Thermotolerance and *hsp70* heat shock response in the cold-stenothermal chironomid *Pseudodiamesa branickii* (NE Italy)

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Abstract To better understand the physiological capability of cold-stenothermal organisms to survive high-temperature stress, we analyzed the thermotolerance limits and the expression level of *hsp70* genes under temperature stress in the alpine midge *Pseudodiamesa branickii* (Diptera Chironomidae). A lethal temperature (LT₁₀₀) of 36°C and a lethal temperature 50% (LT₅₀) of 32.2°C were found for the cold-stenothermal larvae after short-term shocks (1 h). Additional experiments revealed that the duration of the exposure negatively influenced survival, whereas a prior exposure to a less severe high temperature generated an increase in survival. To investigate the molecular basis of this high thermotolerance, the expression of the *hsp70* gene family was surveyed via semi-quantitative reverse transcription-polymerase chain reaction analysis in treated larvae. The constitutive (*hsc70*) and inducible (*hsp70*)

forms were both analyzed. Larvae of *P. branickii* showed a significant up-regulation of inducible *hsp70* gene with increasing temperatures and an over-expression of both *hsp70* and *hsc70* by increasing the time of exposure. Different from that was shown in many cold-stenothermal Antarctic organisms, *P. branickii* was able to activate *hsp70* genes transcription (equal to heat shock response) in response to thermal stress. Finally, the unclear relationship between *hsp70* expression and survival led us to surmise that genes other than *hsp70* and other processes apart from the biochemical processes might generate the high thermal-tolerance of *P. branickii* larvae. These results and future high-throughput studies at both the transcriptome and proteome level will improve our ability to predict the future geographic distribution of this species within the context of global warming.

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Introduction

Little is known about the tolerance to thermal stress in aquatic cold-stenothermal species of insects. Data were published on *Oxycera pardalina* Meigen, 1822 (Diptera, Stratiomidae) from cold springs from Russia (Garbuz et al. 2008) and on *Belgica antarctica* Jacobs, 1900 (Diptera, Chironomidae) from the Antarctic region (Rinehart et al. 2006). Garbuz et al. (2008) reported a large difference between the lethal temperatures (LT₅₀=41.2°C and LT₉₉=43°C) and the natural environmental temperatures (<10°C) of the larvae of *Oxycera pardalina*. Rinehart et al. (2006) observed that the larvae of *B. antarctica* are strongly thermotolerant with 60% survival after 3 h at 30°C.

The resistance to heat exposure is generally associated with the synthesis of stress proteins, namely heat shock proteins (HSPs). These are a family of highly conserved proteins that act as chaperones to stabilize other proteins, refold partially unfolded proteins, and detect proteins that are irreversibly damaged under stressful conditions (Tomanek 2002; Clark et al. 2008a). HSPs also play a role under physiological conditions by facilitating the correct folding of proteins during translation (Lindquist 1986; Lindquist and Craig 1988; Parsell and Lindquist 1994; Ellis 1996; Feige et al. 1996; Frydman 2001; Hartl and Hayer-Hartl 2002).

Among HSPs, the 70-kDa family is the most studied in relation to thermal stress and are found in all organisms investigated (Morimoto et al. 1994; Feder and Hofmann 1999). This family is traditionally divided into two groups based on their gene expression patterns: those with low or null expression under non-stress conditions, but which can be quickly induced under stress conditions (HSP70), and a second group of constitutive proteins expressed under non-stress conditions (HSC70) (Qin et al. 2003). The heat shock response (HSR) is a reaction that involves the expression of *hsp70* gene in response to high temperature (Morimoto et al. 1997).

The HSR has been demonstrated in almost all organisms examined (Morimoto et al. 1997), with the exception of some aquatic cold-stenothermal organisms: the hydrozoan *Hydra oligactis* Pallas, 1766 (Bosch et al. 1988), the ciliate *Euplotes focardii* Valbonesi and Luporini, 1990 (La Terza et al. 2001, 2004), the crustacean *Paraceradocus gibber* Andres, 1984 (Clark et al. 2008b), the starfish *Odontaster validus* Koehler, 1906 (Clark et al. 2008b), the larvae of the midge *B. antarctica* Jacobs, 1900 (Rinehart et al. 2006), and several notothenioid fishes (Carpenter and Hofmann 2000; Hofmann et al. 2000; Place et al. 2004; Clark et al. 2008c). The absence of HSR in such organisms could be due to a marked adaptation to the low temperatures they constantly face (Carpenter and Hofmann 2000; Place et al. 2004; Rinehart et al. 2006; Clark et al. 2008c).

Several hypotheses have been proposed to explain from a biochemical point of view the absence of induction of *hsp70* in response to heat stress, including one or more types of lesion found either in the *hsp70*-encoding genes themselves or in the complex regulatory mechanisms that govern HSP70 synthesis. It has been suggested that the reading frame could be disrupted or that mutations in regulatory regions of the gene could block transcription (Hofmann et al. 2000). It is also conceivable that mutation could lead to transcription of an instable *hsp70*-encoding mRNA and finally to the absence of protein translation (Petersen and Lindquist 1989; Hess and Duncan 1996).

HSR had not previously been reported for a single species of freshwater insects from the Alps. The aim of this work is to investigate the tolerance to heat stress and the

ability to activate a HSR in larvae of the cold-stenothermal chironomid *Pseudodiamesa branickii* (Nowicki, 1873) from the Italian Alps.

Materials and methods

Animal model and collection

Pseudodiamesa branickii is a holarctic holometabolous species, distributed from Canada to Asia. In Italy, it is one of most frequent freshwater dipterans in the Alps, Prealps, and Apennines. Its distribution is limited only by the strong cold stenothermy of the larvae living in well-oxygenated springs and streams with water temperature lower than 8°C (optimum at about 4°C) (Lencioni and Rossaro 2005; Lencioni et al. 2008). In mountain regions at altitude lower than 2,000 m a.s.l., this species is typically abundant in winter, with a consistent emergence in spring, whereas at high altitudes, it has only one summer generation. The young larvae are detritivorous whereas the mature ones (IV instar) are carnivorous and feed of small invertebrates, frequently young chironomids even of the same species (Ferrarese and Rossaro 1981).

Fourth-instar larvae of *Pseudodiamesa branickii* were used as an animal model in our experiments. Animal collection was carried out on three dates during December 2007 and January 2008 (Table 1) in a glacio-rhithral stream at 1,584 m a.s.l. (Frigidolfo stream, Val Camonica, Lombardy Province, NE Italy 10°30'19.32" N; 46°17'51.07" E). The Frigidolfo stream was characterized by clear waters (3.8 ± 1.4 NTU) and temperatures ranging from 2.8°C to 3.5°C during the sampling period. Percent oxygen saturation, as recorded by the portable oxy-meter WTW Oxi 330i, ranged from 80% to 90%. Larvae were collected with a 30×30 cm pond net (100-μm mesh size) in lentic depositional areas where sand, silt, and organic debris are deposited. Fourth-instar larvae of *Pseudodiamesa branickii* were sampled with tweezers, transferred in 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 specimen per beaker) with filtrated stream water in a thermostatic chamber (ISCO, mod. FTD250-plus) at 4°C with aerator to maintain percent of oxygen saturation higher than 80%. The stabulation temperature corresponds to the mean temperature of the stream water during the period in which the species is present in the nature (October–April) (Bernabò 2010).

Survival following heat shock

The lethal temperatures 50% and 100% values (LT₅₀ and LT₁₀₀) for *Pseudodiamesa branickii* larvae were calculated

Table 1 Sampling date, shock temperature and duration of the three types of treatment, and number of replicates and of specimens employed

Sampling date	Treatment	Shock temperature (°C)	Duration (h)	Number of replicates	Total number of specimens
December 5, 2007	Short-term shock	K ^a , 8, 15 ^a , 26 ^a , 28, 30, 32 ^a , 33, 34, 35, 36	1	33	160
December 12, 2007	Long-term shock	K ^a , 26	1 ^a , 2 ^a , 4 ^a , 8, 16, 24, 32	24	117
December 12, 2007	Long-term shock	K ^a , 32	1 ^a , 2 ^a , 4 ^a , 8	15	75
January 9, 2008	Pre-exposition shock	K ^a , 26 ^a , 32 ^a , 36, 37, 38	1	18	88

K control

^a Temperatures and duration for which *hsps* gene expression analysis was performed

performing short-term shocks (1 h) at 11 different temperatures from 8°C (equal to upper environmental thermal limit according to literature) to 36°C (equal to experimental lethal temperature) and checking for larvae survival. These values were used to evaluate their thermotolerance.

On the basis of the results of this short-time experiments (see Fig. 1), two temperatures (26°C and 32°C) were selected to perform long-term experiments (1–32 h) in order to calculate the L_{Time50} and $L_{Time100}$ (equal to the time (hours) at which the 50% and the 100% of the tested larvae were killed).

Finally, in order to test the hypothesis that a pre-exposition at less severe high temperature would generate higher thermotolerance, the larvae were pre-exposed at 15°C (equal to intermediate temperature between 4°C and 26°C that is the highest temperature at which, after 1 h of exposure, all tested larvae were found alive) for 1 h and recovered at 4°C for 1 h before the short-term shock at 26°C, 32°C, 36°C, 37°C, and 38°C.

All experiments were carried out in thermostatic chambers. For exposure to different temperatures and times, three groups (equal to replicates) of four to five larvae were transferred into three different 25-ml plastic bottles (Kartell, Italy) filled with 10 ml of preheated filtered (on Whatman GF/C, particle retention 1.2 µm) stream water, under aeration to avoid the mortality due to the oxygen depletion. 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 (equal to stabulation temperature) and, 1 h later, examined for survival. The larvae that moved spontaneously were considered active, whereas those that moved only following a tactile stimulus were considered suffering. The survival was determined by the sum of active and suffering larvae. The immobile larvae were considered dead.

Overall, 90 replicates (for a total of 440 larvae) were analyzed (Table 1).

Reverse transcription-polymerase chain reaction analysis

The analyses of the expression level of *hsc70* and *hsp70* in control and heat-shocked larvae were performed by reverse transcription-polymerase chain reaction (RT-PCR) analysis, using *actin* as an internal control. On the basis of survival curves, this analysis was performed only for some temperatures and for some times of each treatment (Table 1).

Total RNA was extracted from alive larvae using a commercial kit (TRIzol, Invitrogen), according to the manufacturer's protocol. RNA concentration and purity were determined by UV absorption (260:280 nm) using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). One microgram of total RNA was treated with DNase using the RNase-Free DNase set (Qiagen) and reverse transcribed using the First Strand cDNA Synthesis Kit (Fermentas). Primers for *actin* (Table 2) were designed from an alignment of *actin* nucleotide sequences of various insect species. For *hsp70* and *hsc70*, degenerate primers were designed from an alignment of aminoacid sequences. The amplified fragments were subcloned into pJET1.2/blunt cloning vector (Fermentas), transformed into *Escherichia coli* strain DH5α, and sequenced. Consensus sequences were controlled by Blastn, and specific primers with an annealing temperature of 56°C were designed for both genes (Table 2). Amplified fragments ranged from 150 to 220 bp. The specificity of each primer pair was checked by PCR amplification and sequencing.

Partial cDNA sequences have been deposited in GenBank under accession numbers HM535779 (*hsp70*), HM535780 (*hsc70*), and HM535781 (*actin*).

Hsps and *actin* cDNAs were amplified by real-time PCR using Kapa Sybr Fast qPCR Mastermix (KapaBiosystems) with Rotor-Gene 6000 instrument (Corbett). PCR conditions were as follows: 95°C for 3 min, 40 cycles each of 10 s at 95°C, 20 s at 55°C, and 20 s at 72°C with a final melting curve analysis (from 72°C to 95°C, increment of 1°C every 5 s).

Fig. 1 Thermotolerance of larvae of *Pseudodiamesa branickii*. Alive and suffering proportions of larvae after **a** short-term exposition (1 h) up to 36°C (survival from 4°C to 26°C=100%, is not shown), **b** long-term exposition at 26°C, and **c** long-term exposition at 32°C

For each of the 45 replicates (Table 1), three real-time PCR amplifications were run.

Each primer pair was controlled for dimer formation (by the analysis of melting curve) and PCR efficiency was calculated over a sixfold 2× dilution series.

Amplification profiles were analyzed using Rotor-Gene 6000 series 1.7 software and Ct values exported to Excel.

Relative expression ratios of the *hsps* genes compared to *actin* between the control and the treated samples were derived using REST-MCS beta software version 2 (2006) with 2000 randomisations (Pfaffl 2001; Pfaffl et al. 2002).

Statistical analysis

According to Bouchard et al. (2006), one- and two-way analyses of variance on the arcsine-square-root-transformed proportion of survival were performed to test for significant differences in survival among temperature treatments (Statistica 8.0, StatSoft Inc, USA). When significant differences were found ($p<0.05$), Tukey'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% and 100% of mortality occurred (LT_{50} , LT_{100} , L_{Time50} , $L_{Time100}$).

Differences in expression levels of *hsps* genes were calculated by REST-MCS beta software version 2 (2006).

Results

Short-term heat shock survival

All larvae of *Pseudodiamesa branickii* were found alive after short exposure at temperatures from 4°C (equal to stabulation temperature) to 26°C. Survival decreased at progressively higher temperatures (Fig. 1a), but it remained higher than 50% up to 33°C ($LT_{50}=32.2\pm1.6^{\circ}\text{C}$). At 36°C, all the tested larvae were killed ($LT_{100}=36.0^{\circ}\text{C}$). The statistical analysis highlighted significant differences between the survival at the different temperatures of shock ($df=7$; $F=29.3$; $p<0.001$). The first significant decrease in survival ($p=0.005$) was observed from 33°C ($60\pm14\%$) to 34°C ($14\pm8\%$). The decrease in survival was accompanied by a progressively increase of the percentage of suffering larvae. At the sub-lethal temperatures (34°C and 35°C), all the survived larvae were suffering.

Table 2 List of primers (5'-3') used to amplify *hsp70*, *hsc70*, and *actin* in RT-PCR analysis

Primer	Sequence (5'-3')
Act forward	GCCTCAACCTCATTGGAA
Act reverse	TGGTTGTAGACGGTTTCGT
Hsp70 forward	AGAGAACATTGTCATCAAGCA
Hsp70 reverse	GAATACGAGTAGAACCACCAACAA
Hsc70 forward	TTGGTGTATGCAGCTAAGAAT
Hsc70 reverse	TGCCTTGACTTGTTTTCACT

Long-term heat shock survival

The survival of the larvae of *Pseudodiamesa branickii* exposed to long-term shock at 26°C (1–32 h) and 32°C (1–8 h) decreased with the increasing duration of exposure, faster at 32°C (Fig. 1b–c).

At 26°C, the survival of larvae was 100% with 8 h of exposition, whereas after 32 h of heat shock, all larvae were killed (Fig. 1b). The $L_{\text{Time}50}$ was 21.5 ± 2.5 h and the $L_{\text{Time}100}$ was 34.6 h. The statistical analysis revealed significant differences among the survival recorded at the different times of exposure ($df=6$; $F=93.1$; $p<0.001$). The first significant ($p=0.001$) decrease in survival occurred between 8 h (100%) and 16 h ($57 \pm 11\%$).

At 32°C, the survival decreased very quickly and after only 8 h that all larvae were killed (Fig. 1c). In addition, the major portions of surviving larvae were suffering. The $L_{\text{Time}50}$ was 2.0 ± 0.6 h and the $L_{\text{Time}100}$ was 6.9 h. The statistical analysis revealed significant differences ($df=3$; $F=13.1$; $p<0.001$) among the surviving larvae recorded at the different times of exposure. At 32°C already between 1 and 2 h of exposition, a significant decrease ($p=0.002$) in survival (from $93 \pm 12\%$ to $25 \pm 10\%$) was present.

Short-term heat shock survival after pre-exposition

Figure 2 shows the comparison between the survival of larvae after 1 h of heat shock treatment with or without a pre-exposition to less severe high temperature (1 h at 15°C). The statistical analysis shows a significant ($p<0.05$) increase of survival of the pre-exposed larvae with respect to the non pre-exposed larvae at 36°C and 37°C. The LT_{50} and LT_{100} of the pre-exposed larvae were $33.8 \pm 0.5^\circ\text{C}$ and 38.2°C respectively. LT_{50} and LT_{100} were respectively 1.6°C and 2.2°C higher than those of larvae without the pre-exposition.

Fig. 2 Survival (mean $\pm$ SE) after 1 h of heat shock between 20°C and 38°C with or without 1 h of pre-exposition at 15°C

Reverse transcription-polymerase chain reaction analysis

For the larvae of *Pseudodiamesa branickii* treated for 1 h at 15°C, the level of expression of both *hsc70* and *hsp70* decreased with respect to the control, significantly only for the latter ($p=0.038$) (data not shown). After 1 h of exposure at 26°C and 32°C, a significant ($p<0.001$) increase with respect to the control only for the inducible form *hsp70* mRNAs was observed (8.75 fold higher at 26°C and 8.72 higher fold at 32°C in 2-logarithmic scale) (Fig. 3). The level of the constitutive form *hsc70* was instead not significantly altered by the high temperatures (Fig. 3).

After 2 and 4 h of exposure at 26°C, the level of expression of both forms (*hsp70* and *hsc70*) increased significantly with respect to the level observed after 1 h of exposure (*hsp70*, 1.21-fold higher after 2 h ($p=<0.001$) and 1.30-fold higher after 4 h ($p=0.005$); *hsc70*, 2.56-fold higher after 2 h ($p=<0.001$) and 2.37-fold higher after 4 h ($p<0.001$)) (Fig. 3). At 32°C, conversely, the level of expression of inducible *hsp70* did not change significantly both after 2 and 4 h of exposition, whereas the level of constitutive *hsc70* decreased significantly after 4 h of exposure (1.60-fold of decrease, $p=0.011$) (Fig. 3).

The pre-exposition at 15°C showed a significant increase of the expression level of both forms only at 26°C with respect to that observed in short direct shocks (a 1.49-fold up for *hsp70* ($p<0.001$) and 2.26-fold up for *hsc70* ($p<0.01$)) (Fig. 3).

Discussion

Similarly to what observed for *Oxyera pardalina* (Garbuz et al. 2008) and *B. antarctica* (Rinehart et al. 2006), also *Pseudodiamesa branickii* can survive short-term heat shocks at substantially higher temperatures than those they normally face in nature, with a lethal temperature 50% of about 32°C and a lethal temperature 100% of 36°C. However, our experiments of long-term heat shock highlighted that this great ability to survive high temperatures is limited to a very short period of exposure ($L_{\text{Time}100}=35$ h at 26°C and 7 h at 32°C). Conversely, the pre-exposition treatment emphasized a positive effect on survival to high temperatures (increase of 2°C of LT_{100}). Such a positive effect has not previously been reported for other aquatic cold-stenothermal insects (Rinehart et al. 2006; Garbuz et al. 2008).

Differently from most aquatic cold-stenothermal organisms studied to date (Carpenter and Hofmann 2000; Hofmann et al. 2000; La Terza et al. 2001, 2004; Place et al. 2004; Rinehart et al. 2006; Clark et al. 2008b, c), *Pseudodiamesa branickii* is able to activate an *hsp70* gene transcription in response to thermal stress (HSR), with

Fig. 3 Relative expression ratio (2-log scale) of the *hsp70* (gray bars) and *hsc70* (white bars) after 1, 2, and 4 h of shock and after 1 h of pre-exposition followed by 1 h of shock (P+1h) at 26°C and 32°C. Asterisk, significant differences ($p \leq 0.05$) of the level of expression after short-term stress (1 h) relative to the level of control larvae; empty circle, significant differences ($p \leq 0.05$)

of the level of expression after long-term stress (2 and 4 h) relative to level of larvae stressed for 1 h; number sign, significant differences ($p \leq 0.05$) of the level of expression after shock (1 h) with pre-exposition relative to the larvae stressed without pre-exposition (1 h)

significant increase of *hsp70* mRNA after 1 h at 26°C and 32°C. Until now, the ability to activate an HSR in aquatic cold-stenothermal organisms was reported only for the mollusks *Nacella concinna* (Strebel 1908) and *Laternula elliptica* (King and Broderip 1831) (Clark et al. 2008a; Clark and Peck 2009), the ciliate *Euplotes nobilii* Valbonesi and Luporini, 1990 (La Terza et al. 2001), and the adults of *B. antarctica* (Rinehart et al. 2006).

The expression level of both forms *hsp70* and *hsc70* were positively affected by duration of exposure and by the pre-exposition treatment only at the lowest temperature of the critical thermal range (26–36°C). At the LT_{50} temperature, no significant alteration was evident.

As previously reported by other authors, the relationship between thermotolerance and *hsp70* expression is not always straightforward (Hoffmann et al. 2003; Dahlgaard et al. 1998; Jensen et al. 2008). Multicellular organisms possess, in fact, various mechanisms to cope with high temperatures apart from the strictly biochemical ones. Behavior, morphology, and physiology may all function to prevent potential damage from temperature stress, and the average level of HSP70 in whole animals cannot be the only critical factor for survival (Dahlgaard et al. 1998). Moreover, the biochemical response comprises not only *hsp70* gene activation but, as reported by Hoffmann et al. (2003), the transcription of other stress protein genes like the *small hsps*, *dnaJ-1*, *hsp60*, and *hsp80* and of some genes not encoding HSP (for example for *Drosophila melanogaster* Meigen, 1830, the gene involved in signaling pathway for epithelial cell polarity *shark* and the unknown function gene *anon-23Da*).

These considerations could explain why resistance of *Pseudodiamesa branickii* larvae was not always proportional to the level of *hsp70* mRNA found. For example, the decrease in survival from 26°C to 32°C for the short-term heat shock and from 2 to 4 h for the long-term heat shock does not correspond to a decrease in the level of *hsp70* mRNA but remains constant. At 26°C, with the increase of the exposure time (from 2 to 4 h), the significant increase of *hsp70* mRNA is combined with a significant increase of *hsc70* mRNA, suggesting a putative role of the constitutive form in the HSR of *Pseudodiamesa branickii*. The constitutive form is known to have a role in regulation of protein metabolism and homeostasis in physiological conditions (Parsell and Lindquist 1994). The increase in the expression level of *hsc70*, together with *hsp70*, after exposure to high temperatures has been observed in other organisms like the diamondback moth *Plutella xylostella* Linnaeus 1767 (Sonoda et al. 2006), the shrimp *Penaeus monodon* (Fabricius, 1798) (Chuang et al. 2007), the hymenopteran *Pteromalus puparum* (Linnaeus, 1758) (Wang et al. 2008), and the killifish *Fundulus heteroclitus* (Linnaeus, 1766) (Fangue et al. 2006). In particular, Wang et al. (2008) suggested that the constitutive form might have a synergic role with the inducible one in the stabilization of the proteins damaged by the heat shock during folding. High-throughput studies at both transcriptome and proteome levels, as well as gene perturbation experiments, are needed to better understand the molecular mechanisms of the HSR response in this and other cold-stenothermal organisms.

Conclusions

In the present study, we have reported results that despite the cold Alpine habitats in which *Pseudodiamesa branickii* larvae live, they show a surprisingly high thermotolerance and a huge gap between the natural temperatures it experiences and the critical limits of their survival.

Moreover, different from the majority of the cold-stenothermal Antarctic organisms, they are able to activate an *hsp70* gene transcription in response to thermal stress (HSR). The non-proportional relationship found between *hsp70* expression and survival rate led us to hypothesize that other genes and other mechanisms, apart from biochemical ones, are involved in the response of *Pseudodiamesa branickii* larvae to high temperature. Overall, these results are useful tools to predict a possible future geographic distribution of this species within the context of global warming.

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