



## Lethal thermal maximum temperature induces behavioral responses and protein expressions (Hsp70 and p53) in juvenile common carp (*Cyprinus carpio* Linnaeus)

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**Abstract:** Temperature is one of the most important environmental factors influencing aquatic organisms at different levels and drastic changes can cause adverse effects on individuals, populations and ecosystems. This study investigated the behavioral response to heating stress and the protein expression of Hsp70 and p53 in muscle, liver, gills and heart at lethal thermal maximum (LTMax) in juvenile of common carp, *Cyprinus carpio*. The fish, acclimated at 25 °C, were submitted to gradual and constant heating up to the LTMax. The results indicated that heating to lethal maximum temperature causes behavioral alterations from temperature of 32.8 °C. In general, the LTMax ranged from 38 to 39.8 °C and 38.8 °C was the temperature at which 50% of the fish reached the LTMax. There were increase of Hsp70 and p53 expression in all tissues analyzed at LTMax in comparison with the control group, indicating loss of functional structure of proteins and DNA damage in juvenile *C. carpio*. This study suggests that heating causes behavioral and protein expression alterations to the fish and these alterations could affect the geographical distributions and survivor of this species.

**Keywords:** Temperature; Denatured proteins; DNA damage; Fish.

**Resumo.** Temperatura letal máxima induz respostas comportamentais e expressões de proteínas (Hsp70 e p53) em juvenis de carpa comum (*Cyprinus carpio* Linnaeus). A temperatura é um dos fatores ambientais mais importantes e que influenciam os organismos

aquáticos em diferentes níveis. Mudanças drásticas na temperatura podem causar efeitos adversos nos indivíduos, nas populações e no ecossistema. Neste estudo, foram investigadas as respostas comportamentais frente ao estresse térmico e as expressões das proteínas Hsp70 e p53 na temperatura letal máxima (LTMax) em carpas juvenis, *Cyprinus carpio*. Os peixes, aclimatados à temperatura de 25 °C, foram submetidos ao aquecimento gradual e constante de temperatura até o LTMax, definida como a temperatura na qual os peixes cessam os movimentos operculares. Alterações do comportamento foram observados durante o aquecimento. Amostras de músculo, fígado, brânquias e coração foram coletados para a imunohistoquímica das proteínas Hsp70 e p53. Os resultados indicam que o aquecimento até a temperatura letal máxima causam alterações no comportamento, perda da estrutura de proteínas e possíveis danos de DNA nos juvenis de *C. carpio*. Este estudo sugere que a compreensão da resposta à temperatura pode ser muito importante para prever o impacto do estresse térmico em organismos aquáticos.

**Palavras-Chaves:** Temperatura; Proteínas desnaturadas; Danos ao DNA; Peixe.

## Introduction

Temperature is one of the most important environmental factors influencing aquatic organisms at different levels such as molecular, biochemical, physiological and behavioral (Pörtner & Knust 2007, Pörtner & Farrell 2008, Dillon *et al.* 2010). Adverse effects of temperature may extend from individuals to populations and ecosystem level. To survive and thrive in a particular thermal habitat, the organisms must be capable to cope with temperature variation (Pörtner 2002).

Vulnerability towards heat stress depends mainly on the organism's thermal tolerance and its upper (or lower) thermal limits (Eme & Bennett 2009). This knowledge is very important for aquaculture and for understanding fish physiology and ecology in a world where the climate change is an actual issue. Physiological studies can assist to predict effects of climate change through determining if the studied species currently live close to its upper thermal tolerance limits, the physiological systems responsible for those limits and the acclimatization capacities for changing their thermal tolerances (Somero 2010). If the species are living close to their thermal limits, the risk of being affected by temperature increase is big (Tomanek 2008, 2010). Additionally, studies at the molecular level can complement this analysis by revealing how proteins or genes can influence in the capacity of adaptation to temperature increase (Somero 2010).

Thermal tolerance and upper (or lower) thermal limits of fish and other aquatic organisms are determined using the dynamic non-lethal method in which the temperature of the medium is changed slowly at a constant rate, from the acclimation level until the animal exhibits the first signs of stress, usually in the form of exaggerated swimming (Elliott 1981, Kilgour & McCauley 1986, Masud &

Singh 2013). In the upper thermal limits, as the temperature keeps increasing during the test, the animal usually displays a sequence of responses: loss of righting response (LRR), sudden onset of muscular spasms (OS), restricted movements of the opercula, coma and death (Lutterschmidt & Hutchison, 1997a). Some of these events can be identified visually and are used as an endpoint for the studies of thermal tolerance in fish. While loss of righting response also known as loss of equilibrium, or onset of muscular spasms are common endpoints for determining critical thermal maximum (CTMax), the cessation of opercular movements is used as end point for determining lethal thermal maximum (LTMax). This thermal point is considered as the lethal maximum or ultimate lethal temperature, as death would follow within 2 to 5 minutes after this observation (Elliott 1995). Lethal Thermal Maximum has been used to study thermal tolerance of a wide range of aquatic animals (Li & Wang 2005, Debnath *et al.* 2006, Takahara *et al.* 2011) as it is easier to determine in comparison to CTMax.

Exposure to thermal stress is known to induce oxidative stress (Lushchak & Bagnyukova 2006) and DNA damage (Anitha *et al.* 2000) in many organisms, mediated by cellular biochemical reactions that produce reactive oxygen species (ROS). Under stable conditions, cells detoxify ROS through a variety of enzymatic and scavenging responses that repair cellular damage, including production of chaperone heat shock proteins and DNA damage repair proteins (De Nadal *et al.* 2011). Hsp70 (Heat shock protein 70kDa) can protect the cell from stress (Ackerman *et al.* 2000, Hofmann 2005, Iwama *et al.* 1999, Iwama *et al.* 2004) by assisting misfolded proteins to regain their native states, preventing the formation of protein aggregates and assisting protein degradation and

translocation (Hartl *et al.* 2011, Proctor & Lorimer 2011). The study of regulation and expression of Hsp70 in natural populations helps to increase the understanding of the thermal biology (Hofmann 2005).

The p53 (Protein 53kDa) is a tumoral suppressor protein which preserves genome integrity through the regulation of relevant cellular pathways (e.g. cell cycle, apoptosis, and cellular senescence) (Dötsch *et al.* 2010, Chillemi *et al.* 2016). When DNA damage is detected, p53 induces the pause of cellular cycle to enables DNA repair (Huang *et al.* 1996) and if the damage cannot be repaired, the apoptotic mechanisms are activated (Matsumura & Ananthaswamy 2004). Both proteins are highly conservative (Kampinga & Craig 2010, Belyi *et al.* 2010) and they are present in variety of organisms. There are only few studies demonstrating the expression of these proteins in fish under stress conditions (Zeng *et al.* 2009).

Fish are expected to be especially sensitive to environmental warming because of their lack of thermal regulation (Donelson *et al.* 2014). Thus, their body temperature is intrinsically linked to the temperature of their surrounding environment, being easily acclimated to controlled thermal conditions in laboratory (Basu *et al.* 2002). Common carp, *Cyprinus carpio*, is a eurythermal freshwater fish whose thermal tolerance ranges from 3 °C to 35 °C, and thermal preference from 23 °C to 30 °C (Froese & Pauly 2016). It belongs to the most widely cultivated group of freshwater fishes in the world, being very important for sportive fishing and pet industry. This species has been increasingly used as a resource of food and as a model for scientific researches to investigate the effects of water pollution and temperature increase on biology, physiology and behavior of aquatic organisms (Gluth & Hanke 1983, 1984, Thoney *et al.* 2003, El-Hakim & Gamal 2009, Masud & Singh 2013, Georgieva *et al.* 2014, Jaxion-Harm & Ladich 2014, Maiditsch & Ladich 2014, Ma *et al.* 2015). Because of the aquaculture potential of *C. carpio*, the aim of the present study is to investigate the thermal tolerance and the effect of heating stress on juveniles through behavior alterations and expressions of the proteins Hsp70 and p53 in the gills, liver, muscle and heart tissues at an acclimation temperature of 25°C.

## Materials and Methods

**Experimental design:** Twenty-four juveniles of *C. carpio* with mean size of 9.0 cm  $\pm$  1.8 cm were purchased from a local supplier. At the laboratory, fish were acclimatized to the water temperature of 25 °C in 200 L tanks for 72 h prior to the onset of experiments. The specimens were distributed into four 40 L aquaria (two experimental and two control groups) and kept at 25 °C for another 24 h (n=6 animal *per* aquaria). Each aquarium was wrapped in a layer of clear plastic and Styrofoam layer with an orifice for the video camera, the intention was to minimize heat loss during experimental trials and avoid stress. They were fed *ad libitum* and continuous aeration was provided. The water of the experimental aquaria was then gradually and constantly heated at a rate of 2 °C h<sup>-1</sup> from 25 °C up to the LTMax, i.e. the temperature at which the fish ceased their opercular movement. When fish reach the LTMax, they were euthanized by spinal transection according with ethical procedures. The total duration of the experiment was of approximately 7.5 hours. Samples of muscle, liver, gills and heart tissues were collected, fractionated (0.5 cm<sup>3</sup>) and fixed for 24 h in 4% paraformaldehyde solution for immunohistochemical analysis (expression of Hsp70 and p53 proteins). Control groups were maintained at constant temperature of 25 °C up to the end of the experiments, then the tissues were subsampled and processed immunohistochemically as stated previously.

The heating system was composed of a sensitive temperature controller (ASLA®, CDP 48U12 – on-off configuration, SP/Brazil), coupled to a 300 W heater and used at 40% of its capacity. Aerators and two submersed aeration pumps were used for better aeration and homogenization of the water temperature in each aquarium.

**Behavioral response:** The behavior of the control and experimental fish was recorded in video and made by different observers. Thus, the behavioral responses of fish to increasing temperature were divided into normal behavior and other three phases (Table I) adapted of behavior studies of Cooking (1959), Beitinger *et al.* (2000) and Lutterschmidt & Hutchison (1997a, b).

In the phase III, fish were removed and returned to aquarium with acclimated temperature, they tried to ventilate by opening and closing their mouth but they did not recover.

**Determination of lethal thermal maximum (LTMax):** Lethal thermal maximum (LTMax) was estimated by application of the dynamic method described in

**Table I.** Table adapted from behavior studies of Cooking (1959), Beitinger *et al.* (2000) and Lutterschmidt & Hutchison (1997a, b). The behavior changes according with temperature increase were classified in stages.

<b>Behavior phases of fishes</b>	
<i>Phase (I)- Initial distress</i>	Fish were agitated, pitching, and swimming around the aquarium
<i>Phase(II)-Sublethal stage</i>	The specimens exhibited a decrease in swimming activity, onset of spasms and loss of equilibrium
<i>Phase (III) - LTMax</i>	Lateral decubitus and cessation of the opercular movements

Debnath *et al.* (2006) using the cessation of the opercular movements as endpoint (phase III).

**Immunohistochemistry:** The subsamples of gills, liver, muscle and heart tissues were fixed in 4% paraformaldehyde solution for 24 h, dehydrated in alcohol, cleared in xylol, embedded in Erv-Plast® (EasyPath/Erviagas, SP/Brazil) blocks, sectioned at 3 µm and placed on silane-treated slides. The histological sections were immersed in citric acid buffer (2mM citric acid and 9mM trisodium citrate, pH 6.0) and kept in microwave during 25 min for antigen recovery. The sections were incubated with Protein Block (DAKO X0909, Dako North America, CA/USA) for 25 min, peroxidase blocking solution for 20 min, and kept overnight with primary antibody p53 Monoclonal [BP53-12, Sigma®, USA], anti-Mouse, dilution 1:2500, and HSP70 Monoclonal [BRM-22, Sigma®, USA] anti-Mouse, dilution 1:6500. Primary antibodies were not used in negative control. The histological sections were incubated once again with biotinylated secondary antibody (15 min), streptavidin–biotin–peroxidase complex solution (15 min), and DAB solution for 90 seconds to amplifier of protein expression (kit DAKO K0679, Dako North America, CA/USA). The tissues were counterstained with Harry's hematoxylin and examined using a light microscope. The efficiency of the antibodies utilized was validated in the study by Cardoso *et al.* (2015) using a quality control test of the immunoreagents on *R. norvegicus* and then utilized in *Trachinotus carolinus* fishes. Photomicrographs were taken in light microscopy and analyzed with a free software ImageJ (Image processing and analysis in Java, <http://rsb.info.nih.gov/ij/>). The percentage area of DAB staining was performed by opening the Tagged Image File Format file and converting the image into 3 colors through the red-green-blue stack function in separate channels by a color deconvolution method (Ruifrok & Johnston 2001). The ImageJ plugin for color deconvolution has a built in predetermined vector values for separating hematoxylin, DAB staining, and a third complimentary channel (Tse & Marson 2013). The percentage area was measured, after this separation, with the threshold tool.

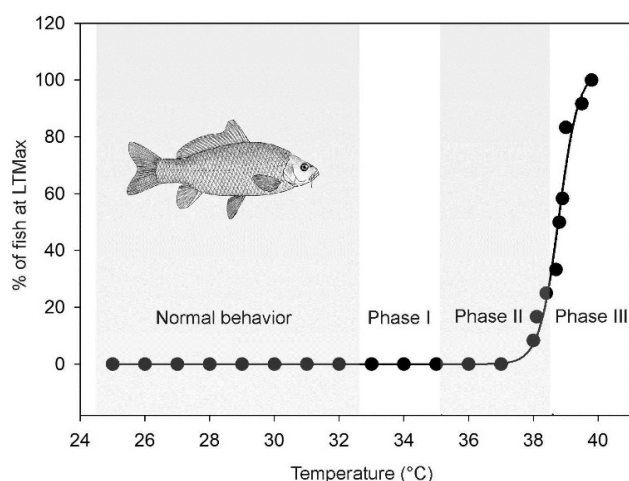
**Statistical analyses:** The statistical analyses of the data presented were checked for normality through Shapiro-Wilk test. The parametric student t or non-parametric ANOVA test ( $p \leq 0.05$ ) were carried out in order to identify differences between groups (Zar, 2009). All the statistical analyses were performed by employing the BioStat software.

## Results

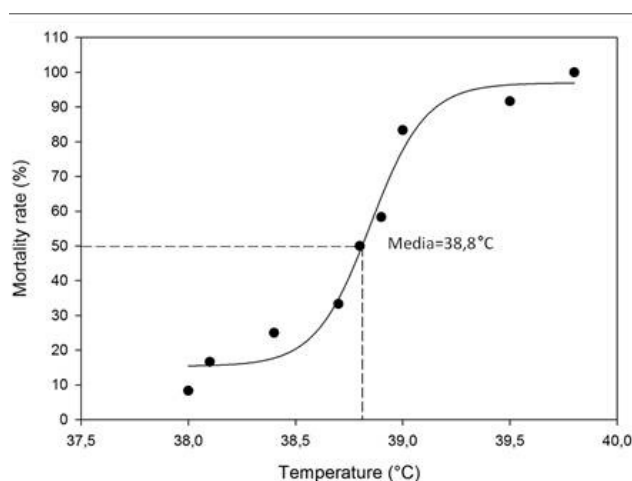
**Physical and chemical parameters of the water:** Control groups had the initial dissolved oxygen concentration of  $7.18 \pm 0.01$  mg/L and final concentration of  $7.21 \pm 0.10$ . LTMax groups had the initial dissolved oxygen of  $7.18 \pm 0.25$  mg/L and final concentration of  $5.80 \pm 0.14$  mg/L. Although at the end of LTMax, experiment the dissolved oxygen had been declined slightly, the concentration was above 5 mg/L.

**Behavioral response:** Fish behavior was considered normal throughout the experiment in the control group, as expected. Experimental fish also exhibited no sign of behavioral alterations in temperatures that ranged from 25 °C to 32.7 °C. However, fishes became agitated, pitching and swimming around the aquarium (phase I) when the temperature increased from 32.8 °C to 35.2 °C. Above 35.2 °C the individuals clearly exhibited low swimming activity, increasing the rate of opercular movement, onset of spasms and loss of equilibrium (phase II). The cessation of opercula movement (phase III) was observed in temperatures that ranged from 38.0 °C to 39.8 °C (Fig. 1).

**Fish at LTMax:** The relationship between the water temperature and the percentage of fish that reached the lethal thermal maximum temperature (LTMax) was well described by a four-parameter logistic (sigmoidal) function ( $R^2 = 0.98$ ,  $p < 0.05$ ). In general, the LTMax ranged from 38 to 39.8 °C; 58.3% of the fish reached their LTMax when the water temperature ranged from 38.7 to 39 °C. Thus, 38.8 °C was the temperature at which 50% of the fish reached the phase III (Fig. 2). In addition, changes in skin pigmentation and increase in mucus secretion were observed in all specimens before reaching the LTMax.



**Figure 1.** Behavior pattern and rate of reaching at LTMax of juvenile *C. carpio* submitted to a gradual and constant water heating (from 25 to 39.5 °C, 2 °C h<sup>-1</sup>). *Phase I*: agitated, pitching, swimming around the aquarium; *Phase II*: decrease in swimming activity, predominantly in the bottom, loss of equilibrium; *Phase III*: lateral decubitus and cessation of opercular movement.



**Figure 2.** Relationship between water temperature and percentage of juvenile *C. carpio* that reached the lethal thermal maximum temperature (LTMax).

**Immunohistochemistry (IHC):** All negative controls did not reveal immunohistochemically staining which corroborates with the specificity of the antibodies used. In gills, the control groups had no p53 expression, while the LTmax groups presented a great number of chloride cells and erythrocyte nuclei positive for p53 (Figs 3A-3B). The percentage of area immunostained for p53 was significantly higher at LTMax than in control fish (Table II). On the other hand, both groups presented Hsp70 expression, but it was significantly higher at LTMax groups than in those from the control group (Fig. 3C-3D and Table I).

In muscle, nuclei exhibited p53 expression in control and LTMax groups (Fig. 4A-4B), being significantly higher at LTMax groups (Table II). Hsp70 expression at LTMax groups occurred in cytoplasm and nuclei of the muscle being significantly higher since control groups did not express these proteins (Fig. 4C-4B and Table II).

The nuclei of the cardiac muscle expressed p53 while the nuclei and cytoplasm expressed Hsp70 at LTMax. Control groups for Hsp70 and p53 did not showed specific expression only for Hsp70 (fig. 5). The percentage of area immunostained were higher in LTMax Groups than control groups for Hsp70 and p53 (Table II).

The hepatocytes had a high p53 expression and low Hsp70 cytoplasmatic expression in fish in LTMax group. p53 and Hsp70 expression were almost undetectable in fish kept at 25 °C (control group). Thus, the percentage of area immunostained for both proteins are significantly higher in LTMax than in control fish (Fig. 6 and Table II).

The present study reports on the effects of the thermal stress on behavior and expression of specific proteins (p53 and Hsp70) in juveniles of common carp at LTMax. The heating rate used here (2 °C h<sup>-1</sup>) was slow and gradual enough to allow observations of behavioral changes and to prevent fish from acclimating to the new temperature, as suggested by Beitinger *et al.* (2000). The heating rate in our experiment was similar to those used in other studies on thermal tolerance of aquatic ectotherms (Beitinger *et al.* 2000, Frederick & Pörtner 2000).

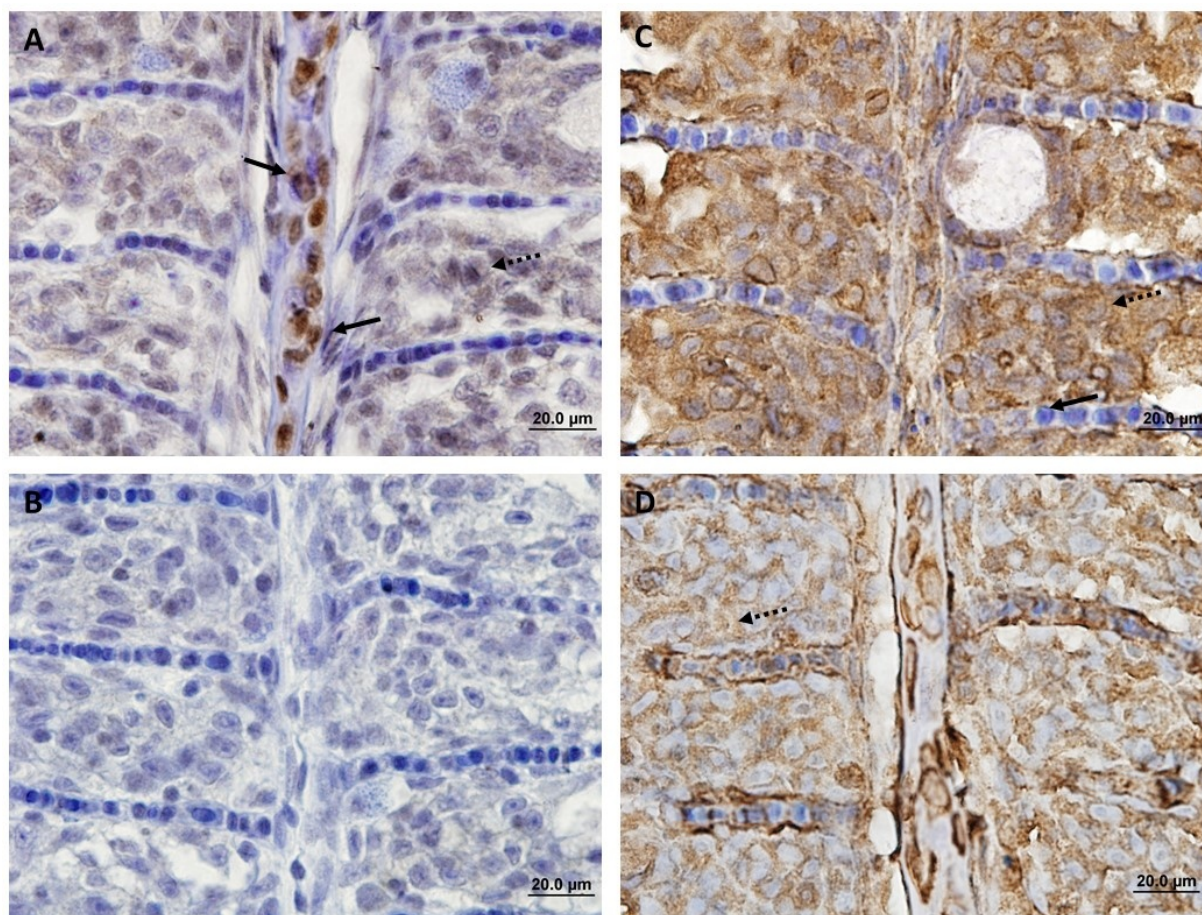
Despite the water at high temperatures diminish the dissolved oxygen in the water, a good system of aeration is enough to maintain the required for carp which can survive in low oxygen concentration (0.3 – 0.5 mg/litre) (Peteri 2004). Even though the amount of dissolved oxygen has not decrease below 5 mg/L, higher temperatures increased the fish metabolism and oxygen consumption. This fact can be visually observed since the frequency of opercular movements were higher as temperature increases.

There are few studies on thermal tolerance of carp and it is highly dependent of the acclimation temperature or the season at which fish are collected. Golovanov and Smirnov (2007) observed that at the heating rate of 40C h<sup>-1</sup>, fishes reached the lethal temperature at 37.4 0.1 ± 0.1 °C in the summer. Wang and colleagues (2007) studies reported that CTMax of common carp at heating rate of 0.3 °C min<sup>-1</sup> was 31.2 ± 0.38 °C for individuals acclimated at 10 °C. Chatterjee and colleagues (2004) observed,



**Table II.** Stained cells in percentage of area (%) in gills, liver, muscle and heart slides. Expression of p53 and Hsp70 in fish from LTMax group are significantly higher than in those of control groups, in all tissues studied.

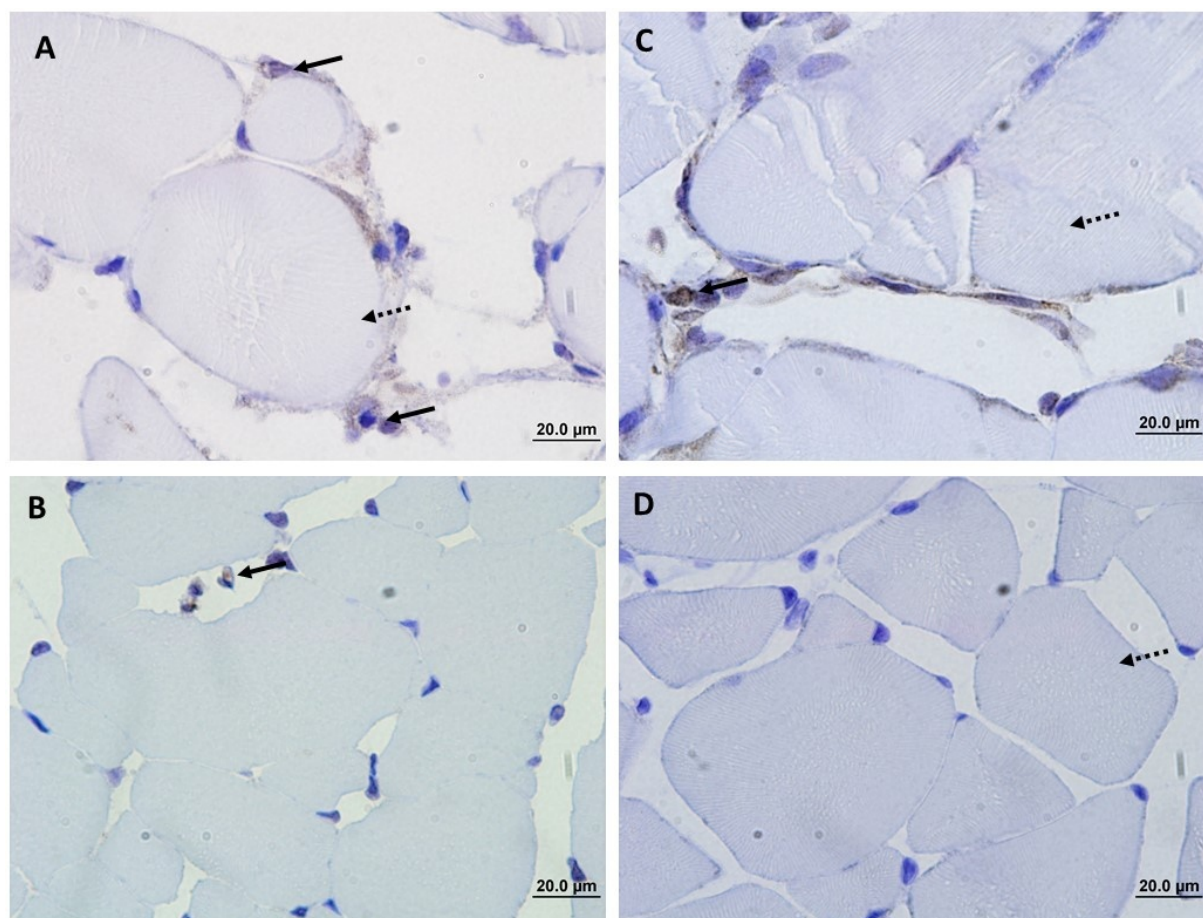
Tissues	p53		Hsp70	
	Control (%)	LTMax (%)	Control (%)	LTMax (%) *
Gills	0.00 ± 0.00	13.67 ± 3.53 *	5.36 ± 4.60	45.27 ± 7.15 *
Muscle	0.10 ± 0.05	1.82 ± 0.76 *	0.00 ± 0.00	2.73 ± 1.00 *
Heart	0.00 ± 0.00	9.67 ± 1.12 *	0.01 ± 0.02	32.23 ± 8.88 *
Liver	0.00 ± 0.00	48.16 ± 2.30 *	1.17 ± 1.79	8.76 ± 3.65 *



**Figure 3.** Immunohistochemical photomicrographs of the gills of juvenile *C. carpio* submitted to thermal stress (A and C) and of control fish kept at 25°C (B and D). A: p53 expression in nucleus (arrows) of the cells; B: almost null expression of p53 expression; C: Hsp70 expression in the cytoplasm (dotted arrows) of the cells; D: Level of Hsp70 expression in the cytoplasm of cells in control fish kept at 25 °C. Blue labeling: Hematoxylin; Brown labeling: proteins.

in a similar study, that at heating rate of 0.3 °C min<sup>-1</sup> and with fish acclimated at 25°C, the *C. carpio* CTMax temperature was of 39.7 °C ± 0.31 °C and the LTMax temperature was of 39.8 °C ± 0.06 °C. In the present study, the LTMax of juvenile carp acclimated at 25 °C was 38.8 °C ± 0.59 °C. Although the slight differences, which can be attributed to differences in acclimation (López-Olmeda *et al.* 2011), thermal history of the animal (Beitinger &

Bennett 2000) and differences of the heating methodology, the temperature in which the fishes can be severely affected is approximately 37 °C to 40 °C. Compared with other cyprinids, *C. carpio* can be considered as one of the most thermal tolerant specie of the family (Golovanov & Smirnov 2007) and can live their entire lives in shallow confined waters (Peteri 2004) that can attain higher temperatures.

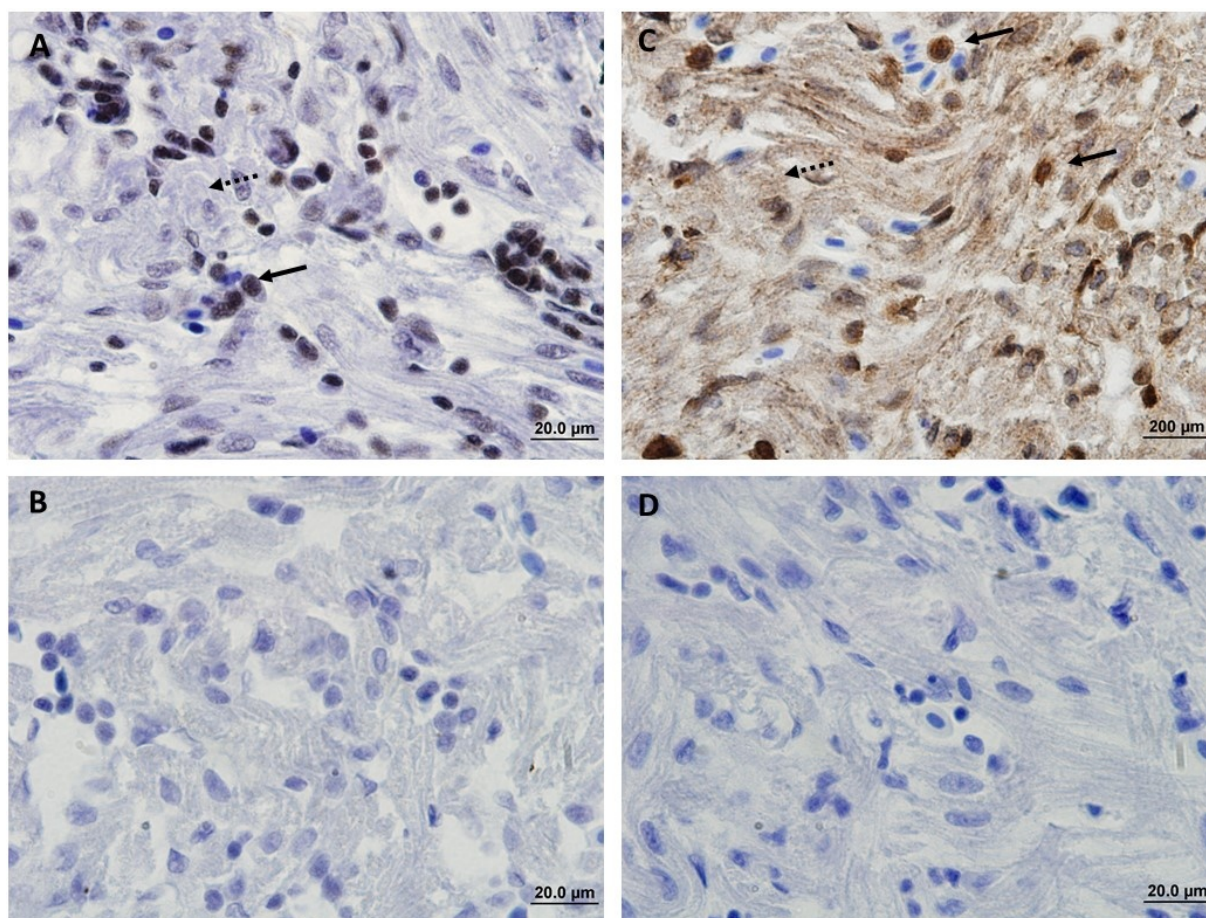


**Figure 4.** Immunohistochemical photomicrographs of the muscle of juvenile *C. carpio* submitted to thermal stress (A and C) and of control fish kept at 25°C (B and D). A: p53 expression in nuclei (arrows) of the cells ; B: almost none expression of p53; C: Hsp70 expression in the cytoplasm (dotted arrows) and nuclei (arrows) of the cells; D: Null expression of Hsp70 cytoplasm and nuclei of cells in fish kept at 25 °C. Blue labeling: Hematoxylin; Brown labeling: proteins.

Even the earlier behaviors changes observed from 32.8 °C on, at phase I may, already have negative effects on fish. Studies show that frantic movements can make organisms more vulnerable to predators (Johnson 2006, Preston & Forstner 2015) or indicate they are seeking to cooler waters (Bellgraph *et al.* 2010). In the phase II, the loss of equilibrium is probably induced by the break down in the central nervous system through the accumulation of acetylcholine in the synaptic cleft, which can disrupt the conduction and integration in the central nervous system (Bilyk & DeVries 2011). The loss of equilibrium is associated to uncoordinated upward and downward movements. By our observations, fishes can go through these phases quickly and soon reach the LTMax or can stay in a phase longer then another before reaching the LTMax. Because of this individual particularity, we collected the samples to analyze the Hsp70 and p53 proteins only when the fish reached the LTMax.

Hsp70s are frequently used as biomarkers of environmental stress in ecological and toxicological studies in fish (Burkhardt-Holm *et al.* 1998, Brun *et al.* 2008, Webb & Gagnon 2009, Metzger *et al.* 2016). In this study, fish submitted to thermal stress showed higher Hsp70 expression than controls in all tissues analyzed, as described in literature (Currie *et al.* 2000, Yamashita *et al.* 2010, Jesus *et al.* 2013). Our results also demonstrated that tissues can have different levels of Hsp70 expression, being more accentuated in the heart and in the gills. The increase in Hsp70 at higher temperature in gills, which are in contact with the water, suggests that *C. carpio* is adapted to deal with high temperatures. Some individuals displayed Hsp70 nuclei expression in cardiac cells as Hsp70 can be displaced from the cytoplasm to the nucleus to protect ribonucleic proteins (RNP) (Seguí-Simarro *et al.* 2003). The nucleic RNPs are associated to RNA and they are sensitive to heat. The role of Hsp70 on RNPs





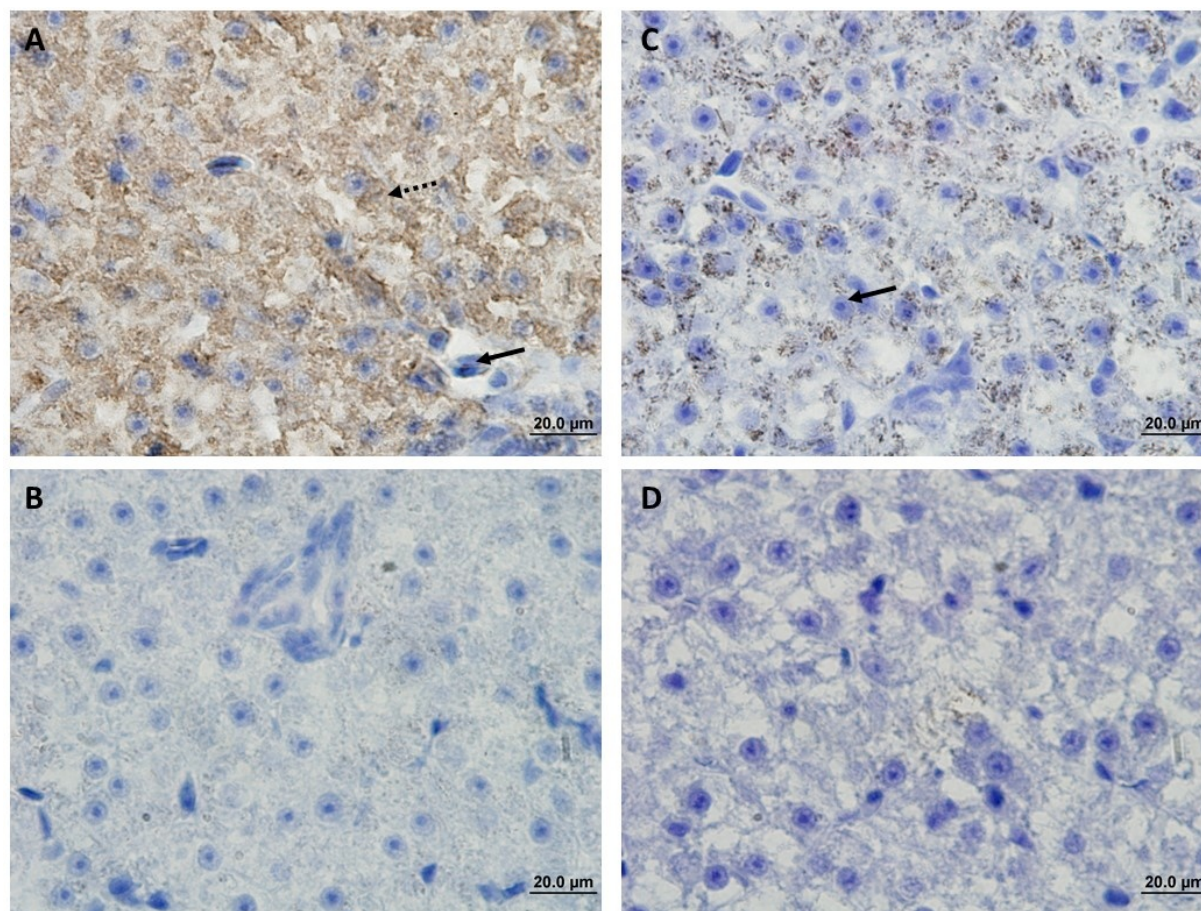
**Figure 5.** Immunohistochemical photomicrographs of the heart of juvenile *C. carpio* submitted to thermal stress (A and C) and of control fish kept at 25°C (B and D). A: p53 expression in nucleus (arrows) of the cells; B: lack of p53 expression; C: Hsp70 expression in the cytoplasm (dotted arrows) and nucleus (arrows) of the cells; D: null expression of Hsp70 expression in cytoplasm of cells in fish kept at 25 °C. Blue labeling: Hematoxylin; Brown labeling: proteins.

seems to be of a great importance to the synthesis of mRNA and rRNA (Bleichert & Baserga 2010). Similar results were obtained by Wang *et al.* (2007), by exposing *C. carpio* to acute heat shock. The fish expressed higher amount of Hsp70 in gills, heart, brain and kidney when compared to control, having mayor expression in gills, heart and kidney. They concluded that different Hsp70 tissue response could have a close relationship with the thermal tolerance of the carp and can facilitate survival when they faced thermal stress. Studies comparing expressions of Hsp70 in mouse and drosophila in different organs also discussed the possible causes of these differences of responses. While Blake and colleagues (1990) believed that this differences reflects the influence of physiologic components in modulating the heat shock response in vivo, Krebs and Feder (1997) believed that some cells respond primarily to damage caused by heat shock independently of the temperature itself and/or that Hsp70 is also damaged by heat and requires time for

recovery in some tissues. Sung and colleagues (2014) described that the heat shock stimulation induces Hsp70 accumulation and confers tolerance to lethal ammonia stress on the common carp *C. carpio* validating the role of Hsp70 in enhancing the stress tolerance. Studies linking Hsp70 and temperature are complex because the past thermal history has a significant impact on the patterns of the Hsp70 response (Hofmann 2005) and because that to compare the diverse studies is a real challenge.

In normal cells, p53 is latent and inactive (Oren *et al.* 2002). However, they become active when exposed to stress situations and usually are expressed in the nuclei. In our study, the high expression of p53 in the nuclei of cells from gill, muscle and heart from fish exposed to thermal stress indicated the occurrence of DNA damage caused by temperature increase. When compared to Hsp70, p53 had lower expression in all tissues, with exception of the liver. Canadillas and colleagues (2006)





**Figure 6.** Immunohistochemical photomicrographs of the liver of juvenile *C. carpio* submitted to thermal stress (A and C) and of control fish kept at 25°C (B and D). A: p53 expression in the cytoplasm (dotted arrow) but not in the nuclei (arrow); B: No expression of p53; C: Hsp70 expression in the cytoplasm (dotted arrow) but also not in the nuclei (arrow). D: Low level of Hsp70 expression in cytoplasm of cells in fish kept at 25 °C. Blue labeling: Hematoxylin; Brown labeling: proteins.

mentioned that p53 have a relative thermodynamical instability. Contrary to what were expected, liver cells had an intense expression in the cytoplasm, which can be involved with the capacity of the p53 trigger the intrinsic apoptotic pathway through the mitochondria. The p53 binds with anti-apoptotic and pro-apoptotic proteins and forms permeable pores in the outer membrane, releasing cytochrome c and activation of caspase-3 (Lu & Lim 2016). Other studies have demonstrated p53 proteins associated with cytoplasmic filaments of actin (Katsumoto *et al.* 1995, Metcalfe *et al.* 1999) and with microtubules (Maxwell *et al.* 1991). If microtubules undergo severe damage, the migration of p53 from cytoplasm to nucleus is impaired (Giannakakou *et al.* 2000); p53 in the cytoplasm can also participate in cell protection mechanisms, binding to the endoplasmic reticulum when this structure is damaged by external agents (Qu *et al.* 2003). There is a lack of data relating p53 and thermal temperature tolerance in *C. carpio* and, although

well characterized in mammals as a DNA damage-protective protein, some works have been done with other organisms. One example is the work of Qian and colleagues (2014) which reported that p53 is involved in shrimp survival in response to acute environmental stresses. This demonstrates that the function of p53 is highly conservative and that it could also be used as a biomarker for genomic damage in aquatic ecosystems (Liu *et al.* 2011). Nevertheless, some precautions have to be taken. Although several studies in fish reported p53 induction after the exposure of cells to thermal stress (Qi *et al.* 2013), chemical stressors (Lee *et al.* 2008, Brzuzan *et al.* 2009, Mai *et al.* 2010, Zhou *et al.* 2015, Zhou *et al.* 2016) or both combined (Ji *et al.* 2012); others studies observed there were no changes in p53 response to the agents (Chen *et al.* 2001; Rau Embry *et al.* 2006, Liu *et al.* 2011). It is important to consider that p53 distribution and activity can differ between different tissues types and species (Liu *et al.* 2011).

Comparing the results of p53 and Hsp70, the tissues which express mayor concentration of these proteins are in both cases the gills and heart, indicating the higher capacity that these tissues have to try to protect themselves from the thermal stress. In normal cells the expression of the Hsp70 is regulated by proteins from the p53 family (Quenneville *et al.* 2002) which inhibit the transcription of Hsp70 by binding to its promoter sites (Szymańska & Zylicz 2009). If the Hsp70 expression increases, it inhibits the p53 ubiquitination (Narayan *et al.* 2015) and the levels of p53 increases. The Hsp70 also can bind directly with p53 when it becomes unfolded during cellular stress or in physiologic condition when the p53 nascent chain is being folded (Fourie *et al.* 1997).

The liver is an exception since it presented a high expression of p53 and a low expression Hsp70. Miova and colleagues (2015) found that human cultured hepatic cells (HepG2 ) after the exposure to heat shock and subsequent recovery had different p53 and Hsp70 expression in time. Perhaps the liver is a sensible tissue and the expression of Hsp70 raised until certain temperature and then dropped at LTMax, but further studies should be done to investigate this. The study conducted by Cardoso *et al.* (2015) in *Trachinotus carolinus* fishes found that Hsp70 expression in the gills at LTMax declined almost at the level of control group. This could be explained by a negative feedback regulation (Abravaya *et al.* 1991, Selvakumar & Geraldine 2005) or even the collapse of the Hsp70 (Hochachka & Somero 2002). These results suggest that the pattern of expression not only varies according with the tissue, but also with the species.

In a general way, there are three categories of stress in fish: the primary consists in a neuroendocrine/endocrine response and is more general; secondary comprises the various biochemical and physiological adjustments associated with stress and the tertiary response represents whole animal and population level changes associated with stress (more details in Iwama *et al.* 1999). Thus, the temperature not only affect the behavior or the physiology of the organism, but also the development, growth, metabolism and reproduction (López-Olmeda *et al.* 2011).

## Conclusions

The temperature at which the *C. carpio* fishes starts the behavior changes is close to the temperature at which they could be find in nature.

This species is eurythermal and can be found very close to their thermal limits. Data indicates that they could be in a great risk of being affected by temperature increase. Carps present cellular responses of Hsp70 and p53 that can vary according to the tissue and this responses can protect them from the thermal stress. These proteins, therefore, are related to cellular process of thermal stabilization, being the liver highly sensitive to temperature changes. Results demonstrated that the expression of both proteins could be used as a biomarker. Results of this work further contributes to the understanding of biological effects of temperature increase on fish, the role of proteins in protecting organs and the organism, as well as their thermal vulnerabilities in an event of environmental warming.

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## Ethical disclaimer

Authors declare that experimental procedures and the manipulation of animals during the investigations reported in this paper complied with ethical protocols and all aplicable national and institutional regulations.

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