Effects of pesticide exposure and thermal stress in a model tropical reef fish, the damselfish *Acanthochromis polyacanthus*

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THESIS DEDICATION

To my parents

STATEMENT OF CONTRIBUTION OF OTHERS

At the time of submission of this PhD thesis, Chapter 2 is available electronically (Botté et al., in press).

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ABSTRACT

Coastal ecosystems including tropical coral reefs are increasingly threatened by land based pollution which is now occurring simultaneously with an increase in ocean temperatures. Despite the high ecological and economical importance of these ecosystems, the impacts of pollutants and thermal stress on tropical marine organisms have only recently been considered and are usually studied in isolation to each other. In South-East Asia and North-Eastern Australia bordering the Great Barrier Reef (GBR), one of the most widely used insecticides is the organophosphate (OP) chlorpyrifos (CPF). In both regions, the wet season produces high rainfall and significant flood plumes transporting CPF onto inshore reefs at a time when reefs also experience high summer water temperatures. A few studies have shown that the damselfish *Acanthochromis polyacanthus* from the Indo-Pacific is sensitive to stress and this fish is becoming a model species in ecophysiology. Still, the underlying mechanisms leading to such sensitivity are largely unidentified. More importantly, very little is known about the effects of simultaneous stressors on reef fish generally. Consequently, this thesis aims to provide new insights into the neurophysiology, oxidative stress response and molecular stress response of a model tropical reef fish from the Indo-Pacific region, the damselfish *Acanthochromis polyacanthus*, after exposure to CPF and/or thermal stress under laboratory-controlled conditions.

Juveniles of *A. polyacanthus* bred at James Cook University were exposed to a) CPF alone, b) temperature stress and c) simultaneous CPF and temperature stresses. Stress markers were used to examine different types of stress response. Activity of the neural enzyme Cholinesterase (ChE) was determined in muscle, as it is recognized as a sensitive indicator for OP exposure. Characterization of ChE was also undertaken to

determine the predominant form of the enzyme in muscle tissue in this model reef fish species. To explore potential oxidative stress responses two key antioxidant molecules were examined. This included measurement of the ratio of the Coenzyme Q antioxidant form ($CoQH_2$) over total CoQ, as well as measurement of the activity of the detoxifying enzyme glutathione-S-transferase (GST) in liver tissue. Finally, expression of candidate stress-responsive genes (heat-shock protein 90, GST, Catalase, Elongation Factor 1α) was examined to explore the molecular response to CPF exposure and thermal stress in *A. polyacanthus*.

Prior to experimental CPF exposure, ChE characterization revealed the presence of a complex mixture of ChEs in *A. polyacanthus* muscle tissue. This mixture was comprised of an atypical vertebrate acetylcholinesterase (AChE) form, as well as a typical vertebrate AChE and an atypical vertebrate butyrylcholinesterase (BChE). Reports in the literature of two other Perciforme species possessing similar ChEs and inhabiting coral reefs suggests evolutionary implications of atypical ChEs and raises the questions of their functional significance in coral reef fish.

Laboratory-controlled CPF exposure revealed that fish exposed to 1 µg/L, 10 µg/L and 100 µg/L of CPF for 96 h (4 days) exhibited a 26%, 49% and 53% decrease in cholinesterase activity, respectively, compared to solvent control fish. This demonstrates that ChE inhibition is a sensitive marker for CPF exposure in *A. polyacanthus*. Furthermore, an increase in the antioxidant CoQ form was found after just 6 h of exposure to 10 g/L CPF, suggesting an early oxidative stress effect and potentially the involvement of $CoQH_2$ to combat CPF-induced oxidative stress.

A thermal stress experiment using a slow increase in temperature revealed that exposure of the fish to 32°C or 34°C compared to 28°C induced a 50% reduction in ChE activity after 96 h of exposure. Most importantly, after 7 days of recovery at 28°C, ChE activity still remained 39% lower in thermally stressed fish, compared to fish only exposed to acclimation temperature (28°C) during the entire experiment. *In vitro* measurements of ChE activity indicated that temperature does not directly impact the activity of the enzyme itself between 26°C and 34°C, which clearly indicates that the *in vivo* decline in *A. polyacanthus* ChE activity associated with temperature increase is the result of a biological stress response in the fish. In addition, CoQ showed a decrease in antioxidant form in the early time points of the experiment in fish exposed to 34°C compared to 28°C, which suggests that this molecule is used to combat temperature-stress induced oxidative stress in liver cells. The results of this chapter demonstrate for the first time a link between thermal stress and a dysfunction of a neural enzyme in a tropical reef fish, which might have important implications for fish fitness and behaviour in the context of changing climates.

CPF exposure and thermal stress were also applied simultaneously on juvenile *A. polyacanthus*. Combined effects were tested with 1 and 10 µg/L CPF at 28°C or 31°C. No interaction between the two factors was found for ChE activity, but ChE activity was decreased by CPF exposure after 7 days of exposure. In addition, the temperature increase of 3°C provided a small measure of protection against CPF at 31°C, since fish exposed to both CPF and 31°C exhibited higher ChE activity than fish exposed to CPF at 28°C. The results obtained for ChE activity across the three experiments indicated a thermal threshold for ChE activity between 31°C and 32°C in

A. polyacanthus, which corroborates whole organism responses in the recent literature on this species. In addition, GST activity also decreased in fish exposed to 31°C compared to 28°C, further supporting the hypothesis of a fine thermal threshold for this species. Finally, four target genes exhibited lower expression in fish exposed to CPF compared to control fish, after 4 days of treatment. This may indicate that a general decrease in gene expression occurred in response to CPF exposure in *A. polyacanthus*. Such results indicate that CPF affects many cellular functions in *A. polyacanthus* over even this short time-frame.

This thesis presents novel information on the response to stress in *A. polyacanthus*, a model species for the Indo-Pacific region. It identifies a strong and prolonged effect of temperature on ChE, a central enzyme in the nervous system of the fish, provides evidence for the use of antioxidant molecule during both CPF exposure and thermal stress, determines a general decline in gene expression as a result of CPF exposure and most importantly suggests that this species might possess a fine thermal threshold at between 31°C and 32°C. Together, these results indicate that *A. polyacanthus* is sensitive to both CPF exposure and thermal stress and that numerous functions are negatively affected by both stressors. While combined pressures of pollution and thermal stress are generally considered to be worse than individual pressures, this study also revealed that fish may in fact cope better with some pollutants at temperatures just below their thermal threshold. However, increases in runoff-associated pollution are likely to negatively affect *A. polyacanthus* and other tropical fish as climate change increases sea surface temperatures beyond their narrow thermal tolerance.

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1. GENERAL INTRODUCTION

1.1. POLLUTION AND CLIMATE PRESSURES

1.1.1. On coastal ecosystems

Natural ecosystems are experiencing increasing pressures due to anthropogenic activities leading to natural habitat destruction on a global scale (Di Poi et al., 2011). Interactions between species and their surrounding environment are changing more rapidly since industrialisation than at any other time. In the marine environment, this is particularly true for coastal ecosystems, which are becoming more accessible to human populations and therefore more readily exploited and damaged by physical impacts and increasing pollution. Pesticides, which comprise insecticides, herbicides and fungicides, are among the many toxicants released into coastal waters that have the potential to affect marine coastal populations (Todd et al., 2010). As such, they are responsible for a large proportion of fish kills reported in estuaries and freshwater systems and constitute a considerable threat to coastal marine ecosystems (La and Cooke, 2010).

In addition to increasing chemical stresses, marine organisms also face rises in sea surface temperatures (SSTs) as the climate changes. Global average temperatures have increased by 0.5°C between 1850 and 2007 (IPCC report, 2007) and it is projected that they will increase by 1.1°C to 6.4°C by 2100, with best estimates ranging from 1.8° to 4°C compared to average temperatures between 1980 to 1999 (IPCC, 2007). Such temperature increases have affected, and will continue to impact, ecological processes such as the phenology, distribution, growth, survival and reproduction of marine species (Roessig et al., 2004, Walther et al., 2002).

1.1.2. On tropical reefs

In light of increased stress levels on marine coastal species, and the deleterious effects of these stresses, inhabitants of marine coastal ecosystems must either adapt to changing conditions or disperse for survival (Berg et al., 2010). From this point of view, species that are restricted to a narrow range of environmental conditions face a greater challenge from climate and pollution impacts than species which can withstand greater variations in their habitat. This is particularly relevant for many tropical coral reef species which have evolved in conditions of high water quality and currently exist close to their upper thermal tolerance (Schiedek et al., 2007). The situation is particularly alarming for South-East Asian reefs. While the South-East Asian region harbours 34% of the planet's coral reefs, 80% are threatened by human activities due to high population density in coastal areas, overexploitation of marine resources, urbanization and associated increases in pollution (Todd et al., 2010).

The Great Barrier Reef (GBR) of Australia is composed of similar habitats as those found in the highly impacted South-East Asian reefs and many coral reef species are common to both regions. Although the GBR does not face the same immediate threat from overexploitation or urban pollution, due in part to the high level of protection afforded by its World Heritage Site status, the GBR is presently experiencing significant degradation of some coastal ecosystems within its confines (Hughes et al., 2003). Terrestrial run-off and associated increase in sediments do occur naturally within the GBR (Wilson et al., 2006), but these phenomena have amplified and have become a greater threat since European settlement of catchments bordering the GBR (Hutchings and Haynes, 2000). Similar to what has happened in South-East Asia, there has been a consistent increase in coastal development, sedimentation, eutrophication and pollution from sewage and contaminated waters

along the length of the GBR since the late 1970's. The dramatic increase in land-clearing to provide space for agriculture, farming and other human activities has resulted in the loss of 50% of land cover since European settlement (Fabricius, 2005, Haynes and Michalek-Wagner, 2000, McCulloch et al., 2003). Overall, the loss of land cover and river contamination has resulted in both the intensification of terrestrial run-off and a change in the chemical composition of the sediments and water entering the coastal reefs of the GBR over the last three decades (Johnson et al., 2000, Lewis et al., 2009, Olafson, 1978, Packett et al., 2009, Richmond, 1993, Shaw et al., 2010).

Most importantly, ocean warming might accelerate the deleterious effects of terrestrial runoff (Schiedek et al., 2007). For example, tropical cyclones are predicted to increase in intensity as a result of climate change, which, as well as causing immediate mechanical stress to reefs, will enhance terrestrial run off leading to increased amounts of sediments, nutrients and chemicals entering the coastal waters (Lough, 2007, Schiedek et al., 2007). Consequently, inshore reef species might be even more impacted in the future and research must address the question of their ability to respond to simultaneous pollution exposure and increasing temperatures.

1.2. PESTICIDE THREAT

1.2.1. Pesticide use in tropical countries

Coastal marine species inhabiting tropical countries have been increasingly exposed to pesticide toxicity in the last two decades. Indeed, many tropical countries have experienced important changes in land use, due to growing economies which have shifted small scale traditional agriculture to high productivity practices. As a

result, pesticide use has become increasingly common in these countries. A striking example is Costa Rica which, despite its small size, became the second ranked country for pesticide use in 2000, with 57,000 tonnes applied that year (Polidoro et al., 2008). The situation is comparable in South-East Asia, where pesticide use is now common practice in countries harbouring coral reefs such as Malaysia, Indonesia, Thailand and the Philippines (Ciglasch et al., 2006, Kaczmarsky and Richardson, 2011, Leong et al., 2007, Moeskops et al., 2010). In South-East Asia, soil erosion is amplified by the high rainfalls that characterize the wet season and participate in increasing terrestrial runoff. Similar weather patterns apply in North Queensland, along the GBR, with an estimated 37% of rainfall ending up as run-off, spreading measurable levels of land-based contaminants into river catchments that directly drain into the GBR lagoon (Johnson et al., 1999). Agricultural chemicals can also enter coastal waters via other routes such as direct spray drift during their application (Schulz, 2004). The Queensland coastline proximate to the Northern GBR is a region of high sugar cane cultivation within 26 GBR associated river catchments, encompassing over 230,000 ha of production within 50 km of the coastline (Humphrey and Klumpp, 2003). To sustain itself this agricultural industry uses pesticides and fertilizers with 30,000 tonnes of pesticides applied in Australia annually (Radcliffe, 2002). Overall, the use of pesticides in proximity to coral reefs, and the similar climate of North Queensland and South-East Asia, highlights the potential for inshore reefs of the GBR to be used as models for impacts of pesticides in the broader Indo-Pacific region.

1.2.2. Chlorpyrifos (CPF), a widely used insecticide

One of the most heavily used agricultural pesticides is the insecticide chlorpyrifos (CPF). In South-East Asian countries, it is mainly used on rice fields. As an example of the contribution of CPF to pollution of Asian waterways, this single toxicant contributed as much as 35% of the pesticides detected in the Selangor River in Malaysia (Leong et al., 2007). Chlorpyrifos is a broad-spectrum organophosphate (OP) compound, also widely used by Queensland's sugar cane industry to protect crops from cane beetles and other insects (National Registration Authority (NRA), 2000). Organophosphates such as CPF act as strong and irreversible inhibitors of cholinesterase (ChE) enzymes. Critically these enzymes play a vital role in the transmission of nerve impulses in the central nervous system (see Mileson et al., 1998 for review). Organophosphates and carbamates have replaced organochlorins, which were banned from agricultural use in the 1970's due to their high persistence in water and the threat they pose to aquatic organisms through bioaccumulation (Vonwesternhagen and Klumpp, 1995). However, OPs are also moderately persistent, particularly in seawater, and themselves represent a potential threat to marine organisms (Bondarenko et al., 2004).

CPF takes as long as 7.5 days to degrade in seawater and takes much longer (up to 24 days) to degrade in sediments (Lalah et al., 2003, Odenkirchen and Eisler, 1988). Of particular concern is, however, that CPF lacks target specificity and affects not only its intended target (insects) but also mammals and numerous aquatic species including molluscs and fish (Fulton and Key, 2001). Chlorpyrifos is rapidly taken up by both oysters and fish and can easily bioconcentrate leading to deleterious physiological effects. As an example of CPF toxicity to fish, two significant fish kills

occurred in Queensland waters in 1995 and 1996 due to CPF-contaminated stormwater, with measured concentrations of the toxic compound in the range of 0.5 to 25 µg/L (NRA, 2000). More recently, the Australian Pesticides and Veterinary Medicines Authority (APVMA) reassessed the risks associated with the use of CPF on crops, according to newer data available on maximum residue limits (APVMA, 2009). This assessment led to the prohibition of this insecticide on crops such as peaches and tree nuts, while CPF usage was restricted to certain timings on crops such as canola, bananas trees or sugarcane. In particular, CPF spraying on sugar cane fields was restricted to the first three months after sugar cane planting or rationing, due to the lack of maximum residue limits data after this period (APVMA, 2009). In Queensland, planting occurs during the wet season in January and February, which enhances the risk of pesticide run-off into the GBR lagoon and onto inshore reefs. Because of CPF's widespread use, its relative persistence in water and lack of target specificity, this pesticide is considered an important threat to the inshore reefs of the Northern GBR and associated fish species (Humphrey and Klumpp, 2004).

1.2.3. Effects of CPF on fish

Fish kills have been reported in the wild as a consequence of exposure to high levels of pesticides (La and Cooke, 2010). At sublethal concentrations, pesticide exposure in fish has reportedly induced a range of adverse effects including changes in enzyme activity, endocrine disruption, impaired reproduction and biased sex ratio (Arcand-Hoy and Benson, 1998, Kime, 1995). In addition, other toxic effects reported from pesticide exposure include immunotoxicity (Galloway and Handy, 2003), cytotoxicity *via* oxidative stress (Livingstone, 2001), genotoxicity *via* DNA strand breaks (Bony et al., 2008) and behaviour impairment (Scott and Sloman, 2004). The

effects of CPF in particular are well-documented on temperate and freshwater fish. Exposure of temperate freshwater fish to sub-lethal concentrations of CPF decreases ChE activity and antioxidant enzyme activities (Kavitha and Rao, 2008, Wang et al., 2009), reduces bone strength (Karen et al., 1998), olfactory capabilities (Sandahl et al., 2005) and swimming performances (Kavitha and Rao, 2008). It also activates the transcription of cytokine genes and genes encoding stress response proteins such as Heat Shock Proteins (HSP) (Eder et al., 2004).

Despite the potential widespread threat of this pesticide to tropical fish, very few studies have tested the response to CPF exposure in a tropical reef fish. To date, only eight studies have investigated the impacts of CPF exposure on tropical fish. Most of these studies have measured only a single marker, namely ChE inhibition, and six have used the freshwater Nile tilapia *Oreochromis niloticus* as the study species (Chandrasekara and Pathiratne, 2007, Gold-Bouchot et al., 2006, Pathiratne et al., 2008, Pathiratne et al., 2009). The very limited work carried out to understand CPF effects in tropical fish also includes a study on a damselfish from the GBR, *Pomacentrus amboinensis.* In this species, CPF exposure induced deformities in newly hatched eggs, decreased survival and reduced length at hatch (Humphrey et al., 2004). However, measurements of ChE activity were not performed, and other cellular effects on antioxidant enzyme activities or gene expression changes remain understudied in tropical reef fish in response to CPF exposure. Interestingly, pesticides, and biochemical signals of OP exposure have even been detected in crab tissues where water sample analysis has failed to detect measurable levels of these pollutants (van Oosterom et al., 2010). In addition, several biomarkers of pollutant exposure were applied to barramundi (*Lates calcarifer*) tissues from Northern

Australia and complemented chemical analysis data (Humphrey et al., 2007). This highlights the substantial value of using biological indicators of exposure in addition to chemical analysis when investigating the presence of contaminants in aquatic ecosystems. Given the importance of South-East Asian reefs and the GBR from both an ecological and economic point of view, and the limited knowledge about CPF effects in tropical fish, enhanced research effort on reef fish toxicology is paramount.

1.3. TEMPERATURE THREAT

1.3.1. Thermal stress on coral reefs

Along with coastal pollution, increases in ocean temperatures are evident worldwide, and tropical reefs are no exception (IPCC, 2007). For instance, the Red Sea has experienced a significant increase in temperature in 1994, with a 0.7°C increase between the averages of 1985-1993 and 1994-2007 (Raitsos et al., 2011), and reefs from the GBR are predicted to be 0.5°C warmer by the end of the present decade compared to the average of 1969-1990 (Lough, 2007). Severe large-scale positive thermal anomalies during unusually warm summers have already resulted in mass mortalities of corals globally and these large-scale events are widely regarded as a result of climate change. Notably in 1998, 16% of the world's coral reefs were killed by coral bleaching associated with unusually high sea surface temperatures (Hughes et al., 2003), highlighting the vulnerability of coral reefs to temperature stress (Hughes et al., 2011). On the GBR, climate change was rated before water quality issues as the greatest threat to coral reef resilience, suggesting that this global scale threat is likely to reduce the ability of coral reefs to recover from natural disturbances and may limit recovery from anthropogenic stressors such as localised pollution events (Johansen and Jones, 2011). The global nature of the climate threat means that

local policies implemented to reduce climate change impacts will have little direct effect at reducing impacts on local reefs (Bohensky et al., 2011). Climate impacts are therefore less manageable than pollution impacts and thermal stress will continue to be a major threat for coral reefs for the foreseeable future.

1.3.2. Effects of upper thermal stress on tropical reef fish

Thermal stress events associated with unusually high temperatures have been regularly reported in the last decade and have led to measurable changes in reef fish community assemblages (Adam et al., 2011, Bellwood et al., 2006, Booth and Beretta, 2002). More recently, data has become available on the direct effects of high temperature stress at the individual level in reef fish. For example, the thermal tolerance of several Indonesian reef fish species was investigated by directly transferring fish from 26°C to 32°C and assessing the Q10, which is a measure of "the relationship between temperature change and metabolic rate" (Eme and Bennett, 2009). The results showed that fish from thermally stable environments such as reef fish are much more sensitive to heat shock than fish living in environments where temperature greatly fluctuates over a day, such as mangrove tidepools. This study therefore demonstrated that the metabolic response of the fish reflected the thermal habitats of the different species studied. Similarly, Kassahn and colleagues (2007) exposed the lemon damselfish *Pomacentrus moluccensis* to heat shock from 28°C to 31°C or 34°C and identified thermal stress responsive genes. Recently, Nilsson and colleagues (2009) exposed several species of cardinalfish and damselfish from the GBR to increasing temperature from 28°C to 31°C, 32°C or 33°C over several days and showed that these fishes exhibit a reduced aerobic scope when exposed to thermal stress. *A. polyacanthus* reared at higher temperatures (+3°C compared to natural

environment temperatures) also showed a decline in both size and health compared to fish reared at natural temperatures (Donelson et al., 2011). In addition, *A. polyacanthus* from the Northern GBR experiences a decrease in growth when exposed to 31°C instead of 28°C (Donelson et al., 2010a, Munday et al., 2008). The higher temperature used in these last two studies (31°C) actually corresponds to the current estimates projected to occur as average summer temperatures by 2100 in waters surrounding the GBR (Lough, 2007). Such data illustrate the impact small rises in temperatures can have on tropical reef fish and highlight the importance of understanding the underlying mechanisms responsible for them. Earlier studies have also provided valuable information on the *A. polyacanthus* endocrine system, its response to handling stress and temperature decrease (Begg and Pankhurst, 2004, Pankhurst, 2001, Pankhurst et al., 1999). However, studies investigating the effects of temperature increase on tropical reef fish at the cellular level remain scarce, especially compared to the abundant literature available on the cellular response to stress in temperate fish (reviewed in Bonga 1997).

1.3.3. The spiny damselfish, an emerging model tropical reef fish species

The increased interest and research carried out on the widespread Indo-Pacific damselfish *Acanthochromis polyacanthus* over the last decade, and particularly over the last 5 years (see Section 1.3.2), illustrates the potential for this tropical reef fish to be considered a model organism with which to study the impacts of natural and anthropogenic stressors, including thermal stress and CPF exposure. *A. polyacanthus* is abundant throughout the Indo-Pacific region (Fig.1.1), where it occurs on tropical reefs within the first 10 m of the water column. The biology of this fish confers significant advantages for both laboratory and field-based research, as it lacks a

pelagic larval phase and is therefore easy to breed in captivity. The species is also very well suited for laboratory studies as its relatively small size (maximum length of 14 cm) makes it easy to house in small aquaria. In addition it survives well in aquarium systems for several years. The life cycle of this species means that *A. polyacanthus* spends its entire life cycle within the confines of tropical reefs and genetic studies suggest very limited dispersal potential of fish from their natal reef (Miller-Sims et al., 2008). This strong site fidelity over the entirety of the life cycle makes this species particularly useful as a bioindicator species for environmental exposure to pollutants or other stressors on individual reefs. However, before the potential of this reef fish species to become a model for toxicology studies can be realised it is vital to develop a solid understanding of its stress responses to a variety of single and combined stressors in the laboratory.

Fig.1.1. *A. polyancanthus* and its geographical range in the Indo-Pacific region.
Photo credit: www.aquarium-photo.com and fishbase.org

1.4. COMBINING PESTICIDE EXPOSURE AND THERMAL STRESS

1.4.1. Increasing pollution in the context of climate change

With globally increasing temperatures and coastal pollution now a reality it is increasingly likely that tropical reef fish such as *A. polyacanthus* will be simultaneously exposed to both pollutants and thermal stress. Increasing temperatures may have direct implications for pollutant exposure as recent reviews indicate that

higher temperatures are likely to increase pesticide volatility and decrease persistence in soil (reviewed in Noyes, 2009). Climate change may therefore lead to an increase in the use of pesticide (Boxall et al., 2009), although pesticide usage in this context will largely depend on a country's policies towards regulation of pesticide use in response to decreased pesticide persistence in soil, or changes in pest distribution and abundance (Bloomfield et al., 2006). In addition, the chemical processes leading to pesticide fate depend on numerous factors, such as soil composition, humidity and soil topology and these characteristics vary considerably from one geographical region to another. As a consequence, it is difficult to predict the general behaviour of a given pesticide in relation to temperature without empirical studies.

1.4.2. Combined effects of pollutants and climate change on fish

Pesticide toxicity is usually increased with increasing temperatures as was demonstrated for many temperate species and recently reviewed in Schiedek et al. (2007). For example, carbaryl, a cholinesterase inhibitor was more toxic to temperate green frog tadpoles at 27°C than at 17°C (Boone and Bridges, 1999). In addition, thermal tolerance can be decreased by exposure to contaminants, as was found in four Australian freshwater fish species, the silver perch *Bidyanus bidyanus*, eastern rainbowfish *Melanotaenia duboulayi*, western carp gudgeon *Hypseleotris klunzingeri*, and rainbow trout *Oncorhynchus mykiss* (Patra et al., 2007). In that study, CPF was actually the most potent compound tested, with exposure to 5 µg/L for 14 days leading to a 5.8°C reduction in thermal tolerance of *Oncorhynchus mykiss.* Despite the multiple threats identified on coastal tropical reefs, very few studies have examined the impacts of simultaneous exposure to multiple stressors, such as pesticide exposure and thermal stress, on the overall stress response in tropical fish.

Humphrey and Klump (2003) provided one of the few studies available on this subject and reported evidence of increased toxicity of CPF in the eastern rainbowfish *Melanotaenia splendida splendida*, a freshwater fish, when exposed to a simultaneous temperature increase. Fish exposed to 500 µg/L CPF at 29°C exhibited 62% mortality after 4 days of exposure, as opposed to only 7% mortality at 25°C. In view of the rapid degradation of inshore reefs worldwide, and in particular in the Indo-Pacific region, empirical studies testing the effects of simultaneous pesticide exposure and thermal stress on tropical reef fish are urgently needed if we are to fully appreciate the impacts of simultaneous increasing pollution and climate change on coral reef fish ecosystems.

1.4.3. Methods used to detect stress in tropical fish

The impacts of both CPF and temperature increase on tropical reef fish can be assessed using stress markers such as those that have been applied on temperate species. The main method used for detecting and quantifying toxicity of CPF in living organisms including fish involves measurement of ChE activity, which is specifically inhibited by CPF and therefore decreases upon exposure. However, using multiple stress markers enables a broader view of the cellular response of an organism to stress. In the last decade, CPF was found to induce oxidative stress in rodents and human cells, suggesting that oxidative stress induction might be a mode of action of CPF (Mansour and Mossa, 2009, Verma et al., 2007). Little is known about this aspect of CPF toxicity in fish however. Consequently, measuring the activity of detoxifying and antioxidant enzymes such as the Glutathione-dependent enzymes, including glutathione-S-transferase (GST) could prove valuable to detect oxidative stress in fish exposed to CPF and/or thermal stress. Typically, GST use glutathione in

order to neutralise cytotoxic compounds and electrophilic molecules such as reactive oxygen species (ROS), which are then excreted from the cell (reviewed in Hayes, 2005). Another oxidative stress marker was also recently used for measuring pollutant-induced oxidative stress in fish (Hasbi et al., 2011): coenzyme Q (CoQ) possesses antioxidant properties and scavenges reactive oxygen species in the mitochondrion, thereby protecting the cell from deleterious molecules, but its efficiency has never been tested under thermally-induced oxidative stress. In addition, the validity of using this molecule to detect stress has not been proven for tropical reef fish species. Finally, molecular-based approaches that target gene expression of classic stress response genes are increasingly being used to better understand the toxicity of CPF and other OPs to aquatic species. A good example is the ubiquitous transcriptional activation of the Heat Shock Protein (HSP) gene family in response to a variety of stressors, including thermal stress and toxicant exposure, as was recently reviewed (Iwama et al., 1998, Kalmar and Greensmith, 2009). For instance, enhanced *hsp* gene expression has been measured in response to contaminants such as pesticides (Eder et al., 2008, Eder et al., 2004) including OP and other insecticides with up-regulation of *hsp* genes reported in Chinook salmon (*Oncorhynchus tshawytscha*), the striped catfish (*Pangasianodon hypophthalmus*) and juvenile striped bass *Morone saxatilis* (Eder et al., 2008, Geist et al., 2007, Sinha et al., 2010). Changes in gene expression were also assessed in response to thermal stress on a GBR fish, the lemon damselfish *Pomacentrus moluccensis*, allowing the identification of several stress-responsive genes in a tropical reef fish (Kassahn et al., 2007b). Given the limited knowledge about the response to stress in tropical reef fish and the availability of stress markers in the literature, the use of ChE activity, antioxidant enzymes and

antioxidant molecules, as well as stress-responsive genes seems extremely valuable to understand the impacts of CPF exposure and thermal stress in tropical reef fish.

1.5. THESIS OBJECTIVES

Considering the chemical and physical stresses that the inshore reefs of the Indo-Pacific region are facing, and their high ecological and economical value, as well as the gaps in knowledge identified in the literature and the available tools to detect stress in fish, the present thesis aims to examine the stress responses to both CPF exposure and thermal stress in the model tropical reef fish *Acanthochromis polyacanthus*. The present work utilizes both well-established and novel stress markers in the field of fish ecotoxicology to fulfil its aim. The specific thesis objectives are therefore:

1) to test the sensitivity of juvenile *A. polyacanthus* to the insecticide CPF
2) to investigate the cellular response to stress in juvenile *A. polyacanthus* exposed to ecologically relevant temperature increase.
3) to provide novel information about the single and combined impact of these stressors on the central nervous system and oxidative stress system of *A. polyacanthus*
4) to investigate the transcriptomic changes occurring in *A. polyacanthus* as a result of exposure to CPF and/or thermal stress.

1.6. CHAPTER SYNOPSES

In the second chapter of this thesis, the response of *A. polyacanthus* to CPF exposure alone is investigated using three different CPF concentrations (1, 10 and 100 µg/L). The composition of the different ChEs present in muscle tissue of this species

is elucidated for the first time and the cellular stress response of *A. polyacanthus* to CPF exposure is assessed via the measurement of ChE activity, CoQ antioxidant ratio and GST activity. The use of ChE activity as a sensitive marker for CPF exposure is validated and signs of early oxidative stress in the liver tissue are detected.

In Chapter 3, the response of *A. polyacanthus* to temperature stress is assessed in the context of realistic temperature increases under future climate change scenarios. In order to do so, the impact of a slow increase in temperature of 4°C and 6°C, from 28ºC up to 32ºC and 34ºC, is used as opposed to a rapid heat shock procedure. This chapter provides the first evidence of a prolonged decline in neural enzyme activity (ChEs) with thermal stress in a fish species. *In vitro* evidence also demonstrates that this decrease is very likely to be due to a stress response within the fish, rather than a change in enzyme-substrate affinity or reaction rate. Finally, it provides evidence for a role of the CoQ redox balance in the pathways used by *A. polyacanthus* to detoxify liver cells from temperature-induced ROS.

In the last data chapter (Chapter 4), both stressors are combined in order to investigate the potential additive or interactive effects of both CPF exposure and thermal stress. Two CPF concentrations (1 and 10 µg/L) and an increase in temperature (31°C versus 28°C) are tested in this chapter. An additive effect between the two stress factors applied is seen on ChE activity. Together with the results of Chapter 2, this chapter provides further grounds for the existence of a fine thermal tolerance threshold between 31°C and 32°C for *A. polyacanthus.* Finally, the expression patterns of four target genes, namely Heat-shock protein 90 (*hsp90*), Elongation Factor 1 alpha (*ef1α*), Catalase (*cat*) and Glutathione-S-Taransferase (*gst*),

indicate a temporary but generally depressive effect of CPF exposure on gene transcription after 4 days of exposure, as well as an effect of temperature on antioxidant enzyme-encoding genes.

The implications of the findings and conclusions of the successive chapters are discussed in the last chapter, which places the present thesis in the context of a global increase in pesticide usage and ocean temperatures and presents the future research directions needed in the field of tropical ecotoxicology.

2. Effects of chlorpyrifos on cholinesterase activity and stress markers in *Acanthochromis polyacanthus*

2.1. INTRODUCTION

The use of chemicals for agricultural pest control has significantly increased over the last 20 years. This is particularly true for developing countries of tropical Asia and South America where a rapidly growing population requires increased agricultural production and where pesticides and fertilizers have become more accessible to farmers. Contamination of aquatic ecosystems by pesticides is now evident in southern and South East Asia (Leong et al., 2007), Central America (Carvalho et al., 2002) and South America (Laabs et al., 2007). Along with estuaries and mangrove habitats, tropical reefs are also threatened by increased population and pollution (Wilson et al., 2006, Hughes and Connell, 1999). For example, reefs along highly populated countries such as Indonesia have degraded dramatically as a result of land-based pollution (Edinger et al., 1998). Although granted World Heritage status, inshore reefs of the Great Barrier Reef, Australia (GBR), are not entirely protected from this form of pollution (Hughes et al., 2003), particularly reefs adjacent to areas of intensive agriculture. In these areas, the annual monsoonal floods increase terrestrial runoff, which has been further exacerbated in the last four decades as more land has been cleared for agriculture (Haynes and Michalek-Wagner, 2000, Fabricius, 2005) and recent monitoring programs have clearly demonstrated how pesticides are delivered into the GBR lagoon from agricultural catchments (Lewis et al., 2009, Packett et al., 2009).

The main agricultural activity on the Australian coast along the Northern GBR (Queensland) is the cultivation of sugar cane, which covers over 230,000 ha and is primarily confined to the low-lying coastal plain within 50 km of the ocean (Humphrey et al., 2004). The sugar cane industry uses pesticides such as organophosphates (OP) to combat cane beetles and other insects (Johnson et al., 1999). The most widely used pesticide to sustain the Queensland sugar cane industry is the OP insecticide chlorpyrifos (CPF). While detected in coastal habitats of the GBR (Cavanagh et al., 1999, Kapernick et al., 2006), CPF use on sugarcane has been restricted in Australia to reduce the threat of it entering coastal waters. It is, however, still applied to these crops for three months after planting (APVMA, 2009). CPF is used even more heavily on rice and other crops in many Asian countries and is commonly detected in waterways entering tropical marine habitats (Atapattu and Kodituwakku, 2009, Leong et al., 2007). Because of its broad spectrum, CPF represents a threat to non-target species including aquatic organisms. This toxicity has been demonstrated by numerous field and laboratory studies on temperate species (Fulton and Key, 2001); however, much less is known of its effects on tropical organisms. Research in coral reef toxicology is very recent and has primarily focused on the effects of contaminants on corals (Peters et al., 1997). Fish are also a vital component of coral reef ecosystems, and should be taken into consideration when investigating the effects of contaminants on reef organisms (Sarkar et al., 2006, Peters et al., 1997). While fish kills have been reported to result from CPF terrestrial runoff in Queensland (NRA, 2000) toxic thresholds and sub-lethal effects of CPF on tropical fish remain largely unknown (Humphrey et al., 2004).

The mode of action of CPF is cholinesterase (ChE) inhibition. Cholinesterases are a class of enzymes composed of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), acting in the nervous systems of invertebrates as well as vertebrates including fish (Odenkirchen and Eisler, 1988). Acetylcholinesterase and BChE can be distinguished by their affinity with synthetic substrates and sensitivity to synthetic inhibitors: a typical vertebrate AChE can hydrolyse acetyl-β-methylthiochline (AcβMeSCh), has higher affinity with acetylthiocholine (ASCh) than with butyrylthiocholine (BSCh) and is inhibited by eserine and 1,5-bis (4-allyldimethylammoniumphenyl) pentan-3-one dibromide (or BW284c51). Conversely, a typical vertebrate BChE cannot hydrolyse AcβMeSCh, has a higher affinity with BSCh than with ASCh and is inhibited by eserine and tetra (monoisopropyl) pyrophosphor-tetramide (or iso-OMPA) (Massoulie, 2002). Both AChE and BChE are inhibited by OP and carbamates, including CPF. Chlorpyrifos induces irreversible ChE inhibition (Fulton and Key, 2001), triggering constant stimulation of the muscles which leads to paralysis and death. Assays to measure inhibition of ChE activity are the most common tool to assess sublethal effects of CPF exposure in fish (Fulton and Key, 2001). More recently, it was shown that exposure to CPF causes oxidative stress in mammals (Verma et al., 2007, Crumpton et al., 2000) and fish (Kavitha and Rao, 2008). It has also been hypothesized that inducing oxidative stress might be the primary mode of toxicity of CPF in some instances, regardless of ChE inhibition (Saulsbury et al., 2009). However, data on CPF-induced oxidative stress in fish species remain scarce. One way of detecting oxidative stress is by studying the state of natural antioxidants in animals. One of the most potent and ubiquitous antioxidant molecules is the electron carrier coenzyme Q (or CoQ), reviewed in Navas et al. (2007). The reduced form of CoQ ($CoQH_2$) can be oxidised

by electrophilic molecules including reactive oxygen species (ROS). This prevents ROS from damaging other vital molecules such as lipids, proteins or DNA. $CoQH_2$ therefore acts as a powerful antioxidant (Bentinger et al., 2007). The ratio of $CoQH_2$ to total CoQ (or CoQ redox balance) is an established marker for oxidative stress and has been used as such in humans, as well as marine bacteria (Dunlap et al., 2002, Yamashita and Yamamoto, 1997). Most importantly, CoQ redox balance was recently used to detect oxidative stress in barramundi exposed to contaminants (polyaromatic hydrocarbons) and was shown to be a reliable and easy tool to apply for measuring oxidative stress in fish (Hasbi et al., 2011). In addition to these effects, CPF can also trigger a response from the detoxifying and antioxidant enzymes glutathione transferases (GST) in animals. As a consequence, GST activity generally increases upon exposure to contaminants in order to eliminate the xenobiotics (Hayes et al., 2005) and has been widely used to measure xenobiotics toxicity in aquatic organisms (Antognelli et al., 2006, Wang et al., 2009).

In an effort to understand the impact CPF may have on tropical reef fish species the response of CPF exposure was investigated in juveniles of the damselfish, *Acanthochromis polyacanthus*, a common and abundant tropical reef fish from the Indo-Pacific region. Here three types of cellular markers (ChE activity, CoQ redox balance and GST activity) were used to determine the effects of CPF exposure on the nervous system, antioxidant defences and detoxification pathway. In order to do so, ChE characterization experiments were conducted, as well as measurements of muscle ChE inhibition, hepatic CoQ redox balance and hepatic GST activity.

2.2. MATERIALS AND METHODS

All fish-handling and experimental procedures conformed to James Cook University ethical guidelines, under the authority of the Queensland Department for Primary Industries and Fisheries (Scientific Registration Number 0013), in compliance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and the Queensland Animal Care and Protection Act 2001. This study was conducted under ethics permit number A1258.

2.2.1. CPF analysis

In order to validate nominal CPF concentrations, water measurements were made in aquaria in the absence of fish. CPF was first dissolved into 100 mL acetone at a final concentration of 400 mg/L. Appropriate volumes of this stock solution were dispensed in triplicates into glass aquaria filled with seawater, in order to achieve final nominal concentrations of 4 µg/L, 16 µg/L and 64 µgL. After 24 h CPF was extracted from seawater samples in triplicates using solid phase extraction columns (Negri et al., 2005), but were further eluted with 1 ml of hexane following the methanol elution step. Triplicate extracts were analysed using a Hewlett Packard Gas Chromatograph 6890 equipped with a DB-5 column (J-and-W Scientific) and coupled to an electron capture detector as per (Cavanagh et al., 1999). Extracts were compared with a standard curve (0 µg/l, 4 µg/L, 16 µg/l and 64 µg/L, $R^2 = 0.99$).

2.2.2. Experimental animals and CPF exposure

Damselfish (*Acanthochromis polyacanthus*) were bred and reared at James Cook University, Townsville, Queensland, using broodfish collected from the GBR (Lizard Island), and at a constant temperature of 28 °C. They were kept at 28 ºC for several months until they reached juvenile stage. These fish will be referred to as

"initial fish stock". To conduct experiments fish from the same size class (62 mm ± 7 mm) were specifically chosen as size is a well-known factor for ChE activity variability in tropical fish (Pathiratne et al., 2008). Ninety juvenile *A. polyacanthus* were chosen from cohorts resulting from the mating of 10 parent pairs to minimise family-specific effects. Animals were acclimated for 3 weeks in a static system consisting of 10 glass tanks (9 individuals per tank) within a temperature controlled water bath (26.6 ± 0.3 ºC). Fish were fed commercial pellets (Primo Aquaculture) daily until satiation. Waste siphoning and a 70% water exchange were performed twice a day to prevent the accumulation of ammonia. Salinity was maintained between 34.2 and 35.0 ppt. Aeration was provided with a glass pipette to avoid absorption of pesticide on airstones. Dissolved oxygen was maintained above 5 mg/L at all times. Fish were not fed the day before exposure started and during the experiment to minimise the impact of feeding during the exposure study. In the pilot study, *A. polyacanthus* showed loss of equilibrium when exposed to 500 µg/L for 96 h; it was therefore decided to use lower concentrations in order to investigate sublethal effects that do not visually impair behaviour. Preliminary measures of CPF in water samples also showed that measured concentrations were approximately 70% of nominal concentrations. During exposure, treatments were run in duplicates and consisted of a seawater control, a solvent control (acetone) and three chlorpyrifos treatments, namely 1 µg/L, 10 µg/L and 100 µg/L (nominal concentrations) in 12 L glass tanks. These concentrations were chosen to reflect those at which fish kills have been observed (N.R.A, 2000) and to allow for the potential detection of oxidative stress as a result of CPF exposure. Stock concentrations were prepared in acetone and delivered in the tanks so that acetone volume did not exceed 0.02% (v/v) in order to minimise potential solvent effects on the fish physiology. Appropriate dosage was

maintained by adding the required volume of stocks (and identical final volumes of acetone carrier) after each water exchange, which occurred once a day. Three fish from each tank (6 fish/treatment) were sacrificed prior to exposure (time = 0), and after 6 and 96 h of treatment. Fish were anaesthetised in a 10% 2-phenoxyethanol solution as described in Begg and Pankhurst (2004). Fish were immediately sacrificed by cervical dislocation and dissected promptly. Liver and muscle tissue were snap-frozen in liquid nitrogen. All samples were stored at -80 ºC and processed within a few weeks.

2.2.3. Cholinesterase (ChE) activity

2.2.3.1. Enzymatic assay

Due to the small size of the fish, muscle tissue was used to assess ChE activity instead of brain tissue in order to ensure that enough biological material would be available for protein extraction and measurements of ChE activity in triplicatesTotal soluble protein was extracted from muscle in a homogenising buffer (0.1 % Triton X100 in 0.02 M sodium phosphate buffer). Supernatant was recovered and centrifuged at 10,000 *g* for 20 min at 4 ºC. The resulting protein extract was diluted to determine protein concentration in triplicates (mean CV < 10 %) based on a previously developed assay (Lowry et al., 1951). Absorbance was read at 750 nm on a Biotek Synergy microplate using the Bio-Rad DC protein assay kit (Richmond) with bovine serum albumin as standards. Protein extracts were used to measure ChE activity at 412 nm at 25 °C for 10 min according to Ellman (Ellman et al., 1961) with 34 µL of muscle protein extract diluted in 0.02 M phosphate buffer, 300 µL of 0.02 M phosphate buffer, 20 µL of 0.01 M 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) solution and 2 mM ASCh final concentration in the assay. Samples were run in

triplicates (average CV = 12 %). ChE activity was determined following the equation described by Bocquené and colleagues (Bocquené et al., 1998). Protein concentrations were used to calibrate ChE activity of each sample. ChE activity is expressed in μmol of substrate hydrolysed per min per mg of protein.

2.2.3.2. Substrate affinity

In order to determine the composition of the spiny damsel muscle ChE(s) (AChE and/or BChE), a series of assays were performed according to established methods (Lundin, 1968) and using several substrates. To determine AchE and/or BChE substrate affinity, four fish of similar size were chosen from the initial fish stock and their muscle tissue recovered as described above. Three substrates were used for ChE characterization, namely acetyl-β-methyl-thiocholine (AcβMeSCh), acetylthiocholine (ASCh) and butyrylthiocholine (BSCh). AcβMeSCh is hydrolysed by AChE only; ASCh is hydrolysed faster by AChE than by BChE; BSCh is hydrolysed faster by BChE than by AChE, with which it reacts very slowly (Frasco et al., 2010, Lundin, 1968, Massoulie, 2002). ChE activity was determined as described above with substrate added at the following concentrations: 0.04 mM, 0.08 mM, 0.16 mM, 0.32 mM, 0.64 mM, 1.28 mM, 2.56 mM, 5.12 mM, 10.42 mM, 20.48 mM or 40.96 mM. The assay was conducted at 25°C and read immediately at 412 nm for 30 min. Each sample was run in quadruplicate with an average CV < 3%.

2.2.3.3. Inhibitors effect and in vitro CPF effect

To refine preliminary results on ChE characterization obtained from substrate affinity measurements, ChE activity was assessed with a series of inhibitors specific to the different ChEs, with eserine, iso-OMPA and BW284c51 used as inhibitors of

ChEs, BChE and AChE respectively. ChE activity measurements remained the same with the following minor changes: protein extracts were incubated *in vitro* with inhibitor for 30 min, after which 2 mM of substrate were added (final assay concentration). The effect of each inhibitor on ChE activity was tested with substrates ASCh, BSCh, or AcβMeSCh successively. Ten inhibitors concentrations were tested and ranged from 10^{-6} µM to 400 µM (final assay concentration). CPF was also used as an inhibitor in order to examine its *in vitro* effects on *A. polyacanthus* muscle tissue. Inhibitors were first dissolved into EtOH and appropriate blanks were run in each plate in order to control for potential solvent effect during measurement.

2.2.4. Co-enzyme Q (CoQ) redox balance

The coenzyme Q redox balance was assessed by calculating the proportion of $CoQH_2$ over total CoQ using High Performance Liquid Chromatography (Hasbi et al., 2011). Briefly, frozen whole livers were crushed in liquid nitrogen to ensure homogenous sampling. A sub-sample (about 20 mg) was retained and suspended in 1 mL of solvent containing 50% isopropanol and 50% ethyl-acetate. Sonication was performed at room temperature until complete homogenisation to ensure cell lysis and was followed by centrifugation at 10,000 *g* for 3 min at 10 ºC. Twenty-five µL of the resulting supernatant was injected into a Phenosphere ODS(2) (Phenomenex) column followed inline by a platinum reduction column (Type RC, Irica). The mobile phase consisted of a methanol/isopropanol mix (3:7 v/v) carrying 50 mM of sodium perchlorate ($NaClO_4$). Eluted peaks were measured by amperometric electrochemical detection (ECD; Model Σ 985, Irica) with an oxidation potential of +600 mV (vs. $Ag/AgCl_2$) on a glassy carbon electrode and recorded on a Waters 746 Data Module. The peak area of both reduced and oxidised CoQ compounds were identified by

comparing the peaks obtained with an external CoQ10 standard and used to calculate the proportion of reduced CoQ over total CoQ (%$CoQH_2$ = 100 x $CoQH_2/(CoQH_2+CoQ_{ox})$).

2.2.5. Glutathione-S-Transferase (GST) activity

Sub-samples of liquid-nitrogen crushed liver samples were homogenized in a 0.1 M potassium phosphate buffer at pH 7.4 and centrifuged at 10,000 *g* for 20 min at 4 ºC. The resulting supernatant was centrifuged at 10,500 g for an hour at 4 ºC. The protein concentration of the supernatant was determined using the same Lowry assay described for the ChE assay. Hepatic protein concentration was measured as follows: 20 µL of sample were added to 150 µL of assay buffer (0.1 M potassium phosphate buffer containing 0.1% Triton-X-100), 20 µL of 20 mM glutathione in milliQ water and 10 µL of 20 mM dichloro-2,4-dinitrobenzene (CDNB) solution. Each sample was run in triplicate. The change in absorbance was read on a Bio-Tek Powerwave spectrophotometer at 340 nm every 20 sec for 10 min. GST activity is expressed as nmol of CDNB conjugated per min per mg of protein.

2.2.6. Statistical analysis

The Statistica software package (Statsoft) was used to perform all analyses. Normality and homogeneity of residues were checked before undertaking tests. Differences between treatments were evaluated by performing a two-way ANOVA with time and CPF concentration as fixed factors, followed by simple main effect tests (Quinn and Keough, 2002), which tested the effects of CPF concentration at each time point using post-hoc Tukey tests to identify heterogeneity among treatment means. Significant difference was acknowledged when $p \leq 0.05$.

2.3. RESULTS

2.3.1 CPF measurements and fish behaviour

Mean measured CPF concentrations at the end of 24 h were 3.53 µg/L (4 µg/L nominal), 13.9 µg/L (16 µg/L nominal) and 42.7 µg/L (64 µg/L nominal), corresponding to approximately 80% of nominal concentrations. No behavioural impairment was observed as a result of CPF exposure. Fish retained their balance and did not appear distressed by the CPF treatments.

2.3.2. ChE activity

2.3.2.1. ChE characterization

Cholinesterase had the highest affinity with ASCh, while BSCh exhibited the lowest activity, indicating that the predominant form of ChE in *A. polyacanthus* muscle is acetylcholinesterase (AChE) (Fig. 2.1). Calculation of Km, Vmax and catalytic efficiency were possible with ASCh and AcβMeSCh via Linewaver-Burke plots and resulting equations (Stefano et al., 2008) (Appendix A, Table A.1). Results of inhibitors effects are summarized in Table 2.1. The complete enzyme activity inhibition by eserine using the three substrates showed that the measured enzyme activity was solely due to ChEs as opposed to other esterases. Similar results obtained with BW284c51, an AChE-specific inhibitor, suggest that if muscle contains BChE it possesses an unusual sensitivity to BW284c51. The complete ChE inhibition with Iso-OMPA (BChE-specific inhibitor) on BSCh was expected. However, the unexpected reduced ChE activity with iso-OMPA on AcβMeSCh and ASCh and the plateau at 17% of control activity at high inhibitor concentrations may indicate the presence of a

predominant (83%), iso-OMPA sensitive AChE along with a typical iso-OMPA resistant AChE.

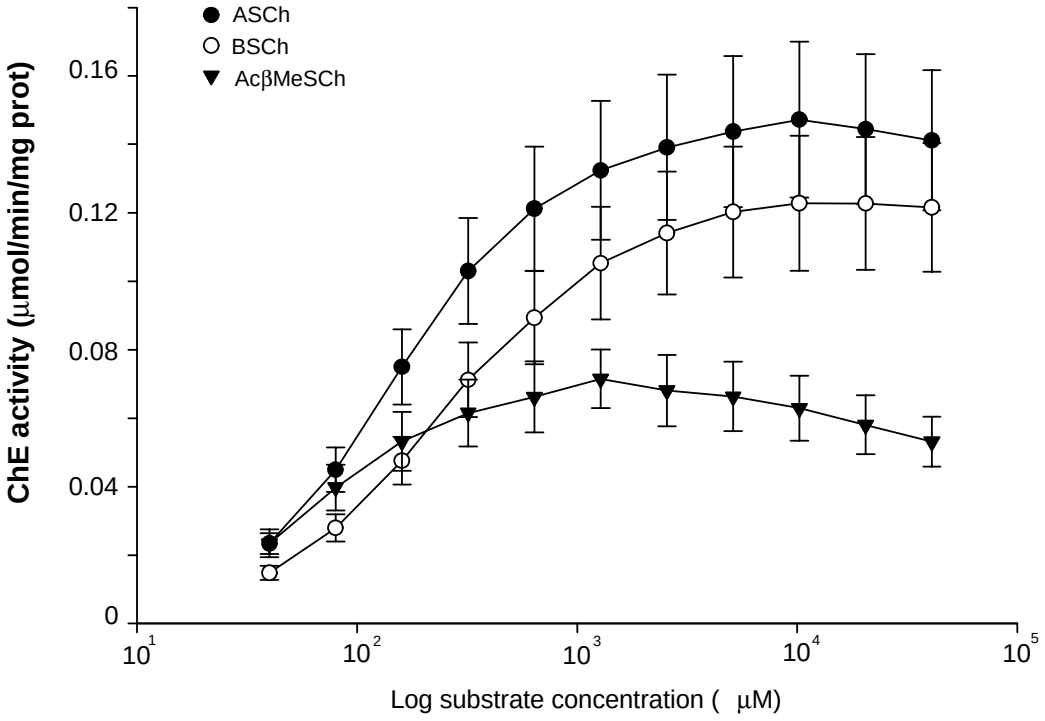


Figure 2.1. Effect of substrate concentration on muscle ChE activity in juvenile *A. polyacanthus* measured with three substrates: ASCh, AcβMeSCh and BSCh.

Table 2.1. Percentage of *in vitro* ChE inhibition in muscle of juvenile *A. polyacanthus* using eserine, BW284c51 and iso-OMPA as inhibitors and ASCh, AcβMeSCh and BSCh as substrates.

Inhibitor	**% ChE inhibition with each substrate**		
	ASCh	BSCh	AcβMeSCh
Eserine	100	100	100
BW284c51	100	100	100
Iso-OMPA	83	100	83

2.3.2.2. ChE activity in response to CPF exposure

In vitro exposure induced 87% inhibition with 2 x 10^8 pM CPF (final assay concentration), regardless of the substrate employed. *In vitro* ChE inhibition with CPF fitted sigmoidal, Hill 4 parameters curves when using ASCh, BSCh and AcβMeSCh

with an R^2 of 0.99 for each substrate (Fig 2.2a). *In vivo* exposures were conducted at the equivalent of 2800, 28,000 and 280,000 pM, corresponding to 1, 10 and 100 µg/L. Micrograms per litre were chosen as units for *in vivo* exposure in order to better compare our results with other studies. *In vivo* exposure induced no mortality in any of the treatments up to and including 100 µg/L CPF over 96 h (Fig.2.2b). The behaviour of fish was not affected by the solvent carrier (0.02 % v/v) or pesticide treatments and no sign of distress was observed over the course of the experiment. Fish exposed to the solvent carrier exhibited ChE activities of 0.22, 0.18 and 0.25 µmol/min/mg prot at t = 0, t = 6 h and t = 96 h respectively, and these activities were not significantly different from activities of the seawater-only control (ANOVA, $F_{1, 23}$ = 1.114,P > 0.2, Appendix B, Table B.1a). Therefore the results obtained with fish exposed to CPF were compared to those obtained from the solvent-control exposures. Chlorpyrifos concentration had a significant effect on ChE activity through time (ANOVA, $F_{8, 74}$ = 2.3854, *P* = 0.024, Appendix B, Table B1.b). Upon fish exposure to CPF, no effect of CPF was detected at t = 6 h, but ChE activity was significantly inhibited after 96 h of exposure compared to solvent control (ANOVA, $F_{4,74}$ = 7.703, *P* < 0.0001 Appendix B, Table B1b) for the three concentrations tested, i.e 1 µg/L, 10 µg/L and 100 µg/L (post-hoc, *P* < 0.05) (Fig. 2.3). After 96 h, these treatments inhibited the fish ChE activity by 26%, 49% and 53%, respectively, relative to solvent control activities (Fig 2.3). The ChE inhibition fitted a sigmoidal curve (Fig. 2.2b), predicting the 96 h IC_{50} in this experiment to be 9.7 µg/L. In comparison, *in vitro* IC_{50} was reached at 55 µg/L with ASCh, at 62 µg/L with BSCh and at 80 µg/L with AcβMeSCh, demonstrating that the whole organism is more susceptible to CPF than protein extract. *In vivo* inhibition also showed a stabilisation around 50%, whereas *in vitro* inhibition started to plateau at 88% inhibition.

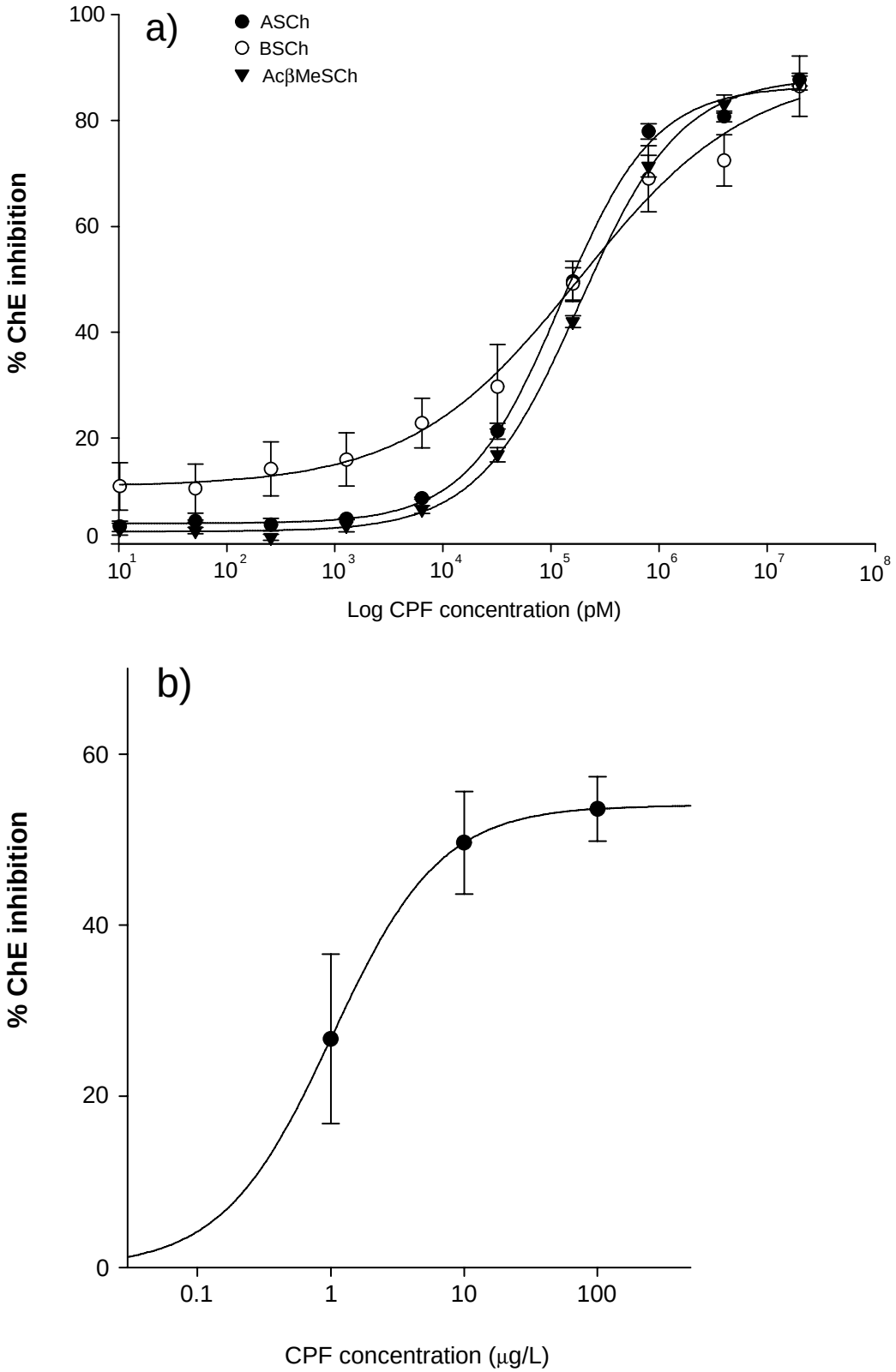


Figure 2.2. Muscle ChE inhibition in *A. polyacanthus* in response to CPF exposure: **a)** *in vitro*, compared to solvent control after 30 min of incubation with CPF; **b)** *in vivo*, compared to solvent control after 96 h of exposure to CPF and measured with ASCh.

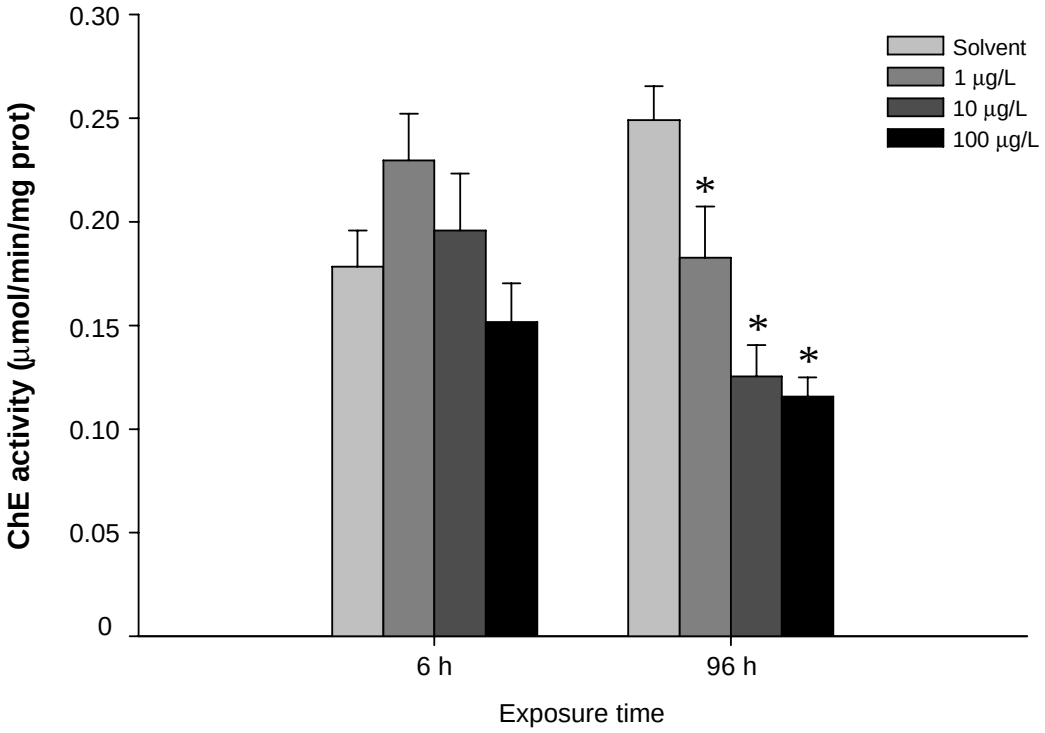


Figure 2.3. Muscle ChE activity in *A. polyacanthus* after 6 h and 96 h of *in vivo* exposure to 1 µg/L, 10 µg/L and 100 µg/L CPF. Datarepresent mean (n = 5-6 ± SE). Asterisks show significant difference with solvent control at each time point.

2.3.3. CoQ redox balance

Fish exposed to solvent carrier (0.02 % v/v) exhibited a $CoQH_2$ ratio of 93.1%, 95.6 % and 93.2% at t = 0, t = 6 h and t = 96 h, respectively. These values were not significantly different from the values obtained from fish exposed to seawater only (ANOVA, $F_{1, 22} = 0.02$, $P > 0.8$, see Appendix B, Table B.2a) and the results from pesticide treated fish were therefore compared to the solvent-treated fish. CPF had a significant effect on the CoQ redox balance through time (ANOVA, $F_{8, 73} = 2.8$, $P = 0.001$, see Appendix B, Table B.2b). Pesticide concentration significantly affected this marker after 6 h of exposure (ANOVA, $F_{4, 73} = 5.7$, $P = 0.012$, Appendix B, Table B.2b), when the $CoQH_2$ form increased in fish treated with 10 µg/L of CPF compared to solvent control fish (Tukey HSD, $P = 0.011$) (Fig. 2.4). No significant effect of the

pesticide was, however, observed for the 1 µg/L and 100 µg/L exposures at 6 h, or for all three exposures at 96 h.

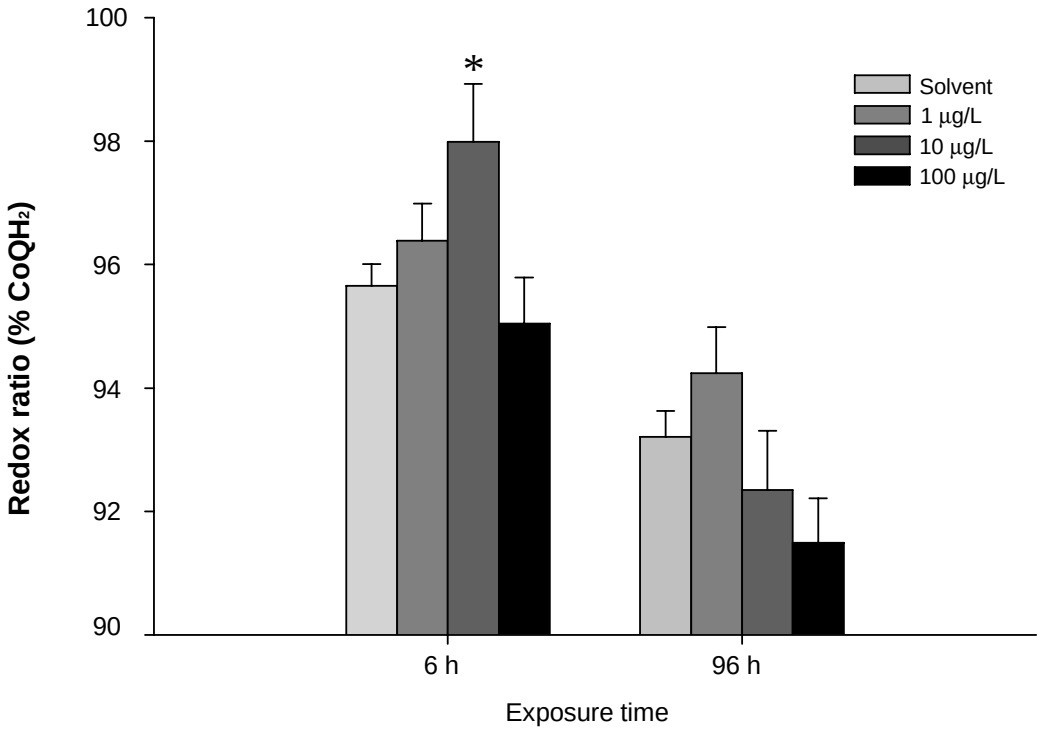


Figure 2.4. Percentage of reduced coenzyme Q (%$CoQH_2$) over the total CoQ in liver in *A. polyacanthus* after 6h and 96 h of exposure to 1 µg/L, 10 µg/L and 100 µg/L CPF. Data represent mean (n = 5-6 ± SE). Asterisks show significant difference with solvent control at each time point.

2.3.4. GST activity

The GST activity was 225 µmol/min/mg prot at 6 h and 226 µmol/min/mg prot at 96 h in the solvent control treatments and were not significantly different from the GST activity measured in fish treated with seawater (ANOVA, $F_{1,34} = 0.197$, $P > 0.6$, Appendix B, Table B.3a). Hepatic GST activity was not significantly affected by the presence of CPF over the course of the experiment (ANOVA, $F_{8,75} = 0.624$, $P = 0.755$, see Appendix B, Table B.3b) and was maintained between 225 and 282 after 6 h of exposure and between 226 and 263 after 96 h of exposure in all treatments (Fig 2.5).

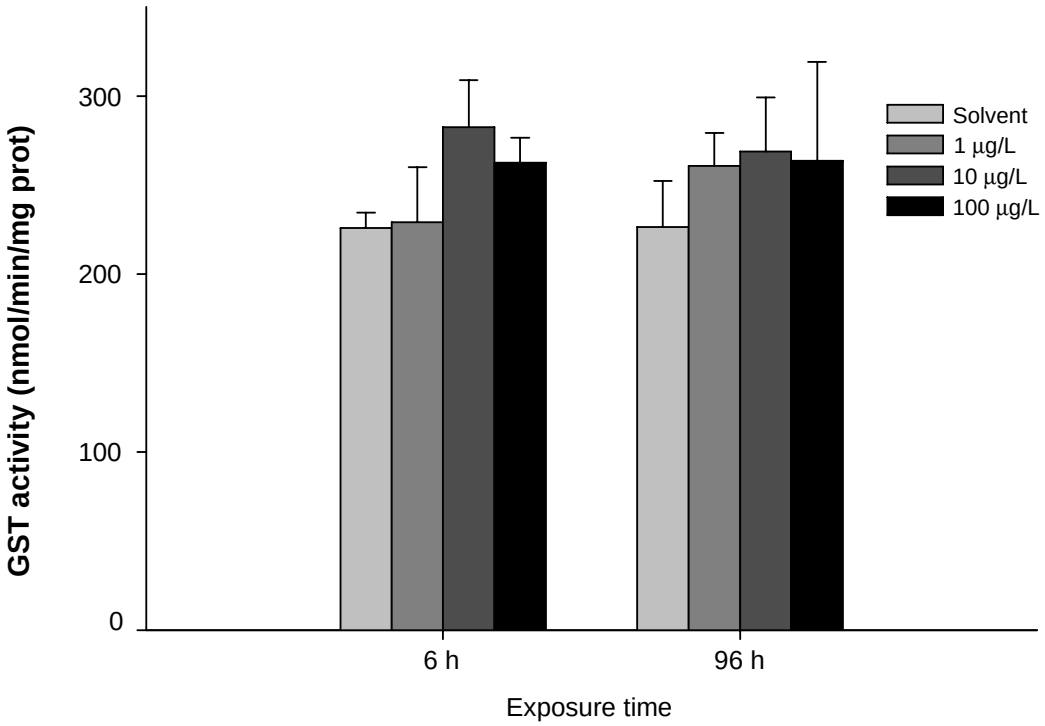


Figure 2.5. Hepatic GST activity in *A. polyacanthus* after 6 h and 96 h of exposure to 1 µg/L, 10 µg/L and 100 µg/L CPF.Data represent mean (n = 5-6 ± SE).

2.4. DISCUSSION

Cholinesterase characterization in *A. polyacanthus* muscle tissue suggested the presence of a typical vertebrate AChE but also revealed a number of unusual properties suggestive of the presence of atypical AChE and BChE as well. ChE activity was shown to be an excellent marker for *in vivo* CPF exposure in juvenile *A. polyacanthus*, with significant reductions in muscle activity observed after 96 h at CPF concentrations far below the lethal dose for this species, and when behavioural symptoms were not apparent. The oxidative stress response to CPF exposure was less sensitive than ChE inhibition, but showed a significant up-regulation of $CoQH_2$ after 6 h at 10 µg/L CPF. GST enzyme activity was the least sensitive marker, with no response observed at 100 µg/L CPF over 96 h. These results demonstrate the

differences in sensitivity between biomarker responses to a key OP insecticide and reveal the possibility of a time-dependent oxidative stress response to CPF in juvenile *A. polyacanthus.*

2.4.1. ChE characterization

Vertebrate AChE and BChE can be distinguished by a number of properties: typical vertebrate AChE can use AcβMeSCh as a substrate, has a greater affinity with ASCh than with BSCh and is inhibited by BW284c51, but not iso-OMPA. Typical BChE cannot use AcβMeSCh as a substrate, has a greater affinity with BSCh than ASCh and is inhibited by iso-OMPA, but not BW 284c51. The present study revealed the presence of an AChE in *A. polyacanthus* muscle, since high ChE activity was measured with the AChE-specific substrate AcβMeSCh. Inhibition of ChE activity on AcβMeSCh after treatment with an AChE-specific inhibitor (BW284c51) confirmed the presence of AChE in the muscle tissue of *A. polyacanthus*. The decrease in ChE activity with the BChE-specific inhibitor iso-OMPA using the substrate AcβMeSCh seems contradictory, since iso-OMPA should not affect enzyme activity in the presence of an AChE-specific substrate. This inhibition could, however, be explained by the presence of two types of AChE in *A. polyacanthus* muscle: a typical AChE, capable of using AcβMeSCh as a substrate and resistant to iso-OMPA, and an atypical AChE, which hydrolyses AcβMeSCh as expected, but is inhibited by iso-OMPA as an atypical characteristic. A hypothesis can be proposed in which the atypical AChE is responsible for the 83% AChE inhibition induced by iso-OMPA in the presence of AcβMeSCh. In addition, complete inhibition of ChE activity was observed with BChE-specific inhibitor when using BSCh. In theory, BSCh can be hydrolysed by AChE. However, the reaction rate is very slow and some authors even

consider that it is negligible (Sturm et al., 1999). In the present case, if AChE was capable of hydrolysing BSCh, the typical AChE should have allowed for a remaining 17% activity at high BChE-specific inhibitors concentration, because it is not sensitive to iso-OMPA. Since no activity remained after exposure to iso-OMPA using BSCh, it suggests that *A. polyacanthus* AChE cannot hydrolyse BSCh, which implies the existence of a BChE responsible for the ChE activity observed when using BSCh. Finally, this BChE possesses the unusual property of being sensitive to BW284c51, as this inhibitor caused complete inhibition of ChE activity when BSCh was used.

The results therefore demonstrate that *A. polycanthus* possesses a complex mixture of ChEs, composed of two types of AChE and a BChe. The AChEs are unable to hydrolyse BSCh; the first AChE is a typical vertebrate form; the second and predominant ChE is an atypical, iso-OMPA sensitive AChE; the third ChE is an atypical form of BChe, which is characterized by sensitivity to BW284c51 and BSCh substrate inhibition at high concentrations. The two atypical types of ChEs have been described in fish muscle in separate studies before: an atypical, iso-OMPA sensitive AChE was found in muscle of three marine teleosts *Platychtis flesus*, *Limanda limanda* and *Serranus cabrilla* (Sturm et al., 1999), and an atypical BW284c51-sensitive BChE exhibiting substrate inhibition was described in the marine fish *Pleuronectes platess*a (plaice) (Lundin, 1968). In the flounder (*P. flesus*), the atypical AChE was largely predominant (92% of total AChE), which is consistent with the hypothesis of a predominant atypical AChE (83% of total AChE) in *A. polyacanthus* muscle tissue. In tropical species, an atypical ChE was described in the surgeonfish *Acanthurus dussumieri*, using enzyme kinetics as well as chromatography techniques to purify the enzyme (Leibel, 1988). Similarly, Leticia and Gerardo (2008) found a

typical AChE and an atypical, BW284c51-sensitive BChE in muscle of the tropical reef fish *Haemulon plumieri* from the Yucatan peninsula. *A. polyacanthus, A. dussumieri* and *H. plumieri* belong to different families (respectively Pomacentridae, Acanthuridae and Haemulidae), but the three species are found on coral reefs and belong to the Perciformes order. This further reinforces the present findings of atypical ChEs in the spiny damselfish. This thesis chapter constitutes the first attempt to characterize ChE from an Indo-Pacific tropical reef fish and, along with similar studies on Perciformes, suggests that tropical reef fish from this order might share common atypical ChEs. Contrary to AChE, BChE does not possess a known natural substrate and although ChEs have been extensively studied in vertebrates, the role of BChE remains hypothetical. The enzyme could act by sequestering or scavenging toxic compounds but evidence of BChE mode of action is scarce (Stefano et al., 2008). In addition, the functional significance of an organism possessing typical or atypical ChE is even less understood (Leticia and Gerardo, 2008) and it is therefore difficult to hypothesize the physiological and ecological implications of the results found here.

2.4.2. CPF exposure and ChE inhibition in muscle

At similar concentrations, *in vivo* CPF exposure induced greater inhibition than *in vitro* exposure. Similar results were found in the freshwater shrimp *Gammarus pulex* (Xuereb et al., 2007). In the present study, the *in vivo* exposures were 96 h-long, which is almost 200-fold longer than the *in vitro* exposures, allowing for accumulated inhibition by CPF which binds irreversibly to ChEs. Although *in vitro* studies are informative on the susceptibility of an animal to CPF, these results stress the importance of using *in vivo* exposure in order to describe biologically relevant effects.

The sensitivity of ChE inhibition to CPF in *A. polyacanthus* (LOEC = 1 µg/L and IC_{50} = 9.7 µg/L) was similar to that reported for other fish species, including small temperate chinook *Onchorhynchus tshawatsha* and coho salmon *Onchorhynchus kisutch* which exhibited 67% inhibition with 2.5 µg/L CPF (Sandahl et al., 2005) and 92% inhibition with 7.3 µg/L (Wheelock et al., 2005). The tropical Nile tilapia *Oreochromis niloticus*, although larger than *A. polyacanthus*, is regularly used for biomonitoring of natural environments (Pathiratne et al., 2009, Silva and Pathiratne, 2008), and showed a 70% muscle ChE inhibition after exposure to 120 µg/L for 48 h (Pathiratne et al., 2008). Fish mortality usually arises when ChE inhibition reaches 80%, but behavioural impairment such as decreased mobility has been observed with 40% to 50% inhibition in the seabass *Dicentrarchus labrax* (Almeida et al., 2010), and the rainbow trout *Onchorhynchus mykiss* (Beauvais et al., 2001), for example. The lack of behavioural changes in *A. polyacanthus* when the fish exhibited 50% inhibition reinforces the use of ChE as a sensitive indicator of sub-lethal stress for this species. Although there is a large body of experimental data on the effects of CPF on fish and aquatic invertebrates, exposure times, tissue types and endpoints are inconsistent, making direct comparison with data from the present study difficult (Mileson et al., 1998). Furthermore, the response of ChE inhibition in fish by OPs can be strongly affected by the developmental stage and body size (Flammarion et al., 2002, Pathiratne et al., 2008). The results from the present chapter, however, clearly demonstrate the utility of ChE inhibition as a biomarker for OP exposure in *A. polyacanthus* and supports the utility of this tropical reef fish as a bioindicator species for toxicology studies in the Indo-Pacific.

The dose-response relationship between CPF exposure and ChE inhibition developed after 6 h and was significant after 96 h. This increasing inhibition of ChE over time is due to the irreversible binding of OPs to ChE (Fulton and Key, 2001) and similar time-dependent activities have been observed in fish previously (Boone and Chambers, 1996). In the present study, inhibition appeared to reach a plateau at approximately 50% inhibition after exposure to 10 µg/L, whereas *in vitro* exposure induced a plateau at 88% inhibition after exposure to 70 µg/L only. Such difference between the *in vivo* and *in vitro* responses could be explained by detoxification mechanisms in the living fish or the continued *de novo* synthesis of ChEs resulting in a "basal" ChE level within fish muscle. The plateau observed therefore might reflect a mechanism by which *A. polyacanthus* can maintain ChE activity. The spiny damsel neuromuscular functions might therefore be relatively well protected against potential CPF exposure and perhaps against other OP and carbamates, enabling the fish to survive. However, other functions might be impaired and in this context, exposure of *A. polyacanthus* to other OPs or a combination of anti-cholinesterase compounds would be beneficial to support this hypothesis. Finally, it is important to note that the results obtained here derive from muscle tissue. As stated in Chapter 1, ChE are neural enzymes and are also found in brain tissue. The sensitivity of *A. polyacanthus* brain ChE to CPF exposure remains unknown, but it is possible that this organ might be more severely impacted by CPF. Future studies on *A. polyacanthus* should therefore include brain ChE in the measurements of ChE activity in order to better evaluate the effects of CPF on this species and determine the most sensitive organ for such measurements.

2.4.3. CPF exposure and detoxification in liver

2.4.3.1. *CoQ redox balance*

In the last decade, numerous *in vitro* studies have found that chlorpyrifos exposure increases cellular ROS in mammals (Giordano et al., 2007), (Bebe and Panemangalore, 2005) and in fish (Kavitha and Rao, 2008), but information is much more scarce for this latter taxonomic group. The CoQ is an electron carrier found as a redox couple, which in the reduced form can be oxidised by ROS. If this happens, ROS impair the CoQ redox balance by decreasing the proportion of reduced CoQ (or $CoQH_2$) over the total CoQ (Yamashita and Yamamoto, 1997). Contrary to this outcome, an increase in $CoQH_2$ was detected after 6 h exposure to CPF at 10 ug/L. This response is therefore more likely to result from an up-regulation of antioxidant defences, a toxicant-induced ROS response also observed in killifish *Fundulus heteroclitus* (Bacanskas et al., 2004). In fact an increase in CoQ biosynthesis has recently been reported as a stress response, whereby newly synthesized CoQ was hypothesised to regenerate other antioxidants such as α-tocopherol (Navas et al., 2007). However, the increase in the reduced form specifically following CPF exposure may also be explained by the cycling of the molecule in plasma membranes. There, the reduced form is regenerated from the oxidised form through the action of several enzymes, including the NAD(P)H:quinone reductase 1, also known as NQO1 or DT diaphorase (Navas et al., 2007). Interestingly, this enzyme was up-regulated in response to xenobiotic contamination in rainbow trout liver (Sturve et al., 2005). More recently, Hasbi et al (2011) found an increase in barramundi CoQ redox balance in response to organic pollutants (benzo[*a*]pyrene) and the authors hypothesized an activation of NQO1 to explain this increase. Oxidative stress can therefore trigger a stress response and stimulate the enzymatic system that regenerates and increases the

antioxidant $CoQH_2$ in order to cope with increasing ROS and this may have occurred in the spiny damsel fish as a response to CPF exposure.

While an increase in $CoQH_2$ ratio was observed when the fish were exposed to 10 µg/L CPF, this increase was not observed at higher CPF concentrations, or after 96 h of exposure. The same bell-shape response is seen for barramundi when toxicant exposure increases (Hasbi et al., 2010). The decline of $CoQH_2$ at the highest concentrations tested could result from the depletion of the necessary cofactor NADPH, or the fact that the replenishing enzyme machinery might be overwhelmed once concentrations reach a certain level. Likewise, NQO1 activity has been shown to increase in response to toxicant-induced oxidative stress when rats were exposed to medium pro-oxidant concentrations only, possibly as a consequence of the enzyme being damaged by excessive cellular ROS produced in the presence of higher contaminant concentrations (Sturve et al., 2005). This may explain the (non-significant) trend for reduced $CoQH_2$/CoQ ratios observed in the spiny damselfish at 100 µg CPF/L after 6 h, and at 10 and 100 µg/L CPF after 96 h, potentially indicating a shift towards the oxidised form as a result of excessive ROS. Other protective mechanisms may act simultaneously, preventing complete depletion of $CoQH_2$. The fact that the response of this antioxidant balance occurred after only 6 h exposure to CPF, before inhibition of ChE was detected, may reflect a primary mode of action of CPF, which may induce oxidative stress and trigger a response by elevating the CoQ redox balance in liver, in order to protect this detoxifying organ from damaging ROS. Although supported by a growing literature (Saulsbury et al., 2009), this hypothesis is relatively novel and in the case of *A. polyacanthus*, further analyses should be carried out in order to support it**,** (for example measurements of antioxidant enzyme activities

such as catalase, SOD or GPx). The results presented here nevertheless suggest that the role of ROS production in CPF toxicity might be under reported for fish species, and that enhanced production of ROS may be an early onset mechanism of toxicity in the spiny damselfish.

2.4.3.2. *GST activity*

Hepatic GST activity in juvenile spiny damselfish was not affected by the current exposures to chlorpyrifos, despite abundant literature demonstrating increases in the GST activity of aquatic organisms in response to a range of xenobiotics (Hayes et al., 2005). It is likely that the response of GST activity to CPF is highly species dependent. For example, CPF inhibited GST activity of the mosquitofish *Gambusia affinis* after 96 h of exposure to 297 µg/L (LC_{50} of CPF for this species, which is 3-fold higher than that tested here) (Kavitha and Rao, 2008) and GST activity also decreased in the goldfish *Carassius auratus* exposed for 5 days to a mixture of pesticides including CPF (Wang et al., 2009). These examples illustrate the possibility that different species may use different mechanisms to cope with CPF exposure. As phase II enzymes, GSTs catalyse the conjugation of glutathione (Woo et al., 2006) with xenobiotics or electrophilic compounds in order to produce a less toxic molecule which is subsequently excreted from the cell (Eaton and Bammler, 1999). In the presence of CPF, GSTs were found to form five different compounds when conjugated with GSH (Fujioka and Casida, 2007), demonstrating the role of GST in the CPF detoxification process. In addition, the promoter region of GST genes caries an antioxidant response element (ARE), which is a shared characteristic with the NQO1 enzyme and other phase II detoxifying enzymes (Navas et al., 2007). In the spiny damselfish, the concentrations of CPF delivered might have been below the

GST-activation threshold, and it is possible that elimination of CPF may have occurred without the need for the fish to enhance this pathway. It is also possible that changes in the GST activity are time-dependent and the sampling regime employed here did not match these changes in activity.

2.4.4. Environmental relevance

The hydrophobic nature of CPF and its immobility in soils means that this insecticide is unlikely to contaminate groundwater through leaching. However, its binding affinity to soil colloids means that CPF can be easily transported into aquatic environments in sediments via erosion and terrestrial runoff (Johnson et al., 1999). Agricultural and urban runoff during monsoonal rainfall delivers the highest loads of contaminants into tropical marine environments (Haynes and Michalek-Wagner, 2000, Fabricius, 2005). Recently, low concentrations of CPF were detected in sediments from inshore reefs of the GBR (11 ng/g dry weight on average) (Kapernick et al, 2006) and in water samples using passive samplers deployed over a month (15 to 37 ng/sampler, corresponding to 5 to 270 pg/L) at two out of 12 river mouths surveyed along the GBR (Shaw et al., 2010, Shaw et al., 2009). These concentrations remain low and the recent restrictions imposed for CPF usage on sugar cane crops (APVMA, 2009) aim at reducing these levels in Australia. However, in other tropical regions, OP concentrations can be much higher: up to 92 ng/L CPF were detected in water samples from a river mouth in Malaysia (Leong et al., 2007) and up to 47.5 μg/L OPs in was observed in Thailand rivers which poses a serious threat to aquatic life in those ecosystems (Thapinta and Hudak, 2000).

Unlike banned organochlorine insecticides DDT and dieldrin, CPF does not bioaccumulate in marine organisms and is difficult to directly detect in animal tissue samples (Varó et al., 2002, Shaw et al., 2009). However, recent field studies have identified depressed ChE activity in two marine species inhabiting contaminated GBR estuaries, namely barramundi *Lates calcarifer* and the mud crab *Scylla serrata* when activities were compared with reference sites (Humphrey et al., 2007, van Oosterom et al., 2010). There is often an apparent discrepancy between the low or patchy detection of CPF in the environment and biomarker indications of exposure in living organisms. This inconsistency is likely due to low-level chronic exposures, as the non-reversible binding of CPF induces ChE inhibition with exposure duration (Fulton and Key, 2001, Boone and Chambers, 1996). In fact, decreases in ChE activity in living organisms might be one of the few reliable ways to effectively detect exposure to OPs such as CPF. To date, only one other study has investigated the effects of CPF on a GBR reef fish (the ambon damselfish *Pomacentrus amboinensis*) and developmental abnormalities were detected in eggs of this species at very high concentrations (750 µg/L), well above typical environmental exposure levels (Humphrey et al., 2004). Depression of ChE activity therefore seems to be a more suitable biomarker for OP exposure as it is generally more sensitive to insecticides such as CPF. In freshwater fish from GBR river catchments, ChE activity in eggs was depressed at more modest CPF exposures of 20 µg/L (Humphrey and Klumpp, 2003). Consistent with the rainbowfish example and the present study on *A. polyacanthus*, the tropical mudskipper *Periophthalmus novaeguineaensis* also exhibits ChE inhibition in response to CPF exposures and this occurs at lower concentrations (LOEC 4 µg/L) (Flores, 2007).

Although the results from this chapter indicate that juvenile *A. polyacanthus* would survive for 96 h at high CPF concentrations, sub-lethal effects on the fitness of this fish and other organisms are possible, especially if the duration of exposure is increased. In addition, other species may be much more sensitive to CPF than fish. For example, some Mexican shrimp species are impacted by CPF at concentrations as low as 10 ng/L (Carvalho et al., 2002). The present study also highlights differences between biomarker responses and how the duration of exposure can affect the detection of a biochemical response. Future work including functional and behavioural activities should be combined with the present biomarker studies, for a more complete understanding of the sub-lethal effects of OPs on fish.

2.5. CONCLUSION

This first data chapter investigated the cellular sublethal effects of exposure to CPF on juveniles of the tropical damselfish *A. polyacanthus*. The results suggest the presence of both typical and atypical ChEs in muscle tissue. They also demonstrate the sensitivity of muscle ChE inhibition as a cellular marker of CPF exposure in the spiny damselfish and reveal signs of an oxidative stress response to CPF exposure. The response of ChE activity occurred at CPF concentrations at least 100-fold less than the lethal dose and was 10-fold more sensitive than the oxidative stress assays (CoQ balance and GST activity) in this potential bioindicator reef fish species. The response of biomarkers used in the present study were, however, very dependent on exposure duration and this should be further investigated for each assay type. Oxidative stress should be further investigated, but the results suggest it may play an early role in the toxicity of CPF in *A. polyacanthus* and thereby add to the recent literature hypothesizing an important role for ROS production in CPF toxicity in fish.

3. Temperature Stress Decreases Neural Enzyme Activity in *Acanthochromis polyacanthus*

3.1. INTRODUCTION

Elevated temperatures due to climate change have the potential to greatly impact marine ecosystems, with studies already finding adverse effects of increasing temperatures on the phenology, distribution, growth, survival and reproduction of marine species (Roessig et al., 2004, Walther et al., 2002). Whilst temperature rise is projected to be more dramatic under polar and temperate climates than in tropical regions (IPCC, 2007), the thermal tolerance range of organisms which have evolved in stable tropical environments may be limited. Tropical species are therefore vulnerable to even relatively small changes in environmental conditions (Hoegh-Guldberg et al., 2007). For example, damselfish species from the tropical northern GBR, including *Acanthochromis polyacanthus,* show a smaller aerobic scope than the same species from the southern GBR, where the fish experience cooler temperatures and greater annual temperature fluctuations (Gardiner et al., 2010). This highlights the sensitivity of tropical fish species to temperature increases. In the last few decades, global climate change has resulted in more frequent episodes of high temperatures on tropical reefs (Schmidt, 2008, Walther et al., 2002, Webster et al., 2005). This has led to rising concerns about the influence of temperature on the life history traits of tropical species and to the investigation of temperature impacts on tropical fish growth (Meekan et al., 2003, Munday et al., 2008), size (Green and McCormick, 2005), survival (Gagliano and McCormick, 2007), metabolic rate and loss of balance (Eme and Bennett, 2009, Eme et al., 2011), swimming performance (Green and Fisher, 2004), reproduction (Hilder and Pankhurst, 2003, Donelson et al., 2011), and

recruitment (Booth and Beretta, 2004). However, studies of the effects of temperature-induced stress in tropical reef organisms at the level below that of the organism (i.e the cellular scale) have mainly been restricted to corals (Przeslawski et al., 2008) and only recently have molecular studies begun in tropical reef fish (Kassahn et al., 2007a). Consequently, basic information on the physiological and cellular response to temperature stress in tropical reef fish is needed in the context of warming tropical waters (Kassahn et al., 2007b).

The long-term knowledge in temperate fish thermal biology has allowed the definition of basic concepts in fish stress physiology. In general, finfish respond to temperature-induced stress via an established stress response mechanism (reviewed in Bonga (1997). In particular, biochemical and physiological changes occur at tissue and cellular levels to increase metabolic rate and alter blood chemistry. These changes are referred to as the secondary stress response. In fish, the secondary response to thermal stress has been assessed in several species using indicators such as gene expression (Iwama et al., 1998), enzymatic activities (Dahlhoff, 2004), or quantification of stress-related compounds such as glutathione, succinate or plasma glucose (Kindle and Whitmore, 1986, Leggatt et al., 2007, van Dijk et al., 1999). At the protein level, key temperature response proteins identified in fish include the well documented heat shock protein family (Dahlhoff, 2004, Feder and Hofmann, 1999, Iwama et al., 1998, Timofeev et al., 2008), but also metabolic and antioxidant enzymes. The level and activity of antioxidant enzymes are affected by temperature stress because they act as important cytoprotectants against the increased cellular production of reactive oxygen species (ROS) that occurs in heat stressed fish (Feidantsis et al., 2008, Heise et al., 2003, Lushchak and Bagnyukova, 2006a). As a

result of ROS production, damage to DNA, proteins and lipids becomes more likely, thus requiring the use of antioxidant defences (Cooper et al., 2002). Accordingly, temperature stress studies on fish often find a change in the activity of the antioxidant enzymes glutathione peroxidase, glutathione-S-transferase (GST), superoxide dismutase or catalase, but whether the activity is increased or decreased depends on the temperature regime applied and the species studied (Bagnyukova et al., 2007, Heise et al., 2006b, Kaur et al., 2005, Leggatt et al., 2007, Lushchak and Bagnyukova, 2006c).

While antioxidant enzymes have been studied for a long time in relation to temperature stress, very little is known about the behaviour of enzymes belonging to the nervous system during the stress response. Neural enzymes may also be affected by thermal stress as part of the secondary stress response. Among these enzymes, Cholinesterases (ChE) are particularly important. Mainly composed of Acetylcholinesterase (AChE) and Butyrylcholinesterase (BChE), these proteins ensure the recycling of the neurotransmitter acetylcholine. The behaviour of ChE in response to temperature has been studied *in vitro* in humans (Cengiz et al., 2002), but although it is widely used as a marker for organophosphate contamination in aquatic organisms very little is known about the impact of temperature stress on these enzymes *in vivo* (Fulton and Key, 2001). Interestingly, in the few studies involving freshwater and estuarine fish, the effect of temperature on ChE activity has produced disparate results whereby some fish exhibit increased ChE activity (Hogan, 1971, Pfeifer et al., 2005), and others show no significant temperature effects (Beauvais et al., 2002, Phillips et al., 2002). No such study has, however, investigated temperature effects on ChE in marine fish species.

The behaviour of antioxidant enzymes and neural enzymes can vary greatly with the species studied and the temperature regime employed. As a result, studies on temperature stress often include examination of non-enzymatic compounds as well, as these can also prove valuable stress indicators in fish. For example, oxidative stress resulting from increasing temperatures can affect molecules such as lipids and vitamins making these useful bio-indicator compounds also (Cooper et al., 2002, Leggatt et al., 2007, Lushchak and Bagnyukova, 2006b). One of the most potent antioxidant compounds is, however, the electron carrier Coenzyme Q (CoQ). This molecule is found in membranes (including the mitochondrial respiratory chain) as a redox couple, composed of the reduced form ($CoQH_2$) and the oxidised form (CoQ_{ox}). The ratio of $CoQH_2$ over total CoQ (or CoQ redox balance) can decrease as a result of ROS oxidising $CoQH_2$, and this change is recognized as a reliable and ubiquitous oxidative stress marker (Bentinger et al., 2007, Beyer, 1992, Crane, 2007, Miles et al., 2005, Navas et al., 2007). Interestingly though, in two tropical fish species, the barramundi *Lates calcarifer* and the spiny damselfish *A. polyacanthus*, CoQ showed a response to contaminant exposure, which induced an increase in the reduced form as a response to oxidative stress as seen in Chapter 2 of the present thesis and in the literature (Botté et al., in press, Hasbi et al., 2011). This underlines the potential for tropical fish species to use CoQ in a different fashion compared to other organisms. In fact, the sensitivity of this oxidative stress marker to temperature stress is largely unknown in fish and this remains an important knowledge gap given the proven production of ROS in heat stressed fish and the central role of CoQ as antioxidant (Feidantsis et al., 2008, Heise et al., 2003, Lushchak and Bagnyukova, 2006a)

In the Southern Hemisphere, average temperatures have increased by 0.6°C over the last 100 years (Lough, 2007). On the Great Barrier Reef of Australia (GBR), average sea surface temperatures have increased by 0.4°C the last 30 years and are projected to increase by 1°C to 3°C by 2100 (Lough, 2007). Recent studies have shown that growth and reproduction are impeded in the Indo-Pacific damselfish *A. polyacanthus*, at temperatures projected to occur as maximums on the GBR by 2100 (31°C) (Munday et al., 2008, Donelson et al., 2011). Individuals from this species exposed to 31°C temperatures were less efficient to convert food into growth than fish exposed to 26° and 28°C, demonstrating the sensitivity of this species to even mild temperature increase (Munday et al, 2008). However, little is known about the cellular mechanisms leading to such negative effects. Applying the cellular stress markers available for temperate fish to this model tropical reef fish species would substantially build upon the existing literature on the stress responses of *A. polyacanthus* and enhance our present understanding of the underlying mechanisms for thermal sensitivity of this reef fish species. The increasing literature on *A. polyacanthus* stress response therefore needs to be reinforced by applying the cellular stress markers available for temperate fish in this model tropical reef fish species, in order to understand the underlying mechanisms for its thermal sensitivity. The impacts of thermal stress were therefore assessed on *A. polyacanthus* at the cellular level by measuring ChE Activity, CoQ redox balance and GST activity, in a controlled laboratory experiment during which the fish experienced thermal stress conditions (32°C or 34°C) compared to acclimation temperatures (28°C).

3.2. MATERIAL AND METHODS

All procedures conformed to James Cook University ethical guidelines (Permit number A1258), under the authority of the Queensland Department for Primary Industries and Fisheries (Scientific Registration Number 0013), in compliance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and the Queensland Animal Care and Protection Act 2001.

3.2.1. Fish maintenance

Juvenile *A. polyacanthus* were bred and reared at James Cook University, Townsville, Queensland, using broodfish collected from the GBR. Here, 90 juveniles (between 54-67 mm standard length) were randomly chosen from a cohort resulting from the mating of 10 parent pairs, again to minimise confounding interpretation of results due to family-specific effects. All fish were kept in 2 L individual glass aquaria in a semi flow-through system (allowing 100% water exchange several times a day) at a constant temperature of 28°C for 8 weeks. During this time *A. polyacanthus* were maintained in visual isolation from each other through the use of black plastic sheets around aquaria to minimize social interactions that might have produced a confounding effect on individual fish stress levels. During the 8 week acclimation period, fish were fed commercial pelleted food once daily (Primo Aquaculture, Australia) until satiation and were visually assessed for disease, distress or mortalities on a daily basis.

3.2.2. Temperature stress experiment

After acclimation to the experimental tanks, fish were divided into three temperature treatments. One group of 30 fish were kept at the acclimation temperature of 28°C (control), while temperature was raised to 32°C (± 0.6°C) or 34°C (± 0.6°C)

in another two groups also each comprising 30 fish. These temperatures were chosen to test both mild and more acute thermal stress in *A. polyacanthus*. Temperatures were raised slowly over a 3.5 day period. All other important water quality parameters remained the same during the course of the experiment (34 ppt salinity, < 1mg L^{-1} NH_3, > 4 mg L^{-1} dissolved oxygen). Once temperatures had reached experimental targets, fish were held at the appropriate temperature for 4 days before temperatures were lowered over 2 days back to those of the control (28°C). Fish were then held at 28°C for a further 7 days in order to detect potential changes in the chosen stress indicators after a week of recovery.

3.2.3. Sample collection

Six fish were sampled from each temperature treatment when experimental temperature was reached ($t_{instant}$) in order to detect early responses, as well as after 6 h, 24 h and 96 h. The first two time points ($t_{instant}$ and t = 6 h) were chosen to assess the early physiological response of the fish; t = 24 h and t = 96 h were chosen to assess the potential for subsequent acclimation of the markers to thermal stress. The last sampling of fish was performed after a 1 week recovery period at 28°C. During sampling, fish handling was kept to a minimum (less than 5 sec) and animals were anesthetized in 0.05% 2-phenoxyethanol solution (Begg and Pankhurst, 2004). Fish were then immediately and humanely sacrificed by cervical dislocation and whole liver and all muscle tissues excised and snap frozen in liquid nitrogen. All samples were stored at -80ºC for future use.

3.2.4. ChE inhibition assay

The methods used to determine ChE activity followed the same procedure as that detailed in Chapter 2, Section 2.2.3.1, page 42 The assay was read immediately at 412 nm at 25°C for 10 min. Each sample was run in triplicate with an average CV of 12%. ChE activity was determined according to Chapter 2. ChE activity is expressed in μmol of ACTC hydrolysed\ min\ mg protein. In order to test for direct thermal denaturation of the enzyme, as opposed to the existence of a complex stress response, *in vitro* experiments were also conducted. For this purpose, muscle samples of 12 fish acclimated to 28ºC for several months were used to measure ChE activity as described previously, except that temperature of the measuring chamber was modified and set at 26ºC, 28ºC, 30ºC, 32ºC and 34ºC.

3.2.5. CoQ redox balance

Details of the methods employed have been provided in Chapter 2, Section 2.2.4, page 44. Liver tissues were chosen to achieve consistency with the previous experiment and studies reporting the use of CoQ redox balance as an indicator of stress for fish (Hasbi et al, 2010; Gagliano et al, 2007).

3.2.6. GST activity

GST activity was measured as described in Chapter 2, Section 2.2.5, page 45.

3.2.7. Statistical analysis

Statistica (Version 7.0, StatSoft 2004, Tulsa, OK, USA) was used to check for normality and homogeneity of the data before statistical analyses. No transformation of data was necessary. Differences between treatments were analysed by performing a

two-way ANOVA with time and temperature as fixed categorical orthogonal variables and a Type III decomposition model to account for unequal number of samples, followed by simple main effect tests (Quinn and Keough, 2002). Post-hoc Unequal N HSD tests were used to discriminate heterogeneous treatment means where appropriate. Significance was accepted when $P < 0.05$.

3.3. RESULTS

3.3.1. ChE inhibition assay

Both temperature and time had a significant effect on ChE activity ($F_{2,68}$ = 16.97, $P < 0.001$ and $F_{4,68}$ = 3.47, $P = 0.009$ respectively, Appendix C, Table C.1), as well as temperature through time ($F_{8,68}$ = 8, $P < 0.001$). Cholinesterase activity did not vary with time in the 28°C (control) treatment group, but exhibited a significant decline in fish exposed to 32°C and 34°C (Fig 3.1). Fish exposed to 32°C showed significantly depressed ChE activity compared to control group fish at both t = 24 h (Unequal N HSD, $P = 0.002$) and t = 96 h (Unequal N HSD, $P = 0.0002$), with a decrease of 43% and 52% respectively (Fig. 3.1). A significant difference between control and 34°C-treated fish was found at $t_{instant}$ (Unequal N HSD, $P = 0.008$) and t = 96 h (Unequal N HSD, $P << 0.001$), with a 48% and 56% decrease respectively (Fig 3.1). It therefore appears that 4 days of consecutive thermal stress at 4°C and 6°C higher than control temperature approximately halved ChE activity in juvenile *A. polyacanthus*. In addition, this effect was prolonged even after removal of the stressor as animals exposed to 32°C or 34°C during the 4 days of treatment still exhibited a 39% reduction in ChE activity compared to that of controls after one week of recovery at the control temperature (Unequal N HSD, $P = 0.025$ and $P = 0.009$) (Fig 3.1).

In vitro measurements were also carried out to examine if temperature could negatively affect muscle ChE activity without the presence of a whole-animal response. Measurements showed no effect of temperature ($F_{4,13}$ = 1.22,P > 0.3, Appendix C, Table C2), suggesting that ChE thermal denaturation and impairment of enzyme-substrate affinity are not responsible for the decrease in ChE activity observed in *A. polyacanthus* at high temperatures (Fig. 3.2).

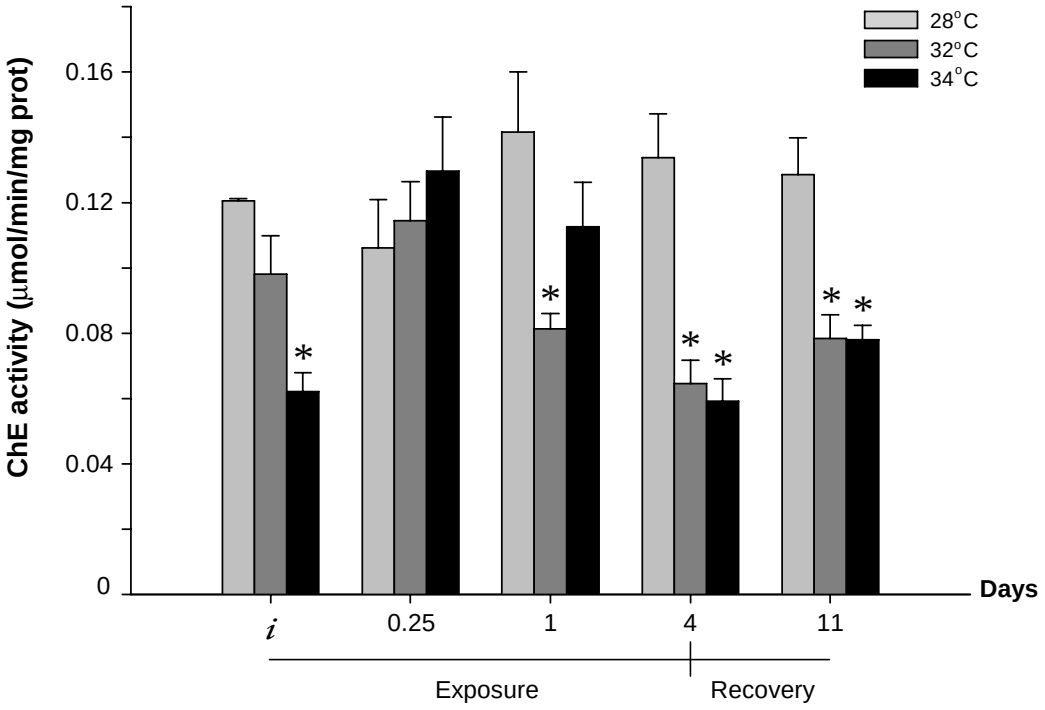


Figure 3.1. *In vivo* muscle ChE activity in *A. polyacanthus* in response to increasing temperature from 28 °C to 32 °C and 34 °C. Time is expressed in days for both exposure and recovery. Data represent mean (n = 5-6 ± SE). Asterisks show significant difference with control temperature at each time point (28 °C).

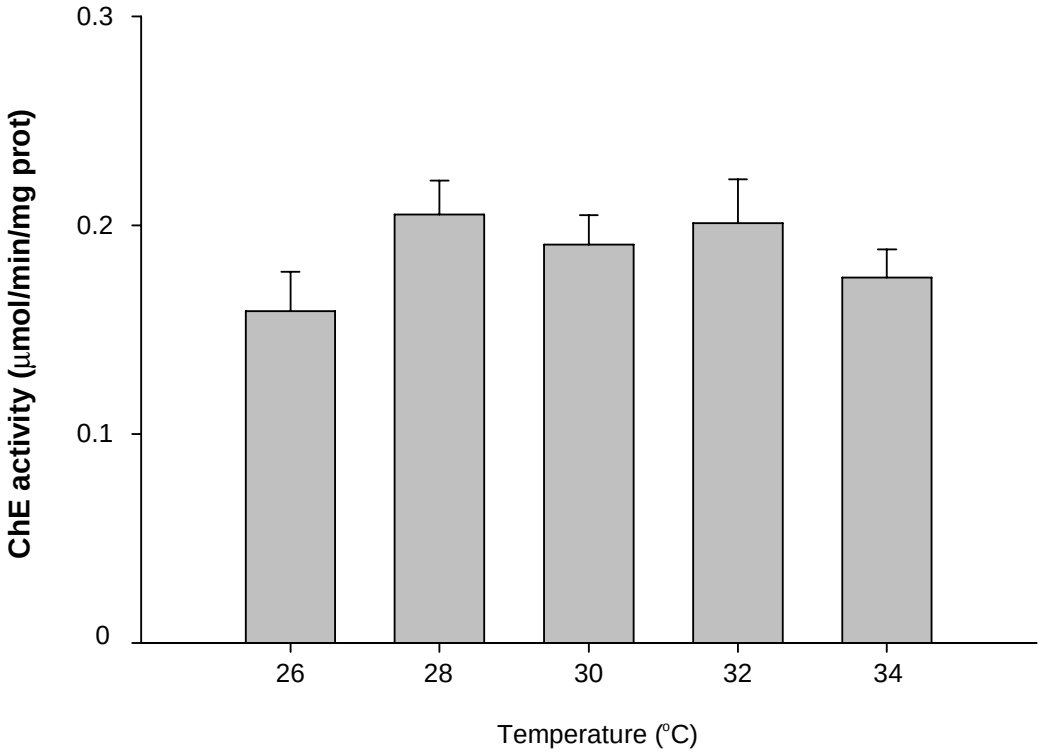


Figure 3.2. *In vitro* muscle ChE activity in *A. polyacanthus* muscle in response to increasing temperature from 28 °C to 32 °C and 34 °C. Data represent mean (n = 12 ± SE).

3.3.2. CoQ redox balance

In *A. polyacanthus*, $CoQH_2$ accounted for more than 85% of the total CoQ throughout the experiment in all control and treatment groups. Temperature had a significant effect through time ($F_{8,71}$ = 2.7, P = 0.012, see Appendix C, Table C.3) with a decrease in $CoQH_2$ at $t_{instant}$ when fish were exposed to 34 °C compared to 28 °C. This decrease was not found at other time points and might reflect a transient response to combat increasing oxidative stress in liver cells. Analysis revealed that only the 34°C treatment induced changes in CoQ redox balance at the times sampled during the course of the experiment (Fig. 3.3 and Appendix C, Table C.3).

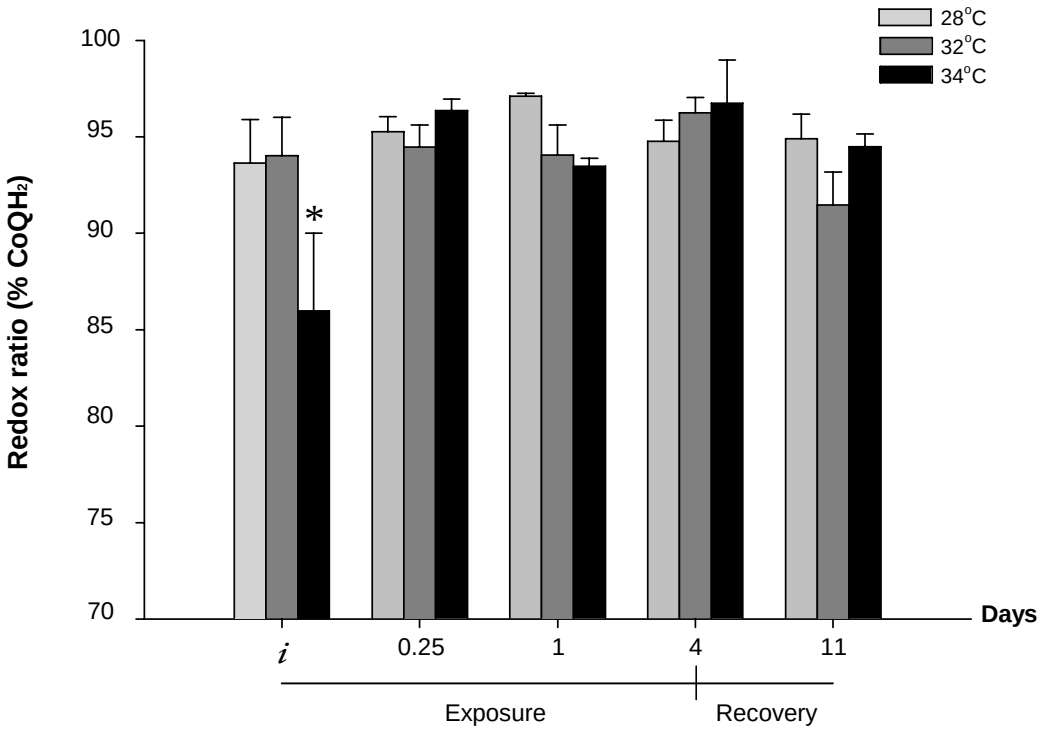


Figure 3.3. Percentage of reduced coenzyme Q (%$CoQH_2$) over the total CoQ in liver of *A. polyacanthus* in response to increasing temperature from 28 °C to 32 °C and 34 °C.

Time is expressed in days for both exposure and recovery. Data represent mean (n = 5-6 ± SE).

3.3.3. GST activity

Temperature had no effect on measured *A. polyacanthus* GST activity ($F_{2,68}$ = 1.392, P = 0.256, see Appendix C, Table C.4). Similarly, there was no effect of time or temperature through time over the course of the experiment ($F_{4,68}$ = 2.076, P = 0.094 and $F_{8,68}$ = 0.187, P = 0.992, respectively). Values ranged from 109-263 nmol min^{-1} ml^{-1} for the control treatment; from 114-382 nmol min^{-1} ml^{-1} for the 32°C treatment group and from 119-274 nmol min^{-1} ml^{-1} for the 34°C treatment group (Fig 3.4.).

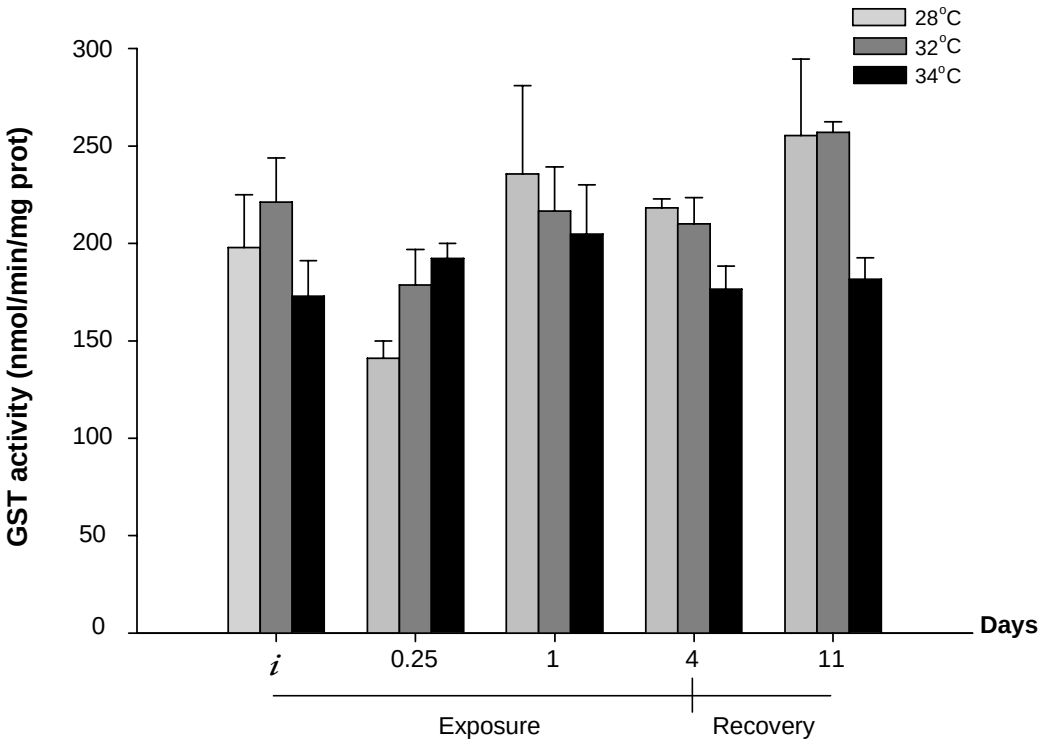


Figure 3.4. Hepatic GST activity in *A. polyacanthus* in response to increasing temperature from 28 °C to 32 °C and 34 °C. Time is expressed in days for both exposure and recovery. Data represent mean (n = 5-6 ± SE).

3.4. DISCUSSION

In this chapter, the effects of mild and acute temperature stress on *A. polyacanthus* were investigated, using three cellular stress markers: muscle ChE activity, hepatic CoQ redox balance and hepatic GST activity. Hepatic GST activity was insensitive to thermal stress at the times sampled. The results show, however, that *A. polyacanthus* muscle ChE activity and CoQ redox balance are sensitive to thermal stress with a decrease of both markers compared to control temperature. Muscle ChE activity was particularly affected with a prolonged reduction for up to 7 days after recovery at control temperature.

3.4.1. ChE activity

This study is the first to measure the impact of temperature increases on ChE activity in a tropical reef fish. As detailed in the previous chapter, Cholinesterases are major neural enzymes which allow the recycling and availability of the choline neurotransmitter in the nervous system and at neuromuscular junctions (Fulton and Key, 2001). Because of their importance for the functioning of the nervous system, ChEs have been studied in detail from a molecular and structural point of view (Massoulie et al., 2008, Silman and Sussman, 2008). In addition, ChE inhibition has been widely used in numerous fish species as an important marker of organophosphate exposure in ecotoxicology studies. The broad utilization of ChE activity as a contaminant marker in field based studies therefore requires examining its behaviour under different environmental conditions, such as in response to elevated temperature.

This is increasingly important in the context of warming waters on coral reefs. But quite surprisingly, little is known of the impact of temperature on ChE activity, particularly in tropical reef fish. Being poikilotherms, the vast majority of fish species are unable to regulate their body temperature. Therefore, their enzymatic reaction rates, as any chemical reaction, are highly temperature dependent. Here it was found that muscle ChE activity was halved in *A. polyacanthus* when the fish were subjected to a slow increase in water temperature to levels projected to occur by the end of the century on the GBR. After 96 h of exposure to both 32°C and 34°C, ChE activity was decreased by approximately 50% compared to control temperature treatments (28°C). In addition, seven days of recovery at control temperature only resulted in a partial recovery of ChE activity, with activity still reduced by 39% compared to control

levels in both of the elevated temperature treatments. This demonstrates an important prolonged effect of relatively short duration heat-stress events is possible on ChE activity in *A. polyacanthus*.

The relationship between ChE inhibition and mortality or sublethal effects is species dependent in fish (Fulton and Key, 2001), but for most species studied, mortality seems to occur only when ChE inhibition reaches 80%. However, impaired stamina in swimming performance trials arises after 50% of ChE inhibition is reached in salmonids such as *Salvenilus fontenalis, Onchorhychus kitusch* and *Salmo gairdneri* (Fulton and Key, 2001). No such test has been performed on a tropical reef fish and the interpretation of the results obtained here would greatly benefit from studies investigating the link between ChE inhibition and swimming behaviour of *A. polyacanthus,* or other aspects of whole-organism fitness. However, it can be hypothesized that a prolonged and substantial inhibition of ChE activity, as was observed here, after a relatively short duration thermal stress, is likely to be associated with other adverse effects that together may negatively impact the general long-term fitness and behaviour of this tropical reef fish species.

As temperature can affect enzyme activity in a number of direct ways, including *via* thermal denaturation of the proteins themselves and/or *via* reduced enzyme-substrate affinity, as was found in *Salmo gairdnerii* in early studies (Baldwin and Hochachka, 1970) for example, it is first necessary to rule out these causes of the decline in ChE activity. The *in vitro* heat-stress experiments on muscle protein extracts from *A. polyacanthus* undertaken here strongly indicate that the ChE molecule itself and the enzyme-substrate affinity are not affected over the temperature

range used for whole animal experiments, since no decrease in ChE activity was found for muscle protein extracts at temperatures up to 34ºC. It is most likely therefore, that the drop in ChE activity in thermally stressed *A. polyacanthus* resulted from a complex stress response potentially including reduced expression of ChE-encoding genes, leading to a reduced amount of ChE enzymes being produced. Gene expression of ChEs has rarely been investigated; however, the little information available suggests a role of temperature in the expression of ChE-encoding genes. For example, Perrier et al. investigated AChE enzyme activity and the expression of one AChE variant in rat (*Rattus rattus*) neuroblastoma cells after heat shock and found no effect on enzyme activity, but did observe a significant increase in AChE mRNA transcripts levels (Perrier et al., 2005). This result suggests that, at least in this particular cell type, temperature may have a significant impact on expression of ChE-encoding gene. In *A. polyacanthus*, the significant decline observed for 34°C-treated fish as experimental temperature was reached ($T_{instant}$) indicates an early response to the more acute stress treatment. However, this strong initial response was followed by a temporary recovery of ChE activity over the period 6 - 24 hrs which might have resulted from a transient upregulation of ChE gene expression or protein translation in response to this acute stress, as a means to restore homeostasis. In fish, ChE gene expression has not been investigated and the possibility of the regulation of ChE activity *via* gene expression in response to temperature stress in the damselfish *A. polyacanthus* cannot be excluded and should be explored in the future.

Seasonal variations in ChE activity should also be examined in *A. polyacanthus*, both under normal temperature fluctuations and during thermal stress events. Indeed, few studies have investigated this question, especially in marine species but a positive correlation was found between temperature and ChE activity in

the natural environment in the Russian freshwater fish *Rutilus rutilus L* (Pfeifer et al., 2005). *Acanthochromis polyacanthus* ChE is not affected by temperature itself *in vitro*, but might follow seasonal variations, which cannot be tested with *in vitro* experiments only. It will therefore be of great interest to determine whether the levels of *A. polyacanthus* ChE activity are relatively higher or lower in summer (compared to winter) when the fish experience high water temperatures and occasional thermal stress, as this could impact the ability of this tropical reef fish to maintain homeostasis during and sustain prolonged thermal stress events in the Indo-Pacific region.

3.4.2. CoQ redox balance

Coenzyme Q is a ubiquitous and central component of cellular membranes and of the mitochondrion respiratory chain where it is present as a redox couple. Changes in the CoQ redox balance have been used as an indicator for oxidative stress in humans (Yamashita and Yamamoto, 1997) and marine bacteria (Dunlap et al., 2002). Only recently has the CoQ redox balance been explored in fish, but to date this has only been in the context of contaminant exposure (Hasbi et al., 2011).

As oxygen consumption and metabolic rates generally rise with increasing temperatures, an increased production of ROS by mitochondria is expected in thermally stressed *A. polyacanthus.* Such an increase in ROS production has been demonstrated for several other fish species, such as the North Sea eelpout *Zoarces viviparous* exposed to laboratory-controlled heat shock from 12ºC to 18ºC, 22ºC or 26ºC and the goldfish *Carassius auratus* exposed to 35ºC compared to 21ºC (Heise et al., 2006a, Heise et al., 2003, Lushchak and Bagnyukova, 2006a). Correspondingly, a shift in the CoQ balance from the reduced form towards the oxidised form (producing

a drop in %$CoQH_2$) in temperature stressed *A. polyacanthus* compared to control fish was both expected, and indeed observed. In the present study, a decrease in $CoQH_2$ was found at the initial time point of the more acute 34ºC treatment, perhaps indicating the use of this compound in *A. polyacanthus* liver cells to scavenge temperature-stress related reactive oxygen species. Interestingly, no significant difference in *A. polyacanthus* hepatic CoQ redox balance compared to controls was found after 4 days of thermal stress. These results indicate that at 34 ºC (+6 ºC of thermal stress above controls) there appears to be an early oxidative stress response in the liver of juvenile spiny damselfish, but that the CoQ redox balance is only perturbed or utilized for ROS neutralization at early stages of the stress event. In this context, other cellular defences such as the activation of antioxidant enzymes are likely to ensure cellular defences against oxidative stress in *A. polyacanthus* liver exposed to thermal stress. The lack of a shift in the hepatic CoQ redox balance in fish exposed to the more chronic 32 ℃ stress suggests that a 4 ℃ temperature increase may not induce a cellular level oxidative stress of substantial magnitude. However, given the complex and multi-faceted oxidative stress responses possible, it is difficult to evaluate the level of oxidative stress experienced by heat stressed *A. polyacanthus* without additional measures of alternative antioxidant responses and/or measures of the actual level of ROS production in experimental fish.

3.4.3. GST activity

Glutathione-S-Transferases are another important component of the antioxidant pathway of most living organisms and consist of a family of detoxifying enzymes which eliminate xenobiotics and ROS from cells (Dinkova-Kostova and Talalay, 2008, Hayes et al., 2005). In rats and other mammals GST genes possess an

antioxidant response element (Rushmore et al., 1991) and enhanced transcription has been observed under oxidative stress conditions (Dinkova-Kostova and Talalay, 2008). It is not known whether GST genes in fish possess the same antioxidant response element, but if so it might be expected that increasing the environmental temperature of *A. polyacanthus* from 28°C to 34°C would lead to an oxidative stress response including an increase in the production of GSTs and/or increase in cellular GST activity. However, no significant effect of temperature on spiny damselfish *A. polyacanthus* GST activity was observed. Considered together, the lack of substantive or prolonged changes in both CoQ and GST activity for *A. polyacanthus* may imply that temperatures of 34 ºC or less do not induce significant oxidative stress in this species.

Alternatively, hepatic GST activity might not be sensitive to oxidative stress in this species. In other studies, GST activity in response to temperature stress varies between species, tissues and with the temperature regime employed. For example, in the temperate goldfish *Carassius auratus* GST activity in kidney did not vary when fish experienced a 14 °C heat shock (Lushchak and Bagnyukova, 2006c), but increased with a 20 ºC heat-shock (from 3 ºC to 23 ºC) (Bagnyukova et al., 2007). In this same study, increased GST activity was observed in brain and kidney but no change was seen in liver. Finally, not all species exhibit the same GST activity pattern in response to heat shock, as the spotted snakehead (freshwater fish) *Channa punctata* showed a significantly reduced hepatic GST activity after 3 h of 12 °C heat shock from 20 ºC to 32 ºC (Kaur et al., 2005, Goel et al., 2005, Mansour and Mossa, 2009), contrary to what was observed in *Carassius auratus* as reported above. Such disparities illustrate the complex behaviour of GST in fish exposed to thermal stress

and indicate that future studies on *A. polyacanthus* thermal stress response should examine GST activity in different tissues as well as in liver.

3.5. CONCLUSION

This chapter explored the response of juvenile *A. polyacanthus* to temperature stress at the cellular level. It provides novel evidence that neural enzyme activity is affected by thermal stress with muscle ChE activity decreased by approximately 50% when the fish are exposed to 4 days at 32 ℃ and 34 ℃ compared to 28 ℃. The results also reveal a prolonged negative impact, with ChE activity still reduced by 39% after 7 days of recovery. *In vitro* measurements verify the decrease in ChE activity is not due to changes in enzyme-substrate affinity and suggests that the depression in ChE activity reflects a true cellular stress response mechanism. ChE activity is therefore a highly sensitive marker not only for CPF exposure but also for temperature stress in *A. polyacanthus*. Further research should be undertaken to better understand the impact of thermal stress on other aspects of the nervous system of this fish species, as such data will shed more light on the degradation of the overall health and condition of thermally-stressed *A. polyacanthus* at relatively modest temperature elevations, as was found in recent studies.

4. Combined Effects of Chlorpyrifos Exposure and Thermal Stress on *Acanthochromis polyacanthus*

4.1. INTRODUCTION

Coastal marine ecosystems are facing a decrease in water quality worldwide (Rabalais et al., 2009). Until recently, most pollution and ecotoxicology studies have focused on temperate regions and species, but tropical marine environments are also affected by significant anthropogenic contamination (Carvalho et al., 2002, Ng Kee Kwong et al., 2002, Readman et al., 1992) The development of agricultural lands, industries and urban zones along tropical coastlines is increasing and contaminants from these sources reach and impact vulnerable marine ecosystems such as mangroves and coral reefs via terrestrial runoff (Larsen and Webb, 2009, Ramade and Roche, 2006). For example, recent surveys have detected a wide range of pesticides within the GBR lagoon (Kapernick et al., 2006, Shaw et al., 2009) and negative impacts of pollution on the physiology of marine organisms have been described across water quality gradients. These include reduced enzyme activity in the barramundi *Lates calcarifer* (Humphrey et al., 2007) and mud crab (*Scylla serrata*) (van Oosterom et al., 2010) and altered reproduction of the sponge *Rhopaloeides odorabile* (Whalan et al., 2007). The effects of contaminants on marine life in other tropical regions, such as in South-East Asia, are likely to be greater still due to the rapid increase in human population and anthropogenic activities along the coast.

Sea surface temperatures (SSTs) are also projected to increase by between 1.8–4.0 ºC by 2100 (IPCC 2007). An increase in SST of 0.5 °C has already been recorded on the GBR since the 1970's (Lough, 2007). Increased SST can affect

tropical fish life history traits such as growth (Meekan et al., 2003), survival (Gagliano and McCormick, 2007) and reproduction (Hilder and Pankhurst, 2003), and this has implications for community assemblages and species distributions (Adam et al., 2011, Bellwood et al., 2006, Booth and Beretta, 2002). In addition to these effects at the whole organism and ecosystem level, thermal stress also affects fish at the cellular or biochemical level. For example, thermal stress results in increases in stress hormones like cortisol, increased plasma glucose and free fatty acids levels as well as decreases in immune defences (reviewed in Bonga, 1997).

While fish commonly face seasonal episodes of thermal stress that may become more intense or prolonged under projected climate change scenarios, thermal stress is unlikely to impact upon fish in isolation. Rather, thermal stress will increase against a background of other stressors such as enhanced contaminant loads. For example, in the Indo-Pacific, the majority of contaminants are transported into reef waters by monsoonal flood events which occur in the warm summer months (Fabricius, 2005); therefore, marine organisms are usually exposed to the highest concentrations of contaminants and thermal stress simultaneously. So far, the few laboratory-based studies of combined insecticide exposure and thermal stress on fish have focused on freshwater species. In Australia, four freshwater fish species showed decreased upper thermal tolerance when exposed to the organophosphate insecticides endosulfan and chlorpyrifos (CPF) (Patra et al., 2007). In addition, Humphrey and Klumpp (2003) showed that temperature increased CPF toxicity in freshwater fish eggs from the rainbowfish *Melanotaenia splendida splendida* which exhibited higher mortality rates at 29 ℃ compared with 25 ℃ for a given CPF concentration. The recent evidence for insecticide exposure in tropical marine habitats (Humphrey et al.,

2007, van Oosterom et al., 2010) highlights the need for studies on the effects of these contaminants on enzymatic and molecular processes in tropical marine organisms and at summer temperatures relevant to future climate scenarios.

The insecticide chlorpyrifos (CPF) is one of the most commonly applied pesticides on farms in the tropics (Hamilton and Haydon, 1996) and it acts by inhibiting cholinesterase enzymes (ChEs) in insects, interfering with signal transduction in nerve and muscle cells and ultimately causing death. Non-target species such as fish are equally at risk from CPF exposure and the ChE enzyme assay has become a primary tool to measure exposure of fish to CPF in the environment (see Chapter 2) (Botté et al., in press, Fulton and Key, 2001). CPF exposure has also been shown to induce oxidative stress in mammals (Crumpton et al., 2000) and fish (Botté et al., in press, Kavitha and Rao, 2008). The assessment of the proportion of redox form of the antioxidant CoQ was demonstrated as a useful oxidative stress marker in the liver of *Acanthochromis polyacanthus* (in Chapter 2 and 3) and an early means to detect oxidative stress induced by either CPF exposure or thermal stress. Other relevant stress biomarkers are enzymes such as glutathione-S-transferases (GST) that are recognised for their detoxifying role after CPF exposure (Fujioka and Casida, 2007) and their susceptibility to temperature stress (Bachinski et al., 1997, Kaur et al., 2005). Toxicant exposure is generally associated with increasing GST activity (Eaton and Bammler, 1999, Hayes et al., 2005), although with a few exceptions (Goel et al., 2005, Mansour and Mossa, 2009). Temperature effects on GST activity are variable and typically depend on the species and tissue studied as well as the temperature regime employed, making it necessary to evaluate stress responses of each potential bioindicator species individually.

Changes at the molecular level, including gene expression patterns, may further reveal the fundamental stress response pathways leading to biochemical and whole-organism responses in fish. Thermal stress effects on gene expression in fish are increasingly being studied using candidate gene expression assays, in which levels of target gene transcripts are measured by quantitative polymerase chain reaction (qPCR) (Filby et al., 2007, Pérez-Casanova et al., 2008, Weber and Bosworth, 2005). Broader techniques such as microarrays, which quantify transcript levels for thousands of genes, are also now being applied, although usually on far fewer individuals (Logan and Somero, 2010, Podrabsky and Somero, 2004). A number of genes that respond to thermal stress have been identified from a microarray study of the GBR damselfish *Pomacentrus moluccensis* (Kassahn et al., 2007a). In contrast, most studies investigating exposure to organophosphates (OP) contamination in fish have assessed protein activity alone, with very few studies assessing the response to stress at the transcriptional level (Tilton et al., 2011). This is surprising because along with the hypothalamus/pituitary pathway, the transcriptional response to a variety of other stressors has been characterized in fish and transcriptional changes are in fact considered the frontline of stress defence mechanisms (reviewed in Bonga, 1997).

The present chapter examined the effects of CPF exposure and thermal stress on the expression of several candidate genes. These genes were chosen to reflect different biological processes potentially affected by CPF and thermal stress. For instance, the general stress response was represented by the heat shock protein 90 (*hspP90*) and elongation factor 1 alpha (*ef1α*) genes. The heat shock activation pathway is a ubiquitous and rapid stress defence mechanism and a good example of how gene transcription can quickly change in an attempt to maintain homeostasis (Roberts et al., 2010). Often referred to as chaperones, HSPs facilitate protein

synthesis and proper protein folding and are among the most well studied stress-response pathways due to their almost ubiquitous activation in response to stress, recently reviewed (Iwama et al., 1998, Kalmar and Greensmith, 2009, Roberts et al., 2010). In particular, HSP90 ensures proper folding of newly synthesized proteins and activates other HSPs (Iwama et al., 1998). EF1α is a protein that promotes elongation of newly synthesized proteins by recruiting transfer RNA (tRNA) to the mRNA/ribosome complex (Thornton et al., 2003). Traditionally, the EF1α-encoding gene has been used as a "housekeeping" or reference gene in qPCR experiments as it often displays stable expression across many tissues and under different conditions (Jorgensen et al., 2006, McCurley and Callard, 2008, Olsvik et al., 2005, Small et al., 2008). However, in parallel, the last decade has seen several studies in the medical field extend the role of *ef1α* to other processes, such as oncogenesis, cytoskeleton regulation, apoptosis and protein degradation (Ibarz et al., 2010, Thornton et al., 2003). The recent deeper understanding of *ef1α* expression patterns led these authors to hypothesize that this gene regulates protein turnover, and the cellular stress response, by selectively enhancing or repressing elongation of stress-responsive proteins, making this gene a potentially valuable stress biomarker. Finally, two additional genes were also selected as potential indicators of an oxidative stress response, namely catalase (*cat*) and glutathione transferase (*gst*). Catalase is an antioxidant enzyme often used in toxicology studies and GSTs are specialised in cell detoxification and antioxidant response, especially in liver (see previous chapters).

In view of the multiple stressors that tropical reefs currently face, the biochemical and molecular effects of the combination of both temperature stress and concurrent CPF exposure were investigated on a potential bioindicator species for the

Indo-Pacific, namely the spiny damselfish *Acanthochromis polyacanthus*. Concentrations of 1 and 10 µg/L CPF were tested with a simultaneous temperature increase from 28 ºC to 31 ºC, for 4 and 7 days. Effects on enzyme activity and oxidative stress at the biochemical level were investigated by measuring muscle ChE activity, hepatic $CoQH_2$ redox balance and hepatic GST activity. Transcriptional changes were explored by measuring the expression of *hsp90*, *ef1α*, *cat* and *gst* candidate genes (Dahlhoff, 2004, Hayes et al., 2005, Iwama et al., 1998, Kassahn et al., 2007a, Podrabsky and Somero, 2004). This study is the first to investigate the effects of simultaneous thermal stress and toxicants exposure on a tropical reef fish by examining a wide range of stress response biomarkers at both the protein and gene transcription level.

4.2. MATERIALS AND METHODS

All procedures conformed to James Cook University ethical guidelines, under the authority of the Queensland Department for Primary Industries and Fisheries (Scientific Registration Number 0013), in compliance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and the Queensland Animal Care and Protection Act 2001. This study was conducted under ethics permit number A1258.

4.2.1. Fish maintenance

Juvenile *A. polyacanthus* ranging from 53 to 62 mm were provided by the Marine and Aquaculture Research Facility of James Cook University in Townsville, Australia. Juvenile fish from across 10 cohorts, originated from the mating of 10 different parent pairs, were used in order to minimise the chances of introducing bias

due to possible genetic differences in physiological responses between fish families. A total of 102 individual fish were maintained at 28 °C in a 1000 L tank at the Australian Institute of Marine Science in an aerated, flow-through system for 2 months prior to the commencement of experimentation during which they were fed daily to satiation and were regularly inspected for signs of distress or diseases. Fish were randomly selected and transferred to test chambers 3 days before the experiment started.

4.2.2. Experimental set-up and sampling

A flow-through, climate-controlled dosing facility allowed the simultaneous exposure of replicate fish to one of two temperatures and one of four concentrations of CPF with two replicate chambers of seven fish for each of the eight treatment combinations. Incoming seawater (24 °C) was filtered (1 µm) into four 600 L sumps which each delivered seawater into multiple treatment chambers using submersible pumps (one pump per sump). The sumps were heated to different temperatures using 2 and 3 kW titanium heating bars controlled with a CR1000 Measurement and Control Datalogger (Campbell Scientific). The logger's precision sensors were placed inside the 18 L glass treatment tanks. The seawater flow rates for treatment tanks were maintained at 400 mL/min, measured using in-line flow meters, and controlled with manual valves. Two Masterflex L/X multichannel peristaltic pumps (Extech Equipment) delivered CPF stock (see below) into each of the treatment tanks at a constant flow rate of 1.0 mL min^{-1}. The experiment was conducted under 12 h light:dark photoperiods using adjustable racks of 55 W, 420 nm compact fluorescent globes (Catalina).

Treatments included a control SST of 28 ºC and a high SST treatment at 31 ºC crossed with four pesticide treatments composed of a seawater control, a solvent (acetone) control, a 1 μg/L CPF treatment and a 10 μg/L CPF treatment. The 31 ºC temperature was chosen over the 32 ºC temperature used in Chapter 3 in order to better reflect projections of future SST on the GBR (Lough, 2007) and to investigate the cellular processes taking place at temperatures which are known to affect *A. polyacanthus* growth and health (Munday et al., 2008, Donelson, 2011). Acetone concentration was less than 0.02% in test chambers (v/v). One fish from each chamber was sampled prior to exposure and three fish were sampled from each replicate chamber (six fish per treatment combination) at each of two time points, namely 4 days and 7 days after starting CPF exposure. During sampling, fish were rapidly removed from the chambers and anaesthetized in 0.05% 2-phenoxyethanol (2-PE) as described in Begg and Pankhurst (2004) after which they were sacrificed by cervical dislocation. Muscle and liver were promptly excised and immediately frozen in liquid nitrogen for later analysis. Since liver tissue was subsequently used for three different assays including CoQ ratio measurements, GST enzyme activity and gene expression quantification, entire livers (both lobes) were taken and snap frozen in liquid nitrogen. Prior to subsampling for different assays the entire livers were crushed in liquid nitrogen and well mixed to ensure homogenization of the sample before biochemical and molecular analyses.

4.2.3. Biochemical analyses

All methods used to measure ChE activity, GST activity and CoQ redox balance followed the procedures described in Chapter 2, Sections 2.2.3.1 (p.42), 2.2.4 (p.44) and 2.2.5 (p. 45).

4.2.4. Quantitative PCR (qPCR) assay

4.2.4.1. mRNA extraction

Messenger RNA was extracted from liver tissue with Dynabeads mRNA direct kit (Invitrogen) using 150 µL of beads, 400 µL of Lysis buffer, two washes with 800 µL of Wash Buffer A and two washes with 500 µL of Wash buffer B. DNA shearing was induced by passing the initial cell lysate through a 21 G syringe until the homogenate lost viscosity. At the end of the extraction, beads containing the mRNA were resuspended in 20 µL of Tris-HCl and mRNA eluted from the beads during a 2 min 80ºC incubation step. The concentration and purity of mRNA was checked on a Nanodrop ND-1000 Spectrophotomoeter. Samples retained for the remainder of the experiment exhibited 260/280 ratios between 1.97 and 2.04 and 260/230 ratios between 1.91 and 2.27. Additional quality control checks were performed for three random samples using a Bioanalyzer (2100 Bioanalyser, Agilent) and revealed very high concentrations of high quality mRNA, with very little degradation. Each mRNA extract was diluted with nuclease free water to a final concentration of 15 ng/µL.

4.2.4.2. cDNA synthesis and quantification

cDNA synthesis was performed using the Invitrogen Superscript III kit by following the manufacturer's instructions and with the following quantities: 75 ng of mRNA, 25pmol of oligo dT and 25 pmol of random hexamers in a final volume of 25 µL. RNA was digested from the resulting cDNA using RNase Cocktail™ Enzyme Mix (Ambion). cDNA was cleaned-up *via* resin columns (Ambion Nuc Away™ Spin Columns) to ensure removal of enzyme, unincorporated dNTPs and oligonucleotides. cDNA concentration was measured in triplicate for each sample on a Wallac Victor2 1420 Multilabel Counter (Perkin Elmer) using the Quant-it™-Oligreen ssDNA assay

kit (Invitrogen) following the manufacturer's instructions. Briefly, oligonucleotides were diluted 1:50 in order to obtain a 20 µM working solution. This stock was further diluted to produce a standard curve ranging from 10 ng/mL to 1 µg/mL. cDNA samples for quantification were also diluted 1:50. Fifty microliters of diluted standard or sample were loaded in triplicates in a 96 well plate along with 50 µL of reagent. Fluorescence was read at 480 nm excitation and 520 nm emission wavelengths. Measurements were corrected for background fluorescence. A standard curve was constructed for each plate (minimum R^2 = 0.975) and used to determine sample cDNA concentration. Each cDNA was subsequently diluted to a final concentration of 0.06 ng/µL for qPCR.

4.2.4.3. qPCR primer design

The target genes for analyses were heat shock protein 90 (*hsp 90*), elongation factor 1 alpha (*ef1α*), catalase (*Cat)* and glutathione-s-transferase (*gst*). In order to design *A. polyacanthus* specific primers in absence of molecular data for this species, universal primers were first designed in conserved gene regions by aligning cDNA sequences of several fish species available in Genbank, using Clustal W software. Primers were designed with Pearl Primer (http://perlprimer.sourceforge.net) to minimize secondary structures (Table 4.1) and manufactured by Sigma-Aldrich. cDNA from liver tissue obtained as described above were used to amplify species-specific PCR products using 1 µL of cDNA (0.06 ng/µL), 5 µL of buffer, 1 µL of dNTPs, 1 µL of each primer and 0.5 µL of DyNAzyme^{TM} I (Finnzymes) in a 50 µL reaction. The following thermocycling program was run on a 2720 Thermal Cycler (Applied Biosystems) : 94 ºC for 1 min followed by 30 cycles of 94 ºC for 30 sec, 56 ºC for 30 sec and 72 ºC for 1 min, followed by a 10 min final extension step at 72 ºC.

PCR product quality was checked on a 1% agarose gel. Although a single band was observed on the gel in each instance, three of the four candidate genes (*hsp90, gst* and *ef1α*) belong to multigene families and different variants could have been co-amplified. The PCR products were therefore cloned into *E. coli* using the TOPO TA Cloning® Kit (Invitrogen) prior to sequencing. A clone library was constructed prior to sequencing of 96 clones, carried out by Macrogen. Resulting sequences from all clones were aligned for each gene using Sequencher software in order to distinguish the gene variants present. Discriminative *A. polyacanthus*-specific primers were then designed with Perl primer to perform qPCR (see Table 2 for primer information).

4.2.4.4. qPCR optimization

In order to optimize the qPCR conditions and to validate the assay, three fish liver samples from the control group (28 ºC and no pesticide) and three liver samples from fish from experimental treatments were pooled and used to obtain cDNA as described above. After quantification with RiboGreen (Invitrogen), cDNAs were diluted in a 10 fold dilution series covering six orders of magnitudes. Quantitative PCR primers were used to amplify the target genes using SYBR® Premix Ex Taq (Perfect Real Time) from Takara using the following program: 95 ºC for 15 sec and then 40 cycles of 95 ºC for 5 sec, annealing temperature for 15 sec, 72 ºC for 30 sec, followed by a melt curve between 72 ºC and 95 ºC. Runs were performed on a Corbett Rotor Gene 3000 designed to ensure homogenous heat circulation in the chamber. Samples were loaded by a robotic liquid-handling workstation CAS-1200 (Corbett Robotics) to minimise human error. The cDNA dilution series was used to calculate the PCR efficiency for each gene (see Table 4.2 for PCR efficiency). Melt curves revealed a single PCR product for each gene amplified in all samples, therefore

verifying the lack of primer dimer formation and the specificity of the assay for a single gene locus.

4.2.4.5. qPCR runs and analysis

As described earlier, cDNA was measured and diluted to the same concentration prior to qPCR analysis so that each sample contained 0.06 ng of cDNA. Each sample was analysed in triplicate for all genes as described in the qPCR optimization section. Samples from the same time point and both temperatures (28 ℃ and 31 ℃) were analysed at the same time (i.e. on the same run). A calibrator positive control sample (28 ℃ and 10 µg/L CPF concentration after 7 days of exposure) was included in each experimental run in order to assess run to run variability. The use of this calibrator sample was set in the qPCR run set-up prior to starting the assay, so that raw data collected after each run were directly provided relative to this calibrator. Variability due to cDNA synthesis was minimal in our experimental procedure, since cDNA was quantified prior to PCR reaction, when most authors quantify RNA only, which introduces variability and requires the use of several housekeeping genes to quantify candidate gene expression. The robustness of expressing gene expression relative to cDNA amount has been demonstrated previously (De Santis et al., 2010, Filby et al., 2007). Relative expression was calculated with the following formula: $E^{(Ctsample-Ctsolvent)}$ where E represents the efficiency of the reaction (calculated during optimization – see Table 4.2), Ctsample is the average cycle number at which fluorescence reached the set threshold for individual samples run in triplicate and Ctsolvent is the average Ct for 28 ℃ solvent control samples after 4 days of exposure. In this way, gene expression of all samples is expressed relative to the solvent control fish sampled at the control temperature (28 ℃) after 4 days of exposure.

4.2.5. Statistical analysis

The Statistica software package (Statsoft) was used to perform all analyses. Assumptions of normality and homogeneity of residues were checked prior to undertaking tests. Gene expression data were square-root transformed in order to meet these assumptions, while all other biochemical marker data did not require transformation. Differences between treatments were evaluated by performing a factorial ANOVA with time, CPF concentration and temperature as fixed factors. Wherever appropriate, simple main effect tests (Quinn and Keough, 2002) and post-hoc Tukey tests were carried out to assess the effects of CPF concentration and/or temperature at each time point and identify heterogeneity among treatment means. Significant difference was accepted when $P \leq 0.05$.

Table 4.1. Universal fish PCR primers and their properties to amplify *hsp90*, *ef1α*, *cat* and *gst* genes.

Primer name	Sequence	Tm (°C)	Annealing temperature (°C)	Amplicon size (bp)
gstF	AGGACATGACTCTGCTGTG	61.59	56	610
gstR	TGGCTTTGATGCTGGGTCT	64.2		
ef1αF	GAYCCACATYAACATCGTG	61.03	56	1020
ef1αR	GGTGGTTCAGGATGATGAC	60.06		
catF	GATTTTCGTGAAGACGTTG	62.6	61	678
catR	GCATATCATCTTGTCGCAGT	60.09		
hsp90F	GGATGAGGAAGTGGAGACCTT	63.3	61	950
hsp90R	GATTTCCTCCACAGCTGAGTC	62.8		

Table 4.2. *Acanthochromis polyacanthus*-specific qPCR primers and their properties to assess gene expression of *hsp90*, *ef1α*, *cat* and *gst*

Primer name	Sequence	Tm (°C)	Annealing temperature (°C)	qPCR efficiency (%)	Amplicon size
qhsp90F	AACCCCGATGACATCAC	59.2	58	101	90
qhsp90R	CATGTAGCCCATGGTAGAG	58			
qgstF	CTGTGGGAGGGATACC	57.2	56	97	132
qgstR	AAACAAGCACATCAGCC	58			
qcatF	ATGGTGTGGACTTCTGGAG	63.7	61	99	175
qcatR	ATGGAACTTGCAGTAGAAACG	63.1			
qef1αF	CGGTGTGAAGCAGCTC	60.3	58	97	85
qef1αR	TGATGTAGGTGCTCATTC	59			

4.3. RESULTS

4.3.1. ChE activity

Carrier solvent had an effect on ChE activity after 4 days of exposure but not after 7 days (see Appendix D, Table D.1a) and subsequent analysis was therefore carried out with solvent control treated fish only. No significant interaction was found between CPF and temperature over the course of the experiment. CPF concentration significantly decreased the ChE activity in damselfish, regardless of time ($F_{3,75}$ = 75, P = 0.002, Appendix D, Table D.1b) (Fig 4.1). The 10 µg/L treatment resulted in significantly lower ChE activity compared with solvent-control fish ($F_{3,75}$ = 23.838, P < 0.001). After four days of CPF exposure, ChE activity decreased by 27% in fish exposed to 10 µg/L at 28ºC and by 37% at 31 ºC, compared to solvent controls (Fig. 4.1). After 7 days fish exposed to 10 µg/L exhibited 59% reduced ChE activity at 28°C and 45% at 31 °C, compared to solvent control fish. Temperature had a significant effect on ChE activity over time ($F_{1,75}$ = 13.917, P < 0.001, see Appendix D, Table D.1b). Simple main effect analysis revealed that temperature had no effect after 4 days of exposure ($F_{1,75}$ = 2.3460, P = 0.130, Appendix D, Table D.1b), but in fact led to significantly higher ChE activity in heat treated relative to control fish after 7 days of exposure ($F_{1,75}$ = 12.5434, P < 0.001, Appendix D, Table D.1b). After 7 days, fish exposed to solvent and to 1 µg/L CPF exhibited a 26% increase between 28 °C and 31 °C, and fish exposed to 10 µg/L showed a larger 67% increase between the control temperature and 31 °C. Although ChE activity was higher at 31 °C, the activity in 10 µg/L CPF treated fish still exhibited reduced activity compared to controls at 28 °C (Fig. 4.1).

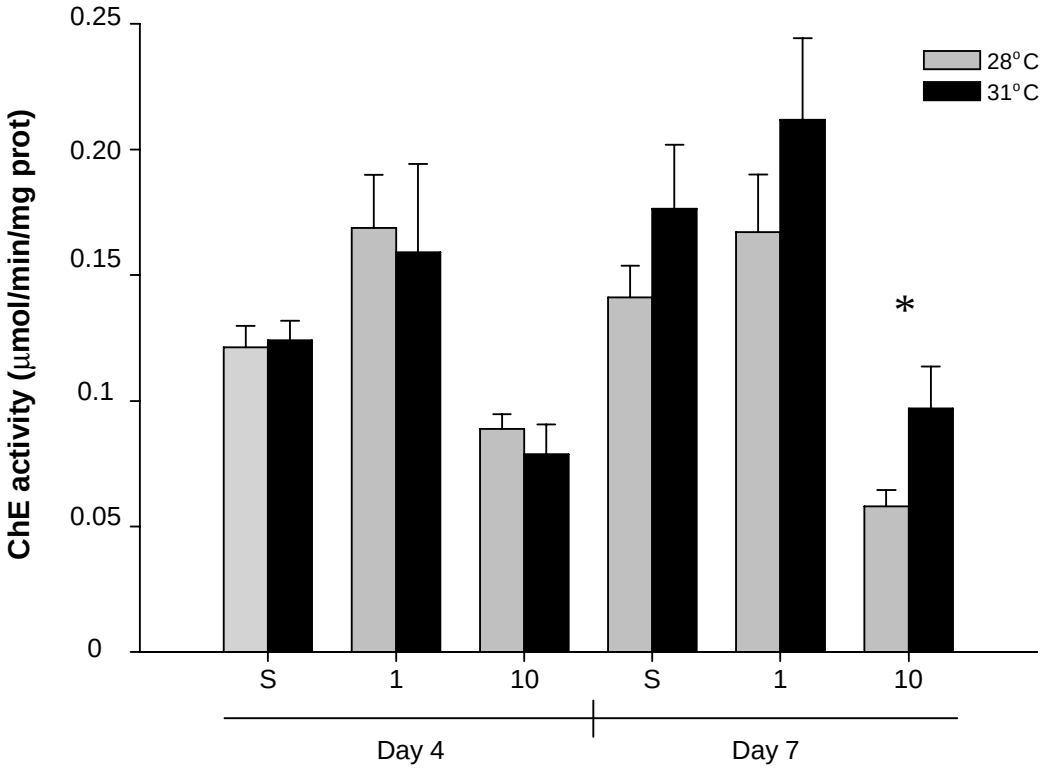


Figure 4.1. Muscle ChE activity in *A. polyacanthus* in response to simultaneous CPF exposure (1 µg/L and 10 µg/L) and temperature increase from 28 °C to 31 °C. Data represent means (n = 6 ± SE). S: solvent; 1: 1 µg/L; 10: 10 µg/L. Asterisks represent significant difference between CPF concentration and solvent control after 7 days of exposure, regardless of temperature. Temperature had a significant effect on ChE after 7 days of exposure (not represented on graph, see Section 4.3.1, p. 97)

4.3.2. CoQ redox balance

CoQ redox balance was affected by time ($F_{1,75}$ = 4.03, *P* = 0.048, see Appendix D, Table D.2b) but not by the solvent carrier or any of the treatments (p > 0.2 see Appendix D, Table D2a and Table D.2b). Percentage $CoQH_2$ over total CoQ ranged between 87.2% and 91.2% after 4 days of exposure and between 85.4% and 88.9% after 7 days of exposure. Although not significant, there was a trend towards a decline in CoQ redox balance with increasing CPF concentration at 28 ºC after 4 days of exposure (Fig. 4.2) with a 3% decrease for fish exposed to 1 µg/L and 10 µg/L CPF compared to control fish. This non-significant response was no longer evident after 7

days of exposure, nor was it evident for fish exposed at 31 ºC for either 4 or 7 days (Fig. 4.2).

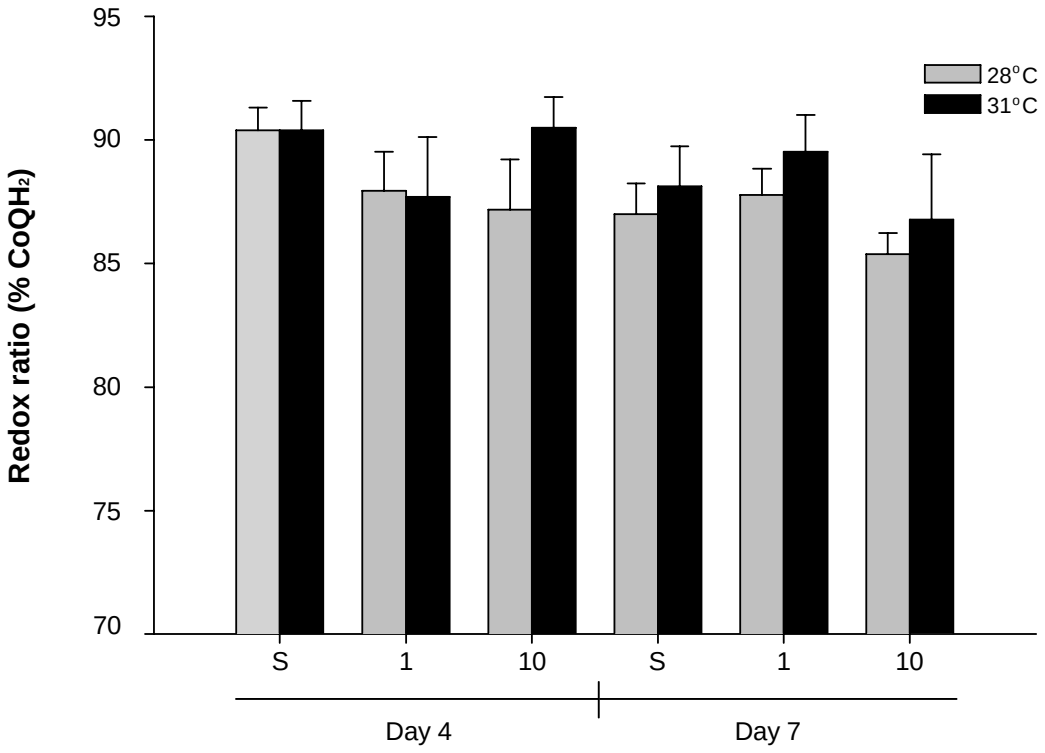


Figure 4.2. Percentage of reduced coenzyme Q (%$CoQH_2$) over the total CoQ in liver of *A. polyacanthus* in response to simultaneous CPF exposure (1 µg/L and 10 µg/L) and temperature increase from 28 °C to 31 °C. Data represent means (n = 6 ± SE). S: solvent; 1: 1 µg/L; 10: 10 µg/L. No significant difference was detected between all treatments over the course of the experiment.

4.3.3. GST activity

GST activity was not affected by solvent carrier throughout the experiment (Appendix D, Table D.3a). GST activity was, however, significantly affected by temperature over time. After 4 days of exposure, GST activity significantly decreased in fish from the 31ºC treatment groups compared to those at 28 ºC ($F_{1,72}$ = 14.85, P = 0.0003, Appendix D, Table D.3b) (Fig. 4.3). After 4 days of exposure, mean GST activity averaged 244, 260 and 239nmol/min/mg prot in fish maintained at 31 ºC and

exposed to solvent, 1 µgl/ and 10 µg/L respectively, while fish exposed to 28 ºC showed an average GST activity of 373, 337 and 296nmol/min/mg prot. The effect of temperature was not apparent after 7 days of exposure ($F_{1,72}$= 0.0460, P = 0.831), at which time values ranged from 242nmol/min/mg prot and 306nmol/min/mg prot for fish exposed to 28 ºC and from 240nmol/min/mg prot to 289nmol/min/mg prot for fish exposed to 31 ºC, possibly indicating acclimation to the higher temperatures. Exposure to CPF did not affect GST activity after 4 days or 7 days of treatment and no interaction between temperature and CPF concentration was found (see Appendix D, Table D.3b).

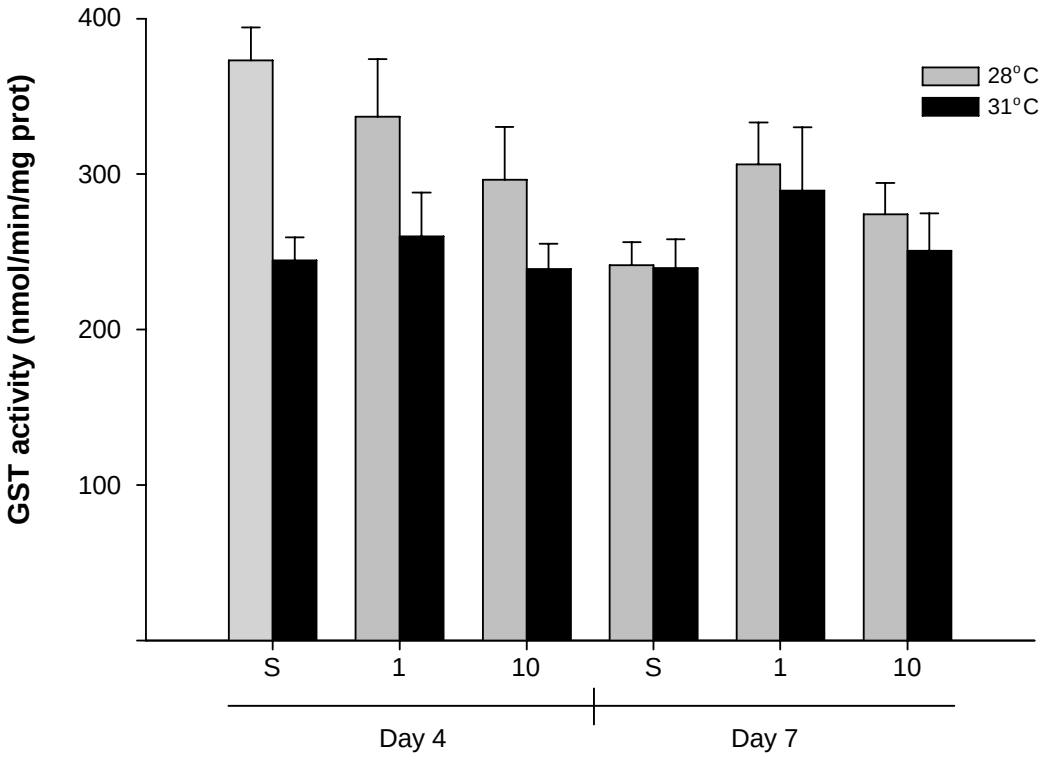
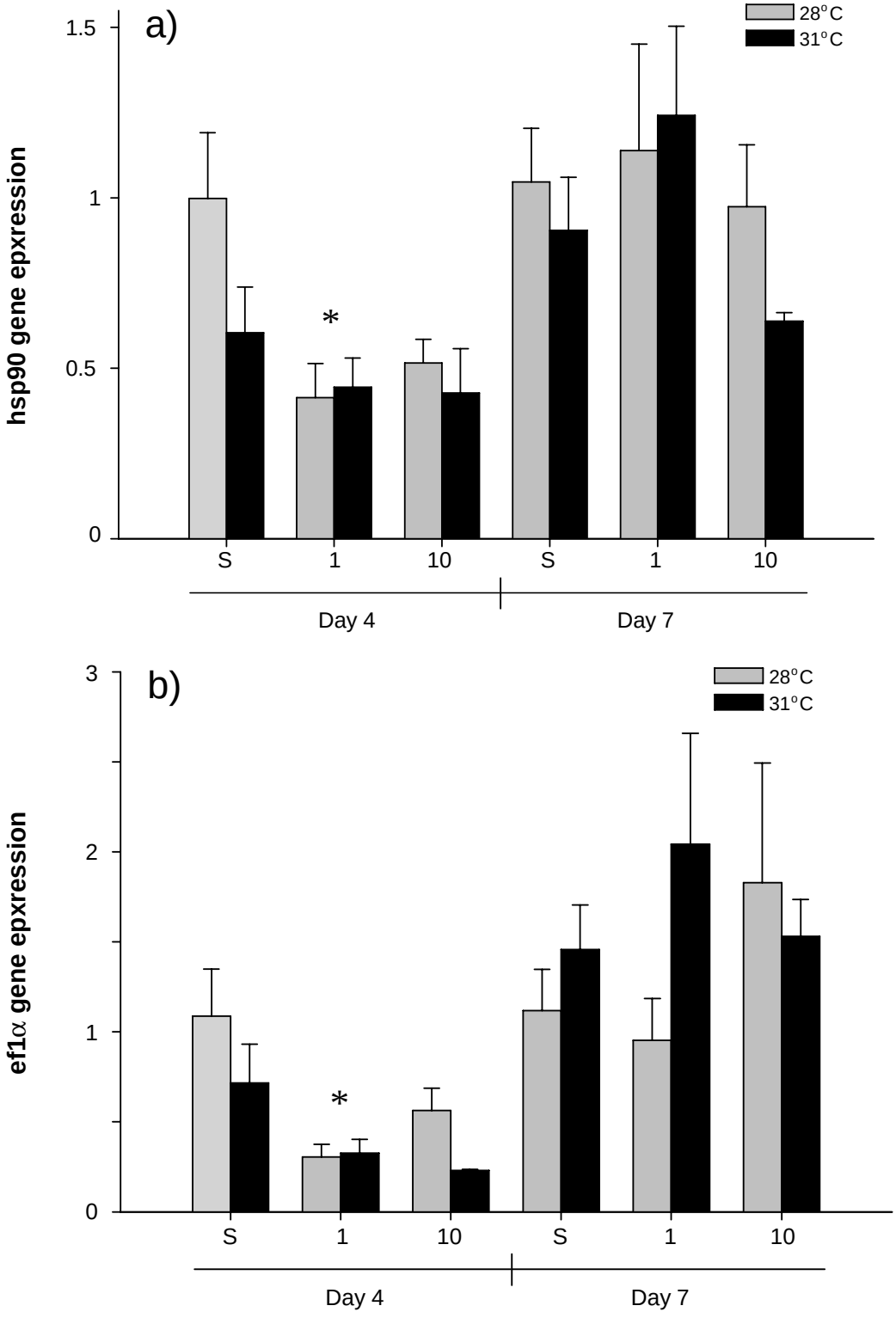


Figure 4.3. Hepatic GST activity in *A. polyacanthus* in response to simultaneous CPF exposure (1 µg/L and 10 µg/L) and temperature increase from 28 °C to 31 °C. Data represent means (n = 6 ± SE). S: solvent; 1: 1 µg/L; 10: 10 µg/L. Temperature had a significant effect on GST activity after 4 days of exposure.

4.3.4. Gene expression

Solvent control had no effect on gene expression (Appendix D, Tables D.4a, D.5a, D.6a and D.7a) Time, however, had a significant effect on the expression of all four candidate genes (Appendix D, Table D.4b D.5b, D.6b and D.7b); therefore each analysis was performed for 4 days and for 7 days separately, following the initial factorial ANOVA. After 4 days of exposure, gene expression was lower in 1 µg/L CPF-treated fish than in solvent control fish for all candidate genes, namely *hsp90* ($F_{2,48}$ = 3.95, P = 0.032, Appendix D, Table D.4b), *ef1α* ($F_{2,49}$ = 7.422, P = 0.003, Appendix D, Table D.5b), *cat* ($F_{2,45}$ = 6.700, P = 0.005, Appendix D, Table D.6b), and *gst* ($F_{2,24}$ = 7.452, P = 0.023, Appendix D. Table D.7b), perhaps indicating a general reduction in gene expression in response to CPF exposure. After 4 days of exposure, *gst* gene expression was also lower in fish exposed to 10 µg/L CPF than in solvent-treated fish (post-hoc, P = 0.031) but this was not the case for *hsp90* (post-hoc P = 0.108), *ef1α* (post-hoc, P = 0.127) or *cat* (post-hoc, P = 0.158). CPF had no effect on gene expression after 7 days of treatment (Appendix D, Table D.4b, D.5b, D.6b and D.7c). An interaction between CPF and temperature affected *gst* transcript level after 4 days of exposure ($F_{2,31}$ = 4.498, P = 0.019, Appendix D, Table D.7c). Further analysis revealed that at this time point, CPF only had a significant effect on *gst* gene expression at 28 °C ($F_{2,24}$ = 16.041, P < 0.001, Appendix D, Table D.7c) but not at 31 °C. At 28 °C after 4 days, solvent-control fish exhibited a significantly higher *gst* mRNA level than fish treated with 1 µg/L and 10 µg/L (post-hoc analysis, P < 0.04 for both concentrations). In addition, temperature significantly decreased *gst* gene expression in solvent-treated fish at this same time point (4 days) ($F_{1,24}$ = 17.619, P < 0.001, Appendix D, Table D.7c). Temperature also affected *cat* gene expression through time ($F_{1,45}$ = 11.12, P = 0.002, Appendix D, Table 6.b). No effect of

temperature was evident after 4 days, but after 7 days of exposure, *cat* gene expression was significantly higher in fish exposed to 31 °C compared to 28 °C ($F_{1,45}$ = 8.129, $P < 0.001$, Appendix D, Table D.6b).



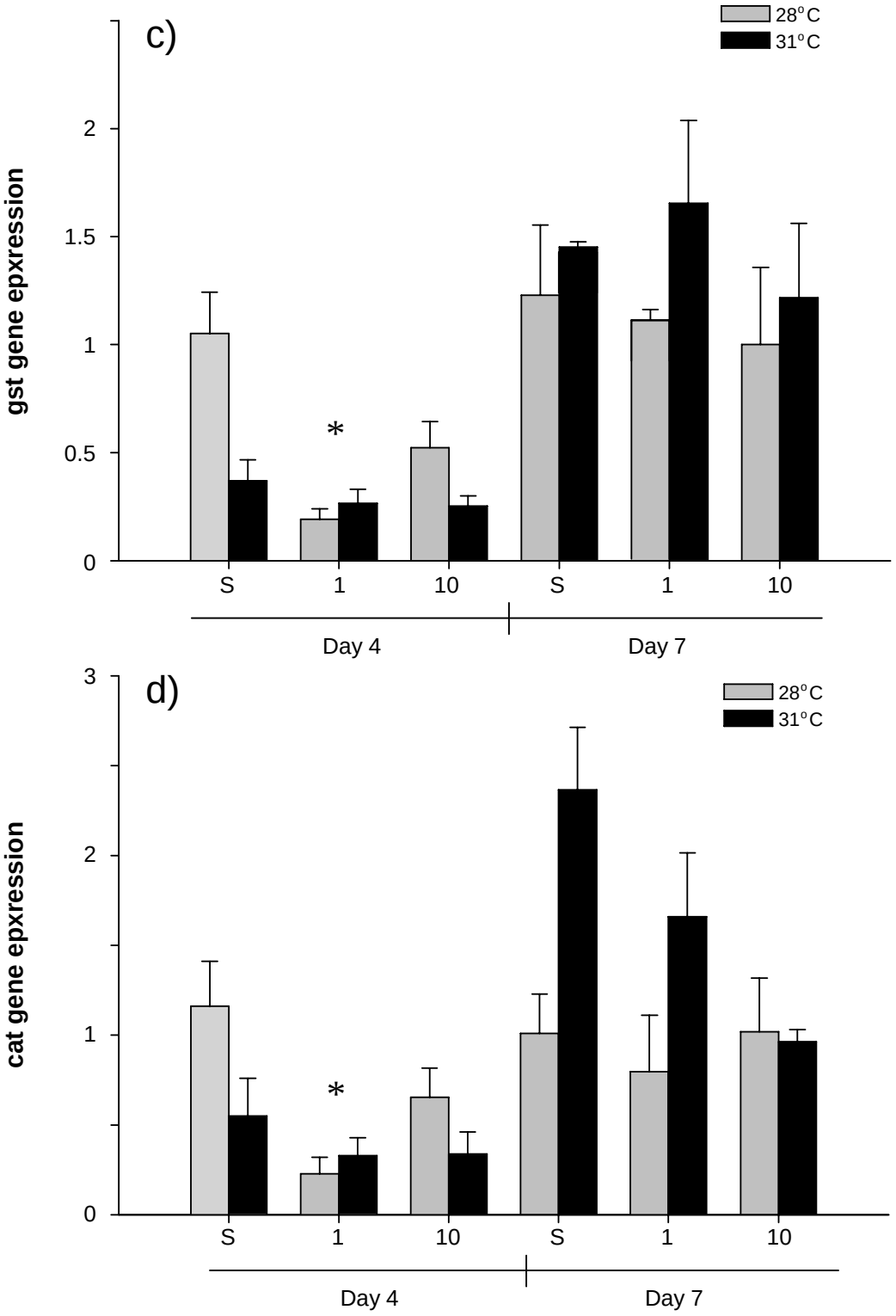


Figure 4.4. Hepatic gene expression relative to solvent control after simultaneous exposure to 1 µg/L and 10 µg/L CPF and temperature increase from 28 °C to 31°C for **a)** *hsp90*; **b)** *ef1α* **c)** *gst*; **d)** *cat*. S: solvent; 1: 1 µg/L; 10: 10 µg/L. Asterisks represent significant difference between CPF and solvent control at each time point regardless of temperature (See section 4.3.3 for detail). Temperature significantly decreased *gst* gene expression after 4 days and significantly increased *cat* gene expression after 7 days.

4.4. DISCUSSION

The present study examined the combined effects of CPF and thermal stress exposure on the tropical reef fish *Acanthochromis polyacanthus*, by exploring the effects on the nervous system (ChE activity), antioxidant defences (GST activity and CoQ redox balance), and gene expression (expression of four candidate stress-responsive genes: *ef1a*, *hsp90*, *cat* and *gst*). It provides novel information on the cellular impacts of multiple stress exposure in a potential bioindicator fish, a topic which is rarely investigated, especially in tropical species. Each of the three biochemical stress indicators (ChE activity, GST activity, CoQ redox balance) responded differently in this tropical damselfish. The activity of the neural enzyme ChE was substantially inhibited by CPF after 7 days of exposure and increased by temperature over 7 days. GST activity decreased at the higher temperature (31 °C) after 4 days, but returned to control levels after a further 3 days. CoQ redox balance was not affected by either temperature or CPF during the course of the current laboratory exposure. After four days, gene expression of *hsp90*, *ef1*a, *gst* and *cat* was lower in fish treated with CPF than in solvent-control fish, reflecting a general decline in hepatic gene expression. This study is the first to report additive effects of CPF and temperature in a tropical reef fish, as well as a reduction of gene expression as a result of CPF exposure. It provides evidence of a complex stress response in this model tropical fish species at the cellular and molecular levels.

4.4.1. ChE activity

4.4.1.1. CPF exposure decreases ChE activity

Chlorpyrifos was originally designed to inhibit insect ChE activity, but has also been shown to have negative effects on aquatic species including fish (Fulton and Key, 2001). In the present study, ChE activity was lower in *A. polyacanthus* exposed to 10 µg/L CPF than in solvent-treated fish after 7 days. The 59% ChE decline at 28 °C for the 10 µg/L treatment is consistent with the usual decrease in ChE activity reported in fish after CPF exposure (Botté et al., in press, Fulton and Key, 2001). However, the response of *A. polyacanthus* to CPF differed somewhat in the present experiment to that observed following static exposure to CPF (Chapter 2). In that chapter, a significant decrease in ChE activity was observed at 1 µg/L and the magnitude of this decrease was greater at 10 µg/L, representing a typical dose-response pattern. CPF concentrations were not measured in this chapter and may have been subtly different due to the application of a flow-through system; however the consistency of ChE activity inhibition at 10 µg/L with other studies indicates that the actual CPF concentrations were close to the nominal concentrations reported here. Different parent-pairs were used to produce juvenile fish for these two experiments and therefore it is not possible to rule out a genetic basis for some of the differential responses observed in this cohort of fish. This disparity between experiments was only apparent at the lowest concentration but highlights the difficulty in applying such methods in fish toxicology due to natural variation. It also highlights the need for further studies in controlled laboratory conditions before any field application can be strongly supported and underlines the need for standard methods in toxicology studies.

4.4.1.2. Thermal threshold in A. polyacanthus ChE

In the present study, increasing temperature by 3 °C had a positive effect on ChE activity in both solvent controls and CPF-exposed fish after 7 days of exposure. This represents an important observation. Indeed, while ChE activity was higher in fish exposed to 31 °C compared to 28 °C (present chapter), *A. polyacanthus* exposed to a further 1 °C increase (to 32 °C, Chapter 3 of the present thesis) exhibited lower ChE activity than control fish, after 4 days of exposure. Taken together, these two results suggest that *A. polyacanthus* ChE possesses a fine thermal threshold between 31 °C and 32 °C. The recent studies showing reduced reproduction of *A. polyacanthus* at 31 °C compared to 28 °C (Donelson et al., 2010b) support this hypothesis and the current study provides some insight into the cellular mechanisms that might lead to an overall decrease in fitness for this species when it is exposed to thermal stress. Results from the third chapter of this thesis also demonstrated that the ChE enzyme itself (*in vitro*) is not affected by temperature between 26 °C and 34 °C; therefore the delayed positive impact observed in the present chapter at 31°C might indicate an upregulation of ChE gene expression at that temperature. This positive impact further illustrates the sensitivity of ChE to temperature in the fish as the result of complex regulatory pathways as opposed to simpler changes in chemical reaction rate. Increases in metabolic rate was also observed at higher temperatures in *A. polyacanthus*, due to increases in water temperature (Nilsson et al., 2009). The positive impact of temperature on ChE activity found in the present study might therefore reflect the requirement for the nervous system to cope with these changes in metabolism.

A temperature-induced increase in fish ChE after 7 days of exposure has not been reported previously. There are several reasons for this. First, ChE activity in fish is investigated almost exclusively in toxicological studies which do not address the impacts of temperature on ChE activity. Second, most ecotoxicology studies employ a 96 h sampling method and therefore miss the more prolonged effect that was observed in this study. The outcomes of the present chapter therefore clearly emphasize the need for examining ChE activity in fish exposed to environmental stressors other than organophosphates and over more realistic exposure periods (weeks rather than days). There is a particular need for further studies on tropical reef fish, as they may have developed an acute sensitivity to thermal stress as a result of the relatively stable temperature conditions in the tropics. For example, a recent study showed that the aerobic scope of the damselfish *A. polyacanthus* and *Pomacentrus moluccensis* exposed to a 4 ℃ increase was more narrow in fish from warm tropical waters (Lizard Island, Northern GBR) than in the same species from cooler, sub-tropical waters (Heron Island, Southern GBR) (Gardiner et al., 2010). In particular, *A. polyacanthus* from cooler sub-tropical waters exposed to 32 ℃ exhibited a 30% reduction in aerobic scope, compared to 27 ℃, whereas individuals from tropical waters exposed to 32 ° exhibited a 60% reduction in aerobic scope compared to 29 ℃. In the current study, the fact that *A. polyacanthus* ChE activity exhibits such a dramatic difference in its thermal stress response between such similar temperatures (31 ℃ and 32 ℃) further indicates that small increases in sea surface temperatures will be critical in the response of tropical reef fish such as *A. polyacanthus* to climate change. This is particularly important, considering the fact that the parent pairs producing the fish used in this study originated from the Northern GBR (Jennifer

Donelson, personal communication) and might therefore be particularly sensitive to thermal stress.

4.4.1.3. Additive effect of CPF exposure and thermal stress

Although the mechanisms are not fully understood, increases in temperature generally lead to higher toxicity of pollutants in many animals and in humans (reviewed in Leon, 2008). An example is the Australian tropical freshwater fish *Melanotaenia splendida splendida* (Eastern Rainbowfish) which was more sensitive to CPF at higher temperatures (Humphrey and Klumpp, 2003). In this chapter, the opposite effect was observed (ChE activity was enhanced as the temperature increased by 3 ℃ even at the highest concentrations of CPF). This result implies that, in the absence of CPF *A. polyacanthus* can sustain temperatures up to 31 °C, without experiencing negative impacts on ChE activity, and that in fact the negative impacts of CPF exposure are somewhat reduced at this temperature. Overall there were no interactions detected between CPF and temperature on ChE activity indicating that the negative impact of CPF and the positive impact of temperature are effectively additive (Dunne, 2010). Such observations are critical in the context of rising sea surface temperatures and simultaneous increase in CPF exposure on tropical reefs. Similar additive effects of herbicides and thermal stress were observed in corals (Negri et al., 2011). In that study a slight protective effect of temperature was also found when corals were simultaneously exposed to herbicides and temperature just below their thermal threshold of ~30 ℃. Similarly, the results of the present study might indicate an optimum for ChE activity in *A. polyacanthus* at 31 ℃. It is particularly important to note, however, that even at temperatures which do not have a negative effect on *A. polyacanthus* ChE activity, the activity of this neural enzyme is still drastically

reduced when the fish is exposed to CPF. The possible protective effect of a 3 ℃ increase on *A. polyacanthus* ChE activity is therefore only effective if the fish is not exposed to other stressors such as CPF or to increases in temperature beyond this range. Future studies examining the response of ChE activity to CPF and thermal stress exposure above 31 ℃ or 32 ℃ will be beneficial to determine the consequences of small but deleterious temperature increases concurrent with CPF exposure and thereby provide information on this species response to thermal events and CPF exposure that it may experience on the Indo-Pacific inshore reefs.

4.4.2. GST activity

4.4.2.1. Temperature decreases GST activity

Glutathione-S-Transferase is commonly used as a marker for contamination and oxidative stress in fish. In the current experiment, GST activity in juvenile *A. polyacanthus* significantly decreased with temperature (31 ºC compared to 28 ºC) after 4 days, regardless of CPF concentrations. The return of GST activity to control levels after 7 days of exposure suggests that exposure to 31 ºC induced an initial stress response in liver over the shorter period, and that acclimation occurred between 4 and 7 days of exposure. Reduction in GST activity in response to temperature has been shown in other aquatic organisms: the freshwater fish *Channa punctata* exposed to a 12 ºC heat shock showed a decline in GST activity after 3 h (Kaur et al., 2005), as did the goldfish *Carassius auratus L*. exposed to 35 ºC compared to 21 ºC (Lushchak and Bagnyukova, 2006) and marine sponges (*Suberites domuncula*) exposed to a 10 ºC heat shock after 30 min (Bachinski et al., 1997). It is worth noticing that such findings were obtained with severe stress using direct immersion of the animal in much warmer water than their acclimation temperature. The present results demonstrate that

a similar pattern is observed when applying more realistic heating rates with a tropical reef fish and over a much narrower temperature range (3 °C). Declining GST activity in response to temperature changes is poorly understood and often counterintuitive. In case of heat stress, it was shown that temperature generally induces an increase in fish metabolic rate, leading to the overproduction of reactive oxygen species which may be excreted from the cell *via* the action of antioxidant enzymes such as GST (Lushchak, 2011). An increase in GST activity for *A. polyacanthus* was therefore expected along with increasing temperatures. However, with several studies finding the opposite outcome, it appears that other mechanisms may operate in the liver of some fish species, when these animals are exposed to increasing temperatures. A compensatory mechanism was reported between different antioxidant enzymes in the goldfish *Carassius auratus L* (Bagnyukova et al., 2007). In this species, different antioxidant enzymes (catalase, superoxide dismutase and GST) are activated in order to minimise the effects of temperature-induced oxidative stress. However, all three enzymes are not activated at the same time (some show lower activity when others exhibited higher activity) (Bagnyukova et al., 2007), perhaps as a way of defending the cell against oxidative stress while conserving enough energy for other functions. With several antioxidant enzymes available to detoxify liver cells, it is possible that *A. polyacanthus* may also possess some other compensatory antioxidant responses that may be activated in liver during thermal stress. In future studies, investigation of the activity levels of additional antioxidant enzymes would be useful to better determine the extent of *A. polyacanthus* defences against oxidative stress and its sensitivity to this type of stress.

4.4.2.2. Thermal threshold in A. polyacanthus GST

The decrease in GST activity reported in the current chapter further supports a thermal threshold between 31 °C and 32 °C for *A. polyacanthus*. Indeed, increasing temperature from 28 °C to 31 °C induced a lower GST activity in heat-treated fish than in control fish while increases from 28 °C to 32 °C did not alter GST activity (see Chapter 3 of the current thesis). These results indicate that, as for ChE, GST does not behave in a similar fashion when the fish are exposed to 31 °C than to 32 °C. This further underlines the fact that if episodes of thermal stress events increase in frequency due to climate change in the Indo-Pacific region, the thermal stress response of *A. polyacanthus* might differ if waters reach 31 ºC or 32 ºC. The results on GST and ChE activity therefore complement each other in defining the thermal tolerance threshold in *A. polyacanthus* cells.

4.4.2.3. CPF does not alter GST activity

The lack of response of GST activity in *A. polyacanthus* exposed to CPF at concentrations up to 10 µg/L for 7 days may imply that this pathway is not used by *A. polyacanthus* liver cells to detoxify organophosphates, or that the sampling time points were not appropriate to detect a significant effect of contamination in the fish. However, GST activity does not always respond predictably following exposure to CPF. For example, CPF exposure inhibited GST activity in the mosquitofish *Gambusia affinis* (Kavitha and Rao, 2008) and in the goldfish *Carassius auratus* (Wang et al., 2009), but increased GST activity was not detected in the mosquitofish *Gambusia yucantana* (Rendon-von et al., 2005). The response of GST activity levels to CPF exposure is therefore highly species-specific and it might be useful to explore possible compensatory mechanisms as discussed earlier and better understand how *A.*

polyacanthus combats CPF-induced oxidative stress. It appears unlikely from the results presented here that GST is the primary antioxidant enzyme used in the liver of *A. polyacanthus* to combat CPF-induced stress. In addition, future studies should investigate direct indices of CPF-induced and thermal stress-induced oxidative stress, such as DNA damage, lipid peroxidation and glutathione levels.

4.4.3. CoQ redox balance

CoQ redox balance was not affected by temperature or CPF over the course of the current 7 day experiment. This suggests that the 3 ºC temperature increase and 1 µg/L and 10 µg/L CPF concentrations used in the present study were not high enough to induce oxidative stress in *A. polyacanthus* liver and activate the CoQ antioxidant response, after 4 days of exposure. CoQ redox balance has been used as an oxidative stress marker for fish exposed to contaminants including Benzo-a-Pyrene (BaP) for 48 h (Hasbi et al., 2011). The authors reported a correlation between hepatic CoQ redox balance and EROD activity (a widely recognised antioxidant enzyme) and showed that $CoQH_2$, the antioxidant form of CoQ, increased with high Benzo-a-Pyrene concentrations, suggesting an increase in antioxidant molecules as a response to elevated ROS in liver. Interestingly, in this thesis an increase in $CoQH_2$ after exposure of juvenile *A. polyacanthus* to 10 µg/L CPF after 6 h (see Chapter 2) was also demonstrated, with the effect disappearing after 4 days. In the present study, the fact that CoQ redox balance is maintained to control levels after 4 days of stress was confirmed, and it is further demonstrated that the combination of two ROS-producing stressors does not change this outcome. Future studies should include more time-points, CPF concentrations and temperatures, together with additional oxidative stress markers in order to better understand the mode of action of CPF toxicity and the

impact of CPF exposure on the CoQ redox balance as well as other antioxidant molecules, and the overall cell integrity.

4.4.4. Gene expression

4.4.4.1. CPF induces a transient decline of gene expression in A. polyacanthus liver.

This study is the first to investigate the changes in gene expression of a tropical reef fish simultaneously exposed to an insecticide and thermal stress. The candidate genes are representative of various functions in the cell, such as general stress response (*hsp90*), oxidative stress (*cat* and *gst*) and gene transcription (*ef1α*). The expression patterns of all four candidate genes were relatively consistent in the present experiment. First of all, significant effects of both stressors were observed on all four candidate genes in *A. polycanthus* after 4 days, but not after 7 days of exposure. The recovery of transcript levels at 7 days may suggest a transient role of gene expression regulation in the response of this species to CPF exposure. Similarly, in the brown trout *Salmo trutta, cat* transcript abundance was affected after 1, 2 and 4 days of exposure to cadmium and zinc, but not after 7 and 15 days (Hansen et al., 2007). Likewise, in the catfish *Pangasianodon hypophthalmus*, *hsp*70 gene expression was altered at 4, 7 and 14 days of 0.5 mg/L organophosphate exposure, but not after 28 and 56 days (Sinha et al., 2010). Interestingly, however, these two previous fish studies reported an up-regulation of gene expression, whereas CPF triggered an initial decrease in transcript levels for the four genes examined here in *A. polyacanthus*. Such results are not uncommon in fish toxicology studies. For example, a reduction in transcript abundance of *hsp90* was also found in gills of the Chinook salmon (*Oncorhynchus tshawytscha*) exposed to CPF for 96 h (Eder et al., 2008). Transcript

levels of *hsp90* were reduced following exposure to 0.64 µg/L of esfenvalerate (a non-organophosphate insecticide) for 24 h in both spleen and gills of juvenile striped bass (*Morone saxatilis*) (Geist et al., 2007). Similarly, the mRNA levels of the transcription factor *ef1δ*, homologous to *ef1α*, was lower in fish inhabiting contaminated water compared to a reference site (Williams et al., 2003). The recent interest in gene expression in fish toxicology studies reveals that molecular changes associated with exposure to contaminants vary considerably from species to species. Very little information is available on the regulation of gene transcription in tropical reef fish in response to toxicant exposure. The results reported in this chapter may indicate that an overall decline in mRNA levels initially occurs as a result of CPF exposure in *A. polyacanthus*. This suggests that complex processes take place in the fish liver cells within 4 days of exposure to CPF. The four targeted genes exhibited a similar pattern after 4 days of exposure with a lower expression in fish exposed to 1 µg/L CPF, but this did not reduce further in fish exposed to 10 µg/L CPF (except for *gst*). A similar result was found in the Chinook salmon *Oncorhynchus tshawytscha* exposed to CPF for 96 h: increases in *hsp90* mRNA level were more pronounced in fish exposed to 1.2 µg/L than in fish exposed to 7.3 µg/L (Eder et al., 2004). In the zebrafish *Danio rerio*, induction of *cat* gene expression was observed in liver when the fish was exposed to 10 and 100 µg/L of the herbicide atrazine, but not when it was exposed to 1000 µg/L (Jin et al., 2010). The lower transcript abundance found in 1 µg/L CPF-exposed damselfish compared to solvent-treated individuals could be the result of a decrease in transcription rate and/or an increase in mRNA degradation. Such regulatory mechanisms cannot be discriminated from gene expression data only. Additional toxicology studies carried out with *A. polyacanthus*, and other tropical fish species, on additional candidate genes and time points will be beneficial to our

understanding of the molecular pathways affected by CPF and the potential regulatory mechanisms used by tropical fish to cope with CPF toxicity.

4.4.4.2. Temperature impacts gene expression of antioxidant proteins

Interestingly, temperature impacted both antioxidant genes (*cat* and *gst*) in their transcript abundance, but in an opposite fashion and at different time points. Gene expression of *gst* was indeed lower in fish exposed to 31 °C compared to 28 °C after 4 days of exposure, while *cat* transcript level was higher in fish exposed to 31 °C compared to 28 °C after 7 days of exposure. The fact that both *gst* transcript level and GST activity are lower in heat-stressed fish than in control fish suggests that the regulation of GST activity occurs *via* gene expression. The delayed and positive response of *cat* to temperature stress (day 7) compared to *gst* (day 4) supports the idea of a compensatory mechanism as hypothesized above (see section 4.4.2.1), by which liver cells of *A. polyacanthus* may use different antioxidant molecules at different times to cope with this stress.

4.4.5. Environmental relevance

In the last decade, marine ecosystems have experienced a rise in sea surface temperatures and chemical load. This has led to the study of the potential interaction between the two types of stressors from a chemical point of view, mainly in temperate regions of North America and Europe and the lack of research in other regions is well recognised (Noyes et al., 2009). From data collected on temperate organisms, it appears that the outcome of stress combination depends on the species studied, and on the balance between uptake and excretion processes, as well as general metabolism (Noyes et al., 2009). The results of the present study indicate that elevating water

temperatures by 3 °C triggers many complex stress response pathways in *A. polyacanthus*, from gene expression regulation to alterations in enzyme activity. Populations of this species are likely to encounter such temperature elevations in the coming decades much more frequently and may well be adversely affected if projections from the last IPCC report (2007) are accurate and laboratory based thermal thresholds hold up under field conditions. It remains difficult to predict the effects of climate change on tropical coral reefs such as those in the Indo-Pacific due to the high variability of heavy rains associated with the summer monsoonal season and the resulting river flows and subsequent terrestrial run off into the lagoon (Lough, 2007). The well-studied Great Barrier Reef (GBR) ecosystem of Australia provides an excellent model to investigate the impacts of these combined pressures. Most rivers from the GBR catchment reach their maximum flows in March, after the wet season, while the highest sea surface temperatures occur on the GBR from January to February (Lough, 2007). At first glance, GBR marine organisms might therefore avoid the most intense and stressful combinations of high temperatures and pesticide contamination. However, it is the earlier first-flush flood events that usually contain the highest concentrations of pollutants. Marine species from the inshore GBR are therefore exposed to maximum levels of terrestrial run-off during the warmest months of the year. Here it was demonstrated that the sensitivity of species to insecticides such as CPF can be increased or decreased depending on exposure duration. However, these results need to be interpreted with caution. For instance, increases in temperature and potentially associated compensatory physiological changes that may assist a species to deal with organophosphate contamination at the cellular level, could have other detrimental effects at the whole organism level, such as reductions in growth or impaired reproduction. Recent studies have already shown that growth

capacity of adult *A. polyacanthus* was reduced at 31 ℃ compared to 26 °C (Munday et al., 2008), and that this species showed reduced aerobic scope when exposed to 33 °C compared to 29 °C (Munday et al., 2009).

Enhancing the adverse impacts of terrestrial run-off and pollution is the predicted increase in frequency of violent cyclones along the GBR with increasing SST (Lough, 2007), potentially enhancing terrestrial run off and pollution. More generally, however, changing climate can affect the distribution and life history traits of agricultural pests (Porter et al., 1991, Schiedek et al., 2007), which might alter the use of pesticide on crops. It is generally thought that increasing temperatures will result in higher pest prevalence, requiring greater application of pesticides which will in turn increase the toxicity of terrestrial runoff (Bloomfield et al., 2006). Increased volatility of pesticides with increasing air temperatures might also result in heavier usage of pesticides. The changes that could occur as a result of intensified thermal and combined chemical stress are therefore far more complex and intricate than conditions used in the laboratory-based experiment conducted in the present study. Additional work should be carried out, testing more combinations of stress on tropical reef fish species, such as several types of pesticides in combination with thermal stress in order to add value to the pattern identified here. Field-based surveys, across a range of genetic stocks should also be associated with laboratory-based experiments in order to better characterize the natural response and most importantly the natural variation in the response of *A. polyacanthus* to such stressors. Multiple species should be used in order to evaluate inter-specific variation in the response of tropical reef fish to combined insecticide and temperature increases exposure. Finally the response patterns identified for a specific location and species should not be extrapolated for

another location with different weather patterns, contamination issues and genetic stocks but rather questioned and investigated for local species and ecosystems.

4.5. CONCLUSION

In this chapter, the effects of simultaneous CPF exposure and thermal stress were investigated on juvenile *A. polyacanthus*. The results show a decrease of ChE activity with increasing CPF concentration after 7 days of treatment. Together with the previous chapters of the present thesis, a thermal threshold between 31 °C and 32 °C was found for ChE activity in this tropical fish species. Measurements of hepatic GST activity revealed a decrease after 4 days of treatment at 31 °C compared to 28 °C, but this disappeared after another 3 days of exposure, perhaps indicating acclimation to higher temperatures. Expression patterns of *hsp90*, *ef1α*, *cat* and *gst* suggest a general decrease in transcription in response to CPF exposure, or a common regulatory mechanism for the expression of these genes. The results obtained in the present study illustrate the multiple and complex pathways affected and/or activated as a result of exposure to thermal stress and CPF exposure in this model tropical fish species. Future studies using longer exposures, additional CPF concentrations and temperature regimes, as well as additional stress markers, will be critical to provide a more complete picture of the impacts of simultaneous stressors on tropical reef fish. Of particular value will be studies of reef associated species such as the spiny damselfish *A. polyacanthus* examined here, which possesses a strong potential as a bioindicator species in the Indo-pacific.

5. GENERAL DISCUSSION

5.1. KEY FINDINGS AND THEIR SIGNIFICANCE

Marine coastal environments have experienced considerable changes, including increased pollution, over the past century from developments associated with increasing agriculture, industry and urban populations. The last decade has also seen rising concerns about increasing water temperatures associated with global change, with consequences for species phenology and life history traits. Although coral reefs often attract and service very dense human populations, the effects of anthropogenic impacts on aquatic ecosystems have been primarily focused on temperate ecosystems. The present thesis aimed at providing novel information on how the model tropical reef fish *A. polyacanthus* responds to both chemical and physical stress. It specifically focused on the description of the biochemical and molecular stress responses associated with exposure to the insecticide chlorpyrifos (CPF) and thermal stress.

Because CPF targets the neural enzymes cholinesterases (ChEs), the second chapter of this thesis identified the different ChEs present in muscle tissue. Both well-described and atypical ChEs were found. Detailed analysis of the characterization results provided evidence for the existence of two AChE and one BChE. Similar findings obtained from tissues of several other Perciformes from reef habitats (Leibel, 1988, Leticia and Gerardo, 2008) suggest that this type of ChE might have a functional relevance for tropical reef fish in general. This hypothesis, however, is to be taken with caution for several reasons. Firstly, ChE characterization needs to be performed on a significantly higher number of reef fish species before the extent of

these atypical ChE can be fully appreciated. Secondly, the functional role of the different ChEs is still unclear and functional differences between them, if any, remains to be defined in tropical reef fish. Range-finding experiments conducted between 10 and 400 µg/L over four days CPF showed that *A. polyacanthus* can withstand very high CPF concentrations before showing signs of balance impairment. Since such concentrations are unlikely to be found in the natural environment, the detection of CPF-induced effects was investigated using a much more sensitive cellular biomarker, namely ChE inhibition. The use of ChE inhibition has been demonstrated for many aquatic species as a marker for toxicant exposure and more precisely organophosphates (OP) and carbamates, but has rarely been used in tropical marine fish. The present study validates the use of ChE inhibition as a sensitive marker for CPF exposure in a common reef fish from the GBR and the Indo-Pacific region. It provides evidence that ChE activity is inhibited by 26% after 96 h of exposure to 1 µg/L and is halved after exposure to 10 or 100 µg/L. While these concentrations are high compared to those detected along the GBR, high CPF concentrations have been detected in other tropical countries such as Thailand (Thapinta and Hudak, 2000), reflecting differences in policies towards the use of agricultural chemicals between different countries. The results obtained constitute the first full dataset established on ChE characterization and inhibition for a tropical reef fish from the Indo-Pacific region and the first characterization of ChEs and application of ChE inhibition assays in a tropical marine fish. The study therefore provides a baseline for future research on this particular species or other species from the same habitat.

The third chapter of the present thesis identified a 50% lower ChE activity in fish exposed to 32 °C or 34 °C for 4 days, compared to 28 °C. Most importantly, ChE was still 39% lower in fish exposed to 32 °C or 34 °C than in fish exposed to 28 °C after 7 days of recovery. This chapter therefore presents novel and valuable information, since for the first time ChE is implicated in the temperature-driven stress response. The hypothesis which stipulates a role of stress in this decline, as opposed to a purely chemical change is supported by *in vitro* experiments which confirm that temperature itself does not change *A. polyacanthus* ChE activity at the levels the fish were exposed to: the reduction in activity is part of a whole organism response. The literature in this field is scarce, as although many toxicology studies focus on ChE activity after exposure to OP and other biological stressors, few studies examine the effect of temperature on these enzymes. The little information available on this topic suggests seasonal variation is possible, with generally a positive correlation between temperature and ChE activity, which further reinforces the idea that a decrease in ChE activity as a result of increasing temperatures constitutes a previously unidentified stress response in fish.

The sensitivity of *A. polyacanthus* ChE activity to CPF was further confirmed in Chapter 4, which combined CPF exposure and thermal stress and in which fish exposed to 10 µg/L CPF approximately exhibited a 50% lower ChE activity compared to solvent-control fish. The results also demonstrated that after 7 days of exposure, ChE activity is higher in fish exposed to 31 °C than in fish exposed to 28 °C, both for solvent-treated fish and CPF-treated fish. These results remain to be fully explained, because CPF is known for inducing irreversible binding to ChE. However, detoxification processes or ChE *de novo* synthesis might take place in the appropriate

organs with better efficiency under higher temperatures, which might reduce the negative effects of CPF exposure. The positive effect of 31 °C on ChE activity, along with the results from Chapter 3 (lower *in vivo* ChE activity at 32 °C than at 28 °C) led to the identification of a fine thermal threshold between 31 °C and 32 °C in *A. polyacanthus* ChE activity. In recent studies, this thermal threshold was also identified in this species for other variables such as growth and reproduction. (Munday et al., 2008). The current thesis therefore provides insights into the biochemical processes which possibly take place in *A. polyacanthus* and lead to an overall reduced fitness of the fish as a result of thermal stress. In addition, this last data chapter determined that no interaction takes place between temperature and CPF exposure when both stressors are applied at the same time, but that the effects of these stressors are additive. This constitutes a key finding of this thesis. Indeed, there is a general lack of knowledge about the response of tropical fish to multiple stressors, which implies that management decisions are largely dependent on studies investigating the effects of stressors in isolation. The present thesis therefore provides valuable information for predicting potential impacts of multiple stressors on this species and highlights the need for empirical studies to understand the influence of one type of stressor on another and their overall combined impacts.

The two sets of experiments conducted in Chapter 2 (CPF only) and Chapter 4 (combining CPF and temperature) did not utilize the same delivery method and experimental designs, due to logistic considerations and equipment availability. This led to an apparent difference in sensitivity of ChE inhibition between fish treated in a static system (Chapter 2) and fish treated in a flow-through system (Chapter 4). While the static experimental system induced a decrease in ChE activity with exposure to

just 1 µg/L CPF and higher, this response was not evident with the flow-through system until fish were exposed to 10 µg/L. Although the magnitude of CPF effects on ChE inhibition was similar in the two experiments for fish treated with 10 µg/L, the higher threshold for ChE inhibition in the flow through system highlights the subtle impact of varying experimental procedures on biochemical markers for stress in fish. Successive measurements of the same variable on a given species, with apparently identical toxicant concentrations, but different experimental set-ups, are highly valuable, but very rare. The compilation of Chapters 2 and 4 in the present thesis provides a unique opportunity to highlight the importance of experimental set-ups for toxicology studies and to strongly support the standardization of these protocols in this field of research.

In order to test the hypothesis of the activation of oxidative stress detoxification processes in liver as a result of CPF exposure and thermal stress, activity of the antioxidant and detoxifying enzyme GST was measured, as well as the ratio of CoQ antioxidant form, $CoQH_2$, upon exposure to CPF only (Chapter 2), temperature only (Chapter 3), or CPF and temperature simultaneously (Chapter 4). Measurements of $CoQH_2$ revealed an increase in the antioxidant form after 6 h of exposure to CPF only (Chapter 2) and a decrease at the onset of thermal stress (Chapter 3). This suggests that CPF exposure and an increase in temperature from 28 °C to 34 °C both induce an initial oxidative stress in liver of *A. polyacanthus*. This constitutes novel information, since evidence for the rise of oxidative stress in fish as a result of CPF exposure is absent in the literature to date. In addition, the opposite response (higher or lower $CoQH_2$) in the two experiments (CPF exposure or thermal stress) reflects the different and complex pathways utilized by *A. polyacanthus* to deal

with oxidative stress in liver. The return of $CoQH_2$ to control levels at all other subsequent time points (later than 6 h after first exposure) indicates that the role of $CoQH_2$ in scavenging CPF-induced ROS is most likely transient and occurs early in the stress response, as was confirmed in Chapter 4, where the 4 d and 7 d sampling time points failed to detect any change in CoQ redox balance. It is therefore unlikely that $CoQH_2$ is responsible for long-term detoxification of ROS in liver of juvenile *A. polyacanthus*. Notably, trends were observed in the CoQ redox balance, with a decrease in the antioxidant form $CoQH_2$ after 96 h of exposure to 1 µg/L, 10 µg/L and 100 µg/L. Such results might reflect the fact that the increase in the antioxidant form of CoQ ($CoQH_2$) is only transient and might even be depleted if exposure to CPF occurs for a longer period of time than a few hours (i.e. 96 h). Most importantly, the fact that these results are not supported by statistical results highlight the need for bigger sample size when performing measurements of oxidative stress markers in *A. polyacanthus* in order to minimize natural variation.

Further investigation of oxidative stress responses included enzymatic activity measurements for the antioxidant enzyme GST. Enzymatic activity of GST was not altered in response to CPF (Chapter 2 and 4). This indicates that if detoxification occurs at higher rates after 4 and 7 days of stress exposure, this happens *via* a process other than GST activation. Future work should attempt to characterize oxidative stress response pathways more fully in *A. polyacanthus*. Chapter 4 did, however, identify a decrease in GST activity in response to a 3 °C temperature increase after 4 days of combined temperature and CPF exposure. As was the case for ChE, data compiled from different chapters (Chapter 3 and Chapter 4) led to the identification of a thermal threshold between 31 °C and 32 °C in *A. polyacanthus*, since GST activity was lower

in fish exposed to 31 ℃ compared to 28 ℃ (Chapter 4), but did not change in fish exposed to 32 ℃ compared to 28 ℃ (Chapter 3). This further reinforces evidence for a fine thermal threshold in this tropical fish species, for at least two biochemical stress markers, and supports recent literature on the sensitivity of *A. polyacanthus* to thermal stress.

The last data chapter of this thesis (Chapter 4) also investigated the molecular processes affected by simultaneous CPF exposure and thermal stress, by measuring gene expression of four candidate genes, namely *hsp90*, *cat*, e*f1α* and *gst*. This constituted the first attempt to characterize the impacts of multiple stressors on gene expression of a tropical reef fish. Transcript levels of all target genes were down-regulated in response to CPF exposure after 4 days of exposure to both CPF and temperature increase. The consistency of this pattern for all four genes confers robustness to the data. The fact that genes representing different biological processes (general stress response and antioxidant enzymes) reacted in a similar fashion indicates that a general down-regulation of gene expression might have occurred in *A. polyacanthus* as a result of CPF exposure. Although unexpected, down-regulation of *hsp90* after exposure to CPF was also shown in other fish species such as Chinook salmon, for example (Eder et al, 2009, see Chapter 4). The reasons for this repression require further investigation, but other researchers have hypothesized that high levels of stress might be inhibitory to the transcription of *hsp* genes (Eder et al. 2009). Finally, the two genes encoding antioxidant enzymes were also affected by temperature: *gst* gene expression pattern was in accordance with GST activity, with lower gene expression for fish at 31 ℃ than at 28 ℃ after 4 days of exposure as was seen for GST activity, while *cat* gene expression was higher in fish exposed to 31 ℃

compared to 28 ℃ after 7 days of exposure. This indicates that different genes encoding proteins with similar functions (*gst* and *cat*) exhibit changes in expression at different times during the stress response. Taken together, the results of this chapter highlight the complexity of molecular responses and the need for additional data on oxidative stress defences in response to CPF exposure and thermal stress in *A. polyacanthus*.

5.2. IMPLICATIONS AND FUTURE DIRECTIONS

The findings of this thesis have a number of implications for our understanding of the cellular stress responses to temperature and pesticide exposure in coral reef fish and define the necessary future directions. Firstly, ChE activity can be used as a sensitive marker for OP contamination in *A. polyacanthus*, a common fish from the Indo-Pacific region. Because of its abundance, wide distribution throughout the Indo-Pacific region and suitability for breeding, *A. polyacanthus* clearly has the potential to become a useful model organism for toxicology studies in reef-harbouring coastal environments and should be regarded as such in future ecotoxicology studies on tropical species. Secondly, ChE activity *of A. polyacanthus* is sensitive to environmentally relevant temperatures increase (28 ℃ to 31 ℃ and 32 °C) and this sensitivity to temperature needs to be tested in a number of tropical reef fish species, in order to confirm the general applicability of the findings observed in the present thesis. Third, *A. polyacanthus* possesses a fine thermal threshold between 31 ℃ and 32 ℃ for a number of biochemical markers, which coincides with recent findings in the literature on the same species. This implies that this species' ability to cope with increasing sea surface temperatures due to climate change might be challenged in the coming decades. Fourth, coenzyme Q appears to be involved in ROS detoxification in

the early stages of exposure to CPF, but detoxification processes that occur later on still need to be identified and the rise of oxidative stress in liver of *A. polyacanthus* as a result of CPF exposure needs to be further investigated. Fifth, CPF exposure and temperature increase have additive effects on *A. polyacanthus* ChE activity, which suggests that if water temperatures exceed the 31 °C thermal threshold, as they might by the end of the century, the neural functions of fish exposed to both types of stress might be very seriously compromised, with potential consequences on fitness and behaviour. This coupled with the lack of knowledge about thermal stress impacts of tropical fish nervous systems strongly supports the need for future studies investigating these questions. Finally, liver cells exhibit a down-regulation of transcription after a few days of exposure to CPF, suggesting that many different cellular pathways are affected by CPF exposure. Taken together, the results of the present thesis indicate that the cellular environment of the tropical reef fish *A. polyacanthus* is affected by doses of CPF that are currently found in South-East Asian river systems and by temperatures that are more frequently experienced on tropical reefs as a result of climate change.

Overall, the potential for increasing pesticide usage due to climate change effects on pest insect distribution, abundance and life history traits, and the likelihood for coastal marine fish to be exposed to higher concentrations of land-based toxicants as a consequence of altered and more severe run-off events is difficult to predict today. However, it is critical to understand the factors influencing the response of marine organisms to combined OP exposure and thermal stress. These influences can be classified in two categories, namely the exposure frequency and severity and the plasticity of the biological response itself. Exposure frequency and severity might

change in the coming decades as a result of warming temperatures, which affect pest distribution, abundance, life cycle and life history traits, but also rainfall frequency and intensity and therefore pesticide spraying frequency, pesticide volatility and degradation rates (Noyes, 2009). Exposure frequency is also influenced by agricultural practices and a country's policy towards pesticide use. The biological response depends on the inherent range and efficiency of biological processes that a species is able to employ to counteract stress, in other words the species' resistance and resilience, which are themselves influenced by many other factors (Wilson et al., 2006). The uncertainty, but also the complexity of the intricate relationships between these factors, contributes to the variety of responses observed in living organisms as a result of exposure to both toxicant exposure and abiotic stress. Unfortunately, few studies have so far investigated the effects of combined stressors on tropical reef fish, especially in relation to pollution. The present thesis therefore represents a valuable contribution to this field of research, as it identifies the impacts of such stressors and the potential mechanisms leading to these impacts. Overall, it provides a strong basis for future work on the ecotoxicology of reef fish in an era of global change.

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APPENDIX A
Graphics supporting calculation of ChE kinetic parameters for Chapter 2

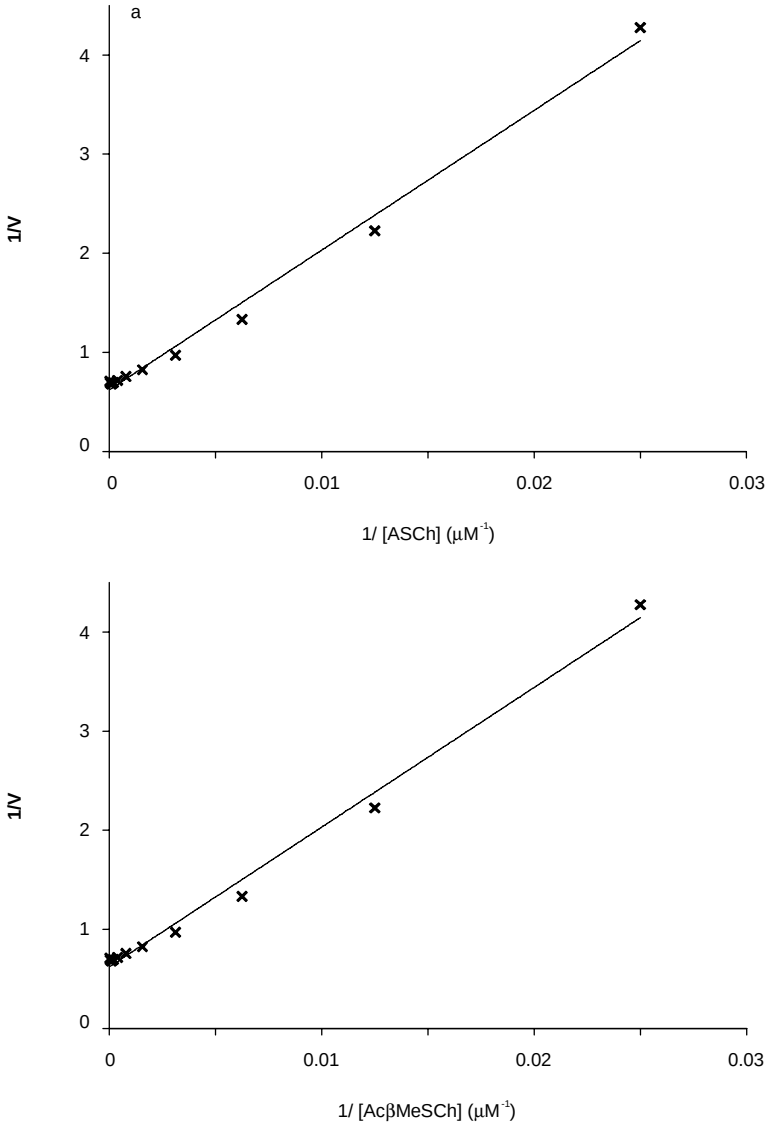

Figure A.1. Linewaver-Burke graphics to determine Km and Vmax of ChE using ASCh and AcβMeSCh as substrates.

Table A.1. Kinetic parameters for ChE activity with ASCh and AcβMeSCh

Substrate	R^2 of Linewaver-Burke plot	Km	Vmax	Catalytic efficiency (Vmax/Km)
ASCh	0.992	230.16	1.609	0.0070
AcβMeSCh	0.998	314.35	1.33	0.0042

APPENDIX B
Statistical tables for Chapter 2

Simple main effects analysis (Quinn and Keough, 2002) testing the effect of CPF and time on *A. polyacanthus* muscle ChE activity, hepatic GST activity and CoQ redox balance. Significant effects are in bold.

Table B.1a. Effects of solvent on *A. polyacanthus* muscle ChE activity.

	Df	MS	F	*P*
Solvent	1	0.002	1.114	0.302
Error	23	0.002		

Table B.1b. Effects of CPF and time on *A. polyacanthus* muscle ChE activity.

	Df	MS	F	*P*
	Effect of **CPF** on ChE activity			
Overall effect	2	0.011	2.945	0.059
After 6 h	4	0.005	0.647	0.631
After 96 h	4	0.029	7.703	< **0.001**
	Effect of **time** on ChE activity			
Overall effect	2	0.011	2.945	0.059
	Effect of interaction			
CPF x Time	8	0.009	2.385	**0.024**
Error	74	0.004		

Table B.2a. Effects of solvent on *A. polyacanthus* hepatic CoQ redox balance.

	Df	MS	F	*P*
Solvent	1	0.1	0.02	0.884
Error	22	2.6		

Table B.2b. Effects of CPF and time on *A. polyacanthus* hepatic CoQ redox balance.

	Df	MS	F	*P*
Effect of **CPF** on CoQ redox balance				
Overall effect	4	8.3	2.7	**0.036**
After 6 h	4	10.7	5.7	**0.012**
After 96 h	4	8.1	1.09	0.368
Effect of **time** on CoQ redox balance				
Overall effect	2	82.9	26.9	**< 0.001**
For solvent	2	2.6	0.848	0.432
For 1 µg/L	2	3	0.966	0.385
For 10 µg/L	2	3.9	1.271	0.287
For 100 µg/L	2	3.0	0.959	0.387
Effect of interaction				
Time x CPF	8	8.5	2.8	**0.001**
Error	73	4905		

Table B.3a. Effects of solvent on *A. polyacanthus* hepatic GST activity.

	Df	MS	F	*P*
Solvent	1	982	0.197	0.660
Error	34	4998		

Table B.3b. Effects of CPF and time on *A. polyacanthus* hepatic GST activity.

	Df	MS	F	*P*
Effect of **CPF** on GST activity				
Overall effect	4	6499	2.123	0.169
Effect of **time** on GST activity				
Overall effect	2	68244	22.298	**< 0.001**
For solvent	2	5341	1.089	0.342
For 1 µg/L	2	23773	4.847	**0.010**
For 10 µg/L	2	22223	4.531	**0.014**
For 100 µg/L	2	2614	0.533	0.589
Effect of interaction				
Time x CPF	8	3061	0.624	0.755
Error	75	4905		

APPENDIX C
Statistical tables for Chapter 3

Simple main effects analysis (Quinn and Keough, 2002) testing the effect of temperature and time on *A. polyacanthus* muscle ChE activity, hepatic CoQ redox balance and hepatic GST activity. Significant effects are in bold.

Table C.1. Effects of temperature and time on *A. polyacanthus in vivo* muscle ChE activity.

	Df	MS	F	*P*
Effect of **temperature** on ChE activity				
Overall effect	2	0.012	16.965	< **0.001**
At $t_{instant}$	2	0.004	6.254	**0.003**
After 6 h	2	0.001	2.026	0.139
After 24 h	2	0.005	7.109	**0.002**
After 96 h	2	0.010	14.778	< **0.001**
After 7 days	2	0.005	6.523	**0.003**
Effects of **time** on ChE activity				
Overall effect	4	0.002	3.471	**0.009**
At 28°C	4	0.002	2.326	0.065
At 32°C	4	0.002	3.122	**0.020**
At 34°C	4	0.005	7.756	< **0.001**
Effect of interaction				
Temperature x Time	8	0.003	4.931	< **0.001**
Error	68	0.0007		

Table C.2. Effects of temperature on *A. polyacanthus in vitro* muscle ChE activity.

	Df	MS	F	*P*
Effect of **temperature** on ChE activity *in vitro*				
Temperature	5	0.004	1.222	0.309
Error	62	0.003		

Table C.3. Effects of temperature and time on *A. polyacanthus* hepatic CoQ redox balance.

	Df	MS	F	*P*
Effect of **temperature** on CoQ redox balance				
Overall effect	2	21.8	1.63	0.203
Effects of **time** on CoQ redox balance				
Overall effect	4	59.0	4.42	**0.003**
At 28°C	4	9.5	0.71	0.588
At 32°C	4	17.5	1.31	0.275
At 34°C	4	96.4	7.25	< **0.001**
Effect of interaction				
Temperature x Time	8	36.0	2.70	**0.012**
Error	71	13.3		

Table C.4. Effects of temperature and time on *A. polyacanthus* hepatic GST activity.

	Df	MS	F	*P*
Effect of **temperature and time** on GST activity				
Temperature	2	0.002	1.392	0.256
Time	4	0.002	2.076	0.093
Temperature x Time	8	0.0002	0.187	0.992
Error	68	0.001		

APPENDIX D
Statistical tables for Chapter 4

Simple main effects analysis (Quinn and Keough, 2002) testing the effect of CPF, temperature and time on *A. polyacanthus* muscle ChE activity, hepatic GST activity and CoQ redox balance, as well as gene expression of the candidate genes *hsp90, gst, cat* and *ef1α*. Significant effects are in bold. In addition to simple main effects, the effect of CPF on gene expression was tested at each time point (4 days and 7 days). Due to more complex interactions between variables, statistical data for *gst* gene expression is presented in two tables for clarity.

Table D.1a. Effects of solvent on *A. polyacanthus* muscle ChE activity.

	Df	MS	F	*P*
Overall effect	1	0.009	5.240	**0.027**
Error	42	0.002		
After 4 days	1	0.017	11.978	**0.002**
Error	20	0.001		
After 7 days	1	0.00	0.0003	0.986
Error	20	0.002		

Table D.1b. Effects of CPF, temperature and time on *A. polyacanthus* muscle ChE activity

	Df	MS	F	*P*
Effect of **CPF** on ChE activity				
Overall effect	3	0.044	75	**0.002**
Effect of **temperature** on ChE activity				
Overall effect	1	0.003	1.841	0.179
4 days	1	0.004283	2.3460	0.129
7 days	1	0.023	12.543	< **0.001**
Effect of **time** on ChE activity				
Overall effect	1	0.003	1.446	0.233
Effects of interactions				
Time x Temperature	1	0.025	13.917	< **0.001**
Time x CPF	3	0.004	2.107	0.106
Temperature x CPF	3	0.003	1.752	0.164
Time x Temperature x CPF	3	0.0005	0.296	0.829
Error	75	0.002		

Table D.2a. Effects of solvent on *A. polyacanthus* hepatic CoQ redox balance.

	Df	MS	F	*P*
Solvent	1	6.1	0.49	0.489
Error	42	12.5		

Table D.2b. Effects of CPF, temperature and time on *A. polyacanthus* hepatic CoQ redox balance.

	Df	MS	F	*P*
Effect of **CPF, temperature and time** on CoQ redox balance				
CPF	3	22.2	1.52	0.217
Temperature	1	13.7	0.94	0.336
Time	1	58.9	4.03	**0.048**
Effects of interactions				
Time x Temperature	1	1.3	0.09	0.763
Time x CPF	3	16.3	1.12	0.348
Temperature x CPF	3	7.3	0.50	0.684
Time x Temperature x CPF	3	4.0	0.28	0.843
Error	73	14.6		

Table D.3a. Effects of solvent on *A. polyacanthus* hepatic GST activity.

	Df	MS	F	*P*
Solvent	1	8609	1.485	0.229
Error	41	5798		

Table D.3b. Effects of CPF, temperature and time on *A. polyacanthus* hepatic GST activity.

	Df	MS	F	*P*
Effects of **CPF** on GST activity				
Overall effect	3	8012	1.892	0.139
Effect of **temperature** on GST activity				
Overall effect	1	37000	8.739	**0.009**
4 days	1	62903	14.8562	< **0.001**
7 days	1	195	0.0460	0.831
Effect of **time** on GST activity				
Overall effect	1	37000	8.739	**0.004**
Effects of interactions				
Time x Temperature	1	25617	6.050	**0.016**
Time x CPF	3	10942	2.584	0.060
Temperature x CPF	3	4088	0.966	0.414
Time x Temperature x CPF	3	2265	0.535	0.660
Error	72	4234		

Table D.4a. Effects of solvent on *A. polyacanthus* hepatic *hsp90* gene expression.

	Df	MS	F	*P*
Solvent	0.061014	1	0.061014	1.916
Error	1.178370	37	0.031848	

Table D.4b. Effects of CPF, temperature and time on *A. polyacanthus* hepatic *hsp90* gene expression.

	Df	MS	F	*P*
Effect of **CPF** on *hsp90* gene expression				
Overall effect	2	0.095	2.105	0.133
4 days	2	0.146	3.951	**0.032**
7 days	2	0.064	1.418	0.252
Effect of **temperature** on *hsp90* gene expression				
Overall effect	1	0.083	1.844	0.181
Effect of **time** on *hsp90* gene expression				
Overall effect	1	0.88127	19.5195	< **0.001**
Effects of interactions				
Time x Temperature	1	0.002	0.043	0.837
Time x CPF	2	0.122	2.697	0.078
Temperature x CPF	2	0.024	0.533	0.590
Time x Temperature x CPF	2	0.016	0.353	0.704
Error	48	0.045		

Table D.5a. Effects of solvent on *A. polyacanthus* hepatic *ef1α* gene expression.

	Df	MS	F	*P*
Solvent	1	0.003	0.013	0.911
Error	40	0.241		

Table D.5b. Effects of CPF, temperature and time on *A. polyacanthus* hepatic *ef1α* gene expression.

	Df	MS	F	*P*
Effect of **CPF** on *ef1α* gene expression				
Overall effect	2	0.154	1.629	0.206
4 days	2	0.332	7.422	**0.003**
7 days	2	0.046	0.486	0.618
Effect of **temperature** on *ef1α* gene expression				
Overall effect	1	0.002	0.023	0.880
Effect of **time** on *ef1α* gene expression				
Overall effect	1	3.143	33.297	< **0.001**
Effects of interactions				
Time x Temperature	1	0.367	3.882	0.054
Time x CPF	2	0.300	3.182	0.050
Temperature x CPF	2	0.155	1.645	0.203
Time x Temperature x CPF	2	0.008	0.087	0.917
Error	49	0.094		

Table D.6a. Effects of solvent on *A. polyacanthus* hepatic *cat* gene expression.

	Df	MS	F	*P*
Solvent	1	0.008	0.052	0.821
Error	36	0.159		

Table D.6b. Effects of CPF, temperature and time on *A. polyacanthus* hepatic *cat* gene expression.

	Df	MS	F	*P*
Effect of **CPF** on *cat* gene expression				
Overall effect	2	0.451	6.362	**0.004**
4 days	2	0.351	6.700	**0.005**
7 days	2	0.184	2.598	0.086
Effects of **temperature** on *cat* gene expression				
Overall effect	1	0.113	1.593	0.213
4 days	1	0.169	2.393	0.128
7 days	1	0.765	8.129	< **0.001**
Effect of **time** on *cat* gene expression				
Overall effect	1	2.35321	33.1760	< **0.001**
Effects of interactions				
Time x Temperature	1	0.789	11.118	**0.002**
Time x CPF	2	0.062	0.878	0.423
Temperature x CPF	2	0.154	2.172	0.126
Time x Temperature x CPF	2	0.143	2.014	0.145
Error	45	0.07093		

Table D.7a. Effects of solvent on j *A. polyacanthus* hepatic *gst* gene expression.

	Df	MS	F	*P*
Solvent	1	0.205	1.463	0.237
Error	28	0.140		

Table D.7b. Effects of CPF, temperature and time on *A. polyacanthus* hepatic *gst* gene expression after 4 and 7 days of exposure to both stressors.

	Df	MS	F	*P*
Effect of **CPF** on *gst* gene expression				
Overall effect	2	0.073	1.958	0.158
Effects of **temperature** on *gst* gene expression				
Overall effect	1	0.013	0.347	0.560
Effect of **time** on *gst* gene expression				
Overall effect	1	2.326	62.001	< **0.001**
Effects of interactions				
Time x Temperature	1	0.201	5.364	**0.027**
Time x CPF	2	0.107	2.851	0.073
Temperature x CPF	2	0.169	4.498	**0.019**
Time x Temperature x CPF	2	0.040	1.075	0.354
Error	31	0.038		

Table D.7c. Effects of CPF and temperature on *A. polyacanthus* hepatic *gst* gene expression after 4 days of exposure to both stressors.

	Df	MS	F	*P*
Effect of **CPF** on *gst* gene expression				
Overall effect	2	0.279	7.452	**0.023**
At 28°C	2	0.415	16.045	**< 0.001**
At 31°C	2	0.011	0.419	0.673
Effects of **temperature** on *gst* gene expression				
Overall effect	1	0.261	6.958	**0.013**
For solvent	1	0.456	17.619	**< 0.001**
For 1 µg/L	1	0.016	0.609	0.443
For 10 µg/L	1	0.096	3.699	0.664
Effects of interactions after 4 days				
Temperature x CPF	2	0.169	4.498	**0.019**
Error	24	0.02587		

Table D.7d. Effects of CPF, temperature and time on *A. polyacanthus* hepatic *gst* gene expression after 7 days of exposure to both stressors.

	Df	MS	F	*P*
Effect of **CPF** on *gst* gene expression				
Overall effect	2	0.016	0.432	0.653
Effects of **temperature** on *gst* gene expression				
Overall effect	1	0.040	1.069	0.309
Effects of interactions				
Temperature x CPF	2	0.006	0.166	0.848
Error				