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 ectotherms 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 ectotherms, 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
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 andLoeschcke, 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).

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.

![Figure 1 - Geographical distribution of S. torgalensis and S. carolitertii in Portugal, with the respective sampling sites marked with triangles.](Image)
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 manufacturer’s 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 TTGAAGGCCTAAGACTCCAG (reverse) and the hsc70-specific primers GTTCAAGCAGCCATCTTAGC (forward) and TGACCTTCTCCTTGAGGC (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 ATCAGGCTAATGGTTAAAGTTGG (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; Brelijak and Gabrilovac 2005). The amplification conditions for the pair hsp70/gapdh were those described in the manufacturer’s instructions (QIAGEN multiplex PCR kit, Qiagen Inc., Valencia, CA, USA) (final concentration: 1 PCR master mix with 3 mM MgCl₂, 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 GoTaq Flexi DNA polymerase (Promega, Madison, WI, USA) with 0.3 μM of each primer, 0.25 mM of each dNTP and 2 mM of MgCl₂. 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 AATTCACCTGCACCCACG (forward) and TCTCCTTTTGCCTCAGTCTG (reverse) and TTTGCTTTGGATGTCACCT (forward) and GTGGGAATGTGCTGGTTC (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 GTACAAGGGTGAAGGTTAAGGC (forward) and GTGATGCGAGGTGCTACATACGT (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 as follows:

\[ I = \frac{\sum x_i}{n\bar{x}_{20}} \]

where \( I \) 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
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 ($H = 15.351, df = 3$).
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 Xiphophorus 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).

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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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.