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HSP70 expression in blood cells in diploid and triploid rainbow trouts as short-term stress thermal and welfare statement model

Expresión de HSP70 en células sanguíneas en truchas arcoíris diploides y triploides como modelo de estrés térmico a corto plazo y estado de bienestar

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ABSTRACT | In triploid fish physiology there is a lack of information about its molecular responses under stress and how to quantify the stress levels or intensity. Thus, the goals of this trial were to evaluate the pattern of HSP70 in the gills, heart and muscles of non-stressed diploid and triploid rainbow trouts, and in the blood cells after the exposure to heat stress in order to assess their stress levels. Similar HSP70 immunohistochemical detection in the gills, heart and muscles samples was seen in both ploidies. However, HSP70 expression in blood varied between ploidies over the experimental time. Control and stressed diploid trouts showed analogous HSP70 levels, but heat stressed triploid trouts showed lower HSP70 levels throughout the experiment with differences observable after 12h and 24h. It was expected higher HSP70 expression in both ploidies once this protein protects the cells against a wide range of disturbing agents. Recent evidences showed that triploid fishes have lower gene expression and this fact must be explored in future investigation in rainbow trouts. In conclusion, comparisons over time between diploid and triploid HSP70 levels in trouts could be used in stress evaluation and welfare statement.

Palabras clave
Oncorhynchus mykiss
Acuicultura
Salud de los peces
Estrés por calor
Proteínas de choque térmico

RESUMEN | En la fisiología de los peces triploides, falta información sobre sus respuestas moleculares bajo estrés y cómo cuantificar los niveles o la intensidad del estrés. Por lo tanto, los objetivos de este ensayo fueron evaluar el patrón de HSP70 en las branquias, el corazón y los músculos de truchas arco iris diploides y triploides no estresadas, y en las células sanguíneas después de la exposición al estrés por calor para evaluar sus niveles de estrés. Se observó una detección inmunohistoquímica de HSP70 similar en las muestras de branquias, corazón y músculos en ambas ploidías. Sin embargo, la expresión de HSP70 en sangre varió entre ploidías durante el tiempo experimental. Las truchas diploides de control y estresadas mostraron niveles análogos de HSP70, pero las truchas triploides estresadas por calor mostraron niveles más bajos de HSP70 durante todo el experimento con diferencias observadas después de 12 h y 24 h. Se esperaba una mayor expresión de HSP70 en ambas ploidías una vez que esta proteína protege a las células contra una amplia gama de agentes perturbadores. Evidencias recientes mostraron que los peces triploides tienen una menor expresión génica y este hecho debe ser explorado en futuras investigaciones en truchas arcoíris. En conclusión, las comparaciones a lo largo del tiempo entre los niveles diploides y triploides de HSP70 en truchas podrían usarse en la evaluación del estrés y de bienestar.

INTRODUCTION

Fish species are exposed to different types of stressors in breeding systems, such as management for biometry, hypoxia and acute changes in the water physicochemical parameters (Wendelaar Bonga, 1997). In response, catecholamines and cortisol released by the chromaffin cells and interrenal tissue, respectively, lead to consequences throughout the body. The magnitude of the physiological response to stress in fish can be influenced by the stressor, as well as genetic, developmental and environmental factors (Iwama *et al*., 2004; Schreck and Tort, 2016).

In addition to the physiological response, the cellular response to stress is related to the action of Heat Shock Proteins (HSP). HSPs are evolutionarily conserved proteins that are organized into families, including the HSP70 family - HSP72, HSP73, HSP75, and HSP78 (Wu *et al*., 2017). Located in the cytosol and cell nucleus, the HSP70 family is present in the normal cell state to aid in the folding of native polypeptides and their translocation to different cellular compartments (Clark and Peck, 2009). Also, they assist mis-folded proteins to attain or regain their native states and also target degraded proteins and regulate their removal from the cell, thus preventing the formation of cytotoxic aggregates (Clark and Peck, 2009). However, the response varies according to the tissue and family of HSP, and the sensitivity of its expression may vary among fish species (Basu *et al*., 2002; Iwama *et al*., 2004; Schreck and Tort, 2016). Several studies have attempted to establish a relationship between physiological and cellular responses to stress, but it seems to be inconsistencies between the two kinds of responses, since a fish may show a physiological response to stress without changes in its HSP cell profile (Iwama *et al*., 2004).

Facing the same stressor, diploid and triploid fishes can express different responses, as many of the effects caused by triploidization are still unknown. For example, triploid rainbow trouts *Oncorhynchus mykiss* exposed to aflatoxin B1 and to carcinoma inducers are more resilient to their effects than diploid ones (Arana *et al.*, 2002; Thorgaard *et al.*, 1999). According to Yamamoto and Iida (1995), the nonspecific defense in rainbow trouts is similar between the ploidies and this fact does not affect the susceptibility to pathogenic microorganisms. However, Pressinoti (2006) observed that the peritoneal phagocytes of triploid trouts phagocyte a greater number of yeasts than the diploid ones; but the phagocytic capacity and phagocytic index showed similar responses.

The evaluation of fish blood provides a general picture of its health and welfare, being a first tool for screening of healthy and sick animals (Fazio, 2019). In this way, it can be used to monitor changes arising from pollution and disease (Peutz *et al*., 1996; Kumar, 2012; Roy *et al*., 2017), without the need for euthanasia or collection of organ samples. Thus, the goals of this trial were to evaluate the pattern of HSP70 in the gills, heart and muscles of non-stressed diploid and triploid rainbow trouts, and in the blood cells after the exposure to heat stress in order to assess their stress levels, demonstrating that the evaluation of HSP70 expression in trout blood cells can be a tool for operational welfare indicator (Segner *et al*., 2012).

MATERIAL AND METHODS

Experiment 1 - HSP70 immunolocalization in trout not exposed to thermal stress

Twenty 12-month-old female rainbow trouts (*Oncorhynchus mykiss*) - 10 diploids (40.65±1.31cm and 1097±213.85g) and 10 triploids (32.65±1.33cm and 902±94.61g) were acquired from the Salmoniculture Experimental Station Dr. Ascânio de Faria, Campos do Jordão, Brazil, belonging to the Paulista Agency for Agribusiness Technology. This research was approved by the Bioethics Committee of the College of Veterinary Medicine and Animal Science of the University of São Paulo/USP # 1337/2008.

For organs collection, rainbow trouts were anesthetized by immersion anesthesia in a 70 mg/L benzocaine-based solution (Benzocaine, Sigma Aldrich, USA), previously dissolved in ethyl alcohol (Silva *et al*., 1998). Upon reaching the deep anesthesia stage with total loss of equilibrium and no reaction to handling or pain stimuli (Ross and Ross, 2008), blood was collected from the caudal vein with a 5 ml syringe with a 22-gauge heparinized needle to confirm the triploidization by blood smear evaluation (Benfey *et al*., 1984). Then, they were euthanized in a 250 mg/L benzocaine solution and samples of the gills (Washburn et al. 2002), liver (Boone and Vijayan, 2002) and skeletal and cardiac muscles (Clarkson *et al*., 2005) were fixed in 10% buffered formalin solution (Paraformaldehyde, Sigma-Aldrich, USA), dehydrated in alcohol and embedded in histologic paraffin (Paraplast, Sigma-Aldrich, USA).

Immunohistochemistry was performed using the LSAB + HRP System kit (DakoCytomation, Carpinteria, CA, USA) following the manufacturer instructions. The primary antibody used was the anti-Hsp 70/Hsp 72 monoclonal antibody (Enzo Life Sciences, USA, code C92F3A-5) at the 1:100 dilution in

PBS buffer 0.1M pH 7.2. After dewaxing, the slides were submitted to antigen recovery from immersion in 0.01 M sodium citrate buffer pH 6.0 and submitted to 3 cycles of 5 minutes each in a microwave oven at maximum power. A 3% hydrogen peroxide in methanol and a 1.5% bovine serum albumin (BSA) diluted in PBS were both used for 30 min to block the action of the endogenous peroxidase and the non-specific reactions, respectively. The sections were incubated with the primary antibody in a humid chamber overnight at 4°C. After the secondary antibody and streptavidin incubations for 1h each, positive reactions were developed with a chromogenic 3.3- diaminobenzidine solution (DAB, DakoCytomation, USA) with different developing times: 1 min for heart, 2 min for gills and liver and 5 min for muscles. The sections were washed in distilled water and counterstained with Harris hematoxylin, dehydrated and mounted in Permount® resin. As negative controls, sample sections were incubated with PBS, rather than primary antibody.

Experiment 2 - diploid and triploid trouts exposed to heat stress

Twenty female rainbow trouts *O. mykiss* (10 diploids and 10 triploids), from the same batch of the experiment 1, were acclimated in tanks with water at 16˚C for seven days (preferred temperature for culture is 10-17°C as reported by Ineno et al., 2005). All trouts were anestethized in a 70 mg/L benzocaine-based solution and blood was collected by caudal vein punction. To heat stress induction, seven diploid and triploid trouts were transferred to a tank with water at 20˚C for 1h (groups Stressed 2n and Stressed 3n, respectively). After, they were retransferred to water at 16˚C, and blood collections were performed 1h, 12h and 24h after heat stress. Control groups (groups Control 2n and Control 3n, n=3 each) were kept at 16˚C and undergone blood collection at the same time of heat stress groups. To ensure that the consequences of the heat stress were not overlapped by hypoxia, pure oxygen was bubbled to keep constant water oxygen levels throughout the trial. Blood samples were frozen in liquid nitrogen and stored at - 80°C until evaluation.

With the Immunoblotting method we propose to qualify and quantify the expression of HSP70 in blood samples using antibody against HSP70 (Stressgen anti-HSP70/HSP72 monoclonal antibody, code C92F3A-5). For this, we use polyacrylamide gel electrophoresis to separate denatured proteins according to its molecular weight. Using microtubes, 1 ml of blood was added to 200 µl of ice-cold extraction buffer solution composed of SDS (Sodium dodecyl sulfate, 0.1M), DTT (DL-Dithiothreitol, 0.1 M), PMSF (Phenylmethylsulfonyl fluoride, 1.10^{-3} M), glycerol (20%) and Tris-HCl (TRIS hydrochloride, 0.12 M pH 6.8) and were boiled in water bath for 5 minutes. After, the microtubes were centrifuged at 18,000 g for 15 min at 4°C. The supernatant was transferred to another tube and frozen at - 20°C. The Bradford protein assay was performed to measure the concentration of total protein in all samples (Bradford, 1976). Aliquots containing 160 µg of protein plus the sample buffer (composed of 240 mM Tris-HCl, 40% glycerol, 0.8% SDS and 200 mM β-mercaptoethanol) were subjected to a voltage of 100 V for 1h (adapted from Deane et al. 2006) in SDS-PAGE gel (resolving 10% and stacking 5%). After running, the samples were transferred to a nitrocellulose membrane (Hybond-P PVDF Membrane, Amersham Pharmacia Biotech, USA) using 120 mA current for 2h30min. At the end of the transfer, we used a secondary antibody (Blotting Grade Affinity Purified, Goat anti-Mouse IgG (H + L) - HRP Conjugate, Bio Rad, USA) and developed the membrane with the ECL kit (ECL Western blotting Analysis System, GE Healthcare, USA) above X-ray films. The X-rays were scanned and analyzed using Image J software (NIH, USA). Gel analyses provided arbitrary values for the HSP70 densitometric intensities, which were normalized from the values for control fish for a semi-quantitative evaluation.

Data analysis

The arbitrary values for the HSP70 intensities were evaluated using analysis of variance (one-way and two-way ANOVA) with Tukey's post test. All values are shown as a mean ± standard deviation. The results were statistically significant when $p<0.05$. Data analysis was performed using GraphPad Prism 6 software (GraphPad Software, San Diego, USA).

RESULTS

Experiment 1

Immunohistochemistry showed cytoplasmic expression of HSP70 in hepatocytes, with no difference in the intensity between diploid or triploid rainbow trouts. Hepatocytes close to vessels with denser nuclei and lipid droplet (Fig. 1a) showed more intense HSP70 cytoplasmic signaling when compared to trout hepatocytes with loose nuclei with no fat deposition (Fig. 1b). Indeed, we noticed a difference between hepatocytes with and without lipid droplet deposition in cytosol (Figs. 1b and 1c). Control livers did not show any detection of HSP70 (Fig. 1d).

We obtained a weak signaling for HSP70 on skeletal (white and red muscles) (Figs. 1e and 1f) and on cardiac muscle fibers (Figs. 1g and 1h), with no difference between ploidies.

In the same way, the signaling on the gills was cytoplasmic without differences between ploidies and no signaling in negative controls. We observed that the mucoid and chloride cells of the gill lamellae had more intense signal than the epithelial cells (Figs. 1i and 1j).

Experiment 2

There was homogeneity in HSP70 levels among all control groups (two-way ANOVA, $p = 0.06$). Yet, there was no statistical significance of HSP70 levels when it was considered the interaction between ploidies and time after heat stress (two-way ANOVA, $p = 0.34$), as showed in figures 2 and 3.

However, the evaluation of HSP70 levels over time for each ploidy showed different scenarios. HSP70 levels in diploid trouts did not show a statistical difference between control and heat-stressed trouts (one-way ANOVA, $p = 0.13$), but heat stressed triploid trouts showed lower HSP70 levels throughout experiment, with significant differences after 12h ($p = 0.030$) and 24h ($p = 0.003$) in comparison to the respective control group (Figs. 2 and 3). Indeed, it was detected a second protein with a molecular weight of approximately 40 kDa characterizing the co-chaperone HSP40 bound to HSP70.

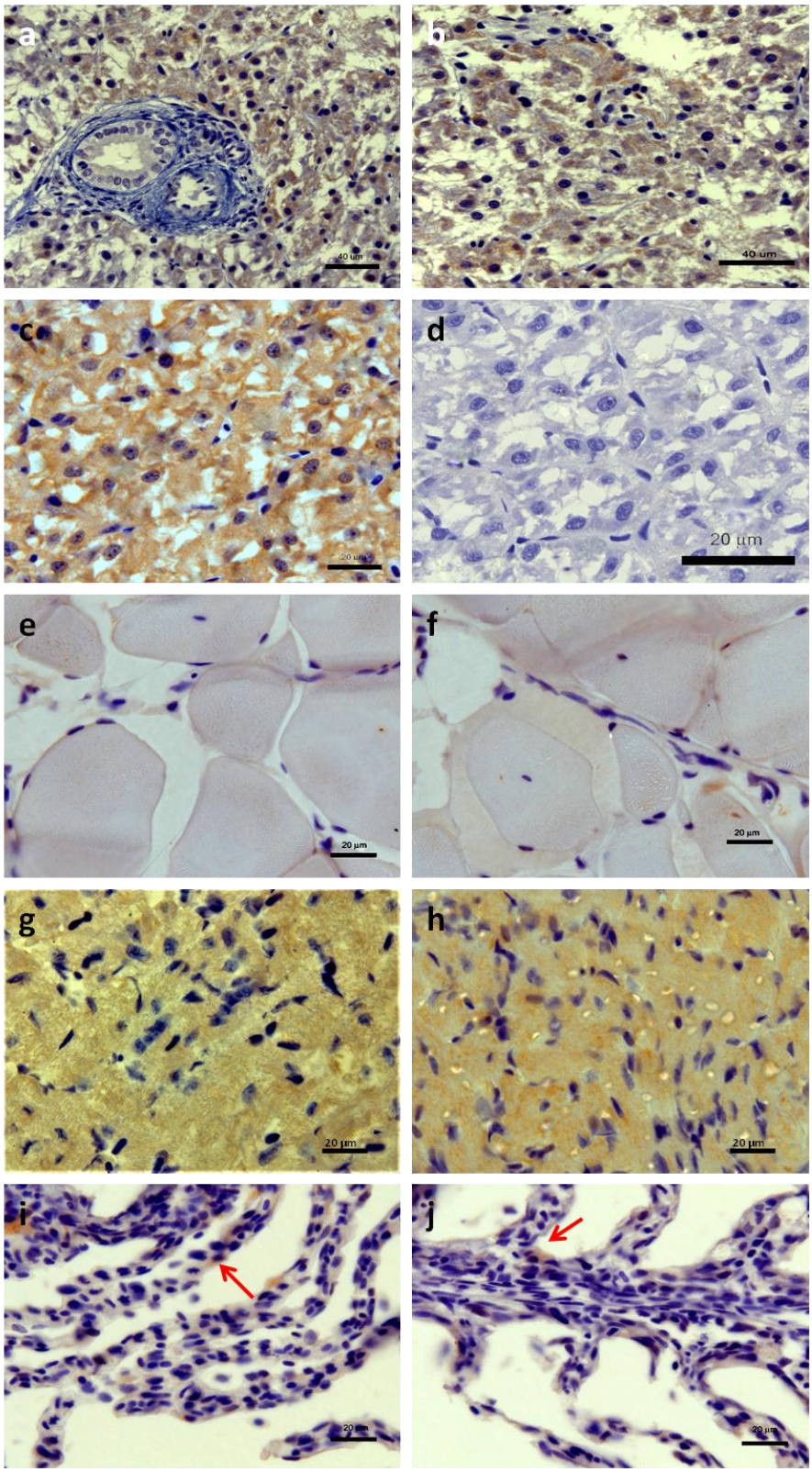

Figure 1. a In the liver of diploid trouts, the hepatocytes near of the portal triad vessels show a more intense HSP70 detection when compared to **b** with hepatocytes without cytoplasmic lipid droplet in the liver of triploid trouts, **c** in the liver of triploid trouts, the hepatocytes with cytoplasmic lipid droplet show intense HSP70 detection, **d** control triploid liver without reaction, **e** and **f** diploid and triploid skeletal muscle fibers with weak HSP70 detection, **g** and **h** diploid and triploid cardiac muscle fibers with weak HSP70 detection, **i** mucoid cell (arrow) in a secondary gill lamella positive for HSP70 in diploid trout, **j** chloride cell (arrow) proximal to primary gill filament positive for HSP70 in triploid trout. Bars: 40 µm (**a** and **b**), 20 µm (**c** to **j**).

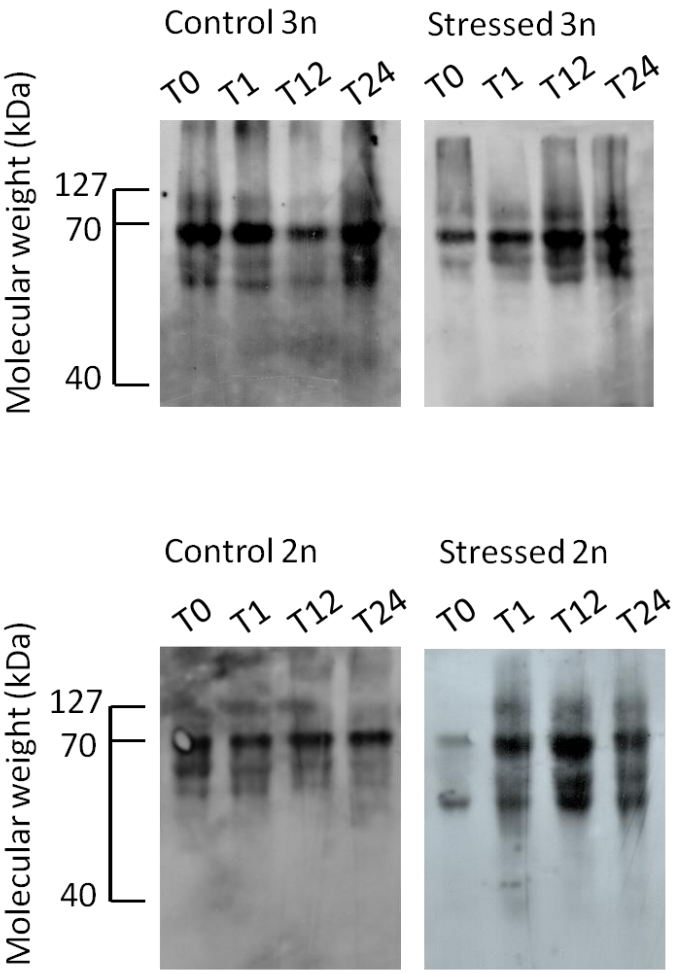

Figure 2. Western blot for HSP70 protein in blood samples of diploid (Control 2n and Stressed 2n) and triploid (Control 3n and Stressed 3n) rainbow trouts *O. mykiss* following heat stress. Immunoprecipitated bands about 40 kDa belong to HSP40 co-chaperone.

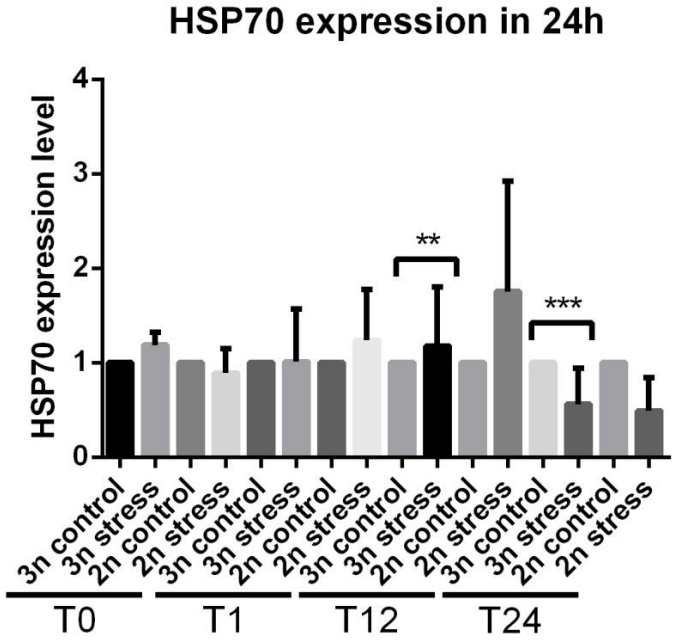

Figure 3. The expression levels of HSP70 were quantified by Image J software densitometric analysis and were normalized to the control groups. $p<0.05$ (**) and $p<0.005$ (***). One-way ANOVA.

DISCUSSION

Experiment 1

No differences in immunohistochemical detection of HSP70 in organ samples between ploidies were observed, although a more intense presence in triploid trout tissue samples was expected. One explanation is the fact that the trouts were under ideal conditions; however, data in the literature are conflicting regarding *hsp70 gene expression* and HSP70 levels in different fish species and organs exposed to stressors. The gills maintain an intimate relationship with the external environment, performing different physiological activities (Evans *et al*., 2005), and are susceptible to harmful actions of pollutants and parasites (Strzyzewska *et al*., 2016). In gills, HSP70 was observed in chloride cells, known for the large number of mitochondria that supply energy to the Na^+K^+ATPase transporter and chloride uptake channels in freshwater fishes, and in mucoid cells which release mucin to protect the gills against pathogens (Goss *et al*., 1998; Wilson and Laurent, 2002; Dymowska et al., 2012). Therefore, the uniform detection of HSP70 in those cells in diploid and triploid trouts is justified by the maintenance of homeostasis. In wild Pacific halibuts *Hippoglossus stenolepis* gills the HSP70 levels are variable (Scofield *et al*., 1999) but, when Atlantic salmons *Salmo salar* are exposed to different kinds of environmental stress (DuBeau *et al*., 1998; Zarate and Bradley, 2003), there is the induction of *de novo* synthesis of HSP70 in the gills, with elevated levels in common carp *Cyprinus carpio* gills after 24 hours of exposure to high temperatures (Wang *et al*., 2007a).

Hepatocytes close to the portal triads are the first cells that interact with blood reaching out the liver parenchyma (Akiyoshi and Inoue, 2004), suffering more intense stressor consequences (Wolf and Wheeler, 2018). Thus, we observed in both ploidies a more intense detection of HSP70 in hepatocytes with dense nuclei in those regions than in hepatocytes with loose nuclei, suggesting *de novo* HSP70 synthesis to avoid unfolded proteins in endoplasmic reticula, once acting as chaperones (Adams *et al*., 2019). Indeed, HSP70 accumulation in primary cultures of hepatocytes of diploid trouts *O. mykiss* were higher after 24h of exposition to heat shock and heavy metals (Boone and Vijayan, 2002), but not observed after acute stress conditions such as handling (Washburn *et al*., 2002) and exhaustive exercise (Clarkson *et al*., 2005). Even under chronic stress such as transport over a long period of time, there are no differences in liver HSP70 levels in diploid and triploid trouts (Leggatt *et al*., 2006).

In heart and skeletal muscles, no difference in HSP70 levels was observed, similar to that reported in diploid brook trouts *Salvelinus fontinalis* exposed to heat shock and thermal tolerance at 25°C (Stitt *et al*., 2014) and in diploid rainbow trouts *O. mykiss* after exhaustive exercise (Clarkson *et al*., 2005). It is likely that HSP70 levels are similar in both ploidies to protect muscle fibers, thus favoring escape behavior under acute stress condition.

Experiment 2

Even though erythrocytes from diploid and triploid trouts have similar mean corpuscular hemoglobin concentrations (Benfey and Sutterlin, 1984; Yamamoto and Iida, 1994), the increase in ploidy in rainbow trout *O. mykiss* results in a reduction in aerobic capacity (Virtanen *et al*., 1990) and survival after exposure to gradual and acute water heating (Verhille et al. 2013). Trout erythrocytes are able to synthesize HSP70 after the beta-adrenergic stimuli, such as short-term acute stress used in this trial (Currie *et al*., 2008; Schreck and Tort, 2016), with regulation at nuclear transcription level (Currie and Tufts, 1997). Unexpectedly, we have detected lower HSP70 levels in triploid trouts 1h after the acute heat stress, which was observed until after 24h post-stress. HSP40 was also detected, as this co-chaperone controls the HSP70 binding to unfolded polypeptides, preventing protein misfolding (Qiu *et al*., 2006). Moreover, HSP70 activity is dependent on ATP hydrolysis by HSP40 (Sugito *et al*., 1995; Ohtsuka and Hata, 2000).

A possible explanation for our results is given by genetic analysis of the naturally allotriploid fish *Squalius alburnoides*. This species has a compensation mechanism undergoing to reduced transcript levels when compared to the diploid state, but it is not a whole haplome that is inactivated. The allelic expression

patterns differing between genes and between different tissues for one and the same gene is evidence of a regulation mechanism involving gene-copy silencing in triploid fishes (Pala *et al*., 2008). Recently, Matos *et al*. (2019) reported that the few genes silenced in *Squalius alburnoides* triploids are related to mitochondria and ribosomes activity regulation, with consequent control of cell size and protein synthesis, respectively. Moreover, analysis of gene expression in farmed triploid fish species revealed that genes related to the heat shock protein family, such as *hsp70* and genes related to glycogenesis enzymes are among the upregulated ones in the bighead catfish *Clarias macrocephalus* (Chatchaiphan *et al*., 2017), and in coho salmon *O. kisutch* those genes are related to nutrition and compensatory growth (Christensen *et al*., 2019). Considering salmonids as polyploid fishes (Otto and Whitton, 2000), it is likely that the silencing of genes contributed to the HSP70 levels in triploid *O. mykiss* rainbow trouts.

Another factor that can influence the allelic expression is the DNA methylation, a situation in which DNA cannot be transcribed. Literature data are scarce, but triploid brown trouts *Salmo trutt*a and Amazon mollies *Poecilia formosa* do not show methylation differences in relation to their diploid counterparts, however *S. alburnoides* presents differences between ploidies, possibly because it is a natural allotriploid species (Covelo-Soto *et al*., 2015; Matos *et al*., 2016). However, there are no data in the literature to confirm the relationship between DNA methylation and gene expression in triploid *O. mykiss* trouts.

Concomitantly, fish skin cell cultures, liver samples and erythrocytes can be used for experimental and applied toxicological assessments, as HSP70 shows elevated levels after exposure to pollutants (Triebskorn *et al*., 1997; Vijayan *et al*., 1998; Kilemade and Mothersill, 2001; Köhler *et al*., 2001), heavy metals (Fulladosa *et al*., 2006) and complex polluted environments (Wang *et al*., 2007b). From the results, ploidy and the time of exposure to the stressor should be considered because, as observed in the present study, the analysis of the HSP70 expression in blood cells can be used as a welfare indicator and biomarker for the integrated effect of all environmental stressors acting on an organism (Köhler *et al*., 2001).

The assessment of HSP70 levels in fish erythrocytes is a useful tool for the operational welfare indicator (Segner *et al*., 2002), as it can reveal changes in the homeostasis of fish exposed to acute changes in their environment, such as sudden peaks in water temperature during winter in regions with subtropical highland climate, e.g. Campos do Jordão, Brazil (Rolim and Aparecido, 2016). The results may also favor the development of more resistant lines to stressors such as water heating (Ineno *et al*., 2005), and its consequences such as hypoxia and water acidification.

CONCLUSION

In conclusion, the detection of HSP70 in both ploidies is more intense in the organs that carry out protein synthesis and release, mainly the liver and gills. Finally, it must be considered that triploid rainbow trouts may present the silencing of genes related to heat shock proteins.

Conflict of interest

The authors declare no conflict of interest.

Ethical approval

This research was approved by the Bioethics Committee of the College of Veterinary Medicine and Animal Science of the University of São Paulo/USP # 1337/2008.

REFERENCES

Adams C.J., Kopp M.C., Larburu N., Nowak P.R., Ali M.M. (2019). Structure and molecular mechanism of ER stress signaling by the unfolded protein response signal activator IRE1. *Frontiers in Molecular Biosciences*, 6: 11.

Akiyoshi H., Inoue A. (2004). Comparative histological study of teleost livers in relation to phylogeny. *Zoological Science*, 21: 841-850.

Arana S., Tabata Y.A., Sabino M., Rigolino M.G., Hernandez-Blazquez F.J. (2002). Differential effect of chronic aflatoxin B1 intoxication on the growth performance and incidence of hepatic lesions in triploid and diploid rainbow trout (*Oncorhynchus mykiss*). *Archivos de Medicina Veterinaria*, 34: 253-263.

Basu N., Todgham A.E., Ackerman P.A., Bibeau M.R., Nakano K., Schulte P.M., Iwama G.K. (2002). Heat shock protein genes and their functional significance in fish. *Gene*, 295: 173-183.

Benfey T.J., Sutterlin A.M. (1984). The haematology of triploid landlocked Atlantic salmon, *Salmo solar* L. *Journal of Fish Biology*, 24: 333-338.

Boone A.N., Vijayan M.M. (2002). Constitutive heat shock protein 70 (HSC70) expression in rainbow trout hepatocytes: effect of heat shock and heavy metal exposure. *Comparative Biochemistry and Physiology - Part C*, 132: 223-233.

Bradford M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72: 248-254.

Chatchaiphan S., Srisapoome P., Kim J.H., Devlin R.H., Na-Nakorn U. (2017). *De novo* transcriptome characterization and growth-related gene expression profiling of diploid and triploid bighead catfish (*Clarias macrocephalus* Günther, 1864). *Marine Biotechnology*, 19: 36-48.

Christensen K.A., Sakhrani D., Rondeau E.B., Richards J., Koop B.F., Devlin R.H. (2019). Effect of triploidy on liver gene expression in coho salmon (*Oncorhynchus kisutch*) under different metabolic states. *BMC Genomics*, 20: 1-14.

Clark M.S., Peck L.S. (2009). HSP70 heat shock proteins and environmental stress in Antarctic marine organisms: a mini-review. *Marine Genomics*, 1: 11-18.

Clarkson K., Kieffer J.D., Currie S. (2005). Exhaustive exercise and the cellular stress response in rainbow trout, *Oncorhynchus mykiss*. *Comparative Biochemistry and Physiology - Part A*, 140: 225-232.

Covelo-Soto L., Leunda P.M., Pérez-Figueroa A., Morán P. (2015). Genome-wide methylation study of diploid and triploid brown trout (*Salmo trutta* L.). *Animal Genetics*, 46: 280-288.

Currie S., Tufts B. (1997). Synthesis of stress protein 70 (Hsp70) in rainbow trout (*Oncorhynchus mykiss*) red blood cells. *Journal of Experimental Biology*, 200: 607-614.

Currie S., Reddin K., McGinn P., McConnell T., Perry S.F. (2008). β-adrenergic stimulation enhances the heat-shock response in fish. *Physiological and Biochemical Zoology*, 81: 414-425.

DuBeau S.F., Pan F., Tremblay G.C., Bradley T.M. (1998). Thermal shock of salmon in vivo induces the heat shock protein hsp 70 and confers protection against osmotic shock. *Aquaculture*, 168: 311-323.

Dymowska A. K., Hwang P. P., Goss G. G. (2012). Structure and function of ionocytes in the freshwater fish gill. *Respiratory Physiology & Neurobiology*, 184: 282-292.

Evans D.H., Piermarini P.M., Choe K.P. (2005). The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. *Physiological Reviews*, 85: 97-177.

Fazio F. (2019). Fish hematology analysis as an important tool of aquaculture: a review. *Aquaculture*, 500: 237-242.

Fulladosa E., Deane E., Ng A.H.Y., Woo N.Y.S., Murat J.C., Villaescusa I. (2006). Stress proteins induced by exposure to sublethal levels of heavy metals in sea bream (*Sparus sarba*) blood cells. *Toxicology in vitro*, 20: 96-100.

Goss G.G., Perry S.F., Fryer J.N., Laurent P. (1998). Gill morphology and acid-base regulation in freshwater fishes. *Comparative Biochemistry and Physiology - Part A*, 119: 107-115.

Ineno T., Tsuchida S., Kanda M., Watabe S. (2005). Thermal tolerance of a rainbow trout *Oncorhynchus mykiss* strain selected by high-temperature breeding. *Fisheries Science*, 71: 767-775.

Iwama G.K., Afonso L.O., Todgham A., Ackerman P., Nakano K. (2004). Are hsps suitable for indicating stressed states in fish?. *Journal of Experimental Biology*, 207: 15-19.

Kilemade M., Mothersill C. (2001). Heat shock protein 70 levels in rainbow trout primary epidermal cultures in response to 2, 4-dichloroaniline exposure: A novel in vitro aquatic toxicity marker. *Environmental Toxicology*, 16: 253-259.

Köhler H.R., Bartussek C., Eckwert H., Farian K., Gränzer S., Knigge T., Kunz N. (2001). The hepatic stress protein (hsp70) response to interacting abiotic parameters in fish exposed to various levels of pollution. *Journal of Aquatic Ecosystem Stress and Recovery*, 8: 261-279.

Leggatt R.A., Scheer K.W., Afonso L.O., Iwama G.K. (2006). Triploid and diploid rainbow trout do not differ in their stress response to transportation. *North American Journal of Aquaculture*, 68: 1-8.

Matos I.M., Coelho M.M., Schartl M. (2016). Gene copy silencing and DNA methylation in natural and artificially produced allopolyploid fish. *Journal of Experimental Biology*, 219: 3072-3081.

Matos I., Machado M.P., Schartl M., Coelho M.M. (2019). Allele-specific expression variation at different ploidy levels in *Squalius alburnoides*. *Scientific Reports*, 9: 1-12.

Ohtsuka K., Hata M. (2000). Molecular chaperone function of mammalian Hsp70 and Hsp40-a review. *International Journal of Hyperthermia*, 16: 231-245.

Otto S.P., Whitton J. (2000). Polyploid incidence and evolution. *Annual Reviews of Genetics*, 34: 401-437.

Pala I., Coelho M.M., Schartl M. (2008). Dosage compensation by gene-copy silencing in a triploid hybrid fish. *Current Biology*, 18: 1344-1348.

Peutz I.L.J.A., Oorschot R.W.A., Johnson G.R., Horney B.S., Boon J.H. (1996). The leucogram as an indicator off marine-cultured rainbow trout, *Oncorhynchus mykiss* (Walbaum), health in The Netherlands. *Aquaculture Research*, 27: 437-445.

Pressinoti L.N. (2006). Efeito da triploidização na imunidade celular inespecífica de trutas arco-íris *Oncorhynchus mykiss* (Walbaum,1792). Master's Thesis. University of São Paulo, Brazil.

Qiu X.B., Shao Y.M., Miao S., Wang L. (2006). The diversity of the DnaJ/Hsp40 family, the crucial partners for Hsp70 chaperones. *Cellular and Molecular Life Sciences*, 63: 2560-2570.

Rolim G.S., Aparecido L.E.O. (2016). Camargo, Köppen and Thornthwaite climate classification systems in defining climatical regions of the state of São Paulo, Brazil. *International Journal of Climatology*, 36: 636-643.

Ross L.G., Ross B. (2008). Anaesthetic and sedative techniques for aquatic animals. Blackwell Science. Oxford, United Kingdom.

Roy S., Kumar V., Kumar V., Behera B.K. (2017). Acute phase proteins and their potential role as an indicator for fish health and in diagnosis of fish diseases. *Protein & Peptides Letters*, 24: 78-89.

Schreck C.B., Tort L. (2016). The concept of stress in fish. In: Schreck C.B., Tort L., Farrell A.P., Brauner C.J.(Eds). Fish Physiology. Elsevier Inc., USA. vol. 35, pp. 1-34.

Scofield E., Bowyer R.T., Duffy L.K. (1999). Baseline levels of Hsp 70, a stress protein and biomarker, in halibut from the Cook Inlet region of Alaska. *Science of the Total Environment*, 226: 85-88.

Segner H., Sundh H., Buchmann K., Douxfils J., Sundell K.S., Mathieu C., Ruane N., Jutfelt F., Toften H., Vaughan L. (2012). Health of farmed fish: its relation to fish welfare and its utility as welfare indicator. *Fish Physiology and Biochemistry*, 38: 85-105.

Silva J.R.M.C., Hernandez-Blazquez F.J., Barbieri R.L. (1998). Induced inflammatory process in the Antarctic fish *Notothenia neglecta*. *Polar Biology*, 20: 206-212.

Stitt B.C., Burness G., Burgomaster K.A., Currie S., McDermid J.L., Wilson C.C. (2014). Intraspecific variation in thermal tolerance and acclimation capacity in brook trout (*Salvelinus fontinalis*): physiological implications for climate change. *Physiological and Biochemical Zoology*, 87: 15-29.

Strzyzewska E., Szarek J., Babinska I. (2016). Morphologic evaluation of the gills as a tool in the diagnostics of pathological conditions in fish and pollution in the aquatic environment: a review. *Veterinárni Medicína*, (Praha). 61: 123-132.

Sugito K., Yamane M., Hattori H., Hayashi Y., Tohnai I., Ueda M., Tsuchida N., Ohtsuka K. (1995). Interaction between hsp70 and hsp40, eukaryotic homologues of DnaK and DnaJ, in human cells expressing mutant-type p53. *FEBS Letters*, 358: 161-164.

Thorgaard G.H., Arbogast D.N., Hendricks J.D., Pereira C.B., Bailey G.S. (1999). Tumor suppression in triploid trout. *Aquatic Toxicology*, 46: 121-126.

Triebskorn R., Köhler H.R., Honnen W., Schramm M., Adams S.M., Müller E.F. (1997). Induction of heat shock proteins, changes in liver ultrastructure, and alterations of fish behavior: are these biomarkers related and are they useful to reflect the state of pollution in the field?. *Journal of Aquatic Ecosystem Stress and Recovery*, 6: 57-73.

Verhille C., Anttila K., Farrell A.P. (2013). A heart to heart on temperature: impaired temperature tolerance of triploid rainbow trout (*Oncorhynchus mykiss*) due to early onset of cardiac arrhythmia. *Comparative Biochemistry and Physiology - Part A.*, 164: 653-657.

Vijayan M.M., Pereira C., Kruzynski G., Iwama G.K. (1998). Sublethal concentrations of contaminant induce the expression of hepatic heat shock protein 70 in two salmonids. *Aquatic Toxicology*, 40: 101-108.

Virtanen E., Forsman L., Sundby A. (1990). Triploidy decreases the aerobic swimming capacity of rainbow trout (*Salmo gairdneri*). *Comparative Biochemistry and Physiology - Part A*, 96: 117-121.

Wang Y., Xu J., Sheng L., Zheng Y. (2007a). Field and laboratory investigations of the thermal influence on tissue-specific Hsp70 levels in common carp (*Cyprinus carpio*). *Comparative Biochemistry and Physiology - Part A*, 148: 821-827.

Wang J., Wei Y., Li X., Cao H., Xu M., Dai J. (2007b). The identification of heat shock protein genes in goldfish (*Carassius auratus*) and their expression in a complex environment in Gaobeidian Lake, Beijing, China. *Comparative Biochemistry and Physiology - Part C.*, 145: 350-362.

Washburn B.S., Moreland J.J., Slaughter A.M., Werner I., Hinton D.E., Sanders B.M. (2002). Effects of handling on heat shock protein expression in rainbow trout (*Oncorhynchus mykiss*). *Environmental Toxicology and Chemistry*, 21: 557-560.

Wendelaar Bonga S.E. (1997). The stress response in fish. *Physiological Reviews*, 77: 591-625.

Wilson J.M., Laurent P. (2002). Fish gill morphology: inside out. *Journal of Experimental Zoology*, 293: 192-213.

Wolf J.C., Wheeler J.R. (2018). A critical review of histopathological findings associated with endocrine and non-endocrine hepatic toxicity in fish models. *Aquatic Toxicology*, 197: 60-78.

Wu J., Liu T., Rios Z., Mei Q., Lin X., Cao S. (2017). Heat shock proteins and cancer. *Trends in Pharmacological Sciences*, 38: 226-256.

Yamamoto A., Iida T. (1994). Hematological characteristics of triploid rainbow trout. *Fish Pathology*, 29: 239-243.

Yamamoto A., Iida T. (1995). Non-specific defense activities of triploid rainbow trout. *Fish Pathology*, 30: 107-110.

Zarate J., Bradley T.M. (2003). Heat shock proteins are not sensitive indicators of hatchery stress in salmon. *Aquaculture*, 223: 175-187.

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