Expression of the 70 kDa Heat shock protein family in Alpine freshwater chironomids (Diptera, Chironomidae) under natural conditions

Valeria LENCIONI*, Deborah BOSCHINI and Lorena REBECCHI

ABSTRACT

Chironomidae represent up to 100% of the fauna of Alpine streams. Because they survive stress conditions such as extremely low temperature (annual mean < 4 °C), these animals represent a good organism model to analyze the relationship between adaptation to cold and expression of stress proteins such as the 70 kDa Heat shock protein family. Fourth instar larvae of ten species of cold-stenothermal chironomids (Pseudodiamesa branickii, Diamesa latitarsis, D. laticauda, D. cinerella, D. insignipes, D. zernyi, D. vaillanti, Orthocladius (O.) frigidus, (Euorthocladius) thienemanni and Paratrichocladius nivalis) were collected in a glacier-fed stream in NE Italy at two stations (1300 and 2600 m a.s.l.) and in two seasons (summer 2005 and spring 2006). Immunodetection and quantification of the relative levels of Hsp70 family were performed via Western blot analysis. Significantly different levels of Hsp70 were detected among species. The highest amounts were recorded in P. nivalis and D. insignipes, the lowest in P. branickii. Within the genus Diamesa, lower levels of Hsp70 were observed in the most cold-stenothermal species than in the less cold-stenothermal ones. These differences may be explained by different species autoecology. The results provide information on biochemical strategies of alpine midges to face cold temperatures under natural conditions and new insights into their possible response to global warming.

Key words: cold-stenothermal species, Diamesinae, Orthocladiinae, glacial streams, stress proteins, Italian Alps

1. INTRODUCTION

In Alpine freshwaters, food-chains are simplified and few organisms are adapted to such environmental constraints (Irons et al. 1993). These habitats are colonised mainly by Chironomidae (Diptera) (Lods-Crozet et al. 2001) which possess adaptations to a variety of environmental rigors such as desiccation, anoxia, high or extremely low temperatures and freezing (Danks 1971; Lencioni 2004). Chironomids are the most widely distributed insect family in freshwaters, with about 3700 species widespread throughout all the zoogeographic regions (Ashe et al. 1987; Cranston 1995). In particular, in Alpine streams fed by ice- and snowmelt they account for the majority of the macroinvertebrate species, accounting for up to 100% of the fauna in the kryal (the first km downstream of the glacial snout) (Füreder 1999; Lencioni & Rossaro 2005). The kryal is characterised by extremely low temperatures (annual mean < 4 °C), coupled with considerable seasonal and daily highly variability in channel stability, turbidity and discharge (Brittain & Milner 2001; Maioolini & Lencioni 2001). For these reasons, chironomids are an appropriate taxa to study the adaptive strategies evolved to survive stresses such as low temperatures and temperature variations (Lencioni et al. 2008).

The involvement of heat shock proteins (Hsps) in resistance towards heat, but also cold and in a range of other stresses such as heavy metals, pesticides, desiccation, anoxia and diseases has been documented for many organisms, from bacteria to plants and animals (e.g., Lindquist 1986; Feder & Hoffmann 1999; Sørensen et al. 2003), including chironomids (e.g., Morcillo et al. 1997; Rinehart et al. 2006). However, there is no reference to cold stenothermal species such as Alpine chironomids. Hsps function as molecular chaperones and play a primary role in folding, assembly, intracellular localization, secretion, and degradation of other proteins.

In many organisms, Hsp of 70 kDa is considered the major Hsp family consisting of inducible (Hsp) and constitutive (heat shock cognate, Hsc) forms. The expression of both forms can be activated and/or increased in heat shock response (HSR) (Fader et al. 1994; Feder & Hoffmann 1999). Recently, the ecological importance of inducible Hsps was also demonstrated in recovery and survival of organisms under stressful conditions (Sørensen et al. 2003).

In a wide range of organisms, the expression of Hsps can be influenced by seasonal and altitudinal temperature variations, or by the different geographical areas in which the organisms occur (Fader et al. 1994; Hofmann & Somero 1995; Feder & Hoffmann 1999; Tomanek & Somero 1999). Because vital cellular processes may be susceptible to temperature, ectothermic organisms that live at thermal extremes have altered Hsp expression and function in order to facilitate protein folding (Hofmann 1999). Antarctic organisms represent a good
Tab. 1. Number of larvae collected at the two sampling sites and in the two seasons in the Noce Bianco stream (NE-Italy). Mean ± standard deviation of water temperature (°C) and mean ± standard deviation of percent oxygen saturation at the two sampling sites during the months of March and of July calculated for the period 1999-2002 are given (Lencioni & Maiolini 2002). After the semicolon, the water temperature and the oxygen saturation recorded during the surveys in 2005 and 2006.

<table>
<thead>
<tr>
<th>Species</th>
<th>No. larvae</th>
<th>Sampling site (m s.l.m.)</th>
<th>Sampling date (°C)</th>
<th>Temperature O₂ saturation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudodiamesa branickii</td>
<td>43</td>
<td>1300</td>
<td>31-Mar-2006</td>
<td>4.2 ± 0.8; 4.9</td>
</tr>
<tr>
<td>Diamesa laticaudis</td>
<td>15</td>
<td>2600</td>
<td>11-July-2005</td>
<td>3.9 ± 1.0; 4.0</td>
</tr>
<tr>
<td>Diamesa laticauda</td>
<td>21</td>
<td>1300</td>
<td>31-Mar-2006</td>
<td>4.2 ± 0.8; 4.9</td>
</tr>
<tr>
<td>Diamesa cinerella</td>
<td>23</td>
<td>2600</td>
<td>11-July-2005</td>
<td>3.9 ± 1.0; 4.0</td>
</tr>
<tr>
<td>Diamesa insignipes</td>
<td>84</td>
<td>1300</td>
<td>31-Mar-2006</td>
<td>4.2 ± 0.8; 4.9</td>
</tr>
<tr>
<td>Diamesa zernyi</td>
<td>21</td>
<td>2600</td>
<td>11-July-2005</td>
<td>3.9 ± 1.0; 4.0</td>
</tr>
<tr>
<td>Diamesa vaillanti</td>
<td>23</td>
<td>1300</td>
<td>31-Mar-2006</td>
<td>4.2 ± 0.8; 4.9</td>
</tr>
<tr>
<td>Orthocladius (O.) frigidus</td>
<td>57</td>
<td>1300</td>
<td>31-Mar-2006</td>
<td>4.2 ± 0.8; 4.9</td>
</tr>
<tr>
<td>Orthocladius (E.) thienemanni</td>
<td>22</td>
<td>1300</td>
<td>31-Mar-2006</td>
<td>4.2 ± 0.8; 4.9</td>
</tr>
<tr>
<td>Paratrichocladius nivalis</td>
<td>76</td>
<td>1300</td>
<td>31-Mar-2006</td>
<td>4.2 ± 0.8; 4.9</td>
</tr>
</tbody>
</table>

example in evaluating the relationship between Hsps expression and temperatures because the temperatures they experience are both extremely cold and extremely stable (Vayda & Yuan 1994; Deegenaaars & Watson 1997; Carpenter & Hofmann 2000; Hofmann et al. 2000; La Terza et al. 2001; Place et al. 2004; Clark & Worland 2008). Among insects, the best studied species from cold regions is the chironomid Bèlgica antarctica Jacobs, a permanent semi-terrestrial Antarctica inhabitant (Benoit et al. 2007). Its larvae, living in a thermally buffered soil environment, constitutively up-regulate their Hsps and maintain a high inherent tolerance to temperature stress. On the contrary, adults do not exhibit constitutive up-regulation of Hsps and thus they have a lower intrinsic tolerance to high temperatures, but they maintain the capacity to thermally activate the synthesis of their Hsps (Rinehart et al. 2006).

Another good example for understanding the role of Hsps in developing cold-resistance is represented by the fauna inhabiting the cold Alpine streams, not previously studied from this point of view. Furthermore, numerous studies have investigated the relationship between Hsp synthesis and various potential stress factors, but very few studies have investigated the Hsp levels under natural conditions (Tomanek & Sanford 2003). The aim of this paper is to analyze the cold adaptation in terms of production of Hsp kDa 70 family under natural conditions (Tomanek & Sanford 2003). The aim of this paper is to analyze the cold adaptation in terms of production of Hsp kDa 70 family under natural conditions (Tomanek & Sanford 2003). The aim of this paper is to analyze the cold adaptation in terms of production of Hsp kDa 70 family under natural conditions (Tomanek & Sanford 2003). The aim of this paper is to analyze the cold adaptation in terms of production of Hsp kDa 70 family under natural conditions (Tomanek & Sanford 2003). The aim of this paper is to analyze the cold adaptation in terms of production of Hsp kDa 70 family under natural conditions (Tomanek & Sanford 2003). The aim of this paper is to analyze the cold adaptation in terms of production of Hsp kDa 70 family under natural conditions (Tomanek & Sanford 2003). The aim of this paper is to analyze the cold adaptation in terms of production of Hsp kDa 70 family under natural conditions (Tomanek & Sanford 2003). The aim of this paper is to analyze the cold adaptation in terms of production of Hsp kDa 70 family under natural conditions (Tomanek & Sanford 2003). The aim of this paper is to analyze the cold adaptation in terms of production of Hsp kDa 70 family under natural conditions (Tomanek & Sanford 2003). The aim of this paper is to analyze the cold adaptation in terms of production of Hsp kDa 70 family under natural conditions (Tomanek & Sanford 2003). The aim of this paper is to analyze the cold adaptation in terms of production of Hsp kDa 70 family under natural conditions (Tomanek & Sanford 2003). The aim of this paper is to analyze the cold adaptation in terms of production of Hsp kDa 70 family under natural conditions (Tomanek & Sanford 2003). The aim of this paper is to analyze the cold adaptation in terms of production of Hsp kDa 70 family under natural conditions (Tomanek & Sanford 2003). The aim of this paper is to analyze the cold adaptation in terms of production of Hsp kDa 70 family under natural conditions (Tomanek & Sanford 2003). The aim of this paper is to analyze the cold adaptation in terms of production of Hsp kDa 70 family under natural conditions (Tomanek & Sanford 2003). The aim of this paper is to analyze the cold adaptation in terms of production of Hsp kDa 70 family under natural conditions (Tomanek & Sanford 2003).
2.2. Protein extraction

After being sorted, larvae were either immediately utilized for protein extraction, or frozen at –80 °C. For each taxon, groups of 3-5 larvae were homogenized in toto in a buffered extraction solution (20% Tris-HCl 0.5 M, 20% glycerol, 5% SDS, pH 6.8, 0.025% mercaptoethanol; 20 µL). Samples were incubated at 100 °C for 5 min, and centrifuged at 15000 × g for 25 min at room temperature. Protein content of the 15000 × g supernatant was determined using the DC Protein Assay kit (Bio-Rad). These samples were stored at –20 °C.

2.3. One-dimensional gel electrophoresis, Western blot analysis and quantification of Hsp70

Detection and quantification of 70 kDa Hsp were performed using one-dimensional-SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis. For comparison, equivalent amounts of total protein (10 µg) for each taxon were electrophoresed. Total proteins were loaded and separated on a 12% SDS-polyacrylamide gel with 4% stacking gel using the buffer system described by Læmmli (1970). Pre-stained SDS molecular weight markers (Sigma) were run on each gel to indicate molecular weight.

Following preparation, proteins were electrophoretically transferred overnight at 90 mA (BioRad semidy blotting apparatus, USA) to a prehydrated nitrocellulose membranes (VWR Protran BA83 300 mm, Schleicher & Schuell) in a transfer buffer (25 mM Tris-base, 0.192 M glycine, 20% methanol; pH 8.3). Transfer conditions were optimized to ensure complete transfer of the protein in the 70 kDa region of the gel.

Hsp70 were detected and quantified by Western blot analysis. Dry membrane was blocked with 3% non-fat dry milk in Tris-buffered saline (TBS; 150 mM NaCl, 20 mM Tris base, pH 7.4, and 0.05% Tween 20) for 1.15 h, subsequently washed four times for 5 min, then transferred for 10 min in TBS. The membrane were then incubated for 1 h with the primary antibody [Hsp70 (K-20): sc-1060 goat polyclonal antibody made in rabbit; Santa Cruz] diluted 1:500 in a solution containing 1% non-fat dry milk in TBS. After incubation with the primary antibody, the membrane was washed four times for 5 min and one time for 10 min in TBS. The membrane was then incubated for 1 h with an anti-goat IgG-HRP (Pierce) secondary antibody, made in donkey and diluted 1:5000 in a solution containing 1% non-fat dry milk in TBS. Finally the membrane was washed four times for 5 min, and one time for 10 min in TBS.

The Western blot was developed using the enhanced chemiluminescence (ECL) detection system (Amersham) according to the instruction of manufacturer. The blot was exposed to Hyperfilm ECL (Amersham) for 5-30 s. The developed films were then densitometrically scanned using a digitising software program (Scion Image). The density of sample bands was standardized by dividing the sample band with the density of a purified Hsp sample (0.5 µg; Hsp70: SPP-758, Stressgen) run on every gel.

Data of the level of Hsp70 expression were analysed with one-way ANOVA after logarithmic transformation and compared with the Student-Newman-Keuls (SNK) test, or with the Kruskal-Wallis test. Statistical analyses were performed using the software program SPSS Version 13.0.

3. RESULTS

The relative levels of Hsp70 in larvae of P. branickii, D. insignipes, O. (E.) thienemanni, P. nivalis and O. (O.) frigidus collected in spring 2006 are shown in figure 1. High significant differences (P <0.001; F(4, 33) = 7.91) in the relative level of Hsp70 among these species were detected. The SNK test showed that the level of Hsp70 of P. branickii differed significantly from those of all other species, whereas the level of Hsp70 of D. insignipes differed from all species except P. nivalis. No differences were detected between O. (E.) thienemanni, O. (O.) frigidus and P. nivalis. P. branickii showed the lowest relative level of Hsp70 and D. insignipes the highest levels (Fig. 1).

The relative levels of Hsp70 in the species of the genus Diamesa collected in spring 2006 (D. insignipes, D. laticauda, D. vaillanti) and in summer 2005 (D. latitarsis, D. cinerella, D. zernyi) are shown respectively in figures 2 and 3. Higher relative levels of proteins were recorded in species collected in summer at 2600 m a.s.l. than in species collected in spring at 1300 m a.s.L, although the differences were not statistically significant (Kruskal-Wallis test: P >0.05).
Fig. 2. Representative Western blot and relative levels of Hsp70 among Diamesinae species collected in spring 2006. Di = Diamesa insignipes, Dl = Diamesa laticauda, Dv = Diamesa vaillanti. The bar shows the mean of 4 replicates ± S.E., except for D. insignipes where the number of replicates was 3.

Fig. 3. Representative Western blot and relative levels of Hsp70 among Diamesinae species collected in summer 2005. Dc = Diamesa cinerella, Dl = Diamesa laticauda, Dz = Diamesa zernyi. The bar shows the mean of 4 replicates ± S.E.

4. DISCUSSION

This investigation represents the first study in which Hsp70 family proteins were detected under natural conditions in cold-stenothermal chironomids, stressing how these proteins confer resistance against cold. Furthermore, the SDS-PAGE and Western blotting methodologies were adapted and applied for the first time to Diamesinae and Orthocladiinae. Existing knowledge of Hsps expression in midges, based on a similar methodology, was restricted to the eurythermal Chironominae Chironomus thummi Kieffer (Morcillo et al. 1982, 1997; Carretero et al. 1986, 1991) and Chironomus tentans Fabricius (Karouna-Reiner & Zehr 1999, 2003), and only for exposure to stressors such as heat or heavy metals. However, the expression of hsp genes after exposure to different temperatures was recently analysed in another orthocladi, B. antarctica, although a different methodology was employed (Rinehart et al. 2006).

Considering the importance of Hsps in biochemical systems, their detection in cold-stenothermal midges that experience very cold water stress was not unexpected, and extends the significance of Heat shock proteins as adaptive strategy against stressors. Cold temperatures can cause protein denaturation and some organisms express Hsps as response to cold stress (Feder & Hofmann 1999). Larvae of the Antarctic midge, B. antarctica, have adopted the strategy of expressing hsp genes continuously, possibly to facilitate protein folding in a habitat more thermally stable than that of the adults, but subject to frequent freeze-thaw episodes or other stresses (Rinehart et al. 2006). A similar pattern of adaptation is shared with another Antarctic species, the notothenioid fish Trematomus bernacchii Boulenge that inhabits waters at cold and constant temperatures (Carpenter & Hofmann 2000) and with several cold-adapted insects from temperate zones (Joplin et al. 1990; Yocum et al. 1991; Denlinger et al. 1992; Lee et al. 1995). Induction was observed in some animals that live at temperatures 1-3 °C higher than the temperature at which the organisms live (Feder & Hofmann 1999). Due to the commercial antibody we used, cross-reacting with several Hsp70 family members, the Hsp70 levels we found may be a reflection of inducible, constitutive or both forms of heat shock proteins (Hsp/Hsc). Notwithstanding, in terms of total amount of heat shock proteins, intra- and inter-species comparisons preserve their validity as suggested by other authors using the same type of antibody (Airaksinen et al. 2003; Chapovetsky & Katz 2006).

Some differences in the level of Hsp70 were detected among taxa investigated. These differences may be explained on the basis of differences in autoecology. The highest protein levels were detected in P. nivalis and D. insignipes, the lowest in P. branickii. All these taxa are known to colonize cold streams but the latter is commonly found in thermally constant water conditions typically found in springs (Lencioni & Rossaro 2005). In other organisms living in quite stable temperature conditions, lower levels of Heat shock proteins have been recorded (Feder & Hofmann 1999; Carpenter & Hofmann 2000). In addition, the higher levels of Hsps measured in P. nivalis and in D. insignipes compared to P. branickii, may be the result of small fluctuations in temperature that occur in their microhabitats, as observed in other aquatic organisms (Fader et al. 1994; Feder & Hofmann 1999). This is consistent with the general rule that in poikilotherm organisms a positive correlation exists between the content of Hsp70 protein in animals under normal non heat-shock conditions and the average temperature of the habitat of that animal (Ulmasov et al. 1992).
As indicated in the results, Diamesa species collected in summer at higher altitude have slightly higher values of protein than in Diamesa species collected in spring at lower altitude. The species living at 2600 m a.s.l. face more stressful conditions due to the shortness of biological window and the higher risk of freezing than species living at 1300 m a.s.l., and therefore they maintain higher levels of Hsps. This could justify the pattern observed.

Furthermore, the levels of Hsp70 detected among the Diamesa species at each site, could reflect different levels of cold-stenothermy being D. insignipes, D. cinerella, D. vaillanti and D. zernyi less cold-steno-thermal than D. laticauda and D. lattitarsis (Ferrarese & Rossaro 1981; Lencioni & Rossaro 2005; Rossaro et al. 2006). These results suggested that under natural conditions the most cold-stenothermal species have lower levels of Hsp70 than the least cold-stenothermal ones.

5. CONCLUSIONS

This study clearly indicate that midges from cold Alpine streams employ the Hsp70 protein family (including constitutive and/or induced proteins) in their physiological adaptation to cold waters of their natural habitat. This information provides new insights in cold-stenothermal adaptation in midges inhabiting Alpine streams which are becoming more and more affected by glacier retreat. The hydrological and thermal regime of alpine streams is likely to change under the global warming scenario at high latitude and altitude, and extremely specialized fauna (such as Diamesa species) are predicted to become extinct as glaciers decline and finally disappear (Rossaro et al. 2006; Brown et al. 2007). Thus, it is important to know the role of metabolites such as Hsps in developing tolerance to the forecasted temperature increases in Alpine fauna. For this purpose, laboratory experiments on cold-stenothermal midges exposed to high temperatures will provide a further approach to study the relationship between temperature and Hsps expression. The knowledge as to how insects will potentially react and adapt in face of global warming is one of the major challenge in prediction of future biodiversity trends.

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