Article

THERMAL STRESS INDUCES HSP70 PROTEINS SYNTHESIS IN LARVAE OF THE COLD STREAM NON-BITING MIDGE *Diamesa cinerella* Meigen

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Laboratory experiments on the cold stenothermal midge Diamesa cinerella (Diptera, Chironomidae) were performed to study the relationship between increasing temperature and heat shock proteins (HSP70) expression at translational level (Western blotting). Thermotolerance of IV instar larvae collected in nature at 1.5–4.3°C during seasons was analyzed through short-term (1 h at ten different temperatures from 26°C to 35°C) and long-term (1–14 h at 26°C and 1–4 h at 32°C) heat shocks. A high thermotolerance was detected (LT_{50} = 30.9–32.8°C and LT_{100} = 34.0–37.8°C). However, survival decreased consistently with increasing exposure time, especially at higher temperature ($LTime_{50}$ = 7.64 h at 26°C and $LTime_{50}$ = 1.73 h at 32°C). The relationship between such heat resistance and HSP70 expression appeared evident because a relationship between HSP70 level and larval survival rate was generally found. A heat shock response (HSR) was consistent only in the summer larvae. The absence of HSR in the other populations coupled with even higher amounts of HSP70 than in summer, led us to hypothesize that other macromolecules and other adaptive mechanisms, apart from biochemical ones, are involved in the response of D. cinerella larvae to high temperature. Altogether these

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results stressed how in this midge the HSP70 protein family confers resistance against cold, being detected under natural conditions in control larvae collected in all seasons, but also against warm under experimental heat shocks. These results give new insights into possible responses to climate changes in freshwater insects within the context of global warming.

Keywords: thermotolerance; heat shock; freshwater insects; chironomids; Alps

INTRODUCTION

The resistance to heat exposure is generally associated with the synthesis of stress proteins, namely heat shock proteins (HSPs). The HSPs act as chaperones that function in all types of cell to ensure the proper folding and compartmentation of proteins (Feige et al., 1996), to prevent the aggregation of heat-damaged proteins and to facilitate their renaturation following a heat shock (Parsell and Lindquist, 1993, 1994). The synthesis of new HSPs is dependent not only on the amount of damaged proteins but also on the amount of existing HSPs in the cells. Furthermore, the HSP 70 family was recognized as a molecular biomarker that determines when a species comes under threat and the extent of such threat (Clark and Peck, 2009). Numerous families of HSPs have been identified, the naming of which is related to their molecular weight. Among these, the 70 kDa family is the most studied in relation to thermal stress and it has been found in all organisms investigated (Parsell and Lindquist, 1993; Morimoto et al., 1994; Feder and Hofmann, 1999). This family is traditionally divided into two groups based on their gene expression patterns. The former group includes those with low or null expression under nonstress conditions, but which can be quickly induced under stress conditions (HSP70); the latter one is made up by constitutive proteins expressed under nonstress conditions (HSC70; Qin et al., 2003).

The expression of *hsp70* gene in response to high temperature is considered the main molecular pathway at the base of the heat shock response (HSR; Morimoto et al., 1997). Nevertheless, how the timing and duration of thermal stress under natural conditions alters HSR remains a complicated issue (Buckley et al., 2001).

In most organisms, the genes encoding HSPs are expressed primarily in response to stress but not in normal conditions, probably because *hsp* expression at normal temperature may be deleterious during development (Sørensen et al., 2003). Conversely, animals from extreme habitats, such as deserts and polar regions, express constitutive *hsp* genes always and show no or modest upregulation of these genes in response to thermal stress (Rinehart et al., 2007). For example, in Antarctic fishes the constitutive expression of *hsp* genes is thought to be an adaptation to the constant cold low temperature of the polar sea (Place and Hoffmann, 2005). Anyway, *hsp70* gene expression is highly plastic and levels of induction are influenced by thermal history such as seasonal temperature cycling, vertical zonation, and biogeography (Somero, 2002).

The knowledge as to how insects will potentially react and adapt in face of global warming is one of the major challenges in prediction of future biodiversity trends. 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 is predicted to become extinct as glaciers decline and finally disappear (Rossaro et al., 2006;

Table 1. General Abiotic Features of Sampling Stations at Noce Bianco Stream

	Summer	*Autumn*	*Winter*	*Spring*
Sampling date	11.VII.2008	14.XI.2008	9.I.2009	31.III.2009
Altitude (m a.s.l.)	2,605	1,260	1,260	1,260
Water temperature (°C)	2.0	3.0	1.5	4.3
Percent saturation O_2	85	89	90	85
pH	7.1	6.9	6.9	7.1
Turbidity (NTU)	80	20	11	49

Brown et al., 2007). Thus, it is important to know the role of proteins such as HSPs in developing tolerance to the forecasted temperature increases in alpine fauna.

Chironomids are the most widely distributed insect family in freshwaters, with about 3,700 species widespread throughout all the zoogeographic regions (Cranston, 1995). They possess adaptations to a variety of environmental rigors such as desiccation, anoxia, high or extremely low temperatures and freezing (Danks, 1971; Lencioni, 2004). Among these midges, Diamesinae is the best adapted subfamily to colonize cold waters, with the genus *Diamesa* Meigen, 1835 accounting for up to 100% of the fauna in glacial streams (Füreder, 1999; Lods-Crozet et al., 2001; Lencioni and Rossaro, 2005). As the cold sthenotermal midges belonging to *Diamesa* are potentially threatened by global warming in alpine area, they are appropriate animals to study the adaptive strategies evolved to survive temperature increasing within the scenario of climate change and glacier retreating (Lencioni et al., 2008, 2009). Previous studies highlighted that midges from cold Alpine streams employ the 70 kDa heat shock family in their physiological adaptation to cold waters of their natural habitat (Lencioni et al., 2009) and as a response to heat stress exposure (Bernabò et al., 2011). In this work, laboratory experiments on the cold stenothermal midge *Diamesa cinerella* Meigen, 1835, exposed to high temperatures, were performed to study the relationship between temperature and HSP70 expression at translational level in order to increase the knowledge about the role of HSPs in developing heat stress tolerance and to predict the fate of cold stenothermal species under global warming.

MATERIALS AND METHODS

Animals and Sampling

Fourth-instar larvae of *D. cinerella* were used as an animal model. *Diamesa cinerella* is a paleartic holometabolous species, well distributed in the mountain regions of central-southern Europe. In Italy, it is abundant in the Alps and Apennines (Rossaro et al., 2006). At altitude lower than 2,000 m a.s.l., this species is typically abundant in autumn/winter, with a consistent emergence in spring, whereas at highest altitudes it has only one summer generation (Ferrarese and Rossaro, 1981). The larvae are detritivorous and live in the sediment, in the moss/algae mats or on submerged rocks in cold running waters fed by glaciers or snow packs, with average annual temperature lower than 12°C (Lencioni et al., 2008).

Animal collection was carried out in 2008–2009 on four occasions (one in each season) at two different altitudes (Table 1) in the glacier-fed stream Noce Bianco (Val de la Mare, Trentino Province, NE Italy, 46°N, 10°E). During the sampling period, the Noce Bianco

stream exhibited low temperatures (≤4.3°C), high percent of oxygen saturation (≥ 85%), turbidity varying between 11 and 80 NTU, and pH around neutrality (Table 1). Larvae of *D. cinerella* were found abundant in mats of the chrysophyte *Hydrurus foetidus* (Villars) Trevison, collected with a 30 × 30 cm pond net (100 μm mesh size). Fourth-instar larvae were sorted on the field with tweezers, transferred to plastic bottles filled with stream water and transported to the laboratory in a cooling bag. Animals were maintained in 500-ml glass beaker (max 50 specimens/beaker) with filtrated (on Whatman GF/C, particle retention 1.2 μm) stream water in a thermostatic chamber (ISCO, mod. FTD250-plus) at 4°C with aerator to maintain %oxygen saturation higher than 80%. The stabulation temperature (4°C) corresponds to the mean temperature of the water of the two stream reaches during the period when the species is present (Boscaini et al., 2004).

Heat Shock Exposure

Larvae of *D. cinerella* were exposed to two different heat shocks, a short-term and long-term shock. Both kinds of experiments were carried out in a thermostatic chamber.

The survival to short-term heat shock was analyzed in *D. cinerella* larvae collected in each season. Short-term shocks of the 1-h duration were performed to evaluate the thermotolerance of *D. cinerella* larvae as lethal temperatures 50% (LT_{50}) and 100% (LT_{100}). Preliminary experiments evidenced that the larval survival was 100% when animals were exposed to increasing heat shock temperatures from 4 to 26°C. Consequently, for this study ten different temperatures were tested, from 26°C (the highest temperature at which, after 1-h exposure, all tested larvae were found alive) to 35°C (the lowest experimental temperature at which, after 1-h exposure, all tested larvae were found dead).

The survival to long-term heat shock was analyzed only on larvae collected in spring. On the basis of the results of the short-term experiments (see Fig. 1), two temperatures (26 and 32°C) were selected to perform long-term heat shock. In order to calculate the time (h) at which the 50% ($LTime_{50}$) and 100% ($LTime_{100}$) of the tested larvae were killed, different durations of exposure to heat stress were tested. In particular, the larvae were exposed to 1, 2, 4, 8, and 14 h at 26°C, and to 1, 2, and 4 h at 32°C.

For each combination of temperature and time exposition, groups (replicates) of larvae were transferred to 25-ml plastic bottles (Kartell, Italy) filled with 10 ml of preheated filtered stream water, under aeration to avoid the mortality due to the oxygen depletion. Three to five replicates of three to five larvae were employed at each tested temperature of the short-term heat shock, while three replicates each of five larvae at each time of exposure were used during long-term heat shocks. In both short- and long-term heat shocks, three replicates of five specimens were maintained at 4°C for the entire period of the treatment as a control. The mortality in the control was used as a correction factor (Abbott, 1925).

After the treatments, the larvae were immediately placed at 4°C (stabulation temperature) and were examined 1 h later to check their survival. The larvae moving spontaneously or moving following a tactile stimulus (suffering larvae) were considered alive. The immobile larvae were considered dead.

Protein Extraction, Western Blotting, and Quantification of HSP70

Immediately after the survival check, alive larvae were kept at –80°C. For the HSP assay, the protocol optimized for midges and described in Lencioni et al. (2009) was used. For each experimental condition and for each control, three to four groups (replicates) of

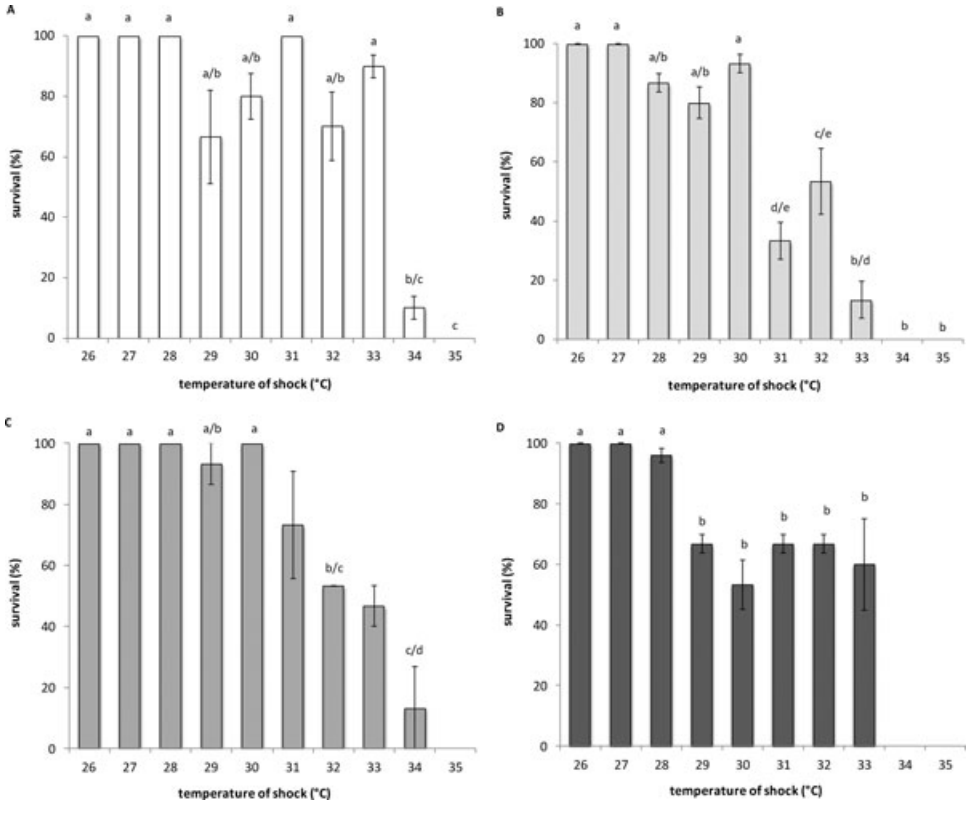

Figure 1. Thermotolerance of *Diamesa cinerella* larvae. Percentages of survived larvae after a short-term exposition (1 h) from 26°C up to 35°C in (A) summer, (B) autumn, (C) winter, and (D) spring. Each bar shows the mean value ± SE. The different letters above the columns indicate significant differences in larval survival exposed to different heat shock temperatures at $P < 0.05$ (LSD Tukey's post-hoc test).

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 15,000 × *g* for 25 min at room temperature. Protein content of the 15,000 × *g* supernatant was determined using the DC Protein Assay kit (Bio-Rad, Hercules, CA).

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 proteins (10 µg) for each heat shock temperature were loaded and separated on a 12% SDS-polyacrylamide gel with 4% stacking gel using the buffer system described in Læmmli (1970). Prestained SDS molecular weight markers (Sigma Aldrich, St. Louis, MO) were run on each gel to indicate molecular weight. Proteins were electrophoretically transferred overnight at 90 mA (BioRad semidry blotting apparatus, Biorad, Hercules, CA) to prehydrated nitrocellulose membranes (Protran BA83, Whatman, Kent, UK) 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. The dry membrane was blocked with 3% nonfat 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, and then kept for 10 min in TBS. The membrane was then incubated for 1 h with the primary antibody [Hsp70 (K-20): sc-1060 goat polyclonal antibody made in rabbit; Santa Cruz, Dallas, TX] diluted 1:500 in a solution containing 1% nonfat dry milk in TBS. To ascertain the relative levels of proteins in the different samples, anti-actin antibody (Sigma Aldrich, St. Louis, MO) was used at a dilution of 1:200. 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 IgGHRP (Pierce antibody, Euroclone, Pero, Milan, Italy) secondary antibody, made in donkey and diluted 1:5,000 in a solution containing 1% nonfat 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 (GE Healthcare, Waukesha, WI) according to the manufacturer's instruction. The blot was exposed to Hyperfilm ECL (GE Healthcare, Waukesha, WI) for 5–30 s. The developed films were then densitometrically scanned using a digitizing software program (Scion Image). The density of each sample band was normalized to each relative actin band.

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 HSPs (HSP/HSC). Notwithstanding, in terms of the total amount of HSPs, intraspecies comparisons preserve their validity as suggested by other authors using the same type of antibody (Airaksinen et al., 2003; Chapovetsky and Katz, 2006).

Statistical Analysis

According to Bouchard et al. (2006), one- or two-way analyses of variance (ANOVAs) on the arcsine-square-root-transformed proportion of survival were performed to test for significant differences in larval survival among temperature treatments for each season. When significant differences were found ($P < 0.05$), LSD Fisher's post-hoc test was used to separate the means. Values with $P < 0.05$ were considered significant. Probit analysis (BioStat© 2007) was used to estimate the temperature and time (95% fiducial limits) at which 50% (LT_{50} and $LTime_{50}$) and 100% (LT_{100} and $LTime_{100}$) of mortality occurred. Data of the level of Hsp70 expression were analyzed with one-way ANOVA on the arcsine-square-root-transformed. Statistical analyses were performed using a software program (Statistica 8.0, StatSoft Inc.).

RESULTS

Short-Term Heat Shock Survival

The survival of *D. cinerella* larvae after short-term heat shock exposure is reported in Figure 1, while LT_{50} and LT_{100} are indicated in Table 2. In all seasons, all larvae of *D. cinerella* were found alive after 1-h exposure at 26 and 27°C: their survival decreased at progressively higher temperatures and in all seasons was zero at 35°C (Fig. 1). In particular, in the summer larvae (Fig. 1A), the percentage of survival was elevated (>60%) until 33°C ($LT_{50} = 32.8 \pm 0.6$°C); then it decreased until 10% at 34°C, and 0% at 35°C. In the autumn larvae (Fig. 1B), the percentage of survival decreased under 50% at 31°C ($LT_{50} = 30.9 \pm 0.4$°C) and reached 0% at 34°C. The survival rate of the winter larvae

Table 2. LT_{50} (Mean ± Standard Error) and LT_{100} Calculated with Probit Analysis (IC 95%)

	LT_{50} (°C)	LT_{100} (°C)
Summer	32.8 ± 0.6	37.8
Autumn	30.9 ± 0.4	34.0
Winter	32.5 ± 0.5	35.9
Spring	31.9 ± 0.5	36.6

progressively decreased from 31 to 35°C, with an LT_{50} value of 32.5 ± 0.5°C. Finally, the survival of spring larvae was elevated (>50%) until 33°C (LT_{50} = 31.9 ± 0.5°C), and then progressively decreased until 0% at 34 and 35°C. The survival percentage of the control larvae maintained at 4°C was always of 100%. In all seasons, the decrease in survival was accompanied by a progressive increase of the percentage of suffering larvae. At the sublethal temperatures of 33–34°C (depending on the season), all the survived larvae were suffering.

The statistical analysis highlighted significant differences among the larval survivals recorded at the different temperatures of shock in each season (Fig. 1; summer: $df = 9$, $F = 2.87$, $P = 0.05$; autumn: $df = 9$, $F = 14.28$, $P < 0.001$; winter: $df = 9$, $F = 19.23$, $P < 0.001$; spring: $df = 9$, $F = 19.53$, $P < 0.001$). The first significant decrease in survival was observed from 33°C (90 ± 4%) to 34°C (10 ± 4%) in summer ($P = 0.04$), from 30°C (93 ± 3%) to 31°C (33 ± 6%) in autumn ($P = 0.002$), from 31°C (73 ± 17%) to 32°C (53.%) in winter ($P = 0.03$), and from 28°C (96 ± 3%) to 29°C (66 ± 3%) in spring ($P = 0.005$).

Significant differences (test post-hoc LSD Fisher) were also found among seasons for some heat shock temperatures: at 30°C between spring and autumn ($P = 0.02$) and between spring and winter ($P = 0.003$); at 31°C between summer and autumn ($P = 0.03$), winter and autumn ($P = 0.04$), summer and spring ($P = 0.03$); at 33°C between summer and autumn ($P = 0.0002$), winter and summer ($P = 0.03$), and finally between spring and autumn ($P = 0.005$).

Seasonal variations were observed in the LT_{50} and LT_{100} values (Table 2) even if not significantly. The highest values of LT_{50} were recorded in summer (32.8°C) and in winter (32.5°C), when the stream water temperature was lower (≤2°C).

Long-Term Heat Shock Survival

The survival of the *D. cinerella* larvae collected in spring and exposed to long-term shock at 26°C (1–14 h) and 32°C (1–4 h) decreased with increasing duration of exposure, faster at 32°C (Fig. 2A–B).

At 26°C, the survival of larvae was 100% until 4-h exposure, about 30% after 8 h, and 0% after 14 h (Fig. 2A). Ten percent of alive larvae were suffering after 4 and 8 h of heat shock treatment.

The $LTime_{50}$ was 7.64 ± 1.37 h and the $LTime_{100}$ was 12.22 h. The statistical analysis revealed significant differences among larval survival recorded at the different times of exposure ($df = 6$; $F = 93.1$; $P < 0.001$). The significant ($P = 0.001$) decrease in survival occurred between 4 h (100%) and 8 h (30 ± 25%) of exposure.

At 32°C, the larval survival was already under 50% after 2-h exposure even though the half part of the larvae was suffering, whereas after 4 h of heat shock all the tested larvae were dead (Fig. 2B). The $LTime_{50}$ was 1.73 ± 0.39 h and the $LTime_{100}$ was 3.33 h. The statistical analysis revealed significant differences ($df = 3$; $F = 13.1$; $P < 0.001$) between

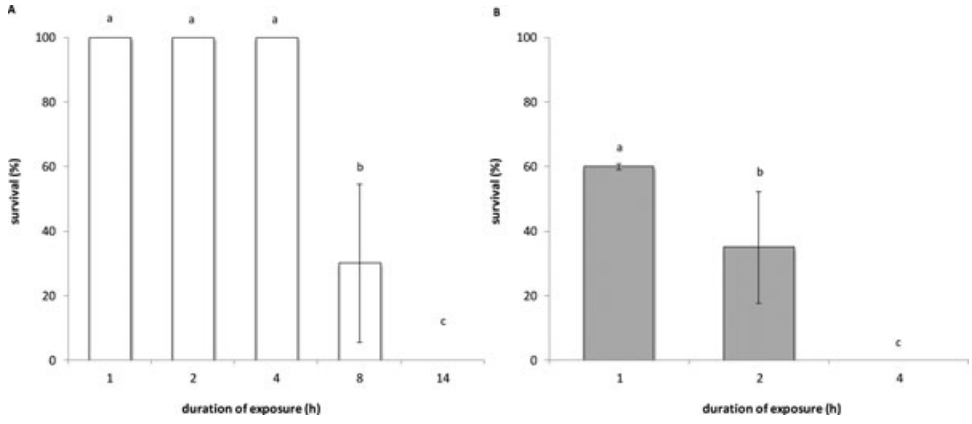

Figure 2. Thermotolerance of *Diamesa cinerella* larvae collected in spring. Percentage of survived larvae after a long-term exposition at 26°C (A) and at 32°C (B). Each bar shows the mean value ± SE. The different letters indicate significant differences in larval survival exposed to different time of heat shock at $P < 0.05$ (LSD Tuckey post-hoc test).

the larval survival recorded at the different times of exposure. A significant decrease ($P = 0.002$) in survival (from 60% ± 1 to 35 ± 16%) was present between 1 and 2 h of exposition.

Heat Shock and HSP70 Expression

Figure 3 shows the actin normalized level of HSP70 in response to short-term heat shock (1 h) in larvae of *D. cinerella* collected during the four seasons. In general, higher levels of HSP70 were observed in the heat-shocked larvae than in the control in all seasons. However, statistically significant differences between control and each stress temperature were recorded only for the larvae collected in summer ($df = 9$; $F = 4.58$; $P = 0.002$). In particular, a significant increase ($P < 0.05$; test post-hoc LSD Fisher)) with respect to control larvae maintained at 4°C was detected at 26, 27, 28, 32, and 34°C at which the level of HSP70 was about twice that detected in the control.

Overall, the higher levels of HSP70 both in the control larvae and consequently in larvae after the heat stress were found in the winter animals than in summer ($P = 0.04$) and in autumn ($P = 0.03$) animals. The lowest levels of HSP70 were found in the autumn ($P = 0.04$).

Figure 4 shows the actin normalized level of HSP70 in response to long-term heat shock at 26°C (1–8 h) and 32°C (1–2 h) in the larvae of *D. cinerella* collected in spring. At 26°C, the levels of HSP70 increased with increasing of the exposure time ($df = 4$; $F = 7.32$; $P = 0.03$); a significant increase ($P = 0.003$ test post-hoc LSD Fisher) was detected between control and 8-h heat shock treatment. No significant differences were found at 32°C.

DISCUSSION

Our experiments highlighted that larvae of *D. cinerella* have a high thermotolerance because they survived short-term heat shocks at substantially higher temperatures than

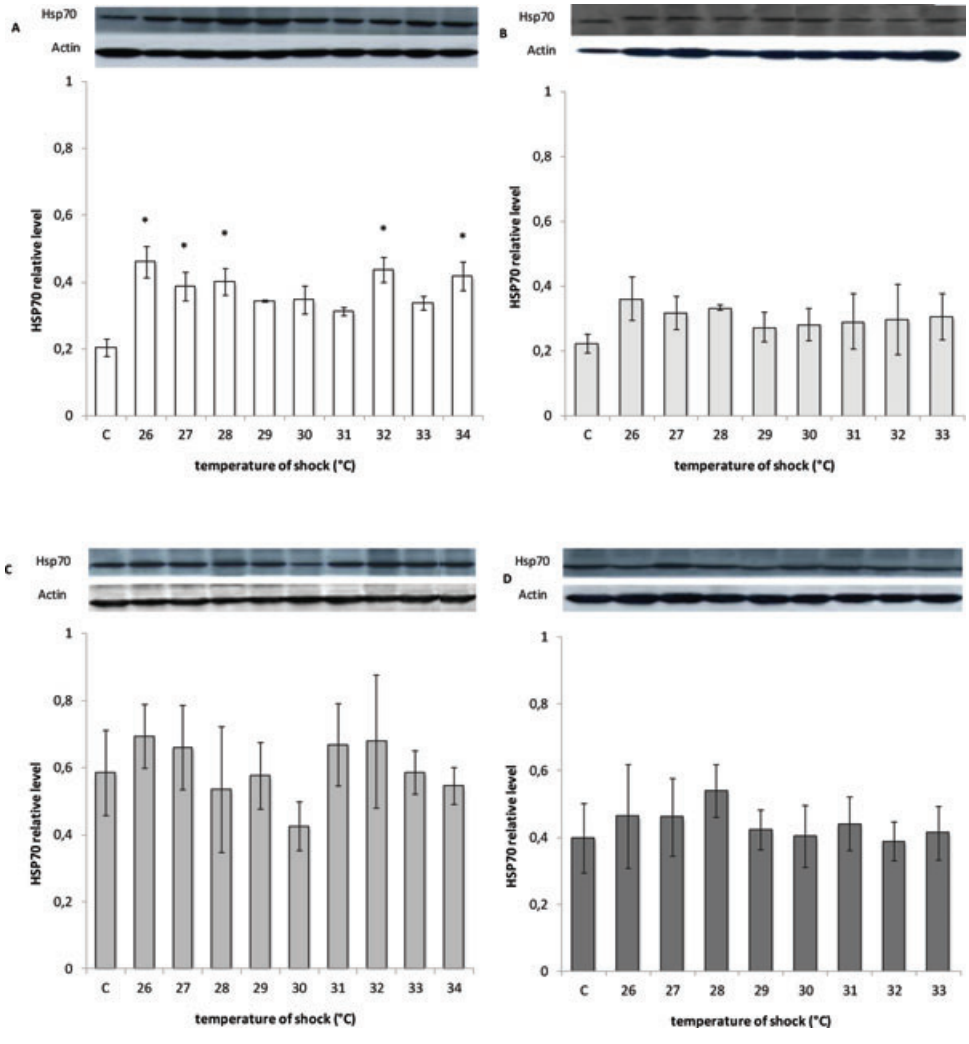

Figure 3. Representative Western blots and relative levels of HSP70 in *Diamesa cinerella* larvae exposed to short-term heat shock collected in (A) summer, (B) autumn, (C) winter, and (D) spring. Each bar shows the mean value ± SE. C = control. $^{*}P < 0.05$ (LSD Tuckey post-hoc test).

those they normally face in nature (LT_{50}: from 30.9 to 32.8°C and LT_{100}: from 34.0 to 37.8°C), similar to what was observed for other cold stenothermal Diptera such as the Stratiomyidae *Oxycera pardalina* Meigen, 1822 (Garbuz et al., 2008), the Chironomidae *Belgica antarctica* Jacobs, 1900 (Rinehart et al., 2006), and *Pseudodiamesa branickii* Nowicki, 1873 (Bernabò et al., 2011). However, the midge survival decreased consistently with increasing exposure time, and this was more evident at higher temperatures ($LTime_{50}$ = 7.64 ± 1.37 h at 26°C and $LTime_{50}$ = 1.73 ± 0.39 h at 32°C). We found evidence that such thermotolerance is associable to HSP70 (including constitutive and/or induced proteins), provided their level is higher at the lower stress temperature (26°C) than in the control. Furthermore, it seems that when the stress is particularly high, evaluable as increasing in mortality, larvae react synthesizing more HSP70 as occurs at 32 and 34°C

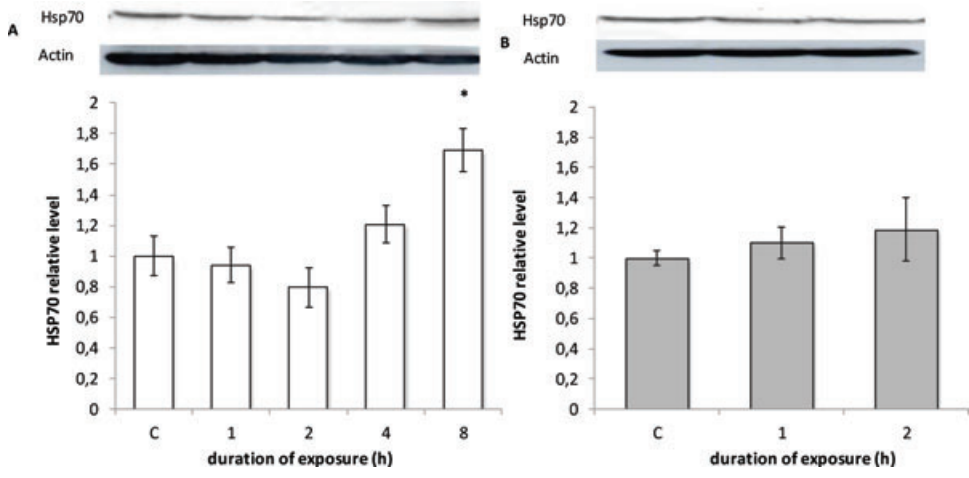

Figure 4. Representative Western blots and relative levels of HSP70 in *Diamesa cinerella* larvae collected in spring and exposed to long-term heat shock at 26°C (A) and 32°C (B). The bar shows mean value ± SE. C = control. $^{*}P < 0.05$ (test post-hoc LSD Fisher).

in larvae collected in summer (Figs. 1A and 3A). However, over a certain temperature (34–35°C) or for prolonged stress, it was evident that HSPs are not sufficient to protect *D. cinerella* larvae against the increasing temperature at which all of them were found dead.

HSP70 were detected already in the control larvae of *D. cinerella* collected in all seasons. This clearly indicates that such midges employ the HSP70 protein family in their physiological adaptation to cold waters of their natural habitat. This was also detected in other alpine cold stenothermal midges (Lencioni et al., 2009) and in the Antarctic midge *B. antarctica* (Rinehart et al., 2006). The strategy of expressing *hsp* genes continuously is adopted by other several organisms belonging to animals and protists living in very cold environments of polar and temperate zones to avoid protein denaturation due to low temperatures (Joplin et al., 1990; Yocum et al., 1991; Denlinger et al., 1992; Lee et al., 1995; Feder and Hofmann, 1999; Carpenter and Hofmann, 2000; La Terza et al., 2001; Place and Hofmann, 2005). According to Chapovetsky and Katz (2006), the presence of constitutively high levels of HSPs confers the capacity to respond promptly to any variation of environmental conditions. Zhang et al. (2011) showed a consistent increase (1.5- to 2.0-fold) of HSP70 and other constitutive HSPs in the freeze-tolerant gall fly larvae of *Eurosta solidaginis* Fitch, 1855 (Diptera, Tephritidae) over late autumn-winter when they enter diapausa than in other periods of the year. This suggested that high constitutive amounts of HSP70 contribute to cell preservation over the coldest months via protection and stabilization of macromolecules.

A heat shock response (as a significant increase in HSP70 level in shocked larvae), highlighted for the time in alpine aquatic insects by Bernabò et al. (2011), was consistent only in the larvae of *D. cinerella* collected in summer at the highest altitude (2,605 m a.s.l.), where the environmental conditions are extremely difficult for life. The larvae collected in summer also have the highest thermotolerance (LT_{50}: 32.8 ± 0.6°C; LT_{100}: 37.8°C).

At high altitude, the stream is free from ice and snow cover for few months a year (from late June to early October), the banks are vegetation less, and discharge is highly variable during the day and between seasons—with peaks of 800–1,000 l/s in late afternoon in

August—according to ice and snow melt. During this period, the quantity of suspended sediments reaches 350 mg/l and the mean summer water temperature is 3.2°C, whereas in winter, when the substrate is partially frozen, it gets down to subzero (Boscaini et al., 2004). Therefore, the growing season is brief and the zoobenthic community is composed mainly of chironomids of the genus *Diamesa* that has the physiological (Lencioni et al., 2004, 2008) and biochemical (present study; Lencioni et al., 2009) adaptations to withstand environmental stresses such as low temperature of water. In the stream reach at lower altitude (1260 m a.s.l.), the growing season is longer, and *D. cinerella* faces its thermal optimum from spring to autumn when water temperature is low (see Table 1) but the substrate is unfrozen. Furthermore, here environmental conditions are milder due to a higher food availability (e.g., periphyton development), substrate and banks more stable, and higher transparency (Boscaini et al., 2004).

The observed seasonal differences in HSR and lethal high temperatures among the four groups of larvae might be associated with different environmental features of the two stream reaches, as described above, and consequently to different stress factors affecting the populations inhabiting them during the seasons. In fact, similar to our data on *D. cinerella*, variation in the level of HSP70 over days to weeks as a function of natural temperature variations was detected in other invertebrates as the leaf beetle *Crysomela aeneicollis* (Schaeffer, 1928) from Sierra Nevada (McMillan et al., 2005) and in intertidal gastropods of the genus *Tegula* (Tomanek and Sanford, 2003). Furthermore, the absence of HSR in the larvae of *D. cinerella* collected in winter, autumn, and spring coupled with even higher amounts of HSP70 than in summer, led us to hypothesize that other macromolecules and adaptive mechanisms, apart from biochemical ones, are involved in the response of *D. cinerella* larvae to high temperature. Behavioral, morphological, and physiological adaptations may in fact, to prevent potential damage from temperature stress since the average level of HSP70 in whole animals, cannot be the only critical factor for survival (Dahlgaard et al., 1998). Finally, the recorded seasonal differences could reflect that larvae of *D. cinerella* collected at the two different stream reaches and altitudes (2,605 m a.s.l. in summer; 1,260 m a.s.l. in autumn, winter and spring) belong to two different populations. In fact, it is known that the level of HSPs can have genetic variation within and between populations, and can vary with developmental and nutritional status of animals being involved in a variety of cell functions (Halpin et al., 2002).

In conclusion, this study stressed how the HSP70 protein family confers resistance against cold, being detected already under natural conditions (control samples of this study; Lencioni et al., 2009), and at the same time how these chaperones may confer high thermotolerance to larvae of cold stenothermal midges under short-term heat shocks. This information provides new insights into cold stenothermal adaptation in midges inhabiting alpine streams, which are affected more and more by glacial retreat and temperature increase.

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