The expression of the *HSP90* gene in response to winter and summer diapauses and thermal-stress in the onion maggot, *Delia antiqua*

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Abstract

The full-length *Hsp90* cDNA in *Delia antiqua* was cloned and sequenced. The deduced polypeptide comprised 717 amino acid residues, with a molecular mass of 82 140 Da. Summer- and winter-diapauses both elevated *HSP90* transcript levels in *D. antiqua* pupae. Levels gradually increased with time in summer diapausing pupae whereas levels fluctuated in winter diapausing pupae. Cold- and heat-stressing summer- and winter-diapausing individuals further elevated *HSP90* expression. mRNA levels gradually increased with time in summer diapausing pupae whereas levels decreased with time after an initial increase in winter diapausing pupae. *HSP90* expression was also up-regulated following cold- and heat-stresses in non-diapausing pupae. Heat-stress gradually increased the mRNA level with time whereas cold-stress gradually decreased levels after an initial increase. These results suggest that the development and physiology of summer- and winter-diapauses, as monitored via variation in *HSP90* transcript levels, can be substantial different.

Keywords: *HSP90*, *Delia antiqua*, expression, diapause, thermal stress.

Introduction

Heat shock proteins (HSPs) function as molecular chaperones by helping in the transport, folding, unfolding, assembly and disassembly of multistructured units, in the degradation of misfolded or aggregated proteins, and by preventing other proteins from getting involved in inappropriate aggregations (Sonna *et al.*, 2002). There are three HSP families in insects: the small HSP (sHSP) family with molecular masses ranging from 20 to 30 kDa, the HSP70 family with molecular mass of approximately 70 kDa, and the HSP90 family with a higher mass (Denlinger *et al.*, 2001). Insect HSP90 proteins are homologues of mammalian HSP90 proteins (Gupta, 1995) and widely referred to as HSP90 (e.g. Rinehart & Denlinger, 2000; Tachibana *et al.*, 2005), and sometimes as HSP83 in *Drosophila* (Goto & Kimura, 2004). *HSP90* is up-regulated in response to heat stress in *Drosophila subobscura*, *D. auraria*, the silverleaf whitefly *Bemisia argentifolii* and the flesh fly, *Sarcophaga crassipalpis* (Arbona *et al.*, 1993; Yiangou *et al.*, 1997; Rinehart & Denlinger, 2000; Salvucci *et al.*, 2000), and in response to rehydration after desiccation in *S. crassipalpis* (Hayward *et al.*, 2004). *HSP90* genes can also be subject to developmental regulation, such as during *Drosophila* oogenesis (Zimmerman *et al.*, 1983), during the dauer stage of developmental arrest in nematodes (Dalley & Golomb, 1992), and during diapause in *S. crassipalpis* (Denlinger, 2002). *HSP90 has* also been implicated in morphological evolution and arrest of reproduction in *Drosophila* (Rutheford & Lindquist, 1998; Marcus, 2001).

Insects that undergo diapause arrest their development and, by doing so, are able to survive harsh environments, exploit seasonal resources, and synchronize the growth pace of populations. Diapausing individuals display striking differences in gene expression when compared with non-diapausing ones, i.e. most of the genes are silenced but a select few are highly up-regulated during diapause (Denlinger, 2002). Whereas in studies with *S. crassipalpis HSP90* transcripts are down-regulated during diapause (Rinehart & Denlinger, 2000), studies in *Drosophila triauraria* and the blowfly, *Lucilia sericata*, have not found evidence of *HSP90*

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GAGTCTTGAAATAAATATTGTGCAAAATACAGCGAGAACTCCAAACGGGAGAAAGTAAAAAACGTTTCTTTTCTGAAGTGTGAATAATCAAAGAAAATAGTGTGGTTGAT 110

TCCATTCAATTGCAAAGTTAATTTAAAAATTTGTGAAAAAAAATCATTAATTTTATTTTAAATTACTACAAATAAACACATCAAGATGCCAGAAGAAGTTGAAACCTTTG 220
                                                                                  M  P  E  E  V  E  T  F  8

CTTTCCAAGCTGAAATTGCTCAGCTTATGTCATTGATCATCAATACATTCTATTCAAACAAAGAAATTTTCTTGCGTGAATTGATCTCCAATGCTTCCGATGCTTTGGAT 330
A  F  Q  A  E  I  A  Q  L  M  S  L  I  I  N  T  F  Y  S  N  K  E  I  F  L  R  E  L  I  S  N  A  S  D  A  L  D  45

AAAATCCGTTATGAATCATTGACCGATCCCACAAAATTGGATTCTGGCAAAGAACTGTACATTAAATTGATCCCCAACAAAGAGGCCGGTACATTGACCCTCATTGATAC 440
K  I  R  Y  E  S  L  T  D  P  T  K  L  D  S  G  K  E  L  Y  I  K  L  I  P  N  K  E  A  G  T  L  T  L  I  D  T  82

TGGTATTGGTATGACCAAATCCGATTTGGTCAACAATTTGGGTACCATTGCCAAATCTGGTACAAAGGCATTCATGGAAGCGTTGCAGGCCGGCGCTGATATCTCTATGA 550
G  I  G  M  T  K  S  D  L  V  N  N  L  G  T  I  A  K  S  G  T  K  A  F  M  E  A  L  Q  A  G  A  D  I  S  M  118

TTGGTCAATTTGGTGTTGGTTTCTATTCCGCCTACTTGGTTGCTGACAAAGTCACCGTGATTTCAAAACACAACGATGATGAACAATACATTTGGGAATCATCGGCTGGC 660
I  G  Q  F  G  V  G  F  Y  S  A  Y  L  V  A  D  K  V  T  V  I  S  K  H  N  D  D  E  Q  Y  I  W  E  S  S  A  G  155

GGTTCATTCACCGTCAAGGCTGACAACTCTGAACCTTTGGGTCGCGGTACCAAGATTGTTTTGTACATTAAAGAAGATCAAACTGATTATTTGGAGGAGGCCAAGATTAA 770
G  S  F  T  V  K  A  D  N  S  E  P  L  G  R  G  T  K  I  V  L  Y  I  K  E  D  Q  T  D  Y  L  E  E  A  K  I  K  192

GGAGATTGTCAACAAGCATTCTCAATTCATTGGCTATCCCATCAAATTGATGGTCCAAAAGGAACGCGACCAAGAAGTCAGCGATGATGAAGCCGAAGAAGACAAGAAAG 880
E  I  V  N  K  H  S  Q  F  I  G  Y  P  I  K  L  M  V  Q  K  E  R  D  Q  E  V  S  D  D  E  A  E  E  D  K  K  228

ATGAAGATAAAAAAGATATGGAAACAGATGAGCCAAGATTGAAGATGTTGGTGAGGATGAAGATGCCGACAAGAAGGACAAAGATGGCAAAAAGAAGAAGACCATCAAG 990
D  E  D  K  K  D  M  E  T  D  E  P  K  I  E  D  V  G  E  D  E  D  A  D  K  K  D  K  D  G  K  K  K  K  T  I  K  265

GTGGCCTACACCGAAGATGAAGAATTGAACAAGACCAAACCCATTTGGACCCGCAATCCCGATGATATGGTGAATTCTACAAATCTCTCACCAA 1100
V  A  Y  T  E  D  E  E  L  N  K  T  K  P  I  W  T  R  N  P  D  D  I  S  Q  A  E  Y  G  E  F  Y  K  S  L  T  N  302

TGACTGGGAAGAACATTTGGCTGTCAAGCACTTCTCCGTTGAAGGTCAACTTGAATTCCGTGCTCTTCTCTTCATTCCCCGTCGCACACCATTCGATCTCTTTGAAAACC 1210
D  W  E  E  H  L  A  V  K  H  F  S  V  E  G  Q  L  E  F  R  A  L  L  F  I  P  R  R  T  P  F  D  L  F  E  N  338

AAAAGAAACGCAACAACATTAAATTGTACGTGCGTGTGTCTTCATCATGGACAACTGTGAAGACTTATTCCCGAATATTGAACTTCATCAAGGGTGTAGTCGACTCT 1320
Q  K  K  R  N  N  I  K  L  Y  V  R  R  V  F  I  M  D  N  C  E  D  L  I  P  E  Y  L  N  F  I  K  G  V  V  D  S  375

GAAGATTTGCCTTTGAACATCTCTCGTGAAATGTTGCAACAAAACAAAGTCTTGAAGGTTATTCGCAAGAATTTGGTCAAGAAGACCATGGAATTGATTGAAGAACTCAC 1430
E  D  L  P  L  N  I  S  R  E  M  L  Q  Q  N  K  V  L  K  V  I  R  K  N  L  V  K  K  T  M  E  L  I  E  E  L  T  412

TGAAGACAAAGAAGAGTACAAGAAATTCTACGATCAGTTCAGCAAGAACTTGAAGTTGGGTGTCCATGAAGATACCAACAATCGTGCCAAGTTGGCCGATTTCTTGCGTT 1540
E  D  K  E  E  Y  K  K  F  Y  D  Q  F  S  K  N  L  K  L  G  V  H  E  D  T  N  N  R  A  K  L  A  D  F  L  R  448

TCCACACATCTGCTTCTGGTGATGATCCTTGCTCTTTGGCCGATTATGTATCTCGCATGAAGGAGAACCAAAAACACATCTACTTCATCACTGGTGAATCCAAGGATCAA 1650
F  H  T  S  A  S  G  D  D  P  C  S  L  A  D  Y  V  S  R  M  K  E  N  Q  K  H  I  Y  F  I  T  G  E  S  K  D  Q  485

GTTGCTCACTCTGCCTTCGTTGAACGTGTTAGAGCTCGTGGCTTTGAAGTTGTCTACATGACTGAACCCATTGATGAATATGTCATCCAACACTTGAAGGAATACAAGGG 1760
V  A  H  S  A  F  V  E  R  V  R  A  R  G  F  E  V  V  Y  M  T  E  P  I  D  E  Y  V  I  Q  H  L  K  E  Y  K  G  522

TAAACAATTGGTATCCGTAACCAAAGAAGGTTTGGAATTGCCCGAAGATGAGGCTGAAAAGAAAAAGTTCGAAGATGACAAGGTTAAATTCGAAAATCTTTGCAAATTGA 1870
K  Q  L  V  S  V  T  K  E  G  L  E  L  P  E  D  E  A  E  K  K  K  F  E  D  D  K  V  K  F  E  N  L  C  K  L  558

TGAAATCTATTTTGGACAGCAAAGTTGACAAGGTTGTCGTATCTAACCGCTTGGTTGAATCACCTTGTTGTATTGTCACCTCACAGTTTGGTTGGTCCGCCAACATGGAA 1980
M  K  S  I  L  D  S  K  V  D  K  V  V  V  S  N  R  L  V  E  S  P  C  C  I  V  T  S  Q  F  G  W  S  A  N  M  E  595

CGTATCATGAAGGCCCAAGCTTTACGTGACACCTCCACCATGGGCTATATGGCCGGCAAGAAACATTTGGAAATCAATCCCGAACATGCCATCATTGAAACTTTGCGTCA 2090
R  I  M  K  A  Q  A  L  R  D  T  S  T  M  G  Y  M  A  G  K  K  H  L  E  I  N  P  E  H  A  I  I  E  T  L  R  Q  632

AAAGGCCGATGCCGATAAAAACGACAAGGCCGTTAAGGATTTGGTGATTTTGTTGTTCGAGACCTCCCTCCTGTCATCTGGCTTCTCTTTGCAAAGCCCTCAAACACATG 2200
K  A  D  A  D  K  N  D  K  A  V  K  D  L  V  I  L  L  F  E  T  S  L  L  S  S  G  F  S  L  Q  S  P  Q  T  H  668

CCAGCCGAATTTACCGTATGATCAAACTTGGTTTGGGTATCGACGAAGACGAGCCCATGCCCACAGAAGACATTCAAAGTGCTGGTGATGCTGCTCCTCCATTGGTTGAT 2310
A  S  R  I  Y  R  M  I  K  L  G  L  G  I  D  E  D  E  P  M  P  T  E  D  I  Q  S  A  G  D  A  A  P  P  L  V  D  705

GATACCGAAGATGCTTCTCATATGGAAGAAGTCGATTAAACACCATTACGCTTAATACATAATGCAAACACTAGGGTTTTAAGTAGTAGTTTTTAAGACAGACTTAGAAG 2420
D  T  E  D  A  S  H  M  E  E  V  D  *                                                                        717

AACGCTGGGTTTTACAAAAAAAAAAGTTTTTTTTTTGTATTTCCCTTTTAAATGTGTAAATTTATGACATTTTTGTTTGCCGCGTTCTAAGCGGTAAAAATCATCAGCAT 2530

CATTATTAAAATTCATTTTTAATTTAAATTTTGTTTATTTTTTTTTTTTTTTATCGTCATGTTTTTTATAAGAGCAGTTTTAAGGTTTAATTTAAAGCAAGCAAAAAAAAA 2640

TCCAAGCTCCAAGTCATATTTTTAGTTTTAACAATTGTTAATTTTATTAACTGTATTTTAGTTCCAATGAATTTAACAATGATATAAAAGGAAATTTGATGTAAAAAAAAA 2750

GAAAATTAAACAAATATAAATGTTTAAAAACAACTTAAATAAAAAGCGTGTATTTAATTTAAAAGTTTGTATATAGGTAAAAAAAAAAAA 2841
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Figure 1. Nucleotide and deduced amino acid sequences of *HSP90* cDNA. The underlined nucleotides represent the presumable polyadenylation signal (AATAAA) and the RNA instability motif (ATTTA). The stop codon (TAA) is indicated by the asterisk. The functional regions are indicated by: '≈' for HSP90 protein family signature (document # PR00775 and PS00298 of database InterPro); '=' for HSP90 protein (InterPro doc PF00183); '~' for histidine kinase-like ATPases (InterPro doc PF02518 and SM00387).

involvement in diapause (Goto & Kimura, 2004; Tachibana *et al.*, 2005). This inconsistency needs to be elucidated with additional species and different types of diapauses.

In this study, we cloned the *HSP90* cDNA of the onion maggot, *Delia antiqua*, and examined its expression in response to both summer and winter diapause as well as to thermal stress during diapause. This particular species diapauses as a pupae in both summer and winter (Nomura & Ishikawa, 2001), making it a good model species for comparisons of the physiological and molecular control of pupal diapause at two temperature extremes.

Results

HSP90 *cDNA in* Delia antiqua

The full-length *HSP90* cDNA of *D. antiqua* is 2841 bp long, and contains a unique open reading frame (ORF) spanning nucleotides 196–2349 (Fig. 1). The cDNA has a poly(A) tail at its 3′ end, and a polyadenylation signal (AATAAA) at nucleotides 2788–2793 (Fig. 1). The polypeptide deduced from the ORF comprises 717 amino acid residues, with a calculated molecular mass of 82 140 Da. Levels of pair-wise HSP90 sequence identity between *D. antiqua*, *Drosophila*
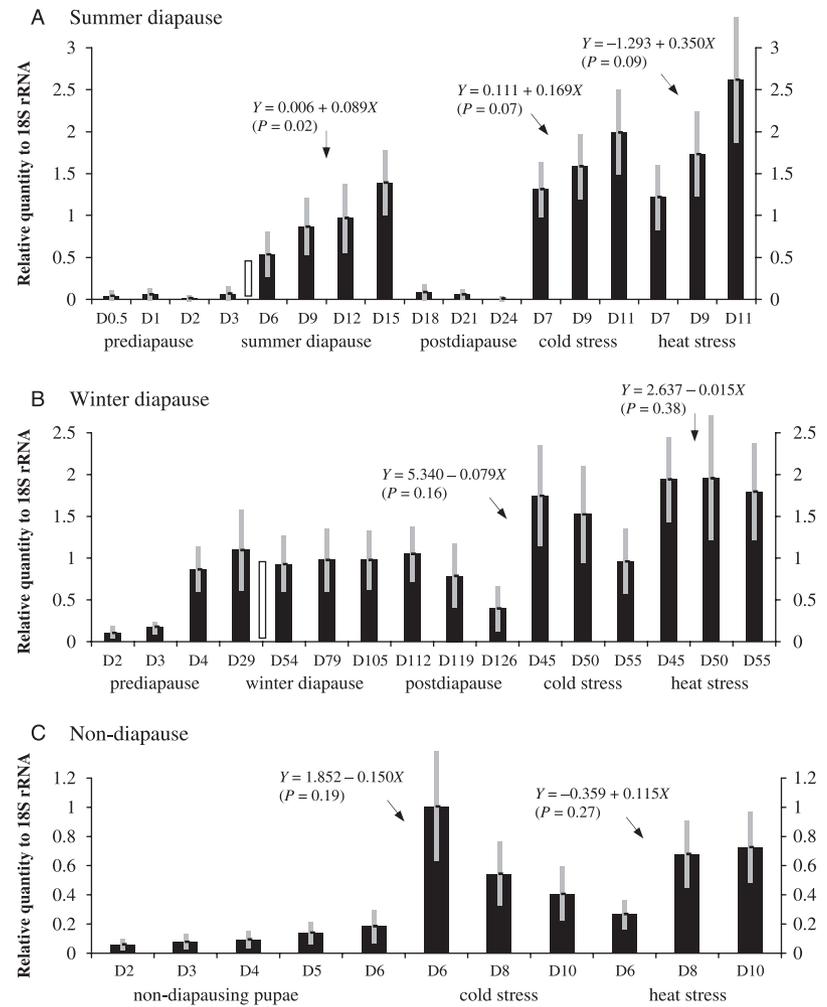
Figure 2. Q-RT-PCR analysis of *HSP90* mRNA levels in SD (A), WD (B) and ND (C) and under cold- or heat-stress. The relative quantities indicate the levels of *HSP90* transcript normalized to the internal standard 18S rRNA. The shaded bars indicate the standard deviation of five repeats. The unfilled bars indicate the D5 pupae in SD and D40 pupae in WD, which were subject to heat- and cold stress. In non-diapause, D4 pupae were subject to heat- and cold stress. Regression equations between the quantity (*Y*) and the day (*X*) are shown above each corresponding treatment group with the *P*-value in parentheses.

melanogaster, *Apis gambiae* and *Bombyx mori* range from 82–92%. The *HSP90* cDNA of *D. antiqua* and its deduced protein sequences were deposited in GENBANK with accession numbers AJ890081.1 and CAI64494.1, respectively.

Expression of HSP90 during diapauses

When onion maggot larvae were reared at 25 °C and 16 L : 8 D, 98% of the pupae enter summer diapause (SD) at 2.2 days after pupariation (D2.2, Ishikawa *et al.*, 2000). Although the SD period is quite variable at 23 °C and 16 L : 8D, lasting between 15 and 45 days, pupae complete SD and start postdiapause development soon after the temperature decreases to 16 °C (Ishikawa *et al.*, 2000). The relative amounts of *HSP90* transcript, normalized to 18S rRNA levels, were very low (between 0.01 for D24 and 0.09 for D18) in both prediapause (D0.5, D1 and D2) and postdiapause pupae (D18, D21 and D24) (Fig. 2). Interestingly, in the first day after entering SD (D3) the amount remained low (0.07); however, in the subsequent stages investigated the amounts were much higher and

gradually increased from 0.54 (D6), 0.86 (D9), 0.97 (D12) to 1.39 (D15) with a slope rate of 0.089/day.

Almost all the pupae enter winter diapause (WD) when larvae are maintained at 15 °C and 12 L : 12 D (Nomura & Ishikawa, 2000). WD starts on day 4.1 and lasts for about 99 days with a postdiapause period of 23.5 days (Nomura, 2001). In a similar pattern to that observed under SD, the relative mRNA levels in the first two days of prediapause (D2 and D3) were low (0.11 and 0.18, respectively), but increased to 0.86 on D4 (Fig. 2). The expression levels then fluctuated between 0.93 and 1.09 throughout WD (D29, D54, D79 and D105) and also during the first stage (D112) of postdiapause. After that, the expression decreased to 0.79 (D119) and 0.40 (D126) in postdiapause pupae.

Thermal stress response

Cold-stressing (−15 °C and 0 L : 24 D) and heat-stressing (35 °C and 16 L : 8 D) the SD pupae from D5, maintained at 25 °C and 16 l:8D, led to an average 2.8 fold increase in *HSP90* expression levels from D5 to D7. After the D7 stage,

the mRNA levels in cold- and heat-stressed pupae gradually increased with treatment duration, consistent with the pattern of unstressed SD pupae. After D7, the rates of *HSP90* increase for heat- (0.169/day) and cold-stressed pupae (0.350/day) were 1.9 and 3.9-fold higher than that for unstressed pupae (0.089/day), respectively (Fig. 2).

Similarly to what is observed in SD pupae, cold-stressing (−20 °C in complete darkness) and heat-stressing (35 °C and 16 L : 8 D) WD pupae from D40 onwards, previously maintained at 15 °C and 12 L : 12 D, sharply increased *HSP90* transcript levels around twofold. In contrast to SD pupae, however, *HSP90* expression levels decreased with treatment duration after the first five days of stresses for both cold-stressed and heat-stressed WD pupae. The rates of *HSP90* decrease for cold- and heat-stressed pupae were −0.079/day and −0.015/day, respectively.

The onion maggot enters SD and WD at the developmental point after finishing about 15% effective accumulate temperature in the entire pupal development (Nomura & Ishikawa, 2000). *HSP90* transcript levels in non-diapausing (ND) pupae are similarly low compared to those in prediapause and postdiapause pupae (Fig. 2). Cold-stressing (−10 °C and 0 L : 24 D) and heat-stressing (35 °C and 16 L : 8 D) ND pupae led to 11 and threefold increases in *HSP90* expression levels, respectively, from D4 to D6. After the D6 stage, mRNA levels in cold-stressed pupae gradually decreased with treatment duration with a slope rate of −0.150/day, whereas those in heat-stressed pupae gradually increased with a slope rate of 0.115/day.

Discussion

The *HSP90* up-regulation before the onset of WD and down-regulation after termination of WD found in *D. antiqua* is not paralleled in other species. In *S. crassipalpis*, for instance, *HSP90* was down-regulated during diapause (Rinehart & Denlinger, 2000), and in *D. triauraria* and *L. sericata HSP90* was not regulated in response to diapause (Goto & Kimura, 2004; Tachibana *et al.*, 2005). In these three species diapause was induced under similar conditions (20 °C and 12 L : 12 D, 15 °C and 10 L : 14 D, and 20 °C and 12 L : 12 D, respectively) to that of WD induction in *D. antiqua* in this study, suggesting that their diapause is a WD as well.

Variation among species is also found in the expression of *HSP23* and *HSP70* genes during diapause. These genes are strongly up-regulated during WD in *S. crassipalpis* (Yocum *et al.*, 1998; Rinehart & Denlinger, 2000), but not regulated as a function of WD in *D. triauraria* and *L. sericata* (Goto & Kimura, 2004; Tachibana *et al.*, 2005). *B. mori* regulates its diapause by production of diapause hormone (DH), and extracts with DH activity from other species, including non-Lepidoptera, are also capable of inducing diapause in *B. mori*. However, attempts to elicit diapause in other species with DH have consistently failed (Denlinger,

2002). Based on the available data it appears that each species uses a specific set of genes in preparation or in response to diapause, and that the up-regulation of *HSP90* transcripts is not a common feature of diapausing insects.

SD and WD prepare insects to survive the seasonally high and low temperatures, respectively. Previous experiments in *D. antiqua* (Nomura & Ishikawa, 2001) have shown that its two types of diapausing pupae (SD and WD) display high survival rates (> 75%) in response to thermal stress (15 days at 5 °C or 35 °C), unlike ND pupae under the same stress conditions (< 10% survival). The up-regulation of *HSP90*, as well as of another gene, *DaTrypsin*, studied previously (Chen *et al.*, 2005), may represent important molecular mechanisms for increasing *D. antiqua* survival during periods of temperature stress during diapause.

While *HSP90* and *DaTrypsin* are both up-regulated in response to SD and WD, there are additional clear differences in their regulation after the onset of diapause. Whereas *HSP90* and *DaTrypsin* expression gradually increased with SD duration, *HSP90* levels fluctuated around an elevated level, and *DaTrypsin* levels gradually decreased in WD pupae.

Both heat and cold-stresses applied during SD and WD sharply raised *HSP90* expression within a short 2–5-day period. After this period, the expression levels gradually increased in both treatments during the temperature stress for SD pupae, whereas levels decreased for WD pupae. This pattern is also consistent with the expression of *DaTrypsin* in *D. antiqua* (Chen *et al.*, 2005). These results consistently suggest developmental and physiological differences between SD and WD pupae, especially in the period following the initial up-regulation of these genes. The up-regulation of *HSP90* in response to cold- and heat-stress would function to help prevent misaggregation of denatured proteins and assist the refolding of denatured proteins back into native conformations, as a common function of HSPs as molecular chaperones (Sonna *et al.*, 2002). The continued up- or down-regulation of *HSP90*, following the initial increase, however, is not understood.

The up-regulation of *HSP90* in ND individuals in response to thermal stress is consistent with patterns seen in *Drosophila*, *S. crassipalpis* and *L. sericata* (Yiangou *et al.*, 1997; Denlinger, 2002; Tachibana *et al.*, 2005). A short exposure (2 or 5 days) to thermal stresses raised the transcript levels in *D. antiqua* by several more fold than the response observed in SD and WD pupae. The relative quantities of transcript, however, were much lower than those observed in the diapausing individuals, as *HSP90* had already been elevated as part of the diapause program.

After the initial period of *HSP90* transcript elevation, ND individuals responded to cold and heat stresses in opposite ways. After a cold stress, the elevated transcript levels gradually decreased with time, whereas when a heat stress was applied, elevated levels gradually increased with time. This pattern is at odds to what is observed after the initial

period of *HSP90* elevation in SD and WD individuals. In SD individuals elevated transcript levels kept increasing with time, whereas in WD individuals elevated transcript levels kept decreasing with time, irrespective of the type of temperature stress applied. These results indicate that diapausal state influences the long-term molecular regulation of *HSP90*.

This study also highlighted a slightly different pattern of *HSP90* regulation between summer and winter diapausing pupae. *HSP90* up-regulation started after onset of SD but before onset of WD, suggesting that while *HSP90* maybe up-regulated as a function of SD onset, *HSP90* up-regulation in the winter may lead to the onset of WD. In the latter case, *HSP90* may contribute to a cell cycle arrest, as described for s*HSP* and *HSP70* (Denlinger, 2002).

This is the second paper to compare the nature of SD and WD at the molecular level, and we believe, the first study on *HSP90* expression in response to SD. Both *HSP90* (this study) and *DaTrypsin* (Chen *et al.*, 2005) showed elevated expression in SD and WD. The pattern of gene activation and late regulation, however, seem to be substantial different between SD and WD and also across genes. The two genes display a similar pattern when pupae respond to a thermal stress during SD and WD, but this pattern is different across SD and WD pupae. Exploring additional genes, and species, both with descriptive as well as functional essays will be necessary to continue to unravel the molecular, developmental and physiological mechanisms of SD and WD.

Experimental procedures

Experimental insects and treatment

The *D. antiqua* ND colony was reared on an artificial diet at 20 °C with a 16 L : 8 D photocycle, as described by Ishikawa *et al.* (1983). The pupae at D4 were subject to cold stress at −10 °C with 0 L : 24 D, or heat stress at 35 °C with 16 L : 8 D, both for 2, 4 or 6 days. Larvae of the SD colony were maintained at 25 °C with 16 L : 8 D. Newly formed puparia were kept under the same conditions as the larvae until D15 and then transferred to 16 °C and 16 L : 8 D to trigger diapause termination (Ishikawa *et al.*, 2000). D5 diapausing pupae were subject to cold stress at −15 °C with 0 L : 24 D, or heat stress at 35 °C with 16 L : 8D, both for 2, 4 or 6 days. These conditions were previously identified as barely allowing survival in this species (Ishikawa *et al.*, 2000). Larvae of the WD colony were reared at 15 °C with 12 L : 12 D (Nomura & Ishikawa, 2000), and pupae were kept under this condition throughout. D40 Pupae were treated either at −20 °C with 0 L : 24 D or at 35 °C with 16 L : 8 D, both for 5, 10 or 15 days. Pupae collected at various stages were snap frozen in liquid nitrogen and stored at −80 °C prior to RNA extraction.

cDNA synthesis, cloning and sequencing mRNA was isolated from the pupae using a MicroPoly(A)Pure™ kit (Ambion, Austin, TX, USA). The first-strand cDNA was synthesized from the mRNA using the oligo(dT)-anchor primer from a 5′/3′ rapid amplification of cDNA ends (RACE) kit (Roche Applied Science, Indianapolis, IN, USA). After purification with a SUPREC™-02 kit (Takara Bio,

Kyoto, Japan), a 476 bp of HSP90 cDNA fragment was amplified by PCR from cDNA using degenerate primers (5′-TCRCART-TRTCCATRATGAA-3′ for forward; 5′-TTCATYGGBTAYCCNATYAA-3′ for reverse), which were designed using consensus sequences of several insect species (degenerate regions underlined). The fragment was then cloned into a pGEM-T vector (Promega, Madison, WI, USA). The HSP90 fragment was sequenced using BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on an ABI PRIM 377HN Sequencer (Perkin-Elmer, Foster City, CA, USA).

For 3′ RACE, the cDNA was amplified with the oligo(dT)-anchor primer and a *HSP90*-specific forward primer (5′-TTCATTGGCT-ATCCCATCAA-3′). The PCR product was reamplified using the anchor primer and another gene-specific forward primer (5′-AGCGATGATGAAGCCGAAGA-3′). For 5′ RACE, template cDNA was synthesized with a *HSP90*-specific reverse primer (5′-TCACAGTTGTCCATGATGAA-3′). After purification, the 3′ end of the cDNA was attached with a poly(A) tail using dATP and terminal deoxynucleotidyl transferase. Then, the cDNA was amplified by PCR using the anchor primer and a gene-specific primer (5′-AGAGATCGAATGGTGTGCGA-3′). The PCR product was amplified once more with the anchor primer and another gene-specific primer (5′-AATGGGTTTGGTCTTGTTCA-3′). The second-round PCR products for both 3′ RACE and 5′ RACE were cloned and sequenced as described above. The initial *HSP90* cDNA fragment and cDNA ends derived from the 5′-and 3′ RACE were edited and assembled to full-length cDNA.

Quantitative real-time PCR (Q-RT-PCR)

Total RNA was isolated from the pupae using a RNeasy Midi/Maxi kit and treated with DNase I (both Qiagen, Valencia, CA, USA). cDNA was reverse-transcribed from total RNA using random nanomers with a RNA PCR kit (Takara Bio). Gene-specific primers 5′-AGCGATGATGAAGCCGAAGA-3′ and 5′-AATGGGTTTGGTC-TTGTTCA-3′ were used to amplify the HSP90 cDNA (186 bp). The 18S rRNA gene was chosen as a reference for normalizing the *HSP90* mRNA levels, as it displays a constant expression level throughout the diapause. Primers for 18S rRNA were the same as Chen *et al.* (2005), which amplified a 333 bp fragment. One cDNA sample, diluted to 1, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}, was employed as an internal standard. Q-RT-PCR was carried out in 20 μl reactions containing 1 μl of template cDNA or the standard, $1 \times$ QuantiTect SYBR Green PCR premix (Qiagen, Valencia, CA, USA), and 0.3 μM of each primer on an ABI PRISM 7700 thermal cycler (Applied Biosystems, Foster City, CA, USA). Thermal cycling conditions were: 95 °C for 15 min, 45 cycles of 95 °C for 15 s, 58 °C for 30 s and 72 °C for 30 s, then 40 °C for 30 s and 95 °C for 1 min with ramp time of 19′59″. The relative molar amounts of *HSP90* and 18S rRNA transcripts were calculated based on crossing point analysis, using standard curves generated from the cDNA standards. *HSP90* mRNA levels were normalized with those of 18S rRNA in the same samples quantified in the same manner, and the final relative mRNA levels of *HSP90* were averages of five replicates. Regression analysis was carried out with the software Statistica v4.5 (StatSoft, Inc., Tulsa, OK, USA).

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