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chironomids (Diptera, Chironomidae) under natural conditions

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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*,* O. *(*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; Maiolini & 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 & Hofmann 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 & Hofmann 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.

Species	No. larvae	Sampling site (m s.l.m.)	Sampling date	Temperature (°C)	O_2 saturation (%)
Pseudodiamesa branickii	43	1300	31-Mar-2006	4.2 ± 0.8; 4.9	80 ± 5; 79
Diamesa latitarsis	15	2600	11-July-2005	3.9 ± 1.0; 4.0	85 ± 3; 81
Diamesa laticauda	21	1300	31-Mar-2006	4.2 ± 0.8; 4.9	91 ± 5; 90
Diamesa cinerella	23	2600	11-July-2005	3.9 ± 1.0; 4.0	85 ± 3; 81
Diamesa insignipes	84	1300	31-Mar-2006	4.2 ± 0.8; 4.9	91 ± 5; 90
Diamesa zernyi	21	2600	11-July-2005	3.9 ± 1.0; 4.0	85 ± 3; 81
Diamesa vaillanti	23	1300	31-Mar-2006	4.2 ± 0.8; 4.9	91 ± 5; 90
Orthocladius (O.) frigidus	57	1300	31-Mar-2006	4.2 ± 0.8; 4.9	91 ± 5; 90
Orthocladius (E.) thienemanni	22	1300	31-Mar-2006	4.2 ± 0.8; 4.9	91 ± 5; 90
Paratrichocladius nivalis	76	1300	31-Mar-2006	4.2 ± 0.8; 4.9	91 ± 5; 90

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; Deegenaars & 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 *Belgica 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 in larvae of cold stenothermal midges (Diamesinae and Orthocladiinae) inhabiting cold streams.

2. MATERIAL AND METHODS

2.1. Animal model and animal collection

Fourth instar larvae of ten chironomid taxa were studied, seven Diamesinae: *Pseudodiamesa branickii* (Nowicki), *Diamesa latitarsis* (Goetghebuer), *Diamesa laticauda* Serra-Tosio, *Diamesa cinerella* Meigen, *Diamesa insignipes* Kieffer, *Diamesa zernyi* Edwards, *Diamesa vaillanti* Serra-Tosio, and three Orthocladiinae: *Orthocladius* (*Orthocladius*) *frigidus* (Zetterstedt), *Orthocladius* (*Euorthocladius*) *thienemanni* Kieffer, *Paratrichocladius nivalis* (Goetghebuer).

Larvae were collected in summer 2005 and spring 2006 in the glacial stream, Noce Bianco (Italian Alps, Stelvio National Park, Trentino, NE Italy, 46°24' N, 10°40' E) (Tab. 1). In spring, larvae belonging to all genera were collected at 1300 m a.s.l. in a stream reach extensively colonized by the chrysophyte, *Hydrurus foetidus* (Villars) Trevison, and characterized by transparent and well oxygenated waters (Tab. 1). *P. branickii* was collected in a lentic depositional area on the left bank, with 70-80% oxygen saturation and a channel free of Crysophyta. In summer, *Diamesa* larvae, almost absent at 1300 m a.s.l., were collected at 2600 m a.s.l., in turbid (turbidity >30 NTU), well oxygenated and cold waters (Tab. 1).

Larvae were collected using a 30×30 cm pond net (100 µm mesh size) and transported alive to the laboratory in cooler bags. Sorting and identification were completed within 24 hours after sampling under the stereomicroscope (50×), according to Ferrarese & Rossaro (1981), Rossaro (1982) and Lencioni *et al*. (2007). Before biochemical analysis, larvae were maintained at 4 °C (= mean water temperature at the sampling site, see table 1) in a thermostatic chamber with an aerator.

Some larvae of each taxon were mounted on permanent slides for species confirmation under the microscope at higher magnification (400-1000×) according to Lencioni *et al.* (2007). In addition, all *Diamesa* pupae and pharate adults collected in the field or obtained from reared larvae were identified to species level according to Langton & Visser (2003) and Serra-Tosio (1971) to confirm the larvae identification. All pupae and pharate adults collected at each sampling site in each season were always ascribed to one *Diamesa* species and this enabled us to treat the collected larvae of each sampling as a single taxonomic entity.

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 semidry 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).

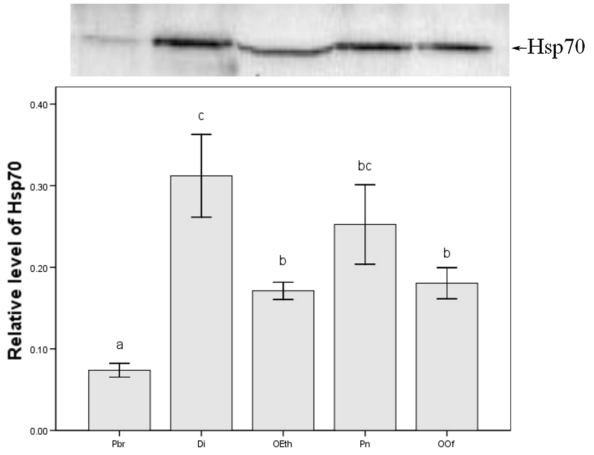

Fig. 1. Representative Western blot and relative levels of Hsp70 in Diamesinae and Orthocladiinae species collected in spring 2006. The different letters above the columns indicate different values at P <0.05 (SNK test). Pbr = *Psudodiamesa branickii*, Di = *Diamesa insignipes*, OEth = *Orthocladius* (*Euorthocladius*) *thienemanni*, Pn = *Paratrichocladius nivalis*, OOfr = *Orthocladius* (*Orthocladius*) *frigidus*. The bar shows the mean of 8 replicates ± S.E., except for *O. (E.) thienemanni* where the number of replicates was 6.

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).

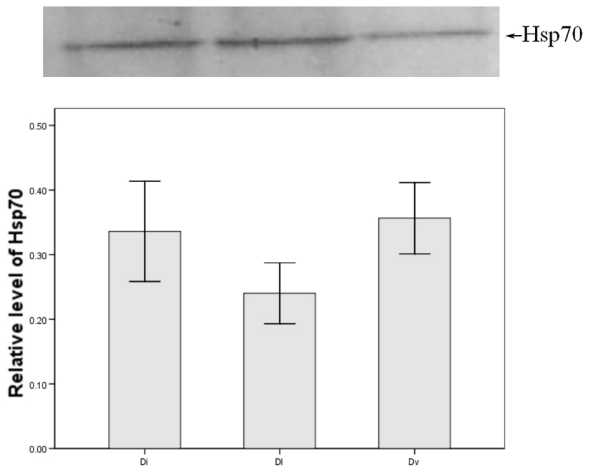

Fig. 2. Representative Western blot and relative levels of Hsp70 among Diamesinae species collected in spring 2006. Di = *Diamesa insignipes*, Dl = *Diamesa laticauda*, Dv = *Diamesa vaillanti*. The bar shows the mean of 4 replicates ± S.E., except for *D. insignipes* where the number of replicates was 3.

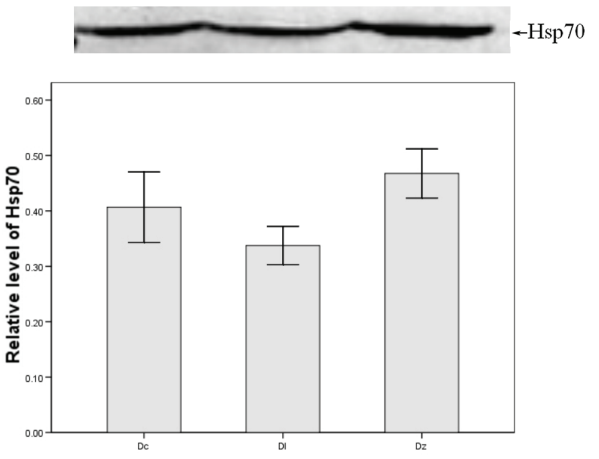

Fig. 3. Representative Western blot and relative levels of Hsp70 among Diamesinae species collected in summer 2005. Dc = *Diamesa cinerella*, Dl = *Diamesa latitarsis*, Dz = *Diamesa zernyi*. The bar shows the mean of 4 replicates ± S.E.

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 orthoclad, *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* Boulenger 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 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 poikoloterm 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-stenothermal than *D. laticauda* and *D. latitarsis* (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 glacial 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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