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Research Article

Different levels of *hsp70* and *hsc70* mRNA expression in Iberian fish exposed to distinct river conditions

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Abstract

Comprehension of the mechanisms by which ectotherms, such as fish, respond to thermal stress is paramount for understanding the threats that environmental changes may pose to wild populations. Heat shock proteins are molecular chaperones with an important role in several stress conditions such as high temperatures. In the Iberian Peninsula, particularly in Portugal, freshwater fish of the genus *Squalius* are subject to daily and seasonal temperature variations. To examine the extent to which different thermal regimes influence the expression patterns of *hsp70* and *hsc70* transcripts we exposed two species of *Squalius* (*S. torgalensis* and *S. carolitertii*) to different temperatures (20, 25, 30 and 35 °C). At 35 °C, there was a significant increase in the expression of *hsp70* and *hsc70* in the southern species, *S. torgalensis*, while the northern species, *S. carolitertii*, showed no increase in the expression of these genes; however, some individuals of the latter species died when exposed to 35 °C. These results suggest that *S. torgalensis* may cope better with harsher temperatures that are characteristic of this species natural environment; *S. carolitertii*, on the other hand, may be unable to deal with the extreme temperatures faced by the southern species.

Keywords: Cyprinidae, heat shock proteins, *Squalius*, thermal stress.

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Introduction

Many organisms are frequently exposed to stressful environmental conditions, such as temperature variations, that pose substantial challenges to their survival and reproduction (López-Maury *et al.*, 2008). Stressful conditions may limit the geographical distribution of organisms by causing them to move to more suitable locations (Hoffmann and Sgrò, 2011). Organisms can also deal with stressful conditions by adapting to them, either through changes in the genetic composition of populations as a result of selection, and/or by phenotypic plasticity; without this adaptability many species would become extinct (Sørensen *et al.*, 2003; Dahlhoff and Rank, 2007; Berg *et al.*, 2010; Hoffmann and Sgrò, 2011). Most animal species (> 99%), including fish, are ecthoterms that cannot regulate their body temperature and this ultimately affects their metabolism (Berg *et al.*, 2010). Since increases in temperature are one of the major consequences of climate change it is important to know how organisms, particularly ecthoterms, respond to high temperatures.

Heat shock proteins (Hsp) are part of an important mechanism that helps organisms to cope with adverse environmental conditions such as thermal stress. This mechanism has a significant ecological and evolutionary role in natural populations (Sørensen *et al.*, 2003; Fangue *et al.*, 2006; Straalen and Roelofs, 2006). In addition to thermal stress, other factors such as insecticides, heavy metals, desiccation, diseases and parasites can also induce Hsp (Lindquist and Craig, 1988; Sørensen *et al.*, 2003; Fangue *et al.*, 2006). Heat shock proteins are vital for proper cell functioning since they facilitate the folding and refolding of proteins and the degradation of misfolded, aggregated or denaturated proteins (Lindquist and Craig, 1988; Ohtsuka and Suzuki, 2000; Sørensen *et al.*, 2003; Wegele *et al.*, 2004).

Several closely related *hsp* genes have been identified and grouped into families based on their evolutionary relationships (Lindquist and Craig, 1988). The extensively studied 70-kDa heat shock protein (Hsp70) belongs to a multi-gene family and its gene expression varies under different physiological conditions (Lindquist and Craig, 1988). The genes that encode the Hsp70 proteins (*hsp70s*) are considered the major *hsp* gene family and consist of exclusively inducible (*hsps*), exclusively constitutive [Heat shock cognates (*hscs*)] and even simultaneously inducible and constitutive genes (Lindquist and Craig, 1988; Ohtsuka and Suzuki, 2000; Place and Hofmann, 2001; Sørensen *et al.*, 2003). The *hsp70* genes and the genes that encode the Hsc70 protein (*hsc70*) belong to the *hsp70* gene family. Whereas *hsp70* genes are

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induced by several types of stress, *hsc70* genes are mainly constitutively expressed under normal (non-stress) conditions (Lindquist and Craig, 1988; Ohtsuka and Suzuki, 2000; Yamashita *et al.*, 2004).

Members of the *hsp70* gene family have been widely studied in many organisms and distinct expression patterns have been found. Several studies have reported a relationship between the expression patterns of *hsp70* and environmental variations throughout a species range (Sørensen *et al.*, 2001; Fangue *et al.*, 2006; Karl *et al.*, 2009; Sørensen *et al.*, 2009; Blackman, 2010; Sarup and Loeschcke, 2010). For example, Fangue *et al.* (2006) detected significant differences in the gene expression levels of *hsp70* between northern and southern populations of *Fundulus heteroclitus* in North America, with the latter being exposed to higher temperatures. Similarly, Sørensen *et al.* (2009) found that southern populations of *Rana temporaria* from Sweden, when exposed to higher temperatures, had the highest levels of Hsp70 protein expression.

The *hsc70* gene was initially described as being constitutively expressed under normal and stressful conditions (Lindquist and Craig, 1988; Place and Hofmann, 2001; Yeh and Hsu, 2002; Yamashita *et al.*, 2004). Fangue *et al.* (2006) reported that individuals from southern populations of *F. heteroclitus* showed enhanced expression of this gene at higher temperatures. This finding demonstrates the importance of studying the expression of *hsp70* genes in closely related species or populations exposed to different temperature regimes in their natural habitats. These findings also suggest that Hsps play an important role in thermal tolerance and that, despite being occasionally paradoxical, the expression patterns of these genes must be interpreted according to the ecological context of each species (Sørensen *et al.*, 2003).

In the Iberian Peninsula, particularly in Portugal, the congeneric freshwater fish species, *Squalius carolitertii* (Cyprinidae) (Doadrio, 1988), a species of least concern (Rogado *et al.*, 2006), and *Squalius torgalensis* (Coelho *et al.*, 1998), a critically endangered species (Cabral *et al.*, 2006), inhabit distinct regions. *Squalius carolitertii* inhabits the northern region whereas *S. torgalensis* is restricted to a small river basin (the Mira river) in the southwestern region (Figure 1) (Cabral *et al.*, 2006). In these areas, the two species are exposed to different environmental conditions with distinct seasonal and even daily water temperature variations. The northern rivers of Portugal have lower temperatures and fewer temperature fluctuations than the southern rivers (Henriques *et al.*, 2010; SNIRH). In northern rivers, the maximum temperature usually does not exceed 31 °C (range: 3-31 °C), whereas southern rivers are characterized by an intermittent regime of floods and droughts in which, during the dry season, freshwater fish are trapped in small pools in which temperatures can reach 38 °C (range: 4-38 °C) (Magalhães *et al.*, 2003; Henriques *et al.*, 2010; SNIRH).

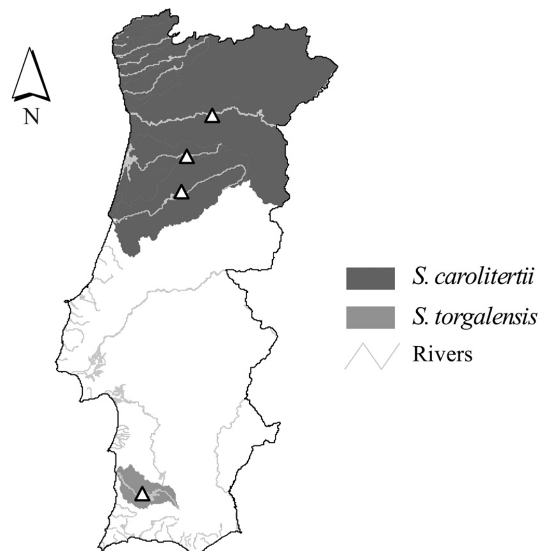

Figure 1 - Geographical distribution of *S. torgalensis* and *S. carolitertii* in Portugal, with the respective sampling sites marked with triangles.

The main goal of this study was to gain insights into the potentially important molecular mechanism involved in the response of *S. carolitertii* and *S. torgalensis* to thermal stress, particularly since these species inhabit regions with distinct environmental regimes. Specifically, we examined the *hsp70* and *hsc70* gene transcription patterns for each species exposed to different temperatures and compared the patterns between the two species; we also tried to correlate our findings with the ecological context of each species. Finally, we examined whether the patterns of transcript expression (for the genes of interest) were similar to those of muscle, which is the most frequently used tissue in such studies (Yamashita *et al.*, 2004). The results described here provide useful insights into the roles of *hsp70* and *hsc70* gene expression in the response of Iberian *Squalius* to thermal stress.

Methods

Sampling and maintenance of fish

Adult fish (6-8 cm long) of *S. carolitertii* and *S. torgalensis* were collected from Portuguese rivers by electro-fishing (300 V, 4 A) (Figure 1). The pulses used were of low duration to avoid killing juveniles. Sampling was done during the spring, when the water temperature in the southern and northern rivers is ~18-22 °C. Fish of both sexes were used since there is no sexual dimorphism in either species. *Squalius torgalensis* individuals were sampled in the Mira river basin since this species is endemic to this region and individuals of *S. carolitertii* were collected in the Mondego, Vouga and Douro river basins of the northern region. The fish were maintained in ~30 L aquaria at 20 °C (mean temperature observed during sampling) on a 12 h photoperiod and were fed daily with commercial flake fish food.

Experimental design

After two weeks of acclimatization (to reduce the stress caused by fishing and confinement), individuals of each species were subjected to four temperature regimens: 20 °C (control temperature) and increases in temperature from 20 °C to 25 °C, 30 °C and 35 °C (testing temperatures). These increases in temperature were achieved with gradual increments of 1 °C per day and, once the testing temperature was reached, individuals were kept at this temperature for 24 h. Six to seven individuals of each species were exposed to each experimental condition, with each individual being exposed to only one experimental condition. After acclimatization at the desired test temperature, fish were anesthetized with 300 mg/L tricaine mesylate (MS-222; Sigma-Aldrich, St. Louis, MO, USA) and fin clips were collected from the pectoral, pelvic and upper caudal fins. The fin clips from each fish were pooled and stored at -80 °C until RNA extraction. To compare the expression patterns of fins and muscle and determine whether fin clips could be used instead of muscle to assess transcript expression, four individuals of *S. torgalensis* (one per test temperature) and 16 individuals of *S. carolitertii* (four per test temperature) were euthanized with MS-222 and muscle tissue was collected. Since *S. torgalensis* is a critically endangered species, our study was designed to minimize the number of individuals euthanized.

RNA extraction and cDNA synthesis

For RNA extraction, TRI Reagent (Ambion, Austin, TX, USA) was added to fin clips and muscle samples. After homogenization with an Ultra-Turrax homogenizer (IKA, Staufen, Germany), RNA was extracted according to the manufacturers protocol and TURBO DNase (Ambion) was used to degrade any remaining genomic contaminants, followed by phenol/chloroform purification and LiCl precipitation (Cathala *et al.*, 1983). Glycogen was used as a co-precipitant in RNA precipitation (Sigma-Aldrich). The quality of the samples was checked using a Nanodrop-1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) based on the 260/280 nm and 260/230 nm absorbance ratios. The concentrations of the samples were determined to ensure a sufficient amount of homogeneous RNA for complementary DNA (cDNA) synthesis. cDNA was synthesized using a RevertAid H Minus First Strand cDNA synthesis kit (Fermentas Inc., Glen Burnie, MD, USA), according to the manufacturer's instructions and stored at -20 °C.

Semi-quantitative RT-PCR

Sixty-one individuals (31 *S. torgalensis* and 30 *S. carolitertii)* were used for quantification of the target transcripts. The *hsp70*-specific primers GGCCCTCATCAAACGC (forward) and TTGAAGGCGTAAGACTCCAG (reverse) and the *hsc70*-specific primers GTTCAAGCAGCCATCTTAGC (forward) and TGACCTTCTCCTTCTGAGC (reverse) were designed using PerlPrimer software v.1.1.19 (Marshall, 2004). The resulting amplicons were sequenced and the sequences then checked manually for errors using SEQUENCHER v.4.2 (Gene Codes Corporation, Ann Arbor, MI, USA). The identities of the genes of interest were confirmed by BLAST searches (Zhang *et al.*, 2000).

Multiplex PCRs were used to amplify the glyceraldehyde 3-phosphate dehydrogenase (*gapdh*) serving as internal control and the gene of interest, which allowed normalized quantification of the mRNAs of interest (*hsp70* or *hsc70*). The primers used to amplify *gapdh* were ATCAGGCATAATGGTTAAAGTTGG (forward) (Pala *et al.*, 2008) and GGCTGGGATAATGTTCTGAC (reverse) (Matos IM*,* unpublished). *Gapdh* has been extensively used as an internal control in several studies and has been validated as a good reference gene for gene expression studies in different experimental conditions (Aoki *et al.*, 2000; Zhou *et al.*, 2010), including those involving temperature changes (Liu *et al.*, 2012). Semi-quantitative RT-PCRs were optimized to ensure the amplification of both cDNAs in the exponential phase (Serazin-Leroy *et al.*, 1998; Breljak and Gabrilovac 2005). The amplification conditions for the pair *hsp70*/*gapdh* were those described in the manufacturers instructions (QIAGEN multiplex PCR kit, Qiagen Inc., Valencia, CA, USA) (final concentration: 1 PCR master mix with 3 mM $MgCl_2$, 0.5 of Q-solution and 0.2 μM of each primer), with an initial denaturation step at 95 °C for 15 min, followed by 30 cycles at 95 °C for 1 min, 58 °C for 1 min and 30 s and 72 °C for 1 min, with a final extension at 72 °C for 10 min. For the gene pair *hsc70*/*gapdh*, the PCR conditions were: 1 unit of Go*Taq* Flexi DNA polymerase (Promega, Madison, WI, USA) with 0.3 μM of each primer, 0.25 mM of each dNTP and 2 mM of $MgCl_2$. The cycling conditions included an initial denaturation step at 95 °C for 5 min, followed by 35 cycles at 95 °C for 1 min, 58 °C for 45 s and 72 °C for 1.5 min, with a final extension at 72 °C for 10 min. Controls without template and without RT (reverse transcriptase) were included to check for PCR contamination and genomic DNA contamination, respectively.

For transcript quantification, 4 μL of each PCR product was loaded onto a 1% agarose gel stained with RedSafe (Chembio Ltd, Hertfordshire, England) and the gels were photographed with a DC290 Kodak digital camera for subsequent image densitometry using ImageJ 1.43 U software (Abramoff *et al.*, 2004). An uncalibrated OD was used (Abramoff *et al.*, 2004) and the band of interest was quantified and normalized against the internal control band (*gapdh*) present in the same lane.

Real-time RT-PCR

To assess whether the results obtained with semi-quantitative PCR corresponded to valid transcript expression patterns, an experiment with real-time PCR was

done. In this experiment, three individuals from each experimental condition for both species were analyzed with two PCR replicates. The primer pairs AATTCCACCTGCACCACG (forward) and TCTCCTCTTTGCTCAGTCTG (reverse) and TTTGCTGTTGGATGTCACTC (forward) and GTGGGAATGGTGGTGTTC (reverse) were used to amplify the *hsp70* and *hsc70* genes, respectively. These specific primers were designed based on the sequences previously obtained from semi-quantitative PCR. The relative expression levels of the genes of interest were measured against *gapdh* (reference gene). The primers used to amplify the *gapdh* gene were GTACAAGGGTGAGGTTAAGGC (forward) and GTGATGCAGGTGCTACATACGT (reverse). All pairs of primers used were designed using PerlPrimer software v.1.1.19 (Marshall, 2004).

Real-time PCRs were done in a final volume of 15 μL containing 7.5 μL of SsoFas EvaGreen Supermix (Bio-Rad, Hercules, CA, USA) and 0.6 μL of each primer (with a concentration of 0.4 μM). The assay conditions included an initial denaturation step at 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s and 55 °C for 5 s. The reactions were done in a Bio-Rad CFX96 system (Bio-Rad). Controls without template and without RT were included to check for PCR contamination and genomic DNA contamination, respectively. The identities of the amplicons were confirmed by melting curve analysis and Sanger sequencing. The PCR efficiency for each sample was assessed using LinRegPCR 11.1 software, which fits a regression line to a subset of data points in the log-linear phase (Ruijter *et al.*, 2009). PCR efficiency ranged from 1.91 to 2 for all primer pairs (1.91 for *hsp70* primers and 2 for *gapdh* and *hsc70* primers). The relative amount of the genes of interest was calculated by the comparative threshold cycle (C_T) method with efficiency correction, using the mean PCR efficiency for each amplicon (Ruijter *et al.*, 2009).

Statistical analysis

In the semi-quantitative PCR analysis, arbitrary values for quantification of the band of interest (*hsp70* or *hsc*70) were divided by the corresponding value for the control band (*gapdh*) to obtain a *hsp70*/*gapdh* or *hsc70*/*gapdh* ratio.

In graphs of the fold change in expression for each transcript a temperature of 20 °C was considered the control condition and assigned a value of 1. The fold variation in the other treatments, relative to the control condition, was calculated as follows: $I_i = \sum x_i / n\bar{x}_{20}$, where I_i is the mean fold increase in expression, x_i is the observed value, $\bar{x}_{20}$ is the mean value of observations at 20 °C for each species and n is the number of individuals of each species per tested temperature.

The data were log transformed [$\log_{10}(x + 1)$] for analysis of variance (ANOVA) in order to test for differences in transcript expression patterns across the experimental conditions for both genes. Whenever the assumptions of homoscedasticity and normality were not met, non-parametric Kruskal-Wallis analyses were done and the results from both analyses were compared. *Post-hoc* parametric and non-parametric comparisons were performed, using the Tukey test and Dunn's test, respectively. The real-time PCR data were analyzed in a manner similar to that used for semi-quantitative PCR, except that the fold change was calculated by the method of Pfaffl (2001). Prior to analysis, the real-time PCR data were transformed as described by Willems *et al.* (2008); the statistical tests used were the same as those used for semi-quantitative PCR. In all cases, a value of $p < 0.05$ indicated significance. All statistical comparisons were done using Statistica 9.0 software (StatSoft, 2009).

Results

Survival in the experiments

Two of seven *S. carolitertii* individuals did not reach the 35 °C experimental condition because they died during the increase from 34 °C to 35 °C. In contrast, none of the *S. torgalensis* individuals died or showed signs of loss of equilibrium during the experiments. In the experiment to compare gene expression in muscle and fins, all individuals of *S. carolitertii* died at 34 °C, before reaching 35 °C.

Expression pattern of the *hsp70* gene

Initially, the identity of each amplicon was confirmed by sequencing. This showed that the *hsp70* primers amplified a fragment with high homology to the inducible form of *hsp70* from other cyprinids, including *Megalobrama amblycephala* (96.5% identity; accession number: EU884290), *Tanichthys albonubes* (96% identity; HQ007352), *Cyprinus carpio* (95.4% identity; AY120894), *Carassius auratus* (94.3% identity; AB092839) and *Danio rerio* (91.7% identity; BC056709). The sequences of the *hsp70* genes of *S. torgalensis* and *S. carolitertii* were deposited in GenBank under accession numbers JQ608477 and JQ608476, respectively.

In both species, the levels of *hsp70* gene expression in muscle and fin clips with increasing water temperature were similar in both tissues (Figure S1, Supplementary material). Consequently, in all subsequent analyses fin clips were used in order to avoid euthanasia of the fish.

In *S. torgalensis* exposed to 35 °C there was a 59-fold increase in the *hsp70* mRNA levels when compared with 20 °C (control condition) and an ~53-fold increase when compared with 30 °C. In contrast, in *S. carolitertii* the corresponding expression increased by no more than three-fold, even at the highest temperature (Figure 2). Statistical analyses indicated a significant difference in *hsp70* mRNA expression among *S. torgalensis* exposed to different temperatures ($F = 29.486$, $df = 3$, $p < 0.001$), with *post-hoc*

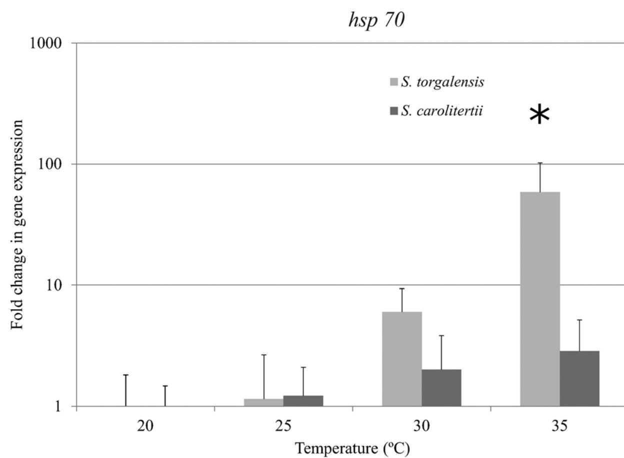

Figure 2 - Fold change in *hsp70* transcript expression in *S. torgalensis* and *S. carolitertii* compared to 20 °C (control condition), as assessed by semi-quantitative PCR. The columns are the mean ± SD of 6 or 7 fish. p < 0.05 compared to all other treatments.

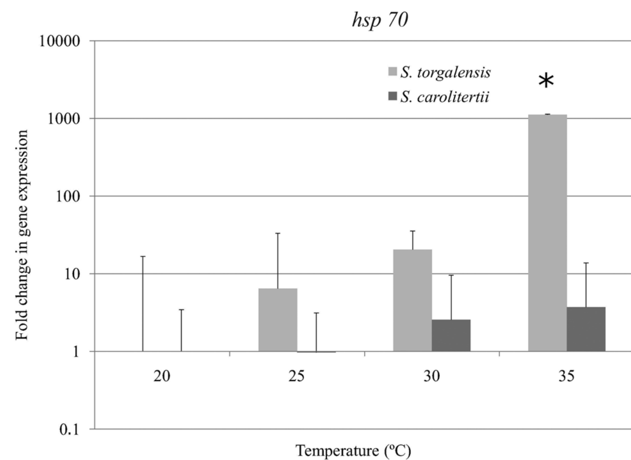

Figure 3 - Fold change in *hsp70* transcript expression in *S. torgalensis* and *S. carolitertii* compared to 20 °C (control condition), as assessed by real-time PCR. The columns are the mean ± SD of 6 or 7 fish. p < 0.05 compared to all other treatments.

comparisons showing that *S. torgalensis* exposed to 30 °C and 35 °C had a significant increase in *hsp70* levels compared with those observed at 20 °C and 25 °C (Table S1, Supplementary material). *Post-hoc* comparisons also demonstrated a significant difference between fish exposed to 30 °C and 35 °C (Table S1, Supplementary material). There were no significant differences in the mRNA levels among the groups of *S. carolitertii* exposed to different temperatures ($H = 3.086$, $df = 3$, $p > 0.300$). As this latter dataset violated the assumption of homoscedasticity the results were also compared with a non-parametric test but the outcome was the same, i.e, there were no differences in the expression of *hsp70* in *S. carolitertii* exposed to different temperatures ($F = 1.220$, $df = 3$, $p > 0.300$).

In general, the real-time PCR results showed similar patterns to those obtained with semi-quantitative PCR for both species, although for *S. torgalensis* the expression pattern of the *hsp70* gene obtained with real-time PCR differed significantly ($F = 92.356$, $df = 3$, $p < 0.001$) among the experimental conditions (Table S2, Supplementary material) (Figure 3). Since this dataset did not satisfy the assumption of homogeneity of variances a non-parametric test was also applied and showed a significant difference in the mRNA expression levels between 20 °C and 35 °C ($H = 9.974$, $df = 3$, $p < 0.050$) (Table S2, Supplementary material).

Expression pattern of the *hsc70* gene

The pair of *hsc70* primers amplified a fragment with high homology to the *hsc70-1* gene from *C. carpio* (78.2% identity; AY120893), followed by *hsc70* from *D. rerio* (81.5% identity; L77146), *M. amblycephala* (80.9% identity; EU623471) and *Ctenopharyngodon idella* (80.1% identity; EU816595). The *hsp70* gene sequences of *S. torgalensis* and *S. carolitertii* were deposited in GenBank under accession numbers JQ608475 and JQ608474, respectively.

The levels of *hsc70* gene expression in muscle and fin clips from *S. carolitertii* were similar in both tissues, but this was not the case for *S. torgalensis* (Figure S1, Supplementary material); the latter species showed higher expression in the fins compared to muscle and all subsequent analyses were done with fins.

Individuals of *S. torgalensis* exposed to 35 °C showed a 14-fold increase in *hsc70* mRNA levels compared to 20 °C (control condition) and an ~12-fold increase compared to 30 °C (Figure 4). One-way ANOVA indicated significant differences in the expression levels of the *hsc70* gene among the four temperatures ($F = 12.504$, $df = 3$, $p < 0.001$) and *post-hoc* comparisons identified a difference between the 35 °C treatment and the other three temperatures (Table S3, Supplementary material). Kruskal-Wallis analysis confirmed the presence of significant differences among the experimental conditions ($H = 15.351$, $df = 3$,

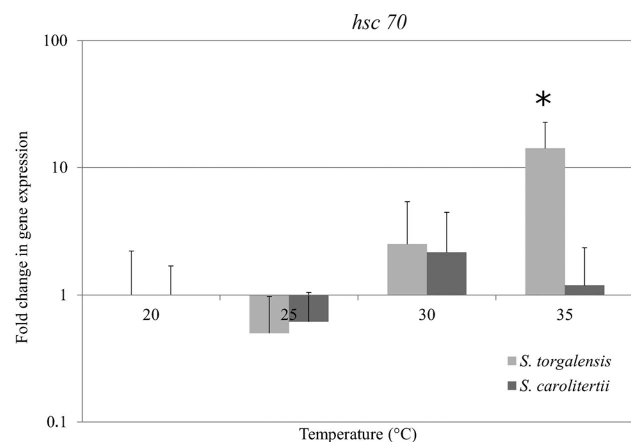

Figure 4 - Fold change in *hsc70* transcript expression in *S. torgalensis* and *S. carolitertii* compared to 20 °C (control condition), as assessed by semi-quantitative PCR. The columns are the mean ± SD of 6 or 7 fish. p < 0.05 compared to all other treatments.

p < 0.005). Although the non-parametric *post-hoc* test showed no significance between the 30 °C and 35 °C treatments, a significant difference was still observed between the 20 °C and 35 °C groups (Table S3, Supplementary material). In contrast, the increase in mRNA levels in *S. carolitertii* was not greater than three-fold, with the greatest increase occurring at 30 °C, although this was not statistically significant ($F = 1.439$, $df = 3$, $p > 0.200$) (Figure 4).

Real-time PCR confirmed the significant increase in *hsc70* expression in *S. torgalensis* at 35 °C ($F = 4.481$, $df = 3$, $p < 0.050$), whereas *S. carolitertii* showed no significant differences among the experimental conditions ($F = 1.391$, $df = 3$, $p > 0.300$) (Table S4, Supplementary material) (Figure 5).

Discussion

In this study, we used fin samples (instead of other organs) to measure *hsp70* transcript expression, thereby avoiding the euthanasia of animals, which is a particularly relevant consideration when studying endangered species. Our finding agree with those of Yamashita *et al.* (2004) who found similar patterns of Hsp70 expression in muscle and in fibroblasts cultured from caudal fin tissue of *Xyphophorus maculatus*. In *S. carolitertii*, fin clips and muscle showed similar patterns of *hsc70* expression, but this similarity was not so evident for *S. torgalensis*. However, this result needs to be interpreted with caution given the small number of muscle samples used from the latter species. Nevertheless, there was an increase in *hsc70* mRNA expression in fins of *S. torgalensis* in response to higher temperatures.

As shown here, there was an increase in *hsp70* mRNA levels in *S. torgalensis* individuals exposed to higher temperatures, as also reported for *hsp70s* in other species (Buckley *et al.*, 2001; Yeh and Hsu, 2002; Yamashita *et al.*, 2004; McMillan *et al.*, 2005; Fangue *et al.*, 2006; Karl *et al.*, 2009; Sørensen *et al.*, 2009; Sarup and Loeschcke, 2010; Waagner *et al.*, 2010). There were significant differences in the expression of this gene between *S. torgalensis* exposed to 20 °C and those exposed to other temperatures, particularly 35 °C. This result was somewhat expected since *S. torgalensis* inhabits an environment that is susceptible to extreme conditions (such as small ponds that can reach high temperatures during the dry season) and should therefore be able to deal with protein denaturation. In contrast, *S. carolitertii* showed no significant increase in *hsp70* expression levels, which suggests that this species is unable to respond to stressful conditions associated with elevations in temperature. Unlike *S. torgalensis*, which showed the largest induction of *hsp70*, some individuals of *S. carolitertii* died at 35 °C, possibly because of this species inability to adjust to thermal stress. The failure of *S. carolitertii* to increase the expression of *hsp70* may reflect its poor ability to adapt to 35 °C; this conclusion agrees with the fact that in its natural environment this species never experiences temperatures > 31 °C (SNIRH).

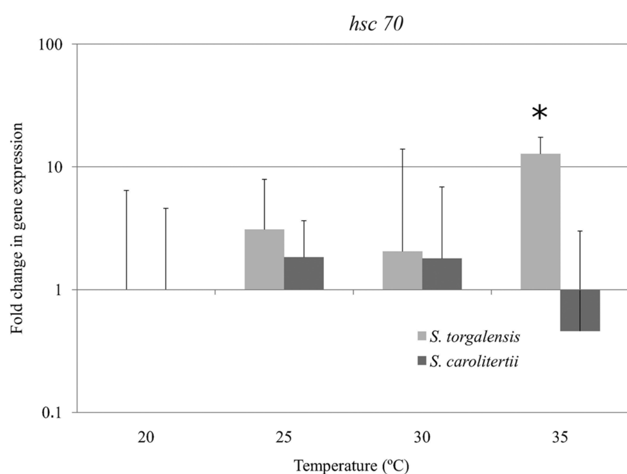

Figure 5 - Fold change in *hsc70* transcript expression in *S. torgalensis* and *S. carolitertii* compared to 20 °C (control condition), as assessed by real-time PCR. The columns are the mean ± SD of 6 or 7 fish. p < 0.05 compared to all other treatments.

However, other mechanisms may also be involved in the responses to thermal stress, including the hormone cortisol, heat shock factors (involved in the regulation of the heat shock response), other *hsps* and even transcripts that encode other proteins (such as the protein Wap65) (Tomanek and Somero, 2002; Frydenberg *et al.*, 2003; Kassahn *et al.*, 2007; Sarropoulou *et al.*, 2010; Tymchuk *et al.*, 2010; Celi *et al.*, 2012). To clarify the molecular mechanisms involved, future experiments should examine how temperature influences cortisol levels in both species since interactions between Hsp and cortisol are known to be involved in stress responses (Celi *et al.*, 2012). The divergent response between the two species may also reflect the more stable environment, with less severe temperature variations, in northern rivers compared to southern rivers (SNIRH).

The *hsc70* gene is often considered to be part of constitutive cell functions in non-stress situations such that an increase in temperature may either decrease or have no effect on the expression of this gene (Yeh and Hsu, 2002; Yamashita *et al.*, 2004; López-Maury *et al.*, 2008). As shown here, there was no significant variation in *hsc70* mRNA expression in *S. carolitertii* at the different temperatures. In contrast, *S. torgalensis* showed a significant increase in *hsc70* expression in fins at 35 °C when compared with the other temperatures. Thus, *S. torgalensis* can enhance the mRNA expression of inducible *hsp70* and constitutive *hsc70* in response to increases in temperature. The latter finding is similar to that of Fangue *et al.* (2006) who reported an increase in *hsc70* mRNA levels during heat stress in *F. heteroclitus* from southern North America. In addition, ATPase activity has been observed in *Gillichthys mirabilis* Hsc70 at high temperatures, suggesting that this protein can function even at extreme temperatures (Place

and Hofmann, 2001). With regard to our findings, the lack of an increase in mRNA expression levels in muscle makes it difficult to conclude that *hsc70* expression confers protection against thermal stress, although the enhanced expression in fins may indicate that the extensive contact surface of this tissue with the external environment might favor this response. Another possible explanation for the variation in mRNA levels between these tissues could be the existence of negative feedback (between Hsp and mRNAs) in the regulation of *hsp* gene expression (Celi *et al.*, 2012).

The increase in *hsp70* expression seen at higher temperatures in *S. torgalensis* may be important in the degradation and re-folding of denatured proteins and suggests that these fish are adapted to deal with high temperatures when they are trapped in ponds during the dry season; in contrast, *S. carolitertii* is unable to deal with such high temperatures. Magalhães *et al.* (2003) stated that *S. torgalensis* has traits typical of species adapted to harsh environments (short life span, earlier spawning age and small body size compared to other *Squalius* that inhabit more stable environments). In addition, species living closer to their thermal tolerance limits may be particularly prone to small changes in their thermal regime (Dahlhoff and Rank, 2007; Reusch and Wood, 2007; Sørensen *et al.*, 2009; Somero, 2010; Tomanek, 2010; Hoffmann and Sgrò, 2011). In this regard, intermittent systems such as that of the Mira river basin are particularly vulnerable to environmental changes. Changes in the seasonal regime of floods and droughts, with the increasing occurrence of severe droughts, may pose new challenges to these fish. Hence, to preserve this species, it would be advisable to promote habitat conservation with a particular emphasis on the conservation of refuges (pools) during the dry season (Sousa-Santos *et al.*, 2009; Henriques *et al.*, 2010).

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Internet Resources

SNIRH. Sistema Nacional de Informação de Recursos Hídricos. http://snirh.pt/ (August 22, 2010).

StatSoft I (2009) STATISTICA data analysis software system, http://www.statsoft.com.

Supplementary Material

The following online material is available for this article:

Table S1 - Semi-quantitative PCR assessment of *hsp70* transcript abundance in *S. torgalensis*.

Table S2 - Real-time PCR assessment of *hsp70* transcript abundance in *S. torgalensis*.

Table S3 - Semi-quantitative PCR assessment of *hsc70* transcript abundance in *S. torgalensis*.

Table S4 - Real-time PCR assessment of *hsc70* transcript abundance in *S. torgalensis*.

Figure S1 - *hsp70* transcript abundance in fin clips and muscle.

This material is available as part of the online article from http://www.scielo.br/gmb.

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Table S1 – Semi quantitative PCR post hoc comparisons for *hsp70* gene expression between treatments for *S. torgalensis*, using Tukey HSD test statistics. Each cell represents the p-value in each pairwise comparison. Significant differences ($P < 0.050$) are marked with *.

	20ºC	25ºC	30ºC	35ºC
20ºC		0.958	0.007*	0.000*
25ºC			0.002*	0.000*
30ºC				0.002*
35ºC				

Table S2 – Real-time PCR post hoc comparisons for *hsp70* gene expression between treatments for *S. torgalensis*, using Tukey HSD test statistics (upper diagonal) and Dunn's test (lower diagonal). Each cell represents the p-value in each pairwise comparison. Significant differences ($P < 0.050$) are marked with *.

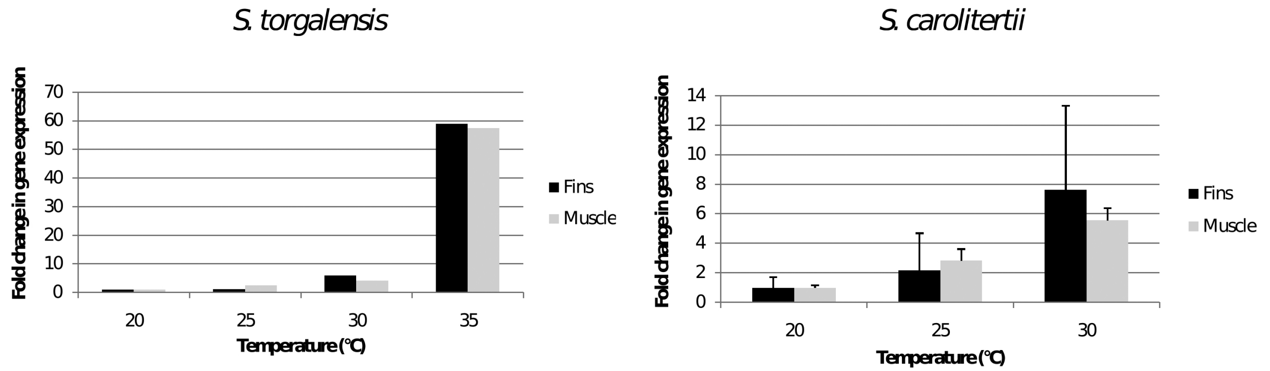
	20ºC	25ºC	30ºC	35ºC
20ºC		0.004*	0.000*	0.000*
25ºC	1.000		0.046*	0.000*
30ºC	0.249	1.000		0.000*
35ºC	0.013*	0.249	1.000	

Table S3 – Semi quantitative PCR post hoc comparisons for *hsc70* gene expression between treatments for *S. torgalensis*, using Tukey HSD test statistics (upper diagonal) and Dunn's test (lower diagonal). Each cell represents the p-value in each pairwise comparison. Significant differences ($P < 0.050$) are marked with *.

	20ºC	25ºC	30ºC	35ºC
20ºC		0.960	0.593	0.000*
25ºC	1.000		0.309	0.000*
30ºC	1.000	1.000		0.001*
35ºC	0.007*	0.004*	0.139	

Table S4 – Real-time PCR post hoc comparisons for *hsc70* gene expression between treatments for *S. torgalensis*, using Tukey HSD test statistics. Each cell represents the p-value in each pairwise comparison. Significant differences ($P < 0.050$) are marked with *.

	20ºC	25ºC	30ºC	35ºC
20ºC		0.445	0.755	0.031*
25ºC			0.938	0.272
30ºC				0.126
35ºC				



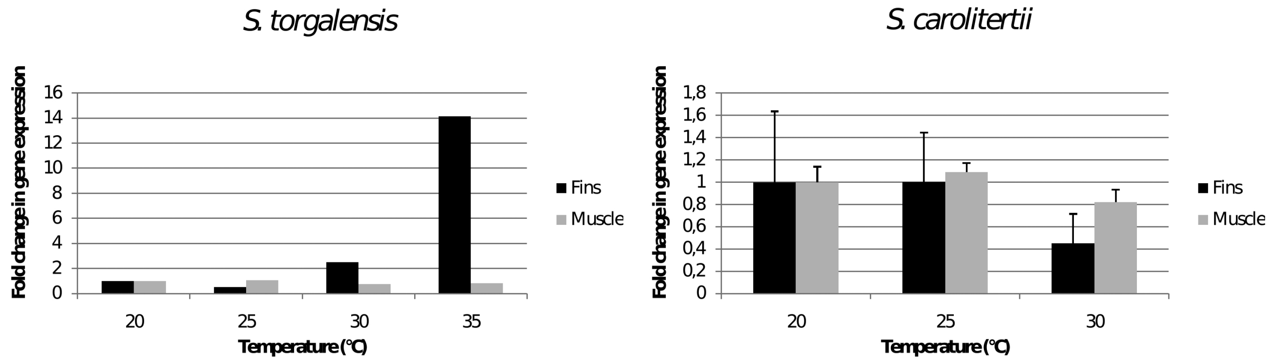


Figure S1 - *hsp70* transcript abundance in fin clips and muscle.