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SHORT COMMUNICATION

Identification of HSP70 gene in *Corythucha ciliata* and its expression profiles under laboratory and field thermal conditions

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Abstract Previous laboratory studies have demonstrated that insects can tolerate high temperatures by expressing inducible heat shock proteins (HSPs). This HSP-based tolerance, however, has seldom been studied under field conditions. Here, we cloned the HSP70 gene of *Corythucha ciliata* (*Cchsp70*), an invasive insect species with substantial thermal tolerance in subtropical China. We also compared the relative mRNA expression levels of *Cchsp70* in response to controlled temperature treatments (2 h at 33–43 °C at 2 °C intervals in the laboratory) and to natural increases in temperature (08:00–14:00 at 2-h intervals, 29.7–37.2 °C) on a hot summer day in the field. The complete cDNA of *Cchsp70* is 2256 bp long and has a 1917 bp open reading frame that encodes a protein (CcHSP70) with 639 amino acids. The expression levels of *Cchsp70* significantly increased in response to high temperatures in both laboratory and field. At similar temperatures, however, the expression levels were much higher in the field than in the laboratory. These results suggest that CcHSP70 contributes to the thermal tolerance of *C. ciliata* and that factors in addition to thermal stress may induce *Cchsp70* expression in the field.

Keywords Heat shock protein 70 · Heat stress · Laboratory population · Field population · Sycamore lace bug

Introduction

Insects have a range of physiological strategies for adapting to high temperatures, such as heat tolerance (Franke and Fischer 2013), warm acclimation (Bowler 2005), and rapid heat hardening (Ju et al. 2011a). Previous laboratory studies have shown that these strategies are often related to the increased production of defensive substances (Huey and Stevenson 1979; Neven 2000). Whether these laboratory results apply to the field, however, remains unclear (Sørensen 2010). Compared to laboratory conditions, field conditions are more complex and variable. Thermal physiology in the field, therefore, may differ from that in the laboratory, even if the intensity of thermal stress is similar under the two conditions. Recognizing these differences is important when researchers attempt to extrapolate from physiological responses observed in the laboratory to physiological responses in the field, particularly with respect to the diurnal increase in temperature that insects regularly encounter in areas with hot summers (Ju et al. 2014a, b).

The sycamore lace bug, *Corythucha ciliata* (Hemiptera: Tingidae), feeds on *Platanus* trees and is now recognized as an invasive pest in China. Native to the temperate regions of North America, this species has invaded many subtropical zones, including middle and eastern China (Ju et al. 2009). In subtropical regions of China, *C. ciliata* has become a common pest that severely damages *Platanus* leaves in urban areas from July to August (Ju et al. 2011a, 2013). During this summer period, daily maximum temperatures always reach or exceed 40 °C and are

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far above the normal developmental temperatures for *C. ciliata*, which range from 26 to 30 °C (Ju et al. 2011b). Although these summer temperatures subject the insect to thermal stress that is not normally encountered in its native ranges, *C. ciliata* can survive, develop, and reproduce well under these conditions, indicating that this species has substantial thermal tolerance (Ju et al. 2011a, 2013, 2015). Our past studies have demonstrated that *C. ciliata* is probably protected from heat injury by antioxidant response, evaporative water loss, and production of triglycerides and polyols (Ju et al. 2014a, b), but we suspect that other thermal mechanisms such as the expression of heat shock protein (HSP) genes may also contribute to thermal tolerance in this insect.

HSPs constitute a supergene family. Based on their molecular weights and homologies, these proteins can be divided into six families (HSP100, HSP90, HSP70, HSP60, HSP40, and small HSPs) (Feder and Hofmann 1999; Sørensen et al. 2003). Among them, HSP70 is the most conserved and most abundant in insects (Neven 2000; Sørensen et al. 2003; Wang et al. 2016). As molecular chaperones, HSPs play a major role in promoting the correct refolding of proteins and in preventing the aggregation of denatured proteins (Feder and Hofmann 1999). HSPs can be overexpressed in response to a variety of environmental stresses, such as heat (Boher et al. 2012), cold (Štětina et al. 2015), dehydration (Teets et al. 2012), UV exposure (Cao et al. 2012), osmolarity (Brigotti et al. 2003), and organic pollutants (Xin et al. 2012). Among these abiotic stressors, thermal stress is perhaps the most important factor that commonly activates the increased expression of HSPs in insects (Cui et al. 2010a; Advani et al. 2016; Cahan et al. 2017).

In this study, we cloned the HSP70 gene of *C. ciliata* and compared its expression profiles under both laboratory and field thermal conditions. We attempted to answer the following two questions: (1) Does the expression of HSP70 gene of *C. ciliata* differ under thermal conditions in the laboratory vs. the field? (2) What is the significance of HSP70 gene expression in the adaptation of *C. ciliata* to high temperatures? We hypothesized that *C. ciliata* is able to increase HSP70 gene expression in response to thermal stress in both the laboratory and field but that the level of expression at similar temperatures may differ in the laboratory vs. the field.

Materials and methods

Laboratory experiment

To clone the full-length complementary DNA (cDNA) of the HSP70 gene of *C. ciliata* and to compare its expression level at high temperatures in the laboratory, we collected *C. ciliata* adults from *Platanus* × *acerifolia* in Changning District of Shanghai, China (31.2°N, 121.5°E) in 2010. These adults were reared in an environmental chamber at 26 ± 0.5 °C with a relative humidity (RH) of 80 ± 5% and a 14 h/10 h (L/D) photoperiod as per Ju et al. (2014a, b). Newly emerged adults were kept at 26 °C for 24 h before they were used in the laboratory experiment. The laboratory-reared adults (regardless of sex) were placed in Petri dishes (diameter = 9 cm; one group of 200 adults per dish) and then were exposed to 33, 35, 37, 39, 41, or 43 ± 0.5 °C for 2 h in a climatic incubator with a relative humidity (RH) of 80 ± 5% (adults exposed to 41 °C for 2 h were also used for molecular cloning of the HSP70 gene, as described in a later section). The adults were then transferred to rearing conditions (26 °C) and allowed to recover for 2 h (Ju et al. 2014a, b). The surviving adults were immediately frozen in a liquid nitrogen canister (YDS-10A, Chengdu Jinfeng Liquid Nitrogen Limited Corporation, China). The specimens were then kept in a low-temperature refrigerator (Thermo702, Thermo Electron Corporation, USA) at − 80 °C for subsequent determination of messenger RNA (mRNA) expression of the HSP70 gene of *C. ciliata*. Each treatment was replicated three times with 200 insects per replicate. For the control, adults were maintained at 26 °C throughout the experiment.

Field study

To quantify the expression of the HSP70 gene under field thermal conditions, adults of *C. ciliata* were cultured on *P.* × *acerifolia* trees, which were growing at the Shanghai Institute of Landscape Gardening Science (31.2°N, 121.5°E) as described in our previous studies (Ju et al. 2014a, b). On 29 July 2011, the adults (regardless of sex) on the upper leaves of the trees were collected at 08:00, 10:00, 12:00, and 14:00. To avoid disturbing the adults on the leaves, the temperature was measured 20 cm below the infested leaves (Ju et al. 2014a); we had previously confirmed that air temperatures were the same adjacent to the infested leaves and adjacent to leaves 20 cm below. The average temperature was 29.7 °C at 08:00, 33.5 °C at 10:00, 35.2 °C at 12:00, and 37.2 °C at 14:00. The collected adults were recovered, frozen, and stored as described for the laboratory experiment. The specimens collected at each collection time were divided into three replicates with 200 individuals per replicate. The adults kept at 26 °C in the laboratory were also considered the control for the field study; this enabled us to compare the relative expression of the HSP70 gene at similar temperatures in the laboratory vs. the field.

Molecular cloning of the HSP70 gene

Newly emerged adults (regardless of sex) were kept at 26 °C for 24 h before they were exposed to 41 °C for 2 h in the laboratory and then to 26 °C for 2 h in a climatic incubator, as described for the laboratory experiment. The surviving

adults were collected for RNA extraction. Total RNA was extracted from one batch of approximately 200 surviving adults of *C. ciliata* using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). A 5-μg quantity of RNA was then used to synthesize first-strand cDNAs with a GeneRacer kit (Invitrogen, Carlsbad, CA, USA). A pair of primers (H70F1 and H70R1; Table 1) was designed to amplify the HSP70 cDNA fragment from *C. ciliata*. The 5′ and 3′ regions of the cDNA were obtained by 5′- and 3′-RACE using an Invitrogen GeneRacer kit with two pairs of gene-specific primers: 5-GSP1, 5-GSP2, 3-GSP1, and 3-GSP2 (Table 1).

Sequence analysis of the HSP70 gene

The full length of the HSP70 gene of *C. ciliata* was assembled from the cloned segments and was named *Cchsp70*. The sequence alignment and identity analysis were implemented with the aid of the DNAMAN software package (Lynnon, Canada). The open reading frame (ORF) was determined and translated into an amino acid sequence using ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder/). Sequence homologous alignment of *Cchsp70* was carried out in GenBank by BLAST (http://www.ncbi.nlm.nih.gov/blast). A phylogenetic tree was constructed on the basis of amino acid sequences of selected HSP70 genes by using the neighbor-joining (NJ) method with MEGA software (http://www.megasoftware.net/).

Quantitative analysis of mRNA expression of the HSP70 gene

To evaluate the thermal induction of *Cchsp70* mRNA expression in the *C. ciliata* adults that survived the temperature treatments in the laboratory or the temperature regimes in the field, a quantitative real-time PCR assay was carried out using an ABI 7900 Real-time PCR System (ABI, USA). Total RNA was extracted as described earlier. A 1-μg quantity of total RNA was reverse transcribed with a Prime Script™ RT reagent kit (Takara, Dalian, China). Paired specific primers (HSP70-qPCR-F and HSP70-qPCR-R; Table 1) were designed to amplify the target fragment from *Cchsp70* cDNA. The other paired primers (Actin-F and Actin-R; Table 1) were designed to amplify the fragment of *β-actin*, which was used as an internal control. The relative mRNA expression levels of *Cchsp70* were calculated using the $2^{-\Delta\Delta Ct}$ method (Long et al. 2015). All data in terms of the relative expression levels of *Cchsp70* are shown as means ± SE (n = 3). Data were subjected to one-way analyses of variance (ANOVAs) by using the general linear model procedure of SPSS 18.0 for Windows (SPSS Inc., Chicago, USA). To satisfy assumptions of normality and equal variance, all data were log-transformed before statistical analyses. When treatment effects were significant ($P < 0.05$), means were compared with Tukey's test.

Results and discussion

The PCR product amplified by the homologous cloning primers contains 703 bp (Fig. S1). The complete cDNA sequence of *Cchsp70* (GenBank accession, KF018929) contains 2256 bp nucleotides with a 1917-bp ORF that encodes a peptide of 639 amino acids with two signature sequences of the HSP70 gene family (IDLGTTYS and IFDLGGGT) (Fig. 1). *Cchsp70* has highly conserved sequences and characteristic motifs of HSP70 genes. For example, its deduced amino acid sequence has HSP70 family signatures, ATP, and the major

Table 1 Primer sequences used in the cDNA cloning and real-time quantitative PCR

Primer type	Primer sequence (5′ → 3′)
cDNA amplification of *hsp70* partial fragment (703 bp)	
H70F1	AGTTCGTGCCACTGCTGGAGACA
H70R1	ATCCTTCGTCATTGCTCGCTCAC
5′ RACE amplification	
5-GSP1	TTCTACCGGCTGCAAGGTTGATCTG
5-GSP2	GCCTCAGTGCTTGACGACAATGTTCGTTTGGCTCTCTCG
3′ RACE amplification	
3-GSP1	GAAGAACTATGCGCCGACTTGTTCAG
3-GSP2	CTACTTGTTGACGTAGCACCGCTATCA
Real-time quantitative PCR of HSP70	
HSP70-qPCR-F	GCAAACGGAATCCTGAATGT
HSP70-qPCR-R	GGCCTGTTTGATTTGGAAAA
Real-time quantitative PCR of *β*-actin	
Actin-F	CAGCCATGTATGTTGCCATC
Actin-R	AGCGGTGGTTGTGAAAGAGT

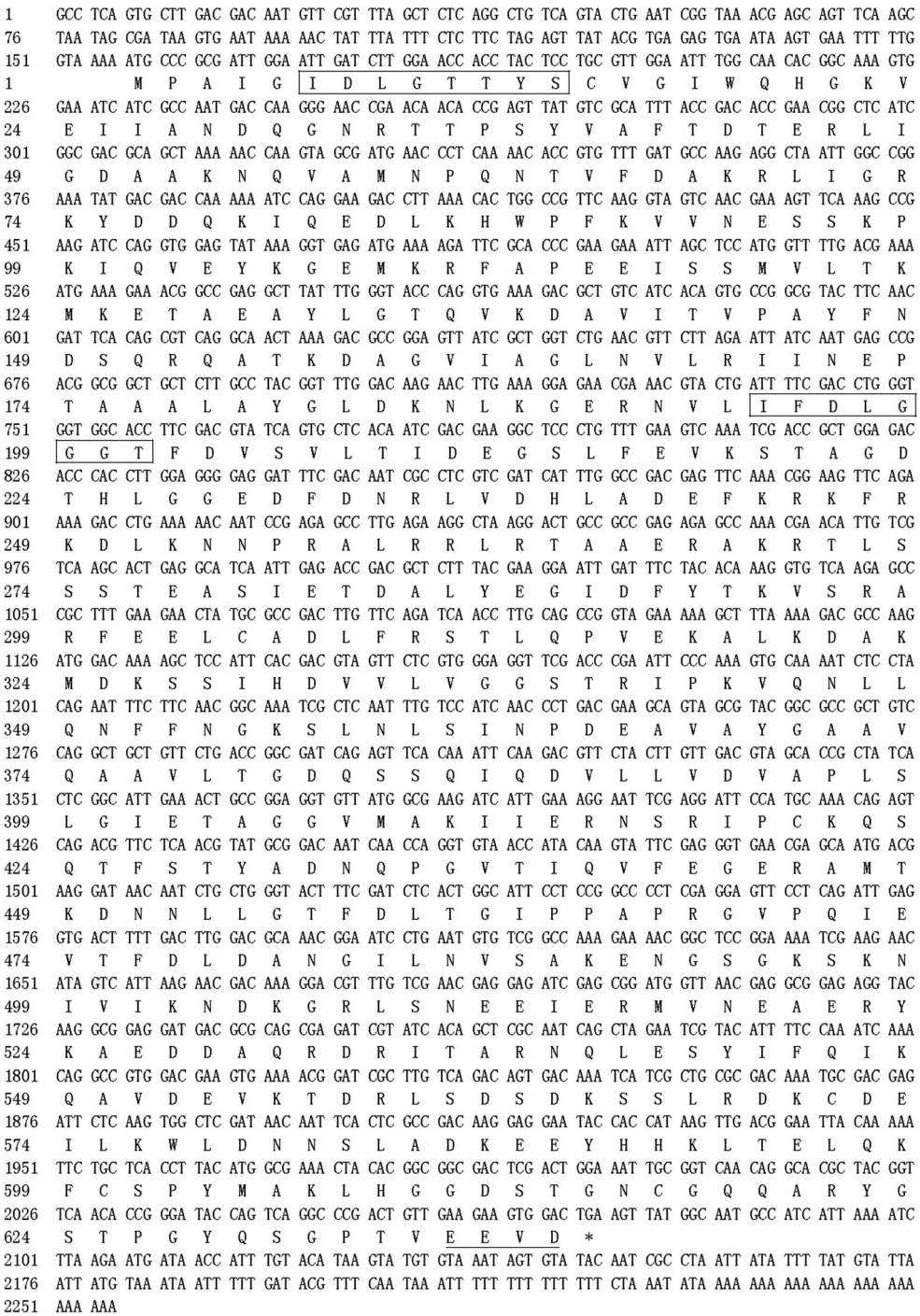

Fig. 1 The complete cDNA sequence of the HSP70 gene of *Corythucha ciliata* and the predicted amino acid sequence of the encoded protein. Nucleotide numbering begins with the adenine in the first methionine codon of the putative open reading frame. The characteristic cytosolic HSP70 sequence, EEVD, is underlined. The asterisk indicates the translational termination codon. The signature sequence of HSP70 family is boxed

structural and functional domains of the HSP70 family (Fu et al. 2009; Cui et al. 2010b). At the C-terminus of *Cchsp70*, the EEVD sequence is strictly conserved and is shared by many other members of HSP families (Cui et al. 2010a; Zhang et al. 2012). This peptide is recognized by TPR domains of HOP (HSP70- and HSP90-organizing protein), which is an adapter protein that regulates the association of HSPs with a multiple chaperone complex (Scheufler et al. 2000). Sequence similarity analysis revealed that the predicted amino acid sequence of *Cchsp70* shares considerable homology with other known HSP70s (Fig. 2), especially with those from species in the Hemiptera (e.g., *Pyrrhocoris apterus*, *Bemisia tabaci*, and *Trialeurodes vaporariorum*), indicating that HSP70 genes of insect species from the same order are closely related. Because highly conserved HSP70s often have similar functions in their protection of cells (Kampinga and

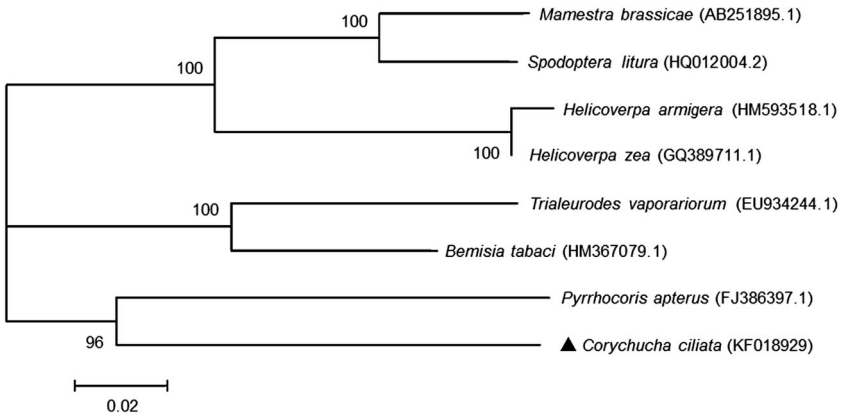

Fig. 2 Phylogenetic analysis of HSP70 homologs in *Corythucha ciliata* and other insects based on amino acid sequences. The GenBank accession numbers for amino acid sequence data are in brackets

Craig 2010), CcHSP70 may act as a molecular chaperone to prevent irreversible misfolding and aggregation of non-native proteins under stressful conditions (Cui et al. 2010b).

HSP70 genes have been found in nearly all organisms, and their expression is usually increased by thermal stress (Feder et al. 1992; De Jong et al. 2006; Sørensen et al. 2003). In our laboratory experiment, the relative mRNA expression levels of *Cchsp70* significantly increased as stressful temperature rose ($F_{6, 20} = 208.3$, $P < 0.0001$) (Fig. 3). The expression level peaked at 41 °C and then significantly declined at 43 °C. The decreased level of *Cchsp70* expression at 43 °C may be caused by inhibition of the enzymes responsible for synthesis of CcHSP70 mRNA when temperature is over 43 °C (Zhang et al. 2012). Previous studies have suggested that the maximum expression of HSP70 genes is usually obtained by temperatures that are 10–15 °C higher than the optimal developmental temperature (ODT) of an organism (Fu et al. 2009; Zhang et al. 2012). Our past studies have shown that the ODT of *C. ciliata* is 26 °C (Ju et al. 2011b). Therefore, the maximum *Cchsp70* expression would be expected to occur at temperatures between 36 and 41 °C. True to this expectation, *Cchsp70* expression levels were very low between 26 and 35 °C and dramatically increased when temperatures increased to 37 °C and higher in the laboratory experiment (Fig. 3). The maximum expression level was at 41 °C, which is 15 °C higher than the ODT of *C. ciliata*. The temperatures that induce *Cchsp70* expression are similar to those that induce the expression of HSP70s in many other insects, such as *Manduca sexta* (Fittinghoff and Riddiford 1988), *Sarcophaga crassipalpis* (Joplin and Denlinger 1990), *Spathosternum prasiniferum*, *Periplaneta americana*, *Heliothis armigera* (Singh and Lakhotia 2000), and *Tribolium castaneum*

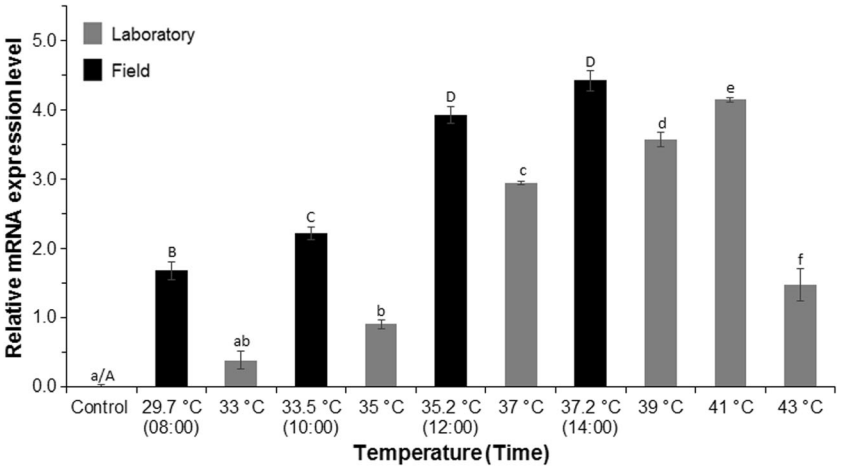

Fig. 3 Relative mRNA expression levels of the HSP70 gene in *Corythucha ciliata* adults after thermal stress in the laboratory experiment (gray columns) and field study (black columns). The expression levels were analyzed by quantitative real-time PCR. The β-actin gene served as an internal control to calibrate the expression levels for all samples. All data have been log-transformed in the figure. In the laboratory experiment, adults were exposed to the indicated high temperatures (33–43 °C) for 2 h. In the field study, the adults were collected from 08:00 to 14:00 (29.7–37.2 °C) on a hot summer day (29 July 2011) in an experimental field in Xuhui District, Shanghai, China (31.2°N, 121.5°E). In both experiments, adults that were kept at 26 °C served as the control. Values are means ± SE (n = 3). Values with different lowercase letters for the laboratory experiment and uppercase letters for the field study are significantly different (Tukey's test after log-transformation at $P < 0.05$, ANOVA)

(Mahroof et al. 2005). This suggests that the thermal physiology, at least with respect to HSP70 expression, is similar in these species.

In previous studies, there have been some but not many cases concerning heat-shock response under field conditions in insects as well as other organisms (e.g., Nath and Lakhotia 1989; Evgen'Ev et al. 2014). In our field study, the mRNA expression levels of *Cchsp70* increased from 08:00 (29.7 °C) to 14:00 (37.2 °C) and were significantly higher than the level for the control (adults kept at 26 °C) (Fig. 3). Levels were higher at noon (35.2 °C) and afternoon (37.2 °C) than at earlier times (29.7–33.5 °C) of the day ($F_{4,\ 14} = 253.3$, $P < 0.0001$). Linking to the laboratory results, the mRNA expression levels of *Cchsp70* at similar temperatures were much higher in the field than in the laboratory, i.e., expression levels were higher at 10:00 (33.5 °C), 12:00 (35.2 °C), and 14:00 (37.5 °C) in the field than at 33, 35, and 37 °C, respectively, in the laboratory (Fig. 3). Moreover, the threshold temperature at which significant increases were detected for *Cchsp70* expression also differed in the laboratory and field, i.e., this threshold temperature was 35 °C in the laboratory and 29.7 °C in the field (Fig. 3). The difference indicates that *Cchsp70* expression may be more readily induced under uncontrolled field conditions than under controlled laboratory conditions. One possible explanation is that insects in the field but not in the laboratory may experience non-temperature stresses (such as UV radiation, osmolarity, and environmental pollution) that may also induce *Cchsp70* expression (Cao et al. 2012; Brigotti et al. 2003; Xin et al. 2012). The differences between the laboratory and field data may also be explained by differences in insect age, acclimation, and behavior; the heat-shock ways (heated by sun in the field but by container in the laboratory); the collecting time; and the humidity changes between laboratory and field treatments.

The increased expression of *Cchsp70* may play an important role in the tolerance of *C. ciliata* to high temperatures. We previously found that *C. ciliata* adults can tolerate temperatures of 35–41 °C, i.e., their survival, development, and reproduction were not reduced by this temperature range in the laboratory. Survival, development, and reproduction were substantially reduced, however, by a 2-h exposure to 43 °C (Ju et al. 2011a, 2013). These previous results are correlated with the *Cchsp70* expression profiles obtained with different temperatures in the laboratory experiment of the current study. This correlation between biological parameters (survival, development, and reproduction) and expression profiles suggests that CcHSP70 is important for the thermal tolerance of *C. ciliata* and that CcHSP70 may be a useful biomarker for detecting thermal tolerance of the insect. Moreover, as we discussed earlier, the antioxidant response and the metabolisms of water, triglycerides, and polyols in bodies of *C. ciliata* also likely counteract the negative effects of high temperatures on the insect (Ju et al. 2014a, b). These combined factors may explain why *C. ciliata* tolerates the high temperatures in subtropical China and why high temperature may not limit the insect's establishment and spread. These findings suggest that *C. ciliata* may spread further south in China, where *Platanus* trees are widely planted. We therefore suggest that measures are urgently needed to prevent the further spread of *C. ciliata*. Further studies are needed to confirm the expected function of HSP70 protein of *C. ciliata* by using relevant antibody to monitor the levels of heat shock proteins present in the cells of insects before and after heat shock and collecting the field data on more separate days to make the data more reliable and applicable. Additional studies are also needed to compare HSP70 expression of *C. ciliata* in response to thermal stress between invasive and native populations. This may explain possible adaptive and evolutionary strategies that help the insect survive in warmer conditions.

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Author's contributions RTJ and BL conceived the experiments. RTJ and QQL performed the experiments. RTJ and QQL analyzed the data. RTJ, LG, JY, and BL wrote the manuscript. All authors read and approved the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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