) were arbitrarily divided into three groups. Solutions that contained ds*GFP*, ds*sNPF*, and ds*NPFR* (55.2 nL, 9 μg/μL) were injected into individuals of each group. After dsRNA injection, adult longevity and fecundity were calculated using 30 aphids per group. These aphids were reared on wheat seedlings, and the adult mortality and number of newborn nymphs per adult aphid were recorded daily. The offspring were removed from each seedling, and the seedlings were replaced every 7 d until each adult aphid died. The wheat seedlings were reared under the conditions described earlier.

Effects of RNAi targeting sNPF and NPFR on aphid intake and larval survival

Newly emerged adult aphids were arbitrarily divided into three groups. Solutions that contained ds*GFP*, ds*sNPF*, and ds*NPFR* (55.2 nL, 9 μg/μL) were injected into individuals of each group. Approximately 0.4 g (W_0) of fresh and detached wheat seedlings were placed into a plastic dish, as described earlier. To maintain freshness, water-soaked filter paper was placed on the bottom of each dish. One day after dsRNA injection, 10 aphids per group were placed into the prepared dish, and four replicates were carried out per treatment. Wheat seedlings of the same weight with no aphids were used as a control. All wheat seedlings were weighed and recorded after 1 (W_1) and 2 d (W_2). "W_0-W_1" represents the percentage of wheat leaf weight reduction from original weight (W_0) to leaf weight after 1 d (W_1); "W_0-W_2" represents the percentage of wheat leaf weight reduction from original weight (W_0) to leaf weight after 2 d (W_2); and "W_1-W_2" represents the percentage of wheat leaf weight reduction from leaf weight after 1 d (W_1) to leaf weight after 2 d (W_2).

To evaluate the effects of RNAi targeting *sNPF* and *NPFR* on the survival rate from 4th-instar nymphs to adults, we selected 3d-instar nymphs for RNAi experiments, because the time of the highest interference efficiency was the day following dsRNA injection. The 15 3rd-instar nymphs per treatment were injected with ds*GFP*, ds*sNPF*, and ds*NPFR* (55.2 nL, 9 μg/μL). The numbers of nymph deaths were recorded until each aphid became an adult. The experiment was repeated four times, and all assays were performed under the conditions described earlier.

Effects of RNAi targeting sNPF and NPFR on aphid survival under different stress conditions

To assess the roles of *sNPF* and *NPFR* in aphid survival under different starvation conditions, newly emerged adult aphids were injected with ds*GFP*, ds*sNPF*, or ds*NPFR* (55.2 nL, 9 μg/μL). Two days after dsRNA injection, 30 injected adult aphids from each treatment were transferred to plastic dishes that contained water-soaked filter paper as described above. These aphids were separately treated with starvation for 24 or 48 h. The mortalities of different treatments were recorded after starvation, and four replicates were carried out per treatment.

Newly emerged adult aphids from the population described above were arbitrarily divided into three groups. ds*GFP*, ds*sNPF*, or ds*NPFR* (55.2 nL, 9 μg/μL) were injected into each of the three groups. Two days after dsRNA injection, 30 injected adult aphids from each treatment were placed on detached wheat leaves in plastic dishes with water-soaked filter paper, heated in a dry bath incubator (Allsheng Instruments, Hangzhou, China) to 36 and 38 °C for 2 h, and then allowed to recover at 24 °C for 1 h. The mortalities of the different treatments were recorded after heat stress, and four replicates were carried out per treatment.

Sublethal LC_{30} concentrations of beta-cypermethrin and imidacloprid were used for the following experiments. Newly emerged adult aphids were injected with ds*GFP*, ds*sNPF*, or ds*NPFR* (55.2 nL, 9 μg/μL). Two days after dsRNA injection, wheat leaves with 30 injected adult aphids from each treatment were dipped into beta-cypermethrin or imidacloprid solutions for 10 sec, and the residual insecticide droplets on the leaves were absorbed with dry filter paper pieces. All treated aphids were then reared on detached wheat leaves in plastic dishes with water-soaked filter paper, and aphid mortality was assessed after 24 h. Three replicates were carried out per treatment.

Statistical analysis

Comparisons of the *sNPF* and *NPFR* expression levels under different treatments were subjected to one-way analysis of variance followed by Tukey's honestly significant difference test ($P < 0.05$). Data on the percentages of survival rate and mortality were log-transformed to meet the assumptions of normality and homoscedasticity required for these analyses. All statistical analyses were performed with SPSS 20 (IBM-SPSS, Armonk, NY, USA).

Results

Toxicity bioassay

The toxicity bioassay results are shown in Table S2. The median lethal concentration values (LC_{50}) of imidacloprid and beta-cypermethrin were 2.56 (95% confidence limit 2.15–3.13) and 1.81 (95% confidence limit 1.53–2.13) mg/L, respectively.

Identification and characteristics of sNPF and NPFR

The *sNPF* cDNA sequence (GenBank accession number MT265223) cloned from *R. padi* was 634-bp long with a single ORF (291 bp) that encoded 96 amino acids (Fig. S1). The first 19 amino acids (MKSIAAVVCTLLLVSTIIS) constituted the signal peptide, and the sNPF protein contained a non-cytoplasmic domain. Sequence alignment revealed that the *R. padi* sNPF sequence shared very high homology with those of other aphids, such as *Myzus persicae* (XP_022169760.1, 100% identity), *Rhopalosiphum maidis* (XP_026813352.1, 98.96% identity), *Melanaphis sacchari* (XP_025197025.1, 98.96% identity), *Acyrthosiphon pisum* (XP_003247250.1, 98.96% identity), and *Sipha flava* (XP_025411079.1, 90.62% identity) (Fig. S1A). The sNPF sequence was further confirmed by the *R. padi* genome published by Thorpe *et al*. (2018). Phylogenetic analysis indicated that the sNPFs clustered with those of other aphids (*M. persicae*, *M. sacchari*, *R. maidis*, *A. pisum*, and *S. flava*) and other hemipteran insects (*Bemisia tabaci*) (Fig. S1B).

NPFR was cloned from *R. padi*, and its ORF contained 1176 nucleotides that encoded 391 amino acids (GenBank accession number MT265224). The predicted molecular weight of NPFR was 44.31 kDa, and the theoretical isoelectric point was 9.57. NPFR contained a common conserved domain (G protein-coupled receptor family 1), which included seven transmembrane helices and a putative peptide ligand-binding pocket (Fig. S2A). The alignment results indicated that the *R. padi* NPFR sequence shared relatively high homology with those of other aphids, such as *R. maidis* (XP_026807386.1, 99.74% identity), *M. sacchari* (XP_025193466.1, 98.47% identity), *A. pisum* (XP_001943708.2, 98.21% identity), *M. persicae* (XP_022171996.1, 97.44% identity), *Diuraphis noxia* (XP_015368577.1, 97.19% identity), *Aphis gossypii* (XP_027843893.1, 96.93% identity), and *S. flava* (XP_025412491.1, 89.03% identity) (Fig. S2A, B). The NPFR sequence was further confirmed by the *R. padi* genome published by Thorpe *et al*. (2018). The NPFR phylogenetic tree revealed that these proteins were divided into two clades, and the proteins from eight aphid species clustered together (Fig. S3).

sNPF and NPFR expression patterns in different developmental stages and wing morphs of R. padi

sNPF and *NPFR* expression were detected in all developmental stages (Fig. 1A). The mRNA expression level of *sNPF* in 1st-instar nymphs was significantly higher than those in the other three nymph instars and in adults, and the expression levels of this gene were lowest in adults ($F = 12.92$; df = 4, 10; $P < 0.001$). The *NPFR* transcript levels were significantly different among 1st-, 2nd-, 3rd-, and 4th-instar nymphs and adults ($F = 48.88$; df = 4, 10; $P < 0.001$). The gene expression was highest in 1st-instar nymphs and lowest in 3rd-instar nymphs.

The *sNPF* and *NPFR* transcript levels were compared between apterous and alate females. The mRNA expression levels of *sNPF* ($P = 0.01$) and *NPFR* ($P = 0.04$) in apterous females were significantly higher than those in alate females (Fig. 1B).

Effects of starvation and refeeding on sNPF and NPFR expression patterns

The *sNPF* and *NPFR* expression levels were determined at several time points after starvation and subsequent refeeding experiments. The expression patterns of these two genes in *R. padi* adult aphids responded similarly to starvation stress and refeeding after starvation stress (Fig. 2). The *sNPF* and *NPFR* transcripts tended to be upregulated following 1 and 2 d of starvation, and significant differences were found in the *sNPF* expression levels between the control and S1 treatment. Furthermore, the *sNPF* transcript levels after refeeding were significantly decreased compared

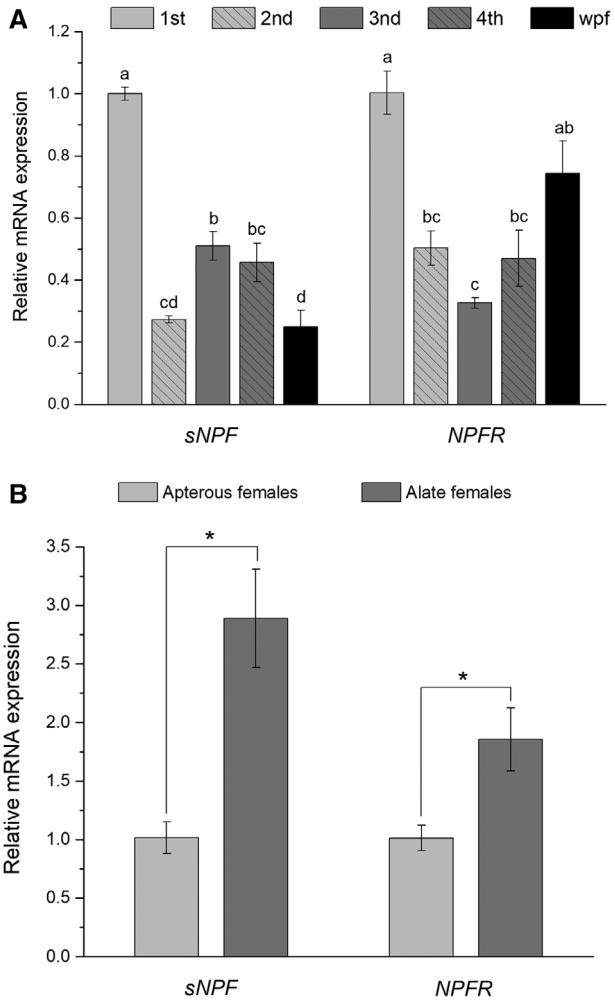

Fig. 1 Relative messenger RNA (mRNA) expression of the *sNPF* and *NPFR* genes in *Rhopalosiphum padi* at five developmental stages (A) and expression profiles of these two genes in apterous and alate parthenogenetic females in *R. padi* (B). 1st, 2nd, 3rd, and 4th indicate the four developmental stages of *R. padi* nymphs; wpf represents adult apterous parthenogenetic females. The values of each gene are normalized to the average expression of that gene. Different letters on the bars indicate significant differences ($P < 0.05$, Tukey's Honestly Significant Difference test). *Significant differences between the two groups were assayed by a *t*-test using a threshold *P*-value < 0.05.

with those after 1 d starvation ($P < 0.001$). A similar situation was found between the S2 and S2F1 treatments ($P < 0.001$). The *NPFR* expression levels were decreased after subsequent feeding compared with those after starvation; however, no significant difference was found between these treatments ($F = 3.59$; df = 3, 8; $P = 0.07$).

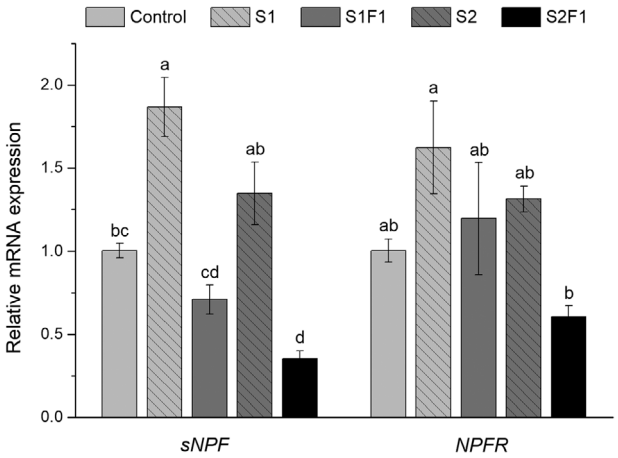

Fig. 2 Expression profiles of the *sNPF* and *NPFR* genes in *Rhopalosiphum padi* adults after starvation conditions and refeeding after starvation. S1, 1 d starvation; S1F1, 1 d starvation and 1 d refeeding; S2, 2 d starvation; S2F1, 2 d starvation and 1 d refeeding. Different letters on the bars indicate significant differences ($P < 0.05$, Tukey's Honestly Significant Difference test).

Effects of heat stress and insecticides on sNPF and NPFR transcript levels

The *sNPF* and *NPFR* mRNA levels were influenced by exposure to sublethal concentrations (1, 2, and 4 mg/L) of beta-cypermethrin and imidacloprid (Fig. 3). After exposure to three different sublethal concentrations of two different insecticides, *sNPF* and *NPFR* expressions in apterous adult aphids tended to be downregulated. After exposure to beta-cypermethrin, the *NPFR* expression levels significantly decreased, regardless of the concentration ($F = 34.63$; df = 3, 8; $P < 0.001$; Fig. 3A). The *sNPF* mRNA level in aphids exposed to 2 mg/L beta-cypermethrin was remarkably decreased compared with that of the control group ($F = 4.63$; df = 3, 8; $P = 0.04$; Fig. 3A). After exposure to 4 mg/L imidacloprid, the transcript levels of *sNPF* ($F = 6.43$; df = 3, 8; $P = 0.02$) and *NPFR* ($F = 5.38$; df = 3, 8; $P = 0.03$) were significantly lower than those of the control (Fig. 3B).

The relative mRNA expression levels of *sNPF* and *NPFR* were quantified in adult aphids that were exposed to heat stress for 2 h. Compared with those in the control group (24 °C), the *sNPF* transcription levels tended to be downregulated as the heat shock temperature increased from 36–39 °C, and the *sNPF* transcript level at 39 °C remarkably decreased ($F = 4.15$; df = 4, 10; $P = 0.03$; Fig. 4). *NPFR* expression in adults treated with different temperatures did not significantly differ between the

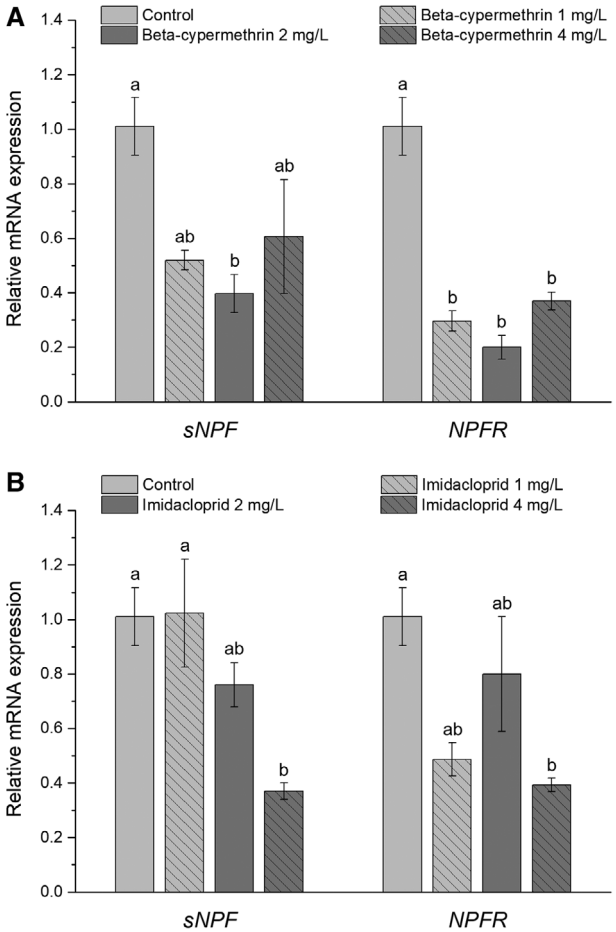

Fig. 3 Relative expression levels of the *sNPF* and *NPFR* genes in *Rhopalosiphum padi* subjected to different sublethal concentrations of beta-cypermethrin (A) and imidacloprid (B). Different letters on the bars indicate significant differences ($P < 0.05$, Tukey's Honestly Significant Difference test).

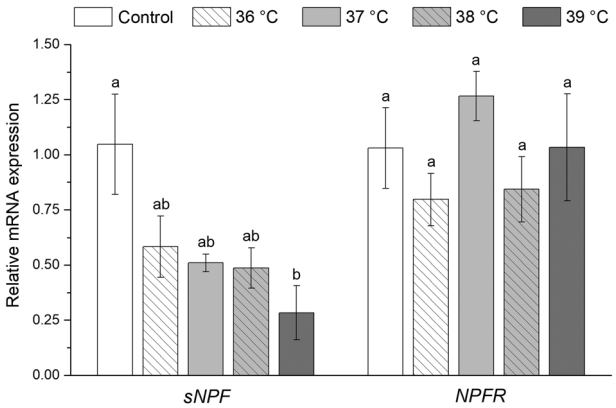

Fig. 4 Relative expression levels of the *sNPF* and *NPFR* genes in *Rhopalosiphum padi* treated with different high temperatures (36, 37, 38 and 39 °C) for 2 h. Different letters on the bars indicate significant differences ($P < 0.05$, Tukey's Honestly Significant Difference test).

control and heat stress groups ($F = 1.23$; df = 4, 10; $P = 0.36$; Fig. 4).

Determination of RNAi efficiency

The optimal dsRNA dosage was determined by injection of four different dsRNA concentrations (3, 6, 9, and 12 μg/μL) and checking the interference efficiency at four different times (1, 2, 3, and 4 d) post-injection. The major results of the dose-response experiments are shown in Figure 5. The *sNPF* transcript level was significantly decreased (reduced by 53.67%) on d 2 after ds*sNPF* injection compared with the control (ds*GFP* injection) ($P < 0.01$, Fig. 5A), and significant differences in *sNPF* transcript levels were found between the ds*GFP* and ds*sNPF* treatments on d 3 ($P = 0.03$). The RNAi efficiency of ds*sNPF* vanished on d 4. Compared with the control, a significant reduction was observed on d 2 after ds*NPFR* injection ($P = 0.02$, Fig. 5A). The RNAi effect was still detected on the 3rd day after ds*NPFR* injection; however, there were no significant differences in *NPFR* mRNA levels between the ds*GFP* and ds*NPFR* treatments on d 3 ($P = 0.24$). The RNAi efficiency was highest on d 2 for both genes. Compared with the control (ds*GFP* injection), the *sNPF* expression levels were significantly decreased at 2 d post-injection with 6 μg/μL ($P < 0.01$), 9 μg/μL ($P = 0.01$), and 12 μg/μL ($P < 0.01$) ds*sNPF*; the *NPFR* expression levels were also significantly reduced at 2 d post-injection with 9 μg/μL ($P = 0.03$) and 12 μg/μL ($P = 0.04$) ds*NPFR*. No significant differences were found in *sNPF* or *NPFR* mRNA levels between the respective treatments of 9 μg/μL dsRNA and 12 μg/μL dsRNA injection. We used 55.2 nL of 9 μg/μL dsRNA for each injection in further RNAi experiments.

The roles of sNPF and NPFR in aphid survival and reproduction

Based on the results of our previous research, adult aphids rarely produce offspring after 15 d. The effects of *sNPF* and *NPFR* on adult longevity and 15-d fecundity per female were examined by injection of ds*GFP*, ds*sNPF*, and ds*NPFR* (Fig. 6). RNAi of *sNPF* significantly decreased adult longevity ($P < 0.01$), whereas ds*GFP* and ds*NPFR* treatments produced no significant variance ($P = 0.07$) in adult longevity (Fig. 6A). The

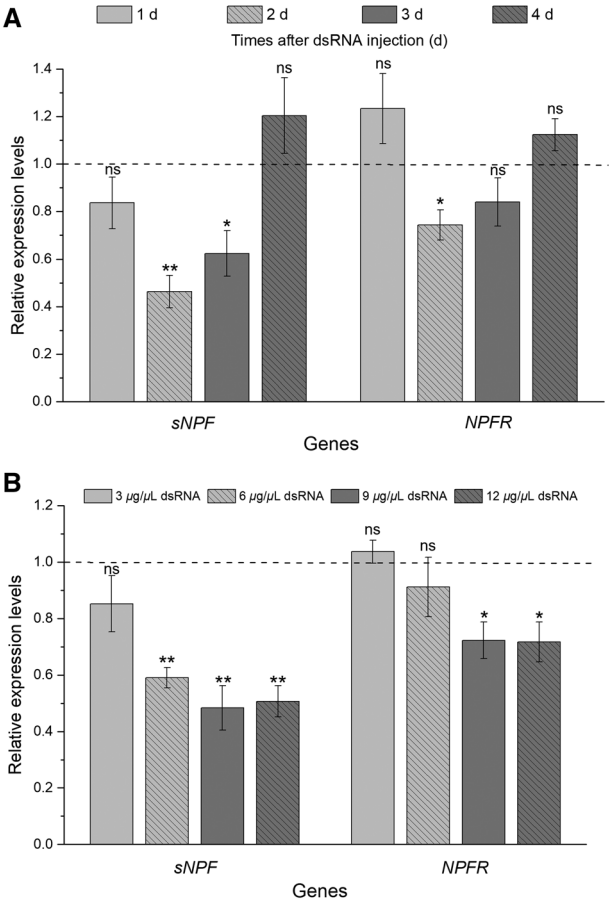

Fig. 5 Relative expression levels of *sNPF* and *NPFR* in *Rhopalosiphum padi* at different times after injection of double-stranded RNA (dsRNA) (A) and the expression of the two genes in *R. padi* injected with different concentrations of ds*sNPF* or ds*NPFR* (B), respectively. Asterisks at the top of the bars show that the values were significantly different (ns, $P > 0.05$; $^{*}P < 0.05$; $^{**}P < 0.01$; *t*-test). The expression of *sNPF* and *NPFR* genes was normalized to the control group (injection of ds*GFP*).

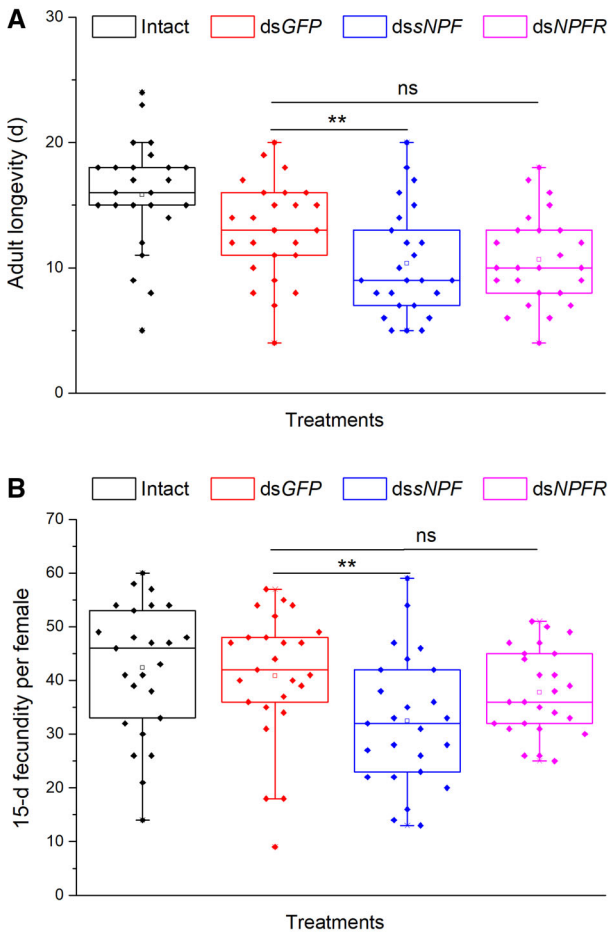

Fig. 6 Effects of RNA interference targeting the *sNPF* or *NPFR* genes on adult longevity (A) and 15-d fecundity per female (B) in *Rhopalosiphum padi*. Asterisks on the top of the bars show that the values were significantly different ($^{*}P < 0.05$; $^{**}P < 0.01$; ns, no significant difference; Tukey's Honestly Significant Difference test).

statistical analysis showed a significant difference in 15-d fecundity per female between the ds*GFP* and ds*sNPF* groups ($P < 0.01$); however, the 15-d fecundity of the ds*NPFR* group was not significantly different from that of the control group ($P = 0.28$, Fig. 6B).

The roles of sNPF and NPFR in aphid intake and larval survival

In all treatments, the percentages of wheat leaf weight reduction (W_0-W_1 and W_0-W_2) were lowest in the control group. Compared with the percentages of wheat leaf weight reduction (W_0-W_1) in the ds*GFP* group, a significant reduction was observed in the ds*sNPF* treatment ($F = 57.08$; df = 3, 12; $P < 0.001$). Compared with the percentages of wheat leaf weight reduction (W_0-W_2) in the ds*GFP* group, significant reductions were found in the ds*sNPF* and ds*NPFR* treatments ($F = 62.68$; df = 3, 12; $P < 0.001$). No significant variance was found in the percentages of wheat leaf weight reduction (W_1-W_2) among the control, ds*GFP*, ds*sNPF*, and ds*NPFR* treatments ($F = 2.13$; df = 3, 12; $P = 0.15$; Fig. 7A). The survival rates from 4th-instar nymphs to adults were not significantly different among ds*GFP*, ds*sNPF*, and ds*NPFR* treatments ($F = 2.50$; df = 2, 9; $P = 0.14$; Fig. 7B).

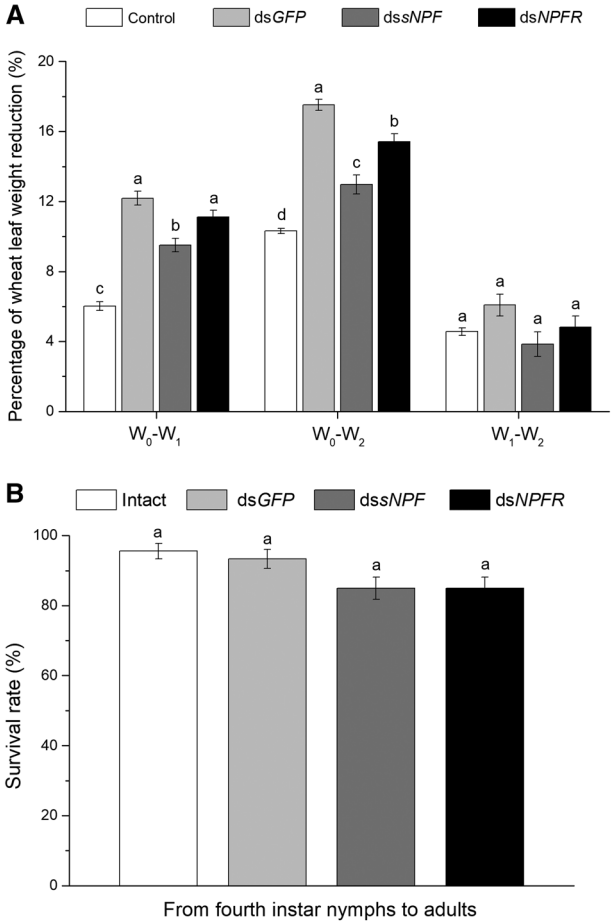

Fig. 7 Effects of RNA interference targeting the *sNPF* or *NPFR* genes on the percentage of wheat leaf weight reduction (A) and survival rate from 4th instar nymphs to adults (B) in *Rhopalosiphum padi*. Different letters on the bars indicate significant differences ($P < 0.05$, Tukey's Honestly Significant Difference test).

The effects of sNPF and NPFR on aphid survival under starvation, insecticide exposure, and heat stress conditions

The mortalities of *R. padi* injected with ds*GFP*, ds*sNPF*, and ds*NPFR* were not significantly different under 24 h starvation ($F = 0.30$; df = 2, 9; $P = 0.75$; Fig. 8A). Compared with aphids injected with ds*GFP*, the mortality of *R. padi* injected with ds*sNPF* ($P < 0.01$) or ds*NPFR* ($P = 0.03$) was significantly increased under 48 h starvation (Fig. 8A). The *sNPF* knockdown resulted in higher aphid mortality after 38 °C heat stress for 2 h ($P < 0.01$), whereas no apparent effect was observed on mortality after infection with ds*NPFR* under 36 °C ($P = 1.00$) or 38 °C ($P = 0.23$) heat stress for 2 h (Fig. 8B).

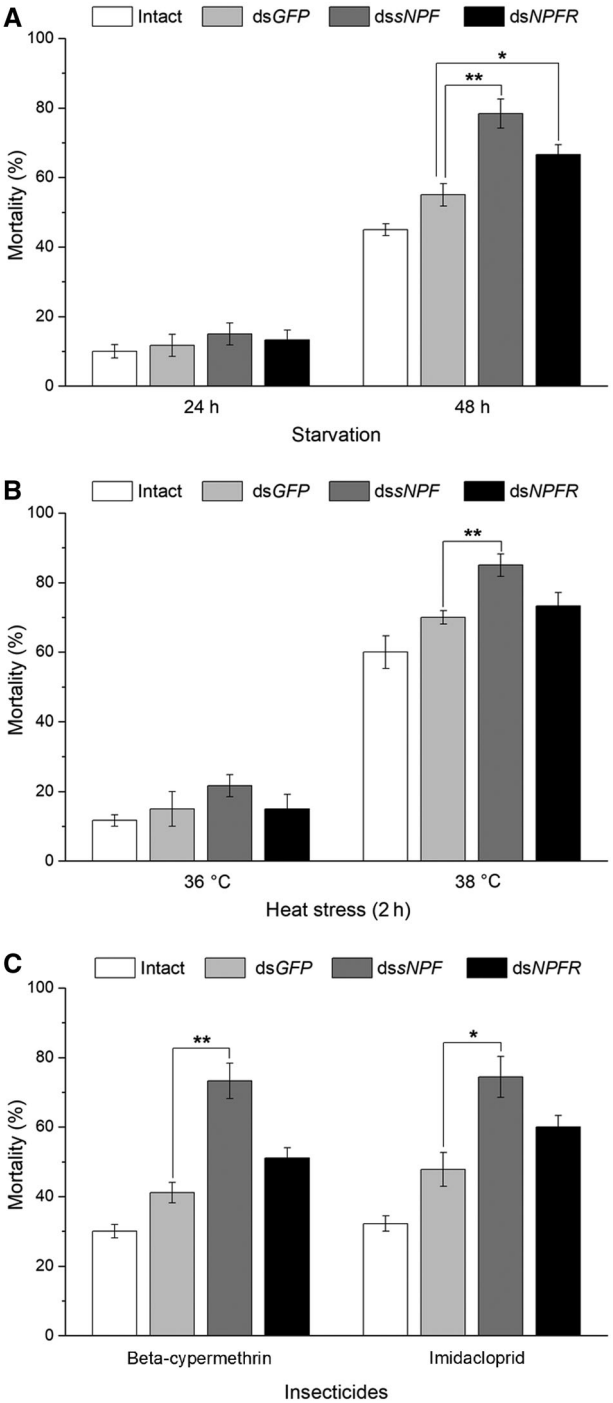

Fig. 8 Roles of *sNPF* and *NPFR* genes in aphid survival after two different starvation periods (A), various heat stress conditions (B) and exposure to sublethal concentrations of two insecticides (C) in *Rhopalosiphum padi*. Asterisks at the top of the bars specify that the values were significantly different ($^{*}P < 0.05$; $^{**}P < 0.01$; *t*-test).

The changes in the mortality of aphids with respect to *sNPF* or *NPFR* silencing treatments with sublethal concentrations (LC_{30}) of beta-cypermethrin (0.93 mg/L) and imidacloprid (1.22 mg/L) are shown in Figure 8C. RNAi targeting *sNPF* significantly increased the susceptibility of *R. padi* to beta-cypermethrin or imidacloprid. After *sNPF* knockdown, the aphid mortality rate (73.33%) under exposure to beta-cypermethrin was significantly increased compared with that after ds*GFP* injection ($P < 0.01$). Compared with that of aphids injected with ds*GFP*, the mortality (74.44%) of *R. padi* injected with ds*sNPF* was significantly increased after exposure to imidacloprid ($P = 0.03$). *NPFR* silencing did not affect the survival of *R. padi* exposed to beta-cypermethrin ($P = 0.07$) or imidacloprid ($P = 0.11$).

Discussion

In this study, the results indicated that the *sNPF* and *NPFR* expression levels were affected by developmental stages, wing morph, starvation, and refeeding after starvation treatments. In addition, *sNPF* knockdown significantly decreased adult longevity, 15-d fecundity, and the percentage of wheat leaf weight reduction, and significantly increased mortality under starvation, insecticide exposure, and heat stress conditions in *R. padi*. Understanding the role of *sNPF* in survival, food intake, and reproduction is essential for developing eco-friendly pest control strategies.

sNPF is evolutionarily conserved, and sNPF and its receptor play vital roles in regulating many physiological processes (Fadda *et al*., 2019). In this study, our results showed that *sNPF* and *NPFR* were differentially expressed in *R. padi* at different developmental stages. Garczynskia *et al*. (2007) found there were *sNPF* and *sNPFR* transcripts in all body regions of *Anopheles gambiae* larvae, pupae, and adults. A similar result was found in *Crassostrea gigas*, and comparison of the copy numbers of Cg-sNPFR-like receptor transcripts revealed that the expression levels of this gene were variable in different life stages (Bigot *et al*., 2014). Differential expression profiles of *BdsNPF* and *BdsNPFR* were detected in the different developmental stages of *B. dorsalis* (Jiang *et al*., 2017). In this study, we specifically compared the *sNPF* and *NPFR* transcript levels in alate and apterous females, and found that both *sNPF* and *NPFR* showed higher transcription levels in alate aphids. Alate aphids usually occur in adverse environmental conditions when they need to search for new host plants (Braendle *et al*., 2006), and alate adults have more sensitive responses to host plants than apterous adults (Peng, 2020, unpublished data). Previous research showed that the sNPF signaling system plays an important role in regulating olfactory sensitivity upon starvation in *B. dorsalis* (Jiang *et al*., 2017). Thus, sNPF might be involved in host plant searching in *R. padi*; however, further research is required to assess the relationships of *sNPF* and *NPFR* expression levels with other physiological functions such as olfactory sensitivity.

Food is a critical source of nutrients for insect survival and reproduction, and intermittent food shortages are commonly encountered under natural conditions (Zhang *et al*., 2019). It is easy to understand that starved insects exhibit an enhanced odor response to food or a desire for food (Jiang *et al*., 2017). We compared the *sNPF* and *NPFR* transcript levels at different time points after starvation and subsequent refeeding, and found that starvation resulted in a tendency toward upregulation of *sNPF* and *NPFR* expression, whereas refeeding after starvation caused a tendency toward downregulation of these genes. These results were similar to those previously reported in several other insect species. In *B. dorsalis*, the *BdsNPF* and *BdsNPFR* transcripts showed significant starvation-induced expression patterns (Jiang *et al*., 2017). *sNPF* was upregulated in starved *A. pisum* and *L. migratoria*, and refeeding experiments decreased the transcript level of these genes (Li *et al*., 2018; Tan *et al*., 2019). These observations indicated that the sNPF signaling pathway might be a key factor in controlling feeding behavior and desire for food.

To further investigate the possible function of the sNPF signaling pathway in aphid feeding, RNAi technology was used. The role of NPF in regulating feeding was first discovered in *Drosophila* (Shen & Cai, 2001). Thereafter, NPF was shown to have a relationship to feeding or foraging in several other insect species, usually playing an active role in stimulating food intake (Gonzalez & Orchard, 2008; van Wielendaele *et al*., 2013; Dillen *et al*., 2014; Li *et al*., 2018; Tan *et al*., 2019). Specifically, pea aphids had a lower appetite for food using electrical penetration graph technology after *sNPF* knockdown, and lower honeydew secretion was found in a group injected with *NPF* dsRNA treatment (Li *et al*., 2018). These results indicated that *sNPF* knockdown resulted in reduced aphid food intake. In this research, we indirectly found that *sNPF* silencing significantly reduced aphid food intake by weighing the isolated wheat leaves. Although we selected three primer pairs to produce dsRNA targets of the *NPFR* gene, the interference efficiency of these dsRNAs was not high. Our results showed that the role of *NPFR* in aphid feeding was less prominent than that of *sNPF*, which may be due to either low *NPFR* interference efficiency or less of an impact of this gene.

The two life table traits, longevity and fecundity, are important aspects of population dynamics and critical to population sustainability and growth (Peng *et al*., 2017). Based on previous research results, we found that *R. padi* mainly produces offspring in its first 15 d of life and rarely produces offspring thereafter. Therefore, we chose 15-d fecundity of females for further comparisons. Our study highlighted that adult fecundity and the 15-d fecundity of females significantly decreased when injected with ds*sNPF*. As mentioned above, aphid food intake could be affected by the *sNPF* expression level. In *R. padi* with *sNPF* knockdown, food intake was decreased. It is possible that there is a trade-off between survival and reproduction in *R. padi*, in which the aphid must sacrifice reproductive potential to maintain normal growth and survival because of the reduced availability of nutrients (Will & Vilcinskas, 2015). *sNPF* silencing can both reduce aphid intake of wheat and help with aphid control.

Starvation, heat stress, and insecticide exposure are common under natural conditions, and insects often encounter these stresses. Our research showed that *R. padi* injected with ds*sNPF* had significantly higher mortality when undergoing prolonged starvation, heat stress, and insecticide treatment. To further understand the underlying mechanisms, we compared the *sNPF* expression levels in *R. padi* after exposure to sublethal concentrations of beta-cypermethrin and imidacloprid, and four different heat stress conditions. The results revealed that the role of *sNPF* in resistance to high temperatures and insecticide exposure may not be direct. Under limited nutritional conditions, aphids may easily die because of stress, and *sNPF* may therefore affect mortality by influencing aphid food intake. However, the specific mechanism needs to be elucidated by further research and investigation. Based on the above results, RNAi targeting *sNPF* can lead to a significant increase in aphid sensitivity to insecticides. These results could be helpful in developing integrated pest management strategies for aphids by directly controlling pests and delaying insecticide resistance development (Niu *et al*., 2018; Shang *et al*., 2020)

Acknowledgments

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Disclosure

The authors declare they have no conflicts of interest.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Amino acid sequence alignment of short neuropeptide F (sNPF) from *Rhopalosiphum padi*, *Myzus persicae*, *Melanaphis sacchari*, *R. maidis*, *Sipha flava* and *Acyrthosiphon pisum* (A) and the phylogeneticrelationships of *R. padi* sNPF with sNPFs from other insect species (B). The phylogenetic tree was established from aligned amino acid sequences using MEGA5. The numbers above the branches represent bootstrap values (%) based on 1000 replicates. The GenBank accession numbers are shown next to the Latin names, and the *R. padi* sequences appear in bold type.

Fig. S2. Schematic diagram of the neuropeptide F receptor (NPFR) domain structure (A) and amino acid sequence similarity and alignment of NPFR from *Rhopalosiphum padi*, *R. maidis*, *Melanaphis sacchari*, *Acyrthosiphon pisum*, *Aphis gossypii*, *Myzus persicae*, *Sipha flava* and *Diuraphis noxia* (B).

Fig. S3. Phylogenetic relationships of the *NPFR* gene of *Rhopalosiphum padi* with those of other insect species. The phylogenetic tree was established from aligned amino acid sequences using MEGA5. The numbers above the branches represent bootstrap values (%) based on 1000 replicates. Latin names are shown next to the GenBank accession numbers, and the *R. padi* sequences are in bold type.

Table S1. Primers used to identify the *sNPF* and *NPFR* genes and primer sequences for real-time quantitative polymerase chain reaction (qPCR) assays and RNA interference with target and reference genes in *Rhopalosiphum padi*.

Table S2. The susceptibility of *Rhopalosiphum padi* to imidacloprid and beta-cypermethrin based on the leaf-dipping bioassay.