



Universidade de Aveiro  
2018

Departamento de Biologia

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**Effects of sub-lethal heat shock in the  
tolerance of *Danio rerio* embryos to cadmium  
exposure**

**Efeitos de um choque térmico sub-letal na  
tolerância a cádmio em embriões de *Danio  
rerio***

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Toxicologia e Ecotoxicologia, realizada sob a orientação científica da Doutora Inês Domingues, Investigadora em Pós-Doutoramento do Departamento de Biologia da Universidade de Aveiro e do Doutor João Pestana, Investigador auxiliar do Departamento de Biologia e CESAM da Universidade de Aveiro.

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## **agradecimentos**

Agradeço a todas as pessoas que me apoiaram durante o decorrer da elaboração desta tese. Em especial ao Nuno Barbosa, ao Ricardo Santos, à Ana Sousa, à Ana Domingues, à Vera Maria e à Anabela Simões pelo apoio e pela boa disposição nos momentos mais difíceis, aos meus pais por fazerem possível a elaboração desta tese e aos meus orientadores por partilharem o seu conhecimento.

## palavras-chave

Choque térmico, cádmio, *Danio rerio*, peixe-zebra, embrião, tolerância induzida, tolerância-cruzada, comprimento total, largura do saco vitelino, comportamento, biomarcadores, stress oxidativo, proteínas de choque térmico, metalotioninas

## resumo

Os organismos aquáticos estão frequentemente expostos a múltiplos *stressores* e sabe-se que um *stressor* pode afetar a tolerância a outro subsequente *stressor* de diferente natureza (processo conhecido como tolerância cruzada). Para determinar os efeitos de um choque térmico na tolerância ao cádmio (Cd), os embriões de peixe-zebra foram expostos a um choque térmico (HS) de 37°C durante 1h e posteriormente expostos a uma gama de concentrações letais de Cd durante 5 dias. Os embriões que foram previamente expostos ao HS mostraram-se mais tolerantes ao Cd, comparativamente aos embriões mantidos à temperatura normal; no entanto, após 48h, esta tolerância não é mais evidente. Para avaliar o impacto da tolerância cruzada no estado de saúde geral dos embriões e os possíveis mecanismos envolvidos neste processo, os organismos foram também expostos a concentrações sub-letais de Cd (após o HS) e parâmetros de vários níveis foram medidos: comportamento, comprimento total e largura do saco vitelino, respostas de vários biomarcadores (Colinesterase (ChE), Glutathione S-Transferase (GST), Catalase (CAT), Glutathione Total (TG), Sistema de Transporte de Eletrões (ETS), Peroxidação Lipídica (LPO)) e também os níveis de proteínas de choque térmico 70 (HSP70) e metalotioninas (MTs). Os nossos resultados mostraram que o HS diminui o comprimento total e a largura do saco vitelino das larvas, provoca hiperactividade e afeta a atividade da ChE e da GST e o conteúdo em TG em larvas de peixe-zebra. Os níveis de MTs não foram afetados pelo Cd ou pelo HS. O Cd também não afetou os níveis de HSP70, no entanto, o HS resultou num aumento do conteúdo de HSP70. Embora o HS tenha aumentado a atividade da GST e o conteúdo em TG, que poderia subsequentemente proteger os embriões da exposição ao Cd nas primeiras 48 horas, o nosso estudo não aponta claramente o envolvimento desta resposta geral ao *stress* na tolerância cruzada ao Cd relatada no ensaio agudo. Por sua vez, embora a exposição ao Cd não tenha tido qualquer efeito sobre os níveis de HSP70, estes resultados sugerem que o aumento dos níveis de HSP70 induzidos pelo HS pode estar envolvido na tolerância cruzada ao Cd. São necessários estudos futuros para confirmar o envolvimento do aumento da atividade da GST, do aumento do conteúdo em TG e do aumento dos níveis de HSP70 no processo de tolerância cruzada e abordar a regulação dos genes destas respostas gerais de stress e as suas funções celulares que culminam na tolerância induzida ao Cd.

**keywords**

Heat shock, cadmium, *Danio rerio*, zebrafish embryo, total length, width of the yolk sac, induced tolerance, cross-tolerance, biomarkers, oxidative stress, heat shock proteins, metallothioneins

**abstract**

Aquatic organisms are often exposed to multiple stressors and it is known that one stressor can affect the tolerance to another subsequent stressor of different nature (a process known as cross-tolerance). To determine the effects of a heat shock on cadmium (Cd) tolerance, zebrafish embryos were exposed to a heat shock (HS) of 37°C during 1 h and then exposed to a range of lethal concentrations of Cd during 5 days. Embryos that were previously exposed to HS were more tolerant to Cd, compared to embryos maintained at normal temperature; however, after 48 hours, this tolerance is no longer evident. In order to evaluate the impact of cross-tolerance on the general health status of embryos and the possible mechanisms involved in this process, organisms were also exposed to sub-lethal concentrations of Cd (after HS) and multilevel endpoints were measured: behavior, total length and width of yolk sac and a set of biomarker responses were assessed (Cholinesterase (ChE), Glutathione S-Transferase (GST), Catalase (CAT), Total Glutathione (TG), Electron Transport System (ETS), Lipid Peroxidation (LPO)) and also the Heat shock protein 70 (HSP70) and Metallothioneins (MTs) content. Our results showed that HS decreases the total length and width of yolk sac of larvae, causes hyperactivity and affects the activity of ChE and GST and TG content in zebrafish larvae. MTs content was not affected by Cd or HS. Cd also did not affect HSP70 levels, however HS resulted in increased HSP70 content in zebrafish. Although HS increased GST activity and TG content, which could subsequently protect the embryos from Cd exposure within the first 48h, our study does not clearly indicate the involvement of this general stress response on cross-tolerance to Cd reported in the acute assay. In turn, although Cd exposure had no effect on HSP70 levels, our results suggest that increased HSP70 levels induced by HS could be involved in cross-tolerance to Cd. Moreover future studies are required to confirm the involvement of increased GST activity, increased TG content and increased HSP70 levels in the cross-tolerance process and address the regulation of these general stress responses genes and their cellular functions that ultimately culminate in induced tolerance to Cd.

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## **List of abbreviations and acronyms**

**ROS** – Reactive Oxygen Species  
**Cd** – Cadmium  
**SOD** – Superoxide Dismutase  
**CAT** – Catalase  
**GSH** – Glutathione  
**GPx** – Glutathione Peroxidases  
**GST** – Glutathione -S- Transferase  
**HSPs** – Heat shock proteins  
**MTs** – Metallothioneins  
**HSP70** – Heat shock proteins 70  
**FET** – Fish Embryo Toxicity Test  
**TG** – Total glutathione  
**LPO** – Lipid peroxidation  
**ChE** – Cholinesterase  
**ETS** – Electron transport system  
**HS** – Heat Shock  
**PMS** – Post-Mitochondrial Supernatant  
**TBARS** – Thiobarbituric Acid Reactive Species  
**LC<sub>50</sub>** – Median Lethal Concentration  
**ELISA** – Enzyme Linked Immunosorbent Assay

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# **Chapter 1 - Introduction**

## **Exposure to multiple stressors**

Stress can be defined as a state in which organisms are challenged by biotic and/or abiotic stressors in their environment, translating into a response, usually at the biochemical and molecular level (Parker, 1999; Van Straalen, 2003). Some of these responses are general and do not depend on the nature of the stressor, such as the development of oxidative stress and the induction of defense proteins (Steinberg, 2012).

In their natural environments, aquatic organisms, like fish, are frequently exposed to multiple stressors that have either a natural or anthropogenic origin, for example, in addition to daily and seasonal temperature variations, fishes are often affected by the release of heated waste water and toxic chemicals such as metals (Hallare et al., 2005; Todgham et al., 2005; Vergauwen et al., 2013a).

## **Temperature**

One of the physical factors that most influence fish is temperature. Several authors even considered temperature as the “abiotic master factor” because it influences the behavior, physiology and distribution of aquatic organisms (Brett, 1971; López-Olmeda and Sánchez-Vázquez, 2011). In addition, the study of its effects became more urgent with the evidence of climate changes and with an increase in the frequency of extreme events like heat waves (Vergauwen et al., 2013a). Anthropogenic activities can affect water bodies’ temperature through global climate change, regional land-use alteration, heated effluents from power generation plants, and summertime urban stormwater runoff (Kinouchi et al., 2007).

Temperatures over and below the range of tolerated ambient temperature generate a state of stress in fish including sublethal physiological and behavioral responses or even death (Gordon, 2005). Fish are particularly vulnerable to environmental temperature variations since they are poikilotherms and have a blood-water countercurrent respiratory system (Beitinger et al., 2000; Schmidt-Nielsen, 1997; Vergauwen et al., 2013b). As a result, temperature has been identified as one of the most vital abiotic factors for fish, since it can potentially affect all the metabolic, physiological

and also ecological aspects and behaviour of fish life cycle (Almeida et al., 2014). Temperatures beyond the optimal limits of a particular fish species adversely influence fish health by increasing the metabolic rate, oxygen consumption, and the invasiveness and virulence of pathogens, which, in turn, will cause a variety of pathophysiological disturbances that can lead to fish death (Dalvi et al., 2009; Gordon, 2005).

Sudden acute changes in temperature act on physiological processes predictably, with cold temperatures slowing, and warmer temperatures accelerating them. Therefore, fish can compensate temperature changes by appropriate alterations in metabolic rates and oxygen consumption. However, these alterations may induce thermal stress with the formation of reactive oxygen species (ROS), which can lead to protein, lipid or DNA damage, and potentially increase energy demand. All these physiological alterations can be measured using biomarkers, which have been considered as useful tools in detecting early adverse effects (Almeida et al., 2014).

In zebrafish, it was reported that temperature above the optimum (26–28°C) affected the rate of development, occurring faster at 33°C (Kimmel et al., 1995), decreased the size of the embryos (Atkinson, 1994) and increased their heart rate (López-Olmeda and Sánchez-Vázquez, 2011).

## **Cadmium**

Chemical pollution is one of the main causes suggested to explain the decline of aquatic species worldwide (Wu et al., 2017). Metals reach the aquatic ecosystems as a consequence of anthropogenic activities, thus this form of pollution is one of the five principal types of pollutants commonly present in surface waters (Atli et al., 2006; Giri et al., 2016; van Dyk et al., 2007). The ubiquity of metal pollution is probably due to some specific characteristics, such as, tendency to accumulate in organisms, persistence in environment due to their chemical stability or poor biodegradability and environmental mobility because they are readily soluble (van Dyk et al., 2007). Metals, despite being found naturally in the ground and surface waters, are considered hazardous pollutants with a significant ecological impact since they can modify the chemical and physical properties of the water bodies, thus affecting aquatic flora and fauna (Giri et al., 2016; van Dyk et al., 2007).

Some metals are essential elements for the normal metabolism of organisms, while others are nonessential and play no significant biological roles (van Dyk et al., 2007). Cadmium (Cd) is a nonessential metal and an important source of contamination to the aquatic ecosystems, since it cannot be degraded, accumulate via the food chain, increasing the risk of environmental exposure (Cuypers et al., 2010; van Dyk et al., 2007). Studies indicate that Cd bioaccumulates in phytoplankton and complex food webs involving aquatic animals such as mollusks, crustaceans and fish (Acosta et al., 2016; Vergauwen et al., 2013b).

Cd contamination has received more importance over the past two centuries since its concentration in aquatic ecosystems have been increased by anthropogenic activities (Wu et al., 2017; Zheng et al., 2016). Some of these activities are the production of nickel Cd batteries, stabilizers, synthetic pigment, metals melting, discharge of municipal effluents, industrial discharges, and mining activities (Guo et al., 2017; Vergauwen et al., 2013b; Wu et al., 2017). Cd pollution is a serious global problem, because Cd causes irreversible toxicity in organisms and is a highly toxic pollutant in rivers, estuaries and nearshore waters (Giri et al., 2016; Ma et al., 2008). Besides that Cd is toxic to humans causing developmental defects, cognitive dysfunction and acting as a carcinogenic agent (classified by the International Agency for Research) (Chow et al., 2008; Vergauwen et al., 2013b; Yuan et al., 2017; Wang et al., 2015).

The most problematic case is in China, where the fossil fuel burning, the waste incineration, the industrial waste discharges and mining activities has contributed to widespread Cd contamination (Ma et al., 2008). Normally, the dissolved Cd levels ranged from 10 to 500 ng L<sup>-1</sup>, but in some industrialized areas in China the levels exceed 1 mg L<sup>-1</sup> and in some waste waters from mines and smelter, the levels can reach 26.5 mg L<sup>-1</sup> (Jin et al., 2015; Ma et al., 2008; Yuan et al., 2017; Zheng et al., 2016).

Since Cd is extremely toxic to humans, animals, and plants even at low concentrations, its toxicity is a well-studied topic in aquatic toxicology (Zheng et al., 2016). Cd can cause diverse effects such as DNA damage and oxidative stress, impairment of reproduction and disruption of ion-osmoregulation, also it is well known that the initial effects of metal pollution may be evident only at cellular or tissue levels before significant

changes can be identified at the whole organism level (Jia et al., 2011; Sellin and Kolok, 2006; Suresh et al., 1995; van Dyk et al., 2007).

Cd is a bivalent cation unable to generate free radicals directly, however exerts its toxicity by the production of ROS leading to impairment of normal oxidative metabolism and oxidative stress (Cuypers et al., 2010; Wang and Gallagher, 2013; Wu et al., 2017). ROS are products of normal cellular metabolism and play an indispensable role in anti-oxidative and anti-microbial defenses, acting directly or as signaling molecules in oxidative stress and inflammatory responses (Zheng et al., 2016). However, excessive ROS can overwhelm cell's intrinsic antioxidant defenses and attack cellular macromolecules including lipids, proteins and DNA leading to oxidative stress and causing lipid peroxidation, protein oxidation and DNA damage (Cuypers et al., 2010; Wang et al., 2015). A proper ROS balance must be maintained for survival, therefore the organisms have developed antioxidant defenses and innate immune systems that helps them cope with the threat (Wu et al., 2017; Zheng et al., 2016). Like other organisms, fish combat elevated levels of ROS with protective antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT) glutathione-dependent enzymes (GSH) namely glutathione peroxidases (GPx) and glutathione-s-transferases (GST). However, once these enzymes are overwhelmed by excessive ROS production, irreversible cellular damage and death can occur (Craig et al., 2007; Lushchak and Bagnyukova, 2006).

### **Cross-tolerance and general stress responses**

Several studies addressed the combined effects of elevated temperature and Cd, concluding that elevated temperature increases the toxicity and bioaccumulation of metals in aquatic organisms, but few studies have investigate the effects when these stressors are applied sequentially, which may also occur in natural ecosystems (Abdel-Tawwab and Wafeek, 2014; Guinot et al., 2012; Olsvik et al., 2016; Piazza et al., 2016).

In 1978, Li and Hahn were among the first researchers to observe that cultures of mammalian cells pre-exposed to sublethal thermal stress acquired greater resistance to subsequent chemical exposure (Li and Hahn, 1978). Thus, it was verified that the phenotypic plasticity in physiological mechanisms of defense against environmental stressors can allow an increased tolerance (Pestana et al., 2016). With the observation of this process, the concept of cross-tolerance or cross-protection emerges, which translates

into the capacity of the exposure to one stressor to alter the tolerance of an organism to other stressors of different nature (Kampinga et al., 1995; Sabehat et al., 1998). Thus exposure to mild stress, which can occur naturally in ecosystems, can be beneficial to the organisms, even if they exhibit typical stress responses, because it can stimulate the stress defense system, increasing the ability of the organisms against future stressors (Minois, 2000; Steinberg, 2012; Suhett et al., 2011).

Based in these investigations, Suhett et al. defined a conceptual model representing a sequential exposure to two stressors. In this model it is possible to verify that the exposure to the first, low intensity, stressor can activate a stress defense system, giving the organism more resistance to a second stressor. However, this phenomenon occurs only in a range of intensities of the first stressor very restricted, below which the defense system is not activated and above which, the energy cost will suppress the possible beneficial effects of the first stressor, leading to synergistic effects of the two stressors. Moreover, above this range of intensities, the stress caused by the first stressor becomes energetically expensive, preventing the occurrence of beneficial effects (Suhett et al., 2015).

Regardless of the intensity of the first stressor, increasing the intensity of the second stressor always reduces the likelihood of beneficial effects of the first exposure (Suhett et al., 2015). Thus, we can conclude that this phenomenon is very limited by the intensity of the stressors involved and by the protective capacity of the stress defense system activated. In addition, adaptive processes of defense require a lot of energy expenditure and the reallocation of energy resources can lead to negative consequences on the suitability of the organisms, for example, more energy is spent in detoxification than in reproduction, leading to a decrease in production of descendants (Haap et al., 2016; Klerks et al., 2011).

Despite the obvious importance of the concept of cross-tolerance for species survival, few studies have been concerned with sequential exposures and investigated how stress induced by an environmental factor, like temperature, may alter the response to another stress, like Cd (Li and Hahn, 1978). The existence of cross-tolerance between these two stressors is only possible if the defense mechanism induced by the first stressor is also

involved in the protection against the second stressor. Although researchers have suggested other mechanisms of common defense against Cd and thermal stress such as increased activity of antioxidant enzymes (Muysen et al., 2010) or metabolic depression (Leung et al., 2000), induction of heat shock proteins (HSPs) (Pestana et al., 2016) and increased metallothioneins (MTs) content (van Cleef-Toedt et al., 2001) are stated as the general stress responses most probably involved. These common mechanisms could allow a cross-induced tolerance because when the second stressor appears, the defense is already present and ready to act.

### **Heat shock proteins**

When temperature is the first stressor most authors suggest the involvement of HSPs in cross-tolerance. These proteins normally represent 5-10% of the total proteins in the cell and increase in amount when cells are exposed to natural or anthropogenic stressors such as temperature, salinity, hormones, nutrient deficiencies, hypoxia or anoxia, diseases, pesticides, metals, desiccation, ultraviolet radiation, parasites, bacterial and viral infections and predators (Basu et al., 2002; Lewis et al., 1999; Pestana et al., 2016; Pirkkala, Lila; Nykanen, Paivi; Sistonen, 2001; Sung et al., 2011). These proteins were first described in cells from *Drosophila melanogaster* during exposures to high temperature (Ritossa, 1962) and so the term “heat shock protein” (Lewis et al., 1999). HSPs are present in all organisms from bacteria to mammals and play important roles in protecting against stressors that can cause cell damage (Pestana et al., 2016; Todgham et al., 2005; Werner et al., 2007). Although most HSPs have a relatively short half-life, some persist in the cell after removal of the stressor and thus may play an important role in long-term adaptation (Dubeau et al., 1998). HSPs are usually classified into different families according to their molecular size and it is known that the family most involved in the responses to stressors is HSP70, the most highly conserved of the HSPs (Lewis et al., 1999; Mahmood et al., 2014). Various studies with zebrafish have already identified and cloned a number of HSPs, including HSP70, HSP47, HSP27, HSP90a and HSP90b (Krone et al., 1997; Krone and Sass, 1994; Lele et al., 1997; Råbergh et al., 2000).

HSPs are involved in essential cell roles such as protein assembly, correct folding and translocation, as well as in regulating interactions between hormones and their

receptors (Iwama et al., 1998). These proteins may thus play a key role in the cross-tolerance process by preventing protein denaturation, by restructuring damaged proteins or by ensuring the degradation of irreversibly damaged proteins, preventing their accumulation and aggregation (Sung et al., 2011). Todgham et al. investigated cross-tolerance to osmotic stress after mild thermal stress in *Oligocottus maculosus* and concluded that this process was associated with the induction of HSP70 in the gills of the organisms (Todgham et al., 2005). Dubeau et al. obtained similar results in *Salmo salar* (Dubeau et al., 1998). In turn, Tedengren et al. examined cross-tolerance to Cd induced by mild thermal stress in *Mytilus edulis* and also detected an increase in HSP70 induction in the organisms (Tedengren et al., 2000). These studies demonstrate that HSPs are a general defense response likely involved in the cross-tolerance process and are therefore central to the survival of natural populations.

### **Metallothioneins**

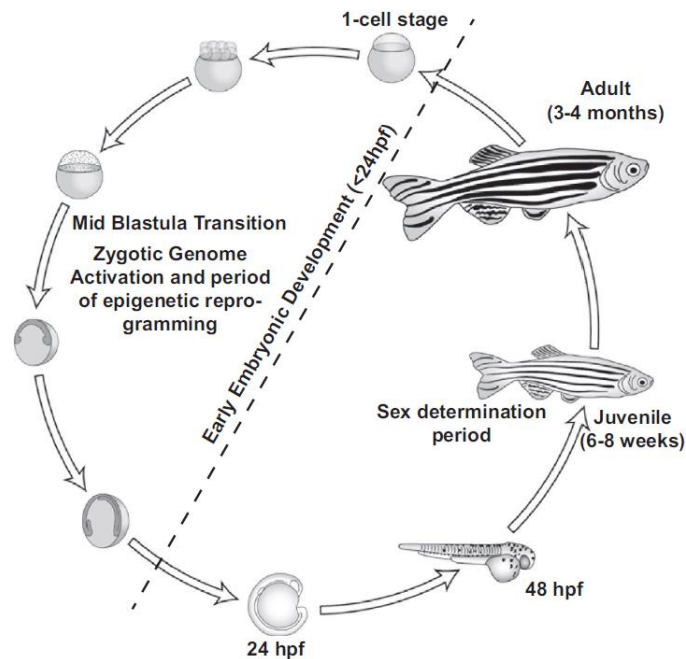
When one of the stressors is a metal the authors suggest the involvement of MTs in cross-tolerance. MTs are a family of low molecular weight cytosolic proteins that contain highly conserved cysteine residues and occur in a large number of phylogenetically diverse organisms. These residues allow these proteins to bind, carry and store various metals. It has been proposed that MTs play an important role in regulating the intracellular availability of essential metals such as zinc and copper and in the detoxification of toxic metals such as Cd and mercury. Metal sequestration may be a mechanism by which MTs confer protection against Cd toxicity (Guinot et al., 2012; Ma et al., 2008; van Cleef-Toedt et al., 2001).

Thus, the increase in cell resistance to Cd toxicity can be achieved by processes that result in increased ability to synthesize MTs (Ma et al., 2008). Since the synthesis of these proteins can be induced by endogenous and exogenous processes as general stress conditions, such as heat stress, their involvement in cross-tolerance processes is very likely (Guinot et al., 2012; Plautz et al., 2013). Some studies have demonstrated that a mild thermal stress causes a significant increase in the induction of MTs in tissues such as liver,

gills and intestine of *Fundulus heteroclitus* and in the liver of *Sparus aurata* (Guinot et al., 2012; van Cleef-Toedt et al., 2001).

## Zebrafish

Zebrafish (*Danio rerio* Hamilton, 1822) belongs to the family of freshwater fishes Cyprinidae and in the wild, it can be found in north-eastern India, Bangladesh and Nepal. These areas have a typical monsoon climate with marked seasonal variations, so there is a wide variation in temperatures, with both daily and seasonal variations being reported (Craig et al., 2007; López-Olmeda and Sánchez-Vázquez, 2011; Spence et al., 2008). Embryonic development of zebrafish has a thermal optimum of 28.5°C and thus laboratory experiments using zebrafish as a test species mostly use 26–28°C as a standard temperature (Kimmel et al., 1995; López-Olmeda and Sánchez-Vázquez, 2011).



**Figure 1.** Representation of Zebrafish life cycle since the egg phase to the adult fish (Aluru, 2017).

Zebrafish has become one of the most highly valued model organism in many fields of research, including genetics, neuroscience, development, physiology, toxicology and biomedicine, and it is frequently used as a model of many human diseases because cardiovascular, nervous and digestive systems of this model are similar to mammals and a high level of resemblance exists among the human and zebrafish genomes (more or less 75% similarity) (Chakraborty et al., 2016; Chow et al., 2008; Fishman, 2001; Haffter et al.,

1996; Langheinrich, 2003; Vascotto et al., 1997). Some characteristics have contributed to the common use of this species, namely its small size (3-5 cm), high fecundity, short generation time (embryo to adult in 3-4 months), external fertilization and development, well-characterized embryonic development (Kimmel et al., 1995), easy maintenance and breeding in laboratory, optical transparency during early development stages and its rapid absorption of substances added directly to the water (Acosta et al., 2016; Aluru, 2017; Craig et al., 2007; Lawrence, 2007).

Zebrafish eggs and larvae' transparency, its fast development and external fertilization allow live embryos to be manipulated throughout their developmental stages, visualization of any developmental abnormalities and enables exposure of embryos to stressors immediately after fertilization, in the absence of any maternal influence. In addition, it is a robust fish, has a mean lifespan of 42 months and large numbers can be kept easily and cheaply in the laboratory. Females can spawn every 2-3 days and a single spawn may contain several hundred eggs, making it possible to obtain high sample sizes for experiments (Aluru, 2017; López-Olmeda and Sánchez-Vázquez, 2011; Spence et al., 2008).

Fish are commonly used for toxicological studies because they are affected both directly through contact with contaminated water and indirectly through their diet. Thus, fish may reflect contamination in other organisms and trophic levels within the aquatic ecosystem and are an important part of the diets of other organisms (Acosta et al., 2016). Zebrafish is a popular vertebrate model system in toxicology and it is recommended as a test species for use in the fish acute, prolonged and chronic toxicity test by OECD (guideline 203, 1992 and 204, 1984) and EPA (OPPTS 850.1075, 1996 and 850.1730, 1996). So there are a number of larval and adult behavioral assays developed to assess the effects of exposure to toxicants (Aluru, 2017; López-Olmeda and Sánchez-Vázquez, 2011; Zheng et al., 2016). Besides that, according to European Union legislation for the protection of animals used for scientific purposes, the use of embryonic stages of vertebrates is not regulated so, the fish embryo toxicity tests (FET) are considered as alternative to animal experiments (Embry et al., 2010; Scholz et al., 2008).

## Objectives

The objective of this work was to increase the knowledge about cross-tolerance in aquatic organisms, using zebrafish embryos (*Danio rerio*) as our research model organism, a sublethal heat shock as the first mild stressor and Cd as the second stressor. Our hypothesis is that an exposure to a nonlethal heat stress in the early stages of development will trigger a biochemical general response to stress that will later increase the tolerance of these embryos when exposed to Cd.

To better understand the embryos health status after previous exposure to heat stress and subsequent exposure to Cd and to elucidate about the general stress responses that are involved with the process of induced tolerance to Cd, several parameters were analyzed: the appearance of malformations during embryo development, the total size of the larvae and the width of the yolk sac, changes of behavior and changes in biomarkers (catalase activity (CAT), glutathione s-transferase activity (GST), total glutathione content (TG), lipid peroxidation levels (LPO), cholinesterase activity (ChE) and the activity of the electron transport system (ETS)) in chapter 2, and changes in MTs and HSP70 contents in chapter 3.

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## Chapter 2 - Previous heat shock affects cadmium tolerance in *Danio rerio* embryos

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### Abstract

Aquatic organisms are often exposed to multiple stressors and it is known that a stressor can affect the tolerance to a different subsequent stressor (process known as cross-tolerance). To determine the effects of a heat shock on cadmium (Cd) tolerance, zebrafish embryos were exposed to a heat shock of 37°C during 1h and posteriorly exposed to a range of lethal concentrations of Cd during 5 days. Embryos that were previously exposed to heat shock showed to be more tolerant to Cd, compared with embryos of the control treatment; however after 48h this tolerance is not evident anymore. To assess the impact of cross-tolerance in the general embryo health status and the possible mechanisms involved in this process, organisms were also exposed to sublethal concentrations of Cd (after the heat shock) and multilevel endpoints were measured: behavior, total length and width of yolk sac and also a set of biomarker responses were assessed (Cholinesterases, Glutathione S-Transferase, Catalase, Total Glutathione, Electron Transport System, Lipid Peroxidation). Our results showed that a heat shock of 37°C during 1h reduces the total length and the width of yolk sac, causes hyperactivity and affects Cholinesterase and Glutathione S-Transferase activity and Total Glutathione content in zebrafish larvae. Although previous heat shock increased the activity of Glutathione S-Transferase and Total Glutathione content which could subsequently protect the embryos from Cd exposure in the first 48h, our study does not clearly point out the involvement of this general stress response on cross-tolerance to Cd reported in the acute assay. Moreover future studies should address the general stress responses responsible for the process of cross-tolerance between heat shock and Cd in *Danio rerio*.

### Keywords

Cadmium, heat stress, induced cross-tolerance, behavior, growth, biomarkers, general stress response, zebrafish embryos

## Introduction

Aquatic organisms are often exposed to multiple, natural or anthropogenic stressors in water bodies, including daily and seasonal temperature changes and metal discharges like cadmium (Cd), a non-essential metal (Hallare et al., 2005; Pan and Hunt von Herbing, 2017; Todgham et al., 2005; Vergauwen et al., 2013b). Contamination by Cd has received considerable attention in the last years because its concentrations in water have been markedly increased by human activities like mining, metal processing facilities, agriculture and discharge of sewage effluents (Abdel-Tawwab and Wafeek, 2014; Bouraoui et al., 2008; Cuypers et al., 2010). It is estimated that approximately 25000 tons of Cd per year are dumped into the environment, from which about half is released by human activities (Zhang et al., 2017). In industrialized areas in China and India, Cd environmental concentrations are much higher than those in normal freshwater ( $<0.0005 \text{ mg L}^{-1}$ ) ranging (e.g.) from  $0.008 \text{ mg L}^{-1}$  in East Lake,  $0.05 \text{ mg L}^{-1}$  in Beijiang River,  $0.06 \text{ mg L}^{-1}$  in Kali River which,  $0.47 \text{ mg L}^{-1}$  in Subernarekha River and  $0.68 \text{ mg L}^{-1}$  in Matla River (Guo et al., 2017; Malarvizhi et al., 2017; Zheng et al., 2016). Moreover Cd is not eliminated by biotransformation mechanisms bioaccumulating and biomagnifying in phytoplankton and complex food webs involving aquatic animals such as mollusks, crustaceans and fish (Acosta et al., 2016; Baudou et al., 2017; Giri et al., 2016; Jia et al., 2011; López et al., 2006). Mechanisms of Cd toxicity involve disruption of ion regulation, oxidative damage, endocrine disruption, genotoxicity, olfactory and renal impairments, histopathological effects and adverse effects on behavior, survival, reproductive parameters and growth (Renieri et al., 2017).

As a primary consequence of global warming, the frequency of extreme events like heat waves and the annual mean of water temperature is increasing, because of that, researchers have been studying, in recent years, the interactions between increased temperature and contaminants (Airaksinen et al., 2003; Klein et al., 2017; Olsvik et al., 2016). Many studies addressed the impacts of increasing temperature in combination with Cd exposure generally concluding that elevated temperatures increase Cd toxicity (Abdel-Tawwab and Wafeek, 2014; Guinot et al., 2012; Muyssen et al., 2010; Piazza et al., 2016; Vergauwen et al., 2013a). This occurs because metal uptake rate and reaction rates

increased with elevated water temperatures. Metabolic rates of poikilothermic organisms are also increased at higher temperatures and physiology, ecology and behavior of fish are affected altering energy consumption. Besides that, elevated water temperature may also become a stressor *per se* especially near the thermal tolerance limits (Beitinger et al., 2000; Dalvi et al., 2009; López-Olmeda and Sánchez-Vázquez, 2011; Vergauwen et al., 2013b). In spite of the relevance of studying the effects of temperature and metal exposure simultaneously, in natural environments sequential exposures of two different stressors also occur (Zhu et al., 2017).

Few studies have investigated how stress induced by an environmental factor can modify the response to subsequent chemical stress (Pestana et al., 2016). However it has been reported that pre-exposure to a sublethal stressor, that frequently occurs in natural ecosystems, may turn out to be beneficial to the overall fitness of organisms, even though they show typical stress responses (Suhett et al., 2015). Cross-tolerance (also known as cross-protection) is the ability of one stressor to transiently increase the tolerance of an organism to a subsequent heterologous stressor that occurs later (Basu et al., 2002; Todgham et al., 2005). It is possible that numerous combinations of stressors can cause such responses but little research has been conducted despite the potentially repercussions for populations maintenance that are sequentially exposed to multiple environmental stressors (Pestana et al., 2016). Some studies have shown that a previous exposure to sublethal heat stress allowed organisms to better resist Cd exposure in microalgae (Tukaj and Tukaj, 2010), brine shrimp (Pestana et al., 2016), mussels (Tedengren et al., 2000), mouse embryos (Kapron-Brás and Hales, 1991) and fish (Vergauwen et al., 2013b; Zheng et al., 2017). The existence of cross-tolerance between these two stressors is only possible if the defense mechanisms induced by the first stressor are also involved in the protection against the second stressor. Several studies have suggested possible mechanisms of common defense against Cd and thermal stress such as increased activity of antioxidant enzymes (Muyssen et al., 2010), increased levels of metallothioneins (MTs) (van Cleef-Toedt et al., 2001), metabolic depression (Leung et al., 2000), and induction of heat shock proteins (HSPs) (Pestana et al., 2016).

Zebrafish, *Danio rerio*, is commonly used for toxicological studies because of its small size, high fecundity, short generation time, external fertilization, well-characterized embryonic development, easy maintenance and breeding in laboratory and optical transparency during early development stages (Chakraborty et al., 2016; Craig et al., 2007; Lawrence, 2007; Spence et al., 2008). Also it is very important to investigate toxicology effects in developing embryos because normally early life stages are the most sensitive periods in the life-cycle of fish, where selective pressures are strong, and when the greatest impact on future adult populations may occur (Aluru, 2017; Hallare et al., 2004; Pan and Hunt von Herbing, 2017). Besides that, according to European Union legislation for the protection of animals used for scientific purposes, the use of embryonic stages of vertebrates is not regulated so, the fish embryo toxicity tests (FET) are considered an alternative to animal experiments (Embry et al., 2010; Scholz et al., 2008). In laboratory, zebrafish are normally kept at 25–28°C and classified as eurythermal, having a particularly high temperature tolerance (Zheng et al., 2017).

Behavior assays with zebrafish larvae have been used to detect neurotoxic effects of various xenobiotics and some authors have stated that neurobehavioral development is the most sensitive indicator of developmental toxicity of Cd (Chow et al., 2008). The neurotoxic nature of metals like Cd had been confirmed by the inhibition of locomotor activity in zebrafish larvae (Hallare et al., 2005; Jin et al., 2015). In turn, heat stress has been reported to cause hyperactivity in fish (López-Olmeda and Sánchez-Vázquez, 2011; Sfakianakis et al., 2012). The determination of a neurotransmission parameter, as the activity of cholinesterase (ChE), may also be useful to demonstrate stress interferences with neural and nervous functions that may compromise behavior and other important physiological functions (Airaksinen et al., 2003) and metals have been reported to inhibit the activity of ChE (Domingues et al., 2010).

Acute changes in temperature act on physiological processes predictably, with cold temperatures slowing, and warmer temperatures accelerating them. Thus, fish can compensate temperature changes by appropriate alterations in metabolic rates. Metabolism is generally measured in terms of an organism's oxygen consumption and was recorded to increase when organisms are exposed to heat stress. Organisms are expected

to use their metabolic energy twice as much at higher than at low temperature, leading to faster energy reserve depletion and thus to increased susceptibility to toxicants (Airaksinen et al., 2003; Piazza et al., 2016). Exposure to metals has also been associated with increases in Electron Transport System activity (ETS) (Baudou et al., 2017; Bednarska and Stachowicz, 2013; Novais et al., 2013; Pedrosa et al., 2017) reflecting metabolic requirements and energy expenditure due to detoxification.

It is well known that the initial effects of metal pollution may be evident at cellular or tissue levels before significant changes are identified in the organism (van Dyk et al., 2007). Metal accumulation causes an increase in highly reactive oxygen species (ROS) leading to oxidative stress in cells and tissues of fish, hence lipid peroxidation (LPO) have been used as endpoint measures of metal toxicity (Atli et al., 2006; Cambier et al., 2010; Cao et al., 2010; Giri et al., 2016) and in fact several studies concluded that Cd exposure lead to elevated LPO in zebrafish (Jin et al., 2015; Ling et al., 2017; Wang et al., 2015; Yuan et al., 2017; Zheng et al., 2016). Alterations in environmental temperature may also induce formation of ROS, which can provoke protein, lipid or DNA damage, and potentially require additional energy (Heise, 2006; Lushchak, 2011). Accordingly to this, fish would develop various enzymatic and non-enzymatic defense mechanisms to counteract oxidative stress, such as: Glutathione (GSH), a widely distributed tripeptide that can either act as a non-enzymatic antioxidant through the direct interaction of the SH group with ROS or serve as a cofactor in the enzymatic detoxification of ROS; Glutathione S-Transferase (GST), an enzyme involved in phase II of biotransformation that catalyzes the conjugation of GSH to several dangerous compounds and Catalase (CAT), an enzyme that degrades hydrogen peroxide into water and oxygen (Bouraoui et al., 2008; Jia et al., 2011). Conflicting results of changes in activity/content of this defense mechanisms have been observed in studies with Cd exposure since it depends on the concentration, the species or the route of exposure (Cao et al., 2010; López et al., 2006; Shi et al., 2005).

The objective of this work was to increase knowledge about tolerance to Cd induced by heat shock, using zebrafish embryos (*Danio rerio*) as our research model organism. Our hypothesis is that an exposure to a sublethal heat shock in the early stages of development will trigger a biochemical general stress response that will later provide increased tolerance

to Cd. In this context, to reach a comprehensive assessment of the impacts of cross-tolerance in general embryo health status and the possible mechanisms involved it seems suitable to consider a multiparameter approach at different levels of biological organization, including individual and sub-individual. This approach consists of several endpoints including a behavior assay, the measurement of total length and width of the yolk sac of zebrafish larvae and the measurement of physiological changes in levels of oxidative damage (LPO), in the activity of ChE, in energetic parameters like ETS activity and in antioxidants involved with detoxification and protection against oxidative stress such as CAT, GST and Total Glutathione content (TG).

## **Material and methods**

### **Test organisms**

Zebrafish (*D. rerio*) eggs were obtained from a culture maintained in carbon-filtered water at the Department of Biology, University of Aveiro. These organisms were kept at  $27.0 \pm 1^\circ\text{C}$  under a 16:8h light/dark photoperiod cycle, with conductivity at  $550 \pm 50 \mu\text{S}$ , pH at  $7.5 \pm 0.5$  and dissolved oxygen at 95% saturation. Adult fish are fed twice daily with commercially available artificial diet (ZM 400 Granular) and brine shrimp.

### **Test chemicals**

Zebrafish embryos were exposed to solutions of Cd obtained from dilution of a cadmium chloride stock ( $\text{CdCl}_2$ ; CAS number 10108-64-2; purity  $\geq 99.0\%$ ). Water of the zebrafish culture was used for dilutions and as control in all tests.

### **Acute assay**

The assay was based on the OECD guideline on Fish Embryo Toxicity Test (OECD, 2013). Reproductive groups of zebrafish adults were placed in aquarium with marbles in the bottom, in the afternoon of the day before the collection of the eggs. Two hours after the beginning of the illumination in the next morning the eggs were collected and cleaned from residues.

Newly fertilized eggs were divided in two groups: in one group (HS) embryos were exposed to a heat shock (HS) of 37°C during one hour while in the other group (N) embryos were kept at 27±1°C. The HS temperature was chosen based on preliminary tests and based on different studies dealing with induction of HSPs in zebrafish, where the control temperature is usually set at 28°C and the temperature used for the HS is commonly set at 37°C (Airaksinen et al., 2003; Hallare et al., 2005; Krone and Sass, 1994; Lele et al., 1997; Råbergh et al., 2000).

After the exposure, Zebrafish eggs from both groups with normal development were selected for the toxicity test (using a Stereoscopic Zoom Microscope-SMZ 1500, Nikon). The embryos were distributed in 6-well plates, where each well contained five organisms in 10 ml of the test solution. Three replicates were made for each treatment. Eggs from HS and N groups were exposed to 0; 3.0; 4.2; 5.9; 8.2; 11.5; 16.1; 22.6; 31.6 and 44.3 mg L<sup>-1</sup> of Cd and kept at 27±1°C. The test lasted five days and embryos and larvae were observed daily with a stereomicroscopic. In the embryo phase, the egg coagulation and the hatching were evaluated whereas in the larvae phase, edemas, spine malformation and mortality were observed.

### **Sublethal assay**

Procedure was the same as for the acute assay but a sublethal range of concentrations was used: 0; 0.02; 0.22 and 2 mg L<sup>-1</sup> of Cd. At the fifth day larvae were photographed with the help of a stereomicroscopic (Stereoscopic Zoom Microscope-SMZ 1500, Nikon). The total length and the width of the yolk sac of larvae was measured using the software NIS Elements D 3.2. Larvae locomotor behavior was analysed using the Zebrabox (Viewpoint, Lyon, France) tracking system over a period of 20 minutes. Dead larvae or larvae exhibiting physical abnormalities were not included in the locomotor analyses. The temperature was maintained stable at 26±1°C and movement was stimulated by alternating light and dark periods. The test consisted of a cycle with four alternating periods: 5 minutes light; 5 minutes dark; 5 minutes light and 5 minutes dark. The swimming distance and the swimming time were measured during each period.

## **Biochemical analyses**

Test procedure was the same as for the acute assay but zebrafish embryos were exposed only to 0 and 2 mg L<sup>-1</sup> of Cd. After HS, the organisms were distributed in petri plates, with 25ml of the test solution. Seven replicates were made for each treatment and were kept at 27± 1°C during the test. Based on the results of the acute test, the test was done using two exposure times: 48 hours of exposure to Cd corresponding to a time point where induced cross-tolerance to Cd was verified or 96 hours of exposure where tolerance of the two groups was similar. At the end of the exposures, samples of 15 embryos from each condition were frozen in liquid nitrogen and kept at -80°C until further analyses.

Each sample was homogenized in 1200µL of ultra-pure water, on ice, using a sonic homogenizer (Sonifier 250, Branson sonicator). The homogenate was divided into different aliquots for the different biochemical analyses: 150µL for LPO, 250µL for ETS and 300µL for analyses of TG, GST, CAT, ChE and protein quantification. To the last aliquot 300µL of K-phosphate buffer (0.2M; pH=7.4) were added followed by its centrifugation at 10000g for 20 minutes at 4°C, originating the Post-Mitochondrial Supernatant (PMS).

## **Lipid peroxidation**

The determination of endogenous LPO levels was performed measuring the thiobarbituric acid reactive species (TBARS) based on the work of Ohkawa et al. (1979) and Bird and Draper (1984) and preventing artifactual lipid oxidation by adding BHT 4% in methanol (2,6-Di-tert-butyl-4-methylphenol) (Torres et al., 2002). To each sample 3µL of BHT, 100µL of cold TCA 100% (trichloroacetic acid) and 1000µL of TBA 0.73% (2-Thiobarbituric acid) were added. The samples were incubated at 100°C during 1 hour, centrifuged at 6000g for 10 minutes at 25°C and the supernatant was pipetted to a microplate (3 replicates of 100µL). LPO levels were determined at 535nm and expressed in nmol TBARS per embryo, using  $\epsilon=1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ .

## **Electron Transport System**

The measurement of ETS was done following a protocol adapted from De Coen and Janssen (1997) and modified by Rodrigues et al. (2015). The fraction for ETS activity measurements was obtained adding 150µL of homogenization buffer and centrifuging at

1000g for 10 minutes at 4°C. To each 50µL of supernatant it was added 150µL of buffered solution and 100µL of INT solution (p-iodonitrotetrazolium). The absorbance was measured kinetically at 490nm during 3min every 20 seconds at 25°C. The oxygen consumption rate conversion was calculated based on the stoichiometric relationship: 2 µmol of formazan formed  $\approx$  1 µmol of oxygen consumed. The formula of Beer-Lambert was applied to quantify the oxygen consumed, using  $\epsilon=1.59\times10^4 \text{ M}^{-1} \text{ cm}^{-1}$ .

### **Protein quantification**

The quantification of the protein on each sample was done following the Bradford method (1976), adapted to 96 well plates, using bovine  $\gamma$ - globuline as a standard. To each 10µL of PMS, 250µL of BioRad solution were added, the plates were placed in the dark, and after 15 minutes the absorbance was read at 600nm.

### **Cholinesterase**

The measurement of ChE activity was done following the protocol defined by Ellman et al. (1961), and adapted to microplate by Guilhermino et al. (1996). To each 50µL of PMS, 250µL of reaction buffer were added and the absorbance was read at 414nm. The formula of Beer-Lambert was then applied to quantify the ChE activity expressed in nmol/min/mg protein, using  $\epsilon=13.6\times10^3 \text{ M}^{-1} \text{ cm}^{-1}$ .

### **Catalase**

The determination of CAT activity was done measuring the decomposition of the substrate hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) (Clairborne, 1985). To each 10µL of PMS, 140µL of K-phosphate (0.05M, pH=7.0) and 150µL of reaction buffer were added. The absorbance was read at 240nm during 2 minutes. The formula of Beer-Lambert was then applied to quantify CAT activity expressed in µmol/min/mg protein, using  $\epsilon=40 \text{ M}^{-1} \text{ cm}^{-1}$ .

### **Glutathione S-Transferase**

The measurement of GST activity was done following the method of Habig et al. (1974), reading the conjugation of GSH with 1-chloro-2,4-dinitrobenzene. To each 50µL of PMS, 250µL of reaction solution were added and the absorbance was read at 340nm. The

formula of Beer-Lambert was applied to quantify GST activity expressed in nmol/min/mg protein, using  $\epsilon=9.6 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ .

### **Total glutathione**

TG content was determined based on the methods of Tietze (1969) using a recycling reaction of reduced glutathione with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) in the presence of glutathione reductase excess. To each 50 $\mu$ L of PMS, 250 $\mu$ L of reaction solution were added. The formula of Beer-Lambert was then applied to quantify TG content expressed in pmol/min/mg protein, using  $\epsilon=14.1 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ . A Labsystem Multiskan EX microplate reader was used for all biochemical determinations.

### **Statistical analysis**

The median lethal concentrations ( $\text{LC}_{50}$ ) at the lethal assay were estimated using three-parameter logistic dose-response curves within the dose response package using the program of analysis R.  $\text{LC}_{50}$  values were statistically compared according to Sprague and Fogels (1976). Shapiro-Wilk and Levene's test were done to assess the normality and homoscedasticity of data, respectively. Two-way Anova's were performed for analysis of the data of the sublethal assay and biochemical analyses using the program SigmaPlot 12.5. The Holm-Sidak method was used for multiple comparisons versus control group. All statistical analyses were performed with a significance level of 0.05.

## **Results**

### **Acute assay**

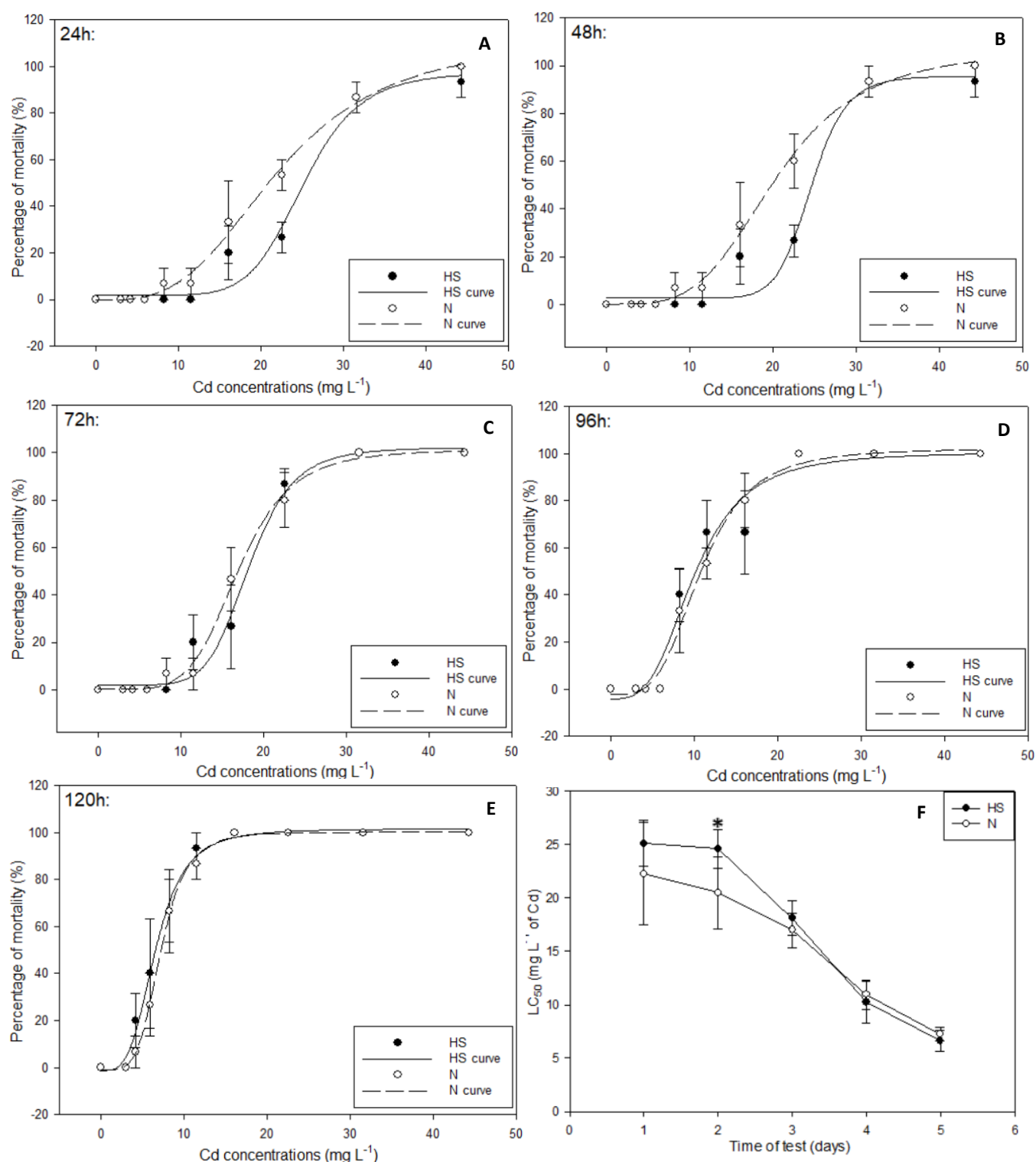
Zebrafish eggs subjected to HS and the control group maintained in the normal temperature conditions were exposed to several concentrations of Cd during 120 hours. Figure 2 displays the dose-response curves of the 2 groups throughout the 5 days of test and the evolution of the  $\text{LC}_{50}$  values. Embryo mortality in the control was always below 10%. As we hypothesized, our results demonstrate that a previous HS affected the tolerance to subsequent Cd exposure and that thermal stress induced cross-tolerance to Cd. In the first two days of exposure the  $\text{LC}_{50}$  of HS embryos is higher than the  $\text{LC}_{50}$  of N

embryos. However we detected significant differences only on the second day. After 72 hours of exposure both groups show similar sensitivity towards Cd and the LC<sub>50</sub> values of the two groups remains similar until the end of the test. We also observed that exposure to Cd caused a delay on hatching and induced malformations on the embryos such as edemas and spinal deformations (Figure 3). We did not find any significant effect of the previous HS on the hatching time or appearance of malformations.

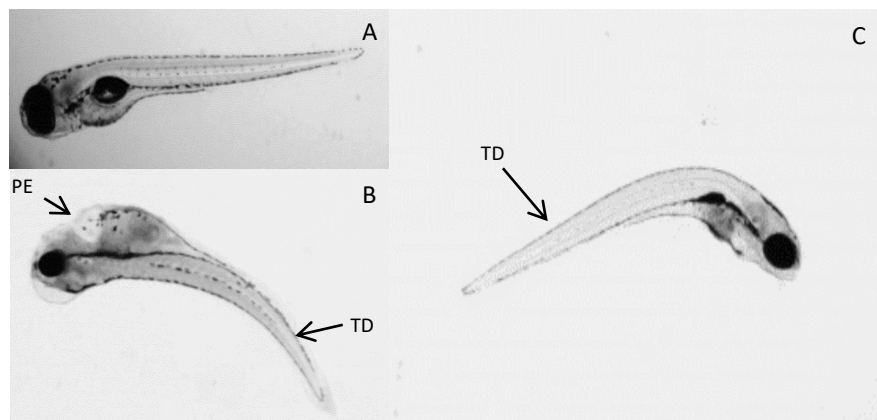
### **Sublethal assay**

The ANOVAs results for endpoints of behavior, total length and width of yolk sac are depicted in table 1. Cd exposure alone affects the growth of zebrafish larvae in a dose-response manner, with larvae exposed to higher concentrations of Cd showing decreased total length (Figure 4A). Previous HS also reduced the total length of zebrafish larvae (Figure 4A). Statistically significant interaction between the two stressors was not detected. Exposure to Cd alone significantly decreased the width of yolk sac of zebrafish larvae in a dose-response manner, with larvae exposed to higher concentrations of Cd showing the highest reduction (Figure 4B). A similar result was also observed for HS alone, where larvae previous exposed to HS exhibited a reduction on width of yolk sac (Figure 4B). Statistically significant interaction between the two stressors was also not detected.

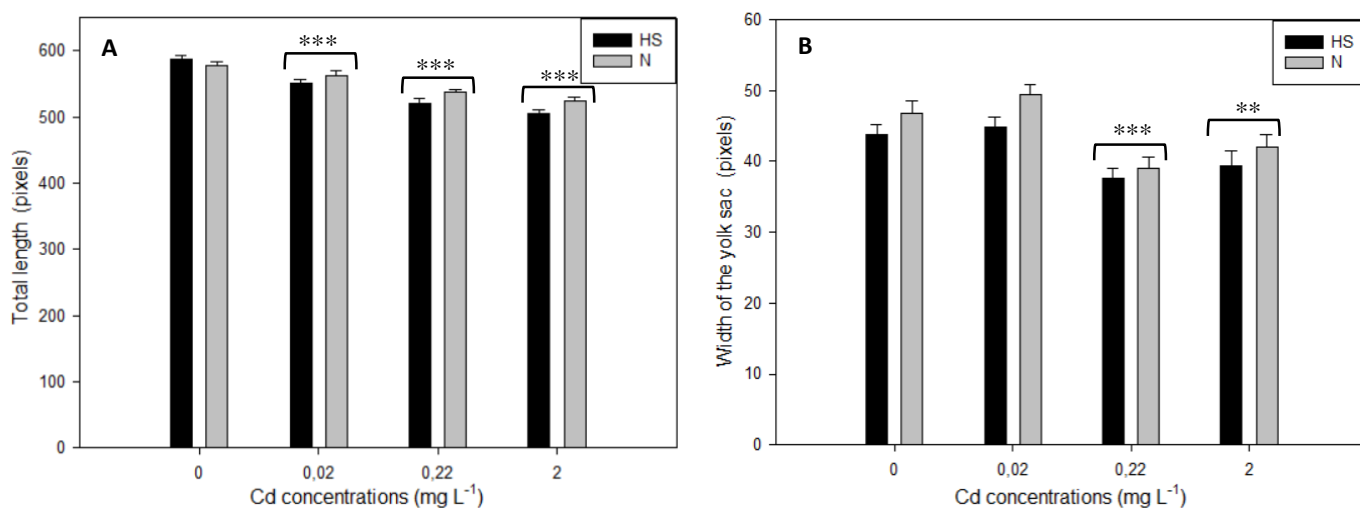
Control larvae exhibited a regular pattern of locomotor activity: in periods of light the larvae decreased the movement but in periods of dark the larvae rapidly increased locomotor activity. This consistent pattern of activity in response to light-dark stimulation was perturbed by Cd in a concentration-dependent manner (see supplemental material, Figure S1). Larvae treated with higher concentrations of Cd ( $\geq 0.22$  mg L<sup>-1</sup>) also showed inhibition of activity with reduced swimming distance in periods of dark and light (Figure 5). The total time spent swimming was also reduced by Cd exposure (data not showed). In turn, larvae treated with previous HS showed a higher degree of hyperactivity visible in the treatment without Cd in dark and light periods, although we only detected significant effect of HS in the light period. Previous HS effect of hyperactivity is completely imperceptible in the presence of Cd, however a statistically significant interaction between the two stressors was not detected.



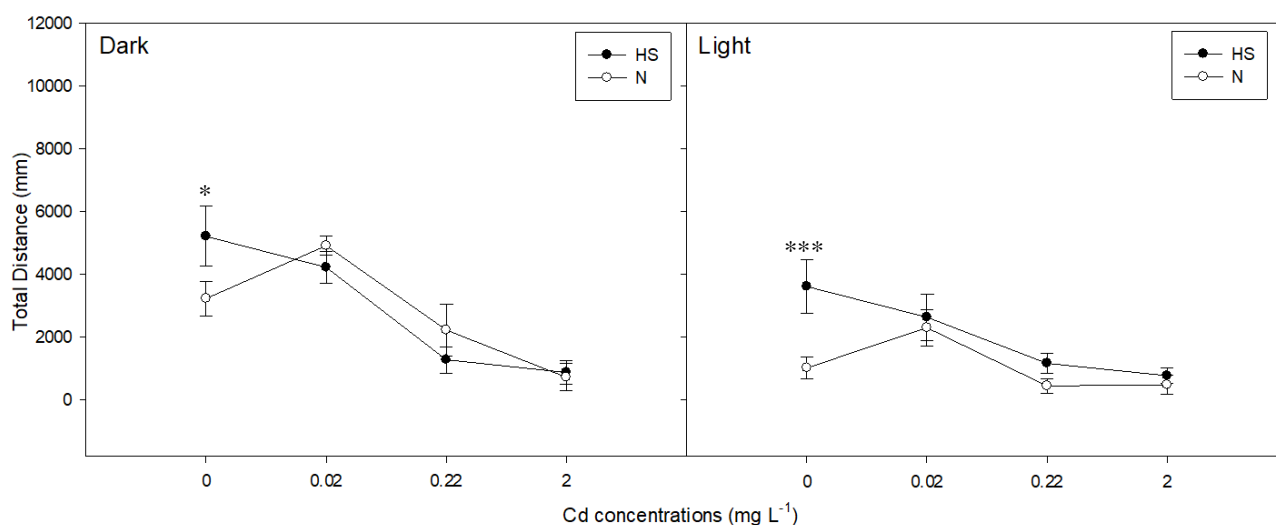
**Figure 2.** Dose-response curves throughout the 5 days of test (A-E). The black dots represent the embryos of the group exposed to HS (HS), and the white dots represent the embryos of the group kept at normal temperature (N). The graphic F represents the evolution of the LC<sub>50</sub> values (mg L<sup>-1</sup> of Cd) of embryos in the 2 groups during the five days of test. All values are presented as mean  $\pm$  standard error. The asterisk (\*) represents the day where the LC<sub>50</sub> values of the two groups (HS and N) were significantly different; \*\*\* indicates  $P < 0.001$ ; \*\* indicates  $P < 0.01$ ; \* indicates  $P < 0.05$ .



**Figure 3.** A) Zebrafish larvae developed in control conditions; B) Larvae exposed to 2 mg L<sup>-1</sup> of Cd showing tail deformation (TD) and pericardial edema (PE); C) Larvae exposed to 2 mg L<sup>-1</sup> of Cd showing tail deformation (TD).



**Figure 4.** Total length (A) and width of the yolk sac (B) of zebrafish larvae after 120h of exposure to Cd. The black bars represents the embryos of the group exposed to HS, and the grey bars represents the embryos of the group kept at normal temperature (N). All values are presented as mean  $\pm$  standard error. Asterisks (\*) denote significant differences compared to the control group, \*\*\* indicates  $P < 0.001$ ; \*\* indicates  $P < 0.01$ ; \* indicates  $P < 0.05$ .



**Figure 5.** Total distance of zebrafish larvae in the periods of dark and light, after 120h of exposure to Cd. The black dots represents the embryos of the group exposed to HS, and the white dots represents the embryos of the group kept at normal temperature (N). All values are presented as mean  $\pm$  standard error. Asterisks (\*) denote significant differences compared to the group N, \*\*\* indicates  $P < 0.001$ ; \*\* indicates  $P < 0.01$ ; \* indicates  $P < 0.05$ .

**Table 1.** ANOVAs results of the effects of Cd alone, HS alone and their interaction (HS\*Cd) on total length, width of yolk sac and behavior; df-degrees of freedom; ss-sums of squares; MS - mean sums of squares; F-F-statistic.

		df	ss	MS	F	p-Value
Total length	Cd	3	0.103	0.034	41.48	<b>&lt;0.001</b>
	HS	1	0.004	0.004	4.682	<b>0.032</b>
	HS*Cd	3	0.004	0.002	1.784	0.151
Width of yolk sac	Cd	3	0.126	0.042	13.54	<b>&lt;0.001</b>
	HS	1	0.021	0.021	6.677	<b>0.011</b>
	HS*Cd	3	0.002	0.0008	0.246	0.864
Behavior (light period)	Cd	3	45242440	15080813	7.407	<b>&lt;0.001</b>
	HS	1	15529904	15529904	7.627	<b>0.008</b>
	HS*Cd	3	14267880	4755960	2.336	0.084
Behavior (dark period)	Cd	3	163913377	54637792	19.92	<b>&lt;0.001</b>
	HS	1	242445	242445	0.088	0.767
	HS*Cd	3	21048349	7016116	2.558	0.064

## Biochemical analyses

The results obtained, after 48h and 96h of test, regarding the biomarkers analyzed, are exhibited on figure 5 and table 2 demonstrate the ANOVAs results. Regarding the effect of HS alone, GST activity demonstrate a significant induction after 48h, with an increase of 50% compared with the control group, however, after 96h, a significant decrease was observed, with a reduction of 36% compared with the control group. Similarly, TG content was increased by HS after 48h, with an increase of 8% compared with the control group, and decreased after 96h, with reduction of 62% compared with the control group. CAT activity, LPO levels and ETS activity were not affected by HS. Regarding ChE activity, a significant induction by HS was observed after 48h of test, with an increase of 42% compared with the control group, however after 96h an inhibition of ChE activity was detected, with a reduction of 9% compared with the control group.

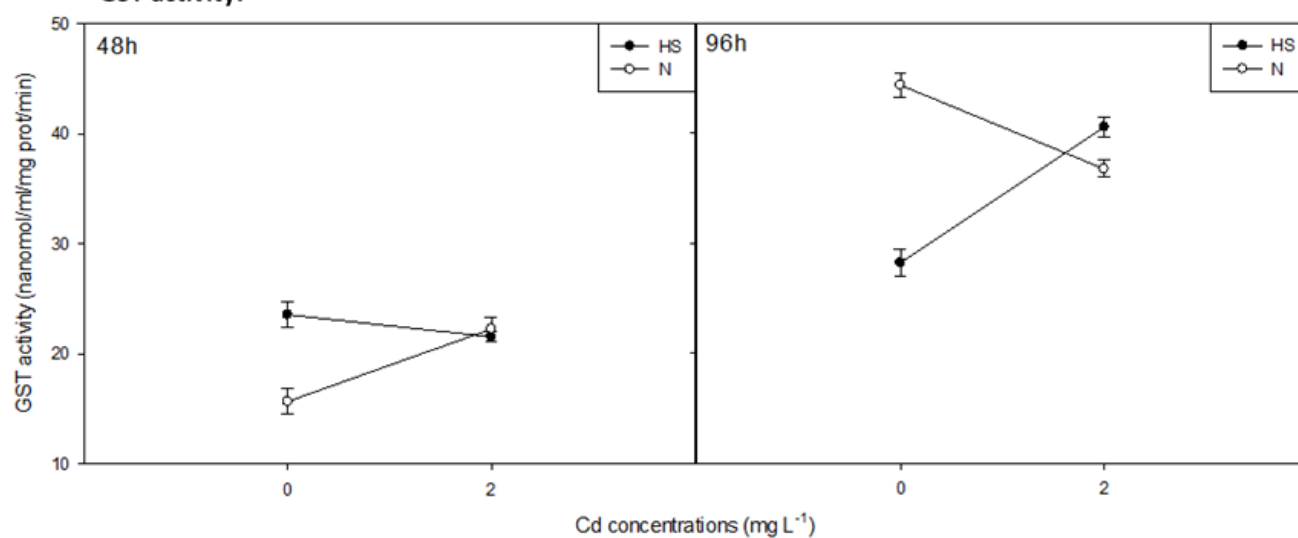
Cd exposure alone resulted in increased GST activity after 48h of test, with an increase of 39% compared with the control group, however, after 96h a significant decrease was observed. Concerning CAT activity a significant inhibition was observed after 96h, with a reduction of 54% compared with the control group. TG content was also decreased by Cd exposure after 48h and 96h, with reduction of 5% and 61% respectively. LPO levels were not affected by Cd after 96h of exposure, but after 48h a significant decrease was observed, with a reduction of 14% compared with the control group. ETS activity demonstrate a significant induction on zebrafish embryos exposed 96h to Cd. The increase in ETS activity was 21% compared with the control group. Regarding ChE activity, a significant induction by Cd was observed after 48h of test, with an increase of 43% compared with the control group, however, after 96h, an inhibition of ChE activity was detected, with a reduction of 25% compared with the control group.

Concerning the biomarker's response when the embryos were exposed to the two stressors, after 48h of test, previous HS influence the Cd effect, with significant interactions, on GST activity, ChE activity and TG content. Larvae previously exposed to HS and posteriorly exposed to Cd showed an induction of GST activity and ChE activity very similar to the effects on larvae only exposed to HS or Cd. However the pattern of response to Cd was altered by HS since larvae without HS had a more pronounced increase in this

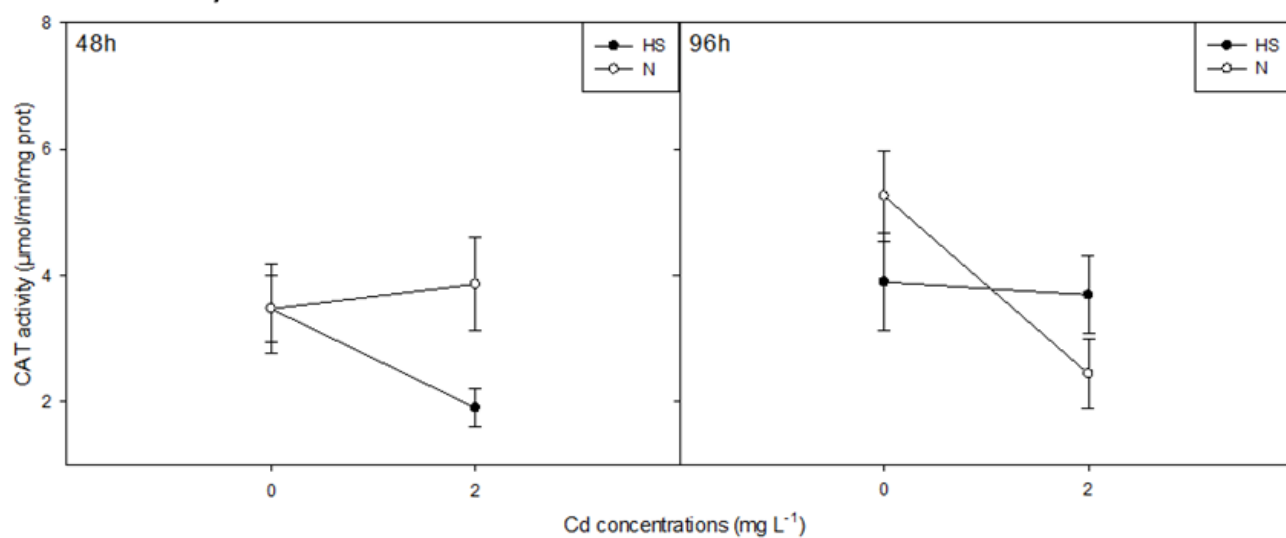
enzymes activity. Regarding TG content, larvae previous exposed to HS and posteriorly exposed to Cd showed a reduction of 36% contrarily to the larvae exposed to HS or Cd alone that have TG contents similar to control group. In this case, the pattern of response to Cd was altered by HS since larvae with HS had a more pronounced reduction on TG content compared with larvae only exposed to Cd.

After 96h, HS influenced the effects of Cd exposure, with significant interactions, on GST activity, LPO levels and TG content. In this three biomarkers the pattern of response to Cd is altered by HS in a similar way. Larvae exposed to HS and posteriorly exposed to Cd increased their GST activity, TG content and LPO levels, while larvae kept at normal temperature and exposed to Cd decreased their GST activity, TG content and LPO levels. Although no significant interaction was detected, CAT activity seems to exhibit the same pattern of response.

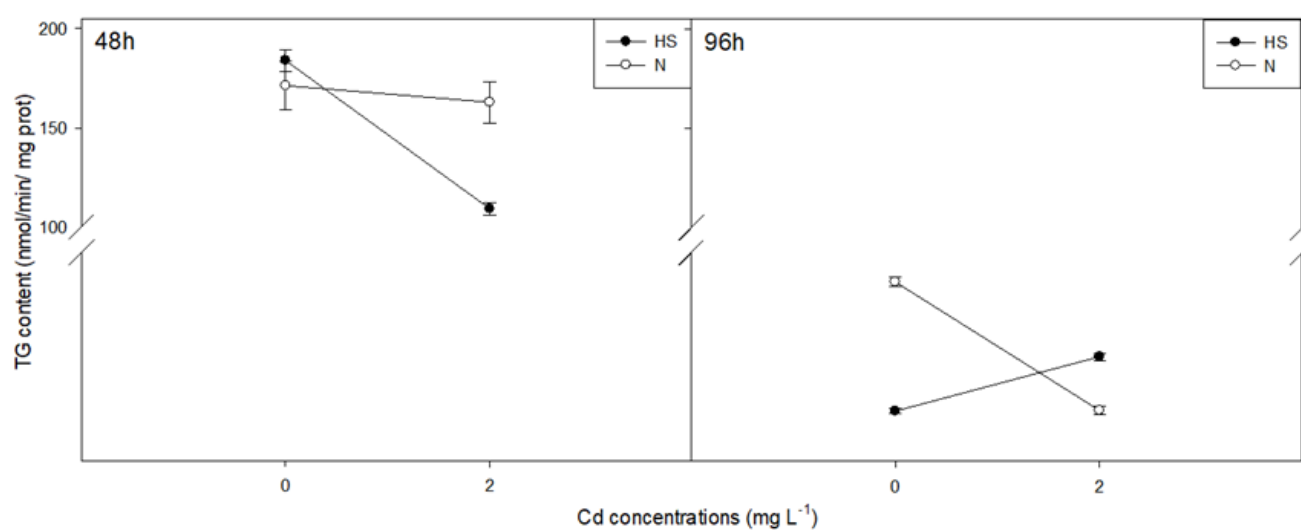
### GST activity:

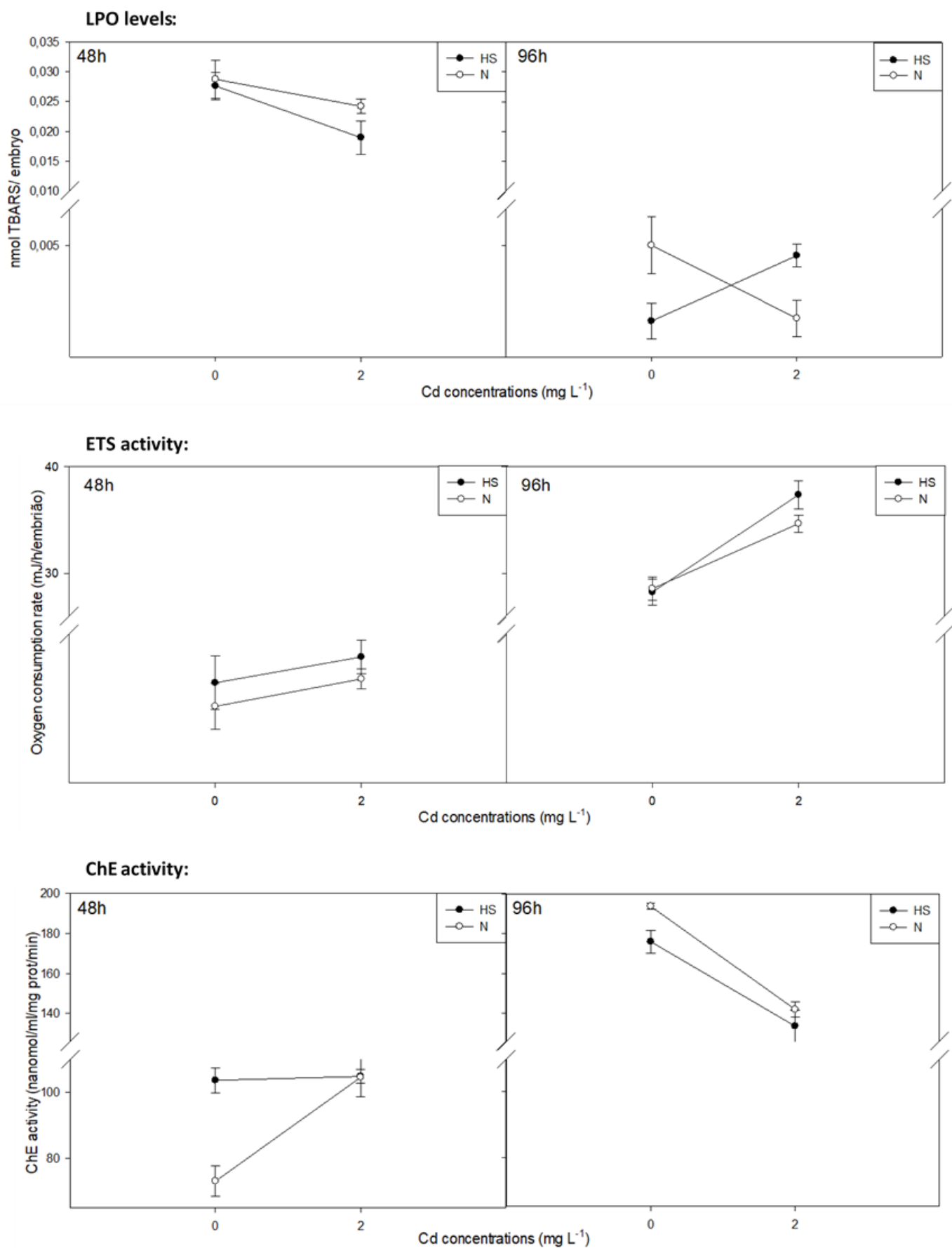


### CAT activity:



### TG content:





**Figure 6.** Biomarker responses of zebrafish larvae kept at normal temperature and exposed to Cd (N) and zebrafish larvae previously exposed to HS and posteriorly exposed to Cd (HS) during 48h or 96h. All values are presented as mean  $\pm$  standard error.

**Table 2.** ANOVAs results of the effects of Cd alone, HS alone and their interaction (HS\*Cd) on the biomarkers analyzed; df-degrees of freedom; ss-sums of squares; F-F-statistic.

		df	ss	F	p-Value
GST activity (48h)	Cd	1	31.21	4.813	<b>0.040</b>
	HS	1	76.25	11.76	<b>0.003</b>
	HS*Cd	1	109.1	16.83	<b>&lt;0.001</b>
GST activity (96h)	Cd	1	31.81	5.451	<b>0.031</b>
	HS	1	217.9	37.33	<b>&lt;0.001</b>
	HS*Cd	1	569.0	97.51	<b>&lt;0.001</b>
CAT activity (48h)	Cd	1	2.330	1.012	0.325
	HS	1	6.450	2.802	0.108
	HS*Cd	1	6.445	2.800	0.108
CAT activity (96h)	Cd	1	14.49	5.191	<b>0.033</b>
	HS	1	0.020	0.007	0.933
	HS*Cd	1	10.85	3.888	0.061
TG content (48h)	Cd	1	0.080	28.11	<b>&lt;0.001</b>
	HS	1	0.024	8.304	<b>0.010</b>
	HS*Cd	1	0.054	19.04	<b>&lt;0.001</b>
TG content (96h)	Cd	1	0.061	33.54	<b>&lt;0.001</b>
	HS	1	0.063	34.56	<b>&lt;0.001</b>
	HS*Cd	1	0.696	383.2	<b>&lt;0.001</b>
LPO levels (48h)	Cd	1	0.0003	6.96	<b>0.014</b>
	HS	1	0.00007	1.611	0.217
	HS*Cd	1	0.00003	0.678	0.418
LPO levels (96h)	Cd	1	6.63x10 <sup>-8</sup>	0.033	0.858
	HS	1	2.00x10 <sup>-7</sup>	0.099	0.756
	HS*Cd	1	0.00002	11.01	<b>0.003</b>
ETS activity (48h)	Cd	1	1.859	1.730	0.202
	HS	1	1.361	1.266	0.273
	HS*Cd	1	0.002	0.002	0.968
ETS activity (96h)	Cd	1	342.8	44.19	<b>&lt;0.001</b>
	HS	1	8.372	1.079	0.311
	HS*Cd	1	13.50	1.740	0.202
ChE activity (48h)	Cd	1	0.034	15.28	<b>0.001</b>
	HS	1	0.033	14.45	<b>0.001</b>
	HS*Cd	1	0.029	13.09	<b>0.002</b>
ChE activity (96h)	Cd	1	0.104	114.4	<b>&lt;0.001</b>
	HS	1	0.018	20.51	<b>&lt;0.001</b>
	HS*Cd	1	0.001	1.633	0.218

## Discussion

Cross-tolerance occurs when previous exposure to a stressor increase the tolerance of an organism to a subsequent different stressor (Todgham et al., 2005). In this study sub-lethal HS seems to induce cross-tolerance to later Cd exposure, since embryos that were pre-exposed to 37°C during 1h exhibited higher LC<sub>50</sub> values than embryos kept at 27°C. Other studies offer evidence of the capacity of thermal stress to increase tolerance to Cd in zebrafish (Hallare et al., 2005; Vergauwen et al., 2013b; Zheng et al., 2017). For example, Hallare et al. exposed embryos to combinations of three temperatures and Cd and reported lower rate of mortality at the highest temperature (33°C). The authors hypothesized that this could be due to the high production of HSPs, which provided greater resistance (Hallare et al., 2005). On the other hand, Vergauwen et al. acclimated adults to different temperatures for 1 month prior to 96h Cd exposure at the respective acclimation temperatures. Based on LC<sub>90</sub> values, the lowest Cd toxicity was detected at the highest temperature tested (34°C). The authors suggested that the warm acclimation provoked a general stress response, such as increased activity of antioxidant enzymes, metabolic depression or induction of HSPs, which protected the organisms against subsequent Cd exposure (Vergauwen et al., 2013b). Zheng et al. exposed adults to 26°C and 34°C for 4 days, and posteriorly to Cd for 1 week at 26°C. The authors discover that preheating in combination with Cd increased survival rate. Once more, they suggested that preheating provoked a general stress response, like MTs increase or HSPs induction, that facilitated a quick response of fish to severe stress situations (Zheng et al., 2017).

However, our results show that this induced cross-tolerance is transient and only observed until 48h of Cd exposure, thus suggesting an energetic/physiological cost of this process. Hallare et al. also reported more susceptibility of larvae post-hatched (after 48h) as compared to embryos pre-hatched (before 48h). Pre-hatched embryos, exposed to higher temperatures were protected from Cd, however, after hatching the larvae showed an increased sensitivity. The author suggested that the production of HSPs have reached its maximum just prior to the time of hatching and thus, the stress induced by both stressors would have overridden the capability of cells to generate more HSPs (Hallare et al., 2005). Biochemical stress response mechanisms, such as the increased of HSP70 or MTs

levels, have been described to follow an optimum curve, so loss of cross-tolerance to Cd could be caused by a reduction in the levels of defense proteins as resulting from damage to the proteosynthetic machinery. The phenomenon of overwhelming biochemical processes could be due to negative effect of Cd on cellular biosynthetic capacity such as transcription and/or translation processes or due to cellular energy deficiency that can limit the energy available for synthesis and/or function of the stress proteins involved (Haap et al., 2016). Zebrafish larvae with less than 120h use only the energy reserves of the yolk sac for their development. Since induced tolerance to Cd probably required induction of a general stress response and since adaptive processes of defense, detoxification, and repair are largely energy demanding, embryos exposed to HS may undergo re-allocation of energy resources with negative consequences for maintenance, growth and reproduction (Bednarska and Stachowicz, 2013; Haap et al., 2016). For example, populations of *Tigriopus japonicus* that were more resistant to copper showed a reduction of reproductive output and growth rate (Kwok et al., 2009).

Differences in the lethality of a chemical between pre and post hatching periods may also be attributed to the interference of the chorion in the embryos (Domingues et al., 2010). This acts as a barrier to Cd transfer to the developing embryo. Matz et al. stated that the protective effect of the chorion is evident in observations that hatched larvae are more susceptible than unhatched embryos and that, once hatched, zebrafish larvae readily accumulate Cd from their environment most likely leading to an increased sensitivity (Matz et al., 2007).

Exposure to lethal concentrations of Cd provoke delay in development and hatching and the appearance of malformed embryos which indicate that Cd can bear a teratogenic risk. Several studies also reported the same effect of Cd on zebrafish embryos (Hallare et al., 2005; Jin et al., 2015; Zhang et al., 2015). Although elevated temperatures are reported to induced abnormalities in zebrafish embryos (Krone et al., 2003; López-Olmeda and Sánchez-Vázquez, 2011), in our study no effects of HS were observed which is in line with previous studies (Hallare et al., 2005).

In this study, a previous HS alone, in early-fertilized zebrafish embryos, had impact in many endpoints analyzed. The reduction in the total length and the width of the yolk sac

of zebrafish larvae caused by HS is in agreement with other studies where zebrafish embryos exposed to increased temperatures developed faster but were smaller (López-Olmeda and Sánchez-Vázquez, 2011). In the light, zebrafish larvae usually show reduced locomotor activity, however, when larvae were pre-exposed to HS they showed a higher degree of hyperactivity, with increased swimming distance. Other studies agreed with our findings that fish are more active at higher temperatures (López-Olmeda and Sánchez-Vázquez, 2011; Sfakianakis et al., 2012). The fact that larvae exposed to HS are smaller and have a smaller yolk sac suggests that these organisms spent more energy on defense processes which limited their growth and reduced their available energy reserves. On the other hand, the hyperactivity caused by HS can have an impact on the metabolism of the larvae and consequently also increase their energy consumption. Either way this effect of HS may lead to future negative consequences.

HS also affected the activity of ChE and GST and the TG content of zebrafish larvae. This effect is noticed even after 96h of the occurrence of the HS. Contrarily to what we expected, HS did not change ETS activity during the test, probably because of the small duration and intensity of the HS or because after 48 and 96h the effect was no longer detected due to recovery. The induction of ChE activity by heat stress at 48h, observed in our study, was already reported in other studies (Airaksinen et al., 2003; Almeida et al., 2014). In agreement with our study, other researchers also reported increased transcript levels of GST after short-term warm acclimation, increased liver GST activity after exposure to increased temperature for 1 day and increased liver GST activity after 24h recovery of HS (Klein et al., 2017; Lushchak and Bagnyukova, 2006; Vergauwen et al., 2013a). Increased TG content due to HS after 48h of test, is in agreement with Heise that detected an increase in TG content after exposure to heat stress in the North Sea eelpout (Heise, 2006). As GST and GSH are involved in detoxification these increases in activity and content may confer advantages to embryos previously exposed to HS in terms of protection against ROS. However, after 96h, we observed that HS has an opposite response: inhibition of GST activity, inhibition of ChE activity and decrease in TG content; which could be related to metabolic costs of heat stress.

Cd exposure alone and as expected also affected several endpoints. The reduction in total length caused by Cd exposure is in agreement with other studies where this effect in growth was also reported (Abdel-Tawwab and Wafeek, 2014; Wold et al., 2017; Wu et al., 2017a; Yuan et al., 2017; Zheng et al., 2016). Smaller larvae with reduced yolk sac suggest that extra energy was consumed to detoxify the accumulated Cd, leading to less energy reserve for development, growth and reproduction (Cao et al., 2010). This could result in several adverse consequences in later life stages since smaller larvae are more vulnerable to predation (Wu et al., 2017b). The normal behavioral pattern produced in response to dark–light stimulation was also impaired by Cd exposure and the average swimming distance and time decreased in a dose-response manner. The neurotoxic nature of metals had been reported in zebrafish larvae (Hallare et al., 2005; Jin et al., 2015) and lead to severe consequences since decreased swimming capacities increase mortality by predation, reduce fish growth by difficulties in prey capture and influence a successful reproduction cycle (Almeida et al., 2014).

Cd affected all the biomarkers analyzed in this study. Although metals are generally associated with inhibition of ChE activity, after 48h of exposure to Cd we detected a significant induction. Other studies also reported similar results (Jebali et al., 2006). Inhibition of ChE activity caused by Cd after 96h of exposure is in agreement with our previous behavior assay where we recorded reduced swimming activity and with previous works reporting inhibition of this enzyme after metal exposure (Domingues et al., 2010). Increased ETS activity caused by Cd exposure observed here was also already recorded in other studies. For example, Baudou et al. reported that fish exposed to 0.8 mg L<sup>-1</sup> of Cd showed a considerable increase in oxygen consumption. ETS increase reflects the allocation of energy resources for defense and repair mechanisms that are necessary to maintain the physiological homeostasis. This energy consumed in detoxification/defense mechanisms is no longer available for maintenance, growth and reproduction, suggesting potential effects on the health status of organisms and the fate of populations (Baudou et al., 2017; Bednarska and Stachowicz, 2013; Novais et al., 2013; Pedrosa et al., 2017).

After 48h, GST activity was increased by Cd. Similarly, other studies recorded an increase in GST activity in fish after exposure to Cd (Bouraoui et al., 2008; Jin et al., 2015).

However, after 96h, we observed an opposite response of inhibition of GST activity by Cd. GST inhibition occurs either via direct action of Cd on the enzyme or indirectly via the production of ROS (Malarvizhi et al., 2017). No significant changes in CAT activity was detected after 48h of exposure to Cd and this may be attributed to the increase in content or activity of other enzymatic or non-enzymatic antioxidants such as GST (Atli et al., 2006). After 96h, the inhibition of CAT activity may be related to the direct binding of metal ions to –SH groups on the enzyme molecule (Atli et al., 2006; Cao et al., 2010). Other studies reported Cd induced inhibition of CAT in zebrafish (Yuan et al., 2017; Zheng et al., 2017). As Cd shows a high affinity for thiols, GSH is a primary target for free Cd-ions, therefore Cd induced depletion of the reduced GSH pool can be related to the reduced TG content observed in our study after 48 and 96h of exposure to Cd (Cuypers et al., 2010). Also, fluctuations on TG content might be related to GST activity changes (Cao et al., 2010). Other studies detected similar results in zebrafish larvae (Jin et al., 2015). Although various studies concluded that Cd exposure increases LPO in zebrafish (Jin et al., 2015; Ling et al., 2017; Wang et al., 2015; Yuan et al., 2017; Zheng et al., 2016) in this study, Cd exposure reduced LPO on zebrafish embryos. This may be due to the ability of antioxidant defenses, like GST, to adequately protect from oxidative stress or because Cd concentration was too low to cause oxidative damage in lipids (Campana et al., 2003).

HS altered Cd effect on GST activity and TG content after 48h. As induced cross-tolerance, observed in the acute assay in the first 48h, is only possible if the defense mechanisms induced by the first stressor are also involved in the protection against the second stressor, increased activity of GST could be involved in this process. GST activity was increased after previous exposure to HS alone and also by Cd exposure alone as detected after 48h of test, which means that is involved in the protection against the two stressors. If previous HS increases this enzyme activity, when zebrafish embryos were exposed to Cd this defense mechanism is already present which is an advantage in terms of defense against Cd. However, after 48h, the levels of GST activity of larvae pre-exposed to HS and later exposed to Cd are very similar to the levels of the larvae kept at normal temperature and exposed to Cd. This may be because GST activity has reached its maximum and could

not be induced anymore due to metabolic costs or because in larvae previously exposed to HS its maximum has happened earlier in time, probably right after HS.

GSH may also be involved in cross-tolerance to Cd. After 48h, HS exposure lead to increased TG content while Cd alone reduced its content. GSH is a very important non-enzymatic antioxidant defense mechanism namely on the direct interaction of the SH group with ROS. The pronounced depletion of TG content after 48h in larvae with HS and posteriorly exposed to Cd may suggest that GSH is in the form of Cd–GSH. This complexation makes free Cd unavailable for the cell metabolism, blocking the mechanisms leading to Cd-induced oxidative stress. The reduced LPO levels also suggest the protective role of this process. The reduction in TG content may also be related with increased GST activity since GSH plays a predominant role as a substrate for GST.

After 96h larvae pre-exposed to HS and posteriorly exposed to Cd increased their GST activity, TG content and LPO levels, while larvae kept at normal temperature and exposed to Cd decreased their GST activity, TG content and LPO levels. The higher GST activity and TG content of larvae with HS could be an advantage in terms of detoxification. Although larvae with HS also had higher levels of LPO than larvae without HS these levels are very similar to control. Despite no significant interactions observed, CAT activity seems to follow the same pattern of response with larvae pre-exposed to HS and posteriorly exposed to Cd increasing CAT activity while larvae kept at normal temperature and exposed to Cd decreasing their activity. Even though the HS seems to ameliorate the effects of Cd on some antioxidants after 96h of exposure this advantage does not translate in cross-tolerance induced to Cd, since at 96h on the acute assay we did not observe any differences in the tolerance of the two groups. However we have to take into account that sublethal concentrations of Cd were used for the biochemical analyses which could result in different patterns of response from what occurs at organismal level when using lethal concentrations.

Zheng et al. exposed zebrafish adults to 26°C and 34°C for 4 days, and posteriorly to Cd for 1 week at 26°C and also concluded that previous acclimation to elevated temperatures had a protective role against the effects of Cd exposure. The authors reported that the treatment previously acclimated to 34°C and exposed to Cd exhibited

higher CAT and SOD activities compared with the treatment of Cd exposure alone, and that the LPO levels were restored to the control levels (Zheng et al., 2017). Other studies reported that previous thermal stress ameliorates effects of Cd in other endpoints, not reported in our study, such as appearance of malformations (Kapron-Brás et al., 1991) and growth rates (Tedengren et al., 2000; Tukaj et al., 2010).

In conclusion the present study demonstrates that a HS modify Cd tolerance in zebrafish embryos. Embryos previously exposed to HS exhibits higher LC<sub>50</sub> values, probably because HS induced a general stress response that protected the embryos from Cd toxicity. However, after hatching, this induced tolerance is not evident anymore. This may be due to the loss of protection of the chorion or the limited protection capacity of the general stress responses caused by negative effects of Cd on cellular biosynthetic capacity or cellular energy deficiency.

Although increased activity/content of enzymatic or non-enzymatic antioxidants, like GST or GSH, is one of the common defense mechanisms related to heat stress and Cd exposure, our results do not allow us to clearly confirm that they are in fact responsible for the observed induced cross-tolerance to Cd. Further studies are necessary to investigate the specific mechanism of cross-tolerance in *Danio rerio* embryos. A possible approach would be to investigate the induction of MTs or HSPs since they also are suggested as general stress responses involved in this process according to the literature.

## Acknowledgments

Authors acknowledge Abel Ferreira from University of Aveiro for the laboratory support.

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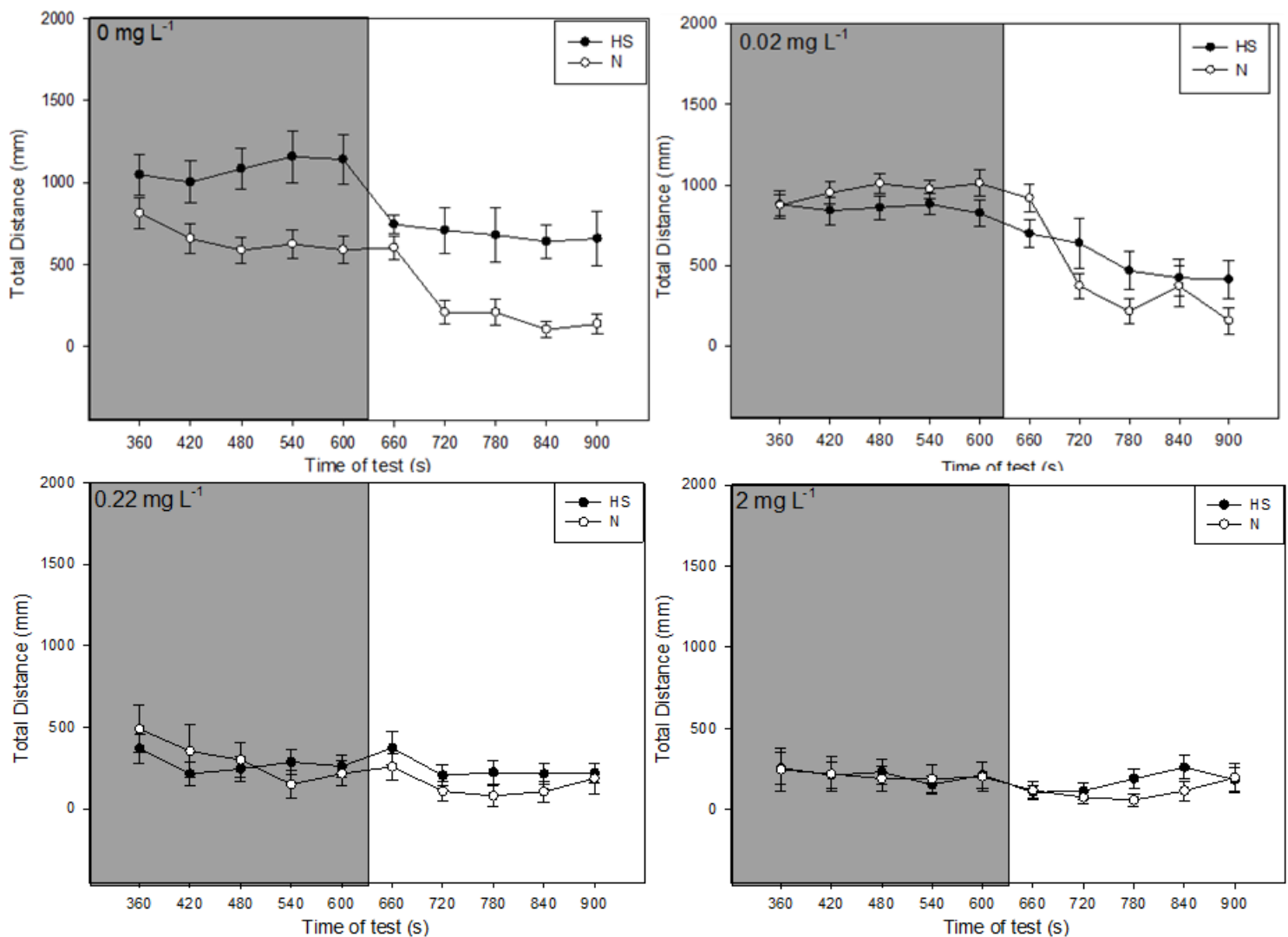
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### Supplementary material:



**Figure S1.** Locomotor behavior of zebrafish larvae after 120h of exposure to Cd. The black dots represents the embryos of the group with thermal stress (HS), and the white dots represents the embryos of the group without thermal stress (N). In all the graphics, white and black areas denote light and dark periods, respectively. All values are presented as mean  $\pm$  standard error.

## Chapter 3 - General stress responses involved in the process of cross-tolerance in *Danio rerio* embryos

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### Abstract

Many studies have suggested the involvement of general stress responses such as metallothioneins (MTs) and heat shock proteins (HSPs) in the process of cross-tolerance. To address the general stress responses responsible for the induced cross-tolerance reported in our last study between a previous heat shock (HS) of 37°C during 1 hour and cadmium (Cd) exposure in *Danio rerio*, we exposed the embryos to four different treatments, control (no HS and no Cd), HS alone (no Cd), Cd alone (no HS) and HS with later exposure to 2 mg L<sup>-1</sup> of Cd. We obtained samples of each treatment and quantified the content of MTs after 48h and 96h of exposure to Cd and the content of HSP70 after 24h, 48h and 96h of exposure to Cd. We did not obtain any significant effects of HS or Cd in MTs content but increased levels of HSP70 were observed in HS treated zebrafish larvae. Although Cd exposure did not had any effect on HSP70 levels, these results suggest that increased HSP70 levels induced by HS could be involved in cross-tolerance to Cd detected in our last study however other mechanisms may also be involved. Moreover future studies should confirm the involvement of HSP70 and address the regulation of HSPs genes and their cellular functions that ultimately culminate in cross-tolerance.

### Keywords

Heat shock, metal exposure, cadmium toxicity, zebrafish embryos, cross-tolerance, metallothioneins, heat shock proteins

## Introduction

Aquatic organisms are constantly exposed to multiple stressors and thus, studying their possible interactions is very important especially within the context of climate change. Cross-tolerance (or cross-protection) occurs when exposure to one stressor alter the tolerance of an organism to other future stressor of different nature and is commonly observed in organisms across a wide range of taxa (Kampinga et al., 1995; Sabehat et al., 1998). In 1978, Li and Hahn were among the first researchers to observe this phenomenon in mammalian cells pre-exposed to sublethal thermal stress. These cells acquired higher resistance to subsequent chemical exposure (Li and Hahn, 1978). Several studies have shown that mild stress, which can occur naturally in ecosystems, even eliciting stress responses, can be favourable to the general fitness of organisms, because it stimulates the stress defense system, increasing the protection of the organisms against future stressors (Minois, 2000; Suhett et al., 2011). Thus, it was verified that the phenotypic plasticity in physiological mechanisms of defense against environmental stressors can allow an increase of tolerance (Pestana et al., 2016). After the discovery of this process, some researchers tried to propose explaining mechanisms. When the stressors involved are elevated temperature and metal exposure, the involvement of increase of metallothioneins (MTs) (Haap et al., 2016; Plautz et al., 2013) and/or induction of heat shock proteins (HSPs) (Dubeau et al., 1998; Pestana et al., 2016; Tedengren et al., 2000; Tukaj and Tukaj, 2010) are usually suggested as main drivers of the induced cross-tolerance.

HSPs were first described in cells from *Drosophila melanogaster* during exposures to high temperature and so the term “heat shock protein” (Lewis et al., 1999). They normally represent 5-10% of the total proteins in the cell and increase in amount when cells are exposed to various stressors such as temperature, salinity, hormones, nutrient deficiencies, hypoxia or anoxia, diseases, pesticides, polycyclic aromatic hydrocarbons, metals, desiccation, ultraviolet radiation, parasites, reactive oxygen species, bacterial and viral infections and predators (Basu et al., 2002; Currie, 2011; Lewis et al., 1999; Madeira et al., 2013; Pirkkala, Lila; Nykanen, Paivi; Sistonen, 2001; Sung et al., 2011). Given that some researchers suggest that all stressors may induce HSPs expression, so these proteins are also known as stress proteins (Augustyniak et al., 2017; Currie, 2011; Dubeau et al.,

1998). HSPs are present in all organisms from bacteria to mammals and they play important roles in protecting against stressors that can cause cell damage (Basu et al., 2002; Pestana et al., 2016; Todgham et al., 2005; Werner et al., 2007). According to their molecular size, there are six main families of HSPs namely HSP100, HSP90, HSP70, HSP60, HSP40, and the small HSPs (Al-Zhgoul et al., 2013; Currie, 2011; Kalmar and Greensmith, 2009). Various studies with zebrafish have already identified and cloned a number of HSPs, including HSP70, HSP47, HSP27, HSP90a and HSP90b (Krone et al., 1997; Krone and Sass, 1994; Lele et al., 1997; Råbergh et al., 2000). HSP70 are stress-inducible highly conserved molecular chaperones and are probably the best characterized and best studied of the stress protein family (Blechinger et al., 2007; Hallare et al., 2004; Lewis et al., 1999; Tedengren et al., 2000).

Although most HSPs have a relatively short half-life, some persist in the cell after removal of the stressor and thus may play an important role in long-term adaptation (Basu et al., 2002; Dubeau et al., 1998). Thus, HSPs are an important component of the cellular stress response and play a critical role in the recovery of cells from stress because they prevent protein denaturation, restructure damaged proteins or ensure the degradation of irreversibly damaged proteins, preventing their accumulation and aggregation (Sung et al., 2011; Todgham et al., 2005). HSPs are also important in routine housekeeping functions associated with protein synthesis and maturation and therefore may exhibit patterns of both constitutive and inducible expression. In fish, constitutive HSPs commonly include heat shock cognate 70 (HSC70) and HSP90 $\beta$ , whereas HSP70, HSP90 $\alpha$ , HSP47 are inducible HSPs whose concentrations increase in response to stress (Currie, 2011; Stefanovic et al., 2016). Some studies that investigate cross-tolerance between thermal stress and exposure to Cd in aquatic organisms have suggested the involvement of HSP70. Tedengren et al. concluded that pretreatment at 20°C in the blue mussel *Mytilus edulis* significantly enhanced the HSP70 response and seemed to confer greater resistance to Cd since they observed maintained filtration rates and only minor reductions in scope for growth (Tedengren et al., 2000). Pestana et al. also investigated the effects of elevated temperature on metal toxicity in *Artemia franciscana* and found that a non-lethal heat shock induced Cd tolerance and enhanced HSP70 production (Pestana et al., 2016). These

studies demonstrate that HSPs are a general defense response likely involved in the cross-tolerance processes. However HSPs expression is sensitive to various factors regarding the stressors and the organism, such as, developmental stage, sex, nutritional status, genetic variability and age of the organism, intensity and duration of the first mild stressor, previous history of exposure to this stressor and recovery time between the two stressors (Augustyniak et al., 2017; Currie, 2011; López-Olmeda and Sánchez-Vázquez, 2011; Mahmood et al., 2014; Sung et al., 2011; Tedengren et al., 2000; Todgham et al., 2005).

Metallothioneins (MTs) are a family of low molecular weight (6-8 kDa), cysteine-rich (20–30%), inducible, cytosolic proteins well known for their high affinity to metals (Cuypers et al., 2010). Their cysteine residues allow them to bind, carry and store various metals reducing their toxicity (Abdel-Tawwab and Wafeek, 2014; van Cleef-Toedt et al., 2001). These proteins occur in a large number of phylogenetically diverse organisms (Ma et al., 2008). It has been proposed that MTs play an important role in homeostasis of essential metals such as copper (Cu) and zinc (Zn), and in the detoxification of toxic metals such as cadmium (Cd) and mercury (Hg) (Bouraoui et al., 2008; Campana et al., 2003). MTs protect against metal toxicity by three possible mechanisms: reduction of metal uptake into the cells, metal sequestration and enhancing metal export out of the cells (Park et al., 2001). Thus, metal sequestration may be a mechanism by which MTs confer cellular protection against metal toxicity since Cd-MT complexation makes Cd unavailable for cell metabolism, blocking the mechanisms leading to Cd-induced oxidative stress (Cuypers et al., 2010; Guinot et al., 2012; Ma et al., 2008; Park et al., 2001; van Cleef-Toedt et al., 2001).

We can then conclude that the increase in cell resistance to metal toxicity can be achieved by processes that result in increased ability to synthesize MTs (Guinot et al., 2012; Pedrosa et al., 2017; van Cleef-Toedt et al., 2001). It is well known that the induction of MTs occurs in aquatic organisms after exposure to metals, including Cd (Eroglu et al., 2005; Jebali et al., 2006; Maria et al., 2014; Renieri et al., 2017; Šrut et al., 2017; van Cleef-Toedt et al., 2001). However, the synthesis of these proteins can also be induced by general stress conditions, such as heat stress, hunger, desiccation and hypoxia (Guinot et al., 2012; Leung et al., 2000; Plautz et al., 2013). Some studies have demonstrated that thermal stress affects the induction of MTs (Abdel-Tawwab and Wafeek, 2014; Guinot et al., 2012; Olsvik

et al., 2016; van Cleef-Toedt et al., 2001). Thus the involvement of MTs induction as a general stress response in cross-tolerance processes is plausible.

Although the cross-tolerance process is very likely to occur in natural ecosystems, mechanisms underlying this phenomenon are still poorly understood. In our last study we concluded that a previous heat shock of 37°C during 1 hour led to a transient increased tolerance to subsequent exposure to lethal concentrations of Cd in *Danio rerio* embryos. Based on this, it might be hypothesized that MTs and/or HSP70 synthesis induced by the HS provided a protection against Cd exposure. The objective of this work is to elucidate about the role of MTs and HSPs as general stress responses that are involved in the process of induced cross-tolerance.

## **Material and methods**

### **Test organisms**

Zebrafish (*D. rerio*) eggs were obtained from a culture maintained in carbon-filtered water at the Department of Biology, University of Aveiro. These organisms were kept at  $27.0 \pm 1^\circ\text{C}$  under a 16:8h light/dark photoperiod cycle, with conductivity at  $550 \pm 50 \mu\text{S}$ , pH at  $7.5 \pm 0.5$  and dissolved oxygen at 95% saturation. Adult fish are fed twice daily with commercially available artificial diet (ZM 400 Granular) and brine shrimp.

### **Test chemicals**

Zebrafish embryos were exposed to solutions of Cd obtained from dilution of a cadmium chloride stock ( $\text{CdCl}_2$ ; CAS number 10108-64-2; purity  $\geq 99.0\%$ ). Water of the zebrafish culture was used for dilutions and as control in all tests.

### **Experimental design**

The assay was based on the OECD guideline on Fish Embryo Toxicity Test (OECD, 2013). Reproductive groups of zebrafish adults were placed in aquarium with marbles in the bottom, in the afternoon of the day before the collection of the eggs. Two hours after the beginning of the illumination in the next morning the eggs were collected and cleaned from residues.

Half of the newly fertilized eggs were exposed to a heat shock (HS) of 37°C during one hour (group HS) while the other half were kept at  $27 \pm 1^\circ\text{C}$  (group N). These temperatures were chosen based on preliminary tests and based on different studies

dealing with induction of HSPs in zebrafish, where the control temperature is usually set at 28 °C and the temperature used for the HS is commonly set at 37°C (Airaksinen et al., 2003; Hallare et al., 2005; Krone and Sass, 1994; Lele et al., 1997; Råbergh et al., 2000).

After the exposure, Zebrafish eggs from both groups with normal development were selected for the toxicity test (using a Stereoscopic Zoom Microscope-SMZ 1500, Nikon) and unfertilized, irregular or injured eggs were discarded. Zebrafish embryos from each group (with or without previous HS) were then divided and exposed to 0 or 2 mg L<sup>-1</sup> of Cd, totalizing 4 treatments. The eggs were exposed in petri plates and kept at 27±1°C.

For quantification of MTs, ten replicas for treatment were made and the test was done using two exposure times: 48 hours of exposure to Cd corresponding to a time point where, on our last study, induced cross-tolerance to Cd by HS was verified or 96 hours of exposure where tolerance of the two groups was similar. For the test of 48h we exposed 20 eggs per sample, while for the test of 96h we exposed 10 eggs per sample.

For quantification of HSP70 levels, six replicates for treatment were made and the test was done using three exposure times; in addition to 48 and 96 hours, we also performance a test with 24 hours of exposure based on the rapid induction of HSPs reported in other studies (Basu et al., 2002). For the tests of 24h and 48h we exposed 100 eggs per sample, while for the test with 96h we exposed 50 eggs per sample.

The embryos and larvae were observed daily with a stereomicroscopic (Stereoscopic Zoom Microscope-SMZ 1500, Nikon). Embryos were sampled in microtubes after exposure, frozen in liquid nitrogen and kept at -80°C until further analyses.

### **MTs quantification**

The quantification of the metallothioneins content (MT) was done following a protocol adapted from Viarengo et al. (1997). Each sample was homogenized in 650µL of phosphate buffer (0.1M, pH 7.4) with DTT (1Mm) and EDTA (1mM), on ice, using a sonic homogenizer (Sonifier 250, Branson sonicator). The homogenate tissue was centrifuged at 12000g during 10 minutes and divided into two aliquots for the protein and MTs analyses.

The quantification of the protein on each sample was done following the method of Bradford (1976), adapted to 96 well plates, using bovine γ-globuline as a standard. To

each 10µL of post-mitochondrial supernatant 250 µL of BioRad solution were added, plates were then placed in the dark, and after 15 minutes absorbance was read at 600nm.

For the MT evaluation, 500µL of 95% ethanol with 8% chloroform were added to each sample followed by centrifugation at 6000g during 10 minutes. To the supernatant 50µL of RNA (1mg/ml), 10µL of HCl (6M) and 1.2ml of cold ethanol (100%) were added. The mixture was frozen for 15 minutes at -80°C and centrifuged as indicated above. The MT-containing pellet was washed with 300µL of 87% ethanol with 1% chloroform and centrifuged at 6000g during 1 minute. The pellet was resuspended with 150µL of 0.25M NaCl and 150µL of 0.2M HCl with 4mM EDTA. After addition of Ellmans reactive (dithionitrobenzoate 0.4mM, 2M NaCl and 0.2M KH<sub>2</sub>PO<sub>4</sub>, pH 8), the absorbance was measured at 412nm and the MT concentration was estimated using the reduced glutathione as a reference standard.

### **HSP70 quantification**

HSP70 was determined using an Enzyme Linked Immunosorbent Assay (ELISA) kit (CUSABIO) in 96 well microplates and following the procedure described in the kit. This procedure employs the competitive inhibition enzyme immunoassay technique, where a competitive inhibition reaction is launched between HSP70 on the samples and biotin-conjugated HSP70 with the pre-coated antibody specific for HSP70 present in the microplate wells. For quantification purposes, a calibration curve was constructed using the standards in a range from 18.75 pg/ml to 300 pg/ml.

### **Statistical analysis**

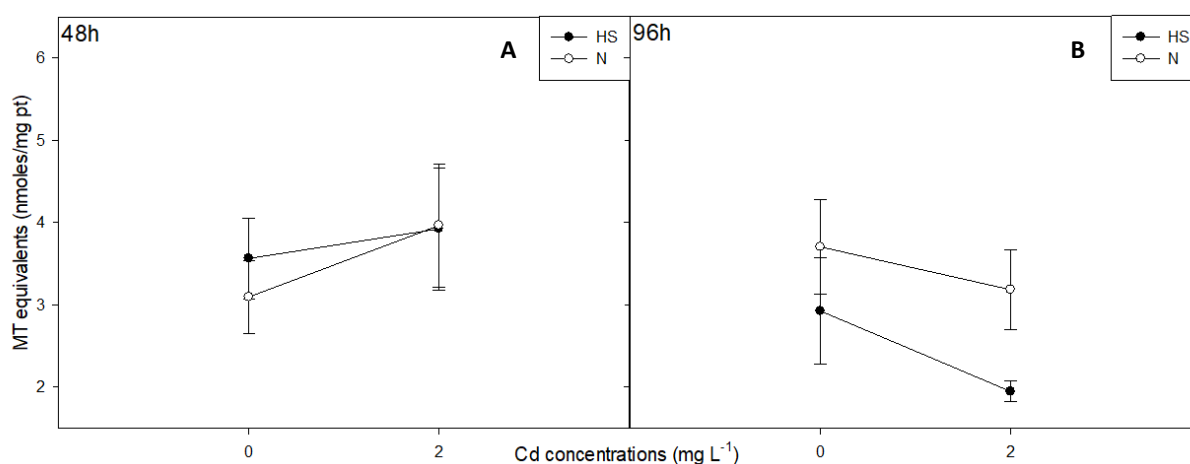
Shapiro-Wilk and Levene's test were performed to assess the normality and homoscedasticity of data, respectively. Two-way Anova's were performed to access significant effects of HS, Cd and their combination on HSP70's and MT's levels using the program SigmaPlot 12.5. The Holm-Sidak method was used for multiple comparisons. All statistical analyses were performed with a significance level of 0.05.

## Results

### MTs content

At 48h, although there is a trend for higher MT-equivalents content in all treatments comparing to control group (Figure 7A), the great variability of the data resulted in no significant effects of Cd, HS or interaction on MT-equivalents content in zebrafish embryos (Table 4).

At 96h, results suggest lower MT-equivalents content in all treatments comparing to control group (Figure 7B); still the great variability of the data resulted in no significant effects of Cd, HS or interaction (Table 4).



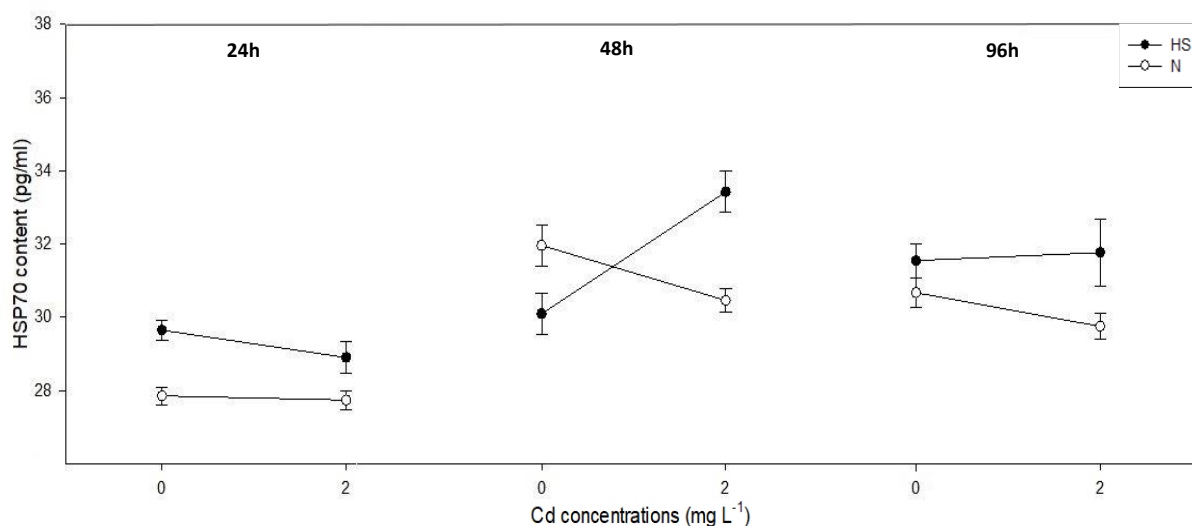
**Figure 7.** MT-equivalents content of zebrafish larvae kept at normal temperature and exposed to Cd (N) and zebrafish larvae pre-exposed to HS and posteriorly exposed to Cd (HS) during 48h (A) or 96h (B). All values are presented as mean  $\pm$  standard error.

**Table 3.** ANOVAs results of the effects of Cd alone, HS alone and their interaction (HS\*Cd) on MTs content; df-degrees of freedom; ss-sums of squares; MS - mean sums of squares; F-F-statistic.

		df	ss	MS	F	p-Value
MTs content (48h)	Cd	1	2.896	2.896	0.973	0.333
	HS	1	0.351	0.351	0.118	0.734
	HS*Cd	1	0.497	0.497	0.167	0.686
MTs content (96h)	Cd	1	3.771	3.771	1.749	0.198
	HS	1	6.804	6.804	3.155	0.088
	HS*Cd	1	0.351	0.351	0.163	0.690

## HSP70 content

At 24h, we detected an effect of HS on HSP70 values; embryos pre-exposed to HS have increased HSP70 content compared to group N (Figure 8). Cd exposure did not affect HSP70 content and a significant interaction between the two stressors was not detected (Table 5). At 48h, embryos pre-exposed to HS and posteriorly exposed to Cd have increasing levels of HSP70 compared to group N (Figure 8). In agreement, although no statistical differences were detected for factors individually a significant interaction was observed (Table 5). At 96h, HSP70 content was increased in the HS treated larvae (Figure 8). No effects were detected for Cd and no interaction occurred between the two stressors (Table 5).



**Figure 8.** HSP70 content of zebrafish larvae kept at normal temperature and exposed to Cd (N) and zebrafish larvae pre-exposed to HS and posteriorly exposed to Cd (HS) during 24h, 48h or 96h. All values are presented as mean  $\pm$  standard error.

**Table 4.** ANOVAs results of the effects of Cd alone, HS alone and their interaction (HS\*Cd) on HSP70 content; df-degrees of freedom; ss-sums of squares; MS - mean sums of squares; F-F-statistic.

		df	ss	MS	F	p-Value
HSP70 content (24h)	Cd	1	0.558	0.558	1.882	0.207
	HS	1	6.545	6.545	22.09	<b>0.002</b>
	HS*Cd	1	0.300	0.300	1.013	0.344
HSP70 content (48h)	Cd	1	5.018	5.018	3.186	0.089
	HS	1	1.810	1.810	1.149	0.296
	HS*Cd	1	34.92	34.92	22.17	<b>&lt;0.001</b>
HSP70 content (96h)	Cd	1	0.996	0.996	0.360	0.555
	HS	1	17.06	17.06	6.170	<b>0.022</b>
	HS*Cd	1	2.649	2.649	0.958	0.339

## Discussion

In our last study, preheating of 37°C during 1h induced cross-tolerance to Cd exposure in *Danio rerio* embryos. Our hypothesis to explain this induced cross-tolerance were that an exposure to a sublethal HS in the early stages of development will trigger a biochemical general stress response, such as MTs synthesis and/or HSP70 induction, which will later provide increased tolerance to these embryos when exposed to Cd. In this study, we provided evidences that a 1h HS of 37°C results in increased HSP70 levels. This increased HSP70 levels may be associated with induced cross-tolerance to Cd.

Although some studies reported that exposure to thermal stress significantly elevated MT induction or MT gene expression (Guinot et al., 2012; Olsvik et al., 2016; van Cleef-Toedt et al., 2001), HS had no influence on MT levels on zebrafish embryos. Zheng et al. acclimated zebrafish adults to 34°C for 4 days and exposed to 0 or 200 µg L<sup>-1</sup> Cd at 26 °C for 1 week and, also reported that preheating alone does not affect MTs levels after 1 week (Zheng et al., 2017). Nevertheless, the lack of response in terms of MTs content to HS in our study could be due to the low intensity and duration of the HS or because the effect occurs earlier and we could not detect it due to recovery to basal levels.

Exposure to metals, like Cd, normally activates the transcription of MT genes via the binding of metal-binding regulatory factors to the metal-responsive elements (Ma et al., 2008). In our study however, MTs levels in zebrafish embryos remain the same after 48 and 96h of exposure to 2mg L<sup>-1</sup> of Cd. The observed lack of MTs induction in response to Cd exposure could be related to the low concentration used. However, Zheng et al. exposed zebrafish adults to 0.2 mg L<sup>-1</sup> Cd during 1 week and reported increased MTs levels. So another factor could be the short-term exposure period. Ma et al. reported that increase in MT levels can be delayed depending on the exposure period (Ma et al., 2008). Maria et al. only detected an increase in MT levels after 6 days of exposure in *Folsomia candida* (Maria et al., 2014) and Marie et al. also did not observe significant increases of MTs after 24h and 72h of exposure to Cd in *Dreissena polymorpha* and *Corbicula fluminea* (Marie et al., 2006).

On the other hand, the method here used produced data with higher variability which could mask the effects of HS and Cd on MTs levels and the differences between the

treatments, so further studies are necessary to improve this method of detection for zebrafish embryos. Besides that, as described by Viarengo et al., the cysteine residues of the measured MT must be free for the DTNB to bind and further MT assessment (Viarengo et al., 1997). Therefore, higher levels of MTs could be present in our samples, but since some are fully bound to Cd in the form of Cd-MT complexes, exerting their protective roles, they were not detected in our study.

Thermal stress among other stressors, like metals, triggers a cellular program called the heat shock response, i.e. elevated expression of HSPs (Airaksinen et al., 2003). Since most of the HSPs genes do not contain introns, the mRNA is translated within minutes following exposure to a stressor (Basu et al., 2002). In this work, a 1h HS of 37°C increased HSP70 levels in zebrafish embryos. This increase is still noticed after 96h of the occurrence of the HS. It seems that HSPs are either relatively stable or continue to be over-expressed due to continued cellular stress (Werner et al., 2007). Airaksinen et al. also reported that HSP70 levels were markedly elevated in zebrafish cell lines after a 1h HS at 37°C and continued to accumulate at 2,4 and 6h (Airaksinen et al., 2003). Boerrigter et al. also exposed zebrafish larvae to a 1h HS of 37°C and observed a 1500-fold increase in HSP70 mRNA expression (Boerrigter et al., 2014). Hallare et al. exposed zebrafish embryos to thermal stress of 33°C and reported a higher induction of HSP70 after 48h of exposure (Hallare et al., 2005). Råbergh et al. also observed a markedly increased HSP70 mRNA levels in zebrafish tissues after 1h HS at 37°C (Råbergh et al., 2000). Krone et al. detected that post-blastula and later stage zebrafish embryos first exhibited inducible HSPs mRNA accumulation following a 1h HS at 34 °C with maximum induction occurring at 37 °C (Krone et al., 2003). Zheng et al. also obtained similar results with up-regulated HSP70 mRNA levels by 1 week after preheating of 34 °C for 4 days in zebrafish adults (Zheng et al., 2017). All these studies confirm that thermal stress strongly induces HSP70 synthesis in zebrafish.

Increased expression of HSP70 is reported as a short-term adaptation to Cd exposure (Renieri et al., 2017). However, we did not detect any effect of Cd alone or after a previous HS on HSP70 levels. This result is in agreement with other studies that also reported no effect of Cd on HSP70 levels or expression (Giri et al., 2016; Tedengren et al., 2000; Zheng et al., 2017). This suggests that HSP70 in zebrafish embryos is primarily induced by

increased temperature and remains unaffected by exposure to other stressors such as Cd (Hallare et al., 2005). However there are some studies that reported an increase in HSP70 transcript levels caused by Cd exposure in zebrafish (Hallare et al., 2005; Vergauwen et al., 2013b), so this absence of Cd effect in our study could also be related to the low concentration used.

After 24h, 48h and 96h we detected the same tendency: larvae pre-exposed to HS and subsequent exposed to Cd had superior levels of HSP70 than larvae kept at normal temperature conditions and posteriorly exposed to Cd. These results suggest a protective role of previous HS and indicates the HSP70 as possible candidates responsible for the process of induced cross-tolerance to Cd reported in our last study. Other studies have also linked the induction of cross-tolerance to Cd with increasing levels of HSP70 in the organisms. Tukaj et al. found that microalgae exposed to 40°C for 1h had higher HSP70 levels and were more tolerant to further exposure to Cd (Tukaj and Tukaj, 2010). Hallare et al. exposed zebrafish embryos to combinations of three temperatures (21°C, 26°C and 33°C) and Cd concentrations and reported that embryos exposed to 33°C have significantly higher expression of HSP70 and were more tolerant to Cd exposure (Hallare et al., 2005). Similarly, Zheng et al. exposed zebrafish adults to 26°C or 34°C for 4 days, and posteriorly to 0 or 200 µg L<sup>-1</sup> of Cd for 1 week at 26 °C and reported that preheating treated fish have decreased mortality and lipid peroxidation and increased mRNA levels of HSP70 (Zheng et al., 2017).

Although in this study lower Cd concentrations (2 mg L<sup>-1</sup> of Cd) did not increase the levels of HSP70, many studies conclude that exposure to Cd increases HSP70 levels and these proteins are involved in protection against this metal, so induction of HSP70 could still be involved in the process of cross-tolerance detected in our first study since there we use higher Cd concentrations (3.0 to 44.3 mg L<sup>-1</sup> of Cd). In our first study, after 48h of exposure to Cd, cross-tolerance was not evident anymore. However in this study after 96h of exposure the levels of HSP70 of zebrafish larvae pre-exposed to HS are still elevated. Although HSP70 levels remain high, these proteins may not be exerting their protective function due to cellular energy deficiency that can limit the energy available for function of the stress proteins involved (Haap et al., 2016).

In conclusion, although Cd exposure did not had any effect on HSP70 levels, these results suggest that increased HSP70 levels induced by previous HS could be involved in cross-tolerance to Cd observed in our last study. The occurrence of cross-tolerance demonstrates that organisms are capable of adaptive responses to stress, which results in an overall enhancement of a cell's tolerance for stress, where all defence mechanisms act synergistically against any subsequent stress (Kalmar and Greensmith, 2009). Thus, in the process of cross-tolerance multiple pathways can be included and not observed in our study, such as oxidative stress, inflammatory responses, and metal transport and we cannot exclude the possibility that other HSPs might also be involved (Pestana et al., 2016; Zheng et al., 2017). As climate change threatens the world's fish stocks and future scenarios of Cd pollution are provably to occur, it is critical that we understand the mechanisms underlying the adaptive responses to stress of fish in cases of exposure to multiple stressors. Future research must establish a direct role for HSP70 in cross-tolerance and integrate the regulation of HSPs genes and their cellular functions that ultimately culminate in this induced tolerance. More studies are also necessary to investigate the involvement of MTs in this process.

## **Acknowledgments**

Authors acknowledge Abel Ferreira and Ana Rita Almeida from University of Aveiro for the laboratory support.

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## Chapter 4 - General discussion

Aquatic organisms exposed to multiple stressors are capable of adaptive responses to stress, which result in an overall enhancement of cell's tolerance, like the process of cross-tolerance (Kalmar and Greensmith, 2009). This process occurs when a previous exposure to a stressor increases the tolerance of an organism to a subsequent stressor of different nature (Todgham et al., 2005). In our study we observed that previous exposure to a sublethal heat shock (HS) of 37°C during 1h, on early fertilized zebrafish embryos, resulted in induced cross-tolerance to subsequent exposure to lethal concentrations of Cd in the first 48h. In other words, mild heat stress resulted in increased tolerance to Cd, since the embryos exhibited higher  $LC_{50}$  compared with embryos kept at normal temperature conditions. These results are in accordance with our hypothesis that an exposure to a sublethal heat shock in the early stages of development will trigger a biochemical general stress response that will later provide increased tolerance to these embryos when exposed to Cd. Other studies also concluded that a previous exposure to sublethal heat stress allowed organisms to better resist Cd exposure in microalgae (Tukaj and Tukaj, 2010), brine shrimp (Pestana et al., 2016), mussels (Tedengren et al., 2000), mouse embryos (Kapron-Brás and Hales, 1991) and fish (Vergauwen et al., 2013; Zheng et al., 2017). As climate change threatens the world's fish stocks and future scenarios of Cd pollution are probable to occur this result is extremely important in the context of population's maintenance.

Considering our initial hypothesis, cross-tolerance between the two stressors can only occur if the previous HS triggered defense mechanisms also involved in the protection against Cd, such as, increased activity of antioxidant enzymes, induction of heat shock proteins (HSPs) and increased metallothioneins (MTs) content (Muyssen et al., 2010; Pestana et al., 2016; van Cleef-Toedt et al., 2001). During our work we tried to discover the general stress responses involved in this process. Although our results do not clearly point out the defense mechanism involved, they give possible candidates, such as GST and TG that are important antioxidants to the organisms and protect against reactive oxygen species (ROS) (Bouraoui et al., 2008; Jia et al., 2011) and HSP70, general stress proteins that protect the cells against numerous stressors since they are involved in prevent protein denaturation, restructure damaged proteins or ensure the degradation of irreversibly

damaged proteins, preventing their accumulation and aggregation (Sung et al., 2011; Todgham et al., 2005).

All these general stress responses, according to the literature, meet the necessary requirements to confer tolerance between these two stressors. In the case of GST activity and TG content, we find that they are induced by HS and Cd, thus, they are involved in protection against the two stressors. However, the levels measured after 48h (when differences in mortality are still detected between the two groups) are similar between the embryos submitted to HS and exposed to Cd and the embryos that remained at control temperature and were subsequently exposed to Cd. This may be because the maximum levels have been reached and could not increase anymore due to metabolic costs or because in larvae previously exposed to HS its maximum has happened earlier in time, probably closer to the HS exposure and at 48h the levels have already been reduced. In the case of HSP70, although many studies conclude that exposure to Cd increases HSP70 levels and these proteins are involved in protection against Cd, in our study this is not verified, probably because we used low Cd concentrations to trigger the induction. Regarding the involvement of MTs, according to the literature it is very likely that these proteins are involved in the cross-tolerance conference to Cd, however, in our study it was not possible to verify any effect of HS or Cd on the levels of these proteins probably due to the method we used that produced data with high variability which could mask the effects of Cd on MTs levels and the differences between the treatments.

However, after 48h of exposure, this tolerance is not evident anymore, which may be caused by a reduction in the protection of the general stress response involved as resulting from damage to the proteosynthetic machinery. This phenomenon of overwhelming biochemical processes could be due to negative effects of Cd on cellular biosynthetic capacity such as transcription and/or translation processes or due to cellular energy deficiency that can limit the energy available for synthesis and/or function of the stress proteins involved (Haap et al., 2016). As zebrafish larvae with less than 120h use only the energy reserves of the yolk sac and the induction of a general stress response for defense, detoxification, and repair is largely energy demanding, embryos exposed to HS may undergo re-allocation of energy resources with consequent metabolic costs which can

negatively affect maintenance, growth and reproduction of the organisms (Bednarska and Stachowicz, 2013; Haap et al., 2016).

Although previous HS seems beneficial for survival against Cd exposure, the analyses of other endpoints at individual level, led us to conclude that negative consequences could also occur. Previous HS influence zebrafish growth and behavior, resulting in smaller embryos with higher degree of hyperactivity, and reduce the width of the yolk sac, which suggest that heat stress increased the energy consumed by zebrafish embryos. Regarding Cd our study indicates clear effects at the individual level, such as development and hatching delay and the occurrence of malformed embryos. Also, Cd reduced embryo growth and yolk sac width which, as in the case of HS, suggests that extra energy is being consumed by the embryos under metal exposure (Baudou et al., 2017). The normal behavioral pattern in response to dark–light stimulation was also impaired by Cd and decreased swimming capabilities were detected. This effect on behavior may lead to severe consequences since decreased swimming capabilities can increase mortality by predation, reduce fish growth by difficulties in prey capture and influence a successful reproduction cycle (Almeida et al., 2014). Nevertheless, at sub-individual level, Cd exposure affected all the analyzed biomarkers in our study but did not influence MTs and HSP70 contents, probably due to the low concentration used or the short-term exposure period.

It is very likely that the process of cross-tolerance involves multiple pathways not observed in our study, such as oxidative stress, inflammatory responses, and metal transport and other HSPs, which result in an overall enhancement of cell's tolerance to stress, where all defence mechanisms act synergistically against any subsequent stress (Pestana et al., 2016; Zheng et al., 2017). Nevertheless more studies are needed to confirm this. Future research must also establish a direct role of increased GST activity, increased TG content and increased HSP70 levels in cross-tolerance and integrate the regulation of their genes and their cellular functions that ultimately culminate in this induced tolerance. More studies are also necessary to improve the method used and investigate the involvement of MTs in this process. Also it could be important to investigate the process of transgenerational transfer of induced cross-tolerance since it can affect the survival of the future generations and influence natural populations survival in future scenarios of climate

change and metal pollution. Hence, since HS can induce a general stress response that can confer later protection to Cd we suggest that heat history should be considered when assessing the effects of metal exposure on aquatic organisms.

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