The Journal of Experimental Biology 213, 4146-4150

doi:10.1242/jeb.051003

Knocking down expression of *Hsp22* and *Hsp23* by RNA interference affects recovery from chill coma in *Drosophila melanogaster*

Hervé Colinet[1,2,*], Siu Fai Lee[2] and Ary Hoffmann[2]

[1]Earth and Life Institute, Biodiversity Research Centre, Université catholique de Louvain, B-1348 Louvain-la-Neuve, Belgium and [2]Centre for Environmental Stress and Adaptation Research, Department of Genetics, Bio21 Institute, The University of Melbourne, Parkville, Victoria 3010, Australia

*Author for correspondence (herve.colinet@uclouvain.be)

Accepted 20 September 2010

SUMMARY

To protect cells from the damaging effects of environmental stresses, all organisms possess a universal stress response involving upregulation of heat shock proteins (Hsps). The mechanisms underlying chilling injuries and the subsequent recovery phase are only beginning to be understood in insects. *Hsp22* and *Hsp23* are both upregulated during the recovery from prolonged chill coma in *Drosophila melanogaster*. This prompted us to investigate the functional significance of these modulations by testing whether expression of these two small Hsps is necessary for recovery after cold stress. We used the GAL4/UAS system to separately knock down expression of *Hsp22* and *Hsp23*, and assayed three aspects of recovery performance in transgenic adults that had undergone 12 h of chill coma at 0°C. The time to recover (short-term recovery) and mobility parameters (medium-term recovery) were significantly impaired in the transgenic flies in which *Hsp22* or *Hsp23* was suppressed. Our findings show that both *Hsp22* and *Hsp23* play important roles in the recovery from chill coma in adult males, and suggest that these contribute to adaptive responses to fluctuating thermal conditions.

Key words: *Drosophila*, *Hsp22*, *Hsp23*, recovery, chill coma, RNAi.

INTRODUCTION

Insects have evolved a range of molecular adaptations to cope with seasonal exposure to stressful (high and/or low) temperatures (Doucet et al., 2009). Heat shock proteins (Hsps) are considered prime candidates for thermal tolerance and adaptation in organisms, including the vinegar fly *Drosophila melanogaster* (Feder and Hofmann, 1999; Hoffmann et al., 2003; Sørensen et al., 2003; Michaud and Denlinger, 2010). Most of the focus of research on these proteins has been on their role in providing heat resistance, while their potential role in non-freezing cold-stress resistance has received less attention (Norry et al., 2007; Sørensen and Loeschcke, 2007), with the exception of *Hsp70* (Michaud and Denlinger, 2004; Sørensen and Loeschcke, 2007; Clark and Worland, 2008). Recently, the effect of low temperatures and diapause on other Hsps has been examined in insects. It appears that a wealth of additional Hsps are responsive to both low temperature and diapause, particularly genes/proteins from the small heat shock protein family (sHsp) (Qin et al., 2005; Li et al., 2007; Rinehart et al., 2007; Huang et al., 2009; Colinet et al., 2010a; Michaud and Denlinger, 2010). A key feature of the response to heat shock is its suppression following the restoration of normal environmental conditions (Parsell and Lindquist, 1993), whereas the response to cold stress is generally observed during the recovery phase (Colinet et al., 2010a; Colinet and Hoffmann, 2010). The molecular mechanisms behind recovery from cold stress are complex and it seems that more genes/proteins are activated during the recovery phases than during the period of the cold stress itself (Colinet et al., 2007; Clark and Worland, 2008).

RNA interference (RNAi)-mediated gene silencing is a powerful tool for exploring gene function. So far only a few studies have used this method to understand how specific genes respond to cold stress in insects. Rinehart et al. (Rinehart et al., 2007) found that suppression of *Hsp23* and *Hsp70* expression by RNAi resulted in a loss of cold tolerance in the flesh fly *Sarcophaga crassipalpis*. Furthermore, Kostál and Tollarová-Borovanská reported that RNAi targeting *Hsp70* negatively affected repair of chilling injuries in the firebug *Pyrrhocoris apterus* (Kostál and Tollarová-Borovanská, 2009). Finally, Colinet et al. found that silencing *Frost* expression impaired recovery from chill coma in *D. melanogaster* (Colinet et al., 2010b).

Drosophila melanogaster is a useful model for understanding the molecular basis of thermal adaptations, as it is found in a range of different thermal environments. Previous results on adult flies suggest that upregulation of cold-responsive Hsps during recovery from cold stress might be related to some undefined repairing functions (Colinet et al., 2010a), supporting the ideas of Kostál and Tollarová-Borovanská (Kostál and Tollarová-Borovanská, 2009). In *D. melanogaster*, there are 11 sHsp genes (Li et al., 2009), although only four members (*Hsp22*, *Hsp23*, *Hsp26* and *Hsp27*) have been studied in detail (e.g. Joanisse et al., 1998; Michaud et al., 2002; Morrow et al., 2006) and these are all upregulated during recovery from cold temperatures (Colinet et al., 2010a). In this study we used the GAL4/UAS system to knock down expression of two of these genes, *Hsp22* and *Hsp23*. We tested whether suppression of this upregulation response affects recovery ability after a prolonged chill coma.

MATERIALS AND METHODS

Drosophila stocks and rearing conditions

RNAi-mediated gene silencing was achieved using the GAL4/UAS system (Duffy, 2002). The UAS-Hsp lines were obtained from the Vienna Drosophila RNAi Center (*Hsp22*, transformant ID 43632; *Hsp23*, transformant ID 111816) (Dietzl et al., 2007). The *actin5C*-GAL4 line (Bloomington Drosophila Stock Center, #4414) was used

to drive the expression of the UAS-Hsp and resulted in ubiquitous Hsp mRNA knockdown. Progeny (*act*-GAL4/UAS-Hsp) were tested in cold recovery assays. To control for genetic background effects, the same GAL4 driver line was crossed to the w^{1118} line (from the Bloomington Drosophila Stock Center) and their progeny (*act*-GAL4/+) was assayed alongside their *act*-GAL4/UAS-Hsp counterparts. Fly stocks were maintained in 250 ml bottles in uncrowded conditions. Bottles were kept at 25°C, 70% relative humidity and continuous light on a standard fly medium as previously described (Hoffmann and Shirriffs, 2002).

RNA extraction and quantitative real-time PCR

To verify the extent of gene silencing, Hsp mRNA levels were measured in: (1) untreated flies, kept at 25°C (i.e. basal expression), and (2) treated flies, recovering for 2 h at 25°C after 12 h of cold stress at 0°C (i.e. during Hsp upregulation). RNA extractions were performed using the RNeasy RNA extraction kit and the RNase-Free DNase Set (Qiagen, Doncaster, VIC, Australia), as described by Colinet et al. (Colinet et al., 2010a). cDNA was synthesized using the Superscript III First-Strand Synthesis System (Invitrogen, Mulgrave, VIC, Australia), according to manufacturer's instructions. Hsp primers were designed with the Primer3 module (http://biomanager.info/) [*Hsp22*, 5′-GCCTCTCCTCGCC-CTTTCAC-3′ (forward) and 5′-TCCTCGGTAGCGCCACACTC-3′ (reverse); *Hsp23*, 5′-GGTGCCCTTCTATGAGCCCTACTAC-3′ (forward) and 5′-CCATCCTTTCCGATTTTCGACAC-3′ (reverse)]. Quantitative real-time PCR (qRT-PCR) was performed on the LightCycler 480 system (Roche Diagnostics, Castle Hill, NSW, Australia) following the method previously described (Colinet and Hoffmann, 2010). The percentage knockdown (i.e. relative expression ratio) was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The ratio of target gene (Hsp) expression in the *act*-GAL4/UAS-RNAi line relative to in the *act*-GAL4/+ control line was calculated and normalized using the housekeeping reference gene *RpS20* [primers described by Colinet et al. (Colinet et al., 2010a)]. Three biological replicates of 20 males each were used and ratios were compared with control values using Student's *t*-test.

Chill coma recovery assays

All tests were performed using synchronized 4-day-old males, sexed visually without CO_2 anaesthesia using an aspirator. Experiments were carried out only on males, in order to relate to expression data of a previous experiment that found that *Hsp22* and *Hsp23* are upregulated during recovery from cold stress in adult males (Colinet et al., 2010a).

Flies were placed in 42 ml glass vials immersed in a 10% glycol solution cooled to 0°C for 12 h (chill coma) before being returned to 25°C to recover. To test whether suppression of Hsp gene expression affects recovery abilities, we used three different measures based on the method previously described (Colinet et al., 2010b). Briefly, 'short-term recovery' compared recovery times (i.e. the time to stand up) at 25°C. Recovery curves were compared between RNAi and control lines using Mantel–Cox analysis with a censoring factor for individuals that did not recover at the end of the experiment. Forty-five flies were monitored for each line. 'Medium-term recovery' assessed climbing activity during a period of 8 h following the end of cold stress. In this negative geotaxis assay, males were individually transferred to a 9.5 cm plastic vial and the height reached within 7 s after a mechanical stimulation was noted. Flies were divided into three categories: (a) 'injured', no climbing; (b) 'recovering', slow climbing without reaching the top of the vial within 7 s; (c) 'fit', fast climbing and reaching the top of the vial within 7 s. The time of observation was chosen based on preliminary assays (see Colinet et al., 2010b). This test was performed repeatedly on the same individuals after 2, 4, 6 and 8 h of recovery at 25°C. Chi-square contingency tests were carried out to compare numbers of flies in the three categories between RNAi and control lines. Seventy flies were tested for each line. Flies were maintained on food during this period.

Finally, 'long-term recovery' measured mortality 24 h after the end of the cold stress. Mortality rates were calculated based on 150 flies per line. Chi-square contingency tests were used to compare mortality rates between RNAi and control lines. All statistical tests were performed using Prism V 5.01 (GraphPad Software Inc., 2007).

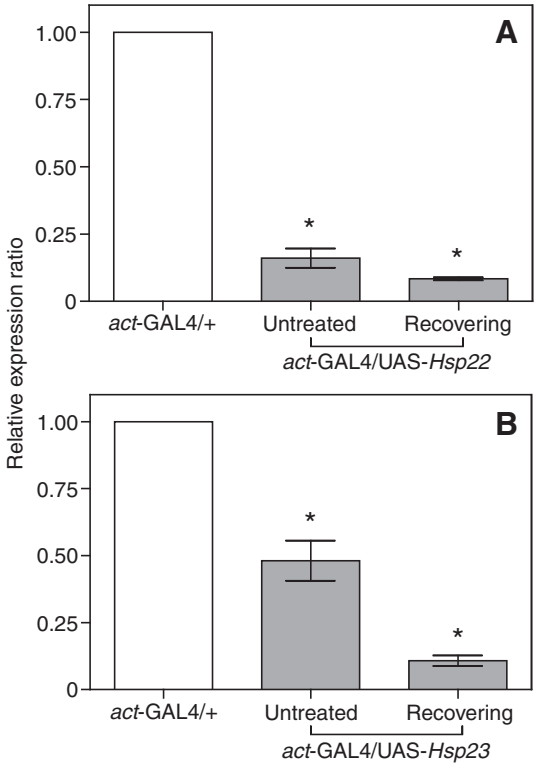

Fig. 1. The mRNA expression of the *Drosophila* heat shock protein genes *Hsp22* (A) and *Hsp23* (B) in untreated (kept at 25°C) and recovering (2 h at 25°C after 12 h at 0°C) adult males. mRNA levels are expressed relative to the control *act*-GAL4/+ line and are normalized against the housekeeping reference gene *RpS20*. An asterisk (*) indicates when the level is significantly different from that of the control (mean ± 95% confidence intervals; *N*=3).

RESULTS

Percentage knockdown

The mRNA level was significantly reduced in the *act*-GAL4/UAS-*Hsp22* line compared with in the *act*-GAL4/+ control line in both untreated (t=75.36, d.f.=2, P=0.001) and recovering (t=557.3, d.f.=2, P=0.001) flies (Fig. 1A). The percentage knockdown reached 84% in untreated males and 92% in treated males. The level of expression was also significantly reduced in the *act*-GAL4/UAS-*Hsp23* line compared with in the *act*-GAL4/+ control line in both untreated (t=22.14, d.f.=2, P=0.0002) and recovering (t=141.9, d.f.=2, P=0.001) flies (Fig. 1B). The percentage knockdown reached 52% in untreated males and 90% in treated males.

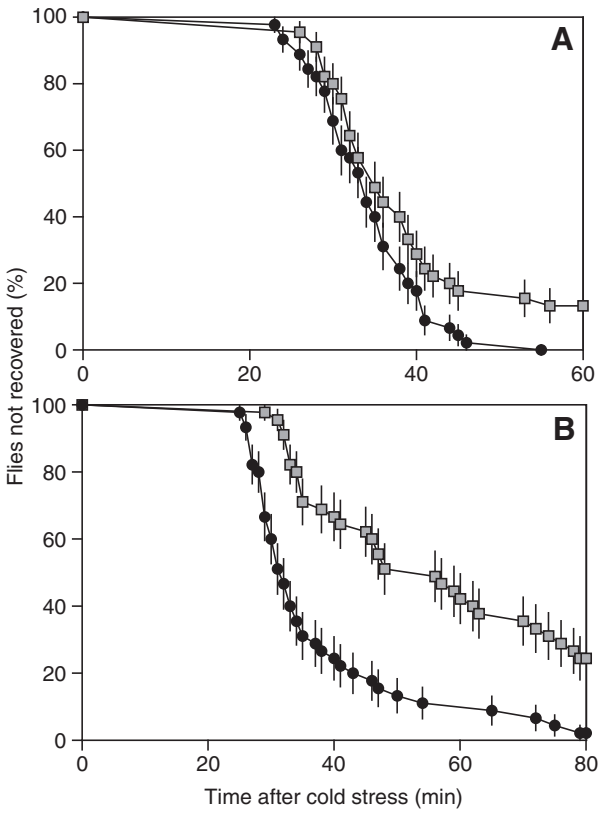

Fig. 2. Comparison of temporal recovery curves in the RNAi line (*act*-GAL4/UAS-*Hsp*; grey squares) *versus* the control line (*act*-GAL4/+; black circles) for *Hsp22* (A) and *Hsp23* (B). The time to recover from chill coma (time to stand up) was monitored in adult males recovering at 25°C after 12 h of cold stress at 0°C. Each data point represents the mean (±s.e.m.) percentage of flies not recovered, based on 45 males per line.

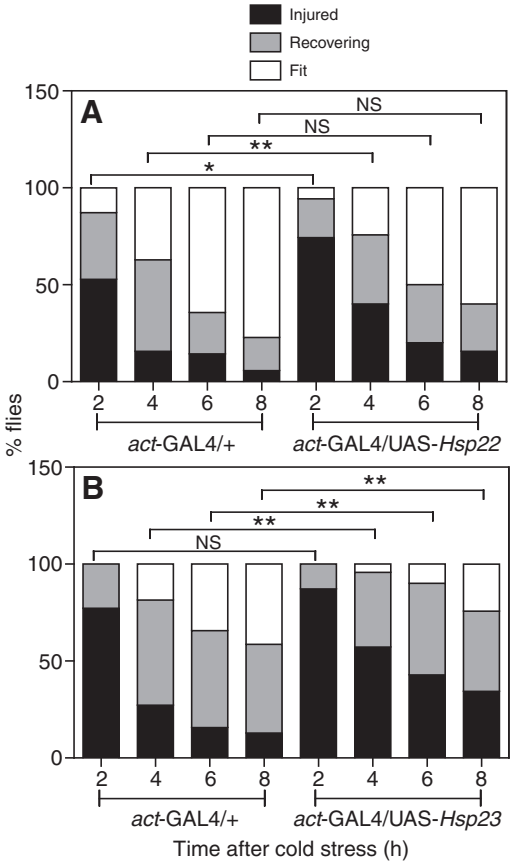

Fig. 3. Climbing activity monitored in the RNAi line (*act*-GAL4/UAS-*Hsp) versus* the control line (*act*-GAL4/+) for *Hsp22* (A) and *Hsp23* (B). Measurements were taken in adult males after 2, 4, 6 and 8 h of recovery at 25°C following 12 h of cold stress at 0°C. Flies were categorized as fit (fast climbing, white bar), recovering (slow climbing, grey bar) or injured (no climbing, black bar). Symbols indicate significant differences (**P*<0.01; ***P*<0.001) in proportions between lines, although some comparisons are not significantly different (NS). Seventy males were tested per line.

Short-term recovery

Short-term recovery was significantly affected in the *act*-GAL4/UAS-*Hsp22* line compared with in the *act*-GAL4/+ control line (Fig. 2A), resulting in significantly different recovery curves (χ^2=5.30, d.f.=1, *P*=0.021). After 60 min, all the *act*-GAL4/+ flies had recovered, whereas 24% of the *act*-GAL4/UAS-*Hsp22* flies remained in coma. The short-term recovery was also significantly affected in the *act*-GAL4/UAS-*Hsp23* line compared with in the *act*-GAL4/+ control line (χ^2=24.69, d.f.=1, *P*=0.001; Fig. 2B). After 80 min, all the *act*-GAL4/+ flies had recovered, whereas 24% of the *act*-GAL4/UAS-*Hsp23* flies still had not recovered. All flies eventually recovered and no mortality was observed at the end of the experiment.

Medium-term recovery

The medium-term recovery tests revealed significant differences in mobility (climbing) between the *act*-GAL4/UAS-*Hsp22* and *act*-GAL4/+ lines (Fig. 3A). Differences were manifested after 2 h (χ^2=7.083, d.f.=2, *P*=0.029) and 4 h (χ^2=10.397, d.f.=2, *P*=0.006) of recovery, with, respectively, 74% and 40% of flies still injured in the *act*-GAL4/UAS-*Hsp22* line compared with 52% and 15% in the *act*-GAL4/+ line. The proportions within each category (fit, recovering, injured) were similar between the *act*-GAL4/UAS-*Hsp22* and *act*-GAL4/+ lines (Fig. 3A) after 6 h (χ^2=2.917, d.f.=2, *P*=0.233) and 8 h (χ^2=5.629, d.f.=2, *P*=0.06) of recovery.

There was initially (after 2 h recovery) no difference in the proportion of injured and recovering flies between the *act*-GAL4/UAS-*Hsp23* and the *act*-GAL4/+ lines (χ^2=2.386, d.f.=1, *P*=0.122) (Fig. 3B). In the *act*-GAL4/+ line, flies recovered progressively with an increasing proportion of fit and a decreasing proportion of injured flies. By contrast, flies from the *act*-GAL4/UAS-*Hsp23* line showed a reduction in their recovery ability with time, which resulted in significant differences between the two lines after 4 h (χ^2=15.586, d.f.=2, *P*=0.001), 6 h (χ^2=18.186, d.f.=2, *P*=0.001) and 8 h (χ^2=10.096, d.f.=2, *P*=0.006) of recovery (Fig. 3B).

Long-term recovery

In the long-term recovery assay, no difference in mortality was observed between the *act*-GAL4/UAS-*Hsp22* and the *act*-GAL4/+ lines (χ^2=0.667, d.f.=1, *P*=0.414), with very low mortality rates in both lines (Fig. 4A). Similarly mortality rates were low and similar between the *act*-GAL4/UAS-*Hsp23* and the *act*-GAL4/+ lines (χ^2=3.447, d.f.=1, *P*=0.067; Fig. 4B).

DISCUSSION

In *D. melanogaster*, chill coma starts around 7°C as a result of neuromuscular dysfunctions (Hosler et al., 2000). At low temperature, chilling injuries accumulate because of various

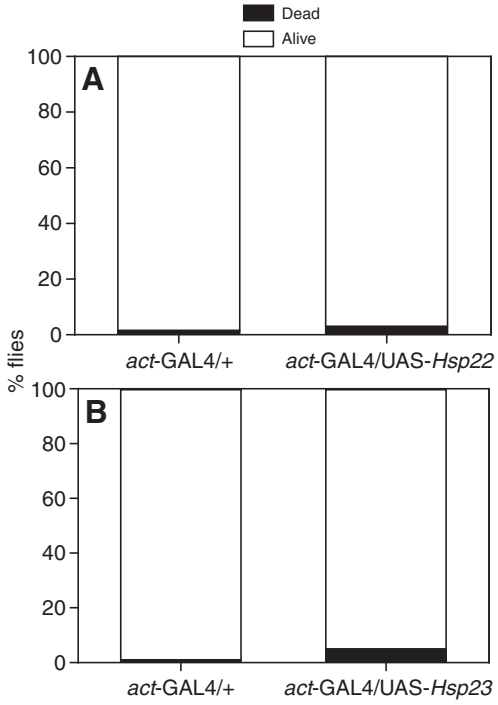

Fig. 4. Mortality rate in the RNAi line (*act*-GAL4/UAS-*Hsp) versus* the control line (*act*-GAL4/+) for *Hsp22* (A) and *Hsp23* (B). Mortality was assessed based on 150 males recovering for 24 h at 25°C after 12 h of cold stress at 0°C.

physiological perturbations, such as thermotropic damage to membranes, complex metabolic disorders, ion homeostasis imbalance and oxidative stress (reviewed by Chown and Terblanche, 2006; Lee, 2010). Within limits, these physiological damages are reversible (Chown and Terblanche, 2006), but an understanding of the molecular mechanisms behind recovery is lacking.

The present results suggest that members of the sHsp gene family are involved in the recovery process. Some sHsp genes are known to be upregulated during the recovery from cold stress in *D. melanogaster* but are not modulated during the cold stress itself (Colinet et al., 2010a). In this study we have now shown that knocking down the upregulation response of *Hsp22* and *Hsp23* by RNAi affects recovery ability. In the case of *Hsp22*, the time to recover was slightly affected and individuals displayed reduced mobility after 2 and 4 h of recovery when compared with controls. However, after 6 h of recovery the *Hsp22*-knockdown flies were as active as their control counterparts. This suggests that the kinetics of the chill coma recovery process was impaired, at least during the first 4 h period immediately after the cold stress. In the case of *Hsp23*, recovery time was also affected, and the reduction of mobility parameters was still manifested even after 8 h of recovery. In general, the phenotypic consequences of *Hsp23* knockdown were more severe than those of *Hsp22* knockdown. Given that the transcript knockdown efficiencies were similar (90–92%) for both genes, our results imply that *Hsp23* might be more strongly involved in cold recovery than *Hsp22*. However, no mortality effect was observed for either gene. Our findings show that upregulation of *Hsp22* and *Hsp23* is required for recovery from prolonged chill coma, at least during the early stages of the recovery process (0–8 h).

The sHsps represent the least conserved subfamily of Hsp genes, and most of the sHsp genes are species specific (Li et al., 2009), so that direct comparison between insects must be made with care. Nevertheless, there are similarities between *S. crassipalpis* and *D. melanogaster*; both of these dipterans express *Hsp70* and *Hsp23* during diapause, whereas *Hsp90* shows an opposite pattern of expression (Rinehart and Denlinger, 2000; Rinehart et al., 2007; Baker and Russell, 2009). Suppressing the expression of *Hsp23* in *S. crassipalpis* by RNAi did not alter the tendency to enter diapause but, as in the case of *D. melanogaster*, there was a negative effect on cold tolerance.

Most organisms have multiple sHsps but their precise identities are often unknown, which makes any functional investigation difficult (Michaud and Denlinger, 2010). Although the identities of *D. melanogaster* sHsps are known, the question as to why there are several structurally similar sHsps remains unclear. *Hsp22* and *Hsp23* are coordinately upregulated during/after heat stress (Bettencourt et al., 2008) and after cold stress (Colinet et al., 2010a). During development, each sHsp gene shows specific stage and cellular patterns of expression without coordination (Michaud et al., 1997; Michaud et al., 2002; Morrow and Tanguay, 2003). This might denote a common function(s) under stress conditions but a more specific role(s) during development. In spite of this, Hsp22 and Hsp23 show different chaperone activity efficiencies, which suggests different modes of action (Morrow et al., 2006). Moreover, overexpression of Hsp22 and Hsp23 both lead to increased lifespan but with different intensity, supporting the idea of specific modes of action (Morrow and Tanguay, 2003; Morrow et al., 2004).

The fact that RNAi directed against *Hsp22* affected recovery less than RNAi directed against *Hsp23* suggests that the two genes contribute to cold tolerance in slightly different ways. The two chaperone products of these genes differ in their subcellular localization, Hsp22 being found in the mitochondria and Hsp23 in the cytoplasm (Joanisse et al., 1998). It is not known whether the proteins Hsp22 and Hsp23 have specific or overlapping activities during the recovery from cold stress, and which functions are required to maintain proper recovery ability. Increased *Hsp22* and *Hsp23* mRNA levels correlate with increased stress resistance in selected *D. melanogaster* lines (Kurapati et al., 2000). A body of evidence supports the idea that *Hsp22* is an oxidative stress response gene (e.g. Bhole et al., 2004; Morrow et al., 2004). Both *Hsp22* and *Hsp23* are involved in protection from the disturbance of normal redox state in *D. melanogaster*. Indeed, *Hsp23* plays important role in hypoxia tolerance (Azad et al., 2009), whereas *Hsp22* has a protective role in hyperoxia (Gruenewald et al., 2009). Maintenance of the oxidant equilibrium might be important during recovery, especially because perturbation of the antioxidant system is known to be related to chilling injury in insects (Rojas and Leopold, 1996; Grubor-Lajsic et al., 1997; Jing et al., 2005). In addition, *Hsp22* expression in motoneurons allows the maintenance of locomotion activity (Morrow et al., 2004). Similarly, *Hsp23* expression within the CNS might confer neuroprotection (Michaud and Tanguay, 2003). These protective functions might also be important during the recovery because chill coma results from neuromuscular dysfunctions (Hosler et al., 2000).

It is clear that molecular chaperones are involved during the recovery from cold stress (Colinet et al., 2010a; Michaud and Denlinger, 2010). Moreover, several other genes show expression changes concomitant with those of Hsps during the recovery phase. This is the case for *Starvin* and *DnaJ-1*, two co-chaperones that interact with members of the Hsp70 family, although the expression of *Starvin* declines more sharply than that of the Hsps after 4 h of recovery (Colinet et al., 2010a; Colinet and Hoffmann, 2010). *Frost* which has no known chaperoning functions also

shows an expression pattern similar to those of the Hsp genes, although its expression is particularly high during cold recovery; knocking down *Frost* expression results in a loss of recovery ability in a fashion similar to that observed following *Hsp22* and *Hsp23* knockdown (Colinet et al., 2010b). It is unclear whether these genes affect cold recovery via independent pathways. Future experiments could aim to simultaneously suppress two or more of these genes and assay for cold recovery performances. The molecular mechanisms behind recovery from cold stress are complex and poorly understood. Genes/proteins involved in stress responses are conserved in all organisms and are related to various key functions, such as cell cycle control, protein chaperoning, DNA stabilization and repair, the removal of damaged proteins, and certain aspects of metabolism (Kültz, 2003; Kültz, 2005).

In conclusion, this study provides evidence that *Hsp22* and *Hsp23* are important for chill-coma recovery in adult *D. melanogaster*. The mechanisms whereby they influence this trait are still unclear, but the chaperone products of these genes might target different cellular and tissue-specific functions important in cold recovery. The role of other sHsp genes upregulated during cold recovery (Colinet et al., 2010a) also remains to be explored.

ACKNOWLEDGEMENTS

We are grateful to Phillip Daborn and Philip Batterham for providing access to the PC2 facility (Melbourne University, Australia), and we thank Steve McKechnie (Monash University, Australia) for assisting in the importation of fly lines. This study was supported by Fonds de la Recherche Scientifique – FNRS, the Australian Research Council via their Discovery and Fellowship schemes, and the Commonwealth Environmental Research Fund. This paper is number BRC189 of the Biodiversity Research Centre.

REFERENCES

Azad, P., Zhou, D., Russo, E. and Haddad, G. G. (2009). Distinct mechanisms underlying tolerance to intermittent and constant hypoxia in *Drosophila melanogaster*. *PLoS ONE* **4**, e5371.

Baker, D. A. and Russell, S. (2009). Gene expression during *Drosophila melanogaster* egg development before and after reproductive diapauses. *BMC Genomics* **10**, 242.

Bettencourt, B. R., Hogan, C. C., Nimali, M. and Drohan, B. W. (2008). Inducible and constitutive heat shock gene expression responds to modification of Hsp70 copy number in *Drosophila melanogaster* but does not compensate for loss of thermotolerance in Hsp70 null flies. *BMC Biol.* **6**, 5.

Bhole, D., Allikian, M. J. and Tower, J. (2004). Doxycycline-regulated over-expression of hsp22 has negative effects on stress resistance and life span in adult *Drosophila melanogaster*. *Mech. Ageing Dev.* **125**, 651-663.

Chown, S. L. and Terblanche, J. S. (2006). Physiological diversity in insects: ecological and evolutionary contexts. *Adv. Insect Physiol.* **33**, 50-152.

Clark, M. S. and Worland, M. R. (2008). How insects survive the cold: molecular mechanisms-a review. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* **178**, 917-933.

Colinet, H. and Hoffmann, A. (2010). Gene and protein expression of *Drosophila* Starvin during cold stress and recovery from chill coma. *Insect Biochem. Mol. Biol.* **40**, 425-428.

Colinet, H., Nguyen, T. T., Cloutier, C., Michaud, D. and Hance, T. (2007). Proteomic profiling of a parasitic wasp exposed to constant and fluctuating cold exposure. *Insect Biochem. Mol. Biol.* **37**, 1177-1188.

Colinet, H., Lee, S. F. and Hoffmann, A. (2010a). Temporal expression of heat shock genes during cold stress and recovery from chill coma in adult *Drosophila melanogaster*. *FEBS J.* **277**, 174-185.

Colinet, H., Lee, S. F. and Hoffmann, A. (2010b). Functional characterization of the frost gene in *Drosophila melanogaster*: importance for recovery from chill coma. *PLoS ONE* **5**, e10925.

Dietzl, G., Chen, D., Schnorrer, F., Su, K.-C., Barinova, Y., Fellner, M., Gasser, B., Kinsey, K., Oppel, S., Scheiblauer, S. et al. (2007). A genome-wide transgenic RNAi library for conditional gene inactivation in *Drosophila*. *Nature* **448**, 151-156.

Doucet, D., Walker, V. K. and Qin, W. (2009). The bugs that came in from the cold: molecular adaptations to low temperatures in insects. *Cell. Mol. Life Sci.* **66**, 1404-1418.

Duffy, J. B. (2002). GAL4 system in *Drosophila*: a fly geneticist's swiss army knife. *Genesis* **34**, 1-15.

Feder, M. E. and Hofmann, G. E. (1999). Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annu. Rev. Physiol.* **61**, 243-282.

Grubor-Lajsic, G., Block, W., Telesmanic, M., Jovanovic, A. and Stevanovic, D. and Baca, F. (1997). Effect of cold acclimation on the antioxidant defense system of two larval lepidoptera (noctuidae). *Arch. Insect Biochem. Physiol.* **36**, 1-10.

Gruenewald, C., Botella, J. A., Bayersdorfer, F., Navarro, J. A., Schneuwly, S. (2009). Hyperoxia-induced neurodegeneration as a tool to identify neuroprotective genes in *Drosophila melanogaster*. *Free Radic. Biol. Med.* **46**, 1668-1676.

Hoffmann, A. A. and Shirriffs, J. (2002). Geographic variation for wing shape in *Drosophila serrata*. *Evolution* **56**, 1068-1073.

Hoffmann, A. A., Sørensen, J. G. and Loeschcke, V. (2003). Adaptation of *Drosophila* to temperature extremes: bringing together quantitative and molecular approaches. *J. Therm. Biol.* **28**, 175-216.

Hosler, J. S., Burns, J. E. and Esch, H. E. (2000). Flight muscle resting potential and species-specific differences in chill coma. *J. Insect Physiol.* **46**, 621-627.

Huang, L.-H., Wang, C.-Z. and Kang, L. (2009). Cloning and expression of five heat shock protein genes in relation to cold hardening and development in the leafminer, Liriomyza sativa. *J. Insect Physiol.* **55**, 279-285.

Jing, X. H., Wang, X. H. and Kang. L. (2005). Chill injury in the eggs of the migratory locust, *Locusta migratoria* (Orthoptera:Acrididae) the time-temperature relationship with high-temperature interruption. *Insect Sci.* **12**, 171-178.

Joanisse, D. R., Michaud, S., Inaguma, Y. and Tanguay, R. M. (1998). The small heat shock proteins of *Drosophila*: developmental expression and functions. *J. Biosci.* **23**, 101-108.

Kostál, V. and Tollarová-Borovanská, M. (2009). The 70 kDa heat shock protein assists during the repair of chilling injury in the insect, *Pyrrhocoris apterus*. *PLoS ONE* **4**, e4546.

Kültz, D. (2003). Evolution of the cellular stress proteome: from monophyletic origin to ubiquitous function. *J. Exp. Biol.* **206**, 3119-3124.

Kültz, D. (2005). Molecular and evolutionary basis of the cellular stress response. *Annu. Rev. Physiol.* **67**, 225-257.

Kurapati, R., Passananti, H. B., Rose, M. R. and Tower, J. (2000). Increased hsp22 RNA levels in *Drosophila* lines genetically selected for increased longevity. *J. Gerontol. Biol. Sci.* **55A**, B552-B559.

Lee, R. E., Jr (2010). A primer on insect cold-tolerance. In *Low Temperature Biology of Insects* (ed. D. L. Denlinger and R. E. Lee, Jr), pp. 3-35. Cambridge: Cambridge University Press.

Li, A. Q., Popova-Butler, A., Dean, D. H. and Denlinger, D. L. (2007). Proteomics of the flesh fly brain reveals an abundance of upregulated heat shock proteins during pupal diapause. *J. Insect Physiol.* **53**, 385-391.

Li, Z. W., Li, X., Yu, Q. Y., Xiang, Z. H., Kishino, H. and Zhang, Z. (2009). The small heat shock protein (sHSP) genes in the silkworm, *Bombyx mori*, and comparative analysis with other insect sHSP genes. *BMC Evol. Biol.* **9**, 215.

Livak, K. J. and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{(-\Delta\Delta Ct)}$ method. *Methods* **25**, 402-408.

Michaud, M. R. and Denlinger, D. L. (2004). Molecular modalities of insect cold survival: current understanding and future trends. *Int. Congress Ser.* **1275**, 32-46.

Michaud, M. R. and Denlinger, D. L. (2010). Genomics, proteomics and metabolomics: finding the other players in insect cold-tolerance. In Low temperature biology of insects (ed. D. L. Denlinger and R. E. Lee, Jr), pp. 91-116. Cambridge: Cambridge University Press.

Michaud, S. and Tanguay, R. M. (2003). Expression of the Hsp23 chaperone during *Drosophila* embryogenesis: association to distinct neural and glial lineages. *BMC Dev. Biol.* **3**, 9.

Michaud, S., Marin, R. and Tanguay, R. M. (1997). Regulation of heat shock gene induction and expression during *Drosophila* development. *Cell. Mol. Life Sci.* **53**, 104-113.

Michaud, S., Morrow, G., Marchand, J. and Tanguay, R. M. (2002). *Drosophila* small heat shock proteins: cell and organelle-specific chaperones? *Prog. Mol. Subcell. Biol.* **28**, 79-101.

Morrow, G. and Tanguay, R. M. (2003). Heat shock proteins and aging in *Drosophila melanogaster*. *Semin. Cell Dev. Biol.* **14**, 291-299.

Morrow, G., Samson, M., Michaud, S. and Tanguay, R. M. (2004). Overexpression of the small mitochondrial Hsp22 extends *Drosophila* life span and increases resistance to oxidative stress. *FASEB J.* **18**, 598-599.

Morrow, G., Heikkila, J. J. and Tanguay, R. M. (2006). Differences in the chaperone-like activities of the four main small heat shock proteins of *Drosophila melanogaster*. *Cell. Stress. Chaperones* **11**, 51-60.

Norry, F. M., Gomez, F. H. and Loeschcke, V. (2007). Knockdown resistance to heat stress and slow recovery from chill coma are genetically associated in a quantitative trait locus region of chromosome 2 in *Drosophila melanogaster*. *Mol. Ecol.* **16**, 3274-3284.

Parsell, D. A. and Lindquist, S. (1993). The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. *Annu. Rev. Genet.* **27**, 437-496.

Qin, W., Neal, S., Robertson, R., Westwood, J. and Walker, V. (2005). Cold hardening and transcriptional change in *Drosophila melanogaster*. *Insect Mol. Biol.* **14**, 607-613.

Rinehart, J. P. and Denlinger, D. L. (2000). Heat-shock protein 90 is down-regulated during pupal diapause in the flesh fly, *Sarcophaga crassipalpis*, but remains responsive to thermal stress. *Insect Mol. Biol.* **9**, 641-645.

Rinehart, J. P., Li, A., Yocum, G. D., Robich, R. M., Hayward, S. A. and Denlinger, D. L. (2007). Up-regulation of heat shock proteins is essential for cold survival during insect diapause. *Proc. Natl. Acad. Sci. USA* **104**, 11130-11137.

Rojas, R. R. and Leopold, R. A. (1996). Chilling injury in the housefly: evidence for the role of oxidative stress between pupariation and emergence. *Cryobiology* **33**, 447-458.

Sørensen, J. G. and Loeschcke, V. (2007). Studying stress responses in the post-genomic era: its ecological and evolutionary role. *J. Biosci.* **32**, 447-456.

Sørensen, J. G., Kristensen, T. N. and Loeschcke, V. (2003). The evolutionary and ecological role of heat shock proteins. *Ecol. Lett.* **6**, 1025-1037.