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Acclimation of killifish to thermal extremes of hot spring: Transcription of gonadal and liver heat shock genes

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ABSTRACT

In this study, we explored the hypothesis that killifish acclimate to thermal extremes through regulation of genes involved in stress and metabolism. We examined the liver and gonadal transcription of heat shock proteins (*hsp70*, *hsp90a*, *hsp90b*), *glucokinase* (*gck*), and *high mobility group b1 (hmgb1) protein* in wild killifish species from hot springs and rivers using quantitative real-time PCR. Moreover, we exposed a river killifish species to a long-term thermal regime of hot spring (37–40 °C) and examined the liver transcription of the heat shock genes. Our results showed that hot spring killifish showed a significant, strong upregulation of liver *hsp90a*. Moreover, the testicular transcript levels of *hsp90a*, *hsp90b*, and *hsp70* were higher in hot spring killifish than the river ones. The results of the common garden experiments showed that the transcripts of *hsp70*, *hsp90b*, and *hmgb1* were mildly induced (> twofold) at the time when temperature reached to 37–40 °C, while the transcripts of *hsp90a* were strongly induced (17-fold increase). The level of *hsp90a* was dramatically more upregulated when fish were maintained in thermal extreme (42-fold change higher than in ambient temperature). Moreover, a significant downregulation of *gck* transcripts was observed at the time when temperature was raised to 37–40 °C (80-fold decrease) and during exposure to long-term thermal extreme (56-fold decrease). It can be concluded that the regulation of heat shock genes particularly *hsp90a* might be a key factor of the acclimation of fish to high temperature environments like hot springs.

1. Introduction

Temperature has quite rightly been termed the 'ecological master factor' as it has a profound effect on the physiology of all ectothermic animals (Gamperl and Farrell, 2004). Environmental temperatures have extensive biological implications for all organisms, but ectotherms in aquatic systems are particularly affected by thermal profiles and climate regimes. This is because aquatic ectotherms primarily exchange heat with their environment through conduction and convection, and their body temperature closely follows the temperature of surrounding water. Thus, environmental temperatures greatly influence the distribution, physiology, and behavior of aquatic organisms, and local adaptation to thermal systems is the norm (Basu et al., 2002; Fangue et al., 2006; Narum et al., 2013).

With reference to temperature, thermal waters are normally considered as those having temperatures sufficiently high so that members of the general freshwater fauna do not usually live in them. Nevertheless, some fish species have successfully survived and adapted to the high temperature of hot springs. The presence of a few cyprinid, cyprinodont, and cichilid species in the hot spring environment (Coad, 1980; Piazzini et al., 2010; Tutar and Okan, 2012) seems to be a remarkable phenomenon as prolonged exposure to such thermal extremes may result in rapid deterioration in physiologic state (LeMoullac and Haffner, 2000). Because these thermal effects have such major consequences for cellular function, fish in thermal waters are assumed to manifest extensive evolutionary adaptations that establish distinct thermal optima and limits for physiological function, as well as the capacity for altering the phenotype in response to constant thermal extremes that could vastly impact various life-history stages (e.g. early development, reproduction).

Thermal acclimation responses in ectotherms commonly result in gene expression changes at a large number of loci associated with protein processing, transcription, and translation (Kassahn et al., 2007). So, identifying differentially expressed genes and pathways under thermal extreme conditions would be important for the understanding of molecular acclimation of fish to extreme thermal waters. Current molecular and genomic tools provide the opportunity to investigate the heat shock response of fishes from varying thermal regimes and link that information with adaptive regions of the genome that may be under selection (Narum et al., 2013). Specifically, the heat shock protein (HSP) response has been demonstrated to be one of the most important cellular mechanisms to prevent the damaging effects of thermal cellular stress (Feige et al., 1996; Werner et al., 2007). Hsp70 and Hsp90 are known to be the

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most strongly upregulated genes in response to chronic high temperatures in fish (Podrabsky and Somero, 2004). The regulatory factors and signaling pathways shaping metabolism and mitochondrial functioning may be also important for thermal acclimation or sensitivity (Windisch et al., 2011). The expression of genes involved in regulating metabolism may be expected to respond to heat shock, as cellular energy pools are accessed to fuel stress response and repair mechanisms. In fact, several genes involved in glycolysis, gluconeogenesis, the tricarboxylic acid cycle, and the electron transport chain could be induced or repressed during thermal stress in fish (Podrabsky and Somero, 2004; Buckley et al., 2006).

In south of Iran, there exist several geothermal waters, some with temperature more than 37 °C in which populations of killifish have been found. For example, a killifish population has adapted to live at temperatures 37–40 °C in a geothermal spring called Geno Hot Spring in Hormozgan provience, unlike the widespread river populations *Aphanius dispar* that live in nearby rivers at temperatures up to 25 °C. The killifish inhabiting Geno Hot Spring has been described as a separate species *Aphanius ginaonis* according to morphological characteristics (Coad, 1980) and otolith morphology (Reichenbacher et al., 2009), but no molecular work supports this species distinction.

Despite the importance of geothermal hot springs as unique environments with constant thermal extremes beyond the tolerance of many fish species, little is known about the acclimation of fish to such high temperature in this ecosystem. Because of its ability to thrive in this high temperature environment, we reasoned that killifish would be an excellent study organism for examining the adaptive responses of fish to extreme environmental conditions of hot springs. Here, as an initial step to elucidate the mechanisms that may be responsible for the capacities of killifish to cope successfully with thermal extremes, we examined the liver and gonad transcription of some candidate genes associated with stress response and metabolic regulation in wild killifish species from hot spring and river using quantitative real-time PCR. Heat shock proteins (*hsp70*, *hsp90a*, *hsp90b*), *glucokinase* (*gck*), and *high mobility group b1* (*hmgb1*) *protein* have been chosen as genes in which the transcription changes were studied. Moreover, we exposed a river killifish species *Aphanius dispar* to a long-term thermal regime of hot spring (37–40 °C) and examined the liver transcription of the heat shock genes as mentioned above.

2. Material and methods

2.1. Experimental animals and sampling

Fish were sampled using a small seine net from wild populations of killifish inhabiting two hot springs with different water temperatures and one river located in Hormozgan province, Iran (25°24', 59°15'E) Sampling sites were Geno hot spring (27°44'59"N, 56°29'87"E) with temperature 37–40 °C, a moderate hot spring called Khorgoo (27°50'91"N, 56°46'22"E) with temperature up to 32 °C and Navand river (27°26'44"N, 56°43'65"E) with temperature up to 25 °C. Liver and gonad tissues were dissected immediately after catching and frozen in liquid nitrogen. As the extreme temperatures can affect gonadal fate in fish (Ito et al., 2008; Breckels and Neff, 2013; Fernandino et al., 2013), gonads were chosen for studying sex-specific transcription of heat shock genes.

A number of killifish individuals from Navand River were then collected and used in common garden experiments to evaluate the degree of acclimation for key traits. The procedure of common garden experiments has earlier been described by Akbarzadeh et al. (2014). Briefly, the samples of killifish were collected from adjacent rivers near to the Geno hot spring Hormozgan, Iran using a small seine net in November 2012. Fish were transferred to the Aquaculture laboratory of University of Hormozgan, where they were held in 300-L circular tanks at ambient temperature (24 °C). Following a week of holding at ambient temperature, 114 uniform-sized animals were equally distributed between two groups including a control group (24 °C) and a treatment group (37–40 °C) with each replicated three times in 100 L tanks with a stocking density of 25 individuals/100 L water. Treatment tanks were raised gradually to 37–40 °C over the course of 7 days at a rate of 2 °C per day. Once at this temperature, 10 individuals were sampled and killed with an overdose of anesthetic (MS-222 tricaine methanesulfonate) and the liver tissue was dissected from each fish and immediately deep-frozen in liquid nitrogen, and stored in a −80 °C freezer until RNA extraction. This temperature regime was designed to mimic the natural temperature experienced daily by *A. ginaonis* in Geno hot spring. The animals in control and thermal tanks were then maintained for a further 44 days on a 12:12 light cycle (12 h dark vs. 12 h light). Water temperature, dissolved oxygen, and pH levels were measured three times a day at 8:00 am, 14.00 pm, and 20:00 pm with a handheld multi-meter (WTW Multi 340i/SET, Germany). Animals were fed on a commercial fish diet two times each day (morning and afternoon). All the fish in all treatments were fed to satiation. One-half of the volume of water in each tank was exchanged for fresh aerated water once a day. Tanks were cleaned daily and any mortality removed and weighed. The mortalities of fish were counted and monitored in both control and thermal stress groups. After 44 days, 10 specimens of killifish from both thermal and control groups were sampled as described above. Prior to the tissue sampling, the length and weight of each fish were measured. The samples were shipped to the Laboratory of Genetics at the University of Turku in Finland for further processing.

Liver tissue has been chosen as a target tissue in this study owing to its importance in metabolic adjustments to stressors and energy balance (Aluru and Vijayan, 2009). Previous research also linked this tissue type to **heat stress** responses (e.g. Hall et al., 2000; Nikinmaa et al., 2013; Rabergh et al., 2000; Smith et al., 2013). The fish care complied with the guidelines of The Iranian Society for The Prevention of Cruelty to Animals, and Iranian Department of Environment.

2.2. Gene expression

For RNA preparation, 100 mg portion of liver tissue and gonads from six individuals from each of the thermal conditions was cut and homogenized in a Qiagen Tissue Lyser using 1 ml Tri Reagent TRI Reagent® (Ambion, Austin, TX, USA), treated with DNase (Promega, USA) in accordance with the manufacturers' instructions. RNA quantification was carried out with a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) reading at 260/280 nm and the quality of the RNA checked with the BioAnalyzer 2100 using the 6000 Nano LabChip kit (Agilent Technologies). All RNA integrity number values obtained were >8, indicative of excellent RNA integrity and quality. One microgram of total RNA was used to synthesize first-strand cDNAs using iScript™ cDNA Synthesis kit (BioRad) for RT-PCR, using a mixture of oligo-dT and random hexamers as primers following the manufacturer's instructions.

2.3. Primer design

The qPCR primers for *hsp70*, *hsp90a*, *hsp90b*, *gck*, *hmgb1*, actin beta (*ACTB*) and elongation factor alpha (*EF1A*) were designed based on the sequences from killifish species available in the GenBank (Accession Nos.: DQ202279.1, DQ202280.1, HM017074.1, DQ202281.1, DQ202282.1, CK817284.1, CD670472.1, CV822354.1, CK817293.1, HM017072.1, AY430091.1, and HM017075.1) and conserved regions of the teleost sequences published from GenBank (Accession Nos.: GU065315.1, GU065314.1, EU518494.1, NM_010439.3, NM_001139629.1, DQ403261.1, AY190702.1, AY222742.1, and L23807.1). Multiple qPCR primer combinations were designed for each gene using Primer3 (Table 1). The specificity and size of the amplicons obtained with primer pairs was checked on a 1.5% agarose gel. The fragments were sequenced using the ABI 3130 Genetic