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Multigenerational heat acclimation increases thermal tolerance and expression levels of Hsp70 and Hsp90 in the rice leaf folder larvae

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ABSTRACT

Physiological response and acclimation to thermal stress is a key strategy of insects to cope with changing climate. The underlying mechanism of heat acclimation in insects is still unclear. Here, the heat selection and transcript level response in the larvae of the rice leaf folder *Cnaphalocrocis medinalis* Güenée, a serious pest of rice in summer, were studied. The survival and fecundity of larvae during multigenerational heat selection at 39 °C were examined, and heat tolerance and mRNA expression of heat shock protein 70 (Hsp70) and 90 (Hsp90) were examined under heat stress. The results showed that survival and fecundity of larvae increased notably and then kept constant after two or three generations of heat selection. Heat selection improved thermal tolerance of larvae. The Hsp70 mRNA expression of the 3rd-instar larvae increased in all five generations of heat selection, but Hsp90 increased only in the first two generations. The response of Hsp70 to 39 °C heat treatment in the larvae kept at 27 °C was different from the larvae exposed to the conditioning heat treatments, but the response of Hsp 90 was similar. Moreover, the Hsp70 and Hsp90 mRNA expression levels were significantly higher in the heat-acclimated larvae than that in the unacclimated larvae at a comparable duration of exposure to 37 and 41 °C. Selection at a high temperature across multiple generations led larvae to heat acclimation, and Hsp70 and Hsp90 were involved in this acclimation process.

1. Introduction

Temperature is the most important physical factor which affects insect biology, population dynamics and geographical distribution (Angilletta, 2009; Parkash and Ranga, 2013). Due to ongoing global warming, insects have to cope with, not only the increased periods of heat stress, but also the more extreme temperature variations (Hoffmann et al., 2013). Insects are small-bodied poikilotherms, and they are highly vulnerable to extreme and fluctuating temperatures (Krijn et al., 2013). Extremely high or low temperature is lethal to insects, and the unsuitable temperature affects development, survival and reproduction of insects (Chidawanyika and Terblanche, 2011; Liu and Zhang, 2013). Based on the significant effects of temperature on insect populations, the ambient temperature has become a key indication for forecasting population dynamics of pest insects. Thermal biology and ecology of insects have also become an attractive theme to reveal the relationship between organisms and environments.

Under the selection pressure of ambient temperature, insects are involved in a series of physiological strategies to cope with temperature variations (Neven, 2000; Elekonich, 2009; Hu et al., 2014). A short period of heat stress induced the increase of water loss, water-soluble protein, and triglyceride in adults of the sycamore lace bug *Corythucha ciliata*, which enhanced their heat tolerance (Ju et al., 2014). Acclimation at 31 °C improved heat tolerance of adult *Drosophila melanogaster*, and the tolerance was related to the changes in protein abundance (Colinet et al., 2013). The production of heat shock proteins (Hsps) is a well-known physiological response of insects to thermal stress (Elekonich, 2009; Diaz et al., 2015; Lu et al., 2016a,b; Cheng et al., 2016; Cai et al., 2017). Four major families of Hsps, which act as chaperones to stabilize and refold denatured proteins, are recognized in insects, including the small heat shock protein sHsp, Hsp60, Hsp70 and Hsp90 (King and MacRae, 2015). Among the Hsp families, Hsp70 and Hsp90 are the most abundant in cells under stressful conditions (Lindquist and Craig, 1988). Hsp70 is highly conservative at the molecular level and it assists in protein folding and mitigates cellular damage during thermal stress (Mayer and Bukau, 2005; Clark and Worland, 2008). Hsp90 is present in the cytosol and nucleus of all eukaryotes. Its functions are in facilitating protein folding to control protein function and activities, binding ligands to their receptors or targets and assembling multiprotein complexes (Schopf et al., 2017). Induction of Hsps coincides with the increase of thermal tolerance of insects and the heat shock response is assumed to be related to the

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subsequent Hsps expression (Cheng et al., 2016). Heat shock at 40 °C induced a higher level of Hsp70 mRNA in the butterfly *Melitaea cinxia* (Luo et al., 2015). The levels of Hsp70 and Hsp90 mRNA in the macropterous *Nilaparvata lugens* increased dramatically after heat shock (Lu et al., 2016a,b). The amounts of both Hsp70 and Hsp90 mRNA in larvae of fruit flies *Bactrocera dorsalis* and *Bactrocera correcta* were increased in response to heat hardening at 35 °C and 39 °C (Hu et al., 2014).

Acclimation can alter thermal tolerance of insects. Heat hardening treatment increased the survival, fecundity and viability of whiteflies *Bemisia tabaci* under heat stress (Diaz et al., 2015). At high temperatures, pre-treatment at 37 °C for 1 h markedly improved survival of the codling moth *Cydia pomonella* at 43 °C for 2 h (Chidawanyika and Terblanche, 2011). Heat tolerance of the oriental fruit fly *B. dorsalis* was significantly enhanced by heat hardening at 35, 37, 39 and 41 °C, and that of the guava fruit fly *B. correcta* was enhanced by heat hardening at 39 °C and 41 °C (Hu et al., 2014). Thermal acclimation is a key physiological strategy of insect populations to cope with changing climate. However, the physiological and molecular mechanisms of heat acclimation are still unclear.

The rice leaf folder *Cnaphalocrocis medinalis* is one of the destructive pests of rice (Miyahara et al., 1981; Khan et al., 1988). This pest insect feeds on rice leaves and leads to greater yield losses (Padmavathi et al., 2013a). Previous studies found that heat exposure to high temperature remarkably reduced longevity and fecundity of the rice leaf folder adults (Liao et al., 2014), but the larvae were able to tolerate the short-term heat stress (Qian et al., 2017). The population density and outbreak events of rice leaf folders significantly increased in the 21st Century (Kwon et al., 2012; Guo et al., 2013). The temperature in summer usually increases gradually, and a high temperature event often lasts for only several days. We hypothesized that the short-term and gradually increasing temperature might enhance the heat tolerance of the rice leaf folder. Therefore, in this study we reared rice leaf folders at a constant laboratory condition and used this laboratory population to explore effects of heat selection on fitness, and then the heat-acclimated and unacclimated strains were set up. Finally, expression levels of mRNA of heat shock protein genes in the heat-acclimated and unacclimated larvae were compared after these larvae were exposed to a high temperature. This study will reveal the mechanism in heat acclimation of the rice leaf folder larvae.

2. Materials and methods

2.1. Insects

The rice leaf folders *Cnaphalocrocis medinalis* were collected from rice fields in Nanjing, China, and reared in the laboratory using wheat seedlings at 27 °C, 60% RH and a photoperiod of 14 L:10D using a method by Zhu et al. (2015). The first- and second-instar larvae of this pest partially roll leaf edges, and the third to fifth instar fold rice leaves longitudinally and settle inside to feed (Islam and Karim, 1997). The leaf folders probably afford protection from harsh weather and natural enemies. The heat tolerance of young (first- and second-instar) larvae was lower than the old ones (third- to fifth-instar), and the third- and fourth-instar larvae had the similar capacity to tolerate high temperature (Qian et al., 2017). Therefore, in this study we chose the third-instar larvae to perform the heat selection experiments.

2.2. Heat selection of larvae

The maximum summer temperature of Nanjing, China, fluctuated between 36 and 40 °C during 2001–2016, with each of the high temperature events lasting for three to five days. Therefore, we performed heat selection for the third-instar larvae of the rice leaf folder at 39 °C. 300 third-instar larvae collected randomly from a laboratory population were transferred onto 10 boxes of wheat seedlings (30 larvae per box of seedlings), and then put into a 39 °C chamber to be exposed for 3 h per day for three successive days. For the remaining time, the larvae were maintained in a climate chamber at 27 °C. Another 300 third-instar larvae (30 larvae per box of seedlings) were reared continuously at 27 °C and acted as a control. During heat selection, the temperature in the chamber was gradually increased at a rate of 3 °C per 30 min and maintained at the target temperature of 39 °C for 3 h. The temperature was then reduced at a rate of 3 °C per 30 min to 27 °C. The survival rates of larvae on each box of seedlings were examined at the end of heat exposure, pupation and adult emergence. After adult emergence, a pair of female and male adults was put into a copulation cup to mate and oviposit, and examined their fecundity (Liao et al., 2014). Ten pairs of adults were observed. Adults from heat shocked larvae were used to produce more larvae and these larvae were again heat shocked and so on for 13 generations. In the first five generations, the survival and fecundity were examined. After 13 generations of heat selection, the population was considered as a heat-acclimated strain. The control population maintained at 27 °C without heat selection was an unacclimated strain.

2.3. Survival of the heat-acclimated larvae exposed to heat stress

Heat tolerance of the heat-acclimated larvae to 41 °C was examined. Twenty third-, fourth- and fifth-instar larvae collected from the heat-acclimated (selected for 13 generations) and unacclimated strains were transferred into a plastic cup (the bottom diameter of 5.5 cm and 17.5 cm height) with 25 wheat seedlings (height 20 cm) which stems were wrapped by wet cotton wool to keep fresh, and then the cup was put into a 41 °C chamber. Five replications were performed for each instar larvae. Larvae survival rate was observed every one hour for six hours. In the first two hours, the survival rates of the acclimated and unacclimated larvae were almost 100%, so only the survival rates of larvae after three, four, five and six hours of exposure were compared between the acclimated and unacclimated strains.

2.4. qPCR of heat shock protein genes

During the first five generations of heat selection, 16 third-instar larvae were collected at the end of heat exposure in each generation, and then stored in liquid nitrogen to measure the amounts of heat-shock protein genes Hsp70 and Hsp90 mRNA by the qPCR method described below. The unacclimated larvae maintained at 27 °C were the control.

To explore the timeframe of Hsp70 and Hsp90 induced by heat shock, 30 third-instar larvae from the heat-acclimated (selected for 13 generations) and unacclimated strains were collected and transferred onto new wheat seedlings in a plastic cup, and then directly exposed to 37 and 41 °C for 0, 15, 30, 60 and 180 min. After heat exposure, the larvae were collected and frozen in liquid nitrogen to measure the amounts of Hsp70 and Hsp90 mRNA using the qPCR method. All the experiments were replicated four times.

Total RNA was extracted from four third-instar larvae using the TaKaRa MiNiBEST Universal RNA Extraction Kit (TaKaRa, Japan). The cDNA was synthesized from 0.5 μg total RNA using the PrimeScript RT reagent Kit (TaKaRa, Japan) following the instructions. The specific primers of Hsp70 and Hsp90 of the rice leaf folder were designed based on the partial sequences we sequenced (Table 1). The β-actin gene (Genbank accession number JN029806.1) was chosen as the reference gene. qPCR was performed on an Applied Biosystems 7500 thermocycler. PCR reactions were performed in a 20 μl total volume containing 10 μl SYBR® Premix Ex Taq II (TaRaKa , Japan), 2 μl of cDNA, 0.4 μl ROX Reference Dye II (50 ×), 0.4 μl of 10 μM sense and antisense primers and 6.8 μl of ddH_2O. The PCR reaction parameters were as follows: 95 °C for 30 s, 40 cycles of 95 °C for 5 s and 60 °C for 34 s, followed by melting curve analysis to determine the specificity of PCR products. Each cDNA sample was measured three times, and four biological replications were performed for each treatment. The relative expression levels of Hsp70 and Hsp90 mRNA were evaluated using the $2^{-\Delta\Delta CT}$

Table 1
Sequence of the primers for real-time quantitative PCR to measure the relative amounts of Hsp70 and Hsp90 mRNA in larvae based on *actin* gene.

Gene	Forward (5′–3′)	Reverse (3′–5′)	Length of amplified fragment
*Hsp*90	TGAGGATGACGATGAGGATAAGA	CAGCATTGCGAGTCCAGAT	113 bp
*Hsp*70	CACCAAGCAGACCCAGAC	CGAACTTTCCGAGGAGGT	116 bp
Actin	ATGGTCGGCATGGGACAG	GAGTTCATTGTAGAAGGTGT	153 bp

method (Livak and Schmittgen, 2001).

2.5. Data analysis

The survival, fecundity and relative expression levels of Hsp70 and Hsp90 mRNA between the heat-acclimated and unacclimated larvae were analyzed using a student *t*-test, and the differences among selection generations or exposure durations were analyzed using the one-way ANOVA followed by Tukey's HSD *post hoc* comparison method. The effects of larval instar, heat acclimation and exposure duration on larvae survival at 41 °C were analyzed using the GLM. These analyses were performed with SPSS statistics software V19.

3. Results

3.1. Heat selection of larvae

Heat exposure to 39 °C for three successive days (3 h per day) in the third-instar larvae significantly reduced survival (Fig. 1A), pupation

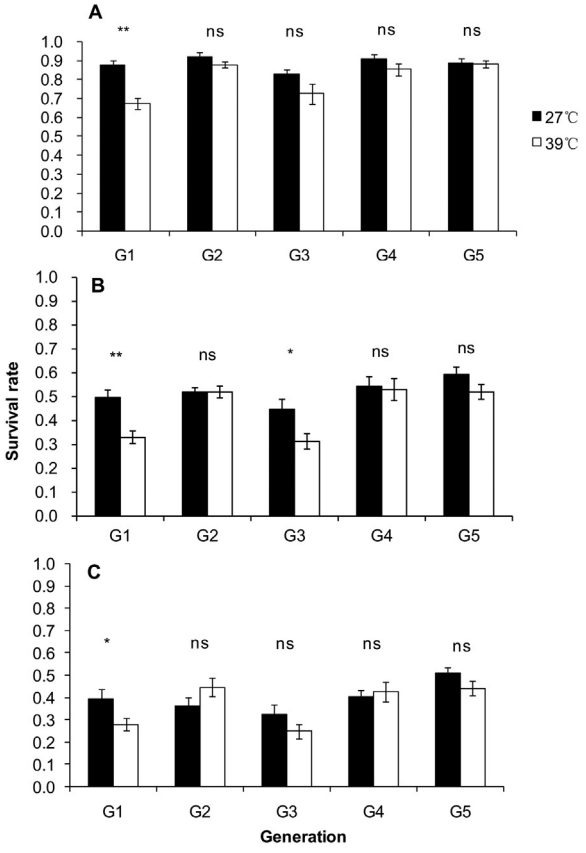

Fig. 1. Survival rates of the third-instar larvae exposed to 39 °C for 3 h per day for three consecutive days over a period of five generations (G1-G5): at the end of exposure (A), at pupation (B) and adult emergence (C). Values are presented as mean ± S.E. * and ** above the bar show significant differences in survival rate of larvae exposed to 39 °C in a generation compared with the control at 27 °C at $P = 0.05$ and 0.01 levels, respectively, and ns means no significant differences at $P = 0.05$.

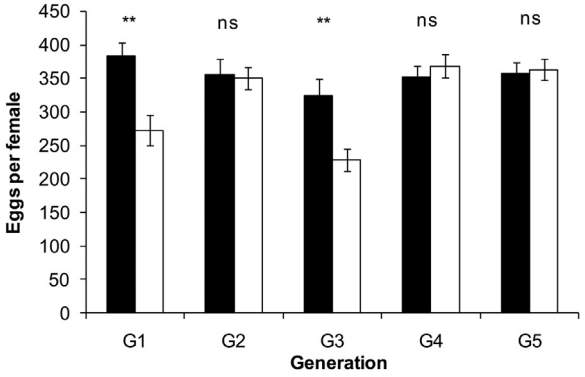

Fig. 2. Fecundity of females which third-instar larvae were exposed to 39 °C for 3 h per day for three successive days over a period of five generations (G1-G5). The control larvae were reared at 27 °C. Values are presented as mean ± S.E. * * above the bar shows significant differences in the number of eggs per female which larvae were exposed to 39 °C, compared with the control at 27 °C at $P = 0.01$ levels, and ns shows no significant differences at $P = 0.05$.

(Fig. 1B), adult emergence (Fig. 1C) and fecundity of the rice leaf folders in the first generation (Fig. 2). However, when the heat stress was imposed again on the third-instar larvae in the following generations (two, three, four, and five generations), it did not affect larval survival (Fig. 1A) and adult emergence (Fig. 1C). Although the larval survival rate at pupation and the fecundity of adults from the heat shocked larvae were significantly lower after three generations of heat selection than that of the control without heat exposure, these values did not significantly differ from the control after four and five generations of heat selection (Figs. 1B and 2). The result indicated that heat acclimation of the rice leaf folder larvae could be induced via multi-generational heat selection.

3.2. Heat tolerance of the heat-acclimated larvae

Survival rate of larvae under the exposure to 41 °C was significantly affected by heat acclimation, larval instar, and exposure duration, and effects of larval instar and heat acclimation on larval survival were also dependent on the exposure duration (Table 2). The survival rates of the third-, fourth- and fifth-instar larvae from the heat-acclimated strain

Table 2
Effects of the larval instar, heat acclimation and exposure duration on the survival of larvae at 41 °C. Survival data were analyzed by the univariate analysis of variance using the GLM and the larval instar, heat acclimation and exposure duration were considered as fix factors.

Source	Type III Sum of Squares	df	F	Sig.
Larval instar (A)	3.006	2	82.543	< 0.001
Heat acclimation (B)	1.090	1	59.833	< 0.001
Exposure duration (C)	2.054	3	37.597	< 0.001
A*B	0.026	2	0.708	0.495
A*C	0.253	6	2.319	0.039
B*C	0.171	3	3.138	0.029
A*B*C	0.079	6	0.721	0.634
Error	1.748	96		
Total	178.985	120		
Corrected Total	8.427	119		

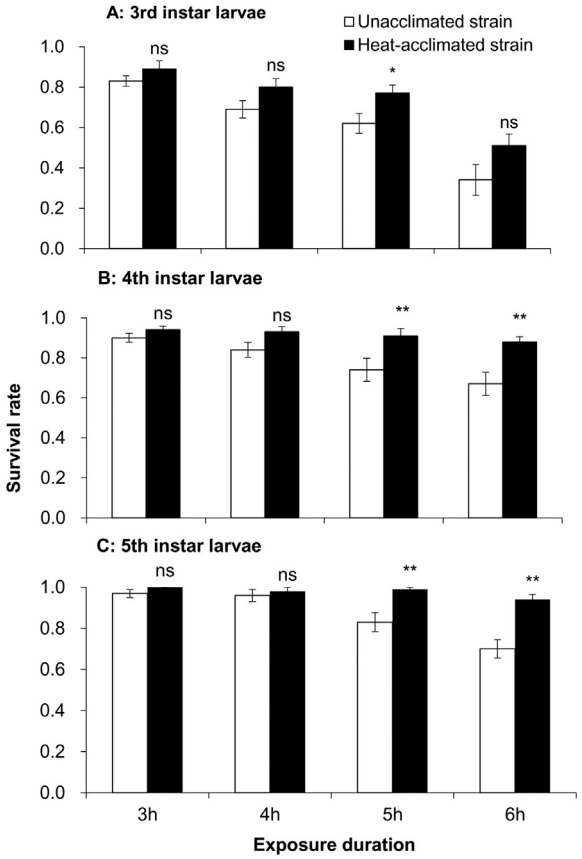

Fig. 3. Survival rates of the third- (A), fourth- (B) and fifth-instar larvae (C) from the heat-acclimated and unacclimated strains exposed to 41 °C for three to six hours. Values are presented as mean ± S.E. * and ** above the bar show significant differences in survival rates of the heat-acclimated larvae compared with the unacclimated larvae at $P = 0.05$ and 0.01 levels, respectively, and ns means no significant differences at $P = 0.05$.

was not significantly different from the unacclimated strain when they were exposed to 41 °C for three and four hours. However, the heat-acclimated larvae exhibited significantly higher survival rate than the unacclimated larvae after five and six hours of exposure to 41 °C (Fig. 3A, B and C) with the exception of the third-instar larvae at six hours of exposure (Fig. 3A). Multigenerational heat selection to 39 °C at the third-instar larvae stage improved the survival rates of the third-, fourth- and fifth-instar larvae under heat exposure to 41 °C.

3.3. The relative amount of Hsp70 and Hsp90 mRNA in larvae during heat selection

During the five generations of heat selection, the relative amount of Hsp70 mRNA in the third-instar larvae after exposure to 39 °C was significantly higher than these of the larvae from control without exposure (Fig. 4A). Moreover, the relative mRNA levels of Hsp70 in the third-instar larvae after exposure to 39 °C were significantly higher in the fourth and fifth generation of heat selection than that in the first to third generation ($F_{4,\ 10} = 5.838$, $P = 0.011$), whereas the mRNA levels in the third-instar larvae from the control at 27 °C were not different among five generations ($F_{4,\ 10} = 1.543$, $P = 0.263$, Fig. 4A). The results showed that the response of Hsp70 to 39 °C heat treatment in the larvae kept at 27 °C was the same as in the larvae selected by the heat treatment for one to three generations, but it differed for the control and heat-acclimated larvae after four and five generations of heat selection (Fig. 4A).

Heat exposure to 39 °C also induced the upregulated expression of Hsp90 mRNA in the third-instar larvae treated with one and two generations of heat selection, but the expression of Hsp90 was not affected by the heat exposure after three to five generations of selection (Fig. 4B). Among the five generations of heat selection, the levels of Hsp90 mRNA expression in the third-instar larvae after exposure did not differ ($F_{4,\ 10} = 1.532$, $P = 0.266$), and the Hsp90 mRNA expression in the larvae kept at 27 °C was also similar among five generations ($F_{4,\ 10} = 0.202$, $P = 0.931$). The response of Hsp90 to heat treatments was similar for the heat-acclimated larvae and control at each of the first five generations (Fig. 4B).

3.4. The relative amount of Hsp70 and Hsp90 mRNA in the heat-acclimated larvae under heat stress

After 13 generations of heat selection at 39 °C, the relative expression levels of both Hsp70 and Hsp90 in the third-instar larvae from the heat-acclimated strain were as similar as the larvae from the unacclimated strain at 27 °C (Figs. 5 and 6, 0 min). However, when the larvae were exposed to 37 °C, the relative expression levels of Hsp70 mRNA in the heat-acclimated larvae significantly increased as exposure duration increased from 15 to 180 min ($F_{4,15} = 4.762$, $P = 0.011$), whereas the expression levels of Hsp70 mRNA of the unacclimated larvae increased after exposure for 15–60 min and then decreased after 180 min ($F_{4,5} = 18.471$, $P < 0.001$). Moreover, after exposure to 37 °C for 180 min, the expression level of Hsp70 mRNA in the third-instar larvae from the heat-acclimated strain was significantly higher than that in the unacclimated larvae ($t = 4.037$, df = 6, $P = 0.007$; Fig. 5A). When the third-instar larvae were exposed to 41 °C, the relative amount of Hsp70 mRNA was increased in both the heat-acclimated ($F_{4,\ 15} = 4.863$, $P = 0.010$) and unacclimated larvae ($F_{4,\ 15} = 11.854$; $P < 0.001$) as the exposure duration extended from 0 to 180 min. However, after 30 min of exposure to 41 °C, the relative amount of Hsp70 mRNA in the heat-acclimated larvae was significantly more than that of the unacclimated larvae ($t = 3.561$, df = 6, $P = 0.012$; Fig. 5B). The heat-acclimated larvae were more sensitive to high temperature in the expression level of Hsp70 mRNA.

Both the heat-acclimated ($F_{4,\ 15} = 34.476$, $P < 0.001$) and unacclimated ($F_{4,\ 15} = 16.584$, $P < 0.001$) larvae increased the relative expression levels of Hsp90 mRNA as larvae were exposed to 37 °C for 15–60 min, and then the expression levels decreased after 180 min of exposure (Fig. 6A). However, the expression level of Hsp90 mRNA in the heat-acclimated larvae was significantly higher than that in the unacclimated larvae when larvae were exposed to 37 °C for 180 min ($t = 3.18$, df = 6, $P = 0.019$, Fig. 6A). When larvae were exposed to 41 °C, the expression levels of Hsp90 mRNA in the acclimated larvae ($F_{4,\ 15} = 75.02$, $P < 0.001$) and the unacclimated larvae ($F_{4,\ 15} = 47.76$, $P < 0.001$) were increased after exposure for 15–60 min, but after 180 min the values decreased (Fig. 6B). Moreover, expression levels of Hsp90 mRNA in the heat-acclimated larvae were significantly higher than that in the unacclimated larvae after 15 min ($t = 5.66$, df = 6, $P = 0.001$) and 30 min ($t = 2.66$, df = 6, $P = 0.037$) of exposure to 41 °C (Fig. 6B). The heat-acclimated larvae were also more sensitive to heat stress in the expression level of Hsp90 mRNA.

4. Discussion

4.1. Heat acclimation of insects

Insects, as small ectotherms, are significantly affected by ambient temperature (Kuo et al., 2006; Radmacher and Strohm, 2011). However, to a certain degree, insects can acquire enhanced thermotolerance via acclimation (Angilletta, 2009). Temperature plays an important role in determining the growth rate, survival, copulation, fecundity and microhabitat selection of the rice leaf folder (Liao et al., 2014; Park et al., 2014; Bodlah et al., 2017). The rice leaf folder cannot complete life history when temperature increases beyond the upper threshold of

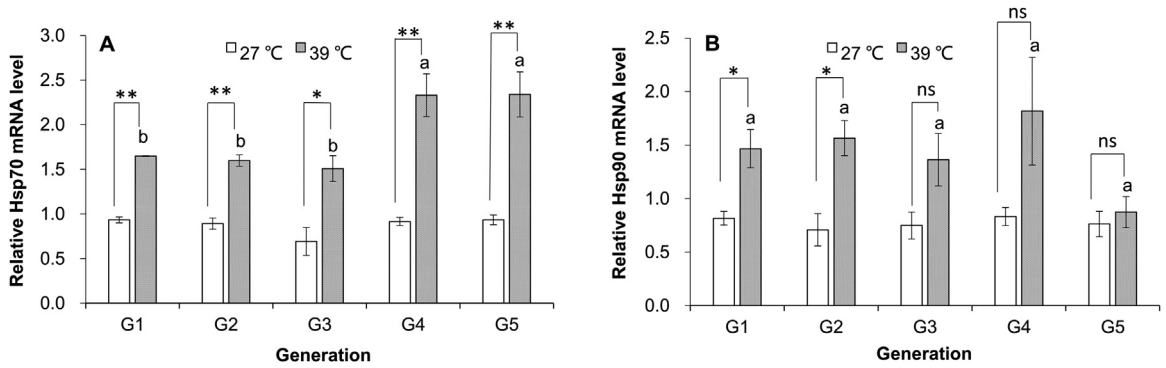

Fig. 4. Relative expression levels of heat shock protein genes Hsp70 (A) and Hsp90 mRNA (B) in the third-instar larvae after heat exposure to 39 °C in five generations (G1-G5). Values are presented as mean ± S.E. * and ** above the bar indicate significant differences in mRNA levels between larvae heat selected at 39 °C and the control at 27 °C at $P = 0.05$ and 0.01 level, respectively, and ns means no significant differences at $P = 0.05$. The different letters above bars indicate the significant differences in expression levels of Hsp70 or Hsp90 mRNA in the heat-acclimated larvae among different selection generations.

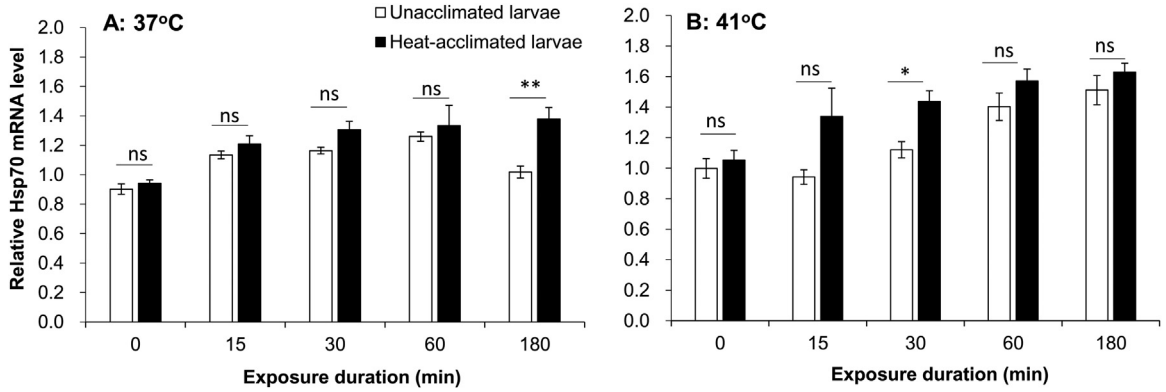

Fig. 5. Relative expression levels of Hsp70 mRNA in the heat-acclimated and unacclimated third-instar larvae exposed to 37 °C (A) and 41 °C (B) for 0–180 min (exposure duration). Values are presented as mean ± S.E. * and ** above the bar indicate significant differences in the expression levels between the heat-acclimated and unacclimated larvae at $P = 0.05$ and 0.01 level, respectively, and ns means no significant differences.

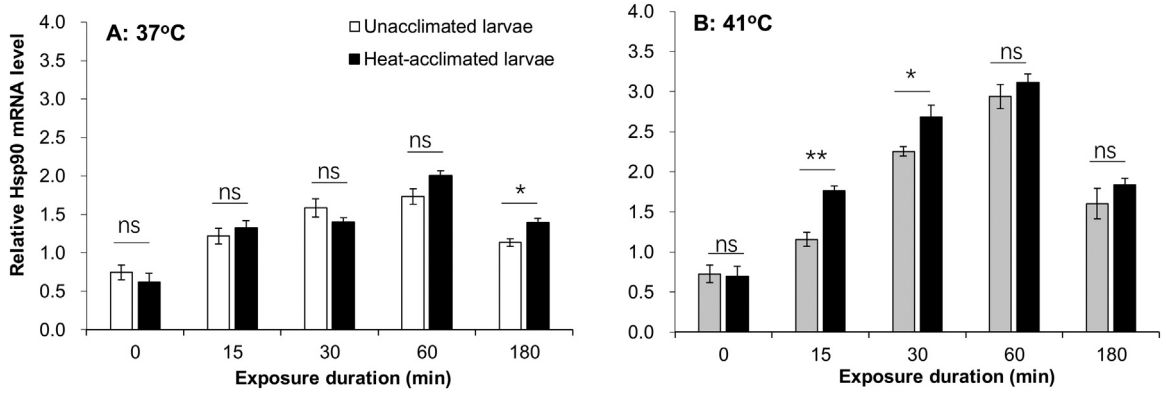

Fig. 6. Relative expression levels of Hsp90 mRNA in the heat-acclimated and unacclimated third-instar larvae exposed to 37 °C (A) and 41 °C (B) for 0–180 min (exposure duration). Values are presented as mean ± S.E. * and ** above the bar indicate significant difference in the expression levels between the heat-acclimated and unacclimated larvae at $P = 0.05$ and 0.01 level, respectively, and ns means no significant differences.

36.4 °C (Padmavathi et al., 2013b). Similarly, in this study, exposure to 39 °C for 3 h per day for three successive days at the third-instar larval stage led to significant declines in survival and reproduction. However, when larvae were selected at 39 °C for two or three generations, their survival and reproduction become similar to larvae maintained at 27 °C. Moreover, the heat-acclimated larvae also had a higher survival rate than the unacclimated larvae when exposed to 41 °C. These results suggest that the rice leaf folder larvae are able to adapt to a short-term heat stress, and heat selection can enhance the heat tolerance of their offspring.

Heat selection resulted in costs to survival and fecundity in the first generation. However, after three generations of heat selection, these costs disappeared. The response of larvae to heat selection supports the beneficial acclimation hypothesis that acclimation to a particular environmental condition provides organisms with performance advantages in that environment (Leroi et al., 1994). It is well known that many insects are able to modify their thermotolerance by thermal acclimation (Angilletta, 2009; Colinet and Hoffmann, 2012; Isobe et al., 2013). For example, the survival rate of *Sitophilus zeamais* adults could be enhanced after exposure to the lethal high temperature if they

experienced prior acclimation (Lü and Zhang, 2016). Acclimation can drastically alter the thermal tolerance of ectotherms and has often been considered as an adaptive response (Brakefield et al., 2007). The rapid adaptive response of the rice leaf folder larvae to heat stress indicates that this pest has a strong potential to cope with high temperature, and global warming might have limited impact on population development. Therefore, population outbreak will occur frequently even in the event of global warming.

Effects of heat acclimation on heat tolerance of insects would be transferred across life stages (Kingsolver et al., 2011; Terblanche and Chown, 2006; Scharf et al., 2015). The higher the juvenile growth temperature, the longer the heat knockdown time of adults had in the red flour beetle *Tribolium castaneum* (Scharf et al., 2015). The larval acclimation of *Culex pipiens* could alter adult phenotypes (Gray, 2013). In the present study, the third-instar larvae of the rice leaf folder were acclimated to 39 °C, and this acclimation improved heat tolerance of not only themselves but also the following fourth- and fifth-instar larvae. Moreover, through one to three generations of heat acclimation, the heat stress of 39 °C did not affect the survival and fecundity of the third-instar larvae. The heat acclimation characteristic across life stages and generations enhances the heat tolerance of the rice leaf folders. The summer temperature of Nanjing usually rises gradually and a high temperature event often lasts for several days. These short-term high temperature events would act as a natural heat acclimatization process and improve the heat tolerance of the rice leaf folders.

Costs or trade-offs of heat acclimation have also been found in insects (Hoffmann, 1995; Hoffmann et al., 2003). Flies *Drosophila melanogaster* acclimated at 36 °C for 75 min had a higher survival rate after a severe stress (39 °C for 100 min), but produced fewer offspring than unacclimated females (Krebs and Loeschcke, 1994). Heat hardening selection led to decreased resistance to ethanol and a reduced dry weight of adult *D. melanogaster* (Hoffmann et al., 1997). There was a trade-off between heat tolerance and reproduction of the butterfly *Bicyclus anynana*, and fecundity was strongly reduced with prolonged heat stress (Franke et al., 2014). These studies suggest that heat acclimation has negative effects on insect fitness. It is expected that the plasticity of upper thermal limits in insects is small in magnitude, and acclimation ability cannot effectively buffer global warming for insects (Sørensen et al., 2016). However, the rice leaf folders can improve heat tolerance via heat selection without obvious costs in survival and fecundity. This would be one of the reasons that population outbreaks of the rice leaf folder have increased in frequency in the last 20 years (Guo et al., 2013).

4.2. Synthesis of heat shock protein mRNA associated with heat acclimation

Heat shock proteins are expressed in most organisms in response to various stressful environmental conditions and are generally considered as one of the defensive mechanisms against thermal stress (Feder and Hoffmann, 1999). Inducible Hsp70 protects cell or tissue against lethal exposure to heat and delays thermal injury (Horowitz, 2001; Ruell et al., 2009). The present study showed that when the larval rice leaf folders were exposed to a high temperature, such as 37 and 41 °C, the levels of Hsp70 and Hsp90 mRNA significantly increased. This result suggests that the heat shock proteins would be involved in the heat tolerance of the rice leaf folders. In this study, we also found that the larvae in all five generations of heat selection at 39 °C exhibited higher levels of Hsp70 mRNA than the control larvae maintained at 27 °C, but Hsp90 mRNA levels were higher only in the first two generations of selection, and after three generations, the Hsp90 mRNA levels were as similar as the control. Similarly, the life-history traits, such as survival and reproduction of the rice leaf folders, became as same as the control after three generations of heat selection. These results suggest that the relative level of Hsp90 mRNA might exhibit the physiological acclimation of insects to heat stress.

However, the levels of Hsp70 mRNA were still significantly higher in the larvae undergone four and five generations of heat selection than in the larvae maintained at 27 °C, although the selected larvae exhibited the same life-history traits as the control larvae. This result indicates that the response of Hsp70 to heat selection is not all the same as Hsp90 in the rice leaf folders, although both the Hsp70 and Hsp90 are upregulated in the first three generations of heat selection. In the wheat blossom midge *Sitodiplosis mosellana*, Hsp70 was most sensitive to heat stress, but the Hsp90 was most sensitive to cold stress (Cheng et al., 2016). The different heat shock proteins may play different roles in the thermal acclimation of insects.

Previous studies usually focused on the response of heat shock protein genes mRNA expression to short-term heat shock (Kim et al., 2008; Cai et al., 2017). For example, heat shock to 40 °C for 1 h resulted in the upregulated expression in the Hsp70 and Hsp90 of the small brown planthopper *Laodelphax striatellus* (Kim et al., 2008). We found that the Hsp70 and Hsp90 in the rice leaf folder larvae were also upregulated in the one hour of heat shock or in the first three generations of heat selection, but when larvae were exposed to 39 °C for 3 h or selected for 4–5 generations, their Hsp90 was not upregulated any more. Moreover, after 13 generations of heat selection, the levels of both the Hsp70 and Hsp90 in the acclimated larvae at 27 °C were not significantly different from the unacclimated larvae (Figs. 5 and 6, 0 h). The correlation between Hsp70 and Hsp90 mRNA expression and heat resistance was not very strong given that very minor differences in Hsp70 and Hsp90 mRNA expression were observed between the unacclimated and heat-acclimated larvae (Figs. 5 and 6). A stronger correlation might be found if levels of Hsp70 and Hsp90 proteins were compared instead of mRNA levels. The multigenerational changes in the amounts of heat shock proteins might be the physiological basis of rice leaf folders to acclimate a high temperature. Based on our knowledge, this is the first time to illustrate the response of heat shock proteins genes of insects to heat stress during the multigenerational heat acclimation.

In conclusion, the rice leaf folder larvae can acclimate to a high temperature environment via multigenerational heat selection. Hsp70 gene is significantly upregulated during heat selection, and Hsp90 upregulated only in the first two generations of selection. Heat-acclimated larvae have the higher sensitivity to heat stress in the Hsp70 and Hsp90 mRNA expression than the unacclimated larvae.

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Disclosure

The authors have no conflict of interests to declare.

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