1 **Growth hormone transgenesis affects thermal tolerance in zebrafish (*Danio rerio*)**

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2 **Highlights:**

- 3 - GH-transgenesis increased the survival rate of juveniles at low temperature;
- 4 - High temperature is more lethal for juvenile and adult GH-transgenic zebrafish;
- 5 - GH-transgenesis increased expression of *hsf1a*, *hsp47*, and *hsp70* genes in juvenile zebrafish;
- 6 - *hsf1a*, *hsp47*, *hsp70*, *hsp90a*, and *hsp90b* genes expression is diminished in adult zebrafish
- 7 GH-transgenesis exposure at high temperature.

Abstract

In fish, growth hormone (GH)-transgenesis may modify physiological mechanisms of adaptation when challenged by biotic and abiotic stressors. Thus, we evaluated whether GH overexpression can alter the thermal tolerance of adult and juvenile GH-transgenic zebrafish (*Danio rerio*). This study compared the thermal tolerance in non-transgenic (NT) and GH-transgenic (T) zebrafish exposed to 13 °C, 39 °C, or 28 °C (control) for 96 h. Mortality rate was checked every 12 h in juvenile (8 week-old) and adult males (6 month-old). Exposure to different temperatures revealed that GH overexpression increases the tolerance of transgenic juveniles exposed to 13 °C and diminishes the tolerance of juveniles and adults, when exposed to 39 °C. Additionally, we have analyzed transcriptional expression from the heat shock proteins (HSPs), which are mainly involved in the thermal tolerance mechanism. The mRNA level analysis results revealed that, under controlled conditions (28 °C), GH-transgenesis upregulates the expression of *hsp47*, *hsp70*, *hsp90a* and heat shock transcription factor (*hsf1a*) in transgenic juveniles, although the same result was not observed in transgenic adults. Exposure to low temperature did not alter the expression of any analyzed gene, both in adults and in juveniles. Exposure to 39 °C decreased the expression of all genes analyzed, in GH-transgenic adults. Furthermore, the HSP expression pattern was analyzed via hierarchical clustering. This analysis revealed two major clusters illustrating the dependency of gene changes related to age. These results indicate that the GH overexpression can alter thermal tolerance of fish, depending of age and temperature.

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1 Introduction

2 Growth hormone (GH) is a single-chain polypeptide that is synthesized, stored, and secreted
3 by the adenohypophysis, which intensifies all major aspects of amino acid uptake and protein
4 synthesis. GH-induced signaling is mediated by a group of tyrosine kinases, Janus kinases
5 (JAKs) and their substrates, and signal transducers and activators of transcription (STATs), with
6 the insulin-like growth factor 1 (IGF-1) being its main biological effector (Kiu and Nicholson,
7 2012).

8 GH is the principal stimulator of somatic growth, and the greatest growth is one of the most
9 desired traits of aquaculture. For this reason, fish have been genetic manipulated, showing
10 significantly enhanced somatic growth via muscle hypertrophy (Kuradomi et al., 2011), or
11 hyperplasia (Hill et al., 2000).

12 GH-transgenesis has proved to increase the growth performance in several aquaculture fish
13 species with impressive results, including tilapia *Oreochromis niloticus* (Rahman et al., 1998),
14 carp *Cyprinus carpio* L. (Guan et al., 2008) and various salmonids such as coho salmon
15 *Oncorhynchus kisutch* (Devlin et al., 2004). However, it is still necessary to assess the potential
16 ecological consequence of growth-enhanced animals, considering that GH-transgenesis may
17 modify physiological mechanisms of adaptation when challenged by biotic and abiotic factors.
18 Among the different effects caused by transgenesis, it is important, both ecologically and for
19 culture, to assess the change in tolerance to temperature. The effect of exposure to temperature
20 was investigated in GH-transgenic coho salmon (*O. kisutch*), respectively (Chen et al. 2015). In
21 this study, Chen and collaborators concluded that genetic modification of growth-related
22 pathways can affect thermal performance, especially at physiological optimum or sub-critical
23 temperatures.

24 To cope with the potential effects of environmental stress, fish respond with different
25 strategies. The cellular stress response, characterized by an increase in the synthesis of heat
26 shock protein (HSPs), is generally exhibited by all organisms (Schlesinger, 1990; Feder and
27 Hofmann, 1999). In order to evaluate early ontogenic effects of GH transgenesis on the
28 respiratory and cellular physiology of Atlantic salmon, Polymeropoulos et al. (2014)
29 investigated the effects of GH transgenesis and polyploidy on metabolic, heart and ventilation
30 rates and HSP levels after exposure to acute hypoxia in post-hatch alevins.

31 The HSP family comprises proteins that were originally identified based on their excess
32 synthesis in cells exposed to elevated temperatures and were subsequently induced by exposure
33 of cells to various stresses (Iwama et al., 1998; Basu et al., 2002). These proteins are named
34 according to their molecular weight, emphasizing those with low molecular weight (16–47
35 KDa), HSP70 (68–73 KDa) and HSP90 (85–90 KDa).

36 Transcription of HSPs depends on the activation of a specific transcription factor, the heat
37 shock factor (HSF-1), which binds to the heat shock element (HSE) in the HSP gene promoters
38 (Morimoto, 1998). Activation of the HSP promoters is mediated via nuclear factors NF-IL6 and

1 STATs, suggesting that HSP transcription can also to mediated by the JAK/STAT pathway
2 (Stephanou et al., 1999; Madamanchi et al., 2001; Schoof et al., 2009). More recently, Jegu et
3 al. (2019) reviewed how HSPs expression is modulated by STAT signaling pathways.
4 Nevertheless, it is necessary to understand whether JAK/STAT pathway GH-induced can be a
5 direct modulator of HSP expression, since the excessive GH presumably affects the (HSPs)
6 expression (Deane and Woo, 2005; Deane and Woo, 2010).

7 Considering all the previously mentioned aspects, our study aimed to test the hypothesis
8 that GH overexpression can alter the thermal tolerance of adult and juvenile transgenic
9 zebrafish. As a result, we exposed juveniles and adults from a GH-transgenic zebrafish lineage
10 (produced as described in Figueiredo 2007) to thermal stress. Tolerance to low and high
11 temperature was related to *hsp* transcriptional expression.

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1 **Material and Methods**

2 *Fish*

3 Transgenic and non-transgenic zebrafish were obtained from crosses between non-
4 transgenic females and hemizygous GH-transgenic males from lineage F0104. The lineage
5 F0104, used in this study, were produced by Figueiredo et al. 2007. Freshly fertilized eggs were
6 collected for microinjection and transgenic zebrafish produced using two transgenes containing
7 the carp (*Cyprinus carpio*) β -actin promoter driving the expression of either the *A. Victoria* GFP
8 gene (pc β A/GFP plasmid) or the marine silverside fish (*Odonthestes argentinensis*) growth
9 hormone (msGH) cDNA (pc β A/msGH plasmid).

10 The fish underwent fasting during temperature treatment period (96 h for thermal
11 tolerance assay; 12 h and 2 h for expression gene assay, in 13 °C and 39 °C, respectively). All
12 experimental procedures were performed as indicated by the Ethics for Animal Use Committee
13 from the Federal University of Rio Grande, where this work was carried out
14 (23116.005574/2013-03).

15 *Temperature tolerance of GH-transgenic zebrafish*

16 Juvenile (8 week-old) and adult males (6 month-old) of non-transgenic and transgenic
17 zebrafish were exposed to 13 °C, 39 °C, or 28 °C (control) for 96 h in an incubator. To avoid
18 rapid changes of water temperature, fish were first put into each incubator at a temperature of 28
19 °C and temperature was increased or decreased in a continuous linear rate of ± 3 °C·h⁻¹.
20 Mortality rate was checked every 12 h. Each experimental temperature was tested in triplicate
21 containing 10 fish in each experimental unit (one fish per liter for adults and five fish per liter
22 for juveniles). A total of 60 adult fishes (30 non-transgenic and 30 transgenic) and 60 juvenile
23 fishes (30 non-transgenic and 30 transgenic). The experimental tank were continuously aerated
24 and parameters such as temperature, pH, nitrate concentration, and photoperiod (14h light/ 10h
25 dark) were daily monitored. Death criteria included the lack of both operculum movement and
26 twitching reflex upon prodding with a needle.

27 *mRNA level*

28 All experimental groups were exposed to 13 °C and 39 °C for 12 and 2 h, respectively. The
29 exposure times were those considered as sublethal according to data from the thermal tolerance
30 experiment. Control animals also exposed to 28 °C, and sampled at the same experimental
31 condition as the treated groups (12 h for control of 13 °C and 2h for control of 39 °C). mRNA
32 level was determined in livers from zebrafish of all groups ($n = 6$ per group). The liver, the hub
33 of metabolism, was selected as the target tissue. Livers were pooled in juveniles (3 independent
34 pools per treatment, 18 fish in total per group). For adults were collected liver individually ($n =$
35 6 fish per group). Total RNA was prepared with Trizol reagent (Invitrogen, Brazil) and purified

1 with RNase free DNase I (Invitrogen, Brazil), following the manufacturer's protocol. For
2 cDNA synthesis, 2 µg of total RNA was reverse-transcribed using a High Capacity cDNA
3 Reverse Transcription Kit (Invitrogen, Brazil). The obtained cDNA was used as a template for
4 gene amplification using specific primers. Gene-specific primers (Table 1) were designed based
5 on sequences available in GenBank using the Primer Express 3.0 software (Applied Biosystems,
6 Brazil). Previously, the PCR amplification efficiency of each primers pair was evaluated by
7 serial dilutions reactions where the efficiency of reactions showed appropriate parameters.
8 Quantitative
9 PCR was performed with an ABI 7500 System (Applied Biosystems, Brazil) using Platinum
10 SYBR Green qPCR SuperMix-UDG (Invitrogen, Brazil). The PCR program consisted of 40
11 cycles of 95 °C for 15 s and 60 °C for 30 s after an initial cycle of 50 °C for 2 min and 95 °C for
12 2 min. The synthesized cDNA (1 µL) was used for the qPCR reactions in a total volume of 10
13 µL and a final concentration of 200 nM per each primer. Triplicates were run for each sample.
14 Melting curve analysis was performed during qPCR to identify the presence of primer dimers
15 and analyze the specificity of the reaction Expression levels of target genes were normalized
16 using the expression of two housekeeping genes, elongation factor 1a (*ef1a*) and β-actin (*bac*).
17 These genes were choosen as reference genes after presented stability when tested with geNorm
18 applet (Vandesompele et al. 2002).

19 20 *Statistical analyses*

21 Fish survival curve (%) among treatments was performed by the log-rank test, where significant
22 differences were considered at $p < 0.05$. The data were analyzed using GraphPad Prism®
23 following the instructions of GraphPad Statistic Guide for comparisons of survival curves. The
24 accumulative survival at 12 h was analyzed by Student's t test, comparing non-transgenic and
25 transgenic, in each temperature separately. Relative mRNA level were analyzed by Relative
26 Standard Curve Method between the transgenic and non-transgenic groups, in each temperature.
27 Expression levels of target genes were normalized by normalization factor using the expression
28 of two housekeeping genes, elongation factor 1a (*ef1a*) and β-actin (*bac*). The values obtained
29 were statistically analyzed by Student's t test. Hierarchical clustering of gene expression and
30 heat maps were produced using PermutMatrix, using McQuitty's method (Caraux and Pinloche,
31 2005).

32

Table

Table 1. Primer sequences used for RT-qPCR analysis of mRNA expression in zebrafish (*Danio rerio*)

Gene	Function	Forward primer 5'-3'	Reverse primer 5'-3'	GenBank Accession Number
<i>hsf1a</i>	Mediator of stress induced transcription of HSP genes	AGCCAGTCTTCCGCCCTGACT	ATGAAGCTCCGTCTTGTCCAGGCA	AF159134
<i>hsp47</i>	Collagen biosynthesis and responsive heat shock	GACGAGCATCTGCATACGGGCT	TCACATTGCGGGTCTGCGGG	U31079
<i>hsp70</i>	Proteins synthesis, translocation, and folding	AAGCGGTGGCTTACGGTGCC	CGTCCAGCAGCAGCAGGTCC	AF210640
<i>hsp90a</i>	Regulator of conformational stability, cellular stress, formation of the steroid receptor complex	ACTCGGACTTGGCATCGATGACG	ACGGGCATATCTTCTCAGCGGGT	AF068773
<i>hsp90b</i>	Developmental stability, cellular stress, formation of the steroid receptor complex	GCTGCAGCAGAGCAAGATCCTCA	CTTGTCTCTCGGCGACGTCCG	AF068772
<i>bactin</i>	Cellular motility, structure and integrity	TCTGTCCACCTTCCAGCAGAT	GATGGACCTGCCTCGTCGTA	AF180887
<i>ef1a</i>	Protein synthesis	GGGCAAGGGCTCCTTCAA	CGCTCGGCCTTCAGTTTG	NM131263

hsf1a, Heat shock transcription factor; *hsp47*, Heat shock protein 47; *hsp70*, Heat shock protein 70; *hsp90a*, Heat shock protein 90 alpha; *hsp90b*, Heat shock protein 90 beta, *ef1a*, Elongation Factor 1-alpha; *bactin*, Beta-actin

1 Results and discussion

2 To evaluated whether GH overexpression can alter the thermal tolerance of adult and
3 juvenile GH-transgenic zebrafish (*Danio rerio*), our compared the thermal tolerance in non-
4 transgenic (NT) and GH-transgenic (T) zebrafish exposed to 13 °C, 39 °C, or 28 °C (control) for
5 96 h. Mortality rate was checked every 12 h in juvenile (8 week-old) and adult males (6 month-
6 old). According the log-rank test, in juveniles, exposed at 13 °C, a significant difference of
7 survival curve was observed, indicating that transgenic juveniles are more tolerant to low
8 temperature than non-transgenic. The higher tolerance of GH-transgenic fish at 13°C may be
9 related to the metabolism of these animals. The metabolic rate is diminished in lower
10 temperature, and changes in oxygen and energy delivery as kinetic processes respond directly to
11 temperature. Low temperature reduces resting heart rate and cardiac output in fish and
12 consequently decreases metabolism (Gamperl, 2011). However, the overexpression of GH
13 increases the metabolic rate in GH transgenic zebrafish (Rosa et al., 2008), this increase may
14 alleviate adverse effects.

15 For adults group, no significant effect was observed on the survival curve under low
16 temperature (13 °C). Similarly, no significant difference was observed in high temperature (39
17 °C) between the survival curve of non-transgenic and transgenic adults and juveniles (Fig. 1).
18 However, if we analyze only the mortality in 12 h of exposure to 39 °C, there are a significant
19 effect (t test, $p < 0.05$) on the final survival of transgenic juveniles and adults (Fig. 1). This
20 finding, at 12 h, indicating thermal tolerance difference between the transgenic and non-
21 transgenic juveniles and adults exposed at 39 °C (t test realized in point 12 h, $p < 0.05$). Thus, the
22 exposure to different temperatures has indicated that GH overexpression can alter the thermal
23 stress tolerance depending on age and temperature in GH-transgenic zebrafish.

24
25 **Figure 1.** Survival percentage of juvenile and adult zebrafish (*Danio rerio*) exposed to 13 °C and 39 °C

1 for 96 h. Data are presented as means $\pm$ SEM ($n = 10$ for each replicate; $n = 3$ replicates). p values
2 represent curve comparison by Log-rank (Mantel-Cox) test. Asterisk (*) indicate significant different
3 between juveniles NT and T in 12 h (t test, $p < 0.05$).

4 In contrast to the results obtained at low temperature, both juvenile and adult transgenic
5 fish were less tolerant to high temperature, where most *hsps* were downregulated. Vergauwen et
6 al. (2010) have reported that zebrafish exposed to high temperatures present diminished protein
7 and lipid content in liver, which evinces that, during warm acclimation, the increased metabolic
8 rate results in increased energy expenditure.

9 Unlike low temperatures, high temperatures tend to increase the metabolic rate
10 (Gamperl, 2011) of fish. Thus, when GH-transgenic zebrafish were exposed to 39 °C, they had
11 the adverse effect of your high metabolic rate potentiated by high temperature, causing
12 transgenic fish to die before death non-transgenic fish.

13 With an aim to explain the observed alteration of thermal tolerance in transgenic
14 groups, we analyzed the *hsps* gene expression profile. In juveniles, under control conditions
15 (Fig. 2), GH-transgenesis promoted a significant rise of 10.6-, 14.3-, 9.5-, and 15.8-fold in the
16 expression of *hsf1a*, *hsp47*, *hsp70*, and *hsp90a*, respectively. In this condition, similar effect was
17 not observed in adults, wherein only the expression of *hsp90a* was altered (decrease by 72%).
18 At low temperature (13 °C; Fig. 2), *hsp47* expression increased in the juveniles (3.9-fold
19 induction), whereas *hsp90b* genes were downregulated (decrease of 60%). In adults, only
20 *hsp90a* gene was upregulated by 1.7-fold in the transgenic fish.

21 Exposure to high temperature (Fig. 2) decreased the expression of a majority of genes in
22 both transgenic groups. *hsf1a*, *hsp47*, and *hsp90a* were downregulated in the transgenic
23 juveniles (49, 74, and 64 %, respectively). Whereas *hsf1a*, *hsp47*, *hsp70*, *hsp90a*, and *hsp90b*
24 were downregulated in the transgenic adults (49, 58, 53, 71, 47 %, respectively).

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2 **Figure 2.** Relative expression of HSP family genes in livers of non-transgenic (white bars) and GH-
3 transgenic (checked bars) zebrafish (*Danio rerio*) kept under control condition (28 °C), low temperature
4 (13 °C) and high temperature (39 °C). mRNA level was normalized by the expression of the elongation
5 factor 1 alpha (*ef1a*) and β -actin (*bac*) gene. Data are presented as median $\pm$ SEM ($n = 6$ per group).
6 Livers of juveniles were pooled (3 livers per n) and liver of adults were collected individually. Significant

1 differences ($p < 0.05$, t test) in mRNA levels are denoted by an asterisk (*). *hsf1a*, Heat shock
2 transcription factor; *hsp47*, Heat shock protein 47; *hsp70*, Heat shock protein 70; *hsp90a*, Heat shock
3 protein 90 alpha; *hsp90b*, Heat shock protein 90 beta.

4 Under normal rearing conditions (28 °C), transgenic juveniles presented higher
5 expression in most HSPs compared to the non-transgenic ones. The ability of organisms to face
6 a stressful situation is associated with its physiological condition, in particular, with its energy
7 status (Tseng and Hwang, 2008). In transgenic juveniles, their entire energy might have been
8 deviated to growth, stimulated by the excess of GH, resulting in increased protein synthesis. The
9 folding of already synthesized proteins depends on the action of molecular chaperones. Thus,
10 the highest growth rate found in transgenic zebrafish (Figueiredo et al. 2007, Studzinski et al.
11 2009) could trigger the HSP transcription. In contrast, in transgenic adult animals, no increase in
12 HSP transcription (in 28 °C) was observed by transgenesis effect. One possible explanation is
13 that, unlike most fish, the zebrafish exhibit determinate muscle growth, with growth plateau
14 after the juvenile phase (Biga and Goetz, 2006), decreasing the need for increased HSP
15 synthesis.

16 In addition to the important role of HSP chaperones in multiple cellular processes such
17 protein translocation and protein folding, they are involved in regulation of the stress response
18 (Lindquist, 1986; Lindquist and Craig, 1988). In fish, HSPs are clearly proven to be involved in
19 thermal responses of fish (Iwama et al., 1998). Considering that overexpression of GH led to
20 increased expression of HSP in juvenile, our hypothesis is that GH overexpression can alter the
21 thermal tolerance of juvenile GH-transgenic zebrafish. This hypothesis was only confirmed for
22 the lowest temperature and, at the transcriptional expression level, only *hsp47* was induced.
23 HSP47 plays a key role in collagen biosynthesis and its structural assembly (Mala and Rose ,
24 2010), and its relation with heat stress has been already demonstrated (Murtha and Keller,
25 2003). Nevertheless, few studies have evaluated the role of HSP47 in cold tolerance response of
26 fish.

27 The hierarchical clustering of gene expression and the heat map are presented in Figure
28 3. The analysis simultaneously considered all genes and all tested temperatures for juveniles and
29 adults groups, emphasizing two major clusters. Cluster I comprises a majority of *hsp* genes from
30 juvenile fish, whereas cluster II majorly includes HSP genes from adults. This heat map
31 demonstrates that age and temperature affect the *hsp* gene transcriptional expression. Similarly,
32 Murtha and Keller (2003) reported that age-related changes were evident on transcriptional
33 expression level of *hsps* in zebrafish. This result emphasizes an age/temperature-related HSP
34 expression pattern.

Figure 3. Hierarchical cluster analysis of heat shock protein (HSP) expression. The relative fold change in gene expression for genes *hsf1a*, *hsp47*, *hsp70*, *hsp90a* and *hsp90b* were compared in GH-transgenic zebrafish normalized by non-transgenic zebrafish, exposed to different temperatures (28 °C; 13 °C and 39 °C). Red indicates the downregulation of gene expression, and green refers to the upregulation of gene expression. *hsf1a*, Heat shock transcription factor; *hsp47*, Heat shock protein 47; *hsp70*, Heat shock protein 70; *hsp90a*, Heat shock protein 90 alpha; *hsp90b*, Heat shock protein 90 beta.

In summary, we assume that the effect of GH excess and thermal stress is crucial in tolerance to different temperatures in zebrafish. The results of the present study emphasize the importance of gathering knowledge concerning the collateral effects of GH-transgenesis for different environmental stressors.

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