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Transcriptome profiling of two Iberian freshwater fish exposed to thermal stress

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ABSTRACT

The congeneric freshwater fish *Squalius carolitertii* and *S. torgalensis* inhabit different Iberian regions with distinct climates; Atlantic in the North and Mediterranean in the South, respectively. While northern regions present mild temperatures, fish in southern regions often experience harsh temperatures and droughts. Previous work with two *hsp*70 genes suggested that *S. torgalensis* is better adapted to harsher thermal conditions than *S. carolitertii* as a result of the different environmental conditions. We present a transcriptomic characterisation of these species' thermal stress responses. Through differential gene expression analysis of the recently available transcriptomes of these two endemic fish species, comprising 12 RNA-seq libraries from three tissues (skeletal muscle, liver and fins) of fish exposed to control (18 °C) and test (30 °C) conditions, we intend to lay the foundations for further studies on the effects of temperature given predicted climate changes.

Results showed that *S. carolitertii* had more upregulated genes, many of which are involved in transcription regulation, whereas *S. torgalensis* had more downregulated genes, particularly those responsible for cell division and growth. However, both species displayed increased gene expression of many *hsps* genes, suggesting that they are able to deal with protein damage caused by heat, though with a greater response in *S. torgalensis*. Together, our results suggest that *S. torgalensis* may have an energy saving strategy during short periods of high temperatures, re-allocating resources from growth to stress response mechanisms. In contrast, *S. carolitertii* regulates its metabolism by increasing the expression of genes involved in transcription and promoting the stress response, probably to maintain homoeostasis. Additionally, we indicate a set of potential target genes for further studies that may be particularly suited to monitoring the responses of Cyprinidae to changing temperatures, particularly for species living in similar conditions in the Mediterranean Peninsulas.

1. Introduction

Temperature is crucial to survival, and thermal adaptation is increasingly of interest given the growing threat of climate change. Freshwater ecosystems are particularly prone to the effects of climate change, such as shifts in thermal, precipitation and flow regimes (Field et al., 2014). Often, this is coupled with an increase in the severity and frequency of droughts, ultimately resulting in an increase in mean water temperature and a decrease in oxygen concentration (Field et al., 2014). Such changes in natural freshwater systems directly influence survival and persistence of extant populations. Ectotherms, such as fish, are especially vulnerable to environmental temperature changes since their body temperature strongly relies on it (Berg et al., 2010). Therefore, to cope with these changes, fish must either exhibit phenotypic plasticity or adapt through micro-evolution, since migration to a more suitable river is often not possible or easily achieved (Bellard et al., 2012).

The Iberian Peninsula presents two distinct types of climate, the Atlantic in the north and Mediterranean in the south (Carvalho et al., 2010). Northern rivers present lower temperatures and fewer temperature fluctuations, ranging from 3 to 31 °C throughout the year. In contrast, southern rivers are characterized by an intermittent regime of floods and droughts in which freshwater fish are exposed to higher temperatures, ranging from 4 to 38 °C, which also results in lower oxygen concentrations (Magalhães et al., 2003; Henriques et al., 2010; Jesus et al., 2013). These

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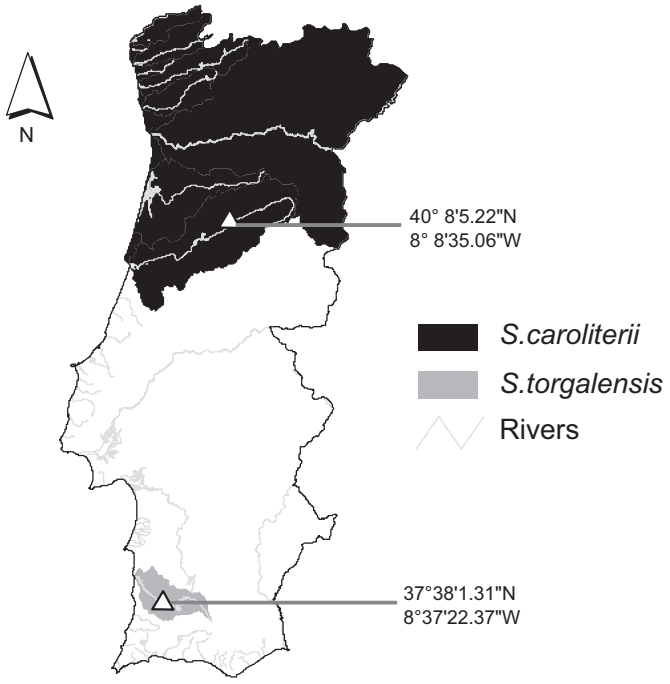

Fig. 1. Species distribution map. Sampling sites are marked with a triangle.

southern rivers are also more likely to be exposed to extreme temperatures and more extended drought periods (Füssel et al., 2012).

The *Squalius* genus (Cyprinidae family) presents an opportunity to study closely related species under distinct climate scenarios because some species are endemic to certain river basins or regions. *S. carolitertii* (Doadrio, 1988) inhabits the northern region of the Iberian Peninsula (Atlantic climate), whereas *S. torgalensis* (Coelho et al., 1998), a critically endangered species (Cabral et al., 2006), is restricted to the Mira river basin in the southwestern region (Coelho et al., 1998) (Mediterranean climate) (Fig. 1). Hence, the two species are adapted to different environmental conditions, with distinct seasonal and even daily water temperature variations (Magalhães et al., 2003; Jesus et al., 2013).

From a physiological point of view, little is known about the responses of these two species to thermal stress, with only one study characterizing changes in gene expression of two Heat Shock Proteins (HSPs) in response to thermal stress (Jesus et al., 2013). In that study, fish of both species were exposed to four temperature treatments (20 °C, 25 °C, 30 °C and 35 °C), with increments of 1 °C per day, and, after reaching the test temperature, fin clips were collected for gene expression. *S. carolitertii* presented no significant changes in the expression of *hsp*70 and *hsc*70, whereas both genes were significantly upregulated in *S. torgalensis* when exposed to a higher temperature (35 °C). Also, two out of seven individuals of *S. carolitertii* did not survive at 35 °C, whereas all *S. torgalensis* individuals survived all treatments. Based on those results, it was suggested that *S. torgalensis* is better adapted to harsher thermal conditions than *S. carolitertii*. However, thermal stress responses are more complex and certainly involve the regulation of other genes (Lindquist and Craig, 1988; Murtha, 2003; López-Maury et al., 2008; De Nadal et al., 2011).

The recent availability of the transcriptomes of both these species, *S. carolitertii* and *S. torgalensis*, (Genomic Resources Development Consortium et al., 2015), comprising 12 RNA-seq libraries and sequence information from three different tissues (fins, liver and skeletal muscle) at two temperatures (18 °C and 30 °C), made it possible for us to perform a more comprehensive analysis of their responses to increasing temperatures. Here, we take advantage of these transcriptomes to profile the gene expression responses to thermal stress in three different tissues of these two species, thereby extending our previous research (Jesus et al., 2013). Specifically, we aimed to (i) characterize the transcriptomic responses of both species to heat stress, both quantitatively and qualitatively; and (ii) search for a set of target genes involved in relevant functional categories for thermal stress responses in fish.

2. Methods

2.1. Data Acquisition

The recently available transcriptomes of *S. carolitertii* and *S. torgalensis* were obtained from Dryad (entry doi:http://dx.doi.org/10.5061/dryad.fm28d) and raw sequences were accessed in NCBI SRA (project accession numbers SRP049802 and SRP049801). For these transcriptomes, adult fish (6–7 cm) of *S. carolitertii* and *S. torgalensis* were captured, by electrofishing (300 V, 4 A), in Mondego and Mira rivers, respectively (Fig. 1). Sampling was carried out during spring, when water temperature varied from 18 °C to 22 °C, approximately. Fish were maintained in groups of seven fish in four aquariums of ~30 L, two for each species. Temperature was kept constant at 18 °C with a 12 h photoperiod and fish were fed once a day with commercial flake food, for two weeks. After these two weeks of acclimation, the temperature was raised 1 °C/h until 30 °C in one aquarium for each species, where fish were kept for 1 h before being euthanized. Temperature was kept constant at 18 °C in the remaining aquaria, and the fish they contained were maintained at acclimation conditions and euthanized at the same time as the test group. In both cases, euthanasia was carried out with tricaine mesylate (400 ppm of MS-222; Sigma-Aldrich, St. Louis, MO, USA), followed by decapitation to guarantee death prior to organ harvesting. In all aquariums, normoxic conditions were maintained (6–8 mg/L of O_2).

RNA was extracted as described in Genomic Resources Development Consortium et al. (2015) and samples of the same tissue were pooled prior to sequencing, comprising 12 RNA-seq libraries (7 pooled individuals per library), with 6 libraries per species. For each species, there are two libraries per tissue (fins, liver and skeletal muscle); one from a control condition of 18 °C, and another from a test condition of 30 °C (the temperature was raised 1 °C/h from 18 °C up to 30 °C). The detailed experimental design, as well as the transcriptome assembly procedure, can be found at Genomic Resources Development Consortium et al. (2015).

2.2. Differential gene expression

Abundance estimation was performed by aligning the raw reads of a given library against the respective species transcriptome (available at Dryad entry doi:http://dx.doi.org/10.5061/dryad.fm28d) using bowtie 0.12.9 (Langmead et al., 2009). Then, RSEM 1.2.8 (Li and Dewey, 2011) was used to compute expression, both in read counts and fragments per kilobase of exon per million fragments mapped (FPKMs) (Trapnell et al., 2010).

In order to assess similarity between tissues, samples were grouped based on hierarchical clustering (Euclidean distance) using expression values ($\log_2$ FPKM) of the 4000 most variable contigs across all samples of each species.

Differential gene expression analyses were performed in EdgeR, Bioconductor R package (Robinson et al., 2010), using the run_DE_analysis.pl script from the Trinity package (Grabherr et al., 2011). For these analyses, we compared two temperatures for each tissue and each species (e.g. Liver 18 °C vs Liver 30 °C). Transcripts with a sum of read counts < 10 in both conditions were discarded in further analyses and we used the statistical cut-off of a false