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COMPOSITION AND EXPRESSION OF HEAT SHOCK PROTEINS IN AN INVASIVE PEST, THE RICE WATER WEEVIL (COLEOPTERA: CURCULIONIDAE)

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ABSTRACT

For poikilothermic groups such as insects, the capacity to adapt to different temperature regimes is particularly important for survival. To investigate the possible role of heat shock proteins (Hsps) in the invasive pest, the rice water weevil (*Lissorhoptrus oryzophilus* Kuschel, Coleoptera: Curculionidae), we first analyzed the composition and expression profile of Hsp families under sub-lethal temperatures of 0 °C and 43 °C, using the quantitative real-time polymerase chain reaction. Eight genes coding Hsp90, Hsp70, and small Hsps were up-regulated under heat stress, while only 1 Hsp70 gene and 1 Hsp90 gene were up-regulated under cold stress. Results indicate that Hsps from all families except Hsp60 are responsible for the capacity of *L. oryzophilus* to tolerate temperature stress, although more genes were up-regulated, and more rapidly, under heat stress than under cold stress. Secondly Hsp expression patterns in diapausing and non-diapausing female adults were investigated. The results showed that rice water weevils in diapause up-regulated no Hsp gene but they down-regulated 4 small Hsps, 2 Hsp90, 1 Hsp70 and 1 Hsp60 genes.

Key Words: diapause, heat shock protein, invasive species, thermal tolerance

RESUMEN

Muchos factores afectan la distribución de especies en la naturaleza y la temperatura es uno de los más profundos. Para grupos de poikilotérmicos como los insectos, la capacidad de adaptación a diferentes regímenes de temperatura es para especies invasoras especialmente importante. Para investigar el posible papel de proteínas de choque térmico (Pcts) en el éxito de la plaga invasiva, analizamos primeramente el perfil de la composición y la expresión de las familias de Pcts bajo temperaturas sub-letales de 0 °C y 43 °C en el gorgojo del agua de arroz (*Lissorhoptrus oryzophilus* Kuschel, Coleoptera : Curculionidae), utilizando la reacción en cadena de la polimerasa cuantitativa en tiempo real. Ocho genes codificantes de Pct90, Pct70, y pequeños Pcts fueron incrementados bajo condiciones de estrés térmico, mientras que sólo 1 gen Pct 70 y 1 gen Pct 90 fueron incrementados bajo condiciones de estrés por frío. Los resultados indican que las Hsps de todas las familias menos la Pct 60 son responsables por la capacidad de *L. oryzophilus* para tolerar el estrés de temperatura, aunque más genes fueron incrementados más rápidamente bajo condiciones de estrés por calor que bajo estrés por frío. Un segundo estudio comparó los patrones de expresión de Pct en hembras adultas en diapausa y no diapausa. Los insectos en diapausa no incrementaron el gen Hsp pero si redujeron 4 genes pequeños de Pcts - 2 Pct 90, 1 Pct 70 y 1 Pct6.

Palabras Clave: diapausa, proteína de choque térmico, especies invasoras, tolerancia térmica

Many environmental factors affect the distribution of species in nature such as temperature and humidity (Gibbs et al. 2003), chemicals and heavy metals (Macnair 1997). Thermal environments often vary spatially and temporally across the geographic range of a species, and such varia-

tion can be an important selection force leading to genetic divergence among local populations (Angilletta 2009). When individuals in a population are exposed to stressful conditions, there are 3 possible outcomes, i.e., they avoid the stress, they adapt to the stress, or they are killed (Hoffmann et al. 1991). There is, however, a particular set of proteins, named heat stress proteins (Hsps), that are preferentially expressed under stress (Peter et al. 2002). These are highly-conserved molecules that are categorized into several subgroups according to size, structure and function (Lindquist et al. 1988; Boorstein et al. 1994; Richter et al. 2010). Expression of Hsps is increased when the cells are exposed to elevated temperatures, and they are induced in cells exposed to sub-lethal heat shock (Kiang & Tsokos 1998). The heat shock response was first discovered by Ritossa (1962) in *Drosophila melanogaster* Meigen (Drosophilidae). The induction of Hsps occurs under circumstances relevant to the environment of the species, such that in arctic fish Hsps are induced around 5 °C and in thermophilic bacteria around 100 °C (Parsell et al. 1993). Hsp families are known to be important in the biology of insects. For example, small heat shock proteins and Hsp70 are related to thermo-tolerance in the silkworm (*Bombyx mori* [L.]; Bombycidae) (Moghaddam et al. 2008).

In addition to their role in thermal tolerance, Hsps can be induced by a variety of other stimuli, and they play broader roles. Members of the Hsp70 family in particular are constitutively expressed in cells under normal conditions and function as molecular chaperones, to keep other proteins from forming inappropriate aggregations (Mbaye 2010). As well as being induced by a variety of stresses, Hsp expression can vary with the physiological state of the organism and developmental stage (Feder et al. 1999). For example, Hsp23 and Hsp70 of the flesh fly, *Sarcophaga crassipalpis* Macquart (Sarcophagidae), are highly up-regulated during diapause (Yocum et al. 1998; Rinehart et al. 2000). Similarly, one of the 2 copies of Hsp70 gene in diapausing adults of Colorado potato beetle, *Leptinotarsa decemlineata* Say (Chrysomelidae), is up-regulated (Yocum 2001). In the gypsy moth, *Lymantria dispar* (L.) (Erebidae), Hsp70 is not expressed at the initiation of diapause, but only after exposure to low temperature (Yocum et al. 1991). Notwithstanding the similarities in the role of Hsps in the foregoing examples, there is no fixed expression pattern of Hsp genes among different species. Accordingly, this study was aimed to elucidate the expression pattern of Hsps in the rice water weevil, *Lissorhoptrus oryzophilus* Kuschel (Coleoptera: Curculionidae), an invasive pest species that has not been the subject of earlier studies of its capacity to tolerate environmental stresses.

The rice water weevil originates from the Mississippi River Valley of the USA (Lange & Grigarick 1959). Parthenogenetic females were found in rice in California in 1959. This species has subsequently come to be considered as an important pest of this crop (Godfrey & Espino 2009). *Lissorhoptrus oryzophilus* has invaded parts of eastern Asia since the 1970s (Way 1990; Kim et al. 1996; Kobayashi et al. 1997; Zhai et al. 1999; Stout et al. 2002; Zhu et al. 2005). In China, the world's largest rice producer, this pest was first detected in Hebei Province in 1988 and is now found over an extensive area from Jilin Province (N 41° - N 46°) to Yunnan Province (N 23° - N 25°) where the climate ranges from temperate to tropical. *Lissorhoptrus oryzophilus* is able to overwinter in the temperate parts of its range as well as to tolerate seasonally high temperatures in summer; thus it possesses effective mechanisms for coping with thermal stress.

Diapause, the arrest of development or normal activity during adverse environmental conditions (Chapman 1998), is suggested as the means for over-summering and over-wintering by the weevil in its large geographical range (Jiang et al. 2004; Zhu et al. 2005). However, whether the Hsps play a role in diapause remains unclear.

To investigate the relationship between the expression pattern of Hsp genes and temperature stress, firstly, we searched for all Hsp genes in the *L. oryzophilus* transcriptome database. Secondly, we exposed insects to the sub-lethal low and high temperatures of 0 °C and 43 °C for 0.5 - 3 h to identify the expression patterns of Hsps under thermal stress. Thirdly, we compared the expression of Hsps between diapausing and non-diapausing *L. oryzophilus* female adults.

Materials and Methods

Insect Collection and Rearing

Rice stubble with attached in roots infested with *L. oryzophilus* cocoons was collected from rice fields in Xinchengfan village, Leping County, Jiangxi Province, China in July 2012 and kept in plastic containers within a cage covered with fine nylon mesh in an insectary. Emerging adults were collected daily and transferred to 100 mL plastic bottles under 16:8 h L:D, 25 ± 1 °C and 70% RH as in Yang et al. (2010). Adults were fed with 20-day-old rice seedlings (cv. 'Xiushui 110', *Japonica*).

Diapausing *L. oryzophilus* adults were collected from the surface soil of a hill nearby rice fields in Yueqing county, Zhejiang Province, China on 2 Nov 2012 and kept in plastic containers within a cage covered with fine nylon mesh. Live individuals were transferred to -80 °C immediately after their arrival at the laboratory. These adults were determined as diapausing weevils according

to Shang (2003). The nondiapausing weevils were collected in 5 July 2012 and frozen at the -80 °C. According to the yearly population dynamics of the weevils indicated in Zhai et al. (1997), this batch of weevils probably were recently emerged and their age probably was 2-3 weeks, and they had not yet entered to diapause.

cDNA Library Preparation and Illumina Sequencing

Total RNA of *L. oryzophilus* adults were extracted by using TRIzol reagent (Invitrogen, California, USA) according to the manufacturer's protocol. According to the Illumina manufacturer's instructions, poly(A)' RNA was purified from 20 ug of total RNA using ologo(dT) magnetic beads and fragmented into short sequences in the presence of divalent cations at 94 °C for 5 min. The cleaved poly(A)+ RNA was transcribed, and second-strand cDNA was synthesized. After the end-repair and ligation of adaptors, the products were amplified by PCR and purified using the QIAquick PCR Purification Kit to create a cDNA library.

The cDNA library was sequenced on the Illumina sequencing platform (GAII). The raw reads were generated using Solexa GA pipeline 1.6. After removal of low quality reads, processed reads with an identity value of 95% and a coverage length of 100 bp were assembled using SOAP de novo software and clustered using TGI Clustering tools. Generated unigenes larger than 350 bp were analyzed by searching the GenBank database with the BLASTX algorithm (data unpublished).

Searching the *Lissorhoptrus oryzophilus* Transcriptome Database for Hsp Sequences

Hsp genes were identified by searching the sequences in this transcriptome database with keywords 'heat shock protein' or 'Hsp'. Further searches of Hsp cDNAs were conducted using BLASTX to compare the sequence against the non-redundant database at NCBI (www.ncbi.nlm.nhi.gov/). Also, known insect hsp genes in GenBank were used to search for similar genes in the transcriptome (TBLASTN).

Alignment of Multiple Sequences of Deduced Amino Acids

Comparisons of deduced amino acid sequences of Hsp60, Hsp70 and Hsp90 of *L. oryzophilus* and *Tribolium castaneum* were conducted at www.ebi.ac.uk/Tools/msa/clustalw2/ and motifs were searched at www.genome.jp/tools/motif/.

Temperature Exposure

For exposure to different temperatures, adults were placed as groups of 30 into 6-cm diam Petri dishes. Each sample was run in triplicate. The low temperature, 0 °C, was obtained in a refrigerator (Siemens, Germany) and the 43 °C environment was obtained in a climate chamber (Saifu Co., Ningbo, China). Weevils were exposed for 0.5, 1, 2 and 3 h, after which, all, except 1 weevil from the 3 h high temperature treatment, were live. Three live individuals were transferred to -80 °C from each run immediately after treatment. The heat-treatment temperature was selected according to the previous results in which a constant 40 °C resulted in 0% hatchability (Raksarart & Tugwell 1975), and the 50% lethal time of adult females under constant 38 °C was 25 days (Shang 2003). The real daily temperature fluctuation in the rice field in Asian subtropics showed that 2-3 h of just under 40 °C and 0.5-1 h of > 43 °C occur on some summer days (Wei & Chen 2009; http://lishi.tianqi.com/xinchang/201308.html) and these short intervals of extreme temperatures might be a major mortality factor (Shih & Cheng 1993).

RNA Extraction and cDNA Synthesis

Total RNA of 3 individuals for each treatment was extracted by using TRIzol reagent (Invitrogen, California, USA). Single-stranded cDNA was synthesized from 1 µg RNA in a 50 uL reaction system with ReverTraAce qPCR RT Master Mix with gDNA Remover (Toyobo, Japan) according to manufacturer instructions.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

To measure expression of Hsp genes, qRT-PCR was used with the primer pair detailed in Table 1. A pre-run test was carried out to confirm the constant expression of the *actin* and *tubulin* genes in each sample. *Tubulin* was used as a normalization gene with the forward primer 5'- GCCTGCTGGGAACTGTATTGTT -3' and the reverse primer 5'- CGCCGAAAAACGTGTTAAAAC -3' with an expected product size of 100 bp. Amplifications were performed using Thunderbird SYBR qPCR Mix (Toyobo, Japan) and 5 pmol of each primer. To ensure the validity of the data, the expression of each gene was tested in triplicate in each of 3 biologically independent experiments. Each RNA sample in each replicate was prepared from 3 individuals. The cycling conditions were: (1) 95 °C, 30 s; (2) 95 °C, 5 s; (3) 60 °C, 30 s; (4) go to 2, 40 cycles. A CFX96 machine (Bio-Rad, USA) and the accompanying software were used for qRT-PCR data normalization and quantification.

After the qRT-PCR assay, results were normalized to the expression level of the *tubulin* reference gene. The relative quantitative method

TABLE 1. PRIMERS FOR QRT-PCR OF HEAT SHOCK PROTEINS OF THE RICE WATER WEEVIL, *LISSORHOPTRUS ORYZOPHILUS*.

Gene	Forward Primer	Reverse Primer
RT-*LoHsp21*a	AGCACTCTAGCCCAGATCGATAA	TTCGTGTTTGCCTTCCACAGT
RT-*LoHsp21b*	TCATCCAGTTGTTCACCATCGT	TTAGTCCGCCAGCTTCTCAAA
RT-*LoHsp20.6*	CGACCAGGTCTTACGAGTCTGA	CGTCGTACCAAGTTCTGAGTTCTC
RT-*LoHsp21c*	ACTGTAGAAGGCAAACATGAGGAA	CGGTGCCTCAATAACCAAAAC
RT-*LoHsp21d*	CGATCTCCTTCAGCCTCTCAA	ACATCCAAATTCGCCTGGAA
RT-*LoHsp21e*	AGAAGGGACGACTTGCTTGAAT	ACTGTTGCGTTCTGAAATTTGC
RT-*LoHsp90a*	ATTGCCAAATCTGGAACCAAA	TTCCCAGTCATCATTGTGCTTT
RT-*LoHsp90b*	TGGTTGGACTGCCAATATGGA	TTGTCTGCTTCAGCCTTTTGAC
RT-*LoHsp70e*	CACAATTTTTGATGCCAAACGA	TGGTTAGTTTTGGTTTCTCCTTTGTA
RT-*LoHsp21f*	CGGAAGAAGATGGAACCACTTG	TTGGGTTGGAATCTGTCCTTATC
RT-*LoHsp21g*	TCATTTTTCATTCAGATCGTTTTGAG	GCGACATCCGTTTTTGAACA
RT-*LoHsp21h*	AGCTTCAACGCCTCCTGTCA	CCGGAACAGAATTGGCACTAG
RT-*LoHsp21i*	GACCCATTCACAAGAAGAATTCG	GTGGTCTTTGTCCACCGTGAT
RT-*LoHsp23*	ATTTTCCACGGATTGTTGGATT	CGGGAGGGACAACTAAACCA
RT-*LoHsp12.2*	CATAATTGGCAGGCGGAAGA	GTGATCACCAGCAACTTTTACTTCA
RT-*LoHsp60*	CCCGGAGGTGGTACTGCTT	GCCATCTACGCCTGCGTTT
RT-*LoHsp90c*	TGATTGGTCAGTTTGGTGTAGGTT	TCCCAAATGTATTGCTCGTCAT
RT-*LoHsp70a*	GACTGTTATAGGTATTGATTTGGGAACTAC	AATACGGTTACCTTGGTCGTTAGC
RT-*LoHsp70b*	TGATGAAGCTGTCGCATATGG	CGGAGCGACATCTACCAAGAG
RT-*LoHsp70c*	ACAAGGATGCCCAAGGTACAGT	CGGCAGCACCTACAGCTACA
RT-*LoHsp70d*	CGCCGTTACGATCCAGGTAT	CCTTGGAGCCGGAGGAATT

(ΔΔCt) was used to evaluate the expression levels (Lival et al 2001). Arbitrary cut-offs of 2.0 × and 0.5 × relative expression ratios compared to that of the reference gene were set as thresholds of up-regulation and down-regulation, respectively.

RESULTS

In order to facilitate clarity, the deduced alignments of amino acids of heat shock protein families of the rice water weevil are shown with color coding of the various amino acids in Suppl. Fig. 1, which can be found in supplementary material for this article in Florida Entomologist 97(2) (June 2014) online at http://purl.fcla.edu/fcla/entomologist/browse. Also a colored version of each of several other figures are found in the supplementary material.

Analysis of Hsp Genes from the Transcriptome

Twenty-one cDNA sequences were obtained from the transcriptomes, including 12 small Hsps, 1 Hsp60 gene, 5 Hsp70 genes and 3 Hsp90 genes (Table 2). Hsp60s, Hsp70s and Hsp90s were more conserved than small Hsps.

Multiple Sequence Alignment

Small Hsps showed relatively low identities, the most divergent of protein sequences were identical at 36% (Table 2). Hsp60, Hsp70 and Hsp90 family members were more conserved. The most divergent of these protein sequences were 83%, 86% and 62% identical, respectively (Table 2). Deduced amino acid sequences of Hsp60,

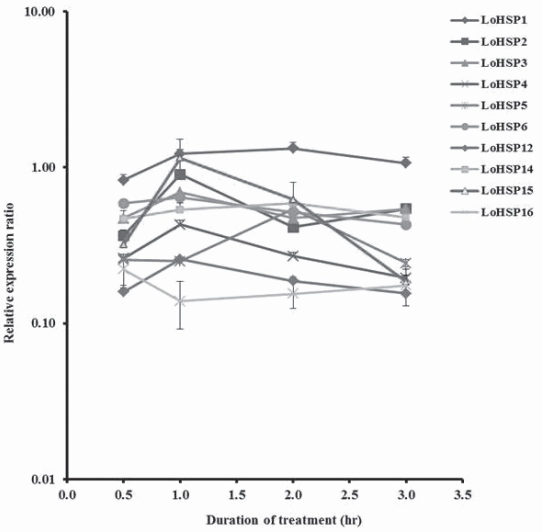

Fig. 1. Relative expression of *L. oryzophilus* small Hsps under cold stress (0 °C) compared to non-stressed controls. A colored version of this figure can be found in supplementary material for this article in Florida Entomologist 97(2) (June 2014) online at http://purl.fcla.edu/fcla/entomologist/browse

TABLE 2. *LISSORHOPTRUS ORYZOPHILUS* HEAT SHOCK PROTEIN (*LOHSPS*) GENES AND THEIR SIMILARITIES WITH THOSE OF OTHER SPECIES.

GenBank	Name	Detail	Similarity [species]	Max dentity (%)	E value
KC620420	*LoHsp21*a	Full length	small Hsp21 [*Tribolium castaneum*]	57	2e-41
KC620421	*LoHsp21b*	Full length	Small Hsp21 [*Gastrophysa atrocyanea*]	67	2e-77
KC620422	*LoHsp20.6*	Full length	Small Hsp 20.6 [*Tribolium castaneum*]	79	1e-104
KC620423	*LoHsp21c*	Full length	Small Hsp 21 [*Tribolium castaneum*]	61	2e-35
KC620424	*LoHsp21d*	Full length	Small Hsp 21 isoform 1 [*Tribolium castaneum*]	66	1e-65
KC620425	*LoHsp21e*	Full length	heat shock protein 1 [*Tribolium castaneum*]	64	7e-54
KC620426	*LoHsp90a*	partial	Hsp90 [*Macrophomina phaseolina* MS6]	62	2e-74
KC620427	*LoHsp90b*	Full length	Hsp 90-alpha [*Camponotus floridanus*]	86	0.0
KC620428	*LoHsp70e*	Full length	heat shock protein 70 [*Mantichorula semenowi*]	95	0.0
KC620429	*LoHsp21f*	partial	Small Hsp 21 [*Tribolium castaneum*]	65	6e-42
KC620430	*LoHsp21g*	Full length	Small Hsp [*Trichinella pseudospiralis*]	36	6e-08
KC620431	*LoHsp21h*	partial	Small Hsp [*Anopheles gambiae*]	54	2e-28
KC620432	*LoHsp21i*	Full length	Small Hsp 21 [*Tribolium castaneum*]	49	8e-45
KC620433	*LoHsp23*	Full length	heat shock protein 1 [*Tribolium castaneum*]	71	6e-79
KC620434	*LoHsp12.2*	partial	Hsp-12.2 [*Ascaris suum*]	63	6e-40
KC620435	*LoHsp60*	Full length	heat shock protein 60 [*Pteromalus puparum*]	83	0.0
KC620436	*LoHsp90c*	partial	heat shock protein 90 [*Apis mellifera*]	88	1e-46
KC620437	*LoHsp70a*	Full length	Hsp70 [*Danaus plexippus*]	93	0.0
KC620438	*LoHsp70b*	partial	Hsp70 B2 [*Tribolium castaneum*]	91	0.0
KC620439	*LoHsp70c*	partial	Hsp70 cognate [*Pediculus humanus corporis*]	90	0.0
KC620440	*LoHsp70d*	partial	heat shock protein 70 [*Anatolica polita borealis*]	86	0.0

Hsp70 and Hsp90 of *L. oryzophilus* were compared with corresponding genes of *Tribolium castaneum* (Herbst) (Tenebrionidae) (Suppl. Fig. 1).

Response to Cold Stress

Hsp genes which determine to small Hsps (*LoHsp23, LoHsp21i, LoHsp21f, LoHsp21c*), Hsp60 (*LoHsp60*) and Hsp90 (*LoHsp90a*) were down-regulated when the insects were exposed to the low temperature for 3 h. One Hsp90 (*LoHsp90b*) and one Hsp70 (*LoHsp70e*) were up-regulated after exposure to 0 °C for 3 h (Figs. 1 and 2).

Response to Heat Stress

Under heat stress, more Hsps were up-regulated including Hsp70, Hsp90 and the small Hsp families (Figs. 3 and 4), than under cold stress (Figs. 1 and 2). Four small Hsp genes (*LoHsp21h, LoHsp20.6, LoHsp21b, LoHsp21e*) were up-regulated when exposed for 0.5 h, and up-regulation reached its peak at 1 h, then declined though remaining higher than in the control except for *LoHsp21e*. These 4 small Hsps showed a similar expression pattern (Fig. 3). One Hsp70 gene (*LoHsp70d*) was especially strongly up-regulated starting from 0.5 h, reached a peak of expression at 2 h, then declined but was still up-regulated compared to the control. In total, 2 Hsp70 genes (*LoHsp70d* and *LoHsp70e*) and 2 Hsp90 genes (*LoHsp90b* and *LoHsp90c*) were up-regulated after heat treatment (Fig. 4).

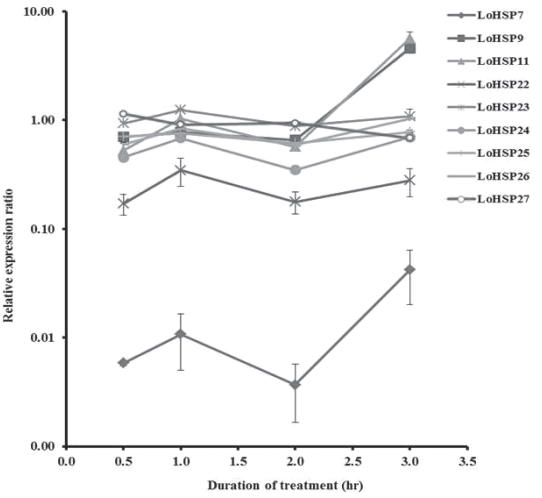

Fig. 2. Relative expression of *L. oryzophilus* Hsp60, Hsp70 and Hsp90 genes under cold stress compared to non-stressed controls. A colored version of this figure can be found in supplementary material for this article in Florida Entomologist 97(2) (June 2014) online at http://purl.fcla.edu/fcla/entomologist/browse

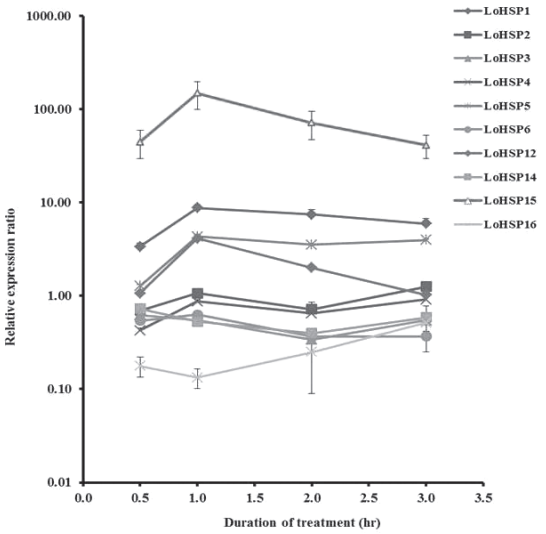

Fig. 3. Relative expression of *L. oryzophilus* small Hsps under heat stress compared to non-stressed controls. A colored version of this figure can be found in supplementary material for this article in Florida Entomologist 97(2) (June 2014) online at http://purl.fcla.edu/fcla/entomologist/browse

The weevils underwent a series of physiological changes during the diapause stage. No Hsp gene was up-regulated in the diapausing adult females, while 4 small Hsps (*LoHsp20.6, LoHsp21d, LoHsp21i, LoHsp23*), 1 Hsp60 (*LoHsp60*), 1 Hsp70 (*LoHsp70a*) and 2 Hsp90s (*LoHsp90a, LoHsp90b*) were down-regulated (Fig. 5).

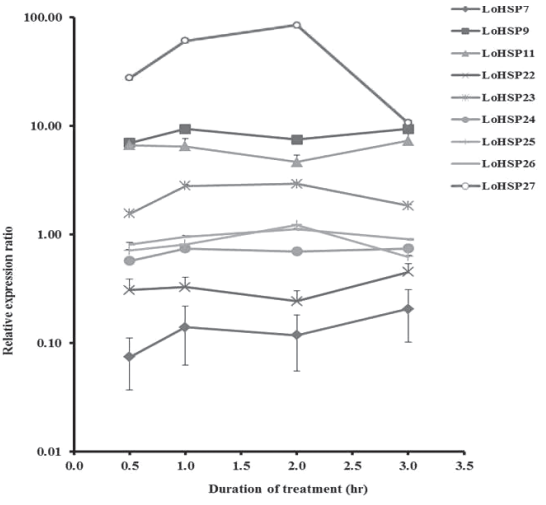

Fig. 4. Relative expressions of *L. oryzophilus* Hsp60, Hsp70 and Hsp90 genes under heat stress compared to non-stressed controls. A colored version of this figure can be found in supplementary material for this article in Florida Entomologist 97(2) (June 2014) online at http://purl.fcla.edu/fcla/entomologist/browse

Discussion

In all organisms examined, from Archeobacteria to humans, temperature elevation above the normal physiological temperature leads to a heat shock response, which consists of a profound alteration of gene expression (Morimoto et al. 1994). In the present study, the cDNA sequences of 12 small Hsp, 1 Hsp60, 5 Hsp70 and 3 Hsp90 genes were determined. These Hsp genes are the first determined in the largest family (Curculionidae) of the largest insect order (Coleoptera). Of specific importance are the 8 genes belonging to the Hsp90s, Hsp70s, and small Hsp families that were up-regulated under heat stress and the 1 Hsp70 and 1 Hsp90 gene that were up-regulated under cold stress. Results indicate that Hsp70s, Hsp90s and small Hsps were responsible for adapting to the temperature stress of *L. oryzophilus*. Hsp expression under thermal stress provided an interesting contrast to the typical stress response in which all Hsps are up-regulated. The experimental system was specifically designed to focus on heat and cold shock response to short stressful conditions, which demonstrated that *L. oryzophilus* has the capacity to adapt to temperature stress under extreme unpredictable environmental events, either during their invasion process by non-intentional human transportation or in overwintering post-arrival in new habitats. Our results showed that certain Hsp70, Hsp90, and small Hsp genes were strongly up-regulated under heat stress and this up-regulation can extend over several hours. Under cold stress, in contrast, only 1 Hsp70 and 1 Hsp90 genes were slightly up-regulated. The expression of 2 small Hsps (*LoHsp12.2* and *LoHsp21g*) was not affected by temperature, suggesting that they are involved in other, non-thermal tolerance roles in the insect's biology. For example, Gu et al. (2012) found that Hsp70 and small Hsps are involved in midgut metamorphosis in the common cutworm, *Spodoptera litura* (F.) (Noctuidae). Small heat shock proteins also help to protect cells from apoptosis, stabilize the cytoskeleton and contribute to proteostasis as housekeeping proteins (Morrow & Tanguay 2012).

Hsp70 proteins have been discovered in a wide range of species and their structure and function is highly conserved (Schlesinger 1990). Up-regulation of Hsp70 mRNA levels in response to low temperature has been reported in insects from various orders (Sinclair et al. 2007; Huang et al. 2007; Yocum 2001; Wang et al. 2008; Elekonich 2009; Yang et al. 2012). In the present study, 1 of the 5 Hsp70 genes was up-regulated in *L. oryzophilus* exposed to low temperature for 3 h. In contrast, 2 Hsp70 genes and 2 Hsp90 genes

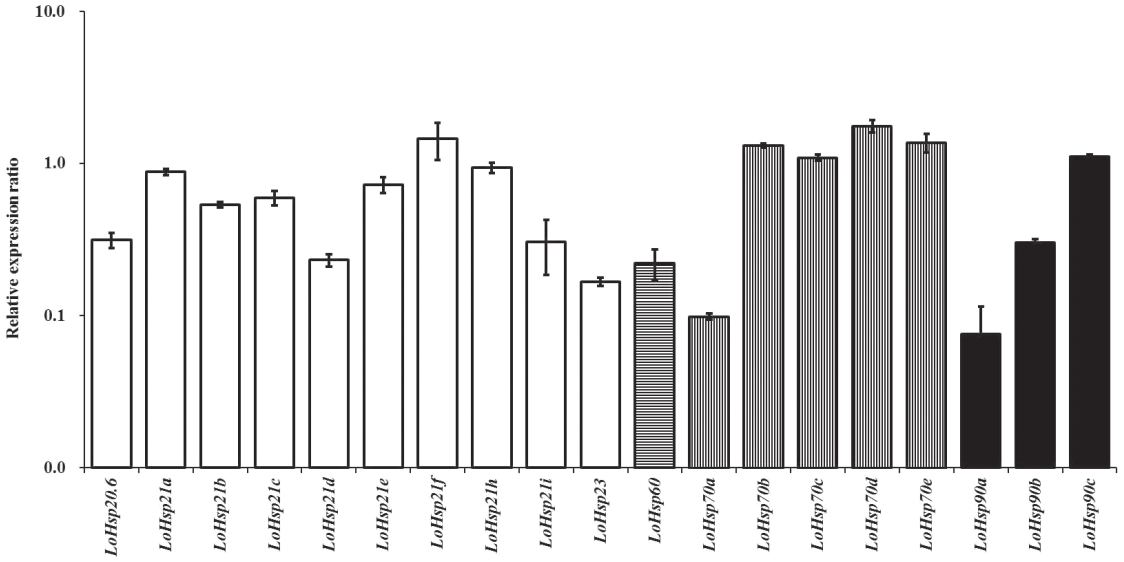

Fig. 5. Relative expression of Hsp genes in diapausing adult females compared to non-diapausing controls.

were up-regulated in *L. oryzophilus* exposed to high temperature. More small Hsp genes were involved in the adaptation to the heat stress, and those 2 Hsp70s and Hsp90s were continuously up-regulated across the stress. Yang et al. (2012) showed that Hsp70s were more critical to coping with heat stress than cold shock. Hsp70 responds dramatically to either heat or cold shock, which can induce a thousand-fold response (Velazquez et al. 1983; Huang & Kang 2007).

The involvement of Hsp in insect diapause has been studied in several species. In the flesh fly, *S. crassipalpis*, most but not all Hsps are up-regulated during diapause. Although many reports suggest that Hsps are associated with diapause (Rinehart et al. 2000, 2007; Yocum et al. 1998), this is not the case in all studied insects. For example, Hsps do not appear to be up-regulated in *Drosophila triauraria* Bock & Wheeler during diapause (Goto et al. 1998, 2004), but were in larval diapause of *Lucilia sericata* Meigen (Calliphoridae) (Tachibana 2005) and adult diapause of *Culex pipiens* L. (Culicidae) (Rinehart et al. 2006). Expression patterns of heat shock protein genes are highly variable among species that undergo diapause. The present study showed that no Hsp gene was up-regulated but 4 small Hsps, 1 Hsp60, 1 Hsp70 and 2 Hsp90 genes were down-regulated during the diapause of *L. oryzophilus*. Rinehart & Denlinger (2000) reported 1 Hsp90 is down-regulated during pupal diapause in the flesh fly, *S. crassipalpis*, but remained responsive to thermal stress, indicating that even a down-regulated protein can be involved in thermal stress.

There is no fixed pattern for the expression of Hsps. Further, heat shock is not the only environmental stress that can induce the expression of Hsps. Other factors such as heavy metals (Levinson et al. 1980); protein kinase C stimulators (Ding et al. 1996); Ca^{2+} increasing agents (Ding et al. 1998) also can induce Hsp expression. Different proteins in the same family can show different expression patterns under the same treatment. In the present study *LoHSP70e, LoHSP70b, LoHSP70c* and *LoHSP70d* belong to Hsp70 family, but showed varied expression ratios after cold and heat shocks. When the weevils were exposed to 43 °C, the expression ratios for these 4 Hsp70 genes varied strongly. *LoHSP70d* and *LoHSP70e* were strongly up-regulated while the other 2 Hsp70 genes showed no difference between treatment and no-treatment. The precise roles of different Hsps are still not fully understood. Further study should consider this group of proteins in coping with thermal stress and possibly other environmental challenges encountered by invading species.

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