Universidade de Aveiro Departamento de Biologia
Ano 2015

Joana Isabel da Silva Andrade

Efeitos da exposição ao cianeto em peixes marinhos, com ênfase em A*mphiprion* spp*.*

Effects of cyanide exposure on marine fish with emphasis to *Amphiprion* spp.

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Marinha, realizada sob a orientação científica do Prof. Dr. Ricardo Jorge Guerra Calado, Investigador Principal do Departamento de Biologia, Universidade de Aveiro e coorientação do Dr. Rui Afonso Bairrão da Rosa, Investigador Auxiliar, MARE – Marine and Environmental Sciences Centre, Laboratório Marítimo da Guia.

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palavras-chave

Amphiprion ocellaris, pesca com cianeto, aquecimento global, Pomacentridae, vulnerabilidade, biomarcadores

resumo

Os recifes de coral tropicais têm uma grande importância nos ecossistemas marinhos, devido à sua produtividade e biodiversidade. No entanto, estes ecossistemas estão a enfrentar um crescente número de ameaças naturais e antropogénicas. A pesca com cianeto é uma técnica destrutiva para capturar peixes vivos de recife, tanto para o consumo humano como para abastecer a indústria de aquários marinhos. O peixe-palhaço e as donzelas (Família Pomacentridae) são o grupo de peixes mais comercializados em aquariofilia e, consequentemente, são dos peixes mais capturados com esta pesca ilegal. O cianeto (CN^-) é considerado um potente inibidor das vias enzimáticas envolvidas na respiração e outras funções fisiológicas no peixe, que quando utilizado em doses agudas pode acumular-se em tecidos e fluidos corporais, afetando negativamente várias enzimas. No presente estudo, foram analisados os efeitos das diferentes concentrações de CN^- (imitando a pesca com cianeto) na sobrevivência e atividade dos biomarcadores em dois tamanhos diferentes de *Amphiprion ocellaris* e calculado o respetivo LC50. Posteriormente, a mesma espécie foi exposta a uma concentração sub-letal de CN^- (25 mg L^{-1}) sob o cenário de aquecimento global previsto para 2100 (29 °C e 32 °C). Oito espécies diferentes de pomacentrídeos foram expostos a uma concentração de 50 mg L^{-1} de CN^- para investigar a variabilidade interespecífica na sua tolerância ao envenenamento por cianeto. Os nossos dados revelaram que o LC50 de peixes pequenos é quase a metade do que o estimado para os peixes de tamanho médio (28,45 mg L^{-1} e 50 mg L^{-1} de CN^-, respetivamente). Também foi possível demonstrar que o aumento da temperatura da água do mar, por si só, pode causar mortalidade no peixe-palhaço, e que a mortalidade promovida pela pesca com cianeto será aumentada a temperaturas mais elevadas. Finalmente, confirma-se que a vulnerabilidade ao envenenamento por cianeto pode variar interespecificamente, mesmo em espécies que estão estreitamente relacionadas filogeneticamente. O peixe-palhaço *A. ocellaris* apresentou a maior tolerância ao envenenamento por cianeto relativamente a todos os pomacentrídeos testados. Por isso, é obrigatório proibir eficazmente a pesca com cianeto nos recifes de coral tropicais, pois o dano que esta prática faz nos oceanos de hoje será largamente ampliado nos oceanos de amanhã.

keywords

Amphiprion ocellaris, cyanide fishing, global warming, Pomacentridae, vulnerability, biomarkers

abstract

Tropical coral reefs have a high importance on marine ecosystems due to their high productivity and biodiversity. However, these ecosystems are facing a growing number of both natural and anthropogenic threats. Cyanide fishing is a destructive technique to capture live reef fish, both for human consumption and to supply the marine aquarium industry. Clownfish and damsels (Family Pomacentridae) are the most heavily traded group of marine aquarium fish and consequently also some of the most commonly targeted fishes by this illegal fishery. Cyanide (CN^-) is considered to be a potent inhibitor of enzymatic pathways involved in respiration and other physiological functions in fish and when used in acute doses can accumulate in body tissues and fluids, negatively affecting several enzymes. In the present study, we analyzed the effects of different concentrations of CN^- pulse exposures (mimicking cyanide fishing) in survival and biomarker activity in two different sizes of *Amphiprion ocellaris* and calculated their respective LC50. Subsequently, the same species was exposed to a sublethal concentration of CN^- (25 mg L^{-1}) under global warming scenarios predicted for 2100 (29 °C and 32 °C). Eight different species of pomacentrids were also pulse exposed to a concentration of 50 mg L^{-1} of CN^- to investigate interspecific variability in their tolerance to CN^- poisoning. Our data revealed that LC50 of small fish is nearly half of that estimated for medium sized fish (28.45 mg L^{-1} and 50 mg L^{-1} of CN^-, respectively). It was also possible to demonstrate that the increase of seawater temperature, by itself, can cause mortality in clownfish, and that mortality promoted by CN^- fishing will be magnified at higher temperatures. Finally, it is confirmed that vulnerability to CN^- poisoning may vary interspecifically, even in species which are closely related phylogenetically. The clownfish *A. ocellaris* displayed the highest tolerance to CN^- poisoning among all tested pomacentrids. Overall, it is mandatory to effectively ban CN^- fishing from tropical coral reefs, as the damage this practice already causes in the oceans of today will be largely magnified in the oceans of tomorrow.

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Chapter 1

1.1. General Introduction

1.1. General Introduction

1.1.1. Coral reefs

Tropical coral reefs are marine biodiversity hotspots that support hundreds of thousands of animal and plant species (Birkeland, 1997; Veron, 2000), providing humankind with a range of ecological services and economic activities. Specifically, the values/goods provided by corals can be divided into three categories/dimensions, namely: i) direct values, which result from the exploitation of coral reefs products and services (e.g. snorkeling, diving, sightseeing, fishing, prospection of bioactive compounds, trade of coral reef species, natural jewelry), ii) indirect values, such as coastline protection against erosion and storms and habitat, spawning and nursery functions to marine species and iii) preservation values associated to the ecological/cultural importance of these ecosystems (it is estimated that corals harbor around one third of marine known diversity) (Cesar, 2000; Reaka-Kudla, 2001; Brander et al., 2007). Overall, the global value of reefs have been estimated to range from $172 billion to $375 billion per year (Moore and Best, 2001; Wilkinson, 2002; Fischlin et al., 2007). These important ecosystems are susceptible to a number of threats, which, in 58% of the cases, are consequences of human activities, such as global warming, ocean acidification, pollution, overfishing, irresponsible dive tourism and destructive fishing practices – e.g. the use of cyanide fishing (see section 1.1.2) (Hughes et al., 2003; Fox et al., 2005; Leao and Kikuchi, 2005; Tissot et al., 2010). According to Burke et al. (2011), actually more than 60% of the world's reefs are under immediate and direct threat from one or more local sources, and about 55% of world's reefs are being affected by overfishing. Also, around 25% of the world´s coral reefs have already been destroyed or seriously degraded due to global warming (Goreau et al., 2000). Nonetheless, destructive fishing practices are still considered the most pervasive immediate threat to coral reefs. In fact, the most affected ones are located in Southeast Asia, where almost 95% are threatened, mostly because of overfishing and destructive fishing pressure (Burke et al., 2011). Over the past 30 years, local successes to protect coral reefs have failed to regional-scale and global management. However, with a correct management and effective policies, coral reefs can continue to provide goods and services to actual and future generations (Wilkinson, 2008) and, as already advocated,

conservation actions to tropical reefs should continue to be a priority for marine ecosystems (Robert et al., 2002).

1.1.2. Cyanide Fishing

Considering the direct values of coral reefs (see previous section), it is important to highlight the role played by non-edible fisheries, mostly for ornamental purposes and the curio trade (Costanza et al., 1997). Specifically, the trade of tropical marine fishes for the marine aquarium market started in Sri Lanka in the 1930s, and expanded during the 1950s, with an increasing number of places issuing permits for the collection of species destined for this industry (Wabnitz et al., 2003). Nonetheless, this activity has also fostered one of the most destructive fishing practices impacting coral reefs – cyanide fishing. In the last decades, cyanide fishing has been recognized as a major cause for coral reefs destruction, representing one of the most negative techniques that can be employed to collect live reef fish (either for human consumption or the marine aquarium trade) (Barber and Pratt, 1998; Burke et al., 2011). Commonly, this illegal method is used in the Asia-Pacific region where divers release a solution of sodium cyanide (NaCN) or potassium cyanide (KCN) dissolved in seawater directly into the reef, generally employing squirt bottles in a series of short pulses, that temporarily stuns fish which are brought to fishing boats and put into fresh seawater to recover. This technique affects both target and non-target fishes (Rubec et al., 2001) (Fig. 1). These chemicals are classified as ionic-cyanides (Rubec et al., 2003), with NaCN being soluble in water and rapidly decomposed in aqueous solution (IPCS, 2004).

Figure 1. Cyanide fishing (Photo by Howard Hall).

In the export market of marine-aquarium fish, this type of fishing is associated to high mortalities (>80%) of reef organisms (Rubec, 1988). The multimillion dollar nature of this business fostered the spreading of this practice throughout Southeast Asia, with its birthplace assumed to be in Philippines (Sorokin, 1993). At present, Indonesia, Sri Lanka, Philippines, the Maldives and USA supply more than 79% of the fish exported worldwide. The most important countries of destination are: the United States, the United Kingdom, the Netherlands, France, Germany and Italy (Leal et al., in press). The more heavily collected fish groups supplying this trade belong to family Pomacentridae (damsel and clownfish) (Rubec et al., 2003). Cyanide (CN^-) is considered as a powerful inhibitor of enzymatic pathways implicated in respiration and other physiological functions in fish and other organisms (Hanawa et al., 1998; Eisler and Wiemeyer, 2004). This compound, found mostly in plant species, affect the environment in gaseous, liquid or solid forms and have the potential to be transported over long distances from their emission sources (IPCS, 2004). In aquatic environments, CN^- is not accumulated in fish tissues because of their rapid detoxification at sub-lethal doses and death at lethal doses (Pablo et al., 1996). In general, after being absorbed by fish gill membranes this drug quickly induces animal asphyxia. CN^- has a high affinity to the ferric form of heme cytochrome c oxidase, the terminal enzyme in mitochondria respiratory electron transport chain (Yamamoto, 1989). As described by the previous author, during respiration, a stable complex of cytochrome c oxidase – CN^- is formed in the mitochondria, leading to the blockage of electron transfer and consequently the cessation of cellular respiration, which causes a cytotoxic tissue hypoxia and anoxia, induced by the activation of cytochrome oxidase. This shift of aerobic to anaerobic metabolism, results in a depletion of energy rich compounds, such as glycogen, adenosine triphosphate, and phosphocreatine and causes decreased blood pH, due to the accumulation of lactate (Yamamoto, 1989). These effects impact the central nervous system causing respiratory arrest and death (Eisler, 1991). When applied/present in small doses, CN^- acts like an anaesthetic that apparently does not cause any undesirable effects (Hall and Bellwood, 1995). Nonetheless, when the absorption rate is significantly higher than the rate of detoxification, there is a rapid accumulation of free CN^- in body tissues and fluids, with exposed fish showing signs of poisoning (Eisler, 1991).

As referred by the previous authors, in acute cases, several different enzymes are affected, such as metalloenzymes, nitrate reductase, nitrite reductase, myoglobin, peroxidases, catalase and ribulose bisphosphate carboxylase (Eisler, 1991). According to Gracia and Shepherd, (2004), CN^- is known to create more damage in organs with high metabolic requirements, such as heart and brain, although it is also known to be toxic in other organs, such as the liver and spleen. In the detoxification process, the majority of absorbed CN^- is converted in thiocyanate (SCN^-) through the enzymatic action of rhodanese (thiosulfate sulfurtransferase), which requires the presence of a sulphur donor (Isom et al., 2010). SCN^- is then excreted in the urine of fish for several days (Vaz et al., 2012), being possible to detect this compound for a number of days in fish tissues and blood (Bruckner and Roberts, 2008). Recent studies showed that nearly 80% of all CN^- entering the organism is converted to SCN^- and latter being excreted in the urine (Logue et al., 2010). Rhodanese enzyme is known to be most active in the liver and kidneys and has been already isolated from fish gills and intestine as well (Leduc, 1984; Baghshani and Aminlari, 2010). Freshwater fish are more sensitive to CN^- poisoning than marine fish, with high mortality being documented at CN^- concentrations as low as 20 $\mu g\ L^{-1}$ and adverse effects on swimming and reproduction being recorded at level above 5 $\mu g\ L^{-1}$ (Eisler and Wiemeyer, 2004).

It is important to highlight that at certain concentrations and/or exposure times, the toxic mode of action of CN^- can be reversible (Lewis, 1960). Because of this rapid detoxification process, fish can cope with high sublethal doses of CN^- without suffering extensive damage over long periods of time (Mengel et al., 1989). In fish species traded for the marine aquarium industry, the excretion of SCN^- is influenced by several variables, such as initial concentration of CN^- and duration of the pulse exposure (Vaz et al., 2012), as well as fish size, species, post-collection handling and holding time in exporting facilities and shipping time to importing facilities (Hawana et al., 1998; Rubec et al., 2003; Bruckner and Roberts, 2008). Resulting from transulfuration process, SCN^- metabolites are about 120 times less toxic than the parent CN^- compound but when accumulate in tissues can lead to the development of abnormalities and other adverse effects (Eisler, 1991).

1.1.3. Climate Change

Ecosystems are being seriously impacted by climate changes induced by anthropogenic pressure (Walther et al., 2002). The “real” future scenarios are uncertain, however global change predictions, with increasing ocean temperature, raises concerns on ecosystems health and biodiversity (Rosa et al., 2012; Freeman et al., 2013; Anacleto et al., 2014). The global sea surface temperature is expected to increase between 0.3 °C to 4.8 °C by 2100 (IPCC, 2014) and this additional heat will be partially absorbed by oceans, causing negative impacts in seawater quality and consequently fitness and survival of marine organisms (Tewksbury et al., 2008; Nilsson et al., 2009). The more restricted capacity of circulatory and ventilatory systems under thermal stress tend to lead to a reduction in aerobic scope (the difference between maximum and resting metabolism) with consequent cascading effects on growth and developmental rate of juveniles and reproductive capacity of adults (Wood and McDonald, 1997; Pörtner, 2002; Nilsson et al., 2009). Additionally, it is worth noting that this climate-change related stressor not only directly affects lower levels of biological organization (e.g. at cellular and organism levels) but also at population and ecosystem levels, namely in their structure and functionality (Pörtner and Knust, 2007). Tropical organisms, particularly ectothermic vertebrates, are expected to be more sensitive to elevated temperatures because they are adapted to live in a relatively constant environment, having a limited capacity for temperature acclimation (Tewksbury et al., 2008). Furthermore, thermal stress leads to the production of heat shock protein (HSP), a class of molecules responsible for the repair, refold and elimination of damaged or denatured proteins (Sokolova et al., 2011). Higher temperatures, and consequently enhanced metabolic rates, are also associated with increased formation of reactive oxygen species (ROS), which may be followed by an enhancement of antioxidant enzyme activity, such as catalase (CAT) and glutathione peroxidase (GPx), glutathione *S*-transferases (GST) and reduced glutathione (GSH) (Lesser, 2006).

1.1.4. Biomarkers

Exposure to toxic compounds, such as CN^-, affects fish enzymatic pathways (Rubec, 1986) and have negative impacts on fish immune capacity/response, behavior, growth and reproduction (Heath, 2000; Arts and Kohler, 2008; Di Giulio and Hilton, 2008). Such changes compromise cell's functionality and integrity and in worst case scenarios may promote tissue lesions and shifts in organ function. All these responses at cellular and organism levels are essential to detect early signs of contamination, thus making possible to avoid negative impacts at higher biological levels (Moore, 1993; Adams et al., 2000). In the last decades, the use of biochemical biomarkers has been commonly applied in the detection of fish sub-lethal responses to environmental contaminants (Van der Oost et al., 2003). According to Gestel and Brummelen (1996), biomarkers can be defined/understood as a "*biological response to an environmental chemical at a sub-individual level, measured inside the organism or in its products (excretions, hair, feathers), that indicates deviations to the normal baseline and relates to the presence of contaminants that cannot be detected from the intact organism*". At the individual level, contaminants can inhibit or stimulate endogenous enzymes, but their mode of action can differ depending on the exposure period, concentration of the compound being monitored and the developmental status of the organism. Normally, early life stages (embryo and larvae) are more sensitive to contaminants than adults (Guillette et al., 1995, Poellinger, 2000, Kuiper et al., 2007). Each organism is equipped with a range of cellular defense mechanisms and biotransformation processes to protect themselves against the action of toxins/toxic compounds (Varanasi et al., 1989). This last process has the objective to make chemicals less toxic and easier to excrete. In general, the toxicity associated with most contaminants is triggered by the enhancement of intracellular chemical species named reactive oxygen species (ROS). These compounds result from the incomplete reduction of oxygen, and production of peroxides species, which may damage a number of cellular components (e.g. cytoskeleton, cytomembranes, mitochondria, proteins, lipids and DNA) (Regoli et al., 2002; D'Autreaux and Toledano, 2007). When an organism is under oxidative stress conditions, it normally shows an imbalance of ROS

levels and is unable to cope with such situation in a fast and effective way (D'Autreaux and Toledano, 2007). These reactive oxygen species can be generated in the electron transport chain (ETC) in the mitochondria or in other cell sites such as endoplasmic reticulum and microbodies (Van der Oost et al., 2003; Lesser, 2006). Commonly, fish have a complex mechanism of detoxification to protect cells against oxidative stress, inhibiting oxyradical formation, including an interacting network of antioxidant enzymes such as glutathiones or catalase (Cadenas and Davies, 2000; Van der Oost et al., 2003). GST (Glutathione *S*- transferases), belongs to the first enzymes that act in this detoxification process (Henson et al., 2001), acting as catalysts for the conjugation of various electrophilic compounds with the tripeptide glutathione (Gulick and Fahl, 1995). This enzyme is located in the cytosol, although it can also occur in microsomes and mitochondria, and plays an important role in the protection pathway against oxidative damage and peroxidative products of DNA and lipids (Henson et al., 2001). The activity of this enzyme can be increased by exposure to compounds such as nitro compounds, organophosphates and organochloride, and both increased or inhibited in certain pollutants such as PAHs (Van der Oost et al., 2003). Another enzyme responsible for detoxification is calatase (CAT), a hematin-containing enzyme, that facilitate the removal of hydrogen peroxide (H_2O_2), which is metabolized to molecular oxygen (O_2) and water. Mostly this enzyme is located in peroxisomes, involved in the fatty acid metabolism and also appears connected to glutathione peroxidase (GPx), as it acts against oxidant stress (Diesseroth and Dounce, 1970; Huggett et al., 1992) (Fig. 2). Catalase, glutathione *S*-transferases and glutathione peroxidase, are examples of enzymes used in toxicology studies, normally measured in gills and liver (Ogunji et al., 2007). In vertebrates, liver is the main organ involved in detoxification process while gills are the main barrier to the entry of contaminants in the organisms (Koca et al., 2005). Other important biomarker that can be used to measure the exposure to neurotoxic compounds, such as organophosphates, carbamates or similar molecules, is the enzyme acetylcholinesterase (AChE) (Sancho et al., 2000). Located in the anterior part of nerve terminals, this enzyme acts through the hydrolysis and inhibition of the neurotransmitter acetylcholine, into two compounds, choline and an acetate group (Purves et al., 2008). The most common

symptoms associated to the inhibition of this enzyme are system atrophy, deterioration of cognitive autonomy, neuromuscular functions and respiratory failure (Soreq and Seidman, 2001). Several studies addressing the exposure to organophosphates, carbamate insecticides and metals, have shown the suitability of AChE as a biomarker (Stanek et al., 2006; Calisi et al., 2009). Concerning biomarkers of effect, lipid peroxidation (LPO) has been considered an important endpoint because it results in the formation of highly reactive and unstable hydroperoxides of both saturated and unsaturated lipids, which can be measured through the presence of a metabolite - malondialdehyde (MDA). Lipid peroxidation can disrupt membrane functionality, inactivate proteins and the formation of DNA adducts (Valavanidis et al., 2006) (Fig. 2).

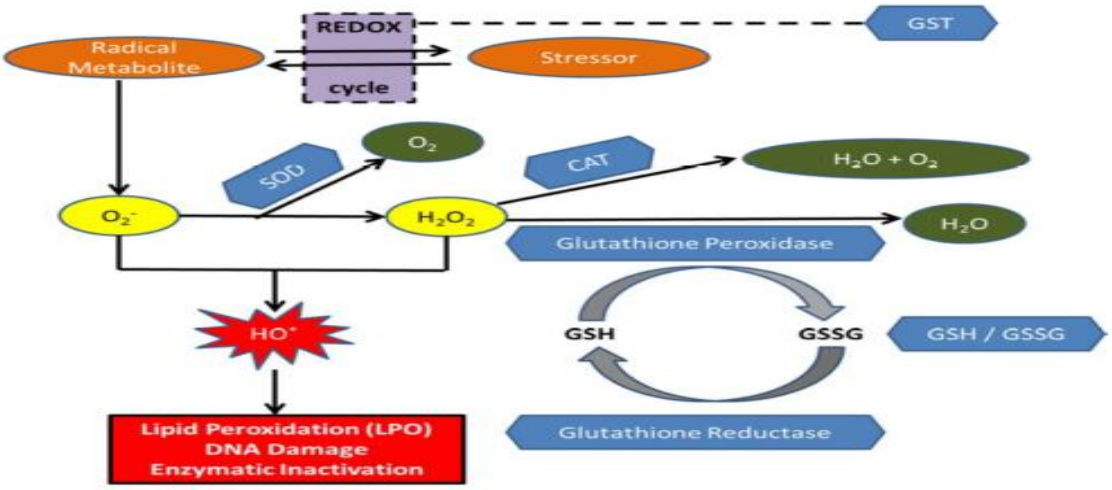

Figure 2. Scheme of oxidative stress generated by reactive oxygen species (ROS) (From Ferreira, 2014).

1.1.5. Acute tests

Toxicity tests are used to evaluate the effects of contamination on the survival, growth, reproduction, behaviour and/or other attributes of organisms, and determine whether the contaminant concentrations in a site's media are high enough to cause adverse effects in organisms (EPA, 1994). These important tests have many applications such as, demonstrating whether contaminants are bioavailable, evaluating the toxicity of substances whose biological effects may not have been well characterized, illustrating the nature of a toxic effect and having a role in the monitoring and measurement of lethal or

sublethal effects, or both (EPA, 1994). There are two types of toxicity tests: acute toxicity test (test to determine the concentrations of chemicals that cause an adverse effect (usually death) and chronic toxicity test (short-term test in which sublethal effects (e.g., reduced growth or reproduction) are usually measured in addition to lethality) (EPA, 2001).

The principle of acute tests is to expose an aquatic organism, test substances preferably for a period of 96 h, record mortalities at specific endpoints (e.g. 24, 48, 72 and 96 hours) and calculate concentrations which kill 50% of the study species (LC50). Before performing an acute test, it is required to know some information about test subtances, validity of test and procedure, which is regulated by entities such OECD (OECD, 1992). There are few studies that have used these toxicity tests to evaluate CN^- toxicity in tropical marine fish, with most available literature focusing on fresh water fish given their higher vulnerability to CN^- poisoning (Eisler and Wiemeyer, 2004).

1.1.6. Family Pomacentridae

Reef fish communities are important components of the structure of reef environments contributing to their functional pathways through competition and territoriality. This type of communities' stands out for its diversity in morphology and number of species (Ceccarelli et al., 2001). One important group of these communities are the members of family Pomacentridae (Bonaparte, 1831), which has an important role in energy and nutrient transfer processes, as well as recruitment and growth of corals (Horn, 1989), invertebrates, algae (Ferreira et al., 1998) and other herbivorous fish (Schwamborn et al., 2002). The Pomacentridae includes about 320 species, with 33 genera, mostly occurring in tropical and sub-tropical waters and associated with rocky substrates (Allen, 1991) These tropical fishes exhibit a large diversity of living modes, for example they can be symbiotic (e.g. *Amphiprion* spp.), schooling (e.g. *Chromis* spp.) and bottom-dwelling species (e.g. *Chrysiptera* spp.). This acanthopterygian family is characterized to be brightly colored, have similar external morphology, small sized (no species is larger than 300 mm in total length, TL, measured from the tip of the snout to the tip of the longer lobe of the caudal fin), have a single nostril on each side of the head, two anal spines, an incomplete

lateral line, a single dorsal fin that is well-developed, small teeth and scales, and a smooth palate (Allen, 1991; Bellwood and Sorbini, 1996). Among other features, they can be distinguished by their territoriality and also by their different ways of feeding, such as the ability to capture certain preys through morphological modifications of their feeding apparatus and/or locomotion method (Kotrschal, 1988). This family is not only important at ecological level it also represents half of the number of marine ornamental fish collected worldwide (Wabnitz et al., 2003). The top 5 most traded fish species for marine aquariums are all members of the Pomacentridae, which emphasizes the commercial relevance they continue to play in the marine aquarium trade. These five species are, by decreasing order in the number of traded specimens: the blue-green damselfish (*Chromis viridis*), the clown anemonefish (*Amphiprion ocellaris*), the whitetail dascyllus (*Dascyllus aruanus*), the sapphire devil (*Chrysiptera cyanea*) and the threespot dascyllus (*D. trimaculatus*) (Wabnitz et al., 2003).

1.1.7. Amphiprion ocellaris

Amphiprion ocellaris (Cuvier, 1830) is one of the most important marine ornamental fish in the aquarium trade (Wittenrich, 2007), which has become popularly known by the general public after the blockbuster animation movie “Finding Nemo”. The species is brightly coloured (normally described has a bright orange fish with three lateral vertical white bars, the middle one with an anteriorly protruding bulge), easy to keep in captivity and displays a relatively small size (ranges up to 110 mm in TL) (Fig. 3). This species occurs in Indo-West Pacific: Eastern Indian Ocean including Andaman and Nicobar Islands, Thailand, Malaysia, and northwest Australia to Singapore, Indonesia, and the Philippines. To the north it ranges from Taiwan to Ryukyu Islands (Allen, 1991). *A.ocellaris* lives in shallow and calm lagoons and is an obligate symbiont with several sea anemones species, such as *Tichodactyla mertensii* (Brandt, 1835), *Stichodactyla gigantea* (Forsskäl, 1775) and *Heteractis magnifica* (Quoy and Gaimard, 1833) located up to 10 meters deep in reef flats, crests and slope (Nelson et al., 1996; Lim et al., 2008). This species is an oviparous species that can be easily cultured in captivity (Wittenrich, 2007), exhibiting a distinct pairing behaviour during breeding. They are protandrous hermaphrodites, forming

monogamous pairs, laying demersal eggs that adhere to the substrate and are aerated and guarded by males (Fricke and Fricke, 1977; Miyagawa-Kohshima et al., 2014). Compared to other coral reef fishes, *A.ocellaris* has a short larval period (15–20 days) and an unusual social hierarchy (Wellington and Victor, 1989). Because of the ever growing marine aquarium trade, this species has been highly exploited commercially (Wood, 1985). Although 28 different species are currently recognised within genus *Amphiprion*, *A. ocellaris* the most traded one, representing alone more than 15.6% of total exports in the world and over 25% in European countries (Wabnitz et al., 2003). In the more intensively exploited reef areas, reduction in stock size can increase their vulnerability risk to over exploitation, with replenishment being dependent from spawning elsewhere (Munro, 1983). This conservation issues and high market demand have prompted the development of culture methods (Kim et al., 2007; Olivotto et al., 2008), with a significant part of the trade being supplied at present by cultured specimens. As already mentioned before, nearly half of all live reef fish traded in the marine aquarium industry belong to the Pomacentridae. It is therefore no surprise to verify that these species are also target by CN^- fishing, especially in Asia-Pacific region (Rubec et al., 2003; Wabnitz et al., 2003). This fact was used as the rationale to support the select of this family in general and *A. ocellaris* in particular as model organisms for the present dissertation.

Figure 3. *Amphiprion ocellaris* (Photo by Jim Anderson).

1.1.8. Objectives

Due to the lack of information on the lethal toxicity of CN^- for *A. ocellaris*, the aim of this dissertation is to evaluate the median lethal concentration (LC50) of CN^- during pulse exposures mimicking CN^- fishing in two different size classes (small (≈25 mm TL) and medium fish (≈38 mm TL)) of *A. ocellaris´*s and evaluate the physiological effects of pulse exposure to CN^- through the basal activity of the following biomarkers: acetylcholinesterase (AChE), glutathione *S*-transferases (GST), catalase (CAT), lipid peroxidation (LPO) and glutathione peroxidase (GPx) (Chapter 2).

Another aim of this dissertation is to evaluate, for the first time, the combined effects of CN^- poisoning and environmental warming (+ 3 °C and 6 °C degrees) in the detoxification pathways of clownfish *A. ocellaris* (Chapter 3).

Last, the vulnerability to CN^- poisoning of the most heavily collected species of Pomacentridae currently traded to supply the marine aquarium industry (8 species in total) and recurrently captured through the use of this illegal and destructive fishing practice was compared. This evaluation also took into consideration the phylogeny of the Pomacentridae, in order to allow a better understanding of any potential trend in their vulnerability to CN^- poisoning (Chapter 4).

1.1.9. Ethics Statement

All in-vivo studies were carried out under an institutional license for animal experimentation issued by Direção Geral de Veterinária (DGV), Portuguese Ministry of Agriculture, Rural Development and Fisheries). This work was in strict accordance with the recommendations within the Guide for the Care and Use of Laboratory Animals of the European Union, that is represented in Portugal by the Decreto Lei n º 129/92 de 06 de Julho, Portaria Nº 1005/92 de 23 de Outubro de 1992.

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Chapter 2

2.1. Physiological effects of cyanide exposure on different sized clownfish *Amphiprion ocellaris*

Physiological effects of cyanide exposure on different sized clown fish *Amphiprion ocellaris*

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Abstract

Cyanide fishing is one of the most destructive fishing techniques employed to collect live reef fishes. These illegally collected fishes continue to supply the marine aquarium trade, regardless of the high mortalities (>80%) associated with cyanide poisoning. Surprisingly, only a few studies have focused on the toxicity of cyanide poisoning in marine fish. The present study aimed to evaluate the median lethal concentration (LC50) of cyanide (CN^-) during pulse exposures in small (≈25 mm total length, TL) and medium sized (≈38 mm TL) clownfish *Amphiprion ocellaris*, one of the most heavily traded marine ornamental fish in the world. The physiological effects associated with the pulse exposure to CN^- were evaluated through biomarkers activity (acetylcholinesterase (AChE), catalase (CAT), glutathione peroxidase (GPx), lipid peroxidation (LPO) and glutathione *S*-transferases (GST)) 96 h post exposure to different concentrations of CN^- (ranging from 6.25 to 100 mg L^{-1}). The 96 h LC50 values determined were 28.45 mg L^{-1} of CN^- for small-sized and 50 mg L^{-1} of CN^- for medium-sized fishes. In addition, gills, brain, muscle and liver showed significant differences in the activity of the biomarkers surveyed, with these inter tissues differences also being recorded between size classes (less perceptible) and CN^- concentrations. This experimental study revealed three key findings: low concentrations of CN^- are already highly toxic to *A. ocellaris* (apprehended squirt bottles revealed concentrations up 1500 mg L^{-1}), fish size influences their vulnerability to CN^- poisoning, and that the biomarkers selected enable researchers to effectively monitor the physiological effects of CN^- poisoning in this clownfish species. So, it is important that authorities enforce existing laws that already criminalize the use of CN^- fishing and increase the awareness of importing countries on the destructive nature of this practice.

Key-words

Cyanide poisoning; aquarium marine trade; median lethal concentration; biomarkers; tropical coral reef fish

2.1.1. Introduction

Tropical coral reefs are biodiversity hotspots (Hughes et al., 2003), sheltering a large number of fish species and protecting coastline from erosion (Costanza et al., 1997; Veron, 2000). Besides its ecological relevance, they also support many economic activities, providing goods and services to human populations (Costanza et al., 1997; Veron, 2000). For instance, in the Philippines, the trade of tropical reef fish (for food and non-food purposes) by local communities represents around US$ 1 billion of the annual budget (White et al., 2000). However, it is also known that overfishing is affecting about 55% of world's reefs and consequently, destructive fishing practices, such as cyanide fishing, are considered one of the biggest threats to these endangered ecosystems (Hughes et al., 2003; Fox et al., 2005; Leao and Kikuchi, 2005; Tissot et al., 2010). Despite the high mortalities associated with cyanide fishing (>80% of target specimens) (Rubec et al., 2001), and excluding the dramatic effects it has on non-target species (fish and invertebrates), this illegal practice continues to be widely employed in the Asia-Pacific region (Burke et al., 2011). Fisherman uses a repeated squirting of the cyanide (CN^-) solution, in a series of short pulses, over a reef area, that temporarily stuns fish (Rubec et al., 2001). When absorbed across fish gill membranes, CN^-, blocks the oxygen-transporting protein hemoglobin, inducing tissue anoxia, cytotoxic hypoxia and interfering with enzymatic reactions (Rubec, 2001; Eisler and Wiemeyer, 2004; Carberry, 2013). The toxicity of this compound can be reverted through the metabolization of CN^- into a nontoxic soluble compound – thiocyanate (SCN^-) - in the presence of thiosulfate and the enzyme rhodanese (Isom et al., 2010). Recent studies showed that closely 80% of all CN^- entering the organism is converted to SCN^- and excreted in the urine (Logue et al., 2010). Because of this rapid detoxification process, fish can be exposed to high sublethal doses of CN^- without any apparent damage over extended periods (Mengel et al., 1989). It is also known that CN^- toxicity can be influenced by a variety of factors, including concentration, environmental temperature and dissolved oxygen and be more damaging not only to organs known to display high metabolic requirements (e.g., heart and brain), but also in a number of other important organs (e.g. liver and spleen) (Ballantyne and Marrs, 1987; Gracia and Shepherd, 2004). Furthermore, some studies suggest that toxicity

of CN^- differs with fish size and age, among species (Arifin and Hindarti, 2006) and handling stress (Hanawa et al., 1998).

A*mphiprion ocellaris,* is a popular clownfish that is heavily traded for marine aquariums worldwide and occurs in the Indo-west Pacific region (Wood, 1985; Wittenrich, 2007). Although it is commonly targeted by CN^- fishing (Ricardo Calado pers. com., July 2005), little is known on its vulnerability to CN^- poisoning. Physiological shifts can be monitored and quantified through the use of biomarkers (Andersen et al., 2006). Currently, there is still a generalized lack of information concerning the role of size and age on CN^- poisoning in marine fish.

The aim of the present work was to access if differenced sized clownfish *A. ocellaris* would display comparable physiological responses in their different tissues after pulse exposures to a range of CN^- concentrations. The lethal concentration (LC50) of CN^- in two different size clownfish (small (TL ≈25 mm) and medium (TL ≈38 mm)) were calculated after 96h, as well as the activity levels of enzymes implicated on: i) neurological function (acetylcholinesterase – AChE), ii) oxidative stress response (catalase (CAT); glutathione peroxidase (GPx); lipid peroxidation (LPO)) and iii) detoxification pathways (glutathione *S*-transferases (GST)) in their brain, liver, muscle and gills.

2.1.2. Material and Methods

2.1.2.1. Fish Maintenance

Small-sized (n=60, TL ≈25 ± 0.033 mm (measured from the tip of the snout to the tip of the longer lobe of the caudal fin); average wet weigh 0.30 ± 0.092 g) and medium-sized (n=60, TL ≈38 ± 0.024 mm; average wet weight 1.12 ± 0.21 g) *A. ocellaris* were purchased from a Portuguese breeder (Opérculo Lda.) to assure that all specimens had never been exposed to CN^- prior to the experimental procedures. All specimens were acclimated for 2 months under a 12 h ligt : 12 h dark photoperiod in 90-L glass tanks (0.6 m high × 0.6 m long × 0.25 m wide) equipped with a circulation pump providing a water flow of 2500 L h^{-1} (Turbelle nanostream-6025 Tunze, Germany) and connected to a 150 L sump (0.5 m high, 0.7 m long and 0.5 m wide) equipped with a biological filter (submerged bio-balls), a fluidized bed filter (FLF100 ReefSet, Portugal), a 300 watt submergible heater (that kept

the water temperature stable at 26 °C) (Eheim Jäger 300 W, Deizisau, Germany), a protein skimmer (ESC150 ReefSet, São Mamede Negrelos, Portugal) and a submerged pump with the a water flow of 1000 L h^{-1} (Eheim 1262, Deizisau, Germany). Further details on this life support system can be found in Rocha et al. (2013). The system was also equipped with an osmoregulator (Reef SetH or Deltec Aquastat 1000, Delmenhorst, Germany) used to replace evaporated water with freshwater purified by reverse osmosis (RO) to keep salinity stable at 35. The salinity was checked daily using a hand refractometer to detect any potential malfunction of the described automation. Water parameters were maintained within the following optimal ranges for the husbandry of *A. ocellaris*: temperature 26 ± 0.5 °C; not detectable ammonium, nitrite and nitrate; pH 8 ± 0.2. Nitrogenous compounds were monitored every week using colorimetric tests (Salifert), while pH was monitored using a Pinpoint pH meter (PH 370, American Marine). All seawater employed during the acclimation period was prepared by mixing RO water with a commercial synthetic salt mix (Tropic Marine Pro Reef, Wartenberg, Germany). All fishes were fed four times a day until satiation with a commercially produced pelleted diet (Hikari Marine S).

2.1.2.2. Acute Test Validity

To validate the acute test for CN^- toxicity for *A. ocellaris* the following conditions were completely fulfilled: i) fish were fed 24 h before the beginning of the test, ii) at the end of the test, the mortality recorded in the control treatment did not exceed 10% (or one fish if less than ten are used), iii) constant conditions of semi-static procedure were maintained as far as possible, iv) dissolved oxygen concentration were at least 60% of the air saturation value throughout the test, v) maximum loading did not exceed 1.0 g fish L^{-1} (for semi-static test), vi) a 12 h of photoperiod, vii) no food was administrated to the fish during all toxicity tests and viii) a minimum of at least 7 fish was employed for each treatment. In order to fulfil the requirements for this acute test, it was of paramount importance to hold the specimens to be employed correctly (from an husbandry point of view) and to have all essential information concerning the test solution (CN^-) (OECD, 1992) (see Supplementary data).

2.1.2.3. Experimental Design and Treatments

Following the acclimation period, the 60 small-sized *A. ocellaris* were randomly divided into 6 groups of 10 fish each for cyanide (CN^-) pulse exposure at five different concentrations (6.25, 12.5, 25, 50 and 100 mg L^{-1}), plus a control where no CN^- was added. Before the beginning of the pulse exposure to CN^-, a stock solution of 2.060 g L^{-1} of CN^-, was prepared in a 500 mL volumetric flask by dissolving (2.001±0.001) g of sodium cyanide (NaCN) (97% purity; Sigma- Aldrich, St. Louis, MO, USA) in ultra-pure water, obtained from a Milli-Q Millipore system (Milli-Q plus 185). CN^- concentration, in the final volume of 3300 mL used during the pulse exposure was prepared as follows: concentration 6,25 mg L^{-1} (adding 10 mL of the stock solution to 3300 mL of synthetic seawater); concentration 12.5 mg L^{-1} (adding 20 mL of the stock solution to 3300 mL of synthetic seawater); concentration 25 mg L^{-1} (adding 40 mL of the stock solution to 3300 mL of synthetic seawater); concentration 50 mg L^{-1} (adding 80 mL of the stock solution to 3300 mL of synthetic seawater) and concentration of 100 mg L^{-1} (adding 160 mL of the stock solution to 3300 mL of synthetic seawater). The pulse exposure to CN^- was divided in 4 steps: exposure bath, first cleaning bath, second cleaning bath and third cleaning bath. In the first step, all fish from the control treatment were collected with a hand-net and dipped for 60s into a 5-L tank filled with synthetic seawater with no CN^-. The duration of the pulse exposure (60s) was selected according to Hanawa et al. (1998). A preliminary trial revealed that handling the fish with a hand net causes no mortality or significant stress (Vaz et al., 2012). After the pulse exposure to CN^- all fish from the control group were dipped for 60s into a 20-L tank filled with synthetic seawater with no CN^- (first cleaning bath). This procedure was repeated two more times (second and third cleaning bath). Following this procedure, all fishes of the first treatment were collected with a hand-net and dipped for 60s into a 5-L tank filled with synthetic seawater dosed with the concentration of 6.250 mg L^{-1} CN^-. After the pulse exposure to CN^- all fish were dipped for 60s into three cleaning baths (see Supplementary data). This procedure was also repeated to the all fish of the other treatments, increasing gradually the CN^- concentrations (12.5; 25; 50; 100 mg L^{-1}). During the exposure procedure, immobilization time was recorded. In the present work, immobilization time was defined as the time at which the last fish of

the group rested motionless in the bottom of the mesh used for the exposure bath. Following the pulse exposure, all fish were randomly distributed into 1-L glass jars filled with 1L of synthetic seawater (prepared as described above) and the recovery time of normal swimming activity was determined. Recovery time was defined as the time at which the last fish of a species group reacquired its normal swimming ability. The toxicity trial was carried out in a semi-static system, where water was fully replaced every day and constantly aerated through air stones. The photoperiod used was 12 h light: 12 h dark, provided by white fluorescent lamps. According to OECD guideline 203 (OECD, 1992), during this toxicity trial no food was provided to the fish. The jars were placed inside a water bath keeping water temperatures stable at 26 °C. The behaviour of all fish was monitored during the first 30 min inside the glass jars to record recovery and mortality. Temperature, salinity, pH, dissolved oxygen, alkalinity, total ammonia–nitrogen, nitrite and nitrate of each experimental jar were measured daily. Temperature was measured using a thermometer and salinity with a handheld refractometer. Dissolved oxygen and pH were measured using an YSI 85 Model (Yellow Springs Instruments, Yellow Springs, USA), and a pH 100 meter (Yellow Springs Instruments, Yellow Springs, USA), respectively. Total ammonia–nitrogen, nitrite and nitrate were monitored using colorimetric tests (Hach, USA). Temperature was maintained at 26 ± 0.1 °C, salinity at 35 ± 0.1, pH 8 ± 0.1, dissolved oxygen concentration at 6.2 mg L^{-1}, nitrite, nitrate and ammonium were not detectable. Mortality was evaluated daily. Fish were considered dead when they were motionless on the bottom, exhibited no opercular movement and presented no response to mechanical stimuli. After 96 h of exposure to different concentrations of CN^-, fish were euthanized by immerging them in ice and necropsied to remove their liver, gills, muscle and brain. All sampled tissues were placed in liquid nitrogen, and stored at -80 °C for biomarker analysis. The same procedure described above was repeated for the 60 medium-sized of *A. ocellaris*.

2.1.2.4. Biomarkers Analysis

The protocol employed to process all fish tissue samples was previously described by Ferreira et al. (2010) and is thoroughly described in the supplementary data. The activity

of glutathione *S*-transferases (GST), catalase (CAT) and glutathione peroxidase (GPx) were monitored in gill, liver and muscle tissues, while lipid peroxidation (LPO) was solely monitored in gill and muscle tissues (as previously described by Bird and Draper (1984) and Ohkawa et al. (1979) and further adapted to microplate). AChE activity was determined solely in the fish brain tissue. Glutathione *S*-transferases (GST) and glutathione peroxidase (GPx) activities were determined as described by Habig et al. (1974) and Mohandas et al. (1984), respectively. Catalase (CAT) activity was determined based on the method described by Clairborne (1985) and adapted to microplate. Acetylcholinesterase (AChE) activity was assessed according to the Ellman method (Ellman et al., 1961) adapted to microplate (Guilhermino et al., 1996). For all biomarkers, protein concentration was determined according to the Bradford method (Bradford, 1976), adapted from BioRad's Bradford micro-test set up in a 96 well flat bottom plate, using bovine γ-globuline as standard (see Supplementary data).

2.1.2.5. Data Analyses

2.1.2.5.1. Median Lethal Concentration (LC50)

Median lethal concentration (LC50) and their respective confidence intervals (95%) were calculated using Minitab software.

2.1.2.5.2. Biomarkers

A three-way analysis of variance (three-way ANOVA) was performed to check for significant interactions between CN^- concentrations, fish size and tissues for LPO, GST, CAT and GPx. For AChE, two-way ANOVA was performed to check for significant interactions between CN^- concentration and fish size. Statistical analyses were performed using the software STATISTICA version 7.0 (StatSoft Inc.), with a significance level of 0.05. The assumptions of normality were checked prior to analysis through Kolmogorov-Sminov and Lilliefors test. Homogeneity of variance was checked through Cochran, Bartlett Chi-sqr and Levene´s test. Whenever significance was accepted ($p < 0.05$), the Tukey multiple comparison test was used for pairwise comparison of means.

2.1.2.6. Ethics Statement

This study was conducted under an institutional license for animal experimentation and a personal license to fourth author Violeta Ferreira, issued by the Direção Geral de Veterinária (DGV), Portuguese Ministry of Agriculture, Rural Development and Fisheries). This work was in strict accordance with the recommendations of Guide for the Care and Use of Laboratory Animals of the European Union, that is represented in Portugal by the Decreto Lei n º 129/92 de 06 de Julho, Portaria Nº 1005/92 de 23 de Outubro de 1992.

2.1.3. Results

2.1.3.1. Anaesthesia and Recovery

Severe gasping, irregular swimming movements, loss of equilibrium and a complete loss of all respiratory activity, followed by a vertical drop in the water to the bottom of the container, were recorded for all groups of small fish pulse exposed to all concentrations of CN^- tested. For the 6.25 mg L^{-1} treatment, fish immobilization occurred after 37s. For the 12.5 mg L^{-1}, 25 mg L^{-1} and 50 mg L^{-1} treatments, fish immobilization occurred after 30s, while at the highest concentration tested (100 mg L^{-1}) fish immobilization occurred after 20s. Fish specimens in the control did not display any of the responses described above. During recovery from CN^- poisoning, fish initially remained on their sides, with mild to strong opercular movements and some fin movements as well. The higher the concentration, the longer this behaviour endured (e.g, at 50 mg L^{-1}, the longest recovery time recorded was ≈36 min, while at 25 mg L^{-1}, 12.5 mg L^{-1} and 6.25 mg L^{-1} the longest recovery times were 18, 8 and 5 min, respectively) (Table 1). At the end of the recovery period, survivors displayed a normal swimming behaviour, comparable to the one exhibited by control fish.

Table 1. Immobilization time (s), complete recovery time of exposed fish (min) and 96 hours survival (%) of small-sized *Amphiprion ocellaris*.

Treatment (mg L^{-1})	Immobilization time (s)	Recovery time (min)	Survival (%)
6,25	**37**	**5**	**100**
12,5	**30**	**8**	**70**
25	**30**	**18**	**40**
50	**30**	**36**	**20**
100	**20**	*	**0**

* No data available due to the mortality recorded (100%).

During CN^- pulse exposure, the behaviour displayed by medium-sized fish was at all similar to that described above for small-sized *A.ocellaris*. Immobilization time for medium fish was as follows: 6.25 mg L^{-1} treatment: 45s; 12.5 mg L^{-1} and 25 mg L^{-1} treatments: 40s; 50 and 100 mg L^{-1} treatments: 28s. Fish specimens in the control did not display any of the responses described above. During recovery from CN^- poisoning, fish displayed the same behaviour as already described for small fish. The same trend described above for small fish recovery time (the highest the concentration, the longest the recovery time) was also recorded in medium-sized fish (e.g, at 50 mg L^{-1}, the longest recovery time recorded was ≈23 min, while at 25 mg L^{-1}, 12.5 mg L^{-1} and 6.25 mg L^{-1} the longest recovery times were 10, 7 and 2 min, respectively) (Table 2). At the end of the recovery period, survivors displayed a normal swimming behaviour, comparable to the one exhibited by control fish.

Table 2. Immobilization time (s), complete recovery time of exposed fish (min) and 96 hours survival (%) of medium-sized *Amphiprion ocellaris.*

Treatment (mg L^{-1})	Immobilization time (s)	Recovery time (min)	Survival (%)
6,25	**45**	**2**	**100**
12,5	**40**	**7**	**100**
25	**40**	**10**	**100**
50	**28**	**23**	**50**
100	**28**	*	**0**

* No data available due to the mortality recorded (100%).

2.1.3.2. Median Lethal Concentration (LC50)

The toxicity trial showed that median lethal concentration (LC50) and their respective confidence intervals (95%) of small-sized *A.ocellaris* pulse exposed to CN^- and depurated during 96 h was 28.45 mg L^{-1} [lower 20,17 mg L^{-1} and upper 36,73mg L^{-1}] (Table 3).

Table 3. LC50 Values at 96 hours (mg L^{-1}) and respective 95% confidence limits of CN^- in small-sized *Amphiprion ocellaris*.

		Standard	95,0% Normal CI	
Parameter	**Estimate**	**Error**	**Lower**	**Upper**
Mean	28,45	4,22	20,17	36,72
StDev	17,75	4,26	11,09	28,40

The survival of small fish decreased with the increasing concentrations of CN^-, with no specimens being able to survive following a pulse exposure of 100 mg L^{-1} of CN^- (Fig. 4).

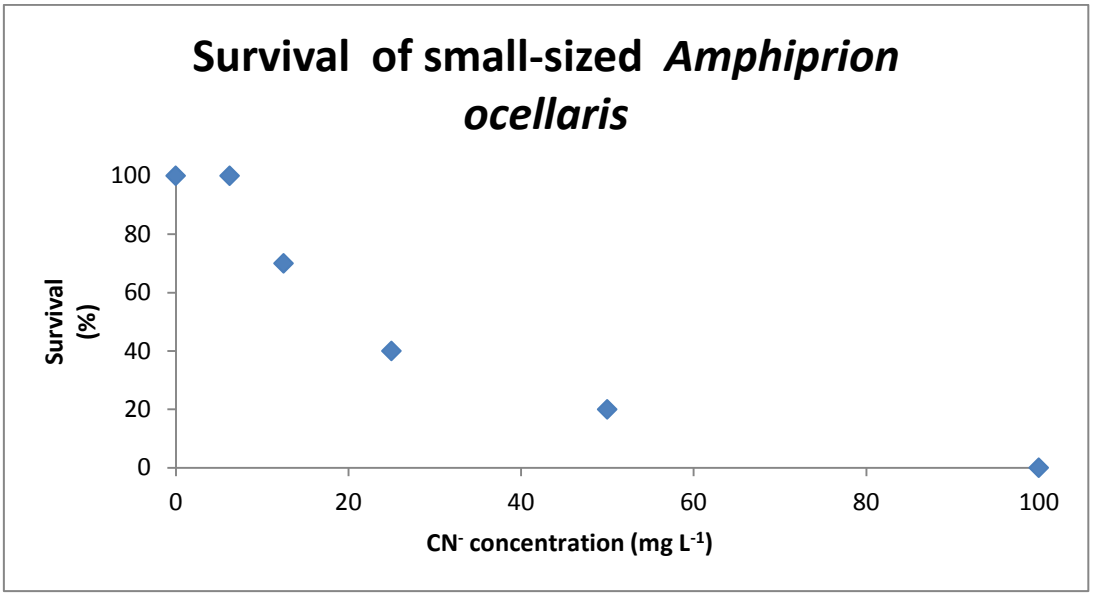

Figure 4. Survival rates of small-sized *Amphiprion ocellaris* for each concentration of CN^- (mg L^{-1}).

The toxicity trial showed that median lethal concentration (LC50) and their respective confidence intervals (95%) of medium-sized *A.ocellaris* pulse exposed to CN^- and depurated during 96 h was 50 mg L^{-1} [lower 37.62 mg L^{-1} and upper 63,49 mg L^{-1}] (Table 4).

Table 4. LC50 Values at 96 hours (mg L^{-1}) and respective 95% confidence limits of CN^- in medium-sized *Amphiprion ocellaris*.

		Standard	95,0% Normal CI	
Parameter	**Estimate**	**Error**	**Lower**	**Upper**
Mean	50	1,65	46,76	53,24
StDev	4,17	1138,11	0,00	-

The survival of medium fish decreased with the increasing concentrations of CN^-, with no specimens being able to survive following a pulse exposure of 100 mg L^{-1} of CN^- (Fig. 5).

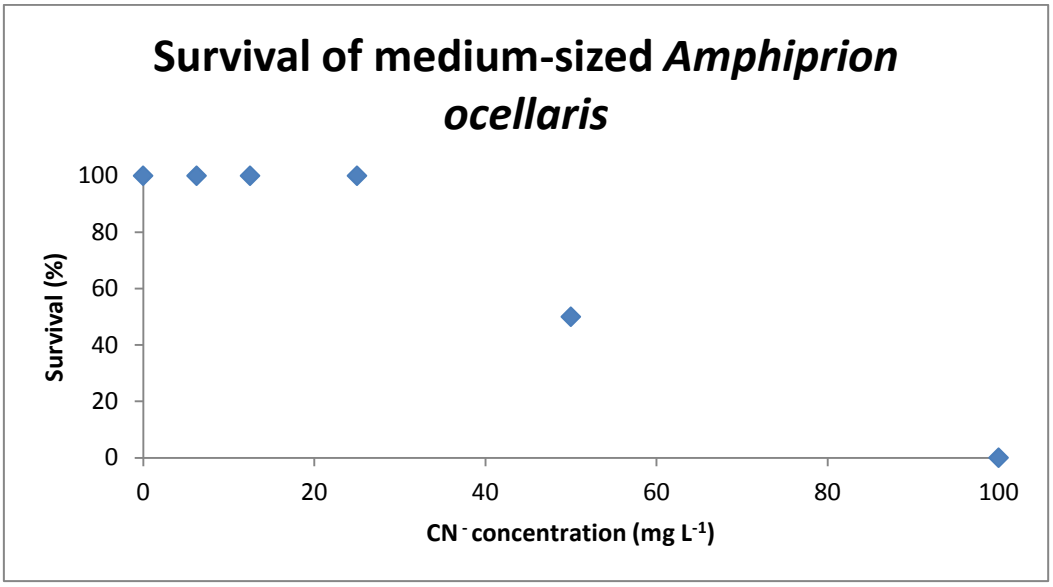

Figure 5. Survival rates of medium-sized *Amphiprion ocellaris* for each concentration of CN^- (mg L^{-1}).

2.1.3.3. Biomarkers

Significant interactions were recorded among the concentration of CN^-, fish size and tissues for LPO activity (three-way ANOVA, log transformation, $F_{4,80} = 9.07$; $p < 0.001$) (for further details on the statistical analysis see Supplementary data). Concerning LPO values recorded in the gills (Fig. 6) no significant differences were detected among treatments and fish sizes. However, the values of LPO recorded in the muscle of medium-sized fish differed significantly ($p < 0.001$) among CN^- concentrations (control, 12.5 mg L^{-1} and 50 mg L^{-1}). Significant differences in LPO rates were also observed among tissues ($p < 0.001$).

For small-sized *A.ocellaris*, LPO rates decreases in the higher concentration in muscle, whereas for the medium-sized *A.ocellaris* they tend to increase in higher concentratrions (with the exception of 25 mg L^{-1}).

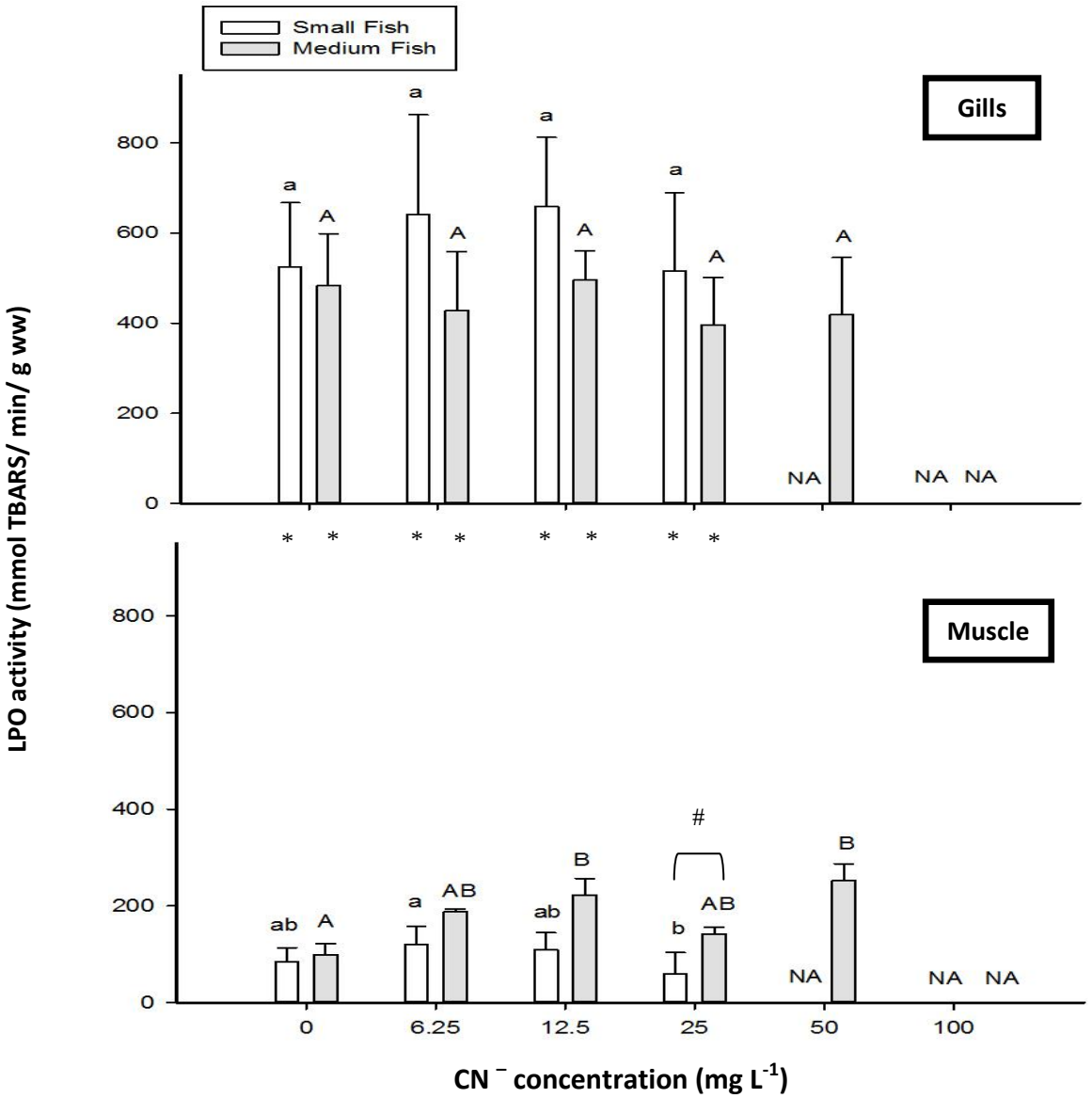

Figure 6. Effect of CN^- in LPO (gills and muscle) of small and medium sized *Amphiprion ocellaris*. Values represent mean ± S.D (n=5 for small and medium size). Significant differences ($p < 0.05$) between concentrations are represented by different small caps for small-sized fish and all caps for medium-sized fish; significant differences ($p < 0.05$) between fish sizes are represented by cardinals (#) and between tissues by asterisks (*). NA – No data available due to the mortality recorded.

Significant interactions were recorded among concentration of CN^-, fish size and tissues for GST activity (three-way ANOVA, log transformation, $F_{8,120} = 27.231$; $p < 0.001$) (for further details on the statistical analysis see Supplementary data). Concerning GST values recorded significant differences between CN^- concentrations were detected ($p < 0.001$) in muscle and in liver (control and 25 mg L^{-1}) for small-sized *A. ocellaris* and in muscle

between control and 50 mg L^{-1} for medium-sized *A.ocellaris* ($p < 0.001$). In muscle, significant differences ($p < 0.001$) were founded among fish size (control, 6.25 mg L^{-1} and 12.5 mg L^{-1}). Significant differences were also founded among tissues ($p < 0.001$).

In small-sized *A.ocellaris* an increase in GST activity was observed in gills and muscle, in contrast to liver where a decrease of this biomarker activity was observed. In medium-sized *A.ocellaris* (Fig. 7) was observed an increase of GST activity in muscle.

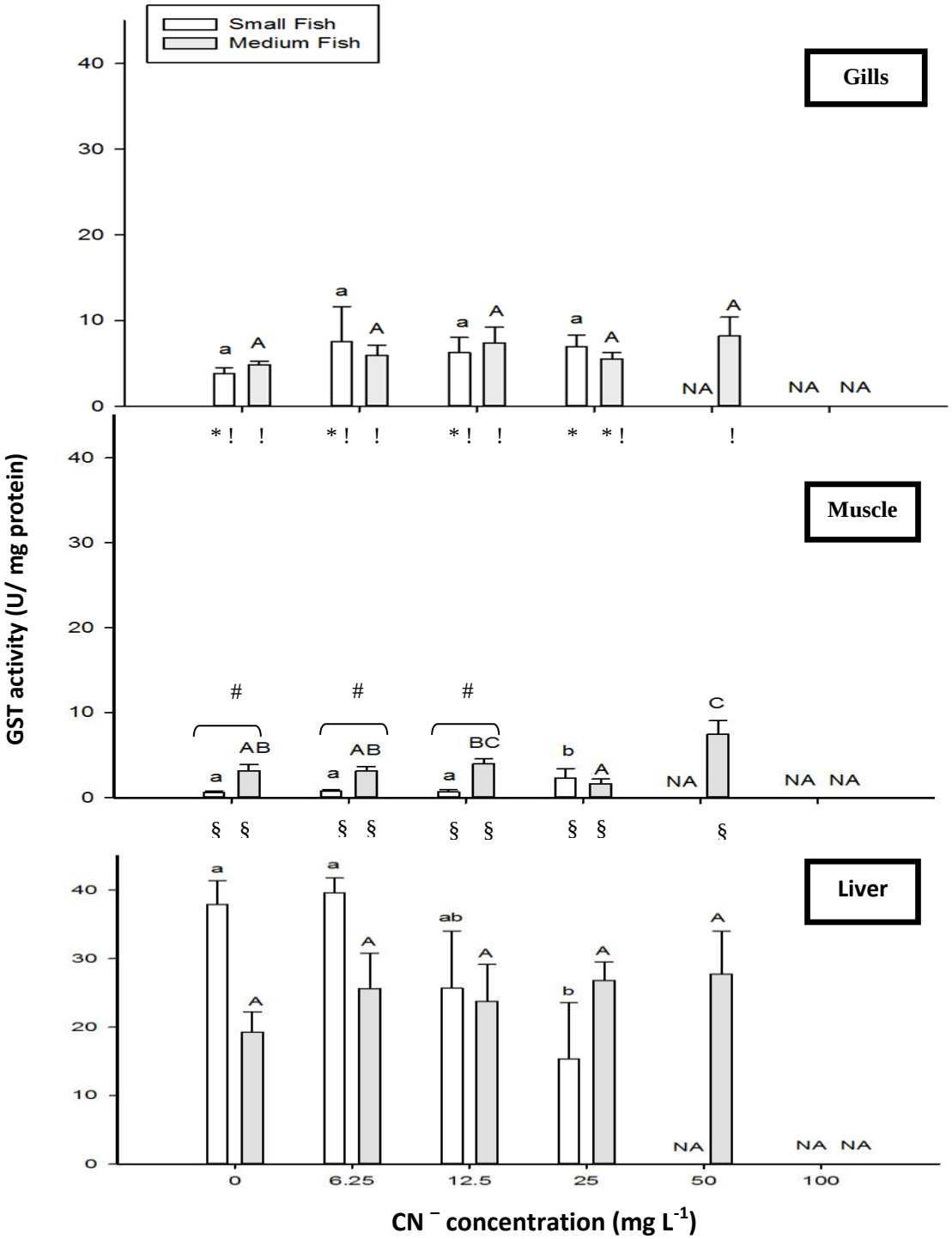

Figure 7. Effect of CN^- in GST activity (gills, muscle and liver) in small and medium sized *Amphiprion ocellaris*. Values represent mean ± S.D (n=5 for small and medium size). Significant differences ($p < 0.05$) between concentrations are represented by different small caps for small-sized fish and all caps for medium-sized fish; significant differences ($p < 0.05$) between fish sizes are represented by cardinals (#); significant differences ($p < 0.05$) between gills and muscle are represented by asterisks (*); significant differences ($p < 0.05$) between muscle and liver are represented by section sign (§); significant differences ($p < 0.05$) between gills and liver are represented by exclamation mark (!). NA – No data available due to the mortality recorded.

Significant interactions were recorded among concentration of CN^-, fish size and tissues for CAT activity (three-way ANOVA, log transformation $F_{8,120} = 13.862$; $p < 0.001$) (further details on the statistical analysis see Supplementary data).

Concerning CAT activity values significant diffences among CN^- concentrations were detected in gills (control and 6.25 mg L^{-1}) and liver (control, 12.5 mg L^{-1} and 25 mg L^{-1}) for small-sized *A. ocellaris* ($p < 0.001$). For medium-sized *A. ocellaris* significant diffences among CN^- concentrations were detected in gills (control and 12.5 mg L^{-1}) ($p < 0.001$), muscle (control and 25 mg L^{-1}) ($p = 0.001$) and liver (control, 12.5 mg L^{-1}, 25 mg L^{-1} and 50 mg L^{-1}) ($p < 0.001$). In gills, significant differences were founded between fish size in control group ($p < 0.001$). Significant differences were also founded among tissues ($p < 0.001$).

In small-sized *A.ocellaris* an increase of CAT activity was observed in lower CN^- concentration in gills and in the higher concentrations in liver. In medium-sized *A.ocellaris*, CAT activity increases in muscle and liver and decreases in 12.5 mg L^{-1} concentration in gills (Fig. 8).

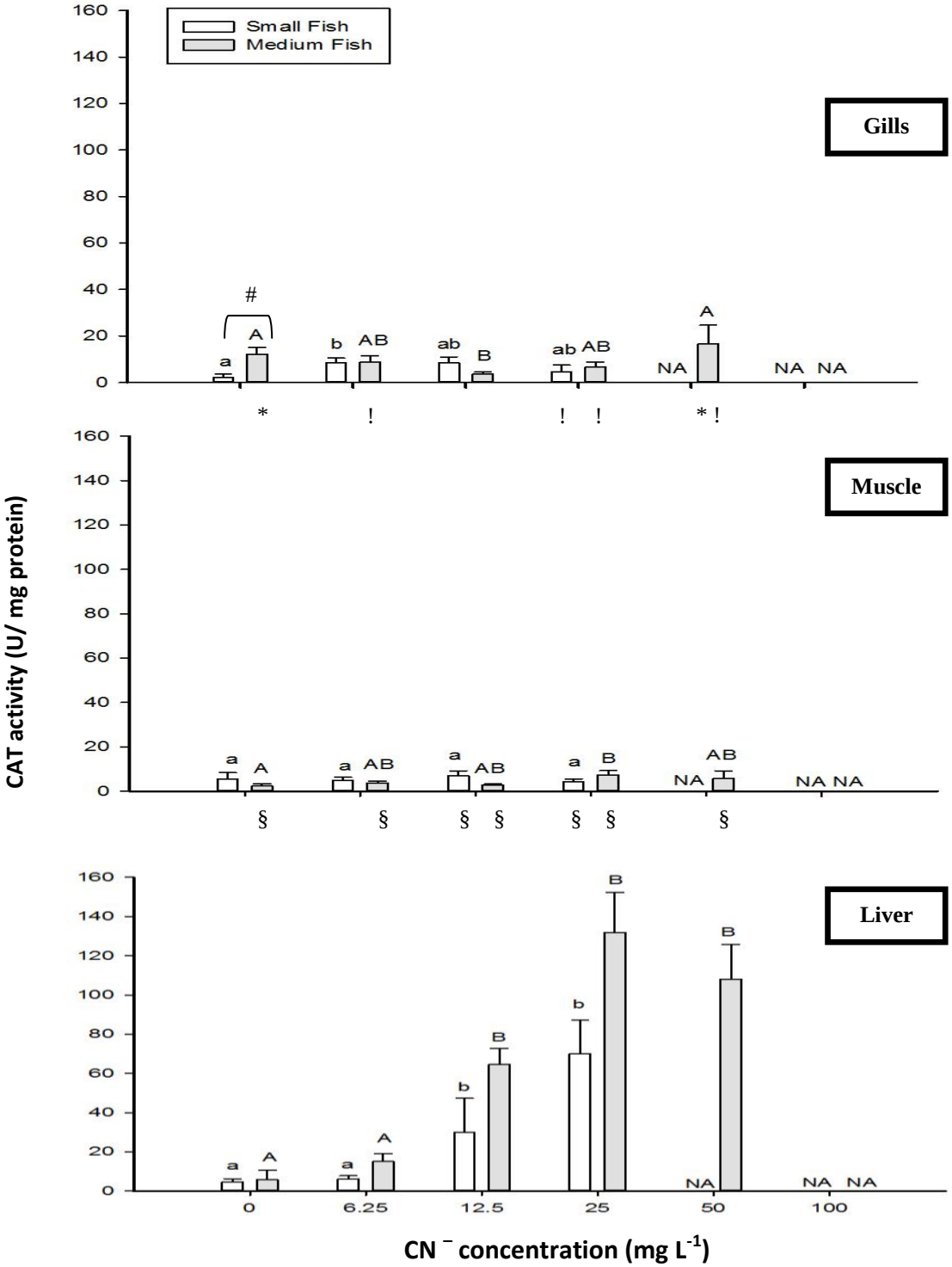

Figure 8. Effect of CN^- in CAT activity (gills, muscle and liver) in small and medium sized *Amphiprion ocellaris*. Values represent mean ± S.D (n=5 for small and medium size). Significant differences ($p < 0.05$) between concentrations are represented by different small caps for small-sized fish and all caps for medium-sized fish; significant differences ($p < 0.05$) between fish sizes are represented by cardinals (#); significant differences ($p < 0.05$) between gills and muscle are represented by asterisks (*); significant differences ($p < 0.05$) between muscle and liver are represented by section sign (§); significant differences ($p < 0.05$) between gills and liver are represented by exclamation mark (!). NA – No data available due to the mortality recorded.

Significant interactions were recorded among concentration of CN^-, fish size and tissues for GPx activity (three-way ANOVA, log transformation $F_{8,120} = 2.987$; $p = 0.04$) (for further details on the statistical analysis see Supplementary data). Concerning GPx values recorded no significant differences among CN^- concentrations and among tissues was detected for small and medium sized *Amphiprion ocellaris*.

In small-sized *A.ocellaris*, a non significant increase in GPx activity was observed in gills and liver. For medium-sized *A.ocellaris* a non significant increase of GPx activity, was observed in gills and muscle (Fig. 9).

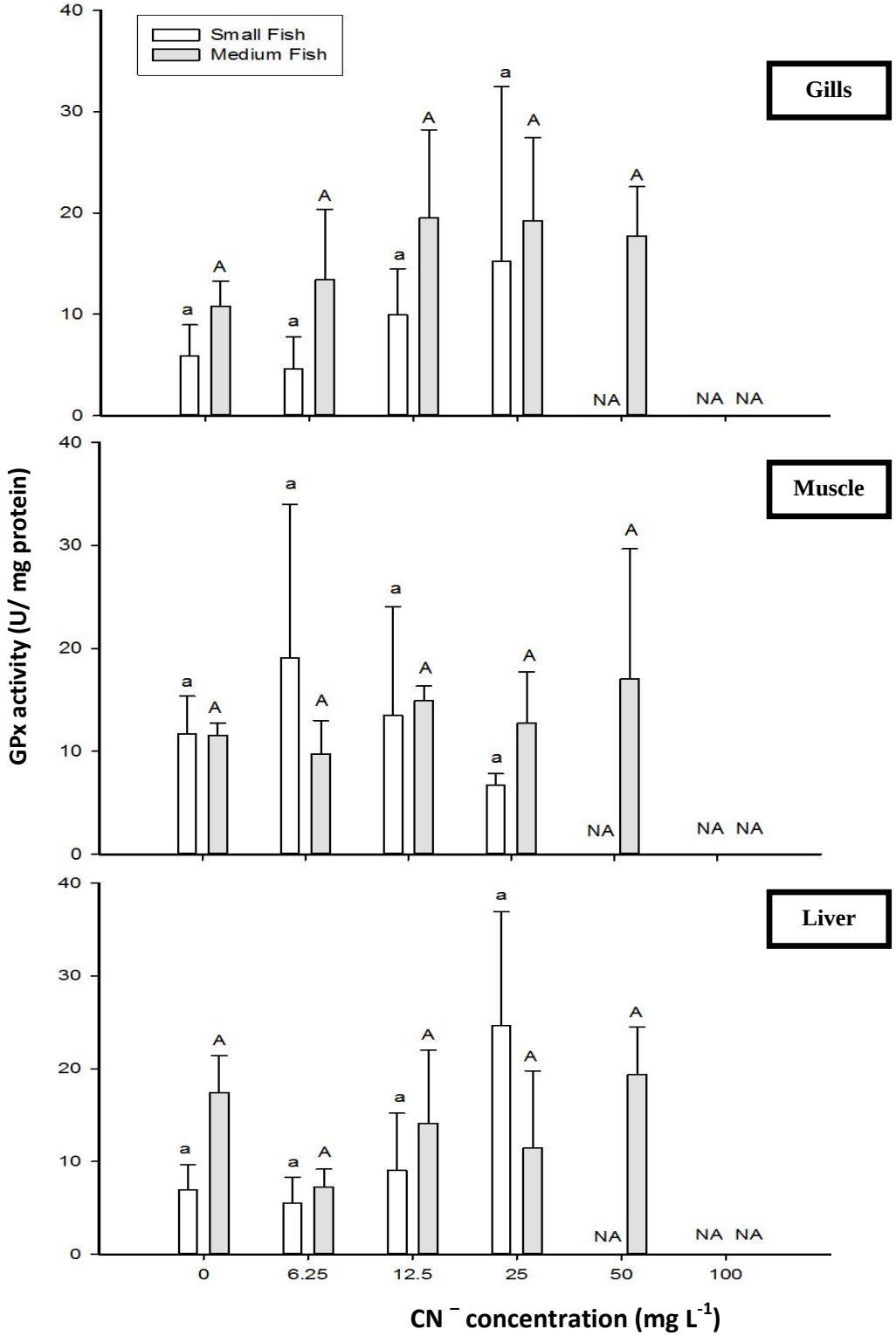

Figure 9. Effect of CN^- in GPx activity (gills, muscle and liver) in small and medium sized *Amphiprion ocellaris*. Values represent mean ± S.D (n=5 for small and medium size). Significant differences ($p < 0.05$) between concentrations are represented by different small caps for small-sized fish and all caps for medium-sized fish. NA – No data available due to the mortality recorded.

Significant interactions were recorded between concentration of CN^- and fish size for AChE activity (two-way ANOVA, log transformation, $F_{4,40}$ = 6.97; p < 0.001) (for further details on the statistical analysis see Supplementary data).

Corcerning AChE values recorded significant differences between CN^- concentrations were detected in brain (control and 6.25 mg L^{-1}) for small-sized *A. ocellaris* (p = 0.0017). Significant differences were founded between fish size in control group (p < 0.001). In small-sized *A. ocellaris,* after the strong decrease in lowest concentration, AChE activity tended to increase with the rise of CN^- concentrations (to values near the control) (Fig. 10).

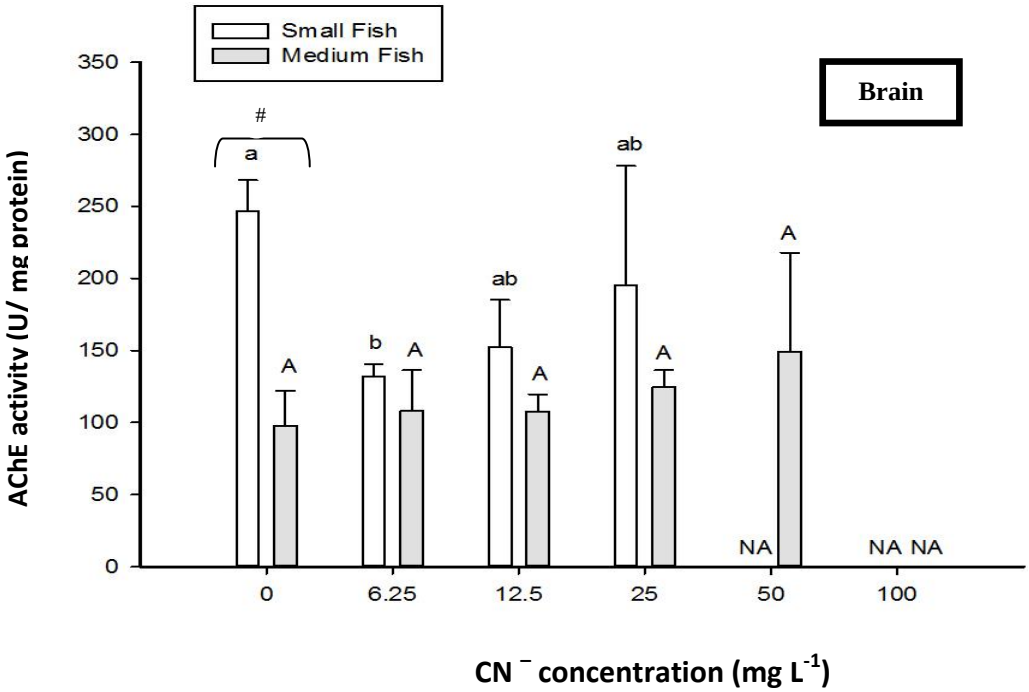

Figure 10. Effect of CN^- in AChE activity (brain) in small and medium sized *Amphiprion ocellaris*. Values represent mean ± S.D (n=5 for small and medium size). Significant differences (p < 0.05) between concentrations are represented by different small caps for small-sized fish and all caps for medium-sized fish; significant differences (p < 0.05) between fish sizes are represented by cardinals (#). NA - No data available due to the mortality recorded.

2.1.4. Discussion

During the present study, fish pulse exposed to CN^- displayed irregular respiratory activity followed by hyperexcitability, loss of balance and ended-up settled in the bottom of the test chamber. The same behaviour was also described by Dube and Hosetti (2010) when exposing the freshwater fish *Labeo rohita* to different concentrations of this compound. According to Kane et al. (2005) and Scherer (1992), changes in locomotor and respiratory activity indicate a common response of fish to toxicant stress, being feasible behavioural indicators of sublethal exposure to xenobiotics. Based on the present findings, we may argue that this behavioural pattern (displayed by fish poisoned by CN^-) is independent on the fact of fish inhabiting marine or freshwater environments. When comparing fish displaying different sizes and exposed to the same concentration of CN^-, the immobilization time recorded was always shorter for smaller fish. The inverse trend was recorded for recovery time, i.e. smaller specimens needed more time to recover from the pulse exposure to CN^- than medium-sized clownfish. Fish mortality also confirmed unequivocally that medium-sized *A. ocellaris* were less vulnerable to poisoning by CN^- than smaller conspecifics, as the median lethal concentration (LC50 96h) calculated for medium specimens was nearly half of that calculated for smaller fish (28.45 mg L^{-1} and 50 mg L^{-1} of CN^-, respectively). These results confirm that size influences the vulnerability of *A. ocellaris* to CN^- poisoning, with smaller specimens being more vulnerable to poisoning by marine aquarium collectors employing this illegal fishing technique. Smaller specimens that are sedated and somehow avoid collection (e.g. by falling into a rock crevice or within the intricate structure of a branchy hard coral) may still be endangered, as longer recovery times render them more vulnerable to the multitude of predators dwelling in coral reefs. Currently, there is still a lack of information about the role of size on CN^- poisoning in tropical marine fish. Hawana et al. (1998) reported no mortality for specimens of *D. aruanus* (TL ≈75 mm) pulse exposed for 60s to 50 mg L^{-1} of CN^-. Our study with *A.ocellaris* (TL ≈38 mm), with the same CN^- pulse exposure conditions, reported fifty percent of mortality. Considering these two species have a similar body plan and shape and belongs to the same family (Pomancentridae), these diffences may somehow be explained by the larger size of *D. aruanus*, which have nearly twice the size of the

specimens of *A.ocellaris* surveyed in the present work. Therefore, size is a fundamental key in vulnerability of species exposed to CN^-.

Other study performed by Vaz et al. (2012), reported 33% of mortality for *Amphiprion clarkii* exposed to 25 mg L^{-1} of CN^-. The vulnerability to CN^- poisoning recorded for *A. ocellaris* (TL≈38 mm) in the same conditions (time of exposure and CN^- concentration) reported no mortality in these specimens. Comparing our study to *A. clarkii*, *A. ocellaris* is less vulnerable to CN^-. Since these two species belongs to family Pomacentridae, the present study reveals the existence of interspecific differences in terms of vulnerability to CN^- poisoning even in species with a close phylogenetic relationship. According to Rubec et al. (2003), CN^- poisoning has already been positively detected in 36 different families of reef fish. If species within the same family and occupying similar ecological and trophic niches display such a contrasting vulnerability to a pulse exposure to CN^-, it is likely that the vulnerability and recovery time displayed by reef fish within other families may be even more variable than that already recorded for the Pomacentridae. Such interspecific variability should not be overlook and future studies on this topic should cover a range of phylogenetically close species (e.g. within the same genus/family), as well as distant related reef fish species (within different families/orders).

A multiple and comprehensive biomarker approach (LPO, GST, CAT, GPx and AChE activities in different tissues) was applied to fully understand the effects of CN^- on *A. ocellaris* physiology. A significant induction of LPO rates was only observed in the muscle of medium-sized specimens, which may suggest that smaller organisms did not show oxidative damage resulting from CN^- poisoning. Nonetheless, given the mortality rates recorded for smaller specimens, the lower levels of LPO recorded may rather result from some type of artefact induced by the toxicity of CN^-. This artefcat is likely to be related to the saturation of the antioxidative enzymatic machinery, as already reported by Pandey et al. (2003). The higher levels of LPO recorded in fish gills, when compared to the levels recorded in the muscle, suggest that this organ, that first contacts with CN^-, can not handle the oxidative stress generated by this toxic. This latter finding was somehow expected, since the muscle tissue usually handles better toxic/toxin-induced oxidative stress than gills.

GSTs play an important role in detoxification processes and have the capacity to convert ROS into less harmful substances (Van der Oost et al., 2003; Vlahogianni et al., 2007). To combat oxidative stress signals, these detoxifying enzymes increase their activity. However, prolonged exposure to contaminants can cause a depletion of GSTs, creating conditions that may favour oxidative damage (Vlahogianni et al., 2007). In this study, a significant induction of GST activity was observed in the muscle of both small and medium-sized *A.ocellaris*, which may reflect an increase of the detoxification efficiency and lower time and/or amount of CN^- in contact with molecular targets (Gravato et al., 2010). Yet, in the liver, small-sized fish showed an inhibition pattern – i.e. the activity of this biomarker decreased. Such dissimilarity between tissues may be due to the fact that the liver is the main organ associated with detoxification processes, while the muscle displays a lower intensity action towards oxidative metabolism (Prieto et al., 2006).

Regarding catalase, the significant induction in the liver (in both size groups) may indicate that there was a compensatory physiological response, adaptive to an oxidative stress situation. A similar trend of variation was observed in the gills of small-sized fish and in the muscle of medium-sized ones.

On the other hand, GPx activity showed no significant differences with CN^- exposure, which suggests that this biomarker was inappropriate to evaluate physiological effects of CN^- in *A. ocellaris*.

When comparing the differences in antioxidant activities among tissues, small and medium-sized specimens always displayed higher levels in the liver than in their muscle or gills. As already referred, the antioxidant defense system acts preferentially in the liver, as this is the main organ associated with detoxification and processing of xenobiotics and metabolic products for degradation (Lushchak et al., 2005). Moreover, in the present study, solley the white muscle was analysed, the one with the lower content of mitochondria and consequently a more reduced potential to handle oxidative metabolism (Prieto et al., 2006). Gills are likely to be the first fish organ being affected by CN^- poisoning, while muscle is only exposed to the amount of toxic which is taken up and distributed through the fish. It is therefore not surprising that all activities of antioxidant enzymes monitored in the present study were much lower in the white fish muscle than

in the liver or gills. This trend has already been recorded in other fish, as reported by Li et al. (2011).

Based on the cativity levels recorded for AChE, it was possible to observe that CN^- neurologically affected small-sized *A. ocellaris*. We argue that CN^- may have enhanced cholinesterase inhibition, which has led to an accumulation of acetylcholine and abnormal postsynaptic excitation. This scenario can trigger an uncontrolled action of neurons and lead to an overstimulation of nerve fibers, which may result in paralysis or even death of poisoned fish (Chebbi and David, 2009). It is worth noting that the inhibition of this enzyme elicits negative effects on neurotransmission, with cascading effects on fish behavior (e.g. ability to avoid predation), growth, reproduction and feeding (Gravato et al., 2010).

In conclusion, we showed that: i) CN^- is highly toxic to the clownfish *A. ocellaris* and has the potential to cause high mortality in this species with the exposure to increasing concentrations of this poison, and ii) fish size influences the way fish can handle their pulse exposure to CN^-. When fish are subjected to sub-lethal concentrations of CN^-, this toxic may induce dramatic shifts in their behavior and metabolic activity. The present study suggests that oxidative stress biomarkers, especially antioxidant enzymes in fish, can provide a useful indicator of the deleterial effects promoted by a sub-lethal pulse exposure to CN^-. Yet, GPx, which has been generally described as a biomarker of pollution-induced oxidative stress, did not provide any useful indication of toxicity load in the present study and therefore its survey may be of little use when screening for CN^- poisoning.

Overall, it is paramount that authorities enforce existing laws that already criminalize the use of CN^- fishing for the collection of live reef fish and increase the awareness of importing countries on the destructive nature of this practice. Reliable methods to detect cyanide-caught fish are urgently needed, as only by truly banning this illegal fishery from coral reefs will it be possible to pursue the preservation of these endangered habitats.

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Chapter 3

3.1. Combined effects of environmental warming and cyanide poisoning on the physiology of the clownfish *Amphiprion ocellaris*

Combined effects of environmental warming and cyanide exposure poisoning on the physiology of the clownfish *Amphiprion ocellaris*

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Abstract

Currently, tropical coral reefs are under immediate and direct threat from one or more local sources, being 55% of world's reefs affected by overfishing and consequently destructive fishing practices like cyanide fishing. Cyanide (CN^-) is a powerful asphyxiant, used to capture fishes in Asia-Pacific region. When applied in high doses during illegal fishing activities, CN^- promotes dramatic levels of mortality in target and non-target species. Another anthropogenic threat to tropical coral reefs is climate change. Around 25 % of the world´s coral reefs have already been destroyed or seriously affected by global warming. By the end of this century, the average global sea surface temperatures are expected to increase by 0.3–4.8 °C, leading to potentially negative impacts in the performance and survival of tropical marine organisms. *Amphiprion ocellaris* is one of the most important marine ornamental fish that has been exploited commercially for the growing marine aquarium trade, representing alone more than 15.6% of total exports in the world. So far, no studies have investigated the toxicity of CN^- compounds in tropical fish under thermal stress scenarios. Therefore, the aim of this study was to evaluate, for the first time, the toxicity of a pulse exposure to a sub lethal dosage of CN^- (25 mg L^{-1}) in clownfish *Amphiprion ocellaris* in a warming context (+ 3 °C and + 6 °C), namely to determine the potential existence of combined effects between these two stressors in the fitness (survival) and antioxidant enzymatic machinery in different tissues (liver, brain, muscle and gills) of this tropical fish species. This study reported that both thermal stress and CN^- exposure, independently of each other, elicited negative effects on the metabolic physiology and mortality of fish and the combination of two stressors can lead to fail detoxification process and damage repair, resulting to an elevated mortality and a whole-organism physiological stress that can limit their survival and distribution in the oceans of tomorrow. There is still data gap on the vulnerability of CN^- exposure to tropical fish. Therefore, some important studies should be developed in order to have a more in depth understanding of this illegal practice in coral reef species and consequently in coral reefs ecosystems.

Keywords

Global warming; vulnerability; cyanide; oxidative stress; mortality

3.1.1. Introduction

Coral reefs are highly productive ecosystems, found primarily in subtropical and tropical regions that provide a variety of valuable goods and services to humankind (Cesar, 2000; Veron, 2000). However, there are currently several natural and anthropogenic factors, such as global warming and cyanide fishing, that are negatively impacting such ecosystems (Hughes et al., 2003; Bellwood et al., 2004; Fox et al., 2005; Leao and Kikuchi, 2005; Tissot et al., 2010). Cyanide (CN^-), a powerful asphyxiant for fish, acts quickly by inducing tissue anoxia and cytotoxic hypoxia (Bridges, 1958; Rubec et al., 2001). Collectors that capture fish with CN^-, normally in the Asia-Pacific region, use squeeze bottle containing CN^- dissolved in seawater and applied it directly onto the reef (Bridges, 1958; Rubec et al., 2001). This fishing technique contribute to the high mortality (>80%) of these organisms (Rubec et al., 2001). CN^- is known to create more damage in organs with high metabolic requirements (e.g. heart and brain), but is also toxic in other organs, such liver and spleen (Gracia and Shepherd, 2004). When applied in small doses, CN^- acts like an anaesthetic that apparently does not cause mortality (Hall and Bellwood, 1995), but when applied at high doses, there is evidence of increased mortality (Hawana et al., 1998). On fishes, CN^- toxicity can be influenced by a variety of factors including concentration, environmental temperature, dissolved oxygen content, pre-exposure and age (Ballantyne and Marrs, 1987). Comparing to the other aquatic organisms, freshwater fishes were the most sensitive group to CN^- with sublethal effects detected over 5 µg L^{-1} CN^- and high mortality at concentrations >20 µg L^{-1} (Eisler and Wiemeyer, 2004). An important tool to evaluate the sensitivity of aquatic organisms to stress conditions such CN^- fishing is acute toxicity tests. The principle of this test is exposing an aquatic organism, to the test substance preferably for a period of 96 hours and record mortalities (OECD, 1992).

By the end of the century, the average global sea surface temperatures are expected to increase 0.3 - 4.8 °C (IPCC, 2014), leading to potentially negative impacts in seawater quality and consequently the performance and survival of organisms (Nilsson et al., 2009). Tropical organisms are expected to be particularly sensitive to elevated temperatures because they inhabit a relatively thermostable environment and more close to their

thermal maxima (Tewksbury et al., 2008) and are close to the thermostable threshold. Moreover, temperature plays a crucial role in the physiology of tropical ectotherms, since their body temperature changes with the temperature of the environment, resulting in alterations of the rates of all physiological and biochemical reactions and stability of biological molecules (Hochachka and Somero, 2002). For some tropical reef fishes, recent data has shown that temperature affects not only aerobic scope (Rosa and Seibel, 2008) but also developmental rate of juveniles, reproductive capacity of adults and circulatory and ventilatory systems capacity (Pörtner, 2002; Nilsson et al., 2009). When the specific windows of thermal tolerance are exceded fish can be affected also in growth, body size, behavior, immune defense and feeding (IPCC, 2014).

Amphiprion ocellaris (Cuvier, 1830), is one of the most important marine ornamental fish having a high market value (Wittenrich, 2007). This species had been exploited commercially for the growing marine aquarium trade (Wood, 1985) facing fishing pressure. Even though research on this species has been conducted on reproduction, early live stages and larviculture techniques (Kim et al., 2007; Olivotto et al., 2008) any study have focused on combined effect of CN^- and environmental warming to this clownfish species. Therefore, the objective of this study was to evaluate the toxicity of CN^- in a sub lethal concentration (25 mg L^{-1}) on clownfish *A. ocellaris´*s in a warming scenario and determine sublethal effects using conventional biomarkers related to oxidative stress [catalase (CAT); glutathione peroxidase (GPx); glutathione *S*-transferases (GST); lipid peroxidation (LPO)] and neurological function (acetylcholinesterase – AChE).

3.1.2. Material and Methods

3.1.2.1. Fish Maintenance

Sixty *A. ocellaris* (Total Lenght ≈36 ± 0.045 mm (measured from the tip of the snout to the tip of the longer lobe of the caudal fin); average wet weigh 0.94 ± 0.38 g) were purchased from a Portuguese breeder (Opérculo Lda.) to assure that all specimens had never been exposed to CN^- prior to the experimental procedures. All specimens were acclimated for 2 weeks under a 12 h ligt: 12 h dark photoperiod in a 270 L glass tank (1.20 m high x 0.45 m long x 0.50 m wide) equipped with a circulation pump providing a water flow of 2500 L h^{-1}

(Turbelle nanostream-6025 Tunze, Germany) and connected to a 125 L sump (1.20 m high X 0.35 m long X 0.40 m wide) equipped with a biological filter a biological (submerged bio-balls), 50µm mesh bag (mechanical filtration), a protein skimmer (Deltec® APF600), a 300 watt submergible heater (that kept the water temperature stable at 26 °C) (Eheim Jäger 300 W, Deizisau, Germany), and a submerged pump with the water flow of 3400 L h^{-1} (Eheim 1262, Deizisau, Germany). This system was also equipped with an osmoregulator (Reef SetH) used to replace evaporated water with freshwater purified by reverse osmosis (RO) to keep salinity stable at 35. The salinity was checked daily using a hand refractometer to detect any potential malfunction of the described automation. Water parameters were maintained within the following optimal ranges for the husbandry of *A. ocellaris*: temperature 26 ± 0.5 °C; not detectable ammonium, nitrite and nitrate; pH 8 ± 0.2. Nitrogenous compounds were monitored every week using colorimetric tests (Salifert), while pH was monitored using a Pinpoint pH meter (PH 370, American Marine). All seawater employed during the acclimation period was prepared by mixing RO water with a commercial synthetic salt mix (Tropic Marine Pro Reef, Wartenberg, Germany). All fishes were fed four times a day until satiation with a commercially produced pelleted diet (Hikari Marine S).

One week before the beginning of the experience, all fish were divided in three different groups, and acclimated at three different temperatures, 26 °C, 29 °C and 32 °C (the average global sea surface temperatures expected to increase by the end of the century), provided by a water bath with a 300 watt submergible heater (Eheim Jäger 300 W, Deizisau, Germany) (that kept water temperature stable at 26 °C, 29 °C and 32 °C). The water was changed in 50 % every day until the beginning of experience.

3.1.2.2. Acute Test Validity

To validate the acute test for CN^- toxicity for *A. ocellaris* the following conditions had to be completely fulfilled: i) fish were fed 24 hours before the beginning of the test, ii) constant conditions of semi-static procedure were maintained as far as possible, iii) dissolved oxygen concentration were at least 60 per cent of the air saturation through the test, iv) exposure duration were 96 hours, v) maximum loading did not exceed 1.0 g fish L^-

^{1} (for semi-static test), vi) a 12 hours of photoperiod, vii) no food administrated to the fish during all toxicity tests and viii) a minimum of at least 7 fish was employed for each treatment. At the end of the toxicity test, mortality in the control should not exceed 10 percent. In order to fulfil the requirements for this acute test, it was of paramount importance to hold the specimens to be employed correctly (from an husbandry point of view) and to have all essential information concerning the test solution (CN^-) (OECD, 1992) (see Supplementary data).

3.1.2.3. Experimental Design and Treatments

After the acclimation period, the 60 medium-sized specimens of *A. ocellaris* were randomly divided into 6 groups of 10 fish for cyanide (CN^-) pulse exposure: first group was used as a control (no CN^- was added); second group was exposed to a 25 mg L^{-1} of CN^-; third group was exposed to 29 °C with no CN^-, fourth group was exposed a concentration of 25 mg L^{-1} of CN^- and 29 °C; fifth group was exposed to 32 °C; and the last group was exposed to a concentration of 25 mg L^{-1} of CN^- and 32 °C. Before the beginning of pulse exposure to CN^- a stock solution of 2.060 g L^{-1} of CN^-, was prepared in a 250 mL volumetric flask by dissolving (1.001±0.001) g of sodium cyanide (NaCN) (97% purity; Sigma- Aldrich, St. Louis, MO, USA) in ultra-pure water, obtained from a Milli-Q Millipore system (Milli-Q plus 185). CN^- concentration in the final volume of 3300 mL used during the pulse exposure was prepared adding 40 mL of the stock solution to 3300 mL of synthetic seawater (25 mg L^{-1}). The pulse exposure to CN^- was divided in four steps: exposure bath, first cleaning bath, second cleaning bath and third cleaning bath. In the first step, all fish from the treatments 1, 3, and 5 were collected with a hand-net and dipped for 60s into a 15-L tank filled with synthetic seawater with no CN^- (with the respective temperature). The duration of the pulse exposure (60s) was selected according to Hanawa et al. (1998). A preliminary trial revealed that handling the fish with a hand net causes no mortality or significant stress (Vaz et al., 2012). Then, all fishes of treatments 2, 4, and 6 were collected with a hand-net and dipped for 60s into a 15-L tank filled with synthetic seawater dosed with 25 mg L^{-1} of CN^-. After the pulse exposure to CN^- all fish from the same group were dipped for 60s into a 20-L tank filled with synthetic seawater

(with the respective temperature) with no CN^- (first cleaning bath). This procedure was repeated two more times for all fish from all groups (second and third cleaning bath) (see Supplementary data). For treatments with no CN^-, was used the same procedure of cleaning baths. During the exposure procedure, immobilization time was recorded. In the present work, immobilization time was defined as the time at which the last fish of a species group rested motionless in the bottom of the mesh used for the exposure bath. Following the pulse exposure, all fish were randomly distributed into 1-L glass jars filled with 1 L of synthetic seawater (prepared as described above) and the recovery time of normal swimming activity was determined. Recovery time was defined as the time at which the last fish of a species group reacquired its normal swimming ability. The toxicity trial was carried out in a semi-static system, where water was fully replaced every day and constantly aerated through air stones. The photoperiod used was 12 h light: 12 h dark, provided by white fluorescent lamps. According to OECD guideline 203 (OECD, 1992), during this toxicity trial no food was provided to the fish. The jars were placed inside a water bath keeping water temperatures stable at 26 °C, 29 °C and 32 °C. The behaviour of all fish was monitored during the first 30 min inside the glass jars to record recovery and mortality. Temperature, salinity, pH, dissolved oxygen, alkalinity, total ammonia–nitrogen, nitrite and nitrate of each experimental jar were measured daily. Temperature was measured using a thermometer and salinity with a handheld refractometer. Dissolved oxygen and pH were measured using an YSI 85 Model (Yellow Springs Instruments, Yellow Springs, USA), and a pH 100 meter (Yellow Springs Instruments, Yellow Springs, USA), respectively. Total ammonia–nitrogen, nitrite and nitrate were monitored using colorimetric tests (Hach, USA). Temperature was maintained at 26 ± 0.1 °C, 29 ± 0.1 °C, 32 ± 0.1 °C, respectively, salinity at 35 ± 0.1, pH 8 ± 0.1, dissolved oxygen concentration at 6.2 mg L^{-1}, nitrite, nitrate and ammonium were not detectable. Mortality was evaluated daily. Fish were considered dead when they were motionless on the bottom, exhibited no opercular movement and presented no response to mechanical stimuli. After 96 h of exposure to different concentrations of CN^-, fish were euthanized by immerging them in ice and necropsied to remove their liver, gills, muscle

and brain. All sampled tissues were placed in liquid nitrogen, and stored at -80 °C for biomarker analysis.

3.1.2.4. Biomarker Analysis

The protocol employed to process all fish tissue samples was previously described by Ferreira et al. (2010) and is thoroughly described in the supplementary data. The activity of glutathione *S*-transferases (GST), catalase (CAT) and glutathione peroxidase (GPx) were monitored in gill, liver and muscle tissues, while lipid peroxidation (LPO) was solely monitored in gill and muscle tissues (as previously described by Bird and Draper (1984) and Ohkawa et al. (1979) and further adapted to microplate). AChE activity was determined solely in the fish brain tissue. Glutathione *S*-transferases (GST) and glutathione peroxidase (GPx) activities were determined as described by Habig et al. (1974) and Mohandas et al. (1984), respectively. Catalase (CAT) activity was determined based on the method described by Clairborne (1985) and adapted to microplate. Acetylcholinesterase (AChE) activity was assessed according to the Ellman method (Ellman et al., 1961) adapted to microplate (Guilhermino et al., 1996). For all biomarkers, protein concentration was determined according to the Bradford method (Bradford, 1976), adapted from BioRad's Bradford micro-test set up in a 96 well flat bottom plate, using bovine γ-globuline as standard (see Supplementary data).

3.1.2.5. Data Analyses

3.1.2.5.1. Biomarkers

A three-way analysis of variance (three-way ANOVA) was performed to check for significant interactions between CN^- concentrations, temperature and tissues for LPO, GST, CAT and GPx. For AChE, two-way ANOVA was performed to check for significant interactions between CN^- concentration and temperature. Statistical analyses were performed using the software STATISTICA version 7.0 (StatSoft Inc.), with a significance level of 0.05. The assumptions of normality were checked prior to analysis through Kolmogorov-Sminov and Lilliefors test. Homogeneity of variance was checked through

Cochran, Bartlett Chi-sqr and Levene´s test. Whenever significance was accepted ($p < 0.05$), the Tukey multiple comparison test was used for pairwise comparison of means.

3.1.2.6. Ethics Statement

This study was conducted under an institutional license for animal experimentation and a personal license to fourth author Violeta Ferreira, issued by the Direção Geral de Veterinária (DGV), Portuguese Ministry of Agriculture, Rural Development and Fisheries). This work was in strict accordance with the recommendations of Guide for the Care and Use of Laboratory Animals of the European Union, that is represented in Portugal by the Decreto Lei n º 129/92 de 06 de Julho, Portaria Nº 1005/92 de 23 de Outubro de 1992.

3.1.3. Results

3.1.3.1. Anesthesia, Recovery and Mortality

During the CN^- exposure (25 mg L^{-1}), all fish behaved sereve gasping, irregular and erratic movements and a complete loss of balance and all respiratory activity, followed by vertical drop in the water to the bottom. For the 26 °C and 29 °C treatments exposed to CN^-, fish immobilization occurred after 37s, while in 32 °C treatment exposed to CN^-, fish immobilization occurred after 40s. Fish specimens in the control did not display any of the responses described above. During recovery from CN^- poisoning, fish initially remained on their sides with mild to strong opercular movements and some fin movements. The higher the temperature, the longer this behaviour endured (e.g, at treatment 6, the longest recovery time recorded was ≈10 min, while at treatment 4 and 2, the longest recovery times were 3 and 2 min, respectively) (Table 5). At the end of the recovery period, survivors displayed a normal swimming behaviour, comparable to the one exhibited by control fish.

Table 5. Immobilization time (s), complete recovery time (min) and survival (%) on *Amphiprion ocellaris* exposed to CN^- in a global warming scenario.

Treatment	Immobilization (s)	Recovery (min)	Survival (%)
1 – 26 °C	*	*	100
2 – 26 °C; 25 mg L^{-1} CN^-	37	2	100
3- 29 °C	*	*	100
4 -29 °C; 25 mg L^{-1} CN^-	37	3	60
5- 32 °C	*	*	80
6 – 32 °C; 25 mg L^{-1} CN^-	40	10	20

* No data available in treatments with no CN^-.

In Figure 11, it is shown a decrease of survival rates with temperature rise. Moreover, when fish were exposed to both CN^- and thermal stress the percentage of survivors was even lower.

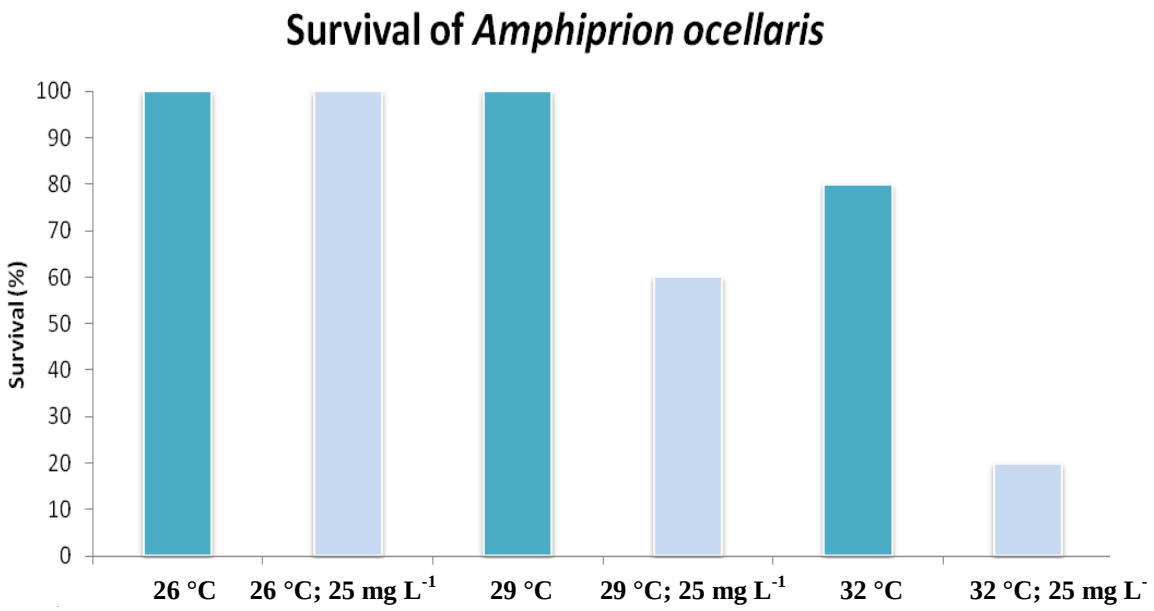

Figure 11. Survival rates in *Amphiprion ocellaris* specimens, exposed to CN^- under two different global warming scenarios (+3 °C and +6 °C).

3.1.3.2. Biomarkers

No significant interactions were recorded among concentration of CN^-, temperature and tissues for LPO activity (three-way ANOVA, log transformation, $F_{2,48} = 0.76$; $p = 0.47$) (for further details on the statistical analysis see Supplementary data). Concerning LPO values recorded in gills (Fig. 12) significant differences were detected among treatments. The values of LPO recorded in the gills differed significantly ($p < 0.005$) between control group and the other treatments. Significant differences in LPO rates were also observed between tissues ($p < 0.001$).

In both gills and muscle, LPO rates decreases with temperature rise and CN^- exposure.

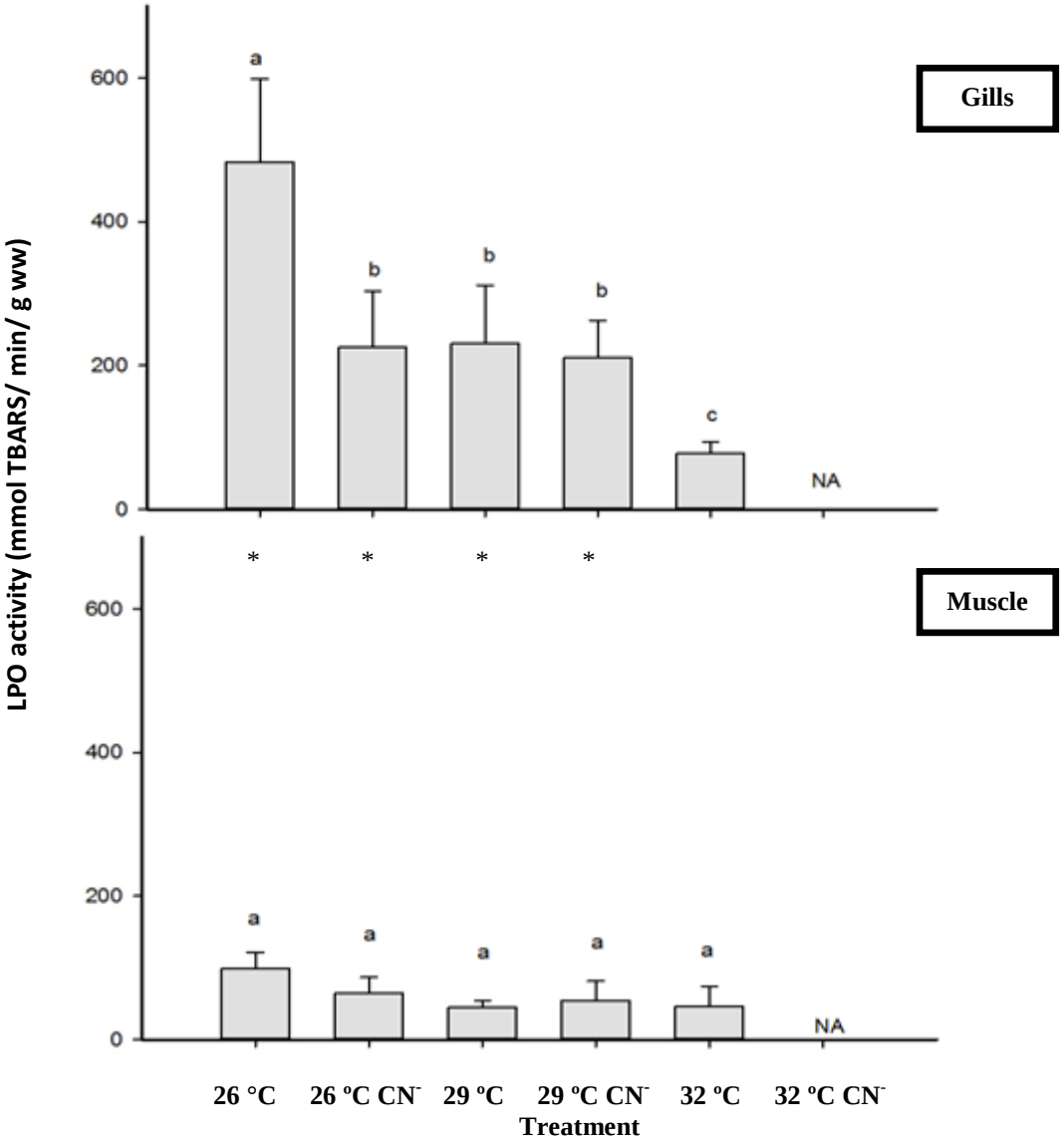

Figure 12. Effect of CN^- exposure and the increment of temperature in LPO activity (gills and muscle) in *Amphiprion ocellaris*. Values represent mean ± S.D (n=5). Different letters represent significant differences ($p < 0.05$) between treatments; different asterisks (*) represent significant differences ($p < 0.05$) between gills and muscle. NA - No data available due to the mortality recorded.

Significant interactions were recorded among concentration of CN^-, temperature and tissues for GST activity (three-way ANOVA, log transformation, $F_{2,72} = 18.674$; $p < 0.001$) (for further details on the statistical analysis see Supplementary data). Concerning GST values recorded in the gills, muscle and liver, significant differences were detected among treatments. The values of GST recorded in the gills differed significantly ($p < 0.001$) among control group and the rest of the treatments (except with 32 °C). In muscle, significant

differences ($p < 0.001$) were recorded among control group and treatments with CN^-. In liver, significant differences ($p < 0.001$) were recorded between control group and the highest temperature (32 °C). Significant differences in GST activity were also observed between tissues ($p < 0.001$).

For *A.ocellaris* GST activity was possible to observe an increase with the temperature rise and CN^- exposure in gills and liver. However, in muscle an increase of this biomarker activity was observed only with CN^- exposure (Fig. 13).

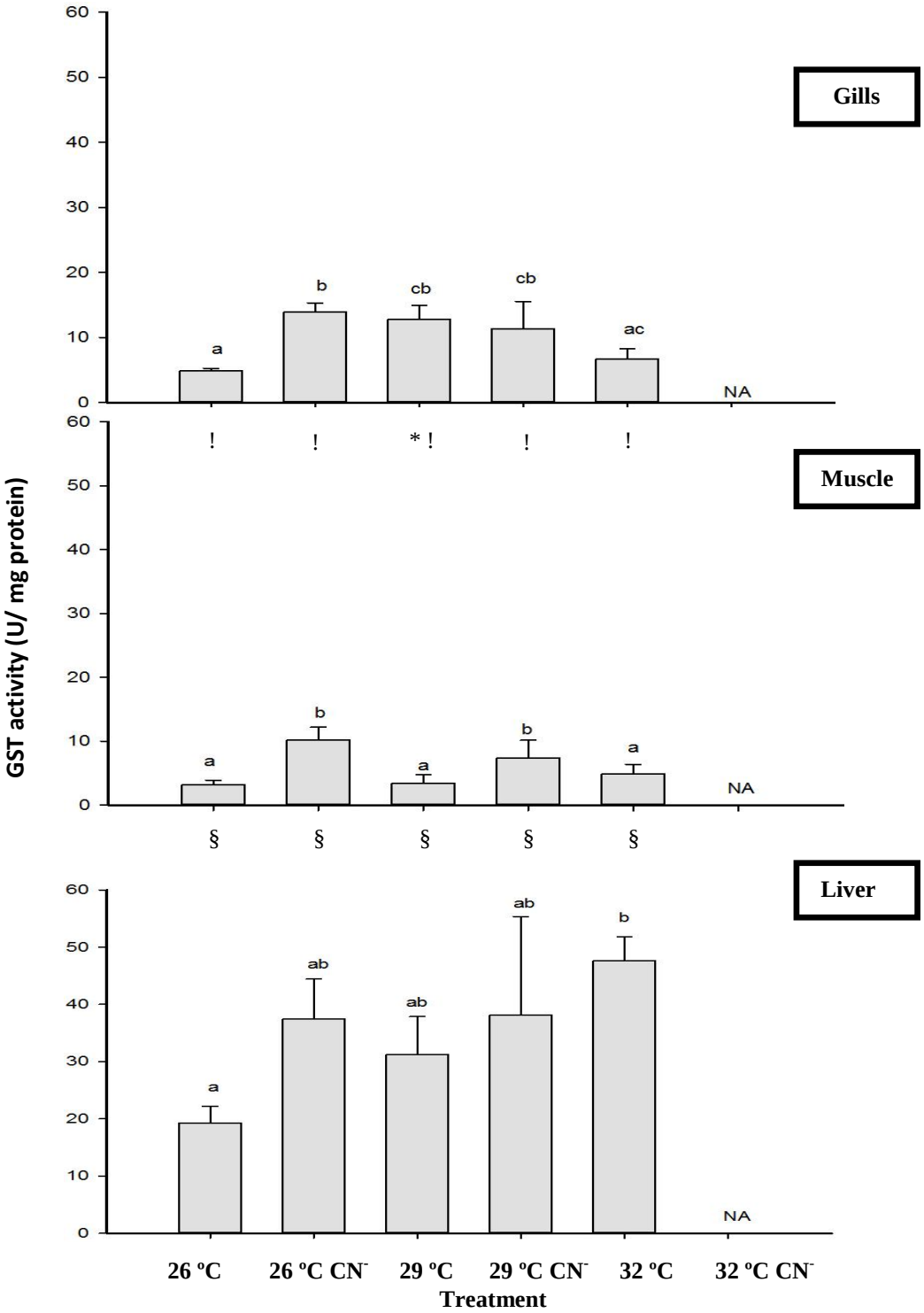

Figure 13. Effect of CN^- exposure and the increment of temperature in GST activity (gills, muscle and liver) in *Amphiprion ocellaris*. Values represent mean ± S.D (n=5). Different letters significant differences ($p < 0.05$) between treatments; significant differences ($p < 0.05$) between gills and muscle are represented by asterisks (*); significant differences ($p < 0.05$) between muscle and liver are represented by section sign (§); significant differences ($p < 0.05$) between gills and liver are represented by exclamation mark (!). NA – No data available due to the mortality recorded.

Significant interactions were recorded among concentration of CN^-, temperature and tissues for CAT activity (three-way ANOVA, log transformation, $F_{4,72}$= 33.485; $p < 0.001$) (for further details on the statistical analysis see Supplementary data). Concerning CAT values recorded in the gills, muscle and liver significant differences were detected among treatments. The values of CAT recorded in the gills differed significantly ($p < 0.001$) between control group and the higher temperature (32 °C). In muscle, significant differences ($p < 0.001$) were recorded among control group and two other treatments (26 °C with CN^- and 32 °C). In liver, significant differences ($p < 0.001$) were recorded among control group and the rest of the treatments. Significant differences in CAT activity were also observed among tissues ($p < 0.001$).

For *A.ocellaris*, CAT activity decreases with the temperature rise and CN^- exposure in gills. However, in liver an increase of this biomarker activity was observed with CN^- exposure and temperature rise. In muscle, an increment of CAT activity was observed with temperature rise, but was also observed a decrease of the biomarker activity when fish was exposed to CN^- (Fig. 14).

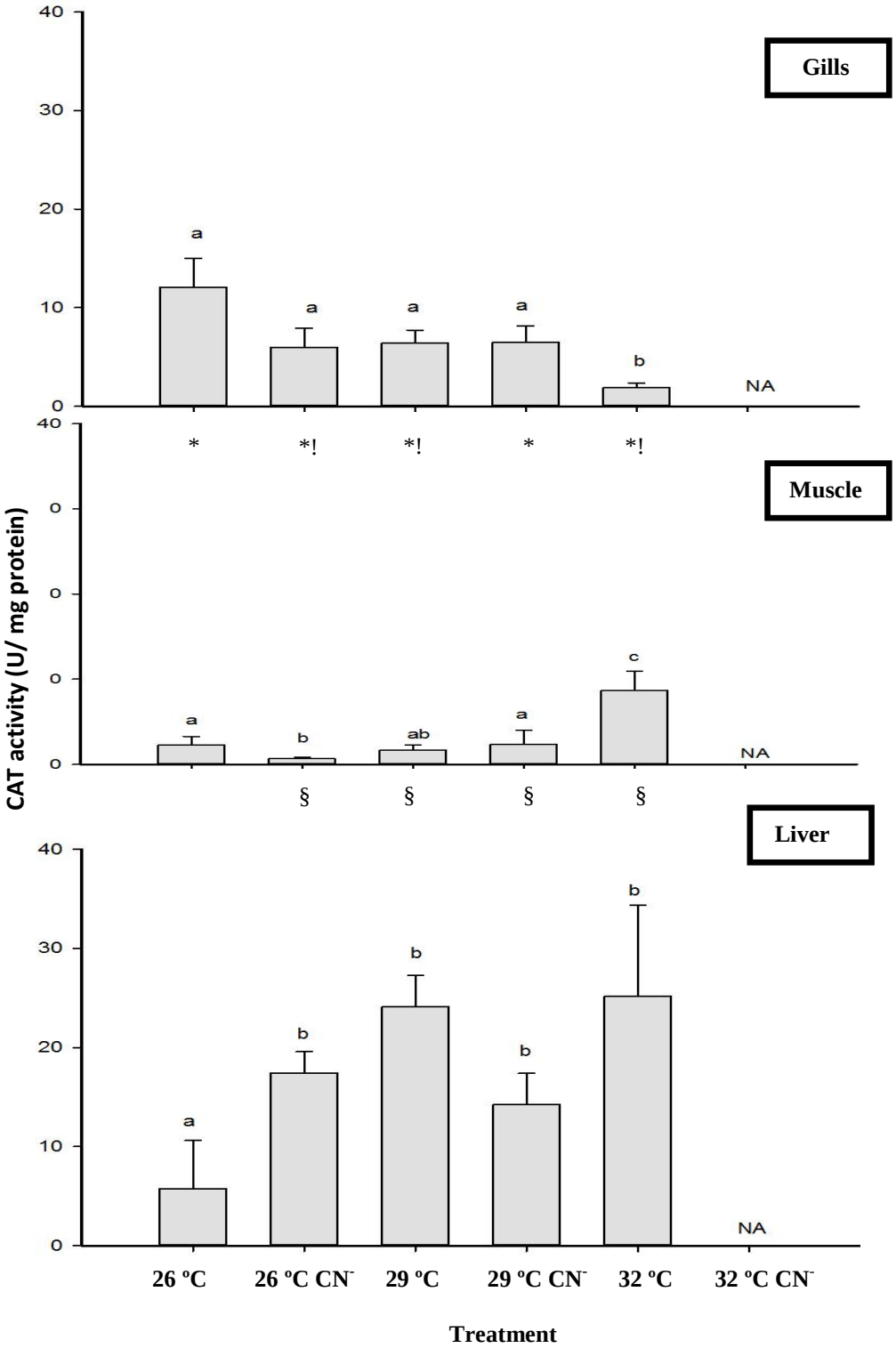

Figure 14. Effect of CN^- exposure and the increment of temperature in CAT activity (gills, muscle and liver) in *Amphiprion ocellaris*. Values represent mean ± S.D (n=5). Different letters means significant differences ($p < 0.05$) between treatments; significant differences ($p < 0.05$) between gills and muscle are represented by asterisks (*); significant differences ($p < 0.05$) between muscle and liver are represented by section sign (§); significant differences ($p < 0.05$) between gills and liver are represented by exclamation mark (!). NA – No data available due to the mortality recorded.

Significant interactions were recorded among concentration of CN^-, temperature and tissues for GPx activity (three-way ANOVA, log transformation, $F_{4,72}$= 8.769; $p < 0.001$) (for further details on the statistical analysis see Supplementary data). Concerning GPx values in gills, muscle and liver (Fig. 15) no significant differences were detected among treatments. Significant differences in GPx activity were observed among tissues ($p < 0.001$).

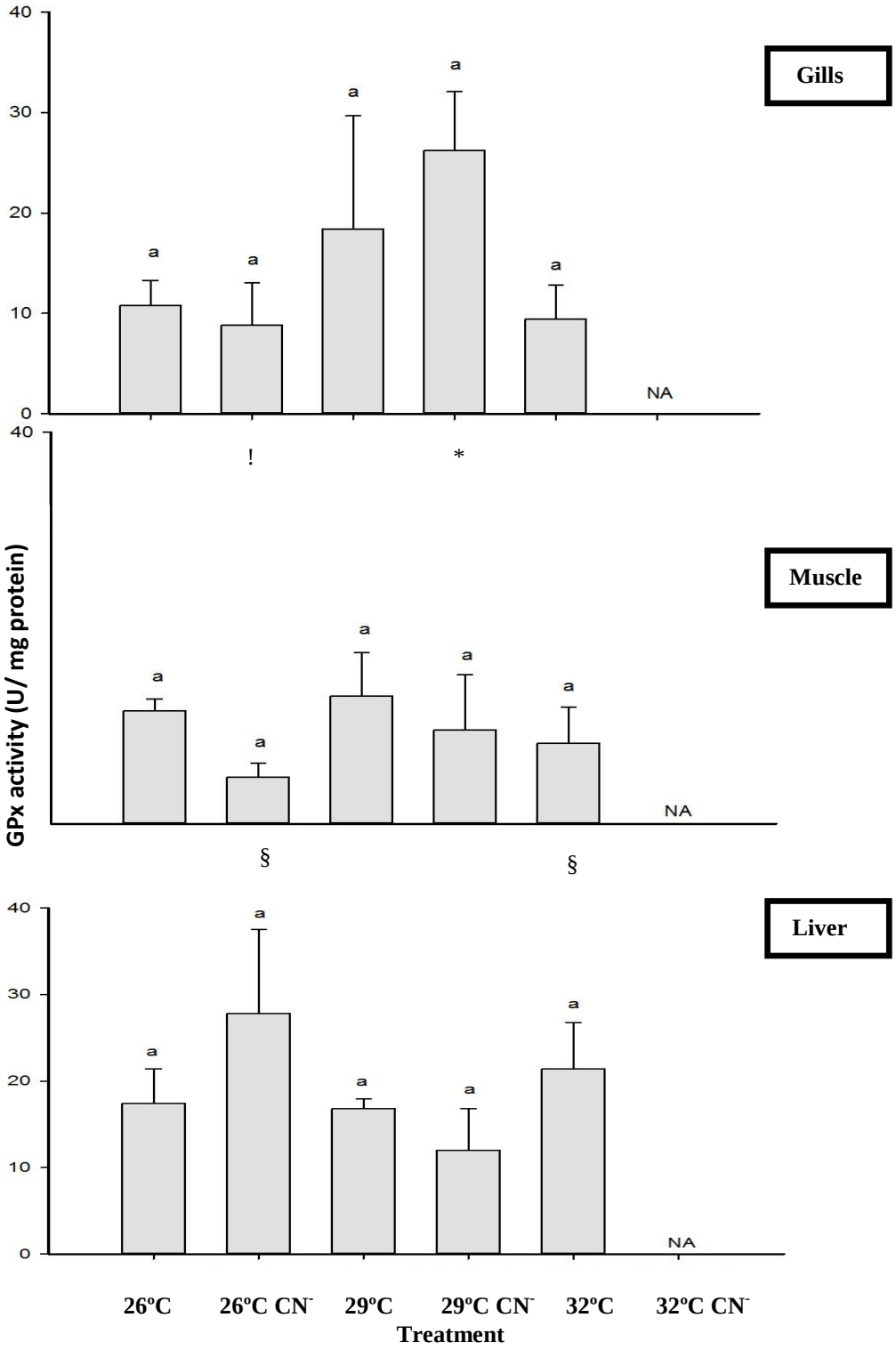

Figure 15. Effect of CN^- exposure and the increment of temperature in GPx activity (gills, muscle and liver) in *Amphiprion ocellaris*. Values represent mean ± S.D (n=5). Different letters means significant differences ($p < 0.05$) between treatments; significant differences ($p < 0.05$) between gills and muscle are represented by asterisks (*); significant differences ($p < 0.05$) between muscle and liver are represented by section sign (§); significant differences ($p < 0.05$) between gills and liver are represented by exclamation mark (!). NA – No data available due to the mortality recorded.

No significant interactions were recorded among concentration of CN^- and temperature for AChE activity (three-way ANOVA, log transformation, $F_{4,72}$= 8.769; p < 0.001) (for further details on the statistical analysis see Supplementary data). Concerning AChE values recorded in brain (Fig. 16) no significant differences were detected among treatments.

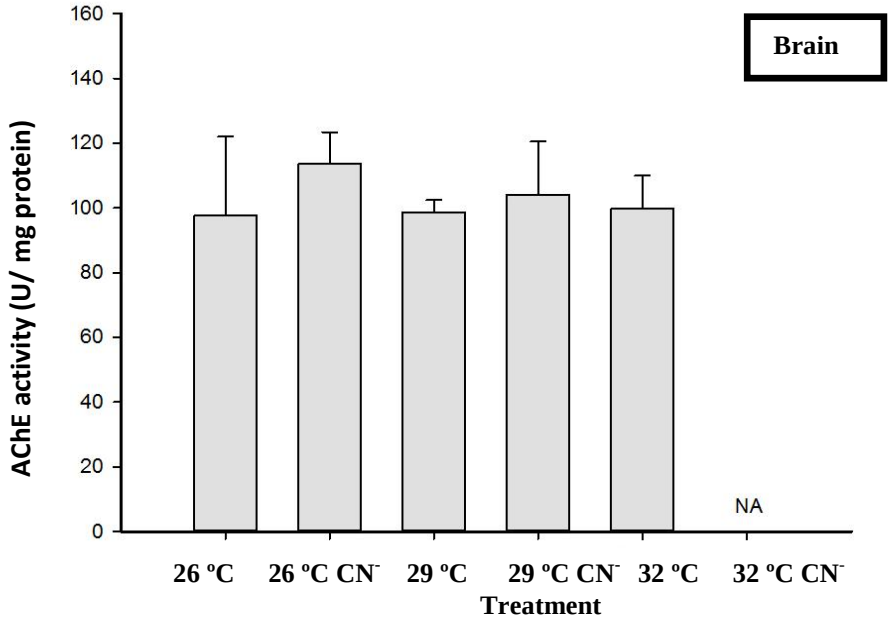

Figure 16. Effect of the interaction of CN^- exposure and the increment of temperature in AChE activity (brain) in *Amphiprion ocellaris*. Values represent mean ± S.D (n=5). NA – No data available due to the mortality recorded.

3.1.4. Discussion

In this experiment, fish exposed to CN^- under thermal stress behaved with irregular respiratory movements followed by hyperexcitability, loss of balance and settling to the bottom of the test chamber. This behaviour was similar to a preliminary study with *Amphiprion ocellaris* exposed to different concentrations of CN^- (Chapter 2). The thermal acclimation did not affect normal swimming capacity but the combined effect of CN^- and temperature has extended the behavior observed with CN^- exposure. This type of changes in locomotor and respiratory functions represent a recurrent and integrated response of fish to contaminants and constitute important indicators of sublethal exposure to toxics (Scherer, 1992; Kane et al., 2005). Using the same concentration of CN^- and different temperatures, immobilization and recovery time was shorter in lower temperatures and vice-versa. Regarding mortality, our study showed that temperature had a considerable effect on CN^- toxicity in *A. ocellaris*. Similar findings were observed by Cairns and Scheier (1963) with bluegill sunfish in various concentrations of CN^- after 48, 72, and 96 hours exposure at diffent temperatures.

A possible explanation may be related to the fact that CN^- (in certain concentrations) forces fish to metabolize anaerobically and at higher temperatures this physiological change is more pronounced (Brigdes, 1958). The same occurs with the interaction metals-temperature. When organisms are exposed to metals at higher temperatures, metal rates uptake and accumulation increases with rising temperature. Environmental warming affect the bioavailability of metals making them more soluble (Cairns et al., 1975; McLusky et al., 1986; Hutchins et al., 1996; Heugens et al., 2002).

Biomarkers of oxidative stress have been largely used to determine the effects induced by several contaminants on organisms (Nunes et al., 2008).

A large number of toxicological studies use lipid peroxidation (LPO) as a biomarker of structural and functional changes in the membranes that lead to cell death (Lackner, 1998; Ramos et al., 2000; Berntssen et al., 2003; Hermes-Lima, 2004). Here, we observed a decrease in lipid peroxidation, in both tissues, indicating that fish showed no cell damage in any of the different treatments. However, the most pronounced decrease was noted with increasing temperature. The decrease in LPO rates can be explained by the

activity of antioxidant enzymes such as CAT and GST. GSTs, enzymes of phase II detoxification processes, have the function to catalyze the conjugation of glutathione to electrophilic xenobiotics, and increase their activity to combat oxidative stress caused by toxicants (Van der Oost et al., 2003; Buet et al., 2006; Vlahogianni et al., 2007). Results showed an increase of the GST activity in all tissues not only under thermal stress or CN^- exposure but also under the combined conditions. This result reflects two important points: temperature induces oxidative stress in fish and its combined effect with CN^- enhances oxidative stress. Similar findings were observed in other marine fish (e.g., *Anguilla anguilla* and *Mugil cephalus*) exposed to different toxic compounds under increasing temperature, with the increment of activity of GST under higher temperature suggesting the occurrence of major pro-oxidant pressure (Gorbi et al., 2005).

Catalase has the primary function to degrade hydrogen peroxide that results from superoxide degradation. Our study showed an increase in catalase activity in the liver, under CN^- exposure and increasing temperature, probably due to a compensatory physiological response to oxidative stress. The results recorded in this study are supported by several authors who reported that this enzyme activity is usually higher at elevated temperatures (Viarengo et al., 1991; Orbea et al., 2002). In gills, catalase activity was inhibited under CN^- exposure and increase of the temperature. This result is in accordance with Alavandi and Hosetti (2014), who showed that CAT activity, is more inhibited in gills than other tissues surveyed in our study. On the other hand, GPx activity showed no significant differences with CN^- exposure under the warming scenarios, thus suggesting that this biomarker may not be appropriate to evaluate physiological effects of these two stressors.

Regarding tissue differences, higher activities of GST, CAT and GPx were observed in the liver followed by gills and muscle. In fact, these antioxidant enzymes act preferentially in liver - the main organ in detoxification and processing metabolic products for degradation (Lushchak et al., 2005). Moreover, the muscle analyzed in the present study was only the white muscle, the one with the lower content of mitochondria and consequently a reduced role in oxidative metabolism (Prieto et al., 2006). Gills are the first system affected by CN^- while muscle is exposed only to the amount of toxin which is assimilated

and distributed through the fish. It is therefore not surprising that activities of all antioxidant enzymes in fish were much lower in white muscle, with reduced role in oxidative metabolism, than in liver and gills, as already reported by several authors (Hermes-lima, 2004; Atli et al., 2006; Sampaio et al., 2010). As in Chapter 2, the AChE activity did not vary significantly with CN^- exposure, neither with thermal stress. In opposition, Forget et al. (2003) and Ricciardi et al. (2006) have shown an induction in its activity with increasing temperature.

In conclusion, this study showed that interactions between temperature and CN^- will promote the occurrence of negative effects on the metabolic physiology of coral reef fish. Higher water temperatures and CN^- poisoning *per se* can induce significant levels of physiological stress to clownfish. However, the combination of these two stressors can lead to a generalised failure of detoxification pathways and damage repair processes, resulting in higher physiological stress and generalised mortality. It is therefore mandatory to effectively ban CN^- fishing from tropical coral reefs, as the damage this practice already causes in the oceans of today will be largely magnified in the oceans of tomorrow.

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Chapter 4

4.1. Contrasting vulnerability to cyanide poisoning in eight species of Indo-Pacific damselfish (Pomacentridae)

Contrasting vulnerability to cyanide poisoning in eight species of Indo-Pacific damselfish (Pomacentridae)

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Abstract

Coral reefs are the most biologically diverse of shallow water marine ecosystems under optimal physical and chemical conditions. However, these ecosystems are susceptible to cyanide fishing, recognized as a major cause for coral reefs destruction. One important group of reef fish communities is the family Pomacentridae that not only plays an important key role in coral reef food webs but also represents half of the ornamental marine species trade. The aim of the present study was to understand if exists differences in vulnerability to cyanide poisoning in eight (very closed phylogenetically) species belonging to this family (*Amphiprion clarkii*, *Amphiprion frenatus, Amphiprion ocellaris, Chromis margaritifer, Chromis viridis, Chrysiptera cyanea, Dascyllus melanurus* and *Dascyllus trimaculatus).* After acclimation period fishes were divided into groups per species for cyanide pulse exposure (50 mg L^{-1}). Interspecific variability in terms of vulnerability to cyanide poisoning was exhibited by coral reef fish, even when these are phylogenetically closely related. Sub-family Chrominae demonstrates more vulnerability to cyanide fishing problem. This work highlights the need to enforce the true ban of this practice from coral reefs.

Keywords

Cyanide exposure; interspecificity; phylogeny; mortality; tropical coral reefs

4.1.1. Introduction

Tropical coral reefs are one of the most important ecosystems in the world, being known by their remarkable biodiversity and for providing a number of goods and services to humankind (Brander et al., 2007; Burke et al., 2011). Often referred to as the "rain forests of the sea" (Burke et al., 2011), coral reefs currently face an unprecedented number of both natural and anthropogenic threats (Tissot et al., 2010). While some of these threats affect coral reefs at a global scale, such as ocean warming and acidification, other threats of no lesser concern impact these ecosystems at a more local scale (e.g., coastal development and unsustainable tourism, marine-based pollution, overfishing and destructive fishing) (Hughes et al., 2003; Fox et al*.,* 2005; Leao and Kikuchi, 2005; Tissot et al., 2010). Destructive fishing, one of the types of overfishing, is one of the most pervasive immediate threats, affecting more than 55 percent of all this ecosystems in world (Burke et al., 2011). This fishing practice, declared illegal since 1985, has raised concern for its impacts on aquatic marine life but unfortunately the practice is still prevalent (Arifin and Hindarti, 2006; Tissot et al., 2010). Normally, the potent cyanide (CN^-) toxin is available in the commercial market in two distinct forms, sodium cyanide (NaCN) and potassium cyanide (KCN) that have differences in terms of wave action or currents, and length of stay in the vicinity of a cloud of CN^- solution but do not differ significantly in the strong capacity to affect fish (McAllister et al., 2001). According to Rubec (1986), CN^- are extremely toxic to fish and expose that organisms to concentrations greater than 5 mg L^{-1} in several minutes are lethal. Another study showed that fishers use CN^- concentrations ranging from 1.5 to 120 g L^{-1} (Arifin and Hindart, 2006). Cervino et al. (2003) demonstrated that CN^- exposure (1 or 2 minutes) at concentration of 50 mg L^{-1} of CN^- cause mortality to some corals (*Acropora millepora, Goniopora sp., Favites abdita*) and anemones (*Aiptasia pallida*). Tropical reef fish communities are very important, working in community structure through processes as competition and territoriality (Ceccarelli et al., 2001). Herbivorous fish, such as damselfish, (family Pomacentridae), have an important role in tropical reef communities, not only because contribute for energy transfer and nutrient but also have a massive influence on recruitment and growth of other type of organisms, such as corals (Horn, 1989), invertebrates, and algae (Ferreira et

al., 1998). Apart from the ecological relevance, this family represents a half of the trade of ornamental marine species, included in a global multi-million dollar industry, worth an estimated US$200-330 million annually, and operating throughout the tropics (Wabnitz et al., 2003). These ornamental marine species are collected and transported mainly from Southeast Asia to consumers in the main destination markets such as United States, European and Japan. Within this family, the most traded species are the blue-green damselfish (*Chromis viridis*), the clown anemonefish (*Amphiprion ocellaris*), the whitetail dascyllus (*Dascyllus aruanus*), the sapphire devil (*Chrysiptera cyanea*) and the threespot dascyllus (*D. trimaculatus*) (Wabnitz et al., 2003).

There is still data gap on the vulnerability of CN^- to tropical fish and, to our knowlwdge, there are no interspecific studies addressing the vulnerability to the CN^- fishing threat. Therefore, the present study aims to investigate the vulnerability of eight different Pomacentridae species to CN^- exposure.

4.1.2. Material and Methods

4.1.2.1. Fish Maintenance

All fishes were purchased from a local producer (Opérculo Lda., Portugal), to assure that all specimens had never been exposed to CN^-. Before the acclimation period, all fish were measured in terms of weight and size. Fishes were held for one week, with a 12 h L: 12 h photoperiod in two 270 L glass tanks (1.20 m long; 0.50 m high; 0.45 m wide), with an internal circulation pump (Turbelle nanostream-6025 Tunze, Penzberg, Germany; approximate flow of 2500 L h^{-1}) and it was connected to a 125 L sump (1.20 m long; 0.35 m wide; 0.40 m tall). The sump was equipped with a biological filter (submerged bio-balls), a 50 mm mesh bag for mechanical filtration, a submergible heater (Eheim Jäger 300 W, Deizisau, Germany), a protein skimmer (DeltecH APF600) and a submerged pump (EheimH 1262, 3400 L h21). This system was also equipped with an osmoregulator (Reef SetH) used to regulate the water level by replacing evaporated water with freshwater purified by reverse osmosis to keep salinity at 35 (salinity was checked daily using a hand refractometer to detect any potential malfunction of the described automation). The fish maintenance system used seawater prepared by mixing RO water with a synthetic salt

mix (Tropic Marine Pro Reef, Wartenberg, Germany). All fish were fed four times a day until satiated with a commercial pelleted feed (HikariH Marine S). Water parameters were maintained within the following optimal ranges for the species: temperature 26 ± 0.5 °C; not detectable ammonium, nitrite and nitrate; pH 8 ± 0.2. Nitrogenous compounds were monitored every week using colorimetric tests (Salifert), while pH was monitored using a Pinpoint pH meter (PH 370, American Marine).

4.1.2.2. Experimental Design and Treatments

After the acclimation period, ten fishes from 6 different species (N=10; *Amphiprion ocellaris* (total length (TL) ≈40 ± 0.02 mm, measured from the tip of the snout to the tip of the longer lobe of the caudal fin); weigh 1.57 ± 0.3 g), *Chromis viridis* (N=10; TL ≈30 ± 0.013 mm; weigh 0,5 ± 0.05 g); *Chrysiptera cyanea* (N=10; TL ≈45 ± 0.01 mm; weigh 1.57 ± 0.17 g); *Chromis margaritifer* (N=10; TL ≈45 ± 0.02 mm; weigh 1.58 ± 0.3g); *Dascyllus trimaculatus* (N=10; TL ≈42 ± 0.03 mm; weigh 2.17 ± 0.4g); *Dascyllus melanurus* (N=10; TL ≈39 ± 0.036 mm; weigh 1.54 ± 0.34 g), *Amphiprion clarkii* (N=9; TL ≈35 ± 0.01 mm; weigh 0,9 ± 0.2 g) and *Amphiprion frenatus* (N=9; TL ≈35 ± 0.015 mm; weigh 1.11 ± 0.15 g) were randomly divided into groups per species for CN^- pulse exposure using a hand net. The duration of the pulse exposure (60s) was selected according to the work by Hanawa et al. (1998). A preliminary trial revealed that fish handling with a dip-net causes no mortality or significant stress (Vaz et al., 2012).

All groups were exposed to a concentration of 50 mg L^{-1} of CN^- at 26 °C, a sub-lethal concentration calculated in a preliminary study with *Ampriprion ocellaris* (TL 3.77 ± 0.23 cm; weight 1.12 ± 0.2 g) performed by the authors. Before the beginning of pulse exposure of CN^- a stock solution of 2.060 g L^{-1} of CN^-, was prepared in a 500 mL volumetric flask by dissolving (2.001±0.001) g of NaCN (97% purity; Sigma- Aldrich, St. Louis, MO, USA) in ultra-pure water, obtained from a Milli-Q Millipore system (Milli-Q plus 185). The concentration of CN^- employed during the pulse exposure (50 mg L^{-1}) was prepared by adding 80 mL of the stock solution to 3300 mL of synthetic seawater. The pulse exposure was divided in four steps: exposure bath, first cleaning bath, second cleaning bath and third cleaning bath. In the first step, all fishes from one species were

collected with a hand-net and dipped for 60s into a 15-L tank filled with synthetic seawater dosed with 50 mg L^{-1} of CN^-. After the pulse exposure all fish from the same species were dipped for 60s into a 20-L tank filled with synthetic seawater with no CN^- (first cleaning bath). This procedure was repeated two more times (second and third cleaning bath). During the exposure procedure, immobilization time was recorded. In the present work, immobilization time was defined as the time at which the last fish of a species group stopped swimming and rested motionless in the bottom of the mesh used for the exposure bath. Following the third cleaning bath during the pulse exposure, all fish were randomly distributed into 1-L glass jars filled with 1 L of synthetic seawater and ere determined the recovery time of normal swimming activity. In the present work, recovery time was defined as the time at which the last fish of a species group reacquired its normal swimming ability. An air stone was placed inside each glass jar to provide water aeration during the depuration stage. The behaviour of all fish was monitored during the first 30 minutes inside the glass jars to record recovery and mortality promoted by the cyanide pulse exposure. The jars were placed inside a water bath keeping water temperature at 26 °C. All fishes were exposed to a photoperiod of 12 h light: 12 h dark provided by white fluorescent. The toxicity trial was carried out in a semi-static system, where water was fully replaced every day. According to OECD guideline 203 (OECD, 1992), no food was provided to the fish. Temperature, salinity, pH, dissolved oxygen, alkalinity, total ammonia–nitrogen, nitrite and nitrate of each experimental tank were measured daily. Temperature, salinity and dissolved oxygen were measured using an YSI 85 Model (Yellow Springs Instruments, Yellow Springs, USA), and the pH was measured with a pH 100 meter (Yellow Springs Instruments, Yellow Springs, USA). The other parameters were measured using colorimetric assays. Temperature was maintained at 26 ± 0.1 °C, salinity at 35 ± 0.1, pH 8 ± 0.1, dissolved oxygen concentration at 6.2 mg L^{-1}, nitrite, nitrate and ammonium were not detectable. Mortality was evaluated daily. Fish were considered dead when they were motionless on the bottom, exhibited no opercular movement and presented no response to mechanical stimuli.

4.1.3. Results

During cyanide exposure, severe gasping, followed by loss of balance and a complete loss of all respiratory activity was observed in all fish when dipped in 50 mg L^{-1} of CN^-. Fish total length amplitude was ≈14 mm and relative to weigh 1.67 g (Fig. 17. and fig. 18). By ordering the species by decreasing time of immobilization, *Dascyllus trimaculatus* was immobilizated after 40s, *Chrysiptera cyanea* 37s, *Dascyllus melanurus* 32s, *Amphiprion ocellaris* 28s, *Amphiprion frenatus* 23s, *Chromis viridis* 20s and both *Amphiprion clarkii* and *Chromis margaritifer* 12s (Fig. 19). During recovery from anaesthesia, the fish initially remained on their sides with mild to strong opercular movements and some fin movements. Only two species were able to recover from the pulse exposure to CN^-: *A. ocellaris*, with a complete recovery time of 23 min; and *Chrysiptera cyanea*, with complete recovery time of 15 min (Fig. 19). At the end of the recovery period, specimens displayed a normal swimming behaviour, not evidencing any signs of loss of balance or disorientation.

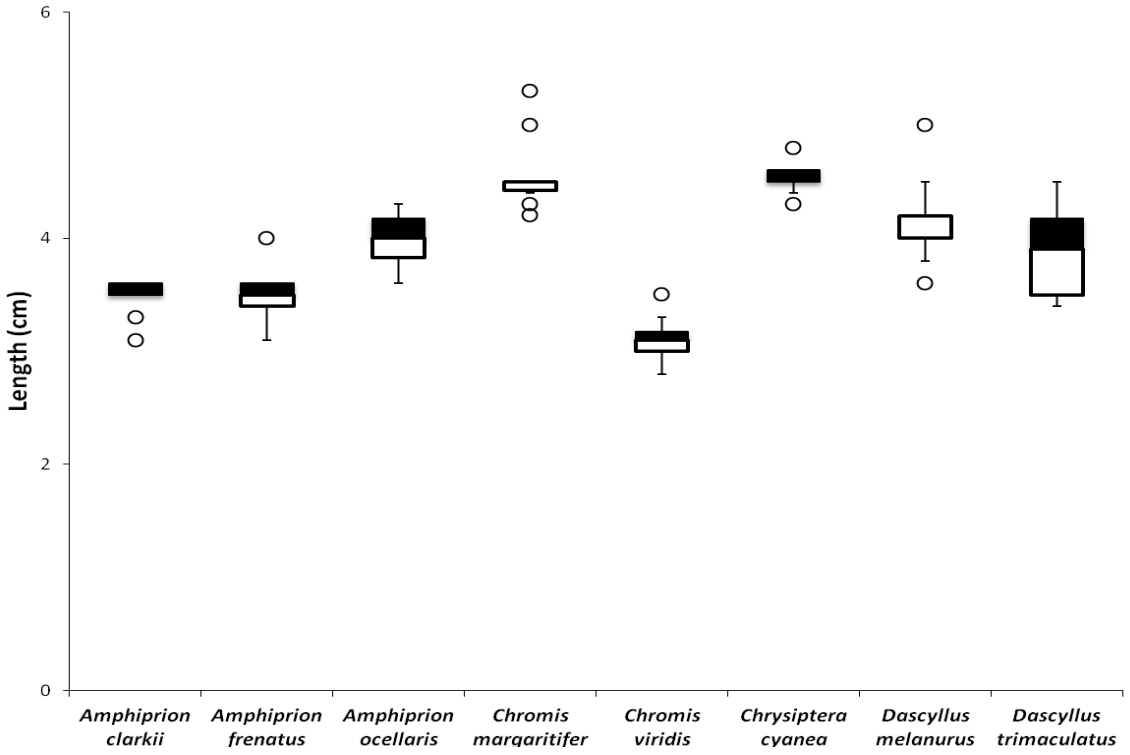

Figure 17. Total length (cm) of species *Amphiprion clarkii, Amphiprion frenatus, Amphiprion ocellaris, Chromis margaritifer, Chromis viridis, Chrysiptera cyanea, Dascyllus melanurus* and *Dascyllus trimaculatus* exposed at 50 mg L^{-1} of CN^-.

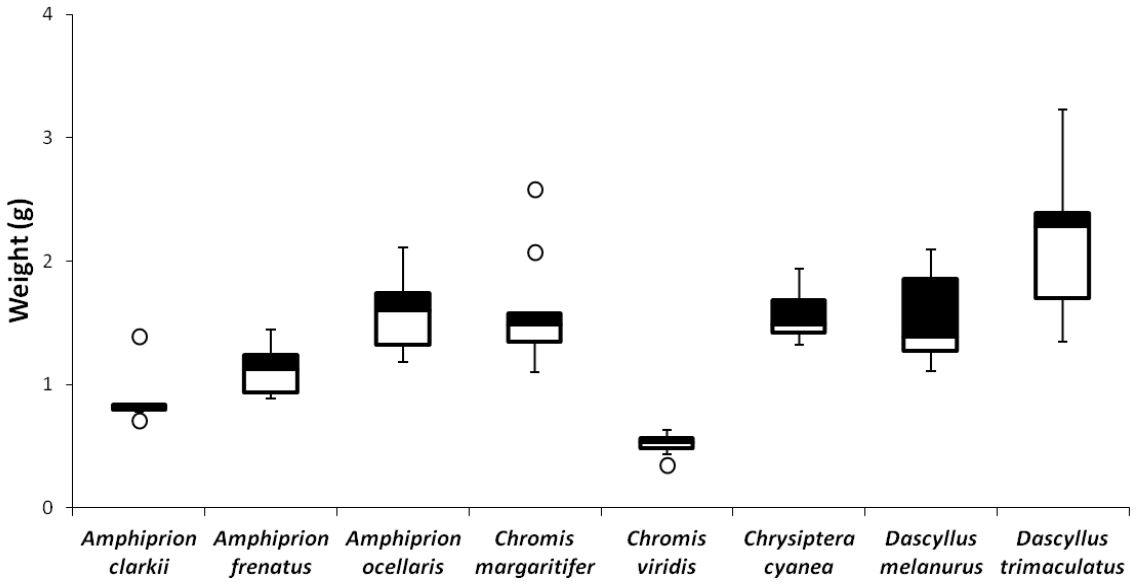

Figure 18. Weight (g) of species *Amphiprion clarkii, Amphiprion frenatus, Amphiprion ocellaris, Chromis margaritifer, Chromis viridis, Chrysiptera cyanea, Dascyllus melanurus* and *Dascyllus trimaculatus* exposed at 50 mg L^{-1} of CN^-.

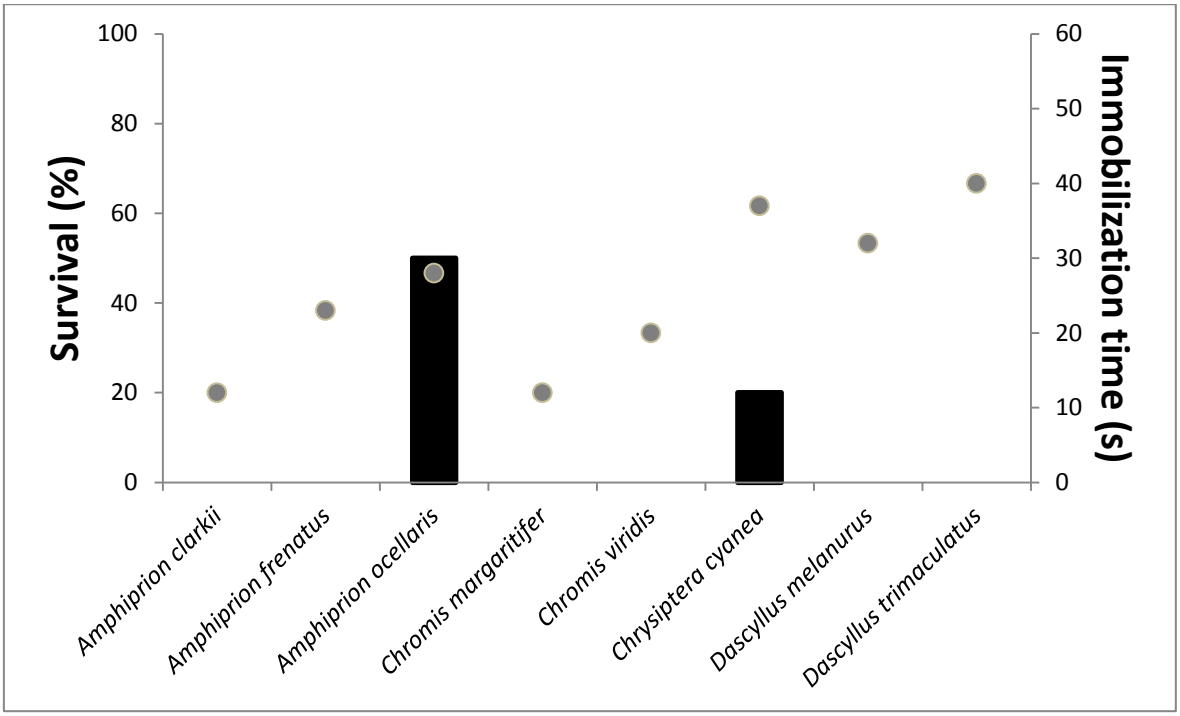

Figure 19. Survival rate and immobilization time (s) in species *Amphiprion clarkii, Amphiprion frenatus, Amphiprion ocellaris, Chromis margaritifer, Chromis viridis, Chrysiptera cyanea, Dascyllus melanurus* and *Dascyllus trimaculatus* exposed at 50 mg L^{-1} of CN^-.

4.1.4. Discussion

In the present study, all species displayed a similar behaviour when exposed to CN^-, namely irregular, erratic, and dartic movements, followed by loss of balance and settling to the bottom of the test chamber (as in Dube and Hosetti, 2010). Here we also showed that there is large interspecific variation regarding vulnerability to CN^- poisoning even in species with a close phylogenetic relationship (e.g. within the same family – the Pomacentridae). Only two species, *Amphiprion ocellaris* and *Chrysiptera cyanea*, were able to survive a CN^- exposure of 50 mg L^{-1}. These results may somehow be related with the phylogenetic proximity of the different genera of family Pomacentridae surveyed in the present work. According to Cooper et al. (2009) this family can be divided in two important sub-families: the Chrominae, restricted to the genus *Chromis* and *Dascyllus*, and the Pomacentrinae, which includes genus *Chrysiptera* and *Amphiprion*. In our study, solely species within sub-family Pomacentrinae were able to endure the pulse exposure to the concentration of CN^- tested in the present work. It is worth highlighting that the different vulnerability to CN^- poisoning recorded within genus *Amphiprion*, may somehow be explained by the polyphyletic nature of this group of clownfish. Indeed, several authors have already reported the polyphyletic nature of genus *Amphiprion* (e.g. Quenouille et al., 2004; Santini and Polacco, 2006). In the study by Cooper et al. (2009), *A. ocellaris* is placed in group separated from *A. clarkii* and *A. frenatus*. Therefore, the lower vulnerability to CN^- poisoning displayed by *A. ocellaris* may eventually be related with subtle (but apparently significant) differences in the metabolic pathways involved in CN^- toxicity.

Despite the persistence of CN^- fishing in Indo-Pacific coral reefs (Sadovy et al., 2003; Bruckner and Roberts, 2008), accurate information on reef fishes vulnerability to this illegal practice is still scarce. Although no LC50 (96 h) was calculated in the study by Hawana et al. (1998) on *D. aruanus*, by Vaz et al. (2012) on *A. clarkii* or in the present study for any of the studied species, the vulnerability to CN^- poisoning recorded for *A. ocellaris* in the present study are somehow in line with data from Vaz et al. (2012), as these authors report 33% mortality for specimens exposed to 25 mg L^{-1} of CN^- (a pulse exposure to twice this concentration of CN^- could therefore be expected to result in a

generalized mortality of poisoned fish, as recorded in the present work). Our data are also in line with those of Hawana et al. (1998), for *D. aruanus*, a close relative (see Cooper et al., 2009) of the species surveyed in the present work, *D. melanurus*. Hawana et al. (1998) reported no mortality for specimens of *D. aruanus* with an average TL of about 75 mm pulse exposed for 60s to 50 mg L^{-1} of CN^-. These specimens were nearly twice the size of the specimens of *D. melanurus* surveyed in the present work. As recorded in the preliminary study referred above (see materials and methods) performed with *A. ocellaris*, double sized fish require twice the concentration of CN^- to induce mortality.

Overall, this study evidences the level of interspecific variability in terms of vulnerability to CN^- poisoning exhibited by coral reef fish, even when these are phylogenetically closely related. Despite already being considered as an illegal practice in most countries affected by cyanide fishing, this work highlights, from a conservation point of view, the need to enforce the true ban of this practice from coral reefs. If a given species commonly targeted by fishermen illegally using CN^- poisoning displays a lower vulnerability to the toxic than other accompanying species (e.g., *A. ocellaris*), the negative impacts of this practice will be even more destructive. By targeting a less vulnerable species, higher dosages of CN^- must be administered to achieve the desired effect, thus exposing all other reef fish and invertebrates to potentially lethal dosages of this toxic.

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Chapter 5

5. Conclusions and Final considerations

5. Conclusions and Final considerations

The present study highlighted that the vulnerability to cyanide (CN^-) of coral reef fishes can vary intraspecifically (e.g., with specimens size), as well as interspecifically. Indeed vulnerability to CN^- poisoning can vary even between phylogenetically close species (within the same family and even within the same genus). CN^- can be classified as highly toxic to members of family Pomacentridae, with sub-lethal doses of this poison being enough to induce dramatic shifts in the behavior and metabolic activity of fish. Interactions between increasing water temperature (under scenarios predicted by the Intergovernmental Panel of Climate Change – the IPCC) and CN^- poisoning strongly affects the physiological tolerance of coral reef fish to these two stressors and can limit their survival and distribution in the oceans of tomorrow. In fact, the already recognised destructive impact of CN^- fishing today will dramatically be magnified in tomorrow's coral reefs. There are still some important studies to be developed in order to have a more in depth understanding of CN^- poisoning in coral reef species in particular, and coral reefs ecosystems in general. In this way, priority actions should be taken to understand the synergistic effects of CN^- fishing, additional sources of anthropogenic pollution and ongoing climate change in individuals and populations as well, with emphasis to carry-over and Transgenerational effects. Effective methods to detect coral reef fishes collected illegally with CN^- poisoning are also urgently needed. Only when such detection methods are fully reliable and operational will it be possible to take legal action on those exporting and importing CN^- caught coral reef fish and strengthen the fight to ban CN^- fishing from coral reefs.

Supplementary Data

Materials and Methods (Chapter 2 and 3)

Acute Tests

All fish used in the acute test must be held in the laboratory for at least 12 days before they are used for testing, under the following conditions: light (12 to 12 hours photoperiod daily); temperature (appropriate to the species); oxygen concentration (at least 80 per cent of air saturation value); feeding (three times per week or daily until 24 hours before the test is started). In relation to test solutions, chosen concentrations are prepared by dilution of a stock solution. The test should be carried out without adjustment of pH. If there is evidence of marked change in the pH of the tank water after addition of the test substance, it is advisable that the test should be repeated, adjusting the pH of the stock solution to that of the tank water before adding of the test substance. This pH adjustment (normally with HC1 and NaOH) should be made in such a way that the stock solution concentration is not changed to any significant extent, and that no chemical reaction or precipitation of the test substance is caused. The exposure should have the following conditions: duration (preferably 96 hours); the maximum loading (1.0 g fish/litre for static and semi-static tests); photoperiod (12 to 16 hours); temperature (appropriate to the species and constant within a range of 2 °C); oxygen concentration (not less than 60 per cent of the air saturation value); aeration (can be used provided that it does not lead to a significant loss of test substance); feeding (none); disturbance (that may change the behaviour of the fish should be avoided); number of fish (at least 7 fish must be used at each test concentration and in the controls); controls (one blank and, if relevant, one control containing the solubilising agent are run in addition to the test series); and observations (the fish are inspected at least after 24, 48, 72 and 96 hours). Fish are considered dead if there is no visible movement (e.g. gill movements) and if touching the caudal peduncle produces no reaction. Dead fish are removed when observed and mortalities are recorded. Observations at three and six hours after the start of the test are desirable. Records are kept of visible abnormalities (e.g. loss of equilibrium, swimming behaviour, respiratory function, pigmentation, etc.).

Measurement of pH, dissolved oxygen and temperature should be carried out at least daily (OECD, 1992).

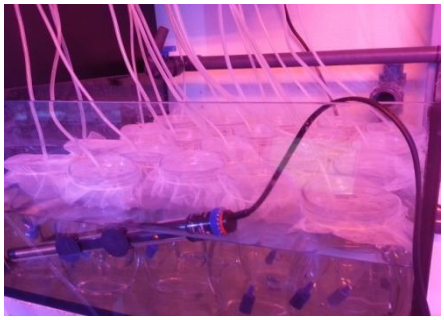
Experimental Design and Treatments

1. Acclimation period

2. Cyanide pulse exposure

3. Cleaning baths

4. Recovery



5. Post-exposure depuration

Schematic representation of experimental procedures for cyanide (CN-) pulse exposure and depuration of *Amphiprion ocellaris.*

Biomarkers

Post-Mitochondrial Supernatant (PMS)

The post-mitochondrial supernatant was obtained using the protocol described by Ferreira et al. (2010). Gills, muscle and liver removed previously were homogenized using a sonicator (Kika Labortechnik, V200Scontrol, Germany) in a homogenization buffer (K-Phosphate 0.1 M buffer, pH 7.4). The homogenate obtained were separated: 150 µl in a 2 ml microtube and 2.5 µL butylated hydroxytoluene (BHT) 4% in methanol, for lipid peroxidation (LPO) determination. The remaining tissue homogenized was centrifuged (10,000 g for 20 min at 4 °C) and the antioxidant enzyme activities (glutathione *S*-transferases, glutathione peroxidase and catalase) were quantified through the Post-Mitochondrial Supernatant (PMS). The PMS was divided into three microtubes for posterior analysis of biomarkers and protein quantification. All microtubes were stored at -80 °C until analysis.

Lipid Peroxidation (LPO)

Based on the methods described by Bird and Draper (1984) and Ohkawa et al. (1979) and adapted to microplate, the lipid peroxidation assay was performed measuring thiobarbituric acid-reactive substances (TBARS). The reaction included a mixture of 150 µl homogenized tissue, 500 µL trichloroacetic acid sodium salt (TCA) 12% (w/v), 400 µl Tris-HCl 60mM with diethylenetriaminepentaacetic acid (DTPA) 0.1 mM and 500 µL 2-thiobarbituric acid (TBA) 0.73% (w/v). The reaction mixture was then incubated in boiling water (100 °C) for 60 minutes. Subsequently, samples added in microtubes were centrifuged for 5 min at 11,500 rpm (25 °C). Quadruplicates of samples were kept away from light and putted into a 96-well microplate, to read the absorbance at 535 nm. LPO is expressed as nmol TBARS hydrolyzed per minute per mg of wet weight, using a molar extinction coefficient of 1.56 x10 M^{-1} cm^{-1}.

Glutathione *S*-Transferases (GST)

According to the procedure described by Habig et al. (1974) and optimized for 96-well microplate. Glutathione *S*-transferases total activity was determined using 100 µL of the

PMS, added to 200µL of a reaction solution and the result/substrate produced was measured at 340 nm**.** 1-Chloro-2,4-dinitrobenzene (CDNB) was used as substrate and, upon conjugation of the thiol group of glutathione to the CDNB substrate, there is an increase in the absorbance. Therefore, the enzyme activity was determined spectrophotometrically by measuring the formation of the conjugates of glutathione (GSH) and l-chloro-2,4-dinitro-benzene (CDNB). The reaction solution was a mixture of 4950 µl of phosphate buffer 0.1 M (pH=6.5),900 µl of L-glutathione reduced (GSH) 10 mM and 150 µl of 1-chloro-2,4- dinitrobenzene (CDNB) 10 mM. Bovine y– globulin (Sigma-G5009) was used as positive control to validate the assay. The absorbance at 340 nm was recorded every 20 seconds for 5 min, using a plate reader (Bio-Rad, Hercules, CA, USA). The increase in absorbance is directly proportional to GST activity. The enzymatic activity of the GST was expressed as unit (U) per mg of protein and was calculated using a molar extinction coefficient of 9.6x 10^{-3} M^{-1} cm^{-1}, where a U corresponds to a nmol of substrate hydrolyzed per minute.

Glutathione Peroxidase (GPx)

Glutathione peroxidase activity was determined according to the method described by Mohandas et al. (1984). To perform the assay, 15 µl of the PMS, were added to 282 µl of a previous prepared solution and 3 µL H_2O_2 0.5 mM in each well of a 96-well microplate. The previous prepared solution was a mixture of 22.680 ml of K-phosphate buffer 0.05 M (pH 7.0), in a EDTA 1mM solution, Sodium azide 1mM and glutathione reductase (GR) 1 U/mL, with 1.350 ml glutathione reduced (GSH) 4 mM and 1.350 ml of NADPH 0.8 mM). The absorbance at 340 nm was recorded at 1 and 3 min, using a plate reader (Bio- Rad, Hercules, CA, USA). The enzymatic activity is expressed as unit (U) per mg of protein. A U corresponds to one nmol of substrate hydrolyzed per minute, using a molar extinction coefficient of 6.22x$10^{-3}$$M^{-1}$ cm^{-1}.

Catalase (CAT)

The activity of Catalase was determined based on the method of Clairborne (1985). In this assay, CAT activity is assessed by measuring the rate of H_2O_2 removal. Consequently, the

reaction can be followed by a decrease in the absorbance as H_2O_2 is turned into oxygen and water. To perform the assay, 15 μL of the PMS, were mixed with 135 μL of K-Phosphate 0.05 M (pH 7.0) and 150 μL H_2O_2 0.03M and placed in each well of a 96-well microplate (Nunc-Roskilde) and the absorbance at 240 nm was recorded for 1 min, using a plate reader. The enzymatic activity is expressed as unit (U) per mg of protein. A U corresponds to one mmol of substrate hydrolyzed per minute, using a molar extinction coefficient of 40 M^{-1} cm^{-1}.

Acetylcholinesterase (AChE)

The AChE activity determination was performed according to the Ellman method (Ellman et al., 1961) adapted to microplate (Guilhermino et al., 1996). Each brain per organism was homogenized using a sonicator in potassium phosphate buffer (0.1 M, pH 7.2), and the supernatants obtained after centrifugation of the homogenates (4 ºC, 6000 rpm, 3 min) were removed and stored at -80 °C until enzymatic analysis. To perform the assay, in a 96 well microplate were added 250 μl of the reaction solution and 50 μl of the sample. The absorbance was recorded at 414 nm, after 10, 15 and 20 min, using a plate reader. The reaction solution had 1 ml of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) 10 mM solution, 1.280 ml of 0.075 M acetylthiocholine iodide solution and 28.920 ml of 0.1 M phosphate buffer. The enzymatic activity is expressed as unit (U) per mg of protein. A U corresponds to one nmol of substrate hydrolyzed per minute, using a molar extinction coefficient of $1.36x10^{-3}M^{-1}cm^{-1}$.

Protein Quantification for Biomarkers

For all biomarker measurements protein concentration was determined according to the Bradford method (Bradford, 1976), adapted from Bio-Rad's Bradford micro-assay set up in a 96 well flat bottom plate, using bovine g-globuline as standard.

Statistical Analysis (Chapter 2)

Results of three-way ANOVA evaluating the interaction of CN^- (different concentrations), size (small and medium) and tissues (gills and muscle) in LPO of *Amphiprion ocellaris*.

	SS	***Degr. of Freedom***	***MS***	***F***	***p***
Intercept	577,0196	1	577,0196	43201,01	0,000000
Size	0,0906	1	0,0906	6,78	0,010960
CN-	0,7561	4	0,1890	14,15	0,000000
Organ	7,1939	1	7,1939	538,60	0,000000
Size*CN-	0,1145	4	0,0286	2,14	0,082994
Size*Organ	0,4283	1	0,4283	32,06	0,000000
CN-*Organ	1,3374	4	0,3344	25,03	0,000000
Size*CN-*Organ	0,4844	4	0,1211	9,07	0,000004
Error	1,0685	80	0,0134		

Results of three-way ANOVA evaluating the interaction of CN^- (different concentrations), size (small and medium) and tissues (gills, muscle and liver) in GST of *Amphiprion ocellaris*.

	SS	***Degr. of Freedom***	***MS***	***F***	***p***
Intercept	99,17386	1	99,17386	7428,484	0,000000
Size	1,10678	1	1,10678	82,902	0,000000
CN-	0,75142	4	0,18785	14,071	0,000000
Organ	26,37751	2	13,18876	987,886	0,000000
Size*CN-	0,32193	4	0,08048	6,028	0,000187
Size*Organ	1,10934	2	0,55467	41,547	0,000000
CN-*Organ	3,88286	8	0,48536	36,355	0,000000
Size*CN-*Organ	2,90840	8	0,36355	27,231	0,000000
Error	1,60206	120	0,01335		

Results of three-way ANOVA evaluating the interaction of CN^- (different concentrations), size (small and medium) and tissues (gills, muscle and liver) in CAT of *Amphiprion ocellaris*.

	SS	***Degr. of Freedom***	***MS***	***F***	***p***
Intercept	131,6694	1	131,6694	4708,432	0,000000
Size	0,7873	1	0,7873	28,155	0,000001
CN-	5,6476	4	1,4119	50,489	0,000000
Organ	12,6562	2	6,3281	226,290	0,000000
Size*CN-	0,9407	4	0,2352	8,410	0,000005
Size*Organ	2,4508	2	1,2254	43,820	0,000000
CN-*Organ	6,9733	8	0,8717	31,170	0,000000
Size*CN-*Organ	3,1011	8	0,3876	13,862	0,000000
Error	3,3558	120	0,0280		

Results of three-way ANOVA evaluating the interaction of CN^- (different concentrations), size (small and medium) and tissues (gills, muscle and liver) in GPx of *Amphiprion ocellaris*.

	SS	***Degr. of Freedom***	***MS***	***F***	***p***
Intercept	158,9252	1	158,9252	3643,625	0,000000
Size	1,0718	1	1,0718	24,573	0,000002
CN-	1,0416	4	0,2604	5,970	0,000205
Organ	0,0389	2	0,0194	0,445	0,641595
Size*CN-	0,1739	4	0,0435	0,997	0,412053
Size*Organ	0,3463	2	0,1732	3,970	0,021404
CN-*Organ	0,9638	8	0,1205	2,762	0,007758
Size*CN-*Organ	1,0423	8	0,1303	2,987	0,004347
Error	5,2341	120	0,0436		

Results of two-way ANOVA evaluating the interaction of CN^- (different concentrations) and size (small and medium) in AChE of *Amphiprion ocellaris*.

	SS	***Degr. of Freedom***	***MS***	***F***	*p*
Intercept	228,3719	1	228,3719	26245,16	0,000000
Size	0,3397	1	0,3397	39,04	0,000000
CN-	0,1039	4	0,0260	2,99	0,030107
Size*CN-	0,2424	4	0,0606	6,97	0,000236
Error	0,3481	40	0,0087		

Statistical Analysis (Chapter 3)

Results of three-way ANOVA evaluating the interaction of CN^- (0 or 25 mg L^{-1}), temperatures (26 °C, 29 °C and 32 °C) and tissues (gills and muscle) in LPO of *Amphiprion ocellaris*.

	SS	***Degr. of Freedom***	***MS***	***F***	***p***
Intercept	247,4032	1	247,4032	16333,73	0,000000
Temperature	0,9510	2	0,4755	31,39	0,000000
CN-	0,0014	1	0,0014	0,09	0,765038
Organ	3,2951	1	3,2951	217,55	0,000000
Temperature *CN-	0,7451	2	0,3726	24,60	0,000000
Temperature *Organ	0,8293	2	0,4147	27,38	0,000000
CN-*Organ	0,1059	1	0,1059	6,99	0,011023
Temperature *CN-*Organ	0,0231	2	0,0115	0,76	0,472169
Error	0,7270	48	0,0151		

Results of three-way ANOVA evaluating the interaction of CN^- (0 or 25 mg L^{-1}), temperatures (26 °C, 29 °C and 32 °C) and tissues (gills, muscle and liver) in GST of *Amphiprion ocellaris*.

	SS	***Degr. of Freedom***	***MS***	***F***	***p***
Intercept	100,1931	1	100,1931	9342,423	0,000000
Temperature	0,0487	2	0,0243	2,270	0,110703
CN-	0,7039	1	0,7039	65,637	0,000000
Organ	7,1850	2	3,5925	334,980	0,000000
Temperature *CN-	0,6964	2	0,3482	32,468	0,000000
Temperature *Organ	0,3989	4	0,0997	9,299	0,000004
CN-*Organ	0,9647	2	0,4824	44,977	0,000000
Temperature *CN-*Organ	0,8011	4	0,2003	18,674	0,000000
Error	0,7722	72	0,0107		

Results of three-way ANOVA evaluating the interaction of CN^- (0 or 25 mg L^{-1}), temperatures (26 °C, 29 °C and 32 °C) and tissues (gills, muscle and liver) in CAT of *Amphiprion ocellaris*.

	SS	*Degr. of Freedom*	*MS*	*F*	*p*
Intercept	48,52921	1	48,52921	2087,824	0,000000
Temperature	0,38127	2	0,19063	8,201	0,000618
CN-	0,18532	1	0,18532	7,973	0,006137
Organ	7,71675	2	3,85838	165,995	0,000000
Temperature *CN-	0,01627	2	0,00814	0,350	0,705852
Temperature *Organ	4,25224	4	1,06306	45,735	0,000000
CN-*Organ	0,26629	2	0,13315	5,728	0,004914
Temperature *CN-*Organ	3,11329	4	0,77832	33,485	0,000000
Error	1,67356	72	0,02324		

Results of three-way ANOVA evaluating the interaction of CN^- (0 or 25 mg L^{-1}), temperatures (26 °C, 29 °C and 32 °C) and tissues (gills, muscle and liver) in GPx of *Amphiprion ocellaris*.

	SS	*Degr. of Freedom*	*MS*	*F*	*p*
Intercept	106,8250	1	106,8250	4660,357	0,000000
Temperature	0,1682	2	0,0841	3,669	0,030387
CN-	0,0292	1	0,0292	1,272	0,263139
Organ	1,0932	2	0,5466	23,845	0,000000
Temperature *CN-	0,0860	2	0,0430	1,875	0,160722
Temperature *Organ	0,7845	4	0,1961	8,556	0,000010
CN-*Organ	0,1617	2	0,0808	3,527	0,034579
Temperature *CN-*Organ	0,8040	4	0,2010	8,769	0,000008
Error	1,6504	72	0,0229		

Results of two-way ANOVA evaluating the interaction of CN^- (0 or 25 mg L^{-1}) and temperatures (26 °C, 29 °C and 32 °C) in AChE of *Amphiprion ocellaris*.

	SS	*Degr. of Freedom*	*MS*	*F*	*p*
Intercept	120,9256	1	120,9256	37111,27	0,000000
Temperature	0,0014	2	0,0007	0,21	0,809022
CN-	0,0090	1	0,0090	2,77	0,109017
Temperature *CN-	0,0061	2	0,0030	0,93	0,407863
Error	0,0782	24	0,0033		

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