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Differential expression of two *HSP70* transcripts in response to cold shock, thermoperiod, and adult diapause in the Colorado potato beetle

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Abstract

Partial clones for two members of *Leptinotarsa decemlineata* inducible 70 kDa heat shock protein family (*LdHSP70A* and *B*) were developed using RT-PCR. *LdHSP70A*, but not *LdHSP70B*, was upregulated during adult diapause. The ability of *L. decemlineata* to express these two genes in response to subzero temperatures depended on the thermal history of the beetles. Chilling diapausing beetles increased the rate at which both *LdHSP70A* and *B* were expressed following a cold shock at -10°C . Following cold shock at -10°C , *LdHSP70B* expression peaked after 3 h at 15°C for chilled diapausing individuals, decreasing to near background levels by the sixth hour. In contrast, nonchilled diapausing beetles expressed their highest level of *LdHSP70B* only after 6 h at 15°C . Diapausing beetles exposed to a thermoperiod with a mean temperature of either 0 or -2.5°C expressed significantly higher levels of both *LdHSP70A* and *B* than beetles exposed to constant 0 or -2.5°C . These results demonstrate that the expression of *LdHSP70A* and *B* is differentially regulated in response to diapause and environmental conditioning. Published by Elsevier Science Ltd.

Keywords: Diapause; Cold shock; Heat shock; Thermoperiod; *Leptinotarsa decemlineata*

1. Introduction

Various forms of dormancy occur across a wide spectrum of organisms. Dormancy is a key life strategy enabling organisms to survive adverse environmental conditions and to synchronize their life cycles with both abiotic and biotic factors needed for development and reproduction. One common characteristic of different forms of dormancy is the expression of heat shock proteins as a part of the dormancy developmental program (Denlinger et al., 2001). The heat shock proteins are an ubiquitous group of highly conserved proteins that are expressed in response to stress-induced protein denaturation and developmental cues. In nonstressed cells, one of their major functions is as molecular chaperones, assisting in protein folding and the assembly of protein complexes; during stress, they inhibit formation of and help dissolve protein aggregates, refold proteins, or tar-

get proteins for degradation (reviews: Parsell and Lindquist, 1993; Denlinger et al., 2001).

A growing body of literature supports a role for heat shock proteins in insect diapause and overwintering mechanisms. Diapausing first instar larvae of the gypsy moth, *Lymantria dispar*, express 90 and 75 kDa heat shock proteins during recovery at 4°C from 24 h exposure to -20°C (Yocum et al., 1991). In the pupal diapause of the flesh fly, *Sarcophaga crassipalpis*, 70 and 23 kDa heat shock protein transcripts are expressed at very high levels as part of the normal diapause program (Yocum et al., 1998; Rinehart et al., 2000). The effect of diapause upon the expression pattern of the heat shock proteins appears to be gene specific. The expression of the heat shock cognate 70 is unaffected by diapause, but the 90 kDa heat shock protein is down regulated in *S. crassipalpis* (Rinehart et al., 2000; Rinehart and Denlinger, 2000). The role of specific heat shock proteins may vary between types of diapause. *HSP70* was highly expressed during the pupal diapause of *S. crassipalpis* (Rinehart et al., 2000), yet expression of *HSP70* was not detected

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during the reproductive diapause of *Drosophila triauraria* (Goto et al., 1998).

What is the ecological relevance of the heat shock proteins in insect diapause and overwintering? A more extensive survey of insects must be undertaken to answer this question, as currently only three species have been investigated: *L. dispar* (Yocum et al., 1991; Denlinger et al., 1992), *S. crassipalpis* (Yocum et al., 1998; Rinehart et al., 2000; Rinehart and Denlinger, 2000), and *D. triauraria* (Goto et al., 1998). The Colorado potato beetle, *Leptinotarsa decemlineata*, is an excellent model system to strengthen our understanding of the ecological relevance of the heat shock proteins because it is a worldwide agricultural pest with an extensively studied diapause (de Kort, 1990). In this investigation, two basic questions were asked: (1) Are the heat shock protein transcripts expressed as a normal part of the adult reproductive diapause program of the Colorado potato beetle? (2) Does the thermal history of the beetles affect their expression of heat shock protein transcripts at ecologically relevant temperatures?

2. Materials and methods

2.1. Insects

Colonies of *L. decemlineata* were established with beetles collected from the Red River valley of North Dakota in 1996 and reared according to Yi and Adams (2000). Insects were reared in walk-in environmental chambers in screened plastic cages (38×38×40 cm³) and fed potato (*Solanum tuberosum* 'Luther Burbank') plants. To obtain nondiapausing adults, the beetles were reared throughout larval development at 17 h light:7 h dark, 26±2°C and 65% relative humidity. To produce diapausing adults, larvae were reared at 8 h light:16 h dark, 24±2°C and 65% relative humidity. Within 24 h of adult emergence the beetles were collected and placed in plastic containers (18.5×11.5×21 cm³) covered with cheesecloth and fed fresh potato leaf bouquets held in water bottles. The diapause-destined adults fed for about 17–18 days and then left the potato leaves. Diapausing beetles on the bottom of the rearing container on day 20 were collected, placed in moist vermiculite and stored at 10°C in constant darkness.

Nondiapausing beetles used for experimental purposes were separated from the rearing colony on adult emergence, fed fresh potato leaf bouquets and kept under 17 h light:7 h dark, 26±2°C and 65% relative humidity. These individuals were used within 3–6 days. Nonchilled diapausing adults used in these experiments were 23–26 days post-emergence and maintained under normal diapausing inducing conditions. Chilled diapausing adults were stored at 10°C for 60–120 days.

2.2. Supercooling point determination

Diapausing beetles ($n=9$) were individually inserted into 1 ml disposable pipette tips and the sensing junction of a copper/constantan 36-gauge thermocouple was placed against the ventral surface of each beetle. The pipette tips were sealed with foam rubber plugs to anchor the thermocouple and prevent the egress of the beetles. The thermocouples were connected to a multichannel data logger (DAS-TC/B, Omega Electronics, Stamford, CT) that recorded the beetles' temperatures at 2 s intervals. The beetles were inserted into a Nalgene™ Cryo 1°C freezing container, the lid was lightly screwed on and the container was placed in a freezer set at -70°C. Under these conditions the beetles' cooling rate was approximately 1°C/min.

2.3. Temperature stress

To expose beetles to high and low temperatures, three adult beetles were placed in a polystyrene tube (13×100 mm², Falcon) sealed with a cotton plug. Precise temperature control was achieved using adjacent refrigerated glycerol baths. Thermoperiod regimes were carried out by transferring the tubes of beetles between the cryophase temperature and thermophase baths at 12 h intervals.

2.4. RNA isolation

Total RNA was isolated from adults by mincing a single beetle in a 2 ml RNase-free microcentrifuge tube with 0.5 ml of TRIzol® reagent (Gibco-BRL), then adding another 0.5 ml of TRIzol®. Samples were centrifuged at 12 000g for 10 min, supernatant was transferred to a new 2 ml RNase-free microcentrifuge tube, and another 0.5 ml of TRIzol® was added. Samples were then stored at -70°C until the RNA was purified following the manufacturer's protocol. The isolated RNA pellets were stored under absolute ethyl alcohol at -70°C then dissolved in either 30 µl of formamide for northern blot analysis or 30 µl of diethyl pyrocarbonate-treated water for use in RT-PCR reactions.

2.5. Clone development

The initial clones of *L. decemlineata* HSP70A and B were isolated by RT-PCR using the sense primer 5'-GCAAAGAAYCARGYYGATGAAC-3' and the antisense primer 5'-GTDGMYTTVACVTCRAAGAT-3', each designed to anneal within the 5' conserved region of the transcripts. Total RNA was isolated from nondiapausing adult beetles exposed to 39°C for 1 h and single stranded cDNA was then generated using Superscript II and oligo(dT) primer (Gibco-BRL) according to the manufacturer's protocol. The PCR reaction using plati-

num TAQ DNA polymerase (Gibco-BRL) yielded a 450 bp amplicon that was then cloned into pCR 2.1 (Invitrogen). Sequence data revealed that our PCR product contained two different *HSP70* clones.

3'-RACE was performed using single stranded cDNA generated as above except the primer 5'-GGCCACGCGTCTGACTAGTACT₁₇-3' was substituted for the oligo(dT) primer. PCR was conducted using the gene-specific primers 70A, 5'-GGAAAGTTTCAC-TAGTCG-3' and 70B, 5'-CTTTTGGCTTTTAAGTC-3' and the anchor primer, 5'-GGCCACGCGTCTGACTAG-TAC-3'. The 3'-RACE products were cloned into pCR 2.1 (Invitrogen) and these clones were used to generate probes for northern analyses.

2.6. Probe labeling

Two 50 µl PCR reactions using the 3'-RACE clones as templates were combined and run on 1% TAE agarose gel. The PCR products were isolated from the excised gel band using GENE CLEAN® II (BIO 101). The clones were random primed with biotin using the NEBlot Phototope™ kit (New England BioLabs). The labeled probes were stored at a concentration of 20 ng/µl at -20°C in TE buffer (pH 8.0).

2.7. Northern blotting

Twenty micrograms of total RNA per sample were separated on a 1% agarose denaturing gel (0.41 M formaldehyde, 1X MOPS-EDTA-sodium acetate). The RNA was then transferred overnight onto a noncharged nylon membrane (millipore) using downward capillary action in 20X SSC (3 M NaCl, 0.3 M Na citrate, pH 7.0) transfer buffer (Schleicher and Schuell). After completion of the transfer the blots were air-dried and then UV crosslinked at 20 000 µJ/cm² and the filters stored at 4°C. To ensure that equivalent RNA was loaded for each sample, ethidium bromide was added to all samples and a photograph was taken at the end of the gel run to compare rRNA band intensity. Prehybridization and hybridization were carried out in 1X hybridization buffer (0.1 ml/cm²; 0.5 M NaCl, 0.1 M NaPO₄ [pH 7.0], 6 mM EDTA, 1% SDS) at 65°C. The final probe concentration was 20 ng/ml of hybridization buffer. Biotin labeled probes were detected with the Phototope™-Star Detection kit (New England BioLabs). To ensure accurate comparison of the expression patterns of *LdHSP70A* and *B*, duplicate northern blots were made and processed in parallel throughout the experiment. Both blots were exposed simultaneously to a single piece of BioMax film (Kodak). Each northern blot represents duplicated experiments.

2.8. DNA sequencing

Clones were sequenced using the vectors internal primers sites and custom primers internal to the inserts. DNA sequencing was performed at the University of Georgia on an AB1373A DNA sequencer using dye terminator chemistry according to the manufacturer's standard protocol. The BlastX program (Altschul et al., 1997) was used to search the GenBank sequence repository for identity.

2.9. Genbank

The nucleotide sequences for *LdHSP70A* and *B* were deposited in GenBank and assigned the accession numbers AF288978 and AF322911, respectively.

3. Results

3.1. Clones

3'-RACE yielded clones of 1863 and 1823 bp in length for *LdHSP70A* and *B*, respectively. The clones were 70% identical at the nucleic acid level. Northern blot analysis determined that both genes had a transcript size of 2.3 kb. A BlastX search of the GenBank sequence repository revealed that *LdHSP70A* had the highest score at 75% identity and 84% positives to *Drosophila melanogaster HSP68* (accession number AF096275). The closest match of *LdHSP70B* was with *Anopheles albimanus HSP70* (accession number AF096275) at 74% identity and 83% positives.

3.2. Supercooling point determination

Insect supercooling points are not a fixed physiological parameter, but will vary depending upon environmental conditions (Costanzo et al., 1997). The diapausing Colorado potato beetles reared under the conditions of this study had a mean (±S.E.) supercooling point of -17.6±1°C. The supercooling value recorded in this study is within the range of the values reported for diapausing Colorado potato beetles (Costanzo et al., 1997). The low temperatures used in these experiments are thus above the beetle's supercooling point.

3.3. *LDHSP70A* expression during diapause

The heat shock positive control revealed that both *LdHSP70A* and *B* were highly expressed during a 1 h exposure to 39°C, but were not detectable in nonstressed nondiapausing adults (Fig. 1A). Though not detectable with more optimal exposures (i.e. low background), longer exposures revealed low levels of expression of *LdHSP70A* in nonchilled diapausing beetles (Fig. 1B).

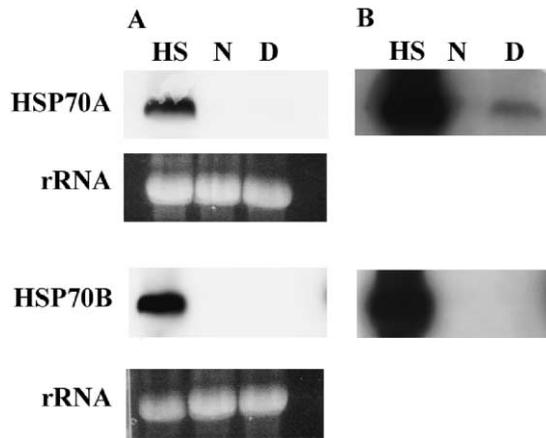


Fig. 1. Expression of *LdHSP70A* and *B* in nondiapausing and diapausing adults of *L. decemlineata*, optimal (A) and extended (B) exposure of the northern blots. Total RNA (20 μ g) isolated from heat shocked (HS: 39°C for 1 h), nondiapausing (N) and diapausing (D) beetles was separated on a 1% formaldehyde–agarose gel and transferred onto noncharged nylon membrane. The northern blot was hybridized using a biotin random primed DNA probe. RNA concentrations were determined spectrophotometrically and stained with ethidium bromide to ensure equal loading. Ethidium bromide stained ribosomal RNA is shown below its respective northern blot.

LdHSP70B was either not expressed as a part of the diapause program or was below the level of detection. These results show that both *LdHSP70A* and *B* are highly induced by a heat shock and that *LdHSP70A* is expressed as a normal part of the diapause program.

3.4. Expression of *LdHSP70A* and *B* following a cold shock

Cold shocking diapausing beetles at -10°C for 2 h induced only a minor upregulation of *LdHSP70A* during recovery at 15°C . In sharp contrast, *LdHSP70B* was highly upregulated (Fig. 2). This demonstrates differential expression of two members of the 70 kDa heat shock protein family in response to cold shock.

Nonchilled diapausing beetles expressed low levels of *LdHSP70A* and no detectable levels of *LdHSP70B*; chilling beetles at 10°C for 90 days had little to no effect on expression of either *LdHSP70A* or *B* in nonstressed individuals (controls, Fig. 2). However, chilling greatly increased the rate at which both *LdHSP70A* and *B* were expressed during recovery at 15°C from a cold shock at -10°C . One hour after removal from -10°C , the chilled individuals expressed significantly higher levels of both transcripts. By the sixth hour of recovery the expression of *LdHSP70A* was equal in chilled and nonchilled beetles, but the expression of *LdHSP70B* was significantly less in the chilled as compared to the nonchilled (Fig. 2). Chilling diapausing Colorado potato beetles thus affect not only the rate at which the 70 kDa heat shock protein transcripts are expressed but also the duration of their expression. Taken together these results

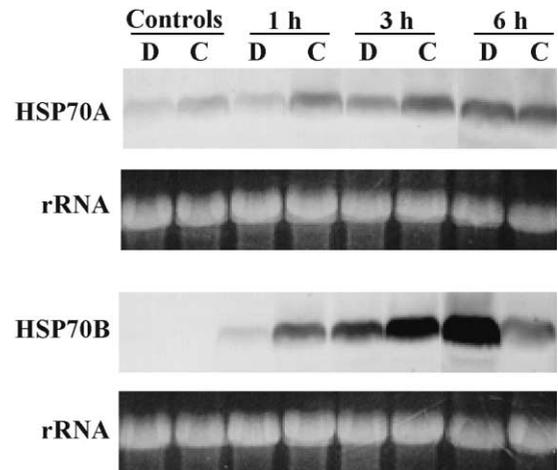


Fig. 2. *LdHSP70A* and *B* expression during recovery at 15°C following a cold shock at -10°C . Total RNA (20 μ g) isolated from diapausing (D) and chilled diapausing (C) beetles exposed at -10°C for 2 h and permitted to recover at 15°C for 1, 3 or 6 h was separated on a 1% formaldehyde–agarose gel and transferred onto noncharged nylon membrane. The northern blot was hybridized using a biotin random primed DNA probe. RNA concentrations were determined spectrophotometrically and stained with ethidium bromide to ensure equal loading. Ethidium bromide stained ribosomal RNA is shown below its respective northern blot.

demonstrate that *LdHSP70A* and *B* are differentially expressed following a cold shock and that the expression patterns are significantly affected by thermal history.

3.5. Thermoperiods

Under field conditions in the Red River valley of North Dakota overwintering diapausing Colorado potato beetles experience fluctuating subzero temperatures. If the heat shock proteins are involved in overwintering survival, diapausing beetles must be able to express these proteins under such conditions. Accordingly, diapausing beetles were exposed to thermoperiods with a mean temperature of 0°C (12 h cryophase (12C):12 h thermophase (12T), $-5^{\circ}\text{C}:5^{\circ}\text{C}$) (Fig. 3) or -2.5°C (12C:12T, $-10^{\circ}\text{C}:5^{\circ}\text{C}$) (Fig. 4) for up to three days. As expected, increasing the severity of the thermoperiod induced not only increased levels of expression of *LdHSP70A* and *B* but also shortened the onset of expression (Figs. 3 and 4). Of more significance, however, are the results from the 0°C thermoperiod northern blot analysis showing clearly that *LdHSP70A* and *B* were differentially expressed in response to both the thermoperiod and the constant 0°C positive controls (Fig. 3). In the thermoperiod samples *LdHSP70A* was expressed throughout the third thermophase, whereas *LdHSP70B* was expressed only in the 1 and 4 h samples of the third thermophase. Minor differences in the expression patterns of *LdHSP70A* and *B* were also seen in the -2.5°C thermoperiod (Fig. 4). During the first thermophase *LdHSP70A* expression increased by the seventh hour, but

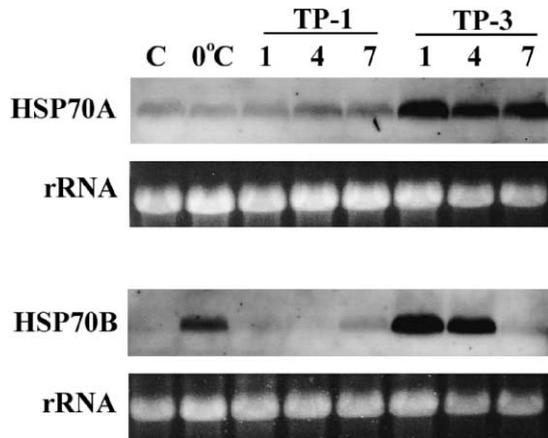


Fig. 3. Expression of *LdHSP70A* and *B* during exposure to a mean 0°C thermoperiod (12C:12T, -5°C:5°C). Diapausing beetles were first exposed to the -5°C cryophase portion of the thermoperiod and samples were collected at 1, 4, and 7 h during the first and third thermophase (TP-1 and 3). Controls were diapausing beetles at the normal storage temperature of 10°C (C) and diapausing beetles exposed to 0°C for 72 h (0°C). Total RNA (20µg) for each sample was separated on a 1% formaldehyde-agarose gel and transferred onto noncharged nylon membrane. The northern blot was hybridized using a biotin random primed DNA probe. RNA concentrations were determined spectrophotometrically and stained with ethidium bromide to ensure equal loading. Ethidium bromide stained ribosomal RNA is shown below its respective northern blot.

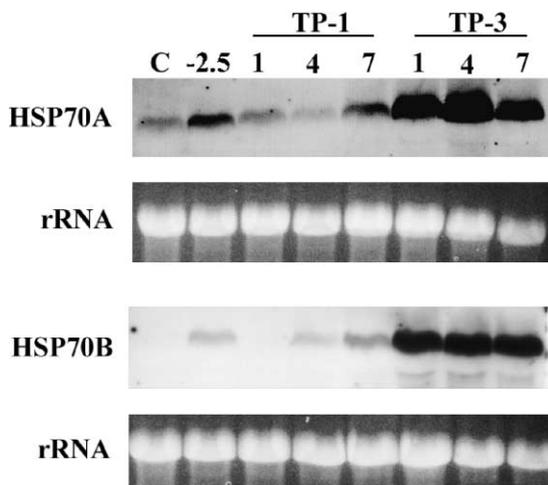


Fig. 4. Expression of *LdHSP70A* and *B* during exposure to a mean -2.5°C thermoperiod (12C:12T, -10°C:5°C). Diapausing beetles were first exposed to the -10°C cryophase portion of the thermoperiod and samples were collected at 1, 4, and 7 h during the first and third thermophase (TP-1 and 3). Controls were diapausing beetles at the normal storage temperature of 10°C (C) and diapausing beetles exposed to -2.5°C for 72 h (-2.5°C). Total RNA (20 µg) for each sample was separated on a 1% formaldehyde-agarose gel and transferred onto noncharged nylon membrane. The northern blot was hybridized using a biotin random primed DNA probe. RNA concentrations were determined spectrophotometrically and stained with ethidium bromide to ensure equal loading. Ethidium bromide stained ribosomal RNA is shown below its respective northern blot.

LdHSP70B expression increased by the fourth hour. Differential expression of *LdHSP70A* and *B* in response to low temperature could also be seen in the beetles exposed to constant 0°C for 72 h (Fig. 3). Expression of *LdHSP70A* was equivalent to the nonstressed control, but *LdHSP70B* was highly expressed as compared to the nonstressed control. These results clearly demonstrate not only that both *LdHSP70A* and *B* are expressed at ecologically relevant temperatures, but they are also differentially expressed in response to low temperatures stress.

4. Discussion

Clones for two members of the *L. decemlineata* 70 kDa inducible heat shock protein family were isolated and used to investigate the expression of *HSP70s* during diapause and upon exposure to ecologically relevant temperatures. This study showed that members of the 70 kDa heat shock protein family are differentially expressed during diapause. *LdHSP70A*, but not *LdHSP70B*, was expressed at detectable levels from the onset of diapause to at least three months into diapause. Storage at 10°C after entering diapause had no marked effect compared to nonchilled beetles on the base level of expression of *LdHSP70A*. Differential expression of members of the *HSP70* family has been shown in the hibernating ground squirrel, *Spermophilus tridecemlineatus*, in which the mitochondrial form of *HSP70* (*GRP70*) is expressed during hibernation but *HSP70* is not (Carey et al., 1999). Transcriptional regulation of the heat shock protein genes in response to diapause has been studied in only two other insects, *D. triauraria* (Goto et al., 1998) and *S. crassipalpis* (Yocum et al., 1998; Rinehart et al., 2000; Rinehart and Denlinger, 2000). In contrast to the current study, Goto et al. (1998) did not detect upregulation of *HSP70* transcripts as a normal part of the adult diapause program in *D. triauraria*. Another species variation in the expression of *HSP70s* occurs between *L. decemlineata* and *S. crassipalpis*. *S. crassipalpis* expresses very high levels of *HSP70* transcripts throughout diapause, and neither cold nor heat shock affects their levels (Rinehart et al., 2000). In contrast, in diapausing *L. decemlineata* *HSP70A* is expressed at very low levels and is highly upregulated in response to cold shock. The differential expression of *LdHSP70A* and *B* during diapause highlights the need to isolate all the members of the *HSP70* family before drawing definitive conclusions as to species variation in the role of *HSP70* during diapause.

The expression patterns of heat shock proteins are not only developmentally regulated in the Colorado potato beetles but can also be altered by the beetles' thermal history. Environmental factors have been shown to alter expression patterns of heat shock proteins in a number

of organisms. Chilling diapausing *L. dispar* larvae increase both cold tolerance and the capacity to express heat shock proteins following a cold shock (Denlinger et al., 1992). Murine tumor SQ-1 cells exposed to 41°C in order to induce thermotolerance synthesized HSP70 at a reduced rate compared to control cells during a second thermal challenge (Li and Mak, 1989). A seasonal variation in the expression of heat shock proteins has been shown in fish (Dietz and Somero, 1992; Fader et al., 1994) and mussels (Hofmann and Somero, 1995; Roberts et al., 1997), though at this time it is not known which factor(s) (photoperiod, thermal history, etc.) may be responsible for these observations. For example, within the brain of the goby fishes *Gillichthys mirabilis* and *Gillichthys seta* HSP90 accumulates at higher levels during summer than in winter. Dietz and Somero (1992) speculated that the summer increase in HSP90 might account for the observed increase in the threshold temperature for the induction of the heat shock response. The thermal history of diapausing Colorado potato beetles plays a significant role in expression of 70 kDa heat shock protein transcripts following an exposure to subzero temperatures. Chilling not only increases the rate at which both *LdHSP70A* and *B* are expressed but also alters the expression pattern of *LdHSP70B* as compared to nonchilled diapausing beetles. A significant component of the thermal history of an overwintering insect is the range of temperatures it experiences over a given time period. Cyclic temperature regimes have been shown to increase cold tolerance in the beet armyworm, *Spodoptera exigua* (Kim and Song, 2000) and in various flies (Chen and Denlinger, 1992; Leopold et al., 1998). In diapausing Colorado potato beetles, thermoperiodism greatly enhanced the expression of *HSP70* transcripts during exposure to ecologically relevant overwintering temperatures compared to constant mean temperature.

The results presented here for Colorado potato beetles and the examples from the literature clearly demonstrate that the regulation of the heat shock response is not a simple on/off response but is finely tuned to developmental and environmental conditions. The differential expression of *LdHSP70A* and *B* during diapause and in response to low temperatures indicates that individual members of the 70 kDa heat shock protein family may well have varying roles in overwintering survival of *L. decemlineata*.

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