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Testing the role of heart mitochondrial stability and function in heart failure of ectotherms exposed to heat stress

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Abstract
Hearts appear to be the first organs to fail in heat stressed animals. Predictions of climate change mediated increases in ocean temperatures suggest that the ectothermic heart may place tight constraints on the diversity and distribution of marine species with cardiovascular systems. For many such species, their upper temperature limits (T\text{max}) and respective heart failure (HF) temperatures (T\text{HF}) are only a few degrees from current environmental temperatures. While the ectothermic cardiovascular system may act as an ‘ecological thermometer’, the exact mechanism mediating HF remains unresolved.

This thesis hypothesised that heat-stressed cardiac mitochondria drives HF in ectotherms and first investigated this in a common New Zealand fish Notolabrus celidotost. This thesis further tested cardiac mitochondria in wrasses from cold temperate (Notolabrus fucicola) and tropical (Thalassoma lunare) habitats to explore the effects of temperature across species from differing thermal habitats. Then, utilising N. celidotost, a species’ capacity to acclimate cardiac mitochondria was assessed following acclimation to mean seasonal temperatures. Finally, this thesis compared the thermal tolerance of heart and cardiac mitochondrial function in native (Ovalipes catharust) and invasive (Charybdis japonica) paddle crabs to test the role of mitochondria in providing a species an ecological advantage.

High resolution respirometry coupled to fluorimeters were used to assess the temperature-mediated changes in cardiac mitochondrial respiration, ROS and ATP production, and these changes were overlaid with the T\text{HF} (27.8 ± 0.4 °C) of N. celidotost. Even at saturating oxygen levels, several mitochondrial components were compromised before the onset of T\text{HF} suggesting that impairment of oxygen consumption by heart mitochondria preceded oxygen limitation. Importantly, the capacity to efficiently produce ATP in the heart was limited at 25 °C, and this was prior to the acute T\text{HF} for N. celidotost. Membrane leakiness increased significantly at 25 °C, as did cytochrome c release and permeability to NADH. Maximal flux rates and the capacity for the electron transport system (ETS) to uncouple were also altered at 25 °C. These data indicate that mitochondrial membrane integrity is lost, depressing ATP synthesis capacity and promoting cytochrome c release, prior to T\text{HF}. It was concluded that mitochondria can mediate HF in heat stressed hearts in fish and plays a significant role in thermal stress tolerance.
The contribution of cardiac mitochondrial dysfunction to heat stress induced HF was compared in cold temperate (*N. fucicola*), temperate (*N. celidotus*) and tropical (*T. lunare*) wrasse species. *T. lunare* had the least scope to maintain heart function with increasing temperature. Heat exposed fish of all species showed elevated plasma succinate, and the heart mitochondria from the cold temperate *N. fucicola* showed decreased phosphorylation efficiencies (depressed respiratory control ratio, RCR), cytochrome *c* oxidase (CCO) flux and ETS flux. *In situ* assays conducted across a range of temperatures using naïve tissues showed depressed Complex II (CII) and CCO capacity, limited ETS reserve capacities and lowered efficiencies of pyruvate uptake in *T. lunare* and *N. celidotus*. Notably, alterations of mitochondrial function were detectable at saturating oxygen levels, indicating that cardiac mitochondrial insufficiency can occur prior to HF without oxygen limitation. These data indicated that species distribution may be related to the thermal limits of mitochondrial stability and function.

While acute thermal stress may drive HF, ectotherms have a capacity to acclimate. The contributing role of compromised cardiac mitochondrial function to HF in *N. celidotus* acclimated to mean winter (cold acclimated CA, 15 °C) and summer (warm acclimated WA, 21 °C) temperatures was assessed. Heat stress mediated HF occurred at a $T_{HF}$ of 26.7 ± 0.4 °C in CA fish, and at 28.2 ± 0.6 °C WA fish. Biochemical analyses revealed that WA *N. celidotus* had elevated plasma lactate indicating increased dependence on anaerobic pathways. When cardiac mitochondria were tested with increasing temperatures, relative to WA fish, CA fish maintained higher RCR values at higher temperatures. However, apparent breakpoints in the RCR with substrates supporting Complex I (CI) oxygen flux occurred below $T_{HF}$ for both acclimated groups. WA cardiac mitochondria were less sensitive to increasing temperature for respirational flux supported by CI, CII, and chemically uncoupled flux through the ETS. These findings concluded that while acclimation to summer temperatures alters cardiac mitochondrial function in *N. celidotus*, these may come at an energetic cost, thereby increasing susceptibility of this species to further habitat warming.

To understand if more thermo-stable mitochondria provide an advantage to invasive species in warming oceans, the influence of temperature on heart function and cardiac mitochondria were compared between the native New Zealand paddle crab *Ovalipes catharus* and the invasive paddle crab *Charybdis japonica*. Doppler ultrasound showed that with increasing
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temperature *O. catharus* and *C. japonica* elevated their heart rates (*p*≤0.05). However, *C. japonica* showed greater plasticity in heartbeat duration, and contraction rate with increasing temperature, while *O. catharus* was more inclined to increase heart rate, and already had a shorter more rapid contraction at 19 °C. *In situ* testing of mitochondrial function showed that Leak-I was highest for *O. catharus* at all temperatures. *C. japonica* showed a greater inner mitochondrial membrane integrity which suggested tighter coupling of OXP. Although CI lost function on exposure to 30 °C in both species, this occurred more rapidly in *O. catharus*. Additionally, *O. catharus* had higher CCO rates at all temperatures compared to *C. japonica* indicating elevated concentrations of CCO in *O. catharus*. The scope to increase CCO flux was greater in the more stenothermal *O. catharus* than in *C. japonica*. Overall, the substantial differences in heart function and cardiac mitochondria between crab species indicated that mitochondrial integrity may limit survival and future distributions of the native species.

Based on the above investigations, it can be concluded that heart mitochondrial dysfunction contributes significantly to HF in marine ectotherms exposed to increasing temperatures, thereby defining the thermal limits of the ectothermic heart. All mitochondrial components were studied at maximum oxygen saturation and therefore, the changes observed were a direct impact of elevated temperature stress. This finding is contrary to the previously held notion that whole-animal tolerance at upper thermal limits resulted from a mismatch between oxygen demand and oxygen supply to the tissues of ectotherms (Pörtner and Farrell, 2008; Pörtner and Knust, 2007; Pörtner et al., 2004). Currently, emerging studies indicate that at temperatures where aerobic scope and cardiac function are maximised, ectotherms face restricted growth (Healy and Schulte, 2012), a decrease in population abundance (Clark et al., 2013; Gräns et al., 2014) and based on evidence from this thesis, cardiac mitochondria of the ectotherm heart is also compromised (Iftikar and Hickey, 2013; Iftikar et al., 2010; Iftikar et al., 2014).
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The contributing role of cardiac mitochondrial dysfunction to heart failure in a wrasse (Notolabrus celidotus) acclimated to seasonal temperatures.

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Chapter 2

Published as:


Nature of contribution by PhD candidate

All animal studies, mitochondrial assays, plasma and tissue metabolite determination, data and statistical analysis; primary author of manuscript

Extent of contribution by PhD candidate (%)

90%

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1. CHAPTER ONE – GENERAL INTRODUCTION

1.1 Climate change

Historical changes in global climate have involved significant changes in temperature, both warmer and colder than the present climate. The Eocene epoch that began 55 million years ago saw a rise in global temperature of 5–7 °C and atmospheric carbon dioxide (CO₂) levels 8–16-fold higher than current values that resulted in ice free polar regions (Kerr, 2011). Current global temperatures are also predicted to increase by 3 °C by 2050 (Rowlands et al., 2012). While these conditions appear less extreme compared to those of the past, current global climatic changes are unique because recent changes can be largely attributed to one species, namely humans. The rapid onset of current global temperature increase is much faster than those driven by natural mechanisms.

The Milankovitch theory attributes historical climate change to the variation in solar radiation the earth receives (Pidwirny, 2008). The earth’s orbital eccentricity, the orientation of the rotational axis, and the axial tilt, are all parameters of this model that explain climatic variability. Changes in these components are cyclical occurring over 100,000, 26,000, and 41,000 years, respectively (Muller and MacDonald, 1997). The oscillation of the earth’s orbit towards and away from the sun coincides with the troughs and peaks of these cycles, and their summed effects account for the earth’s mass cooling or warming events. Volcanic eruptions that cause changes in atmospheric greenhouse gases can also impact the earth’s climate alongside Milankovitch oscillations (Pidwirny, 2008). Large amounts of aerosol particles and CO₂ are released into the atmosphere with volcanic activity. While this initially cools the earth, following aerosol clearance from the atmosphere within 3 years, CO₂ causes a secondary warming by the greenhouse gas effect (Forster et al., 2007).

CO₂ is essential for life, as it is a requirement for photosynthesis and without CO₂ the earth’s climate would be ~30 °C colder (FSBI, 2007). However, the increase in fossil fuel consumption due to the industrial revolution has increased the atmospheric CO₂ concentrations from approximately 310 ppm to 400 ppm (figure 1.1). This concentration has not been seen since the Pilocene era 3–5 million years ago (Scripps Institution of Oceanography, 2013). This enormous increase in CO₂ and other greenhouse gases traps infrared radiation (heat) within the atmosphere, preventing heat release from the stratosphere.
and thereby warming the earth (Haywood et al., 2009). Global surface temperatures have increased by 0.74 ± 0.18 °C during the 20th century, and the warmest decade on record was January 2000 to December 2009 (Solomon et al., 2007). While future climate change scenarios primarily depend on predictions of atmospheric greenhouse gases and aerosols, 40 future climate scenarios have been drafted by the Intergovernmental Panel on Climate Change (IPCC) (Solomon et al., 2007). However, despite the ambiguity in how societies will cope with future carbon emissions, global warming is currently the most important environmental concern.

Figure 1.1: The Keeling Curve as of May 15, 2013. Atmospheric CO2 concentration from 1958 to 2013 recorded daily by Scripps Institution of Oceanography at UC San Diego (Scripps Institution of Oceanography, 2013).

1.1.1 Ocean warming
Steady increases in air temperature will inevitably lead to the warming of oceans because oceans absorb up to 90% of the heat in the atmospheric system (Bindoff and Willebrand, 2007). Indeed, the Southern Ocean has increased in temperature by 1 °C between 1955 and 1995, and with no continental barriers, heat is transmitted between the Pacific, Atlantic, and Indian Oceans (figure 1.2) (Gille, 2002). By the end of this century, average sea surface temperatures are predicted to increase by 3 °C due to global warming (Donelson et al., 2011; Meehl et al., 2011; Solomon et al., 2007). Greater increases in ocean temperatures are also expected in shallower coastal waters (Nicholls et al., 2007), and in the ocean, these areas
have the greatest abundance of fish species (Helfman et al., 2009; Moyle and Chech, 2004). This can impact the 11,000 km coastline of New Zealand that spans from sub-tropical to sub-Antarctic waters, with annual water temperatures ranging from 18.9 °C in the north to 10.1 °C along the southern-most coast (Schiel, 2011).

![Figure 1.2: Changes in sea surface temperature between the averages recorded in 1950–1969 and that in 1988–2007. Modified from Sumaila et al. (2011).](image)

Because the oceans act as a heat sink, warming oceans will intensify storm systems, alter hydrological cycles (Knutson et al., 2010), and increase ice sheet and glacier melting to elevate ocean volumes (Solomon et al., 2007). Greater water column stratification is being reported in the Southern Ocean due to warming of the upper layers, and this reduces mixing of nutrients (Lyman et al., 2010). Since the oceans cover 71% of the earth’s surface, increases in water temperature will have a significant impact on marine animal and plant species (Hoegh-Guldberg and Bruno, 2010). These impacts can be understood best by investigating the effects increasing temperature has on abiotic variables of seawater.

1.1.2 Impacts of increasing temperature on the abiotic environment

Oceans are the earth’s largest carbon sink, and seawater solubilises and therefore buffers approximately half of all anthropogenic atmospheric CO₂ (Pörtner, 2008; Roessig et al., 2004). However, as water temperatures increase, CO₂ solubility decreases. With continued increases in CO₂ emissions to the atmosphere (figure 1.1), the effectiveness of the natural CO₂ buffering system will decline (Pörtner, 2008). In addition, elevated dissolved CO₂ produces carbonic acid, which decreases the water pH. A global oceanic pH decline of
greater than 0.1 units over the last 200 years has been reported, and this is projected to
decline by 0.3-0.4 units by the middle of the 21st century (Munday et al., 2008; Pörtner,
2008). One of the major consequences of ocean acidification is that it affects the ability of
calcifiers such as corals to form shells and skeletons although, its impact on species with
internalised skeletons such as fish needs to be further investigated (Munday et al., 2010).

Global warming will further decrease the oceanic dissolved oxygen concentrations. Oxygen
minimum zones (OMZ) have also been expanding in tropical seas (Stramma et al., 2008).
Importantly the oxygen content is approximately 33-fold less in water than air, and the
solubility factor of oxygen decreases as water temperatures increase (Dejours, 1975). This
relative deoxygenation in warmer waters can decrease the oxygen supply in the ocean interior
due to upper ocean stratification described previously (Solomon et al., 2007). Most
importantly, systematic deoxygenation will affect the aerobic life of the oceans as dissolved
oxygen is fundamental to most marine animals.

1.1.3 Impact of ocean warming on marine ectotherms
Predicting the impacts of climate change on marine species and ecosystems has been
relatively difficult compared to terrestrial ecology because of the size and complexity of the
oceans (Solomon et al., 2007; Wood and McDonald, 1997). In contrast to terrestrial
ecosystems that are bound by geographical barriers, the redistribution of species due to
climate change may have fewer constraints in oceans. Warming of oceans can disrupt
important marine species interactions between functional groups and trophic levels by
changing the timing between seasonal cycles and this mis-match can further affect survival
(Rosenzweig et al., 2008). For example, prey species with temperature-sensitive distributions
can disperse away from non-shifting predators restraining the predators’ major source of
nutrition. Increased water temperatures have advanced the spring spawning of the north-
western European bivalve, *Macoma balthica* (Philippart et al., 2003). However, advanced
spawning does not coincide with the phytoplankton blooms of spring. This asynchrony
between animal and an abundant food source has led to increased mortalities of juvenile
bivalves due to competition for food. Important ocean food webs can also be disrupted due to
temperature driven changes in food sources such as reduced plankton biomass, and
decreasing plankton size (Hoegh-Guldberg and Bruno, 2010; O’Connor et al., 2009). Thus the
functions and productivity of ecosystems are greatly affected by climate driven changes ultimately affecting species abundance and distribution in marine environments.

Ocean warming has clear and profound impacts on species involved in habitat-formation such as corals, mangroves, sea grass and oysters (Hoegh-Guldberg and Bruno, 2010). Of these, corals have received wide-spread attention due to mass bleaching and mortality resulting from increased temperatures (Hoegh-Guldberg et al., 2007). This has decreased the diversity of coral reef fishes and other organisms that are dependent on this habitat (Donelson et al., 2011; Munday, 2004; Munday et al., 2008). Furthermore, the predicted increase in the sea level is expected to decrease the area of mangrove forests, consequently impacting species requiring brackish habitats (Gilman et al., 2008). In addition, oysters infected with the parasite, *Perkinsus marinus*, were found to spread during an increased warming of the waters across the north-eastern United Sates between 1990 and 1991 (Ford and Smolowitz, 2007). This indicates that diseases of habitat forming species have also been linked to climate change.

Ocean warming can also promote ‘exotic’ or invasive species invasions due to altered thermal structure in colder waters (Rahel and Olden, 2008). Some invasive species have broad climatic tolerances, large geographical ranges and thrive in the new warmer environments. Although the initial translocation of some of these species are due to human influence, such as transportation in the ballast water of ships, establishment in the new environment is dependent on the optimal conditions being present at the destination (Hoegh-Guldberg and Bruno, 2010). Exotic species introductions can have serious implications for resident species and native marine habitats. Both groups compete for niche establishment and food sources in which invasive species thrive due to their aggressive physiology (Stachowicz et al., 2002). The observed warming of the eastern Mediterranean Sea since 1924 has facilitated the introduction and establishment of over 100 alien species, which are usually found in warmer and tropical waters (Raitsos et al., 2010).

Increasing temperatures further affect certain species based on phylogenetic distributions. For example, sub-tidal porcelain crabs are sensitive to heat stress compared to their upper inter-tidal congeners that are more thermo-tolerant (Stillman and Somero, 2000). However, these upper inter-tidal crabs are already living at their putative physiological thermal limits and
have only a limited capacity to adjust their physiology to further increases in habitat temperature (Stillman and Somero, 2000; Stillman, 2003). Effects of climate change are also evident at various ontogenetic stages. Temperature changes greatly influence the larval stage duration and therefore dispersal and survival (O'Connor et al., 2009). Developmental rates of larval coral fishes are increased in warmer conditions leading to higher energy requirements that must be obtained from food resources that may also be being depleted rapidly by the pressures of climate change (Munday et al., 2008).

1.2 Thermal physiology of marine ectotherms

The thermal balance of marine ectotherms are influenced by the temperature of their external environment (Withers, 1992). As thermal generalists, marine ectotherms maintain their function over a range of body temperatures. However, *stenothermal* species can only function over a restricted temperature range compared to their *eurythermal* counterparts that can retain function over a larger variation in body temperature (Hill et al., 2008). The physiological responses of marine ectotherms to environmental thermal challenges have been categorised as three different types (Speakman, 2001). The first, termed *acclimation* occurs in a laboratory setting in which all other environmental factors are controlled except for changes in water temperature (thermal stress) (Prosser, 1991). Therefore, the phenotypic changes in the physiology and biochemistry of acclimated ectotherms can be mainly attributed to the manipulated thermal condition (Speakman, 2001; Withers, 1992). The phenotypic responses of ectotherms to seasonal changes in their natural thermal environment are termed *acclimatization* (Prosser, 1991). The change in physiological and biochemical responses caused by acclimatization cannot be attributed only to environmental thermal stress, and can be influenced by other factors correlated to seasonal temperature changes (eg. food availability) (Hill et al., 2008). Acclimation and acclimatization affect ectotherms individually, and the physiology of the individual is reversible once the thermal stress is removed. These chronic responses are in contrast to *adaptation* (Prosser, 1991). This is an irreversible response that genotypically determines thermal characteristics of different species with varying habitats (Speakman, 2001).

Acclimation and acclimatization generally maintain similar rates at varying temperatures, for eg: heart rate, metabolic rate, respiratory rate, enzymatic reaction rate, maximal aerobic rate (Withers, 1992). In marine ectotherms, thermal acclimation/acclimatization also involves
homeoviscous adaptation, which is maintaining the fluidity of biological membranes regardless of body temperature (Guderley and Johnston, 1996; Withers, 1992). These two physiological responses are types of phenotypic plasticity, which is a change in the physiology of the ectotherm that is environmentally induced (Wilson and Franklin, 2002). In comparison, developmental plasticity encompasses a range of phenotypic characteristics that change due to differences in the developmental environment (Wilson and Franklin, 2002).

1.2.1 Impact of increasing environmental temperature on fish

Approximately 32,500 species of bony fishes (teleosts) occupy a range of ecological niches making them the most successful and diverse vertebrate group. Marine fishes are found in environments where water temperatures vary with latitude, vertical thermoclines, and more local effectors such as seasons and tides (Somero et al., 1996). Marine fishes have varying interactions between temperature, oxygen requirements, activity, and metabolic rates (Beitinger and Fitzpatrick, 1979). Water has the second highest specific heat capacity after ammonia, and is a superb heat sink. Given the necessity for fish to pass blood through the enormous gas exchange surfaces of their gills, the gill also acts as an efficient counter-current heat-exchanger. Apart from specialised fishes such as Tunas, billfish and lamnid sharks, which concentrate heat in specific muscles, for the remaining fish species body temperature effectively equals the temperature of their environment (Beitinger and Fitzpatrick, 1979). This means their metabolism is subject to the thermal effects of the surrounding environment and can effect survival at extreme temperatures (Beitinger and Fitzpatrick, 1979). Therefore, extrapolating from how heat stress impacts fish physiology to outcomes at the population or ecosystem levels presents challenges (Wood and McDonald, 1997). Despite the scale of this task, the effects of increasing ocean temperatures on fishes has to be investigated at the varying levels from cellular, tissue, organism, ecophysiology, population, and ecosystem so that resolutions can be made on how to monitor and potentially remediate detrimental challenges of ocean warming (Rijnsdorp et al., 2009).

1.2.2 Population and species level responses

Climactic variables primarily drive the distributions of fish populations (Roessig et al., 2004). The west coast of North America has seen sea surface temperatures rise by 2 °C between 1931 and 1996 (Sagarin et al., 1999), with a concomitant decrease in reef fish species diversity by 15-25%, and an overall drop in abundance of 95% of these species by ~69% in
While decreases were linked to reductions in prey and algal stocks, reductions were mainly attributed to the declining recruitment of age 0 fish (Holbrook et al., 1997). However, fish populations around this coast have seen a significant increase northwards of southern range species, and a corresponding decrease in northern range species that require colder waters to survive (Parmesan, 2006). A similar climactic increase in water temperatures was observed in the European North Sea where winter bottom temperatures increased by 1.6 °C over 25 years (Dulvy et al., 2008). This has led to bottom-dwelling (demersal) fish to deepen their assemblages by ~3.6 m per decade (Dulvy et al., 2008). The Atlantic cod, *Gadus morhua*, is an important demersal species with a population found in the Baltic Sea (Brander, 2007). Warming waters appear to have led to a rapid loss of this cold-water species, which has also been compounded by intense fishing pressures (Brander, 2007; Dulvy et al., 2008). Furthermore, the increased water temperatures of in-shores along northern New Zealand coastline may be responsible for a reduction in growth rates of the red moki, *Cheilodactylus spectabilis* (Neuheimer et al., 2011). These fish increased their growth rate for populations in the cooler Tasman Sea, alarmingly illustrating that increased ocean temperatures can affect species distribution (Neuheimer et al., 2011).

**1.2.3 Individual responses to increasing temperature**

The effects of warming oceans on fishes at the population and species levels ultimately result from physiological changes at organism and cellular levels (Pörtner, 2001; Pörtner, 2002). Temperature is determined to be the single greatest abiotic factor that impacts on fish physiology (Beitinger and Fitzpatrick, 1979). Since most fishes are unable to regulate body heat, biochemical and physiological functions are dependent on their surrounding thermal environment. Therefore, acclimating or adapting to environmental temperature fluctuations is dependent on altering biochemical and metabolic processes to maintain homeostasis (Farrell et al., 2009; Hochachka and Somero, 2002; Pörtner and Farrell, 2008; Somero, 2002; Somero, 2011; Stillman, 2003).

The thermal tolerance range is where organisms survive within a temperature range where functionality is not compromised. The tolerable thermal window is widest for fishes inhabiting the mid-latitudes that experience large seasonal differences in water temperature. Conversely, this window is most narrow for fishes inhabiting extreme latitudes (polar and tropical waters) (Pörtner and Knust, 2007), as these fishes currently live at optimal
temperatures that vary less than those from temperate regions. Consequently it is speculated that tropical species live only a few degrees from their upper habitat temperatures ($T_{\text{max}}$). The onset of ocean warming can alter this gap exposing fishes with narrow thermal windows to their lethal temperature (Pörtner and Knust, 2007; Wang and Overgaard, 2007).

Generally, the metabolism of adult fish increases as habitat temperature increases, and this presses demands on metabolic fuels (Hochachka and Somero, 1968). The $Q_{10}$ relationship predicts that the sensitivity of systems to acute temperature changes typically results in a 2-3-fold increase in rate or flux for a rise in temperature by 10 °C (Van’t Hoff, 1898). Therefore, if metabolism elevates unchecked and/or runs inefficiently with increasing temperature, fuel that is redirected from homeostatic and anabolic processes must then drive catabolic processes (Hochachka and Somero, 2002). This will alter growth (Brander, 1995), circulation (Blaxter et al., 1971), osmoregulation (Rahn and Baumgardner, 1972), gas exchange (Sollid and Nilsson, 2006), reproduction (Planque and Frédou, 1999), swimming speed (Koch and Wieser, 1983) and ultimately animal and species survival (Fry, 1971; Griffiths and Harrod, 2007).

1.2.4 The oxygen limitation of thermal tolerance

Although almost all physiological processes of fish are simultaneously affected by increasing temperature, previous workers state that the system which appears to first fail at the thermal maximum is the heart (Hochachka and Somero, 2002; Somero, 2010; Wang and Overgaard, 2007). The specific cause of heart failure is unknown. One explanation for acute heart failure (HF) is decreased oxygen availability due to limited aerobic scope (Pörtner and Knust, 2007; Pörtner et al., 2004). Aerobic scope is defined as the difference between the resting metabolic rate and the maximal metabolic rate of an animal. This extra energy expenditure at maximal rates (i.e. above basal commitments) can be used for increasing fitness, such as food capture, growth, activity, and reproduction (Farrell et al., 2009). With increasing environmental temperatures, the activity of the autonomic nervous system increases and a $Q_{10}$-related increase is seen in muscle contractions leading to increased heart and respiratory rates (Clark et al., 2008; Farrell and Jones, 1992; Jones, 1971). A greater oxygen demand and energy requirement is then necessary to maintain the elevated metabolic rate, and consequently the extra energy allocation for increasing fitness is limited (Castro et al., 2013).
As discussed above, at extreme temperatures the heart fails and a limitation in oxygen availability has been suggested to drive heart failure. This is because increased oxygen demands conflicts with decreased supply and at temperatures approaching $T_{\text{max}}$, aerobic scope starts to decline (figure 1.3 top panel) (Pörtner and Farrell, 2008). While the importance of oxygen supply to aquatic systems is well recognised, the limitation of oxygen to the heart is debatable (Clark et al., 2013). Fundamentally different views exist on what actually governs oxygen uptake by heat stressed aquatic ectotherms (Gräns et al., 2014).

**Figure 1.3:** (Bottom panel) As temperature increases, population sizes of fishes have shown to decrease. (Middle panel) This decrease has been linked to a limit in the aerobic scope and cardiac output of fish. (Top panel) For many fish species the upper temperature limit ($T_{\text{max}}$) and the heart failure (HF) temperature ($T_{\text{HF}}$) are only a few degrees away from their current environmental temperature. Modified from Wang and Overgaard (2007).

Oxygen solubility can be viewed in two contexts. In an ecological context, environmental oxygen solubility is discussed in terms of simple concentrations (Chapelle and Peck, 1999),
whereas in physiological contexts, more importance is placed on partial pressures and diffusion gradients. The diffusion potential of oxygen is determined by its partial pressure, since gases dissolve, diffuse and react according to their respective partial pressures (Willmer et al., 2005). With the convergence of ecology and physiology, one would expect an integration of both perspectives; however, oxygen solubility is still commonly discussed in terms of simple concentrations in the field of eco-physiology. Oxygen diffusion and therefore, potential uptake rates are dependent on gradients in oxygen partial pressure ($pO_2$), and importantly the rates of diffusion (activities of gases), and the latter increases significantly with temperature (Verberk et al., 2011; Willmer et al., 2005). Therefore, while the absolute amount of oxygen dissolved in water declines with temperature, there is more than adequate compensation until extreme temperatures are reached. This is due to elevated gas diffusion rates in warm water and potential gradients driven by thermally elevated metabolic rates of fish. If limitations in either oxygen supply to the gills or the diffusion of oxygen across the gills into the blood occurs, the arterial $pO_2$ is expected to decrease with increasing temperature (Farrell et al., 2009). However, this has not been the case in all fishes because arterial $pO_2$ remain unchanged as fish were exposed to progressively warming waters (Sartoris et al., 2003; Steinhausen et al., 2008).

1.2.5 Fish cardiac function with changing temperatures

![Figure 1.4](image)

**Figure 1.4**: Representative figure of the fish circulatory system. Deoxygenated blood is shown in blue, oxygenated blood is shown in red. Modified from Farrell et al. (2009).

For most fish species the heart is a simple two chambered spongy myocardium in a single loop circulatory system. (Farrell et al., 2009). Deoxygenated blood from the body is received by the sinus venosus which opens into the atrium. This deoxygenated blood is then pumped
into the larger, muscular ventricle (atrial contraction), which fills and then contracts (ventricular contraction) directing blood through the bulbous arteriosus in to the ventral aorta (Randall, 1970). Fish heart muscle varies considerably. Some highly athletic fish species (e.g. salmonids and tunas), hypoxia tolerant fish (e.g. fresh water eel family, *Anguillidae*), elasmobranchs, and other primitive fishes (e.g. sturgeon, holosteans, and lungfishes) possess an additional blood supply to the heart via a coronary circulation that arises after the blood has passed through the gills (Farrell et al., 2009). In comparison less active fish hearts are often dependent on deoxygenated venous blood as it passes through the two chambers. In these fishes blood flows from the ventral aorta to the gill filaments for oxygenation, this then supports oxygen requirements of systemic tissues and venous blood returns to the heart (figure 1.4) (Randall, 1970).

The mechanistics involved in the collapse of fish hearts has been studied comprehensively (Farrell, 1997; Farrell, 2002; Farrell, 2007; Farrell et al., 2009; Overgaard et al., 2004; Steinhausen et al., 2008). With increasing temperature, heart rate is usually increased over stroke volume in order to maintain cardiac output due to direct innervation of the cardiac pacemaker cells (Clark et al., 2008; Randall, 1970; Sandblom et al., 2009; Steinhausen et al., 2008). At $T_{\text{max}}$, the ability to increase heart rate to maintain cardiac output declines and this inability is thought to limit aerobic performance at high temperatures (Farrell et al., 2009).

The oxygen demand of the teleost heart accounts for approximately 4% of metabolic rate (Farrell and Jones, 1992) and unless exposed to severe environmental hypoxia, the venous $pO_2$ of fish remains high enough to supply oxygen to even heat-stressed hearts (Davie and Farrell, 1991). This is because of a favourable $pO_2$ gradient between the deoxygenated blood and the heart muscle that promotes oxygen diffusion into cardiac cells (Farrell et al., 2009). Moreover, in many fishes the spongy myocardium is arranged in muscular sheets, and this increases the surface area enhancing oxygen uptake (Pieperhoff et al., 2009).

### 1.3 Involvement of cardiac mitochondria in heat stress-induced heart failure (HF)

Since oxygen limitation appears not to be the main cause of HF in fish exposed to warming waters, other factors can also be postulated. In medical contexts there has been a significant increasing interest in mitochondria due to their central involvement in heart metabolism and HF (Huss and Kelly, 2005; Lesnefsky et al., 2001). The ultimate energy source in cells is adenosine
triphosphate (ATP), and in aerobic tissues such as the heart, ATP is provided by oxidative phosphorylation (OXP) by mitochondria (Lemieux and Hoppel, 2009). In vertebrate hearts, mitochondria can occupy 20 to 40% of the cardiomyocyte volume and channel 90% of the energy (Almeida-Val et al., 1994). Sedentary and active fish species have also been reported to have similar amounts of mitochondria in their cardiac tissue despite varying aerobic capacities (Moyes, 1996).

There has been a significant amount of research on mammalian heart mitochondria and their role in HF (Huss and Kelly, 2005; Lemieux et al., 2011; Lemieux et al., 2010a; Lesnefsky et al., 2001). Heart mitochondria appear to be very sensitive to heat stress (increasing in vivo/in vitro/in situ temperatures), ischemic damage, and oxidative stress. Exposure to heat stress has shown to induce heart mitochondrial swelling, with broken cristae and low matrix density indicating severe structural damage (Song et al., 2000). This damage may depress cellular ATP production, driving necrosis and reactive oxygen species (ROS) production, which can trigger programmed cell death (apoptosis) (Borutaite and Brown, 2003; Cereghetti and Scorrano, 2006; Turrens, 2003). While there are gradients in types of cell death, necrosis and apoptosis are classically defined, as the former occurs in the absence of ATP, and the latter is ATP dependent. Indeed, necrosis and apoptosis contribute to HF (Borutaite and Brown, 2003). OXP is also shown to decrease with increased heat stress in isolated mitochondria from cardiomyocytes of environmentally heat-stressed rats (Qian et al., 2004). Heat stress leads to a decrease in the membrane potential of the mitochondrial membranes, which partially uncouples OXP and diminishes ATP synthesis in isolated rodent mitochondria (Žūkienė et al., 2007). This lowered energy conservation due to increased heat induced cardiac function ultimately leads to cardiac fatigue and failure (Qian et al., 2004).

In reality the influence of temperature on the ectotherm heart mitochondria has been explored only superficially (Somero, 2002). Mitochondrial respiration rates, or oxygen flux, at $T_{\text{max}}$ have often appeared to be robust at temperatures well above the $T_{\text{HF}}$ of many ectotherm species. Thus the contributing role of the mitochondria has perhaps been incorrectly discounted (Pörtner, 2001; Somero, 2002; Somero et al., 1996). However, the maximal rates of mitochondrial respiration can be misleading as the actual efficiency and stability of mitochondria has to be tested in order to understand their contribution to tissue function.
One largely ignored line of evidence implicating mitochondria as instigators of HF in fish is that as temperature approaches $T_{\text{max}}$, succinate concentration (a tricarboxylic acid ($TCA$) cycle intermediate) increases in the blood of heat-stressed fish (Pörtner and Knust, 2007). There is no other bulk source of succinate in vertebrates other than that escaping from heat-stressed mitochondria, as succinate feeds electrons into complex II and the plasma membrane is normally impermeable to this metabolite (Stadlmann et al., 2006; Steinlechner-Maran et al., 1997). Consequently, it is an intermediate that accumulates in a state of mitochondrial dysfunction (Grieshaber et al., 1994).

While numerous comparative studies have been conducted on ectotherms, they have mainly tested isolated liver mitochondria that can be extracted very easily from this soft and large organ (Rolfe and Brown, 1997; Wodtke, 1978), and to a lesser extent skeletal muscle mitochondria (Blier and Lemieux, 2001; Guderley and St-Pierre, 2002; Johnston et al., 1994). Moreover, they have been tested with substrates that do not completely test respirational flux (Guderley and St-Pierre, 2002; Johnston et al., 1994), often with non-physiological buffers that do not relate to the ectothermic environment (Birkedal and Gesser, 2004; Blier and Lemieux, 2001), on vastly different genera (Birkedal and Gesser, 2003; Moyes et al., 1992a), and with species from extreme environments (Ansaldo et al., 2000; Hardewig et al., 1999). Few have investigated the pivotal role heart mitochondria from a common species from temperate marine environments (Hilton et al., 2010; Oellermann et al., 2012).

### 1.3.1 The electron transport system and heat stress

[Figure 1.5: Transmission electron micrograph of *Notolabrus celidotus* cardiac ventricle mitochondria viewed at high power. Image courtesy: Sarah Rynbeck.]
The mitochondrion is encapsulated by an outer membrane and the central matrix is surrounded by the inner membrane which invaginates into the matrix forming cristae (Lesnefsky et al., 2001) (figure 1.5). The electron transport system (ETS) and ATP synthase are located on the cristae of heart mitochondria along with membrane transporters. The matrix contains the enzymes of the \textit{TCA} cycle and among other mitochondria-dependent systems, anti-oxidant defence systems (Lesnefsky et al., 2001).

\textbf{Figure 1.6:} The electron transport system. Modified from (Lesnefsky et al., 2001).

The main substrates of mitochondrial oxidation in the vertebrate heart are carbohydrates and fats, where metabolism of carbohydrate generates pyruvate for mitochondrial uptake. This stands true for the teleost heart as well where ATP is generated through the oxidation of glucose and fatty acids (Driedzic, 1992). This is intuitive since pyruvate is readily available to the \textit{TCA} cycle since it can either be provided by glycogen stores, blood glucose, lactate oxidation or protein catabolism (Lemieux et al., 2006). The uptake of pyruvate is set by the availability of the substrate and by the properties of the pyruvate transporter or pyruvate dehydrogenase (PDH) (Lesnefsky et al., 2001). Hard working muscles, such as those in stress will use carbohydrates and thus metabolise pyruvate due to the faster reaction rates, decreased complexity of glycolysis, and increased efficiency in terms of ATP yield over \(\beta\)-oxidation. Substrate oxidation by PDH can control the rate of mitochondrial respiration and is shown to be altered by temperature (Dufour et al., 1996). In the mammalian system, PDH is shown to be thermally sensitive influencing changes in OXP (Lemieux et al., 2010a). Contrastingly, in rainbow trout and Atlantic wolfish, PDH had low thermal sensitivity and there was no correlation between mitochondrial respiration and PDH activity with increasing temperature (Blier and Guderley, 1993; Lemieux et al., 2010b). Ultimately the enzymes of the \textit{TCA} cycle generate NADH and FADH\(_2\) for oxidation by the ETS (figure 1.6).
The ETS comprises of four multi-unit enzyme complexes (Complex I – CI, Complex II – CII, Complex III – CIII and Complex IV – CIV) in the semi-fluid inner membrane (Lesnefsky et al., 2001). Two mobile electron carriers are also part of the ETS, coenzyme Q in the inner membrane and cytochrome \( c \) in the intermembrane space. All these components of the ETS pass electrons from high redox potentials of NADH and FADH\(_2\) to lower redox potential of molecular oxygen (\( O_2 \)) (Lesnefsky et al., 2001). The ETS is sometimes incorrectly referred to as the electron transport chain suggesting the flow of electrons along a linear cascade (Gnaiger, 2007). Instead, CI and CII together pass electrons to CIII through coenzyme Q, and through cytochrome \( c \) from CIII to CIV (Gnaiger, 2008). Simultaneously, CI, III and IV translocate protons from the matrix to the intermembrane space creating a proton gradient across the inner membrane. The build-up of protons then pass through ATP synthase to produce ATP.

A damaged and leaky inner membrane can uncouple the mitochondria by allowing protons to diffuse back into the matrix (termed proton leak) dispersing the electrochemical gradient (Lesnefsky et al., 2001). Membranes of fish heart mitochondria have been shown to become leakier with increases in temperature (Hilton et al., 2010). Generally, higher temperatures are believed to increase membrane fluidity and therefore, proton leak, causing the uncoupling of the ETS from ATP production and depressing mitochondrial OXP (Pörtner et al., 2001). This change in membrane fluidity with increasing temperature can further affect the enzyme complexes of the ETS that are embedded in the inner membrane. The activities of CI, CIII and CIV have been previously shown to decrease with increasing temperature in ectotherm heart mitochondria (Lemieux et al., 2010b; Oellermann et al., 2012).

1.3.2 ROS production and heat stress

It has been contended that the main physiological producers of reactive oxygen species (ROS) in fish are mitochondria (Filho, 2007). Within mitochondria, superoxide (\( O_2^- \)), a type of ROS, is produced by the one electron reduction of \( O_2 \) (Murphy, 2009). Depending on the concentration of \( O_2^- \) in the mitochondria, the mitochondrial enzyme superoxide dismutase (SOD) catalyses the dismutation of \( O_2^- \) to produce another type of ROS, hydrogen peroxide (\( H_2O_2 \)). In reality, regulated formations of ROS by the mitochondria are normal by-products of cellular respiration and can actually assist with intracellular signalling (Turrens, 2003). But an increase in ROS production can lead to deleterious reactions that can target proteins, DNA...
and even start apoptosis in heart mitochondria (Borutaite and Brown, 2003; Turrens, 2003). This imbalance between the excessive formation of ROS and the antioxidant defences of the mitochondria causes ‘oxidative stress’ (Turrens, 2003). The antioxidant defence system that prevents oxidative stress comprises enzymes and metabolites that are free radical scavengers. In marine fishes, the enzymes of this defence system have been found to be higher in active fish compared to sluggish counterparts (Filho, 2007). Moreover, thermal stress is known to cause oxidative stress in marine ectotherms, where ROS production is found to increase as the mitochondria uncouples in the marine mud clam *Mya arenaria* exposed to increasing temperature (Abele et al., 2002; Abele et al., 2007; Abele and Puntarulo, 2004).

### 1.3.3 High resolution respirometry

Mitochondrial respirometry was first developed on the principle of determining the changes in oxygen concentration in a closed chamber as the mitochondria of a biological sample consumed oxygen (Gnaiger, 2008). The initial oxygen concentration was usually set to environmental levels of 21%, and as oxygen was lowered the concentration plotted against elapsed time provided an estimate of oxygen consumption (Hütter et al., 2006). Chance and Williams (1955) were the first to use oxygen sensitive electrodes, high mitochondrial concentrations, and the principle of a closed chamber to successfully determine the OXP of guinea pig liver mitochondria. Modern standards of mitochondrial respiration with Oroboros Oxygraphs® are based on highly sensitive electrodes, a design that minimises oxygen diffusion and back flux and advanced software that instantaneously calculates oxygen consumption rates (Hütter et al., 2006).

A major advantage in the advancement of mitochondrial respirometry is the ability to measure mitochondrial respiration *in situ* using permeabilised tissue fibres and cells as opposed to the traditional method of isolated cells and mitochondria (Gnaiger, 2008). Although isolated mitochondria gives a pure sample and provides the advantage of avoiding confounding factors of the cell environment, mitochondria form networks and are indeed interdependent on the rest of the cell which in turn affects mitochondrial activity (Hütter et al., 2006). Additionally, the isolating process has the potential to yield only viable sub-populations resulting in bias (Jüllig et al., 2008; Kuznetsov et al., 2008; Picard et al., 2011). However, this method is still used widely to study mitochondrial biology in fishes (Blier and Lemieux, 2001; Fangue et al., 2009; Guderley and Johnston, 1996; Johnston et al., 1998;
Moyes, 1996). Most of these studies have worked with large active fish, such as salmonids and tuna, which have large organs that permit adequate mitochondrial yields predominantly from liver. However, using permeabilised fibres allows the advantage of working with smaller fishes that have smaller, more delicate organs and permits analysis with minimal disruption of mitochondrial structures in limited amounts of cardiac tissue.

1.3.4 The SUIT assay, respiration states and ratios

Heart fibres are permeabilised using saponin, a plant glycoside that targets cholesterol in the plasma membrane (0.5 mol cholesterol per mol phospholipid) only (Kuznetsov et al., 2008). Vertebrate mitochondria contain very low levels of cholesterol in their mitochondrial membranes (0.01-0.07 mol cholesterol per mol phospholipid) (Kuznetsov et al., 2008), while in fish mitochondria it has been reported that mitochondrial membranes have undetectable levels of cholesterol (Ballantyne, 2004; Filho, 2007). Therefore, permeabilisation allows mitochondria within the heart fibres to remain intact and able to remain in their intracellular position. The protocol used is a substrate-uncoupler-inhibitor-titration (SUIT) assay that tests the combined effects of mitochondrial complexes in a stepwise fashion while stimulating maximal flux rates (Kuznetsov et al., 2008).

CI is fuelled using malate, pyruvate and glutamate. Malate requires the acetyl-coA that pyruvate provides to form citrate. Pyruvate also produces NADH allowing mitochondrial oxidation (Gnaiger, 2007). Glutamate can also fuel CI oxidation via glutamate dehydrogenase but requires the presence of malate. The addition of all three CI substrates (M+P+G) ensures maximum respiratory flux through CI. The respiratory control ratio (RCR) is a useful ratio to understand the capacity of CI. It is calculated as follows:

\[
(1) \quad \text{RCR} = \frac{\text{CI respiration with ADP (OXP)}}{\text{Respiration with only CI substrates (Leak-I)}}
\]

This ratio provides an understanding of how efficiently electron transport through CI is coupled to ATP synthesis (Weinstein and Somero, 1998). As an arbitrary rule some have suggested that an RCR greater than 4 indicates adequate coupling, while those that are lower indicates substantial leakiness of the inner mitochondrial membrane (Brand and Nicholls, 2011). Following CI saturation, the addition of succinate fuels CII via reduced prosthetic FAD bound to CII (Gnaiger, 2007). With CI and CII fuelled together, the ETS functions as if
the TCA cycle was intact in vivo where both CI and CII channel electrons through coenzyme Q to CIII. This provides the maximal phosphorylating state of the mitochondria in this thesis termed OXP-I, II.

The integrity of the outer and inner membranes can also be tested through this assay by the addition of cytochrome c and NADH, respectively. Cytochrome c is a peripheral protein that is loosely bound to the mitochondrial inner membrane (Kuznetsov et al., 2008). If the outer mitochondrial membrane is intact, exogenous cytochrome c addition does not affect mitochondrial respiration because endogenous cytochrome c remains in the intermembrane space. When the outer membrane is damaged, endogenous cytochrome c is lost to the cytosol and this takes place in cells undergoing apoptosis (Kluck et al., 1997). Moreover, mitochondrial respiration is inhibited because cytochrome c transfers electrons from CIII to CIV, and if the addition of exogenous cytochrome c increases respirational flux, it is indicative of a damaged outer membrane (Kuznetsov et al., 2008). Likewise, if NADH addition increases respiration in the OXP-I, II state, this is indicative of inner membrane damage because the inner membrane is impermeable to this molecule.

The inhibitors atractyloside, rotenone, malonate and antimycin a are used in this assay to block parts of the ETS. Atractyloside inhibits the adenine nucleotide translocase (ANT) and blocks the ETS at CIII providing information on the proton permeability of the inner mitochondrial membrane (Lehninger et al., 1995). Rotenone, malonate and antimycin a inhibit CI, CII and CIII, respectively (Gnaiger, 2007). In this assay, we titrated FCCP (Carbonyl cyanide p-(trifluoromethoxy) phenyl hydrazine) in small amounts to uncouple the phosphorylation system from the ETS. FCCP acts as a proton carrier and transfers protons from the intermembrane space back into the matrix, thereby disrupting, or chemically uncoupling the membrane potential from ATP synthesis (Lehninger et al., 1995). The ETS is no longer constrained and maximal capacity of mitochondrial respiration can then be determined. Finally, CIV respiration is determined by the addition of TMPD (N,N,N’,N’-Tetramethyl-p-phenylenediamine dihydrochloride) and ascorbate. These two chemicals generate an artificial electron donor system that fuels CIV (Cytochrome c oxidase – CCO) activity.
The substrate-inhibitor titration protocol with mitochondrial states is as follows (figure 1.7):

1) Malate + Pyruvate + Glutamate $\rightarrow$ **Leak-I (state II respiration)**
2) ADP $\rightarrow$ OXP-I
3) Cytochrome c $\rightarrow$ Cyt c
4) Succinate $\rightarrow$ OXP-I, II (state III respiration)
5) NADH $\rightarrow$ NADH
6) Atractyloside $\rightarrow$ Leak-I, II
7) FCCP titrations $\rightarrow$ ETS
8) Rotenone $\rightarrow$ Rot
9) Malonate $\rightarrow$ Malo
10) Antimycin c $\rightarrow$ Ant
11) TMPD + Ascorbate $\rightarrow$ CCO

**Figure 1.7:** Representative respiration assay protocol measuring mitochondrial flux (pmol O$_2$. (s. mg)$^{-1}$, black line, left y-axis), and oxygen concentration (nmol. ml$^{-1}$, dotted grey line, right y-axis), over time (mins). Titrations of mitochondrial substrates, poisons and inhibitors are shown with arrows indicating the time of addition, and the resulting respiratory state is noted in parenthesis. Modified from (Iftikar and Hickey, 2013).

### 1.4 Aims and objectives of this thesis

Given that the mechanisms of heat stress induced HF in ectotherms have not yet been completely explored, the primary aim of this thesis was to determine if heart mitochondria
contribute to heat stress induced HF in marine ectotherms. Specifically, it aims to test whether the temperature at which mitochondrial dysfunction (T_{mt}) occurred was before or after the temperature at which the heart failed (T_{HF}). Emphasis is placed on investigating this in a fish model by comprehensively exploring cardiac function, changes in the mitochondrial electron transport and phosphorylation systems, ROS production, and processes that may promote cell death pathways at T_{max}. This thesis is formed as a thesis with publications, and the chapters of this thesis have been structured to develop a logical and ordered outline.

In Chapter 2, the relationship between fish heart mitochondria and HF with rising water temperatures was established in the marine eurytherm, Notolabrus celidotus. The primary hypothesis that heart mitochondrial failure is causal or proximal to HF was explored. Questions addressed in this chapter were: What is the T_{HF} for this species? Do the components and states of the ETS change with increasing temperature and does this take place before T_{HF}? Does ROS production by heart mitochondria change with increasing temperature? Is substrate uptake by heart mitochondria impacted by heat stress? Is ATP synthesis by heart mitochondria limited with increasing temperature?

It is unknown if closely related fish species from contrasting thermal habitats have varying cardiac responses to increasing environmental temperatures. This unknown was addressed in Chapter 3 by exploring cardiac function and the mitochondrial contribution to HF in three fish species from restricted (stenothermal) habitats; the tropical moon wrasse, Thalassoma lunare, the cold-temperate banded wrasse, Notolabrus fucicola, and comparisons were made to Notolabrus celidotus, which has a broad temperate distribution.

In Chapter 4, the ability of fishes to acclimate their heart mitochondria to elevated temperatures was tested. The model, N. celidotus, was acclimated for 6 weeks to extremes of winter low and summer high temperatures. Cardiac mitochondrial function and its role in HF was explored in these acclimated groups.

Climate change is predicted to provide an environment that enhances the introduction of exotic and invasive species and the success of invasive species can be determined by thermal tolerance. This was addressed in Chapter 5 by investigating cardiac mitochondrial function
and its governance of thermal tolerance in the New Zealand native crab, *Ovalipes catharus*, and the invasive species, *Charybdis japonica*.

The findings of this thesis are synthesised in the final chapter (*Chapter 6*) and discussed in context with reference to the wider literature. Gaps in knowledge of this current field and future research avenues and limitations of this thesis are also outlined.
2. CHAPTER 2 – UNDERSTANDING THE CONTRIBUTING ROLE OF CARDIAC MITOCHONDRIAL FUNCTION TO HEART FAILURE IN HEAT STRESSED NOTOLABRUS CELIDOTUS

Published as:


2.1 Introduction

Increase in ocean temperatures globally present concerns for ectotherms that live in these oceans as they are generally sensitive to fluctuating temperatures (Perry et al., 2005; Pörtner and Farrell, 2008; Walther et al., 2002). By definition, body temperatures of ectotherms change with their environment. As a consequence the capacity to adapt to environmental temperature fluctuations is dependent on altering biochemical and metabolic processes in order to maintain homeostasis (Farrell et al., 2009; Galli and Richards, 2012; Hochachka and Somero, 2002; Pörtner and Farrell, 2008; Somero, 2002; Somero, 2011; Stillman, 2003). Metabolic rates of ectotherms typically increase with acute rises in habitat temperature and this elevates demands on precious metabolic fuels (Hochachka and Somero, 1968). If metabolic rates increase drastically or if they run inefficiently, fuel reserves must be redirected from anabolic processes to power catabolism. This will impair growth, physiological equilibrium, and ultimately survival. Therefore, increasing environmental temperatures or heat stress has complex and integrative effects on circulation, respiration, digestion, growth, reproduction, and locomotive capacities of ectotherms (Somero et al., 1996).

For ectotherms with cardiovascular systems the heart is temperature sensitive, and in most cases the critical temperature for heart failure ($T_{HF}$) is only a few degrees above the upper habitat temperatures ($T_{max}$) (Pörtner and Knust, 2007; Somero, 2002; Somero et al., 1996). For marine ectotherms these apparently fine margins between $T_{max}$ and $T_{HF}$ may have restructured species distributions. With respect to fish, the main causative limitation is believed to be cardiac function. Acute heart failure (HF) in ectotherms has been proposed to
result from decreased oxygen availability (Pörtner and Knust, 2007; Pörtner et al., 2004), because rising habitat temperatures decreases blood oxygen solubility while metabolic rates increase. However, oxygen diffusion rates are enhanced at elevated temperatures and to a limit these can offset decreases in oxygen solubility (Verberk et al., 2011; Willmer et al., 2005). Although oxygen solubility ultimately does become limiting, this condition occurs above temperatures experienced by most tropical organisms where coincidentally there is the greatest species diversity (Verberk et al., 2011). Additionally, increasing temperature can further limit ectotherm cardiac function physiologically since temperature affects the cardiac pacemaker directly disrupting both signal production and transduction (Harper et al., 1995). It has also been suggested that hearts fail with elevated temperature because calcium handling rates become inadequate in cardiac myocytes during excitation-contraction coupling (Farrell, 1997). However, calcium dynamics appear to be maintained across broad temperature ranges despite acute temperature effects on individual proteins (Shiels et al., 2003). Therefore, if oxygen solubility and calcium handling are not limiting cardiac function at high temperatures, what other mechanisms could explain the THF?

Another line of evidence implicate mitochondria as instigators of HF. Mitochondria are central to HF in numerous cardiac diseases (Huss and Kelly, 2005; Lane, 2005; Lesnefsky et al., 2001), and as temperature approaches $T_{\text{max}}$, succinate (a tricarboxylic acid (TCA) cycle intermediate) has been reported to increase in concentration in the blood of heat stressed fish (Pörtner and Knust, 2007). Succinate is a mitochondrial electron transport system (ETS) substrate that feeds electrons into complex II and the plasma membrane is normally impermeable to this metabolite (Stadlmann et al., 2006; Steinlechner-Maran et al., 1997). For marine ectotherms the appearance of succinate in blood indicates mitochondrial dysfunction (Grieshaber et al., 1994). Mitochondria occupy 20 to 40% of the vertebrate cardiomyocyte volume and channel 90% of the energy as ATP to contractile machinery and ion pumps (Hochachka, 1994). The proteomes (Mootha et al., 2003), structures, dynamics (Cereghetti and Scorrano, 2006), and energetic outputs (Benard et al., 2006) make heart mitochondria different to mitochondria from other tissues as they are sensitive to heat stress, ischemic damage, and oxidative stress (Borutaite and Brown, 2003; Brand et al., 1994; Brand and Nicholls, 2011; Lesnefsky et al., 2001).
Cardiac mitochondria have been explored in contexts of HF in mammals exposed to heat stress (Huss and Kelly, 2005; Lemieux et al., 2011; Lemieux et al., 2010a). The respiratory control ratio (RCR) and the phosphate/oxygen (P:O) ratio are measures of mitochondrial uncoupling and putatively oxidative phosphorylation efficiency, respectively. These ratios decreased in isolated mitochondria from cardiomyocytes of rats that were thermally challenged (Qian et al., 2004). These alterations to cardiac mitochondria should depress cellular ATP thereby driving necrosis. Thermally challenged heart mitochondria can also elevate reactive oxygen species (ROS) release and promote apoptosis (Borutaite and Brown, 2003; Cereghetti and Scorrano, 2006; Turrens, 2003). Both necrosis and apoptosis can contribute to HF (Borutaite and Brown, 2003). There are no studies to date that examine if cardiac mitochondria contribute to thermally induced HF in ectotherms such as fish.

The majority of studies investigating temperature influences on ectotherm mitochondria have reported stable respiration rates at temperatures equal to or well above species T_max (Somero, 2002; Somero et al., 1996). Therefore, the role of heart mitochondria in thermally-induced HF has been discounted (figure 2.1). In general, these studies have not measured the efficiency or stability of mitochondria. Most studies used substrates that did not fully test respirational flux (Guderley and St-Pierre, 2002; Johnston et al., 1994), and sometimes with non-physiologically relevant buffers (Birkedal and Gesser, 2004; Blier and Lemieux, 2001). Some of these studies compared disparate species (Birkedal and Gesser, 2003; Moyes et al., 1992a) or species from extreme thermal environments (Ansaldo et al., 2000; Hardewig et al., 1999). Significantly, few have investigated the pivotal role of heart mitochondria in a common species from temperate marine environments (Hilton et al., 2010; Iftikar et al., 2010).

This study determined the effects of increasing temperature on cardiac mitochondria from a common and abundant New Zealand marine fish, Notolabrus celidotus (Francis et al., 2005). This species is an appropriate model organism as it is a food source for a number of finfish and therefore, provides insight into the effects that increasing ocean temperatures could present at species and ecosystem levels. The primary aim was to test whether mitochondrial function is potentially a cause or an effect of HF (figure 2.1). The T_HF of N. celidotus was first determined and cardiac mitochondrial function from heat stressed fish following HF was assessed and compared to non-heat stressed fish (controls). Mitochondrial function was
examined using saponin permeabilised cardiac fibres. Permeabilisation leaves cardiac mitochondria and their cytoskeleton contacts intact and provide a more physiologically relevant preparation in terms of ROS production and stability (Lane and Martin, 2010). The oxygen fluxes through different components of the ETS and oxidative phosphorylation (OXP) systems were then tested to identify the most susceptible points following acute heat stress. Lastly, the affinity of heart mitochondria for pyruvate, ROS production and the capacity to synthesise ATP in maximal respiration states was evaluated to understand the mechanistic contributions of mitochondria to HF.

Figure 2.1: Understanding thermal limits of HF. Previous studies showed that mitochondria were robust beyond temperatures at which the heart fails (green dashed line). This study questions whether cardiac mitochondria fail before (a causal mechanism) or after heart failure (an effect, red line)?

2.2 Materials and methods

2.2.1 Experimental animals

Fish were caught using hand-held line fishing from piers around the greater Auckland region. No specific permits were required for this method of acquiring fish because areas frequented were available for public recreational fishing. No permits were required for number of fish caught since *N. celidotus* is not a protected or endangered species. They were kept at 18 ± 0.5
°C in aerated aquaria with recirculating seawater under a 12 hour light photoperiod for four weeks prior to experiments. Fish were fed every two days with green lipped mussel and feeding was suspended 48 hours prior to experiments. All experiments and procedures met with the ethical requirements and recommendations of the Animal Ethics Committee of the University of Auckland, New Zealand ( Permit approval AEC/04/2009/R720 Fish).

2.2.2 Measuring cardiac function

Following a similar protocol described previously (Iftikar et al., 2010), fish heart rates were measured using foetal Doppler probes (Sonotrax B, Contact Medical Systems, China) without anaesthesia as anaesthetics can affect mitochondrial function (Tarba and Cracium, 1990). Fish were secured ventral side up within a submerged sponge holder in a 4 L plastic container that was then immersed in a larger 20 L water reservoir creating a recirculating system with constantly aerated seawater (18.0 ± 0.5 °C). Seawater was pumped across the gills using a small pump (Rio®, mini 150, Taipei, Taiwan) at a rate of 40 ± 0.1 mL min⁻¹ into the buccal cavity to induce atonic immobility (Wells et al., 2005). A damp black cloth with a 2 cm diameter opening was placed over the fish just behind the gills exposing the underside for placement of the Doppler probe. Fish were held prone for 3 hours prior to experiments to ensure the heart rate had settled and was constant. A thermocouple (Digitech QM-1600) was then placed inside the fish’s mouth to record temperature that was gradually increased 1 °C every 10 minutes in the 20 L reservoir tank using glass aquarium heaters (Gollock et al., 2005; Pörtner et al., 2001; Zakhartsev et al., 2003).

Sonograms were measured over one minute immediately after each temperature was reached (N=8, mean mass 35.93 ± 3.44 g). Previous trials found that the fish heartbeat became inconsistent or intermittent just prior to death. The experiment was terminated when the heartbeat became inconsistent and this temperature represented T_	ext{HF}. Control fish (N=8, mean mass 25.88 ± 1.88 g) were maintained at 18 ± 0.5 °C, and sonograms were measured every 30 minutes for the duration of the temperature-exposure experiments. The Doppler audio output was connected to a PC soundcard via an audio jack and recorded using Audacity® 1.2.6 (http://audacity.sourceforge.net/). Fish were then euthanised by concussion and a heparinised caudal blood sample was taken. Plasma was separated by centrifugation (five minutes at 2500 rcf), frozen in liquid nitrogen and stored at -80 °C for metabolomic analysis. The heart was excised for mitochondrial respirometry described below.
2.2.3 Assessing blood oxygen saturation

Haemoglobin saturation was monitored across the heart non-invasively using spectroscopy. As oxygenated haemoglobin absorbs at near infrared wavelengths around 940 nm and deoxygenated haemoglobin absorbs at 600 nm (Zijlstra et al., 1991), a 3 Watt infrared LED (peak wavelength 940 nm) and a 3 Watt red light-emitting LED (peak wavelength 600 nm) were placed on one side of the fish near the base of the pectoral fins. The transmitted light through the fish heart was received on the opposite side by a 1.5 mm Perspex fibre optic cable attached to an Ocean Optics USB4000 spectrometer. Data were acquired using Ocean Optics SpectraSuite software. As haemoglobin de-saturates the absorption peak wavelength at 600 nm increases, whereas the absorption at 940 nm decreases (Zijlstra et al., 1991). Preliminary experiments showed that the 940:600 ratio decreased with haemoglobin desaturation on exposure of fish to brief hypoxia (N_2 exposure). As above, experimental fish (N=6, mean mass 117.75 ± 22.24 g) were exposed to increasing temperatures (1 °C every 10 minutes) until THF, while control fish (N=4, mean mass 92.0 ± 13.80 g) were maintained at 18 ± 0.5 °C and measured over the same time duration as experimental fish.

2.2.4 Plasma metabolic profile

Plasma metabolites were extracted using -30 °C methanol according to a modified protocol (Villas-Bôas et al., 2003). Initially 20 μL of internal standard (10 mM solution of 2,3,3,3-d_4 DL-Alanine) was added to 100 μL of plasma, vortexed and frozen at -80 °C. Plasma samples were freeze dried (Virtis freeze dryer) and the metabolites extracted by adding 500 μL methanol:water (1:1 v/v) at -30 °C. The solution was vortexed vigorously for one minute and then centrifuged at 4 °C for five minutes at 16,000 g. Supernatants were collected and centrifuged again. The pellets were re-suspended and extracted a second time in 500 μL -30 °C methanol:water (4:1 v/v) and pooled with the first extract. Extracted metabolites were freeze dried following the addition of 5 mL of cold bi-distilled water (4 °C). Metabolites were chemically derivatised using methyl chloroformate and the samples analysed by gas chromatography-mass spectrometry (GC-MS) (Villas-Bôas et al., 2003).

2.2.5 Tissue metabolites and enzyme markers

Lactate, citrate synthase (CS, an aerobic marker of mitochondrial content (Howald et al., 1985; Srere, 1969)) and lactate dehydrogenase (LDH, an anaerobic marker enzyme (Hochachka et al., 1983a)) in cardiac tissue from control and heat stressed fish were
measured similar to Iftikar et al. (2010) with modifications from previous studies (Hickey and Clements, 2003; Newsholme and Crabtree, 1986). Glucose-6-phosphate dehydrogenase (G6PDH), a key enzyme in the pentose phosphate pathway, was analysed according to McClelland et al. (2006). All assays were measured at 25 °C.

2.2.6  Mitochondrial bioenergetics

2.2.6.1 Fibre preparation for mitochondrial respirometry

In all mitochondrial assays the preparation of heart fibres followed the same protocol. Fish hearts were rapidly dissected and then immersed in 2 mL modified cold relaxing buffer (BIOPS: 2.77 mM CaK₂EGTA, 7.23 mM K₂EGTA, 5.77 mM Na₂ATP, 6.56 mM MgCl₂·6H₂O, 20 mM taurine, 20 mM imidazole, 0.5 mM dithiothreitol, 50 mM K-MES, 15 mM Na-phosphocreatine and 50 mM Sucrose, pH 7.1 at 0 °C). The dissected heart was teased into fibre bundles using a dissecting microscope and placed in 1 mL cold BIOPS (4 °C) in a plastic culture plate. Fibres were then transferred to fresh BIOPS containing 50 µg. mL⁻¹ saponin in a 12 well-culture plate, and gently shaken on ice for 30 minutes. Consequently, fibres were transferred and washed three times for 10 minutes in 2 mL of modified mitochondrial respiratory medium (Fish-MiRO5: 0.5 mM EGTA, 3 mM MgCl₂·6H₂O, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH₂PO₄, 20 mM HEPES, 160 mM sucrose and 1g. L⁻¹ BSA, essentially free fatty acid, pH 7.24 at 20 °C). Fibres were blotted dry on filter paper and weighed into 2-3 mg bundles for respiration assays. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2.6.2 Testing mitochondrial function in permeabilised cardiac fibres

Four experiments were conducted to assess heart mitochondrial function. The first two experiments applied identical protocols that measured respiration and ROS production simultaneously. However, the first tested the effect of acute heat stress on hearts in vivo and assayed fibres at 20 °C. The second tested mitochondrial function across a range of temperatures using fibres from naive fish. The third experiment tested apparent affinities of fibres for substrates across a range of temperatures, and the last measured ATP synthesis across a range of temperatures.
Figure 2.2: Representative mitochondrial respiration assay traces from permeabilized cardiac fibres measuring (A) mitochondrial flux (pmol O₂. s⁻¹. mg⁻¹, black line, left y-axis) and oxygen concentration (nmol. ml⁻¹, dotted grey line, right y-axis) over time (mins). Titrations of mitochondrial substrates, poisons and inhibitors and their time of addition are shown with arrows, and the resulting respiratory state in parenthesis. Pyr [pyruvate], Mal [malate], Glu [glutamate], Cyt c [cytochrome-c], Succ [succinate], Atr [attractyloside], FCCP [carbonyl cyanide p-(trifluoromethoxy) phenyl-hydrazone], Rot [rotenone], Malo [malonate], Ant [antimycin-a], TMPD [N, N, N', N'-tetramethyl-p-phenylenediamine], Asc [ascorbate], Leak-I (state 2 respiration through CI in the absence of ADP), OXP-I (state 3 respiration), OXP-I, II (parallel electron transport from CI and CII), Leak-I, II (leak respiration flux rate through CI and CII), ETS (maximal flux of the electron transport system), CCO (activity of CIV, cytochrome c-oxidase) and (B) pyruvate affinity at 20°C (see methods section for details).
2.2.6.3 Experiment 1: Testing the effects of acute temperature exposure in vivo

Heart fibres from control and experimental fish were added to 2 mL chambers containing equilibrated Fish-MiRO5 in Oroboros Oxygraph-2k™ respirometers (Oroboros Instruments, Innsbruck, Austria). Oxygen was added into the gas phase above media prior to closing chambers to supersaturate Fish-MiRO5. Oxygen was maintained above 280 nmol. mL\(^{-1}\) throughout assays to maximise flux. Respiration was measured as the weight-specific oxygen flux [pmol O\(_2\) (mg wet weight \cdot sec\(^{-1}\)] following a titration-inhibition protocol outlined below (figure 2.2). The respiratory flux was calculated in real time as the negative time derivative of the oxygen concentration using Oroboros DatLab Software V 4.1.1.84 (Oroboros Instruments, Innsbruck, Austria).

The substrate-uncoupler-inhibitor titration protocol (Gnaiger, 2009; Gnaiger et al., 2000; Kuznetsov et al., 2008) tested mitochondrial function across the OXP system and ETS (figure 2.2). Complex I (CI) substrates (2 mM malate, and 10 mM pyruvate) were added to measure state II respiration through CI in the absence of ADP (denoted “Leak-I”). Excess ADP (2.5 mM) stimulated oxidative phosphorylation (OXP-I, state III respiration), and glutamate (10 mM) was added to saturate CI. Cytochrome c (Cyt c, 10 \(\mu\)M) was added to test outer membrane integrity. An increase in rate following exogenous Cyt c addition indicates outer mitochondrial membrane damage due to the loss of endogenous Cyt c. Phosphorylating respiration with CI and CII substrates (OXP-I, II) was measured by the addition of succinate (10 mM). NADH (0.5 mM) was then added to assess inner mitochondrial membrane damage. Leak respiration rates were also measured on combined CI and CII substrates by addition of atractyloside (750 \(\mu\)M, Leak-I, II) followed with repeated titrations of carbonyl cyanide \(p\)-(trifluoromethoxy)phenyl-hydrazone (FCCP, 0.5 \(\mu\)M) to uncouple mitochondria (denoted “ETS”). By the addition of rotenone (0.5 \(\mu\)M), malonate (15 mM) and antimycin a (1 \(\mu\)M), CI, II and III activities were inhibited respectively. Finally, the activity of cytochrome c-oxidase (CCO) was measured by the addition of the electron donor couple \(N,N,N',N''\)-tetramethyl-\(p\)-phenylenediamine (TMPD, 0.5 mM) and ascorbate (2 mM) (figure. 2.2). Chemical background assays were run to account for the auto-oxidation of TMPD and ascorbate at the seven experimental temperatures and subtracted from CCO flux rates.
2.2.6.4 Reactive oxygen species detection
Purpose built fluorimeters similar to those used by Hickey et al. (Hickey et al., 2012) that consisted of LEDs with a peak excitation of 520 nm were utilised. The sensors were attached to Oroboros O2K oxygraph systems permitting the simultaneous measurement of ROS production and mitochondrial respiration rates. To calibrate the fluorimeter, 400 pmol of resorufin (25 µM) was added to each chamber prior to each assay. Horse-radish peroxidase (HRP, 2.5 U. mL⁻¹) was added to complete the Amplex-Ultrared reaction. Superoxide dismutase (SOD, 24 U. mL⁻¹) was then added to capture mitochondrial produced super-oxide and convert this to hydrogen peroxide (H₂O₂). Steady state rates were followed using DATLAB 4.3 and corrected for tissue mass and background activities.

2.2.6.5 Experiment 2: Determining the cardiac mitochondrial failure temperature
In these experiments, respiration and ROS were measured in heart fibres from naive fish at seven temperatures (N=8 per temperature) to elucidate the temperature when mitochondrial dysfunction (Tₘ₉) occurs and its relation to T₉F (figure 2.1). Respiration was measured at 15 °C (average ocean temperature in winter), 17.5 °C (tank acclimation temperatures), 20 °C (average ocean temperature in summer), 25 °C (maximal summer temperature), 27.5 °C (T₉F), 30 °C and an extreme maximum of 32.5 °C.

2.2.7 Experiment 3: Determining substrate affinity (Apparent Kₗ₉) with increasing temperature
We tested the capacity (pseudo-affinity, or apparent Kₗ₉; Kₗ₉ app) of mitochondria within permeabilised fibres to take up pyruvate or glutamate in the presence of malate (5 mM) and ADP (2.5 mM) at increasing in situ temperatures (20 °C, 25 °C, 27.5 °C, 30 °C and 32.5 °C). The affinity for succinate was not tested as this is a derivative of acetyl-CoA and therefore, is dependent on pyruvate in vivo. Initial trials optimised pyruvate or glutamate concentrations at different temperatures. Based on these data, the respective substrate was titrated by stepwise substrate additions using microinjection pumps (Oroboros Tip O2K) until respiration flux appeared to be saturated (figure 2.2B). Michaelis-Menten curves were generated, and substrate-saturation curve kinetics were applied to determine Kₗ₉ app and Vₘ₉₉ values using nonlinear regression (Sigma Plot 12.0, San Jose, CA).
2.2.7.1 Experiment 4: ATP production with increasing temperature

The production of ATP was determined by following the changes in free extra-mitochondrial [Mg$^{2+}$] indicated by a Mg$^{2+}$-sensitive fluorescent indicator, Magnesium Green (MgG) (Chinopoulos et al., 2009). This method was adapted to the Oroboros O2K oxygraph using a 503 nm LED for excitation and a 530 nm filter for emission. Experiments with permeabilised fish heart fibres were performed at 20 °C, 25 °C, 27.5 °C, 30 °C and 32.5 °C. Fibres were added to Fish-MiRO5 in the oxygraph chambers with blebistatin (a myosin heavy chain inhibitor, 60 μM) and ouabain (Na$^+$-K$^+$-ATPase inhibitor, 50 μM). MgG (5 μM) was then added and chambers were oxygenated. Leak-I was determined by the addition of malate (2 mM), pyruvate (5 mM) and glutamate (10 mM). Mg$^{2+}$ free ADP (5 mM) was then added in excess to saturate mitochondria and obtain maximum ATP production via CI. Succinate (10 mM) was added to obtain ATP production by CI and CII. All complexes were then poisoned by the addition of rotenone (0.5 μM), malonate (15 mM) and antimycin a (1 μM). After the addition of each substrate or inhibitor, the assay was recorded for 5 minutes to obtain a clear signal.

To calibrate the ATP signal, separate titrations were performed to test the linearity and response of the MgG to sequential additions of Mg$^{2+}$ free ADP and in separate titrations to fresh Mg$^{2+}$ free ATP. ADP and ATP bound Mg$^{2+}$ quenched MgG fluorescence linearly within the range the assays were conducted under. As expected, ATP which has a greater affinity for MgG quenched the MgG signal more for an equimolar amount of ADP. Using these data ratios were derived for each temperature of ATP fluorescence relative to ADP fluorescence. The same amount of ADP (10 μM) was added at each temperature and this resulted in a consistent fluorescence. This signal was used to determine the amount of ATP produced by multiplying the ADP signal by the appropriate ratio and this was then used as a re-calibration value.

2.2.8 Calculations and statistical analyses

Heart rate was determined using a script written for Octave®, a numerical computational freeware. The Doppler output signal in Audacity® was converted to WAV format to use with Octave® which calculates the peak frequency heart rate. The signal envelope was first computed using a root mean square approach, and peaks were defined as any part of the signal greater than 50% of the maximum value of the envelope function. Sonogram data were
analysed using a repeated measures ANOVA followed by a post hoc Tukey test with a significance level of $p \leq 0.05$. In experimental fish, ABT was determined by segmented linear regression using the SegReg program (www.waterlog.info). $R^2$ was computed from the sum of the squares of the distances of the points from the best-fit line determined by nonlinear regression using Prism5®. The plasma metabolite levels were determined from base peak height as detected by the GC and normalised to an internal standard (d4-alanine). Metabolites in the plasma samples were identified using an in-house methyl chloroformate MS library of derivatised metabolites. This library contains MS spectra obtained from ultra-pure standards with the mass spectra saved and analysed with AMDIS 2.65 software (www.amdis.net). A comparative metabolite profile was constructed and analysed using R-software (Aggio et al., 2011).

Respiratory control ratios (RCRs) were calculated as OXP-I/Leak-I, and uncoupled control ratios (UCRs) were calculated as ETS/OXP-I, II. A dose-dependent analysis conducted with Prism5® was used to individually test the temperature breakpoint where RCR and UCR change with heat stress. Increasing assay temperature was considered as an inhibitor and the RCR or UCR were considered as the response to the inhibitor. To test if outer mitochondrial membrane damage had occurred, the fractional increase in oxygen flux after Cyt $c$ addition (Cyt $c$/OXP-I - 1) was calculated. The inner mitochondrial membrane damage (NADH/OXP-I, II - 1) was similarly tested. For both calculations a one-sample t-test was employed to test if measures differed from zero. OXP-I, II/Leak-I, II defined as RCR-II (Iftikar et al., 2010) was also used as a proxy for measuring inner membrane permeability (Gnaiger, 2009). Control or limitation by the ETS was determined by the comparison of leak respiration by atractyloside inhibition relative to uncoupled respiration (Flux control ratio FCR; Leak-I, II/ETS) (Domenis et al., 2011). In mitochondrial respiration assays, differences across temperatures and between control and experimental fish were evaluated with a one or two-factor ANOVA as appropriate, followed by a post hoc test (Tukey). Dose dependent analysis with Prism5® was also used to test if ROS production changed with heat stress. In this analysis, a log(agonist) vs. response curve was used where increasing assay temperature was the agonist while ROS production was the response. All statistical tests were run using SigmaPlot® version 12 (Systat Software, Inc., San Jose, California) unless otherwise stated. Data were reported as means ± SEM (N is the number of fish) unless otherwise stated.
2.3 Results

2.3.1 Thermal tolerance limits of cardiac function

With increasing temperature *N. celidotus* maintained heart rate until an average temperature of $27.8 \pm 0.4 \, ^\circ C$ ($R^2 = 0.93$) (figure 2.3). This indicates the critical temperature of heart failure ($T_{HF}$) occurs above $27.5 \, ^\circ C$ (figure 2.3). The heart rate at the beginning of the experiment for the control fish did not change compared to heart rate at the end of the experiment when the experimental fish had attained $T_{HF}$ ($N=8$, data not shown). Haemoglobin oxygen saturation of experimental fish did not differ from control fish ($p \geq 0.05$; figure 2.4). The percentage change in the 940:600 ratio remained relatively constant in control and experimental animals as temperature gradually increased to $T_{HF}$, and 95% confidence intervals (95% CI) for linear regression overlapped (figure 2.4).

Figure 2.3: Arrhenius break temperature (ABT) of heart rates of single individuals of *N. celidotus*. The ABT was $27.81 \pm 0.39 \, ^\circ C$ and values were expressed as individual heart rates per temperature ($N=8$).

Metabolite analysis of plasma from the cardiac function experiment showed that the only glycolytic intermediate detected was lactate (table 2.1). Lactate levels significantly increased
by 1.55-fold in experimental plasma, compared to controls. Five TCA cycle intermediates were identified in plasma; citrate, cis-aconitate, α-ketoglutarate, succinate and malate. Citrate, succinate, and malate were consistently detectable in both control and experimental plasma samples (\(N=6\), table 2.1). Although citrate appeared to increase in acutely heat stressed plasma, only succinate was significantly elevated and malate was significantly lowered. More essential amino acids (EAAs) were significantly elevated in heat stressed plasma (five amino acids) compared to non-essential amino acids (NEAAs; three amino acids) (table 2.1). Similar to measures in plasma, lactate trended higher (33%) in cardiac tissue of acutely heat stressed fish (\(p=0.07\); table 2.2). The activity of LDH significantly increased by 50%, indicating that heat stressed hearts had up-regulated anaerobic capacities during temperature exposure time while CS and G6PDH remained unchanged with heat exposure.

**Figure 2.4:** Changes in haemoglobin oxygen saturation in *N. celidotus* expressed as the change of infrared (940 nm) to red (600 nm) absorbance ratio in control (\(N=4\), grey x) and experimental (\(N=6\), black x) fish. Values are expressed for individual fish and linear regression was fitted for control (grey line) and experimental (black line) data with 95% CI in daggered lines. Control fish did not experience changes in temperature; therefore regressions were performed on absorbance ratios relative to time (bottom x-axis). Regression analysis for experimental fish were performed on absorbance ratios relative to temperature (top x-axis). Goodness of fit is given as \(R^2\).
Table 2.1: Patterns of metabolites (glycolytic, TCA cycle intermediates, amino acids) in control and experimental fish plasma (N=6). Arrows in column B indicate the increase or decrease in accumulation of metabolite in experimental plasma compared to control plasma.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Increase/Decrease in Experimental plasma</th>
<th>Experimental/Control fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Glycolytic Intermediates</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactate</td>
<td>↑</td>
<td>1.55*</td>
</tr>
<tr>
<td><strong>TCA Cycle Intermediates</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citrate</td>
<td>↑</td>
<td>1.55</td>
</tr>
<tr>
<td>Succinate</td>
<td>↑</td>
<td>1.35*</td>
</tr>
<tr>
<td>Malate</td>
<td>↓</td>
<td>0.92*</td>
</tr>
<tr>
<td><strong>Essential Amino Acids (EAAs)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leucine</td>
<td>↑</td>
<td>2.38*</td>
</tr>
<tr>
<td>Lysine</td>
<td>↑</td>
<td>2.80*</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>↑</td>
<td>2.02*</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>↑</td>
<td>2.29*</td>
</tr>
<tr>
<td>Valine</td>
<td>↑</td>
<td>2.46*</td>
</tr>
<tr>
<td><strong>Non-essential Amino Acids (NEAAs)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cysteine</td>
<td>↑</td>
<td>1.84*</td>
</tr>
<tr>
<td>Glutamate</td>
<td>↑</td>
<td>1.66*</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>↑</td>
<td>2.24*</td>
</tr>
</tbody>
</table>

*denotes significant change in metabolite at \(p \leq 0.05\) in experimental plasma compared to control plasma (column C).

Table 2.2: Glycolytic intermediates and enzymes in control and experimental fish cardiac tissue. Unit for lactate and enzymes is µmol. min\(^{-1}\). mg protein\(^{-1}\).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Experimental</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Glycolytic Intermediates</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactate</td>
<td>106.61 ± 18.46</td>
<td>142.39 ± 10.44 ((p = 0.07))</td>
</tr>
<tr>
<td><strong>Enzymes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G6PDH</td>
<td>369.59 ± 76.19</td>
<td>388.83 ± 26.86</td>
</tr>
<tr>
<td>Citrate Synthase</td>
<td>198.06 ± 80.19</td>
<td>176.35 ± 34.83</td>
</tr>
<tr>
<td>Lactate Dehydrogenase</td>
<td>1050.11 ± 92.75</td>
<td>1682.83 ± 168.00*</td>
</tr>
</tbody>
</table>

Values are means ± S.E.M \((N=4)\).
* denotes significant change at \(p \leq 0.05\).
2.3.2 Mitochondrial bioenergetics

2.3.2.1 Mitochondrial function in heart fibres from fish acutely exposed to heat stress

Following an acute exposure to increasing temperature permeabilised heart fibres showed few differences from control fish (figure 2.5A). However, experimental fish showed significantly lower Leak-I and OXP-I fluxes, compared to control fish. This significantly depressed the RCR by ~22% with temperature exposure ($p<0.05$; figure 2.5A insert). The OXP flux fuelled by CI and CII substrates and uncoupled rates (ETS) between control and experimental fish remained unaltered (figure 2.5A). ROS production was significantly
elevated when mitochondria were uncoupled, although acute heat stress did not change ROS production in Leak-I, OXP-I or OXP-I, II states (figure 2.5B).

### 2.3.2.2 Impacts of in situ heat stress on permeabilised heart mitochondria

![Figure 2.6](image-url)

**Figure 2.6**: Cardiac mitochondrial respirational flux measured at increasing assay temperature in permeabilized cardiac fibres of *N. celidotus* (*N*=8 per assay temperature). (A) Leak-I respiration with CI substrates malate and pyruvate; (B) OXP-I respiration with CI substrates malate, pyruvate and glutamate; (C) OXP-I, II respiration with CI and CII substrates; (D) CCO respiration of complex IV; (E) percentage increase of respiration from initial rates at 15 oC for Leak-I (grey circles), OXP-I (black circles), OXP-I, II (white circles) and CCO (grey squares). Values are means ± S.E.M. Points with similar letters are not significantly different at *p*≤0.05. One-site saturation exponential curves (red dotted lines) were fitted for each graph (A-D) and goodness of fit is given as *R*².
All components (Leak-I, OXP-I, OXP-I, II, CCO respiration, RCR, UCR) of mitochondrial phosphorylation were sensitive to increased temperatures (figure 2.6, 2.7A). An increase in temperature showed the expected exponential increase in Leak-I, OXP-I, OXP-I, II and CCO respiration. However, the goodness of fit of the exponential curve for OXP-I, II ($R^2 = 0.58$, figure 2.6C) and CCO ($R^2 = 0.53$, figure 2.6D) were low. This likely resulted from lower than expected flux rates at 30 °C, which fell below predicted values when an exponential curve was fitted (figure 2.6C, D). The thermal mediated increase in flux appeared to plateau between 27.5 and 30 °C, and this was most evident in OXP-I, II and CCO states (figure 2.6C, D).

Leak-I, OXP-I, OXP-I, II and CCO rates were expressed as a percentage increase from those at 15 °C in order to illustrate the relative effects of increasing temperature for each state. Overall, a more substantial increase was observed for Leak-I rates, compared to other measured states (grey circles, figure 2.6E). At 25 °C Leak-I rates had increased by 74%, whereas other mitochondrial states increased by only 50% or less. The OXP-I and OXP-I, II rates had initial increases of 33% at 17.5 °C. At temperatures above 17.5 °C, the percentage increase in flux rates were only marginal (~15%, figure 2.6E). The percentage increase of CCO appeared to be the most constrained below 30 °C, as the percentage increase in rates remained under 40% (grey squares). At 32.5 °C, OXP-I, OXP-I, II and CCO had increased by ~75% (figure 2.6E). This was further reflected in the ratios of CCO/OXP-I, II and CCO/ETS where values at 32.5 °C were significantly higher than all temperatures measured except at 15 °C (table 2.3).
Table 2.3: Ratios based on mitochondrial respirational flux in *N. celidotus* at 15 °C, 17.5 °C, 20 °C, 25 °C, 27.5 °C, 30 °C and 32.5 °C. OXP-I, II/Leak-I, II (also termed RCR-II) ratio is a simple proxy of inner membrane permeability. The Leak-I, II/ETS (termed flux control ratio, FCR) provides a measure of ETS capacity relative to the leak respiration state when phosphorylation is inhibited by atractyloside. CCO/OXP-I, II and CCO/ETS are measures of the capacity of cytochrome *c* oxidase (CCO) relative to maximum phosphorylation (OXP-I, II) or the ETS respectively.

<table>
<thead>
<tr>
<th>State</th>
<th>15°C</th>
<th>17.5°C</th>
<th>20°C</th>
<th>25°C</th>
<th>27.5°C</th>
<th>30°C</th>
<th>32.5°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>OXP-I, II / Leak-I, II (RCR 2)</td>
<td>2.22 ± 0.19&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.28 ± 0.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.33 ± 0.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.06 ± 0.19&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>2.04 ± 0.24&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.77 ± 0.17&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.55 ± 0.11&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Leak-I, II / ETS (FCR)</td>
<td>0.38 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.43 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.40 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.49 ± 0.04&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.55 ± 0.04&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.56 ± 0.04&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.63 ± 0.05&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>CCO / OXP-I, II</td>
<td>2.96 ± 0.35&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>2.37 ± 0.20&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>2.45 ± 0.21&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>2.96 ± 0.35&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>2.45 ± 0.23&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.13 ± 0.10&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.26 ± 0.36&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>CCO / ETS</td>
<td>2.50 ± 0.34&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>2.29 ± 0.11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.15 ± 0.22&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.37 ± 0.27&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.08 ± 0.09&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.94 ± 0.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.24 ± 0.46&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are means ± S.E.M. (*N*=8 at each temperature). Means with the same letter of the same case are not significantly different from one another (*p*≤0.05).
Figure 2.7: Components of cardiac mitochondrial respirational flux measured at increasing assay temperature in permeabilised cardiac fibres of *N. celidotus*. (A) Mean RCR (black circles, OXP-I/Leak-I, $N=8$ per assay temperature) values and mean UCR (grey squares, ETS/Oxp-I, II, $N=8$ per assay temperature) values. A dose-dependent analysis curve was fitted to both RCR and UCR data (dashed line) (B) Mean fractional increase in OXP-I respiration with Cyt c addition (white circles) and mean fractional increase of OXP-I, II respiration with NADH addition (grey circles). The asterisks denote the increase in respiration after Cyt c addition is significantly different from zero. The plus sign denotes increase in respiration after NADH addition is significantly different from zero at $p\leq0.05$.

RCR values remained above 4 at assay temperatures 20 °C and lower, indicating that mitochondria were robust and not damaged (Brand and Nicholls, 2011). A dose-dependent curve showed a breakpoint at ~20 °C (19.97 ± 0.42 °C), indicating a depression in RCR values after this temperature. From 25 °C upwards, RCRs were significantly depressed suggesting that above this temperature OXP capacity with CI substrates is compromised (figure 2.7A black circles). The RCR was depressed to 3.34 ± 0.29 at 30 °C which was similar to values at 32.5 °C (figure 2.7A). The OXP-I, II/Leak-I, II ratio (RCR-II) displayed a similar trend to RCR as it was significantly depressed at and above 25 °C (table 2.3). The UCR (ETS/OXP-I, II) decreased above 20 °C. Furthermore, when increasing assay temperature was considered as an inhibitor and a dose-dependent analysis curve was fitted to UCR values, a breakpoint was found around 17.5 °C (16.68 ± 1.49 °C, figure 2.7A grey squares). Comparison of the flux control ratio (FCR, ETS/Leak-I, II) further indicated that the ETS may have become limiting above 20 °C ($p\leq0.05$, table 2.3). The fractional increase in oxygen flux resulting from Cyt c addition significantly increased OXP-I rates from 20 °C, indicating outer mitochondrial membrane damage (figure 2.7B). While the inner membrane is
normally impermeable to NADH, the addition of NADH increased OXP-I, II respiration above 20 °C demonstrating damage to the inner mitochondrial membrane (grey circles, figure 2.7B). Following an increase in temperature in situ, there was a gradual increase in ROS production in the Leak-I and uncoupled states (figure 2.8A, D). Dose-dependent analysis determined breakpoints after 30 °C for Leak-I (29.96 ± 0.31 °C), OXP-I (30.53 ± 0.53 °C) and ETS (30.12 ± 0.33 °C) states indicating ROS production increased after this temperature (figure 2.8A-B, D). In the OXP-I, II state the breakpoint was found at 23.99 ± 3.26 °C however, ROS production in this state was significantly elevated only at 32.5 °C (p≤0.05) (figure 2.8C).

![Figure 2.8](image.png)

**Figure 2.8:** ROS production by permeabilised heart fibres in (fmol H₂O₂ (mg. s)⁻¹) with increasing temperature. (A) ROS production in the Leak-I state; (B) ROS production in the OXP-I state; (C) ROS production in the OXP-I, II state; (D) ROS production in the ETS uncoupled state. A dose-dependent agonist analysis curves were fitted for all states (black daggered lines). Values are means ± S.E.M for N=8. Means sharing the same letter are not significantly different from one another at p≤0.05.

### 2.3.2.3 Apparent substrate kinetics of heat stressed heart fibres

In the presence of malate and excess ADP the stepwise addition of pyruvate at all temperatures accelerated the oxygen consumption rate of heart fibres (figure 2.2B). The
apparent affinity of heart fibres for pyruvate was similar at all temperatures until 32.5 °C when the $K_m$ app for pyruvate increased approximately 20-fold higher than all other temperatures measured (figure 2.9A). The predicted $V_{\text{max}}$ extrapolated from Michaelis–Menten curves indicated a significant increase with increasing temperatures and plateaued at 27.5 °C (figure 2.9B). When the mitochondrial $V_{\text{max}}/K_m$ app ratio for pyruvate (analogous to the $k_{\text{cat}}/K_m$ measure of enzymatic efficiency) was compared between temperatures, increasing temperatures led to a significant increase in the ratio until 30 °C (figure 2.9C). At 32.5 °C this ratio decreased 14-fold indicating a substantial loss of kinetic efficiency. The affinity for glutamate to initiate CI respiration remained unchanged at all temperatures, and the $K_m$ app of mitochondria within fibres was in the mM range (data not shown).

![Figure 2.9](image-url)

**Figure 2.9:** Pyruvate affinity in cardiac fibres of *N. celidotus* examined at 20 °C, 25 °C, 27.5 °C, 30 °C and 32.5 °C. (A) Pyruvate concentration giving half maximal respiration rate ($K_m$ app); (B) maximal pyruvate stimulated respiratory flux rate ($V_{\text{max}}$) (C) ratio of $V_{\text{max}}/K_m$ app as an indicator of substrate efficiency. Values are means ± S.E.M for $N=8$. Means sharing the same letter are not significantly different from one another at $p \leq 0.05$. 

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2.3.2.4 Relative ATP production

The production of ATP in permeabilised fibres with increasing assay temperature supported mitochondrial phosphorylation trends identified in this study (figures 2.6-2.9). ATP production was measured in OXP-I, II and by 25 °C it was lower than at 20 °C and was severely inhibited by 32.5 °C (figure 2.10A). When expressed as a ratio of ATP production to oxygen consumption in the OXP-I, II state, the ATP/O ratio was significantly depressed at 25 °C; and ATP/O ratios were depressed by 3.5-fold at 32.5 °C relative to values at 20 °C (figure 2.10B).

**Figure 2.10:** The production of ATP across increasing assay temperatures at 20 °C, 25 °C, 27.5 °C, 30 °C and 32.5 °C in permeabilised *N. celidotus* cardiac fibres; (A) at maximal respiration OXP-I, II and, (B) the ratio of ATP production and OXP-I, II respiration. A dashed line at 27.5 °C indicates *T*_{HF}. Values are means ± S.E.M for *N*=6. Means sharing the same letter are not significantly different from one another at *p*≤0.05.
2.4 Discussion

The contributing role of mitochondria to HF has largely been dismissed because oxygen flux through mitochondria at $T_{\text{max}}$ appeared unhindered in most ectotherms studied to date. However, this paradigm has been developed from mechanistic examinations concerned less with mitochondrial efficiencies, integrities and their significant roles in apoptosis (Somero, 2002; Somero et al., 1996; Žūkienė et al., 2010). In contrast, this study provides comprehensive evidence that mitochondrial function is impaired concurrent with or prior to HF in a temperate fish species. In the presence of multiple and non-limiting substrate concentrations to maximise respiration flux, we showed that several mitochondrial components are compromised before the onset of $T_{\text{HF}}$ (27.5 °C). Specifically, both OXP efficiency and the absolute production of ATP are impaired prior to $T_{\text{HF}}$. The association of these decreases coincides with succinate accumulation in heat stressed hearts despite excess oxygen (in vitro) and no loss of haemoglobin oxygen saturation (in vivo). These data suggest that impairment of oxygen consumption by heart mitochondria precedes oxygen limitation. While mitochondrial respiration rates further increased at supra-physiological temperatures, this depended on substrate concentrations that are not biologically relevant. Importantly, the loss of Cyt c and aerobic generation of ATP production should drive apoptosis or promote necrosis. These significant findings provide insight into current descriptions of analogous challenges in mammals; notably mitochondrial induced cytopathic hypoxia. This condition occurs in haemorrhagic shock, hyper-pyrexia and sepsis (Fink, 2001; Fink, 2002). In these pathological settings, even with sufficient blood oxygenation, mitochondrial respiration is impaired.

2.4.1 The limits on heart function by acute heat stress

The heart is considered to be the most temperature sensitive organ in animals with circulatory systems (Farrell, 1997; Farrell and Jones, 1992), and according to the Fick principle cardiac output (CO, the product of heart rate and stroke volume) increases in conditions of high oxygen demand such as heat stress (Willmer et al., 2005). Demonstrably, cardiac function of *N. celidotus* was altered during exposure to increasing temperatures (figure 2.3). Given that there was a limited increase in beat rate, changes were most likely augmented by alterations in stroke volume which is common for fishes (figure 2.3) (Farrell and Jones, 1992). The change in cardiac function was not a response to blood oxygen levels as haemoglobin saturation in vivo did not decrease with acute heat stress (figure 2.4). This is of great interest
because the primary cause of thermally-mediated HF in ectotherms has been generally subscribed to the decrease in oxygen solubility with temperature (Clark et al., 2008; Pörtner and Farrell, 2008; Pörtner and Knust, 2007; Pörtner et al., 2004). Such a decrease in blood oxygen content should lower mitochondrial oxygen supplies and limit OXP. Numerous studies have identified that the solubility of oxygen in water decreases with increasing environmental temperatures (Frederich and Pörtner, 2000; Pörtner, 2001; Pörtner and Farrell, 2008; Pörtner and Knust, 2007; Pörtner et al., 2004; Zielinski and Pörtner, 2000). However, oxygen diffusion rates increase in warm water (Verberk et al., 2011; Willmer et al., 2005), and no immediate decline in blood saturation that can be associated with hypoxia was found.

In general, glycogen and free glucose become the main metabolic fuels in stressed and hard-working vertebrate muscles including the heart. Increased plasma lactate and succinate, and decreased malate of thermally challenged *N. celidotus* indicate enhanced anaerobic metabolism, interrupted ETS, or mitochondrial disruption in this species (table 2.1). This conclusion is supported by an increase in LDH activities in heart tissues from fish exposed to acute heat stress (table 2.2). Elevated glycolytic capacities may represent attempts to offset increased ATP demands under heat stress (Feidantsis et al., 2009), and compensatory energetic responses to loss of ATP production have been observed in other studies on heat stressed ectotherms (Van Dijk et al., 1999). Furthermore, the accumulation of EAAs in experimental plasma could be attributable to increased protein catabolism to fuel metabolism in the presence of heat stress (Podrabsky et al., 2007). However, the accumulation of plasma glutamate, a NEAA that is a TCA cycle intermediate and neurotransmitter, may also be associated with a global decrease in mitochondrial oxidation with acute heat stress (table 2.1). Thus the altered patterns of metabolites in this study imply the onset of mitochondrial dysfunction for this species (table 2.1, 2.2).

### 2.4.2 Thermal sensitivity of cardiac mitochondria

#### 2.4.2.1 Mitochondrial function following acute heat stress in intact fish

Following acute heat stress exposure, *N. celidotus* hearts most likely face decreased OXP efficiencies supported by lower Leak-I and OXP-I fluxes in experimental animals, compared to controls (figure 2.5A). These changes were reflected by decreased RCRs in acutely temperature exposed fish (figure 2.5A insert). This can be caused by irreversible changes in mitochondrial inner membrane integrity with extreme heat stress which has been observed in
mammalian heart fibres, and this increases the permeability to ions, decreases membrane potential, and results in a decreased OXP efficiency (Brand and Nicholls, 2011; Žūkienė et al., 2010). The depression in RCR and relative increase in Leak-I may indicate a response to ROS, as production trended higher in heat stressed fibres in OXP states and was significantly higher in the chemically uncoupled ETS state (figure 2.5B). Mitochondrial uncoupling at high temperatures is thought to result from increased superoxide up-regulating uncoupling proteins (Murphy, 2009). However, the significantly higher production of ROS in the experimentally exposed heart fibres could also be attributed to damage at either CI or CIII (figure 2.5B) as these are the most commonly accepted sites of mitochondrial ROS production (Murphy, 2009).

2.4.2.2 Impacts of increasing in situ temperature on heart mitochondria

Overall, substantial changes to both the inner and outer mitochondrial membranes were evident at 25 °C before THF. Leak-I rates increased by 75% at 25 °C relative to those at 15 °C (figure 2.6E) indicating increased inner membrane permeability. Whereas OXP states were less temperature sensitive and this resulted in a measurable depression of the RCR (figure 2.7A) suggesting increased oxygen flux rates are required to maintain mitochondrial membrane potentials. An initial dose-dependent analysis indicated that RCR values were inhibited by 20 °C when increasing temperature was considered as an inhibitory dose. A further robust ANOVA analysis proved that this depression had started by 25 °C (figure 2.7A, black circles). These depressed RCR indices should decrease phosphorylation efficiencies and capacities (Brand, 1990; Iftikar et al., 2010; Seebacher et al., 2010). Moreover, the addition of NADH increased OXP-I, II flux prior to THF indicating increased permeability of the inner membrane to this otherwise impermeable molecule (Berg et al., 2002) (figure 2.7B).

The integrity of the outer mitochondrial membrane was also compromised above 20 °C as respiration following cytochrome c addition increased before THF (figure 2.7B). The loss of outer mitochondrial permeability leads to the release and depletion of Cyt c from mitochondria (Borutaite and Brown, 2003; Hand and Menze, 2008). A substantial loss of Cyt c clearly depresses OXP flux which can potentially affect superoxide scavenging (Mailer, 1990). At 25 °C this can prove problematic because ROS production increased as Leak-I respiration increased (figure 2.6A, 2.8A). A key producer of ROS is CI of the ETS and its involvement in cardiac failure has been previously studied (Hickey et al., 2009; Ide et al.,
Thus, despite an inferred decrease in membrane potential via increased Leak-I, ETS complexes remain reduced and can elevate ROS production (figure 2.7A, 2.8A).

OXP flux rates fuelled by CI and CII significantly increased up to an assay temperature of 27.5 °C (T_{HF}) and then plateaued until 30 °C (figure 2.6C). The plateau between 27.5 and 30 °C likely indicates a transition in cardiac mitochondrial function. A similar plateau was also apparent for CCO. Given that this assay tests a single component of the ETS, these data suggest the plateau observed in OXP-I, II may result from limits on CCO until 30 °C. A sudden increase in OXP-I, II and CCO rates was seen above 30 °C and this was responsible for the poor fits to exponential curves predicted by a standard Q_{10} relationship (figure 2.6D). CCO is assumed to be abundant or in excess in mitochondria because CCO flux capacity is in excess relative to that required for maximal OXP and ETS flux rates (Blier and Lemieux, 2001; Gnaiger and Kuznetsov, 2002; Lemieux et al., 2010b). In our study CCO flux was variable relative to OXP-I, II, yet declined relative to ETS flux (CCO/ETS, table 2.3) as temperature increased until 32.5 °C. Therefore, maximal ETS electron flux probably does not require support by the total catalytic capacity of CCO. CCO is however regulated \textit{in vivo} by molecules such as NO, H_{2}S, ATP and O_{2} (Boveris et al., 2000; Brown, 2001; Calvert et al., 2009; Gnaiger et al., 1998), consequently the full CCO flux as determined \textit{in vitro} may not be realised \textit{in vivo}. By 30 °C, CCO flux had only risen by 40% of the initial 15 °C flux (figure 2.6D, E). This can be caused by a relative decrease in CCO (table 3) which impairs oxygen binding capacities \textit{in vivo} (Gnaiger et al., 1998), in particular in the presence of regulator molecules. Notably, a proportionate decrease in CCO relative to OXP also occurs in other ectotherms species with increasing temperature (Hilton et al., 2010; Oellermann et al., 2012). In this study CCO flux was measured well above the T_{HF}, and by 32.5 °C, CCO flux had increased by 75% compared to values at 15 °C (figure 2.6E). This sudden rise in CCO flux suggests a transition in CCO function or a change in affinity for its substrates O_{2} or Cyt c (Hilton et al., 2010).

Decreases in the mitochondrial ATP production and in the transfer of energy through the phosphor-transfer kinases contribute to HF in mammalian cardiac muscle (Ventura-Clapier et al., 2004). This study directly tested ATP production from heart mitochondrial fibres with increasing temperature and found that ATP production decreased with increasing temperature.
(figure 2.10). At 25 °C ATP production was lower than synthesis rates at 20 °C, furthermore by 27.5 °C, ATP production rates dropped by 28% compared to rates at 20 °C (figure 2.10A). ATP demands will likely increase with rising temperatures due to increased demands on cardiac output and simple thermodynamic effects on ATPases in general. ATP/O values were already 18% lower at 25 °C, compared to values at 20 °C (figure 2.10B). This indicates that at 25 °C less ATP is made by cardiac mitochondria and they require more oxygen to do so prior to T_HF. These data are the first to directly show that the fish heart is limited by depressed mitochondrial ATP production as T_HF approaches.

2.4.2 Substrate utilisation following heat stress

Pyruvate is an important substrate for heart mitochondrial oxidation and this appears to be the case for *N. celidotus* (figure 2.9). The $K_m$ for pyruvate can be set by properties of the pyruvate transporter or of pyruvate dehydrogenase (PDH) and in ectotherms has been mainly examined in skeletal muscle mitochondria (Guderley et al., 1995). In this study, the $K_m$ values for pyruvate in the 20 – 30 °C range (~81 µM, figure 2.9A) are higher than values for isolated trout red and white skeletal muscle mitochondria (46 and 37 µM, respectively) (Blier and Guderley, 1993; Moyes et al., 1992b), rat heart mitochondria (~40 µM) (Lemieux et al., 2008), and considerably higher than values found in isolated carp red muscle mitochondria (< 5µM) (Moyes et al., 1992a). The affinities of *N. celidotus* mitochondria for pyruvate remained constant between 20 – 30 °C indicating flux rates will be maintained in vivo until 30 °C (figure 2.9A). However, at 32.5 °C $K_m$ values for pyruvate increased by ~20-fold to 1.52 ± 0.03 mM. Although this is similar to values obtained for goldfish muscle mitochondria (1.17 mM) (Mourik, 1983), these concentrations are considerably higher than the physiological intracellular range for pyruvate concentrations across phyla (Guderley et al., 1995).

A gradual increase in both the pyruvate $V_{max}$ and the $V_{max}/K_m$ ratio were observed in this study between 20 – 30 °C (figure 2.9B, C). But $V_{max}/K_m$ ratio was depressed by ~14-fold at 32.5 °C, compared to values at 20 °C (figure 2.9C). Assuming that the $V_{max}/K_m$ is analogous to the $k_{cat}/K_m$, 30 °C would represent the upper limit of mitochondrial respiration in vivo for *N. celidotus*. The sudden change in the mitochondrial affinity for pyruvate at 32.5 °C may again reflect a transitional change in the inner mitochondrial membrane (figure 2.9B) and perhaps is the same as that reflected in CCO flux rates (figure 2.6D). A more fluid inner
mitochondrial membrane can impair pyruvate transporter function and therefore potentially decrease pyruvate affinities (Lemieux et al., 2010b). In addition, given that pyruvate is imported electrogenically a thermally mediated loss of membrane potential may impair respiration at low pyruvate concentrations. This is consistent with the drop in ATP synthesis capacity and RCR values at higher temperatures. Lastly, all previous work exploring mitochondrial function in heat stressed mitochondria used substrates such as pyruvate at saturating concentrations and many studies showed function well above T_{HF} (Somero, 2002; Somero et al., 1996). Our data indicate that in this species mitochondria most likely cannot work above 30 °C as cytosolic concentrations of pyruvate will be too low.

### 2.4.3 Is the hot heart limited by mitochondria?

Biological systems are dependent on the efficiencies and stabilities of systems, and high respiration flux rates are futile if inadequate amounts of ATP are formed. Despite continued mitochondrial respiration at all states during exposure to temperatures far above the upper tolerance limit of *N. celidotus*, the lack of coincident ATP production (figure 2.10) reveals that maximal rates of mitochondrial respiration can be misleading. Thus, previous studies that found ectotherm mitochondrial respiration to be robust well above species T_{max} do not preclude the possibility of a mitochondrial role in thermally induced HF (Somero, 2002; Somero et al., 1996). In fact, given 1) the depression in ATP production and RCRs and 2) patterns of Cyt c and NADH changes, this study suggests that altered mitochondrial function and stability is a critical element in HF.

The deprivation of energy plays a major role in HF in the mammalian model. In this model metabolism of the heart is determined by substrate utilisation, oxidative phosphorylation and ATP transfer/utilisation of the mitochondria (Neubauer, 2007). Inadequacy or a break down in any or all of these components will contribute to HF. Since substrate utilisation by the mitochondria is affected after T_{HF} in *N. celidotus*, the cellular uptake of fuels to support oxidative phosphorylation is probably not disrupted enough to induce HF prior to T_{HF} (figure 2.9). In contrast, heat stress clearly depresses the production of ATP/energy via oxidative phosphorylation which is exacerbated by an increasingly leaky inner mitochondrial membrane and decreased uncoupling capacity (figure 2.6, 2.7, 2.10). Notably all mitochondrial components were studied at maximum oxygen saturation. Therefore changes observed are a direct impact of elevated temperature stress. Heat stress can further limit
contractile function of the heart due to inadequate ATP production leading to mechanical failure (Neubauer, 2007), but further investigation on ATP transfer/utilisation of cardiac mitochondria is needed. Clarifying these specific mechanisms that lead to HF should provide a powerful biomarker for predicting the impacts of temperature change on marine biodiversity.
3. CHAPTER 3 – THE ROLE OF CARDIAC MITOCHONDRIA IN HEAT STRESS INDUCED HEART FAILURE IN FISH SPECIES FROM DIFFERENT HABITATS.

Accepted as:

3.1 Introduction
Critical environmental stressors can affect the dynamics and distributions of fish populations (Booth et al., 2011; Sunday et al., 2012). Currently, ocean temperatures are influenced by climate change and drive changes in the distributions of fish populations and species (Sunday et al., 2011). As ocean temperatures rise, the thermal limitation of fishes is indicated by a decreased capacity in aerobic performance (Pörtner and Knust, 2007). An understanding of the thermal tolerance windows for fishes has been called for so that the effects of ocean warming on temperature related geographic distributions can be predicted (Pörtner, 2002). Therefore a comparative approach provides a powerful means to test physiological mechanisms responsible for differences in thermal tolerances among related taxa with different thermal niches (Somero, 2010; Somero, 2011).

The thermal tolerances of fishes and other ectotherms appear to be reflected by the temperatures at which their hearts fail ($T_{HF}$), and therefore the heart is contended to be the most temperature-sensitive organ in fish (Pörtner and Farrell, 2008; Pörtner and Knust, 2007). The scope of thermal tolerance has also been proposed to result from limitations imposed by mitochondrial function and densities (Pörtner, 2002). Alterations in mitochondrial function with temperature can contribute to trade-offs in energy budgets that in turn affect fish growth and fertility, and can ultimately influence population dynamics (Pörtner, 2002). However, some studies have indicated that the temperature at which liver and skeletal muscle mitochondrial respiration failed ($T_{mt}$) occurs above critical habitat temperatures (Pörtner, 2002; Pörtner et al., 2000; Somero et al., 1998; Weinstein and Somero, 1998). These works however focused on maximal respiration capacities in terms of flux, with
single electron inputs into respiratory chains, and did not use heart muscle or explore respirational efficiencies.

Recent work has demonstrated that $T_{mt}$ can occur below the $T_{HF}$ in heart muscle exposed to increasing environmental temperatures (Iftikar and Hickey, 2013). This work on the wrasse *Notolabrus celidotus* showed that, while increasing temperature elevated oxygen flux through respiring mitochondria, a greater fraction of the flux at high temperatures was to meet elevated inner mitochondrial membrane proton leak (proton leak represents non-phosphorylating respiration). This study was the first to directly measure ATP production simultaneously alongside respiration, and this declined at temperatures well below $T_{HF}$, indicating a loss of oxidative phosphorylation (OXP) system efficiency prior to $T_{HF}$ (Iftikar and Hickey, 2013). This study also showed that mitochondrial coupling – traditionally measured as the respiratory control ratio (RCR), provides a reasonable measure of the coupling between the electron transport (ETS) and OXP systems. Mitochondria from fish acclimated to 17.5°C showed significant depressions of ATP synthesis at 25°C. Additionally the non-OXP respiration fluxes with respiratory Complex I substrates (Leak-I) had increased by 60% relative to Leak-I at 17.5°C, while the RCRs decreased to less than 4, such that Leak-I accounted for 25% of OXP (Iftikar and Hickey, 2013). Therefore, cardiac mitochondria had lost considerable efficiency prior to $T_{HF}$.

The aims of the present study were to determine i) whether heart $T_{mt}$ occurs below $T_{HF}$ in other wrasse species from habitats with different temperatures; and ii) to explore differences in thermal responses among species. The family *Labridae* is a large group of wrasse species occupying reef habitats in both temperate and tropical waters (Cowman et al., 2009; Westneat and Alfaro, 2005). Two species were investigated; the cold temperate *Notolabrus fucicola* (Banded/Purple wrasse), which is most abundant in southern coastal waters of the South Island of New Zealand (winter and summer temperatures of 9°C and 13°C respectively) (Denny and Schiel, 2001; Denny and Schiel, 2002), and the tropical *Thalassoma lunare* (Lunar wrasse), which inhabits Indo-Pacific waters including North-Eastern Australian reefs (winter and summer temperatures of 25°C and 28°C respectively) (Ackerman, 2004; Randall et al., 1990). We also compared these two species to published data from *Notolabrus celidotus* which inhabits temperate reefs of the North Island, New Zealand (winter and summer temperatures of 15°C and 21°C respectively) (Ayling and Cox, 1982).
We first tested the $T_{HF}$ across all three wrasse species exposed to acute heat stress, and then
tested the influence of heat stress on the mitochondria within permeabilised cardiac fibres
from these fish (figure 3.1A, B). Mitochondrial function was further tested at a range of
temperatures across each species’ thermal range. Lastly, the capacity of heart mitochondria to
take up substrates with increasing temperatures was investigated (figure 3.1A). These data
were used to determine whether mitochondrial dysfunction contributes to HF in other related
fish species, and therefore restrict species distributions.

Figure 3.1: Diagram of the experimental protocol for this study. (A) The experimental design
illustrated as a flow chart depicting the three experimental series. (B) The SUIT assay as
described in Iftikar and Hickey (2013) measuring mitochondrial flux (pmol O$_2$. s$^{-1}$. mg$^{-1}$,
black line, left y-axis) and oxygen concentration (nmol. ml\(^{-1}\), dotted grey line, right y-axis) over time (mins). Titrations of mitochondrial substrates, poisons and inhibitors and their time of addition are shown with arrows, and the resulting respiratory state in parenthesis. Pyr [pyruvate], Mal [malate], Glu [glutamate], Cyt c [cytochrome-c], Succ [succinate], Atr [attractyloside], FCCP [carbonyl cyanide p-(trifluoromethoxy) phenyl-hydrazone], Rot [rotenone], Malo [malonate], Ant [antimycin-a], TMPD [N, N, N', N'-tetramethyl-p-phenylenediamine], Asc [ascorbate], Leak-I (state 2 respiration through CI in the absence of ADP), OXP-I (state 3 respiration), OXP-I, II (parallel electron transport from CI and CII), Leak-I, II (leak respiration flux rate through CI and CII), ETS (maximal flux of the electron transport system), CCO (activity of CIV, cytochrome c-oxidase).

### 3.2 Materials and methods

#### 3.2.1 Experimental animals

*N. fucicola* were collected by hook and line and held at the Portobello marine laboratory (University of Otago) on the Otago Peninsula of the South Island, New Zealand. Fish were housed at ambient temperatures (13.0 ± 1.0 °C) for 4 weeks. *N. celidotus* were collected and held according to Iftikar and Hickey (2013) at 18.0 ± 0.5 °C for 4 weeks. *T. lunare* were netted around Flinders reef, Queensland, Australia and held at Moreton Bay Research Station (University of Queensland) on North Stradbroke Island. Fish were maintained at 27.0 ± 0.5 °C for 4 weeks. All three species were fed daily but fasted for 24 hours before experimentation. All experiments were conducted according to the guidelines of Universities of Otago, Auckland, and Griffith animal ethics committees.

#### 3.2.2 Cardiac function protocol

The protocol of Iftikar and Hickey (2013) was followed for both *N. fucicola* and *T. lunare*. Water pumped into the buccal cavity induced atonic immobility (Wells et al., 2005) and fish were then held for 3 hours prior to experiments to dissipate any associated handling stress. Heart rates were measured non-invasively by foetal Doppler probes (Sonotrax B, Contact Medical Systems, China) to avoid anaesthetics. Probes were placed immediately above the heart of fish held supine in a submerged sponge within a 4 L plastic tank. This was further immersed in a larger 20 L water reservoir with a recirculating seawater system at each species holding temperature. The reservoir water was gradually increased by 1 °C every 10 minutes for heat stressed fish and the temperature was determined by placing a thermocouple (Digitech QM-1600) inside the fish’s mouth. Sonograms were measured after each
temperature was reached, for one minute \((N=6)\). The acute \(T_{HF}\) was determined as the heart rate became arrhythmic or failed. Control fish were held in parallel yet with no heat stress and for the same time duration \((N=6)\). Sonograms from control and heat stressed fish were recorded using Audacity 1.2.6 (http://audacity.sourceforge.net/) and analysed to determine heart rates. Heart rate sonogram data were normally distributed and analysed using a repeated measures analysis of variance (ANOVA) followed by a post hoc test (Tukey’s). Fish were euthanised by cephalic concussion and a blood sample was taken from the caudal vein and rapidly frozen at -80 °C for subsequent metabolomics analysis. The hearts were excised for mitochondrial respirometry (figure 3.1A).

### 3.2.3 Metabolite and enzymes analysis from heart tissue or plasma

Metabolites from plasma of control and experimental fish were extracted using -30 °C methanol (Villas-Bôas et al., 2003). Initially 20 µL of internal standard (10 mM solution of 2,3,3,3-d4 DL-alanine) was added to 100 µL of plasma, vortexed and frozen at -80 °C. Samples were then freeze dried (Virtis freeze dryer) and metabolites extracted by adding 500 µL cold methanol: water (1:1 v/v) at -30 °C. The solution was mixed vigorously for 1 minute and centrifuged at 4 °C for 5 minutes at 16,000 g. The supernatant was collected in a separate tube and the pellet was re-suspended and extracted a second time in 500 µL cold methanol: water (4:1 v/v). This second extracted supernatant was then pooled with the first extract. 5 mL of cold bi-distilled water (4 °C) was added to extracted plasma, frozen to -80 °C and freeze dried. Metabolites were chemically derivatised using methyl chloroformate and the samples were analysed by GC-MS with no modifications from the protocol of Villas-Bôas et al. (2003). The relative level of the metabolites in plasma was based on the base peak height as detected by gas chromatography. Values were normalised by the base peak height of the internal standard (d4-alanine). Metabolites in the plasma samples were identified using an in-house methyl chloroformate (MCF) MS library of derivatised metabolites. These contained MS spectra obtained from ultra-pure standards with the mass spectra saved and analysed in the AMDIS 2.65 software (www.amdis.net) (figure 3.1A).

Lactate, CS (an aerobic marker of mitochondrial content; Srere, 1969), and LDH (an anaerobic marker enzyme; Hochachka et al., 1983) in cardiac tissue from control and heat stressed fish were measured similar to Iftikar et al. (2010) with modifications from previous investigators (Hickey and Clements, 2003; Newsholme and Crabtree, 1986).
3.2.4 Mitochondrial respirometry

Three series of experiments were conducted for all fish species (figure 3.1A). A substrate-uncoupler-inhibitor titration (SUIT) protocol that investigates different components of the phosphorylation system was applied in experimental series 1 and 2, which differed only in assay temperatures (figure 3.1B). In series 1, permeabilised fibres from hearts of fish exposed in vivo to acute heat stress were tested. Permeabilised cardiac fibres were assayed close to the fish’s holding temperature where for N. fucicola, N. celidotus and T. lunare this was 12.5 °C, 20 °C and 27.5 °C respectively (figure 3.1A). In series 2, we determined the temperature at which cardiac mitochondrial function appeared to alter significantly (T_m). For N. fucicola mitochondrial function within cardiac fibres in situ was measured at 10 °C, 12.5 °C, 15 °C, 17.5 °C, 20 °C, 22.5 °C and 25 °C, for T. lunare at 20 °C, 25 °C, 27.5 °C, 30 °C, 32.5 °C, 35 °C and 37.5 °C, and previously collected data was used for N. celidotus measured at 15 °C, 17.5 °C, 20 °C, 25 °C, 27.5 °C, 30 °C and 32.5 °C (figure 3.1A) (Iftikar and Hickey, 2013).

3.2.4.1 Permeabilised fibre preparation

Fish hearts were rapidly dissected and immersed in 2 mL modified ice-cold relaxing buffer (BIOPS, 2.77 mM CaK₂EGTA, 7.23 mM K₂EGTA, 5.77 mM Na₂ATP, 6.56 mM MgCl₂·6H₂O, 20 mM taurine, 20 mM imidazole, 0.5 mM dithiothreitol, 50 mM K-MES, 15 mM Na-phosphocreatine and 50 mM Sucrose, pH 7.1). The hearts were then teased into fibre bundles and placed in 1 mL ice-cold BIOPS in a plastic culture plate. 50 µg. mL⁻¹ saponin was then added while the fibres were shaken on ice for 30 minutes. Fibres were rinsed three times for 10 minutes in 2 mL of modified mitochondrial respiratory medium (Fish-MiRO5, 0.5 mM EGTA, 3 mM MgCl₂·6H₂O, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH₂PO₄, 20 mM HEPES, 160 mM sucrose and 1g. L⁻¹ BSA, essentially free fatty acid, pH 7.24 at 20 °C; Gnaiger et al. 2000). The fibres were then blotted dry and weighed into 2 - 3 mg bundles for respiration assays (figure 3.1B). All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

3.2.4.2 The SUIT protocol

Oxygen was added into the gas phase above media prior to closing chambers to supersaturate Fish-MiRO5. Oxygen concentrations were maintained above 280 nmol. mL⁻¹ to ensure saturation and to maximise flux. Complex I (CI) substrates (2 mM malate, and 10 mM pyruvate) were added to measure state II respiration through CI in the absence of ADP
(denoted “Leak-I”). Excess ADP (2.5 mM) stimulated oxidative phosphorylation (OXP-I, state III respiration), and glutamate (10 mM) was added to saturate CI. Cytochrome c (10 µM) was added to test outer membrane integrity. Phosphorylating respiration with Complex I and II substrates (OXP-I, II) was measured by the addition of succinate (10 mM). NADH (0.5 mM) was then added to assess inner mitochondrial membrane damage. Leak respiration rates were also measured on combined CI and CII substrates by addition of atractyloside (750 µM, Leak-I, II), followed with repeated titrations of carbonyl cyanide p-(trifluoromethoxy)phenyl-hydrazone (FCCP, 0.5 µM) to uncouple mitochondria (denoted “ETS”). By the addition of rotenone (0.5 µM), malonate (15 mM) and antimycin a (1 µM), CI, II and III activities were inhibited respectively. Finally, the activity of cytochrome c oxidase (CCO, Complex IV) was measured by the addition of the electron donor couple \( N,N,N',N'-\text{tetramethyl-p-phenylenediamine} \) (TMPD, 0.5 mM) and ascorbate (2 mM) (figure 3.1B). All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

3.2.4.3 Substrate affinity and apparent K_m calculation
In series 3, the affinity of primary substrates in cardiac mitochondria with increasing assay temperature was tested (figure 3.1A). After preliminary tests, we found that OXP respiration of \( N. \ fucicola \) cardiac mitochondria was greater with malate than with pyruvate. The apparent K_m for malate was determined at 12.5 °C, 15 °C, 20 °C, 22.5 °C and 25 °C for \( N. \ fucicola \). For \( N. \ celidotus \) and \( T. \ lunare \) the main oxidative substrate was pyruvate. The apparent K_m for pyruvate was measured at 25 °C, 27.5 °C, 32.5 °C, 35 °C and 37.5 °C for \( T. \ lunare \) and 20 °C, 25 °C, 27.5 °C, 30 °C and 32.5 °C for \( N. \ celidotus \).

The capacity (apparent K_m, K_m app) of mitochondria within permeabilised fibres to take up the primary CI oxidative fuel was measured in the presence of ADP (2.5 mM). The respective substrate was titrated in at minute volumes by step-wise substrate additions using microinjection pumps (Oroboros Tip O2-K), until respiration flux was maximal (saturated). Michaelis-Menten curves were generated and substrate-saturation curve kinetics were applied to determine K_m app and V_max values using nonlinear regression.

3.2.5 Calculations and statistical analyses
A comparative metabolite profile was generated between the control and acutely heat stressed fish and data was analysed using R-software (Aggio et al., 2011). Differences in metabolite
profiles between control and heat stressed fish were compared using principal component analysis (PCA) and data were projected on a 2D plane (PCI vs. PC2). All mitochondrial respiration rates were expressed per mg wet weight of cardiac fibres. In mitochondrial respiration assays differences across temperatures, and between control and experimental fish, were evaluated with a one or two-factor ANOVA, followed by a post hoc test (Tukey’s). Respiratory control ratios (RCR-I) were calculated as OXP-I/Leak-I, and RCR-II was calculated as OXP-I, II/Leak-I, II. RCR-I and RCR-II were graphed as Arrhenius plots and segmented regression analysis was applied to determine Arrhenius break temperature (ABT). While the Arrhenius plot is intended for kinetic data, the purpose of the natural logarithm/reciprocal plot was to linearize exponential data and resolve changes in states such as those associated with thermodynamic transitions. Therefore, in this study, this analysis was applied to RCR-I and RCR-II. Uncoupled control ratios (UCRs) were calculated as ETS/OXP-I, II. The point at which $V_{\text{max}}/K_{\text{m app}}$ peaked was determined using non-linear regression peak analysis. The level of significance for all statistical tests was set at $p<0.05$. All statistical tests were run using SigmaPlot® version 12 (Systat Software, Inc., San Jose, California) unless stated otherwise.

3.3 Results

3.3.1 Cardiac function with acute heat stress

![Graph showing thermal tolerance profile for three fish species. T_{HF} is the mean temperature of heart failure (HF) for each species (N=6 ± s.d.). T_{tank} is the mean holding temperature of each species.](image)

**Figure 3.2:** Thermal tolerance profile for three fish species. T_{HF} is the mean temperature of heart failure (HF) for each species (N=6 ± s.d.). T_{tank} is the mean holding temperature of each species.
species prior to experiments \((N=6 \pm \text{s.d.})\). \(T_{\text{summer}}\) and \(T_{\text{winter}}\) are the average temperatures experienced by each species in the summer and winter, respectively. The temperature range between \(T_{\text{summer}}\) and \(T_{\text{winter}}\) is demarcated with a dashed box. (Insert) Cardiac scope is the difference between \(T_{HF}\) and \(T_{\text{tank}}\) \((N=6 \pm \text{s.e.m.})\). The asterisks indicate significant difference \((p \leq 0.05)\) in cardiac scope from other species.

The \(T_{HF}\) of cold adapted \(N. \text{fucicola}\) was 21.7 ± 0.3 °C, while \(T_{HF}\) of the tropical wrasse \(T. \text{lunare}\) was much higher at 32.8 ± 0.3 °C (figure 3.2). The \(T_{HF}\) for \(N. \text{celidotus}\) falls between these values at 27.8 ± 0.4 °C (Iftikar and Hickey, 2013). The cardiac scopes, i.e. the temperature differences between \(T_{HF}\) and the holding temperatures for \(N. \text{fucicola}\) and \(N. \text{celidotus}\) were 8.7 ± 0.3 °C and 9.1 ± 0.4 °C respectively (figure 3.2 insert). In contrast, the cardiac scope was limited to only 5.8 ± 0.2 °C in \(T. \text{lunare}\) (figure 3.2 insert).

### 3.3.2 Enzyme and metabolite profiles from heart and plasma

Citrate synthase (CS) activities were similar for heart tissues of all three wrasse species (table 3.1), indicating similar mitochondrial volumes assuming that CS is constant across species. The anaerobic glycolytic enzyme marker LDH increased activity with acute heat stress in \(N. \text{fucicola}\) and \(N. \text{celidotus}\), but not in \(T. \text{lunare}\) (table 3.1). Acute temperature exposure significantly elevated lactate in heart tissue of both \(N. \text{fucicola}\) and \(T. \text{lunare}\), but not \(N. \text{celidotus}\) \((p=0.07\), (Iftikar and Hickey, 2013)) (table 3.1).

#### Table 3.1: Metabolite concentrations and enzyme activities in heart tissue of \(N. \text{fucicola}\), \(N. \text{celidotus}\) (Iftikar and Hickey, 2013) and \(T. \text{lunare}\) \((N=6 \pm \text{s.e.m.})\). The asterisks indicate significant differences between control and acutely heat stressed fishes \((p \leq 0.05)\).

<table>
<thead>
<tr>
<th></th>
<th>(N. \text{fucicola})</th>
<th>(N. \text{celidotus})</th>
<th>(T. \text{lunare})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate Synthase</td>
<td></td>
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</tr>
<tr>
<td>(µmol. min(^{-1}). mg(^{-1}))</td>
<td>control</td>
<td>experimental</td>
<td>control</td>
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<tr>
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<td>80.26 ± 8.14</td>
<td>80.38 ± 20.88</td>
<td>126.27 ± 43.76</td>
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<tr>
<td>Lactate</td>
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<tr>
<td>(µmol. g(^{-1}))</td>
<td>control</td>
<td>experimental</td>
<td>control</td>
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<tr>
<td></td>
<td>96.64 ± 14.61</td>
<td>142.29 ± 16.28*</td>
<td>106.61 ± 18.46</td>
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<tr>
<td></td>
<td>(p=0.07)</td>
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<tr>
<td>Lactate Dehydrogenase</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>(µmol. min(^{-1}). mg(^{-1}))</td>
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<td>experimental</td>
<td>control</td>
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<tr>
<td></td>
<td>1086.08 ± 98.42</td>
<td>1382.46 ± 64.10*</td>
<td>1050.11 ± 92.75</td>
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</tbody>
</table>

61
Figure 3.3: Principal Component Analysis (PCA) projection of metabolite profiles for control and heat stressed fish. Data for *N. fucicola* (light grey), *N. celidotus* (Ifikar and Hickey, 2013) (dark grey) and *T. lunare* (black) were projected onto a 2D space and metabolic profiles were generated from mean metabolites that had statistically significant ($p \leq 0.05$) accumulation ($N=6$ per treatment per species).
Two-dimensional PCA showed the largest separation between metabolic profiles of control and acutely heat stressed \textit{T. lunare} (figure 3.3), inferring that heat stress had impacted the intermediary metabolism of this species the most. In contrast, less separation was apparent in \textit{N. fucicola} relative to \textit{N. celidotus} and \textit{T. lunare} (figure 3.3). When metabolite changes were compared across species, lactate accumulation increased in heat stressed \textit{N. celidotus} and \textit{T. lunare} plasma but not \textit{N. fucicola} (table 3.2). Exposure to acute heat stress also induced significant changes in some TCA cycle intermediates in the blood. Succinate was elevated in all three species, while citrate and cis-aconitate accumulation was elevated only in \textit{T. lunare} (table 3.2).

**Table 3.2:** Accumulation of glycolytic and TCA cycle intermediates in control and acutely heat stressed fish plasma for \textit{N. fucicola}, \textit{N. celidotus} (Ifitikar and Hickey, 2013) and \textit{T. lunare} (\(N=6\)). Arrows indicate the increase/decrease in intermediate accumulation in the plasma from heat stressed fish compared to control fish, and the fold change is given in the adjacent column. The asterisks denote that accumulation is significant in plasma from heat stressed fish (\(p \leq 0.05\)).

<table>
<thead>
<tr>
<th></th>
<th>\textit{N. fucicola}</th>
<th>\textit{N. celidotus}</th>
<th>\textit{T. lunare}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>increase/decrease</td>
<td>fold change</td>
<td>increase/decrease</td>
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<tr>
<td><strong>Glycolytic intermediates</strong></td>
<td></td>
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<tr>
<td>Lactate</td>
<td>↑</td>
<td>1.22</td>
<td>↑</td>
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<tr>
<td><strong>TCA cycle intermediates</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Succinate</td>
<td>↑</td>
<td>1.76*</td>
<td>↑</td>
</tr>
<tr>
<td>Citrate</td>
<td>↓</td>
<td>0.98</td>
<td>↑</td>
</tr>
<tr>
<td>Cis-aconitate</td>
<td>↓</td>
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<td>↑</td>
</tr>
</tbody>
</table>

### 3.3.3 Mitochondrial function

#### 3.3.3.1 Series 1: \textit{In vivo} acute heat stress exposure

Acute heat stress lowered RCR-I values in heat exposed fish (figure 3.4A). OXP-I, II respiration (state III) was not impacted by acute heat stress in any of the three species (figure 3.4B), but acutely heat stressed \textit{N. fucicola} had lower ETS and CCO fluxes relative to control fish (figure 3.4C, D).
Figure 3.4: Cardiac mitochondrial function of three fish species exposed to *in vivo* acute heat stress. (a) Respiratory control ratios (RCR-I) determined as OXP-I/Leak-I. (b) OXP-I, II rates. (c) Uncoupled (ETS) rates. (d) Cytochrome *c* oxidase (CCO) rates. White bars are mean data for control fish (*N*=6 ± s.e.m.). Grey bars are mean data for heat stressed fish (*N*=6 ± s.e.m.). The asterisks indicate a significant difference (*p*≤0.05) between control and heat stressed fish for each species.

### 3.3.3.2 Series 2: Impact of increase in *in situ* assay temperature

Although all species increased Leak-I flux with increasing temperature, *T. lunare* showed the greatest elevation in flux (figure 3.5A). *N. fucicola* had the least increase in OXP-I, while *N. celidotus* and *T. lunare* showed similar increases (figure 3.5B). *N. celidotus* had the largest elevation in OXP-I, II flux as assay temperatures increased (figure 3.5C) (Iftikar and Hickey, 2013). Similar to OXP-I, *N. fucicola* had the highest OXP-I, II rates for a given assay temperature compared to *N. celidotus* and *T. lunare*. Arrhenius plots for RCRs indicated different thermal relationships for the RCR-II than the RCR-I because ABTs for RCR-II were found in two species, *N. celidotus* (21.8 ± 1.7 °C) and *T. lunare* (28.5 ± 1.3 °C) (figure 3.5D). These ABTs were close to holding temperatures and below their respective *T* _HF_. Only *N. celidotus* showed an ABT for RCR-I at 24.6 ± 2.8 °C below their *T* _HF_ (figure 3.5D).
Figure 3.5: Cardiac mitochondrial function of three fish species exposed to increasing *in situ* assay temperatures. (a) Leak-I rates. (b) OXP-I rates. (c) OXP-I, II rates. (d) Arrhenius plot depicting respiratory control ratios; RCR-I determined as OXP-I/Leak-I and RCR-II determined as OXP-I, II/Leak-I, II. Solid lines and dashed lines represent linear/non-linear regression fitted for RCR-I and RCR-II, respectively. For all mean rates and percentages (*N*=6 ± s.e.m.) within a species, letters of the same case and style are not significantly different (*p*≥0.05) from one another.

Arrhenius plots for CI respiration showed a greater response to increasing temperature compared to CII respiration in *N. celidotus* and *T. lunare* (figure 3.6B, C), as reflected by steeper slopes, significant in *T. lunare* (-7.7 ± 1.7 CI vs. -3.1 ± 1.3 CII) (figure 3.6C). On comparison, CI respiration of *N. fucicola* (-4.0 ± 0.7) responded less to temperature compared to CII respiration (-5.1 ± 1.4) (figure 3.6A). However, CCO respiration had the greatest temperature driven response in *N. fucicola* (-7.1 ± 0.8, figure 3.6A) relative to *N. celidotus* (-6.0 ± 0.7, figure 3.6B) and *T. lunare* (-5.1± 1.1, figure 3.6C).
Figure 3.6: Arrhenius plots depicting respiration of mitochondrial complexes. CI respiration (calculated as OXP-I – Leak-I), CII respiration (calculated as OXP-I, II – OXP-I), CCO respiration (cytochrome c oxidase, CIV) of permeabilised cardiac fibres from (a) *N.* *fucicola*, (b) *N.* *celidotus* (*Iftikar and Hickey, 2013*), and (c) *T.* *lunare*. Linear regression was fitted to data and respective slopes ± s.e.m. are given within the graph. Statistical analysis comparing mean slopes for CI and CCO respiration across fish species show significant differences (*p*≤0.05) with upper case letters. Mean slopes for CII respiration across fish species showed no significant difference. The asterisks indicate significant different (*p*≤0.05) between the mean slopes for CI and CII respiration within a species. The *E_α* for CCO respiration was calculated as 18.9 ± 1.1 kJ/mol for *N.* *fucicola*, 14.5 ± 2.0 kJ/mol for *N.* *celidotus*, and 13.2 ± 2.2 kJ/mol for *T.* *lunare*. 
Figure 3.7: Mitochondrial respiration of uncoupled permeabilised cardiac fibres from three fish species exposed to increasing in situ assay temperatures. (a) ETS rates. (b) Uncoupled control ratios (UCR) determined as ETS/OXP-I, II. The dotted line at 1 represents when ETS reserve capacity = OXP. (Insert) FCCP concentration required to uncouple cardiac mitochondria. For all mean rates and ratios (N=6 ± s.e.m.) within a species, letters of the same case and style are not significantly different (p≥0.05) from one another.
**N. fucicola** had the greatest fractional capacity to chemically uncouple with FCCP, and increased flux by ~57% at 25 °C relative to rates at the holding temperature (T_{tank}) (figure 3.7A). However, this required a higher FCCP concentration (1.58 ± 0.15 µM, figure 3.7B insert). Additionally, **N. fucicola** maintained UCRs above a value of 1 at all assay temperatures (figure 3.7B) indicating a greater ETS reserve capacity, or stability compared to OXP. **N. celidotus** had also maximised ETS rates by 32.5 °C (figure 3.7A), although were more sensitive to FCCP, requiring only half (0.81 ± 0.07 µM) the concentration used by **N. fucicola** (figure 3.7B insert). **T. lunare** had the lowest uncoupling capacity, requiring on average 0.5 µM of FCCP (figure 3.7B insert) to achieve a maximal ~45% uncoupling compared to rates at holding temperature (figure 3.7A). Moreover, UCR values of **N. celidotus** and **T. lunare** were significantly depressed below their T_{HF} at 25 °C and 27.5 °C, respectively (figure 3.7B). In **T. lunare**, from 27.5 °C upwards UCRs were equal to or below a value of 1, indicating that the total ETS reserve capacity was required to support maximal OXP.

### 3.3.3.3 Series 3: CI substrate affinity with increasing *in situ* assay temperature

OXP respiration of **N. celidotus** and **T. lunare** cardiac mitochondria preferred pyruvate as its main CI substrate, compared to cardiac mitochondria of **N. fucicola** that preferred malate as the main CI substrate. The K_m^app for malate in **N. fucicola** did not change with increasing temperature (figure 3.8A). In **N. celidotus**, the K_m^app for pyruvate remained unchanged up to 30 °C and then increased at 32.5 °C by 19-fold (Iftikar and Hickey, 2013). Similarly, in **T. lunare** K_m^app pyruvate was 3-fold higher at 37.5 °C (figure 3.8A). V_max rates for **N. fucicola** were unaffected by temperature, while they increased with temperature for **N. celidotus** (figure 3.8B). **T. lunare** increased V_max up to 32.5 °C and then decreased at 37.5 °C to rates equivalent to initial rates. Apparent substrate oxidation efficiencies as determined by the V_max/K_m^app ratio (as a proxy for the traditional measure of enzyme efficiency of k_cat/K_M) indicated that for **N. celidotus** and **T. lunare** were depressed at their maximal assay temperatures (figure 3.8C). But predicted optimal temperatures for substrate oxidation were below their T_{HF} at 26.6 ± 1.0 °C and 30.6 ± 0.7 °C, respectively. V_max/K_m^app ratios remained unaffected by heat stress in **N. fucicola** (figure 3.8C), but it should be noted that fibres of this species were oxidising malate.
Figure 3.8: Substrate kinetics for three fish species with increasing assay temperatures. (A) The apparent affinity (K_m app) to primary CI substrate specified in the figure legend. The asterisks indicate within a species the K_m app is significantly different (p≤0.05). (B) V_max rates. (C) K_m app/V_max ratio indicative of enzyme efficiency. Solid and dashed lines represent T_HF and optimal enzyme efficiency temperature determined by non-linear regression (parabola), respectively. For all rates and ratios (N=6 ± s.e.m.) within a species, letters of the same case and style are not significantly (p≥0.05) different from one another.


3.4 Discussion

The present study indicates that while mechanisms differ, some form of cardiac mitochondrial dysfunction occurs below $T_{HF}$, and that species from narrow ranging thermal habitats were more susceptible to heat stress. The tropical *T. lunare* was most greatly impacted by acute heat stress with a limited ability to increase cardiac scope (figure 3.2), and showed the greatest shifts in plasma metabolite profiles (figure 3.3). The TCA cycle intermediate succinate was most elevated in heat stressed *T. lunare*, indicating a more severe mitochondrial insufficiency in this species (table 3.2). The RCR-II was also altered substantially just above $T_{tank}$ for *T. lunare* and *N. celidotus* indicating that OXP was compromised below $T_{HF}$ (figure 3.5E). Furthermore, the tropical *T. lunare* showed limited CII activity (figure 3.6C), and a decreasing ETS reserve capacity with rising temperature, depicted by the inability of their cardiac mitochondria to uncouple with increasing assay temperatures (figure 3.7A, B). Cardiac mitochondria from acutely in vivo heat stressed *N. fucicola* had depressed RCR-I, ETS and CCO fluxes, relative to control fish (figure 3.4A, C, D). In addition, when cardiac fibres from *N. fucicola* were assayed across temperatures (in situ), CI respiration showed a diminished response (figure 3.6A). Overall, this study confirmed that $T_{mt}$ occurs below $T_{HF}$ for *N. fucicola* and *T. lunare* that inhabit relatively stable thermal environments.

3.4.1 The impact of acute heat stress on the wrasse heart

The tropical wrasse *T. lunare* had the lowest scope to increase heart rate relative to holding temperature (figure 3.2). Such a narrow thermally influenced cardiac scope has been established previously in warm adapted ectotherms (Stillman and Somero, 1996; Stillman and Somero, 2000; Vernberg and Tashian, 1959), where the ABT for heart rate in warm-adapted intertidal porcelain crabs was close to the maximal microhabitat temperatures (Stillman and Somero, 1996). Therefore despite higher absolute thermal tolerances, under heat stress warm adapted species have lower cardiac scopes compared to temperate relatives (Stillman and Somero, 2000). PCA analysis also confirmed that in vivo heat stress caused the greatest change in the plasma metabolite profile of *T. lunare* relative to the colder wrasse species (figure 3.3). Specifically, the increase in all TCA cycle intermediates, in particular succinate in acutely heated *T. lunare* plasma indicates that this species must increase its anaerobic dependence with heat stress (table 3.2). The plasma membrane is considered to be impermeable to succinate (Stadlmann et al., 2006). The accumulation of succinate in fish
blood likely indicates mitochondrial disruption at CII, also known as succinate dehydrogenase (SDH) (Grieshaber et al., 1994). Data presented here provides support that the tropical *T. lunare* is closer to HF and is more metabolically limited than its temperate equivalents *N. fucicola* and *N. celidotus*, and as such would be the most susceptible of the three species to rising temperatures given predictions of global warming.

3.4.2 The impact of heat stress on mitochondrial bioenergetics

The RCR provides an indication of mitochondrial efficiency as the constituents used to calculate this ratio are dependent on the components of OXP being intact (Brand and Nicholls, 2011). It is known that *in vitro* mild heat stress will depress OXP coupling and that extreme heat stress will cause irreversible changes in mitochondrial inner membrane integrity in the mammalian heart (Žūkienė et al., 2007). This acts through increased inner mitochondrial membrane proton leakage, and therefore decreased OXP efficiency. Here it resulted in a lowering of the RCR-I in all three heat stressed species when assayed *in vitro* (figure 3.4A).

When tested at various temperatures, RCR-I was most affected in *N. celidotus* (figure 3.5D). Arrhenius plot analysis revealed a breakpoint in the RCR-I for *N. celidotus* at temperatures below T_{HF}, suggesting a lowered OXP efficiency in this species (Hilton et al., 2010). High temperatures are known to increase the inner membrane proton leak while either decreasing membrane potential and, often coinciding with escalating oxygen flux that does not contribute to ATP production (Žūkienė et al., 2007). This depresses OXP capacity in rat heart mitochondria (Žūkienė et al., 2007), and substantially depresses ATP production by 25 °C, below T_{HF} in *N. celidotus* (Iftikar and Hickey, 2013).

Increasing assay temperature impacted the RCR-II of *N. celidotus* and *T. lunare* the most (figure 3.5D). Arrhenius breakpoints occurred at 21.8 ± 1.7 °C for *N. celidotus*, and 28.5 ± 1.3 °C for *T. lunare*, which was just above their respective T_{tank}. As the RCR-II is determined from OXP-I, II, it putatively more closely reflects mitochondrial OXP and its efficiencies *in vivo*. Part of CII is succinate dehydrogenase (SDH), which is reported to be sensitive to heat stress in ectotherm mitochondria (O'Brien et al., 1991). This perhaps explains the more prominent inflexion in the RCR-II and decreased responsiveness in CII flux with increasing temperature in *N. celidotus* and significantly so in *T. lunare* relative to CI fluxes (see slopes
CHAPTER 3

figure 3.6B, C). This may also explain the accumulation of plasma succinate, as inadequate SDH capacity will promote succinate release into the blood (table 3.2), indicating a relative insufficiency of CII in *T. lunare* cardiac mitochondria at temperatures prior to HF.

CCO had a greater overall flux, or excess capacity compared to CI and CII respiration in all three species (figure 3.6A-C), and this is common for ectotherms (Blier and Lemieux, 2001; Dahlhoff and Somero, 1993). CCO is an indicator of maximal aerobic capacity and the activity of CCO in ectotherms is typically highest in the heart because of its high metabolic demands (Ludwig et al., 2001). While warm acclimation has been shown to increase CCO activity in fish hearts (Cai and Adelman, 1990; Foster et al., 1993), the thermal sensitivity of CCO was greatest in the colder *N. fucicola* (figure 3.6A), compared to the tropical *T. lunare* (figure 3.6C). The decreased thermal response of *T. lunare* CCO relative to *N. fucicola* and *N. celidotus* may limit ETS flux as temperatures increase. This may also explain the greater overall CCO capacities in the tropical species.

Both *N. fucicola* and *N. celidotus* showed more robust ETS respiration with greater capacities to uncouple mitochondria with increasing assay temperatures (figure 3.7A). The cold adapted *N. fucicola* also needed higher FCCP concentrations for similar assay temperatures to *N. celidotus* (figure 3.7B insert), indicating a tightly-coupled ETS in *N. fucicola*, or differences in FCCP mobility and inner membrane composition (Gnaiger et al., 2000). This was also reflected in the UCR observed for *N. fucicola* that remained unaffected as assay temperatures increased (figure 3.7B). On comparison both *N. celidotus* and *T. lunare* had significantly depressed UCRs at temperatures well below their *T_{HF}* indicating either less coupled OXP systems or less ETS reserve capacity. *T. lunare* in particular had UCRs at or below the value of 1 (figure 3.7B), signifying a loss in ETS reserve capacity. This also indicates a loss of capacity to generate mitochondrial membrane potentials with increasing temperatures. We note that uncouplers such as FCCP can depress ETS respiration in fish heart mitochondria (Hilton et al., 2010). This may result from effects on the electrogenic importation of substrates (e.g. pyruvate, which requires a membrane potential).

High mitochondrial respirational flux in most animals is fuelled by pyruvate followed by glutamate (Johnston et al., 1994; Lemieux et al., 2008; Moyes et al., 1990). The preferential use of malate for cardiac mitochondrial respiration in *N. fucicola* can perhaps be attributed to
an abundance of malic enzyme activity. Malate supports cardiac mitochondrial respiration in cold adapted teleosts (Skorkowski et al., 1984; Skorkowski et al., 1985) and flux could be attributed to mitochondrial malic enzyme (Skorkowski, 1988), which converts malate to pyruvate. Therefore pyruvate oxidation is still potentially driving OXP-I flux, as it can be derived from malic enzyme. However, heat stress did not impact malate uptake by heart mitochondria from *N. fucicola* for all assay temperatures measured (figure 3.8A), indicating that the malate-aspartate shuttle and subsequent down-stream enzymes are thermostable in this temperature range. In contrast, high temperatures substantially decreased the affinity for pyruvate uptake in *N. celidotus* and *T. lunare* at their respective maximal assay temperatures of 32.5 °C and 37.5 °C (figure 3.8A) (Iftikar and Hickey, 2013). Although these temperature maxima are above the T_HF for both species, the peak temperature for enzyme efficiency derived from the ratio of $V_{max}/K_m$ app pyruvate were below T_HF at 26.6 ± 1.0 °C and 30.6 ± 0.7 °C, respectively (figure 3.8C). Subsequently, substrate transport or turnover efficiency is lost prior to HF in *N. celidotus* and *T. lunare*.

### 3.4.3 Is habitat a limiting factor in a changing climate?

The upper thermal limits on aerobic scope were postulated to govern fish species distributions, as the aerobic scope of fishes declines with increasing temperature (Pörtner and Farrell, 2008; Pörtner and Knust, 2007). It has been contended that this loss of aerobic scope coincides with apparent decreases in tissue oxygen supplies and T_HF (Farrell et al., 2009; Pörtner and Farrell, 2008; Pörtner and Peck, 2010; Wang and Overgaard, 2007). However, recent reappraisals indicate that thermal limitations on aerobic scope occur at temperatures above those constraining other crucial parameters, such as fish growth, reproduction and locomotion (Clark et al., 2013; Gräns et al., 2014; Healy and Schulte, 2012). Moreover, oxygen supply may still be adequate where these parameters fail, and potentially at and above temperatures where aerobic scope fails (Gräns et al., 2014; Iftikar and Hickey, 2013).

Our work shows that while mechanisms vary among species, cardiac mitochondrial function is likely to also be disrupted at temperatures below T_HF for these wrasse species, and may therefore contribute significantly to each species thermal habitat range. The cardiac mitochondria of the cold temperate *N. fucicola* appears to be less stable under *in vivo* thermal stress, while cardiac mitochondria of temperate *N. celidotus* and tropical *T. lunare* showed rapid losses in OXP efficiencies (RCR-I, II) above $T_{acclimation}$. *T. lunare* also has the narrowest...
thermal window for heart function, with decreased ETS reserve capacity, and a lowered mitochondrial substrate affinity that coincides with $T_{HF}$. Additionally, CII and CCO appear to be most limited in terms of thermal plasticity in *T. lunare*. Importantly, these conditions manifest at saturating oxygen concentrations indicating that cardiac mitochondria can become impaired without invoking oxygen limitation. Our data conclude that heat stress can mediate cardiac mitochondrial insufficiency in *N. fucicola, N. celidotus* and *T. lunare*, and therefore cardiac mitochondria can provide insight to species thermal limits. Understanding mitochondrial function, or dysfunction in ectotherms such as fish, still requires study across a greater range of species to better understand the potential ramifications of climate change.
CHAPTER 4 – THE CONTRIBUTING ROLE OF CARDIAC MITOCHONDRIAL DYSFUNCTION TO HEART FAILURE IN *NOTOLABRUS CELIDOTUS* ACCLIMATED TO SEASONAL TEMPERATURES.

Submitted as:


4.1 Introduction

Fish species that occupy temperate ocean zones face large seasonal fluctuations in temperature and acclimating physiological and cellular functions to these changing conditions often incur a cost (Caldwell and Vernberg, 1970; Cossins and Prosser, 1978; Prosser, 1967). When exposed to winter and summer temperatures, temperate fishes have to compensate for the decelerating and accelerating effects of temperature on maintaining the functional balance between ATP formation and ATP demand (Lucassen et al., 2003). How fishes achieve control over mitochondrial metabolism at seasonal temperature extremes is yet to be clearly established, and this will impact their spatial distributions (Pörtner and Farrell, 2008; Pörtner and Knust, 2007; Somero, 2002; Somero, 2011).

Apparent changes in the abundance and distributions of some fish populations have been attributed to warming oceans, and the proximal cause of these changes has been attributed to metabolic limitations, in particular on cardiac function (Pörtner and Knust, 2007). The hearts of fishes appear to be extremely sensitive to heat stress, and may determine the upper thermal limit of most fishes due to limitations on cardiac output (the product of heart rate and stroke volume) (Farrell, 1997; Harper et al., 1995; Pörtner and Knust, 2007; Pörtner et al., 2004; Shiels et al., 2002). The heart is highly dependent on sustained aerobic ATP production by mitochondrial oxidative phosphorylation (OXP), and can contribute to heart failure (HF) at elevated temperatures (Hilton et al., 2010; Iftikar and Hickey, 2013; Rodnick et al., 2014; Strobel et al., 2013). Given the current concerns for increasing environmental temperatures
(Solomon et al., 2007), it is vital to understand cardiac energy metabolism and how plastic cardiac mitochondrial function are in fish acclimated to their seasonal temperatures.

Previous studies on the plasticity of mitochondrial function in fishes acclimated to seasonal temperatures have mainly explored skeletal muscle mitochondria due it’s important role in locomotion (Guderley, 1990; Guderley and Johnston, 1996; Guderley et al., 1997; Guderley and St-Pierre, 2002). Muscle mitochondria in cold-acclimated fish displayed higher OXP rates/oxidative capacities per milligram of mitochondrial protein compared to warm acclimated fishes (Guderley and Johnston, 1996; St-Pierre et al., 1998). This increased oxidative capacity has been mirrored by increased activities of enzymes in the electron transport system (ETS) such as cytochrome c oxidase (CCO) (van den Thillart and Modderkolk, 1978; Wodtke, 1981) and succinate dehydrogenase (SDH) (Hazel, 1972). In addition, the respiratory control ratios (RCR, an indicator of the coupling between the ETS and OXP systems) of rainbow trout skeletal muscle mitochondria, increased with cold acclimation and decreased with warm acclimation (Bouchard and Guderley, 2003). Warm acclimated trout also lowered OXP rates after six weeks of acclimation compared to initial OXP rates at week 0, indicating alterations in mitochondrial capacity, which likely decrease OXP efficiencies are instigated by warm acclimation.

Recent work using a temperate teleost model has reported that the temperature at which cardiac mitochondria fail ($T_{mv}$) through a loss of OXP, occurs at a temperature below the temperature at which the heart fails ($T_{HF}$) (Iftikar and Hickey, 2013). The most obvious loss of function with heat stress was an increase in the proton leak rate within the inner mitochondrial membrane as reflected by elevated respiration in the non-OXP state (termed “Leak” representing non phosphorylating respiration). Mitochondria were also shown to have a lowered capacity to uncouple chemically at higher temperatures, indicating that the residual or reserve capacity of the electron transport system (ETS) was diminished (Iftikar and Hickey, 2013). Most importantly absolute ATP synthesis rate and therefore, the ATP synthesis relative to oxygen flux decreased with rising temperature, to the extent that aerobic ATP synthesis rates were inadequate at temperatures before the heart failed (Iftikar and Hickey, 2013). These data collectively indicate that heart mitochondrial dysfunction can play an important role in HF of heat stressed fish (Iftikar and Hickey, 2013). However, it remains unclear how cardiac mitochondrial ETS and OXP alters at upper and lower limits of a
temperate fish’s natural temperature range and how the components of the respiration system may adjust with temperature acclimation.

This study explores the acclimation capacity of cardiac mitochondria in a temperate eurythermal and non-migratory wrasse species, *Notolabrus celidotus* (Spotty). This species is exposed to significant seasonal and daily temperature fluctuations and has considerable adaptive capacity (Jones, 1980; Jones, 1984). *N. celidotus* were acclimated to their average winter (15 °C, cold acclimated, CA), and summer (21 °C, warm acclimated, WA) sea surface temperatures (Atkins, 2013). This chapter aimed to compare changes in ETS and OXP in CA and WA fish in terms of how mitochondrial properties respond to acute temperature changes, and how these related to T_{HF}. The specific goals were; 1) determine the variation in T_{HF} in acclimated fish, 2) ascertain the different heart mitochondrial states and understand if breakpoints in these states occur before HF, 3) determine ROS production and substrate affinity of cardiac mitochondria with increasing temperature, 4) and to test whether T_{mt} occurs before or after T_{HF}, and if acclimation allows mitochondrial plasticity at mean seasonal temperatures. It was hypothesised that cardiac mitochondria from WA *N. celidotus* will have a lower oxidative capacity compared to CA *N. celidotus* and that in WA fish, cardiac mitochondrial dysfunction can contribute to HF.

### 4.2 Materials and methods

#### 4.2.1 Experimental animals and acclimation protocol

Fish were caught by line and baited hook from piers surrounding Ti Point (36°19’2” S, 174°47’4”E, North Eastern New Zealand). Following capture, fish were held in aerated polybins before transport to the Leigh Marine Laboratory, University of Auckland. Fish were housed in six insulated 36L tanks with 200 μm filtered, flow-through seawater at 15 °C for two weeks prior to acclimation. For acclimation, two temperature treatments (three tanks per temperature) were chosen to expose fish to either a mean winter temperature of 15 ± 0.2 °C, or a mean summer temperature of 21 ± 0.1 °C. Once acclimation temperatures were reached, fish were held at their prescribed temperatures for six weeks. The responses of whole-animal performance traits by acclimation in fish can be observed by 4 – 6 weeks of exposure to the new external condition (Condon et al., 2010; Hammill et al., 2004; Johnston and Lucking, 1978; Wilson et al., 2007). Additionally, mitochondrial oxidative capacities were altered
within two weeks of warm and cold acclimation (Bouchard and Guderley, 2003). Therefore, the six week seasonal temperature exposure duration used in this study was believed to have led to complete acclimation of *N. celidotus*.

Flow rates throughout holding provided a complete exchange of water every 3 hours (i.e. ~36 L. h⁻¹). Dissolved oxygen levels were inspected frequently (3 - 5 times per week) and maintained at 85 - 90% saturation, and water quality was checked weekly (API Saltwater test kit, Mars Fishcare, PA, USA). A thermistor positioned in each holding tank enabled continuous measurement of the temperature profile that could then be viewed in custom designed software (In house design – Leigh Marine Lab). Fish were exposed to a constant 12L: 12D light cycle and fed fresh mussel every two days. All experiments and procedures met with the ethical requirements of the University of Auckland, New Zealand (NZ) (Approval AEC/04/2009/R720 Fish).

4.2.2 Measuring cardiac function

Cardiac function was measured non-invasively using a foetal Doppler probe (Sonotrax B, Contact Medical Systems, China) described previously (Iftikar and Hickey, 2013; Iftikar et al., 2014). Briefly, fish were snugly held supine within a submerged slotted sponge in a re-circulating system. A small aquarium pump (Rio®, mini 150, Taipei, Taiwan) was used to pump water into the buccal cavity of the fish and this induced an atonic immobility as described by Wells et al (2005). The Doppler probe was placed on the skin above the heart and for each acclimation temperature, experimental fish (*N* = 6, mean mass 32.2 ± 6.4 g (CA), 34.5 ± 3.7 g (WA)) had a digital thermocouple (Digittech QM-1600) placed inside the fish’s mouth to record temperature. Glass aquarium heaters gradually increased the water temperature in the reservoir tank by 1 °C every 15 minutes. Heart-rate sonograms were measured over one minute at each temperature till *T_HF*. In parallel to heat stressed fish, control fish (*N* = 6, mean mass 27.0 ± 7.8 g (CA), 30.1 ± 5.4 g (WA)) were maintained in an identical set up and heart rate sonograms were measured every 30 minutes. Fish were euthanised by cephalic concussion and a blood sample was withdrawn by caudal puncture into a heparinised syringe. Plasma was separated by rapid centrifugation (five minutes at 2500 rcf), snap frozen in liquid nitrogen and stored at -80 °C for metabolomic analysis.
4.2.3 Plasma metabolomics

A modified protocol from Smart et al. (2010) was used to extract metabolites. For 100 µL of plasma, 20 µL of internal standard (10 mM of 2,3,3,3-d4 DL-Alanine) was added. Samples were vortexed, frozen at -80 °C and then freeze dried. Metabolites were extracted by adding 500 µL of methanol:water (1:1 v/v) at -30 °C, vortexed and centrifuged at 4 °C for five minutes at 16,000 g. Supernatants were centrifuged again and pellets were extracted a second time by re-suspension in 500 µL of methanol:water (4:1 v/v) at -30 °C. This supernatant was pooled with the first extract and samples were topped to 5 mL with 4 °C bi-distilled water and freeze dried. Methyl chloroformate (MCF) was used to chemically derivatise extracted metabolites and gas chromatography-mass spectrometry (GC-MS) was used to analyse samples (Smart et al., 2010). Plasma metabolites were quantified from base to peak height detected by the GC and normalised to d4-alanine. Quantified metabolites were identified by comparison to an in-house MCF MS library of derivatised metabolites. A comparative metabolite profile between the two acclimated groups was developed and analysed using R-software (Aggio et al., 2011).

4.2.4 Mitochondrial respirometry

4.2.4.1 Fibre preparation

The preparation of heart fibres was identical in all mitochondrial assays. The immediately dissected fish heart was immersed in a 2 mL modified ice-cold relaxing buffer (BIOPS, 2.8 mM CaK2EGTA, 7.2 mM K2EGTA, 5.8 mM Na2ATP, 6.6 mM MgCl2·6H2O, 20 mM taurine, 20 mM imidazole, 0.5 mM dithiothreitol, 50 mM K-MES, 15 mM Na-phosphocreatine and 50 mM Sucrose, pH 7.1) with an osmolarity of 350 mOsm at 0 ºC. The heart was teased into fibre blocks using a dissecting microscope and placed in 1 mL ice-cold BIOPS. 50 µg. mL⁻¹ saponin was added as fibres were shaken in a plastic culture plate on ice for 30 minutes. Fibres were transferred and rinsed three times for 10 minutes in 2 mL of modified mitochondrial respiratory medium (MiRO5, 0.5 mM EGTA, 3 mM MgCl2·6H2O, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH2PO4, 20 mM HEPES, 160 mM sucrose and 1 g. L⁻¹ BSA, essentially free fatty acid, pH 7.2 at 20 ºC; Gnaiger et al. (2000)). The fibres were blotted dry on filter paper and weighed into 2 - 4 mg bundles for respiration assays. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).
4.2.4.2 Mitochondrial respiration assay

Respiration was measured in a Oroboros Oxygraph-2k™ respirometer (Oroboros Instruments, Innsbruck, Austria) utilising a substrate-uncoupler-inhibitor titration (SUIT) protocol at six different temperatures; 15 °C (representative mean ocean winter temperature), 20 °C (representative mean ocean summer temperature), 25 °C (T<sub>HF</sub>), and maximum temperatures of 27.5 °C, 30 °C and 32.5 °C to determine exact breakpoint in mitochondrial function (Gnaiger, 2009; Kuznetsov et al., 2008). Oxygen was maintained above 280 nmol. mL<sup>-1</sup> to ensure saturation. Complex I (CI) substrates (2 mM malate, 10 mM pyruvate) were added to measure state II respiration through CI in the absence of ADP (denoted Leak-I). Excess ADP (2.5 mM) stimulated oxidative phosphorylation (OXP-I), and glutamate (10 mM) was added to saturate CI. Cytochrome c (10 μM) tested outer membrane integrity. Phosphorylating respiration with CI and CII substrates (OXP-I, II, state III respiration) was attained by succinate (10 mM) addition. NADH (0.5 mM) assessed inner mitochondrial membrane damage. Leak respiration on combined CI and CII was measured by atractyloside (750 μM, Leak-I, II) addition, followed by repeated titrations of carbonyl cyanide p-(trifluoromethoxy)phenyl-hydrazone (FCCP, 0.5 μM) to uncouple mitochondria (denoted ETS). By the addition of rotenone (0.5 μM), malonate (15 mM) and antimycin a (1 μM), CI, II and III were inhibited, respectively. Cytochrome c-oxidase (CCO, CIV) was measured by the addition of the electron donor couple N,N,N′,N′-tetramethyl-p-phenylenediamine (TMPD, 0.5 mM) and ascorbate (2 mM).

4.2.4.3 ROS (Reactive Oxygen Species) detection

ROS production and simultaneous comparison to mitochondrial respiration was achieved using purpose-built fluorometers attached to the Oroboros O2K oxygraph (Hickey et al., 2012). To calibrate the fluorometer, resorufin (400 pM) was added to each chamber prior to the SUIT assay. Horse-radish peroxidase (HRP, 2.5 U. mL<sup>-1</sup>), Amplex-Ultrared (25 μM) and super-oxide dismutase (SOD, 24 U. mL<sup>-1</sup>) were used to convert the net super-oxide produced by mitochondria to hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) that can be detected by the fluorometers. Steady state rates were followed using DATLAB 4.3 and corrected for tissue mass and background activity determined prior to substrate addition.
4.2.4.4 Apparent Km for pyruvate

Km (Km\text{app}) for pyruvate was measured in permeabilised heart fibres from CA and WA fish. Initially oxygen was added to saturation and then malate (2 mM) and excess ADP (2.5 mM) were added. The concentrations at which pyruvate was taken into the mitochondria at different temperatures were optimised previously. Based on this data, pyruvate was titrated in at very small volumes (0.2 – 0.5 µL) into the oxygraph using a tip-O2K® up to saturation to give a step-wise increase in respiration. Substrate-saturation curve kinetics were applied to determine Km\text{app} and V_{\text{max}}.

4.2.5 Calculations and statistical analyses

Data are reported as means ± SEM (N is the number of fish), unless otherwise stated and the level of significance for all statistical tests was set at p < 0.05. All statistical tests were run using SigmaStat® version 3.1 (Systat Software, Inc., San Jose, California) unless stated otherwise. Doppler recordings of heart rate were converted to WAV format using Audacity software (www.sourceforge.net) and analyzed with a software script written in Octave® (www.gnu.org). To establish if cardiac function components changed with time, a repeated measures analysis of variance (ANOVA) followed by a post hoc test (Tukey’s) was applied. A comparative metabolite profile was generated between the control and acutely heat stressed CA and WA fish and data was analysed using R-software (Aggio et al., 2011). Differences in metabolite profiles between control and heat stressed fish were compared using PCA and data were projected on a 2D plane (PCI vs. PC2). Differences in plasma metabolite concentrations were determined using an ANOVA followed by a Tukey’s test.

All mitochondrial respiration rates were expressed per mg wet weight of cardiac fibres. Respiratory control ratios-I (RCR-I) were calculated as OXP-I/Leak-I and RCR-II were calculated as OXP-I, II/Leak-I, II. Differences across temperatures in RCRs and mitochondrial respiration states were evaluated by ANOVA followed by a Tukey’s test. A dose-dependent analysis (DDA) with Prism5® was applied to RCR-I and RCR-II to determine the breakpoint where ratios changed with increasing temperature. For each group, ROS production with increasing assay temperature did not significantly change. Hence the total area under the curve was quantified for individual fish for a respiration state to give net ROS production. A one-factor ANOVA was applied to determine if net ROS production differed across mitochondrial states.
4.3 Results

4.3.1 Cardiac function in acclimated *N. celidotus* acutely exposed to in vivo heat stress

The mean $T_{HF}$ for fish acclimated to 15 °C was 26.7 ± 0.4 °C, and for those acclimated to 21 °C was higher at 28.2 ± 0.6 °C ($p=0.07$, figure 4.1). At $T_{HF}$ the mean heart rate for CA fish was 80.8 ± 3.7 beats min$^{-1}$ ($N=3$), and for WA fish was 73.7 ± 5.8 beats min$^{-1}$ ($N=4$) (figure 4.1). Above $T_{HF}$, heart rate declined and became arrhythmic for both acclimated groups.

![Figure 4.1: Heart beat rate (beats. min$^{-1}$) of cold acclimated (CA, 15 °C), and warm acclimated (WA, 21 °C) *N. celidotus*. The solid and dashed line corresponds to temperature of heart failure ($T_{HF}$) in CA and WA fish, respectively.](image)

4.3.2 Metabolite profiles of plasma from acclimated *N. celidotus*

Two-dimensional principal component analysis (PCA) displayed super-imposed plasma metabolite profiles for control CA and WA *N. celidotus*, indicating plasma profiles were not affected by acclimation to seasonal to temperatures (figure 4.2). However, when individual metabolites were analysed, lactate (glycolytic intermediate) was significantly higher in control WA fish plasma compared to CA fish (figure 4.3). Citrate, succinate and cis-aconitate (*TCA* cycle intermediates) were comparable in control CA and WA fish. PCA determined the largest separation in metabolic profiles to be between control and heat-stressed WA *N.
celidotus, inferring that acute heat stress had most impacted the intermediary metabolism of these fish (figure 4.2).

Figure 4.2: Principal Component Analysis (PCA) projection of metabolite profiles for CA and WA N. celidotus. Data for control fish and acutely heat-stressed fish were projected onto a 2D space and metabolic profiles were generated from mean metabolites that had statistically significant ($p \leq 0.05$) accumulation ($N=6$ per acclimation treatment).
Figure 4.3: The relative baseline concentrations of the glycolytic intermediate (lactate) and TCA cycle intermediates (citrate, succinate and cis-aconitate) to the standard, d4-alanine, in CA and WA N. celidotus acclimated to 15 °C (cold acclimated, CA) and 21 °C (warm acclimated, WA). The asterisk * indicates a significant difference (p≤0.05) between metabolite intermediates of CA and WA fish.

4.3.3 The effects of acclimation on cardiac mitochondrial function

Leak-I respiration increased at the same rate for both acclimated groups with increasing assay temperature, but at 25 °C, WA fish had a significantly higher Leak-I compared to CA fish (figure 4.4A). Leak-I expressed as a percentage increase from rates at 15 °C showed that by 25 °C, WA N. celidotus had maximised Leak-I (figure 4.4B). Similar to Leak-I, OXP-I also increased with increasing assay temperature in both groups, but this increase was sharper in CA fish (figure 4.4C), which was more evident when OXP-I was expressed as a percentage increase from rates at 15 °C (figure 4.4D). The fractional increase in OXP-I respiration from 15 °C was significantly lower from 27.5 °C upwards in WA fish compared to CA fish. Respiratory control ratios with CI substrates (RCR-I) in WA fish were significantly depressed by 20 °C and DDA analysis predicted a breakpoint at 19.5 ± 0.3 °C (figure 4.4E). Relative to WA fish, CA fish had significantly higher RCR-I values at 20 °C and 25 °C, and a higher DDA predicted breakpoint at 24.8 ± 0.4 °C.
While OXP-I, II rates in CA and WA groups increased as assay temperatures became warmer, this increase was more prominent in CA fish (figure 4.5A). After 25 °C, WA *N. celidotus* only increased OXP-I, II up to ~40% compared to CA fish that continued to increase OXP-I, II to <60% when rates were expressed as a percentage increase from rates at 15 °C (figure 4.5B). Although the Leak-I, II rates were similar for both groups (figure 4.5C),
WA fish had maximised Leak-I, II by 27.5 °C compared to rates at 15 °C (figure 4.5D). Although DDA analysis predicted similar breakpoints for CA (24.3 ± 0.9 °C) and WA (24.9 ± 0.5 °C) *N. celidotus* (figure 4.5E), these breakpoints were below their respective T_{HF} (figure 5.1). Notably WA fish had significantly lowered their RCR-II at 25 °C to values similar to that measured at the maximal point of 32.5 °C (figure 4.5E).

**Figure 4.5:** Cardiac mitochondrial function related to CI and CII of permeabilised fibres from *N. celidotus* acclimated to 15 °C (CA, cold acclimated) and 21 °C (WA, warm acclimated) exposed to increasing in situ assay temperatures. (A) OXP-I, II rates. (B) OXP-I, II rates expressed as a percentage increase from rates measured at 15 °C. (C) Leak-I, II rates. (D) Leak-I, II rates expressed as a percentage increase from rates measured at 15 °C. (E) DDA applied to RCR-II determined as OXP-I, II/Leak-I, II. The solid and dashed line in each panel indicates the T_{HF} for both CA and WA *N. celidotus*, respectively. For all respiration rates, percentages and ratios, letters of the same case for each acclimated group are not significantly (*p* > 0.05) different from one another. The asterisk * indicates differences between acclimated groups (CA vs. WA) for a given temperature.
Figure 4.6: Arrhenius plots for cardiac mitochondrial function in permeabilised fibres from *N. celidotus* acclimated to 15 °C (CA, cold acclimated) and 21 °C (WA, warm acclimated) exposed to increasing assay temperatures. (A) CI rates determined as OXP-I – Leak-I. (B) CII rates determined as OXP-I, II – Leak-I, II. (C) CCO rates. Linear regression was applied for all data.
Compared to WA *N. celidotus*, Arrhenius plots for CI, CII, and CCO respiration showed a greater response in CA *N. celidotus* to increasing temperature reflected by steeper slopes (figure 4.6A-C). In particular, CII respiration in WA *N. celidotus* presents almost no response to increasing temperatures (-0.8 ± 1.6) compared to CA fish (-3.9 ± 1.6) (figure 4.6B). CCO respiration had the greatest temperature driven response in both CA (-6.1 ± 0.5) and WA *N. celidotus* (-5.3 ± 0.6) when compared to CI and CII respiration within an acclimated group (figure 4.6C).

![Arrhenius plots for CI, CII, and CCO respiration](image)

**Figure 4.7:** Cardiac mitochondrial function related to uncoupling of permeabilised fibres from *N. celidotus* acclimated to 15 °C (CA, cold acclimated) and 21 °C (WA, warm acclimated) exposed to increasing *in situ* assay temperatures. (A) ETS rates. (B) ETS rates expressed as a percentage increase from rates measured at 15 °C. The solid and dashed line in each panel indicates the T_{HF} for both CA and WA *N. celidotus*, respectively. For all respiration rates and percentages, letters of the same case for each acclimated group are not significantly (p>0.05) different from one another. The asterisk * indicates differences between acclimated groups (CA vs. WA) for a given temperature.

As temperatures increased, CA *N. celidotus* had a higher capacity to chemically uncouple their cardiac mitochondria with FCCP compared to their WA counterparts (figure 4.7A).
When uncoupling rates were expressed as a percentage increase from initial rates, CA fish had increased uncoupling ability by 51% at 25 °C compared to the significantly lower 34% in WA fish (figure 4.7B). Moreover, WA *N. celidotus* were unable to uncouple their mitochondria further beyond 27.5 °C which is notably below their T_{HF} (figure 4.7A).

### 4.3.3.1 ROS production

Total ROS production was not influenced by increasing assay temperatures in either CA or WA *N. celidotus* except in the Leak-I, II state when WA fish produced significantly higher ROS (figure 4.8A). Additionally, the non-enzymatic anti-oxidant measured as total glutathione (GSSG) was found to be significantly higher in the WA group, compared to CA *N. celidotus* (figure 4.8B).

![Graph A](image)

**A.**

Area under the curve for ROS production (total area)

![Graph B](image)

**B.**

Relative Concentration of Glutathione

Figure 4.8: ROS production and antioxidant systems in *N. celidotus* acclimated to 15 °C (CA, cold acclimated) and 21 °C (WA, warm acclimated) exposed to increasing *in situ* assay temperatures. (A) Total ROS production determined as area under the curve for each respiration state with increasing assay temperature. (B) Relative concentration of total glutathione (GSSG) to d4-alanine determined by GC-MS in both acclimated groups. The asterisk * indicates differences between acclimated groups (CA vs. WA).
**4.3.3.2 Apparent pyruvate affinity (K_m app)**

K_m app pyruvate was similar between both acclimated groups at each assay temperature measured (table 4.1). Within acclimated groups, K_m app pyruvate significantly increased at 32.5 °C by 13-fold in CA fish and 11.5-fold in WA fish, compared to values at lower temperatures. CA fish increased V_max at 32.5 °C whilst increasing temperature did not influence V_max rates in WA fish (table 4.1). The apparent efficiency for pyruvate uptake as determined by V_max/K_m app was significantly depressed at 32.5 °C in both CA and WA groups (table 4.1).

**Table 4.1**: Apparent affinity of the primary CI substrate, pyruvate in *N. celidotus* acclimated to 15 °C (CA, cold acclimated) and 21 °C (WA, warm acclimated) measured at assay temperatures of 20, 25, 27.5, 30 and 32.5 °C. V_max/K_m app is an indicator of enzyme efficiency.

<table>
<thead>
<tr>
<th>Assay Temperature (°C)</th>
<th>20</th>
<th>25</th>
<th>27.5</th>
<th>30</th>
<th>32.5</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>K_m app (µM)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CA</td>
<td>72.48 ± 7.70</td>
<td>88.36 ± 19.53</td>
<td>76.05 ± 12.53</td>
<td>81.03 ± 10.13</td>
<td>1018.72 ± 144.80*</td>
</tr>
<tr>
<td>WA</td>
<td>80.31 ± 13.44</td>
<td>71.39 ± 16.29</td>
<td>79.05 ± 4.42</td>
<td>88.89 ± 23.27</td>
<td>920.82 ± 297.72*</td>
</tr>
<tr>
<td><strong>V_max (pmol (mg. s)^-1)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CA</td>
<td>18.56 ± 2.13</td>
<td>22.85 ± 3.23</td>
<td>24.04 ± 2.54</td>
<td>24.79 ± 3.32</td>
<td>33.67 ± 3.26*†</td>
</tr>
<tr>
<td>WA</td>
<td>23.80 ± 4.19</td>
<td>18.39 ± 3.79</td>
<td>24.51 ± 3.60</td>
<td>19.92 ± 2.01</td>
<td>22.60 ± 3.64</td>
</tr>
<tr>
<td><strong>V_max/K_m app</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CA</td>
<td>0.27 ± 0.04</td>
<td>0.30 ± 0.08</td>
<td>0.34 ± 0.07</td>
<td>0.33 ± 0.08</td>
<td>0.03 ± 0.003*</td>
</tr>
<tr>
<td>WA</td>
<td>0.31 ± 0.07</td>
<td>0.29 ± 0.09</td>
<td>0.32 ± 0.06</td>
<td>0.34 ± 0.15</td>
<td>0.03 ± 0.011*</td>
</tr>
</tbody>
</table>

Values are means ± s.e.m. (N=6 for each species at each temperature).

*Significant difference between assay temperatures within an acclimation group (p≤0.05).
†Significant difference between assay temperatures for a given acclimation temperature (p≤0.05).

**4.4 Discussion**

Crucial mitochondrial mechanisms contributing to HF were examined in CA and WA *N. celidotus* to understand if cardiac mitochondrial plasticity had occurred during thermal acclimation. This study illustrates that WA *N. celidotus* will likely face critical conditions if increasing environmental temperatures persist. These data support previous observations that cardiac mitochondrial dysfunction can contribute to HF in WA fish (Iftikar and Hickey, 2013;
Iftikar et al., 2014). Several components of mitochondrial function were found to be impaired at temperatures below $T_{HF}$ in WA *N. celidotus*. Specifically, as assay temperatures increased, the ability for WA *N. celidotus* cardiac mitochondria to increase OXP-I and OXP-I, II decreased alongside OXP efficiency measures (RCR-I and RCR-II), that were depressed at 20 °C which was below the $T_{HF}$. This indicates a compromised OXP system. These compromised components of cardiac mitochondrial function in WA *N. celidotus* suggest decreased cardiac mitochondrial capacities to elevate respiration flux and maintain function as environmental temperatures increase.

4.4.1 The effects of acute *in vivo* heat stress on *N. celidotus* acclimated to seasonal temperatures

The completeness of acclimation in fishes can be assessed by a variety of mechanisms including the ability to perform normal functions such as the complete processing of a meal (Peck et al., 2010; Peck et al., 2008). During this study, fish were fed every two days and complete satiation was achieved by animals in both acclimation set ups, signifying that fish had acclimatised to their respective temperatures. Acclimation affects all physiological functions in fishes, including the cardiovascular system (Haverinen and Vornanen, 2009; Lillywhite et al., 1999). WA fishes exposed to rising temperatures are shown to have a lower heart rate due to the pacemaker resetting its activity to a lower rate, which allows cardiac scope to be maintained even as temperatures increase (Farrell, 1997). In this study, although WA fish heart rates plotted lower than CA fish, the differences between CA and WA heart rates were not significant at a given temperature (figure 4.1). Potentially, this is because WA fish were acclimated to their summer temperatures, which are routinely experienced in nature, and therefore basal heart rate responses in WA *N. celidotus* may have not been challenged to their whole extent.

The mean $T_{HF}$ of CA (26.7 ± 0.4 °C) and WA fish (28.2 ± 0.6 °C) did not significantly differ (figure 4.1). However, a significant increase in plasma lactate of control WA fish (figure 4.3) indicates that this group may have increased basal anaerobiosis relative to CA fish. Moreover, PCA analysis confirmed that acute *in vivo* heat stress affected the plasma metabolite profile of WA fish more than their CA counterparts (figure 4.2). Similarly, acute heat stress significantly altered the plasma metabolite profile of the tropical wrasse *Thalassoma lunare* compared to wrasses from temperate habitats (Iftikar et al., 2014). This
suggests that wrasses exposed continually to warmer temperatures may be metabolically limited when faced with acute heat stress compared to wrasses from colder temperatures.

4.4.2 The effects of acclimation on mitochondrial bioenergetics

Acclimating *N. celidotus* to average winter temperatures appeared to provide the most protection for heart mitochondrial CI function with heat induced stress (figure 4.4). This was indicated by robust OXP-I rates and RCR-I values in CA *N. celidotus* even as assay temperatures became warmer (figure 4.4C-E). Similarly, skeletal muscle mitochondria from CA rainbow trout had higher RCR values compared to WA rainbow trout (Bouchard and Guderley, 2003). Cold acclimated ectotherms possess mitochondrial membranes with increased unsaturated lipid contents (Grim et al., 2010; Guderley and St-Pierre, 2002; Irving and Watson, 1976), making the membranes more fluid and leaky. The shifting of phospholipid composition in the inner membrane with cold acclimation can affect the transport activity of the adenine-nucleotide transporter (ANT) (Bouchard and Guderley, 2003). The increased phosphorylation capacity and RCR-I values in CA *N. celidotus* could be attributed to this increased activity of the ANT (figure 4.4E). Conversely, the increased fluidity of mitochondrial membranes in CA fish can also account for the varying activities of membrane-bound enzymes, such as CI which is bound to the inner-mitochondrial membrane (Irving and Watson, 1976). Indeed, cold acclimation has been shown to increase the activities of mitochondrial membrane-bound enzymes (Guderley and St-Pierre, 2002; van den Thillart and Modderkolk, 1978).

In contrast, WA fish had significantly depressed their RCR-I values by 20 °C below their *T_{HF}* (figure 4.4E) and altered their RCR-II by 25 °C (figure 4.5E). CII produces a lower yield of ATP per oxygen molecule because the CII electron input only contributes 6 protons that transfer across the inner-mitochondrial membrane compared to the 10 protons contributed by CI (Hinkle and Yu, 1979). In the OXP-I, II respiration state, the ETS has a greater electron supply than electron inputs from CI or CII alone (Lemieux and Warren, 2012). OXP-I, II in WA fish revealed an apparently lower thermal sensitivity since respiration increased at 25 °C and then plateaued (figure 4.5A). Previous studies have found lower activation energies of mitochondrial enzymes including succinate-dehydrogenase (CII) in warm acclimated fishes, and this was attributed to changes in membrane structures (Guderley and Johnston, 1996; Hazel, 1972). This decreased thermal responsiveness of CII observed in WA *N. celidotus*
may explain relative decreases in CII mediated flux (figure 4.6B). However, given recent discoveries of diet mediated changes in respiratory chain super-complex organisation of Complexes I and II and their associations with ubiquinone III and IV (Lapuente-Brun et al., 2013), interactions with temperature may also occur.

Compared to CI and CII rates, cytochrome \( c \) oxidase (CCO) rates had a greater overall flux in both CA and WA fish (figure 4.6C). The excess capacity of CCO in isolated mitochondria is well known, and is reported to be 10-fold higher than the maximal respiration rates observed for pyruvate in trout muscle mitochondria (Bouchard and Guderley, 2003). In ectotherms, CCO activity appears to be highest in the heart, likely due to the heart’s active role (Ludwig et al., 2001). While such high activities initially indicate that CCO exerts minimal control over respiration, \( \textit{in vivo} \) CCO appears to exert considerable control over respiration flux (Villani and Attardi, 1997; Villani and Attardi, 2001), including within cardiac muscle (Kuznetsov et al., 1996). However, as assay temperatures increased, CCO rates were comparable when compared between CA and WA \( N. \textit{celidotus} \) (figure 4.6C). This suggests a similar capacity of CCO to maintain mitochondrial oxygen binding (Gnaiger et al., 1998), regardless of acclimation. Previously, warm acclimation was found to increase cardiac mitochondrial CCO activity in cod (Foster et al., 1993), and hypoxia-tolerant carp (Cai and Adelman, 1990). However, warm acclimation may cause the cod and carp to face hypoxic conditions during which high CCO activity maximises oxygen affinity in the mitochondria. At summer temperatures, the \( N. \textit{celidotus} \) heart probably still receives an adequate oxygen supply.

WA fish also had a decreased capacity to chemically uncouple their cardiac mitochondria as temperatures increased relative to CA fish (figure 4.7A-B). This could be attributed to a loss in inner membrane barrier function caused by heat stress (Žūkienė et al., 2007; Žūkienė et al., 2010) in warm acclimated fish. Given that CII contributes less to proton pumping, and consequently more to oxygen consumption, a decrease in ETS excess capacity can also be attributed to depressed flux through CII. Moreover, proton leakage from the inter-membrane space to the matrix can also utilize uncoupling proteins (UCP) (Brand et al., 1994; Stuart et al., 2001), and the ANT that is inhibited by atractyloside, which was employed in this present work. Temperature-dependent UCP expression increases in warm acclimated fish indicating an increased potential for proton leakage (Mark et al., 2006). Leak-I, II rates, i.e. in the
presence of atractyloside remained comparable for both groups (figure 4.5C), indicating changes in apparent proton leak can also be attributed to ANT. The decreased capacity of WA fish to uncouple heart mitochondria with increasing assay temperatures lowered OXP efficiency (depicted by RCR-II) by 25 °C prior to HF in these fish (figure 4.5E). In contrast, the more tightly coupled mitochondria observed in CA fish decreases mitochondrial proton leakage and therefore, prevents energy loss at low temperatures.

4.4.2.1 The effects of acclimation on cardiac mitochondrial ROS production and antioxidants

ROS is mainly produced by the ETS which converts ~0.15% of oxygen consumed to ROS (St-Pierre et al., 2002). An increase in ROS production occurs with the disruption of electron transfer in the ETS (Murphy, 2009; Turrens, 2003), and can occur in fishes experiencing temperature changes (Heise et al., 2007; Heise et al., 2006). However, ROS production in CA and WA *N. celidotus* under state III conditions (OXP-I, II) were comparable (figure 4.8A), but total glutathione was 3-fold higher in WA fish compared to CA *N. celidotus* (figure 4.8B). The cellular antioxidant glutathione protects cells from ROS-induced damage, and antioxidant responses closely match ROS production because the mismatch between the two systems can lead organisms to face oxidative stress (Grim et al., 2010). Although mitochondrial ROS production between acclimated groups remained similar (figure 4.8A), the exacerbated elevation of the glutathione-antioxidant system in WA *N. celidotus* may indicate sustained oxidative stress loads in this group.

4.4.2.2 The effects of acclimation on substrate affinity in cardiac mitochondria

Pyruvate is an important substrate for mitochondrial oxidation and the affinity for pyruvate by mitochondria is determined by the properties of the pyruvate transporter or by pyruvate dehydrogenase (PDH) (Guderley et al., 1995). The corresponding $K_{m\text{app}}$ values for pyruvate in CA and WA fish at the lower assay temperatures between 20 and 30 °C were within the range reported previously for *N. celidotus* (Iftikar and Hickey, 2013). Pyruvate uptake was not affected by acclimation in *N. celidotus* as $K_{m\text{app pyruvate}}$ values were similar between CA and WA *N. celidotus* at all assay temperatures measured (table 4.1). Likewise, $K_{m\text{ pyruvate}}$ of liver mitochondria from cold (4 °C and 10 °C) and warm (16 °C) acclimated trout did not differ when measured at their acclimation temperatures; however PDH activity was higher in the warm acclimated group (Voss, 1985). Notably, the temperature difference between the
cold acclimated trout (6 °C) is similar to that of this study, and therefore a change in PDH activity cannot be expected. \( K_{\text{m app}} \) values increased significantly at 32.5 °C for both CA and WA groups (table 4.1), however, these values were much higher than the physiological concentration of pyruvate (Yancey and Somero, 1978).

### 4.4.3 Conclusion

Previously, whole-animal tolerance at upper thermal limits was postulated to result from a mismatch between oxygen demand and oxygen supply to the tissues of ectotherms (Pörtner and Farrell, 2008; Pörtner and Knust, 2007; Pörtner et al., 2004). Specifically, cardiac performance was believed to be maximised at an ectotherm’s preferred temperature and above this temperature the heart was theorised to fail. (Farrell, 1997; Farrell et al., 2009). However, emerging studies are starting to indicate that at temperatures where aerobic scope and cardiac function are maximised, ectotherms face restricted growth (Healy and Schulte, 2012), and a decrease in population abundance (Clark et al., 2013; Gräns et al., 2014). Since cardiac function is an important factor in setting the upper thermal limits of fishes, determining the molecular mechanisms that contribute to heart failure and their plasticity will elucidate how ocean warming will impact the physiology and distributions of fishes.

Overall this study provides an understanding about the acclimation capacity of cardiac mitochondria with seasonal temperature changes in a temperate teleost. The most important observation presented in this study was that components of cardiac mitochondrial function in WA *N. celidotus* were compromised at temperatures below their \( T_{\text{HF}} \). WA fish also appear to be already facing energetic costs at their summer temperatures, and the inability of the WA cardiac mitochondria to overcome thermal stress may become a greater challenge for *N. celidotus* as oceans warm. This presents an area for future inquiry about general mechanisms underlying the acclimatory capacities of eurytherms as they face global warming.
5. CHAPTER 5 – INVESTIGATING THE CONTRIBUTION OF CARDIAC MITOCHONDRIA IN DETERMINING THE THERMAL LIMITS OF NATIVE VS. INVASIVE ECTOTHERMS

Published as:

5.1 Introduction

Although marine organisms are exposed to a variety of environmental stressors, temperature is perhaps the greatest factor due to its profound impact on biological processes such as metabolism (Stillman and Somero, 1996). Species must tolerate environmental temperature changes that fluctuate over short term (diurnal and seasonal variations) (Stillman and Somero, 1996), and potentially due to progressive changes in climates, perhaps resulting from global warming (Pörtner and Knust, 2007). With the latter case, disruption of thermal habitats may outstrip some species capacities to adapt to temperature stress and decrease species ranges (Helmuth et al., 2002) while increasing others (Walther et al., 2002). Studies of species physiological and biochemical tolerances to increasing temperatures will improve our understanding of the impacts of climate change on species distributions (Stillman, 2003). This may be particularly important in contexts of eurythermal invasive species.

In many marine ectotherms, the heart appears to be extremely sensitive to rising temperature and therefore may provide an indicator for whole-animal thermal stress (Braby and Somero, 2006; McMahon and Wilkens, 1983; Pandolfo et al., 2009; Stenseng et al., 2005). Indeed the cardiovascular system may determine the thermal tolerance limits of marine ectotherms, where the heart appears to be the first organ to fail as temperature rises (Farrell, 2002; Pörtner and Knust, 2007; Sandblom et al., 2009). However, the specific mechanisms driving temperature-mediated heart failure in ectotherms remains to be determined.

In marine ectotherms, high-temperature mediated heart failure may result from extrinsic factors, such as disrupted oxygen supply to the heart, or from intrinsic factors within the
heart, such as disrupted ion channel function or ATP supply from mitochondria (Pörtner and Knust, 2007). One such intrinsic factor involves mitochondrial energetics, which are fundamental to heart function given that they supply 90% of the ATP demands, which increase with rising temperature (Cherkasov et al., 2007). Importantly, heart mitochondria of mammals differ considerably from those found in other tissues, in terms of composition and proteomes (Rodriguez-Zavala and Moreno-Sanchez, 1998). Mammalian heart mitochondria are also known to be much more fragile than those of other tissues, and are easily damaged by stress such as high temperature (Huss and Kelly, 2005; Lemieux et al., 2010b; Lesnfsky et al., 2001). Moreover, mitochondrial disruption not only disrupts cellular ATP production, but it can also trigger necrosis and apoptosis, where these small organelles appear to have pivotal roles (Hand and Menze, 2008). Therefore, heart mitochondrial function may provide biomarkers and explanations that are useful for understanding species tolerances to thermal challenges.

The New Zealand paddle crab, *Ovalipes catharus* (Crustacea: Decapoda: Portunidae), is native to the sheltered and exposed sandy shores all around New Zealand. *O. catharus* tends to reproduce in colder waters (~15 °C), with notable depressions in egg production during summer where temperatures reach approximately around 19 - 20 °C (Haddon and Wear, 1993). In contrast, the Asian paddle crab *Charybdis japonica* (Crustacea: Decapoda: Portunidae) is a recently described invasive species in New Zealand (ca. 2000), which is thought to have arrived at Auckland in ballast water (Smith et al., 2003). *C. japonica* may serve as a disease vector to local fisheries (Maeda et al., 1998) and appears to be more aggressive than *O. catharus* (Archdale et al., 2007). Both adult *C. japonica* (Gust and Inglis, 2006) and larvae (Fowler et al., 2010) appear to acclimate to broad temperature ranges with acute maxima at 34 °C and 43 °C respectively. Since temperature is known to affect the distribution ranges of invasive species like *C. japonica* (Baylon and Suzuki, 2007; Storch et al., 2009), the goal was to test whether hearts and cardiac mitochondria from *C. japonica* were more robust at elevated temperatures relative to *O. catharus*. The integrated function of mitochondrial electron transport systems of each species was also compared and discussed in the context of species thermal tolerance limits.
5.2 Materials and methods

5.2.1 Experimental animals
Crabs were collected by local fishermen and affiliated researchers using baited traps north of Auckland, NZ (35° 43’S, 174° 21’E-36° 38’24 S, 174° 43’38 E, Fowler et al. 2010). Crabs were acclimated for four weeks to 18 ± 0.5 °C in aerated aquaria with re-circulating seawater under a 12L: 12D photoperiod prior to experimentation and fed greenshell mussel every two days. Feeding was suspended 48 hours before experimentation. All experiments and procedures met with the ethical requirements of the University of Auckland, New Zealand (NZ) (Approval AEC/04/2009/T720).

5.2.2 Cardiac function measurements
*O. catharus* (*N*=6, mean mass 183.72 ± 11.44 g) and *C. japonica* (*N*=6, mean mass 105.78 ± 16.78 g) were individually placed into plastic boxes containing 4 L of constantly aerated seawater (19.1 ± 0.1 °C) and a black cloth with a 3 cm diameter opening was placed over the crab, leaving the shell immediately above the heart exposed. Metal frames were placed on the crabs to immobilize claws and this kept the crab stationary. Heart rate was measured using a non-invasive pocket foetal Doppler probe (Sonotrax B, Contact Medical Systems, China). The 8 MHz Doppler probe was placed immediately above the heart with a layer of water maintained between the carapace and probe. The audio output of the Doppler system was connected to a PC via the audio jack and sonograms were recorded and analysed using Audacity 1.2.6 ([http://audacity.sourceforge.net/](http://audacity.sourceforge.net/)).

Once a clear heart beat was detected, the Doppler probe was fixed in place using a clamp mounted to a retort stand. Temperatures were recorded using a digital thermocouple (Digitech QM-1600) placed immediately below each crab. Sonograms were generated for at least one minute as temperature was gradually increased 1 °C every 10 minutes using aquarium glass heaters (*N*=6). Control tanks (animals maintained at a constant 18 °C, *N*=6) were measured every half hour for the duration of temperature experiments. Therefore sonograms were generated from 18 - 30 °C in 1 °C increments, or every 30 minutes for controls. Heart rate (BPM) and beat duration (period of an individual beat) were determined directly from sonograms using Audacity 1.2.6. Crabs were euthanised by chilling in ice, and haemolymph sampled from a leg joint and centrifuged. The heart was excised and snap frozen in liquid nitrogen and stored at -80 °C for metabolite and enzyme analysis.
5.2.3 Mitochondrial respirometry

5.2.3.1 Permeabilised fibre preparation of heart muscle

*O. catharus* (*N* = 6, mean mass 126.72 ± 16.96 g) and *C. japonica* (*N* = 6, mean mass 73.04 ± 8.85 g) were killed by chilling in ice for 20 minutes. The heart was immediately dissected and immersed in 2 mL modified ice-cold relaxing buffer (RB, 2.77 mM CaK$_2$EGTA, 7.23 mM K$_2$EGTA, 5.77 mM Na$_2$ATP, 6.56 mM MgCl$_2$·6H$_2$O, 20 mM taurine, 20 mM imidazole, 0.5 mM dithiothreitol, 50 mM MES, and 400 mM KCl, pH 7.1) at 0 ºC. The buffer originally designed for mammalian muscle was modified with the omission of creatine phosphate and the addition of 400 mM KCl to increase the osmolarity to 1000 mOsm. The heart was teased into fibre bundles using a dissecting microscope and then placed into 1 mL RB and 50 µg mL$^{-1}$ saponin was added. The fibres were gently shaken in a plastic culture plate on ice for 30 minutes before being transferred into the modified mitochondrial respiratory medium (RM, 0.5 mM EGTA, 3 mM MgCl$_2$, 100 mM K-lactobionate, 20 mM taurine, 10 mM KH$_2$PO$_4$, 20 mM HEPES, 200 mM sucrose, 250 mM KCl and 1 g L$^{-1}$ BSA, essentially free fatty acid, pH 7.24 at 20 ºC; Gnaiger et al., (2000)). Fibres were rinsed three times for 10 minutes in 2 mL of RM, blotted dry on filter paper and weighed into bundles approximately 8 - 10 mg for respiration assays. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

5.2.3.2 Mitochondrial respiration assay

Fibres were added to 2 mL chambers containing saturated RM at 20 ºC, 25 ºC or 30 ºC in an Oroboros Oxygraph-2k™ respirometer (Oroboros Instruments, Innsbruck, Austria). Respiration was measured as weight-specific oxygen flux [pmol O$_2$ (sec · mg wet weight)$^{-1}$], and respiratory flux was calculated in real time as the negative time derivative of the oxygen concentration using Oroboros DatLab Software V 4.1.1.84 (Oroboros Instruments, Innsbruck, Austria). To explore mitochondrial function in the context of increasing temperature, we used a substrate-uncoupler-inhibitor titration protocol that measures substrate and coupling control of the mitochondria in permeabilised fibres (Gnaiger, 2009; Kuznetsov et al., 2008). With assay development it was found that pyruvate provided lower flux rates than proline. Therefore proline was used, similar to other workers (Gnaiger, 2008; Hochachka et al., 1983a; Mommsen and Hochachka, 1981), to test Complex I (CI).

The titration inhibitor protocol consisted of CI substrates proline (10 mM) and malate (2 mM) to measure state II respiration through CI in the absence of ADP (denoted “Leak-I”, figure...
5.1). Excess ADP (2.5 mM) was added to stimulate oxidative phosphorylation (OXP-I) or state III respiration. Cytochrome c (10 μM) was added to test outer membrane integrity (an increase in rate following exogenous cytochrome c addition provides indication of outer mitochondrial membrane damage due to the loss of endogenous cytochrome c). Subsequently, succinate (10 mM) a CII substrate was added to measure parallel electron transport from CI and CII (OXP-I, II). Leak respiration rates were also measured on combined CI and CII substrates by addition of atracyloside (750 μM, Leak-I, II). To test for limitation by the phosphorylation system and to measure the maximal flux of the electron transport system (ETS) mitochondrial respiration was uncoupled by carbonylcyanide-p-(trifluoromethyl)phenylhydrazone (FCCP, 0.25 μM) addition. By the addition of rotenone (0.5 μM), malonate (15 mM) and antimycin a (1 μM), CI, II and III activities were inhibited respectively. The activity of cytochrome c-oxidase (CIV, CCO) was measured by the addition of TMPD (0.5 mM) and ascorbate (2 mM) (figure 5.1). Chemical background assays were run to account for the auto-oxidation of TMPD and ascorbate at the three experimental temperatures. To ensure saturation of fibres, oxygen was maintained above 280 nmol. mL⁻¹ by the breakdown of diluted hydrogen peroxide by excess catalase (3115.5 U/ 10µL) to release oxygen.

Figure 5.1: Respiration assay protocol measuring mitochondrial flux (pmol O₂. s⁻¹. mg⁻¹, grey line, left y-axis) and oxygen concentration (nmol. ml⁻¹, dotted black line, right y-axis)
over time (mins). Titrations of mitochondrial substrates, poisons and inhibitors and their time of addition are shown with arrows, and the resulting respiratory state in parenthesis. Pro [proline], Mal [malate], Cyt c [cytochrome-c], Succ [succinate], Atr [attractyloside], FCCP [carbonyl cyanide p-(trifluoromethoxy) phenyl-hydrazone], Rot [rotenone], Malo [malonate], Ant [antimycin-a], TMPD [N, N, N', N'-tetramethyl-p-phenylenediamine], Asc [ascorbate], Leak-I (state 2 respiration through CI in the absence of ADP), OXP-I (state 3 respiration), OXP-I, II (parallel electron transport from CI and CII), Leak-I, II (leak respiration flux rate through CI and CII), ETS (maximal flux of the electron transport system), CCO (activity of CIV, cytochrome c-oxidase). Black arrows labeled O2 in the oxygen concentration data indicate when respiration chambers were re-oxygenated.

5.2.3.3 Complex I integrity at 30 °C

Using the titration protocol outlined above it was noted O. catharus showed an apparent depression in CI respiration at 30 °C. Therefore an additional experiment was conducted to test CI function at 30 °C in both species. Heart fibres from both species (N=4) were assayed with CI substrates, proline (10 mM), malate (2 mM) and excess ADP (2.5 mM) to stimulate OXP-I (CI respiration). This was measured over 30 minutes and succinate (10mM) was then added to test whether CII was still viable even if CI was not.

5.2.3.4 Analysis and calculations

The respiratory control ratios (OXP-I/Leak-I = RCR-I, OXP-I, II/Leak-I, II = RCR-II) were used as simple proxies for measuring inner membrane permeability (Gnaiger, 2009). Limitation by the phosphorylation system was determined from the comparison of uncoupled respiration relative to attractyloside inhibited respiration (ETS/Leak-I, II). A simple ratio of CCO contribution to respiration was determined by comparison of CCO/OXP-I, II. Finally, the relative contributions of CI to total respiration (calculated as % OXP-I, II/OXP-I) was also calculated. To better understand the viability of CI at 30 °C over time, values were expressed as a % activity of initial respiration when ADP was first added and then expressed every 5 minutes for a 30 minute time period.

5.2.4 Enzyme and metabolite assays

Approximately 40 - 60 mg of heart tissue was cut and equilibrated within a 1:20 volume (w:v) of ice-cold homogenisation buffer (25 mM Tris-HCl pH 7.8, 1 mM EDTA, 2 mM MgCl₂, 50 mM KCl, 0.5% Triton X100) in 2 mL centrifuge tubes containing metal ball bearings and homogenised with a tissue lyser (Qiagen, New Zealand). Homogenates were
centrifuged at 14,000 g and the resulting supernatant removed and immediately assayed for Citrate Synthase (CS) and Lactate Dehydrogenase (LDH), with modifications from previous workers (Hickey and Clements, 2003; Newsholme and Crabtree, 1986).

CS was determined at 412 nm with 5 μL tissue homogenate in 100 mM Tris-HCl pH 8.0, 0.1 mM acetyl coenzyme A, and 0.2 mM 5,5’-dithiobis-(2-nitrobenzoic acid) (DTNB), and reactions were started following the addition of 5 mM oxaloacetate. LDH was measured at 340 nm with 5 μL tissue homogenate using a reaction mixture containing 100 mM Tris-HCl pH 7.0, 1 mM EDTA, 2 mM MgCl₂, 1 mM dithiothrietol (DTT) and 0.15 mM NADH, and reactions were started following the addition of 1.5 mM pyruvate where measurements were made at 340 nm by observing the disappearance of NADH. LDH was also measured in undiluted haemolymph. Lactate was measured in the haemolymph enzymatically using L-LDH. All measurements were performed using a Molecular Devices Spectramax-340 plate reading spectrophotometer at 25 °C, and had a final volume of 230 μL per well.

5.2.5 Statistical analyses

Data are reported as means ± SE (N is the number of crabs), unless otherwise stated. Data were normally distributed. Cardiac-somatic indices were calculated as a percentage of total body mass. In respiration assays, where repeated temperature measurements were made on the same heart from a single crab, differences between temperatures were evaluated with a repeated measures analysis of variance (ANOVA) followed by a post hoc test (Tukey’s). The level of significance was set at p < 0.05. All statistical tests were run using SigmaStat® version 3.1 (Systat Software, Inc., San Jose, California).

5.3 Results

5.3.1 Cardiac function in O. catharus and C. japonica

The cardiac-somatic index in O. catharus (0.23 ± 0.01%) and C. japonica (0.20 ± 0.01%) were similar (p ≥ 0.05) for both species. With increasing temperature O. catharus and C. japonica significantly raised their heart rates (p ≤ 0.05, figure 5.2A). In O. catharus, this increase was significant at 25 °C compared to initial heart rates observed at 19 – 20 °C. While in C. japonica, even at 27 °C heart rate remained similar to rates observed at 19 – 20 °C, and then diverged at 28 °C (p ≤ 0.05). C. japonica had a lower initial heart rate of 49.73 ± 5.34 BPM at 19 °C (figure 5.2A). Although heart rates were similar between 20 °C and 24 °C for
each species, the heart rate of *O. catharus* increased more dramatically at higher temperatures (1.5 and 1.6-fold at 25 °C and 30 °C respectively), with $Q_{10}$ values consistently higher overall (figure 5.2A insert). In contrast to heart rate, heart beat duration was up to 30% longer in *C. japonica* at lower temperatures (19 °C to 24 °C) (figure 5.2B).

![Figure 5.2:](image)

**Figure 5.2:** (A) The change in mean heart rate (beats/min, $N=6$ for each species) with increasing temperature (°C) in *O. catharus* and *C. japonica*. (Insert) $Q_{10}$ values for *O. catharus* and *C. japonica*. (B) The change in mean heart beat duration (s, $N=6$ for each species) with increasing temperature (°C) in *O. catharus* and *C. japonica*. (Insert) Representative trace of heart rate at 20°C obtained using Audacity® software. Means sharing the same letter of the same case are not significantly different from one another at $p \leq 0.05$. The plus sign denotes a significant difference between *O. catharus* and *C. japonica* for individual cardiac function measures at a given temperature at $p \leq 0.05$. 

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5.3.2 The effects of temperature on mitochondrial respiration

Although Leak-I (State II respiration) initiated by proline and malate in the mitochondrial respiration assay increased with higher temperatures in both species (figure 5.3A, B), Leak-I flux was significantly higher in *O. catharus* (*p* ≤ 0.05, figure 5.2A). OXP-I flux rates (State III, initiated by ADP addition) in *O. catharus* remained similar at 20 °C, 25 °C and 30 °C (figure 5.3A). In contrast, *C. japonica* could elevate OXP-I with increasing temperature (figure 5.3B). Comparison across species indicated that OXP-I was significantly higher in *O. catharus* at 20 °C and 25 °C (*p* ≤ 0.05), while flux rates were similar at 30 °C for both species. Although the respiratory control ratio-I (RCR-I) with CI substrates (OXP-I/Leak-I) decreased with higher temperatures in both species (figure 5.4A), *C. japonica* maintained higher RCR-I’s at all temperatures relative to *O. catharus*. The addition of cytochrome c had no effect on OXP-I rates at 20 and 25 °C, indicating that the outer membrane remained intact for both species at these temperatures (data not shown).

**Figure 5.3:** Mean respirational flux (*N*=6 for each species) in permeabilised heart fibers of (A) *O. catharus* and (B) *C. japonica* at 20 °C (light grey bars), 25 °C (medium grey bars) and 30 °C (dark grey bars).
30 °C (dark grey bars). See methods and figure 5.1 for respiration state details. The asterisks denote a significant difference between temperatures for each species at a given respiration state at $p \leq 0.05$. The plus sign denotes a significant difference between *O. catharus* and *C. japonica* a given respiration state at $p \leq 0.05$.

**Table 5.1:** Ratios based on mitochondrial respirational flux in *O. catharus* and *C. japonica* at 20 °C, 25 °C and 30 °C. CCO/OXP-I and CCO/ETS provide comparative measures of cytochrome c oxidase (Papa et al., 2008) capacity relative to phosphorylation (OXP-I) or the electron transport system (ETS) respectively. The ETS/Leak-I, II ratio provides a measure of ETS capacity relative to the leak respiration state when phosphorylation is inhibited by atracyloside (Leak-I, II). OXP-I, II/OXP-I %age values indicate the capacity of CI compared to total phosphorylation due to CI and CII substrate phosphorylation (OXP-I, II).

<table>
<thead>
<tr>
<th>State</th>
<th><em>O. catharus</em></th>
<th><em>C. japonica</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20 °C</td>
<td>25 °C</td>
</tr>
<tr>
<td>CCO / OXP-I</td>
<td>1.53 ± 0.05</td>
<td>1.46 ± 0.09</td>
</tr>
<tr>
<td>CCO / ETS</td>
<td>1.61 ± 0.06</td>
<td>1.63 ± 0.14</td>
</tr>
<tr>
<td>ETS / Leak-I, II</td>
<td>2.56 ± 0.39*</td>
<td>1.50 ± 0.15</td>
</tr>
<tr>
<td>OXP-I, II / OXP-I (%)</td>
<td>21.53 ± 1.74</td>
<td>21.70 ± 3.08</td>
</tr>
</tbody>
</table>

Values are means ± s.e.m. (N=6 for each species at each temperature).
* Significant difference between temperatures within a species ($p \leq 0.05$).
+ Significant difference between species at each temperature ($p \leq 0.05$)

OXP flux on CI and CII substrates (OXP-I, II) were comparable for all temperatures in *O. catharus* (figure 5.3A), however for *C. japonica*, flux rates were significantly lower at 20 °C relative to those at 25 °C and 30 °C ($p \leq 0.05$, figure 5.3B). Changes in flux with transition from OXP-I and OXP-I, II differed at 20 °C and 25 °C for each species while at 30 °C OXP-I, II flux rates were comparable (figure 5.3A, B). Comparatively, the Leak-I, II flux was significantly lower at equivalent temperatures in *C. japonica* compared to Leak-I, II flux in *O. catharus* (figure 5.3B). Although within *O. catharus* Leak-I, II fluxes at 20 °C differed from those at 25 °C and 30 °C (figure 5.3A), in *C. japonica* Leak-I, II fluxes differed at all
temperatures ($p \leq 0.05$, figure 5.3B). In both species the respiratory control ratio-II (OXP-I, II/Leak-I, II) was significantly lower at 30 °C compared to ratios that occurred at 20 °C (figure 5.4B). The RCR-II was also consistently lower in *O. catharus* compared to *C. japonica* at equivalent temperatures ($p \leq 0.05$, figure 5.4B). Additionally, the contribution of CII oxidation towards total CI and CII oxidation was in the range of 16.5 - 22.3% except in *O. catharus* at 30 °C when this contribution was substantially elevated up to ~44.4% ($p \leq 0.05$, table 5.1).

![Bar chart](image1)

**Figure 5.4:** (A) Mean RCR-I (OXP-I/Leak-I, $N=6$ for each species) values representing the respiratory control ratios determined with CI substrates proline and malate with and without ADP. (B) Mean RCR-II (OXP-I, II/Leak-I, II, $N=6$ for each species) values determined with proline, malate and the CII substrate succinate in the presence of atractyloside. Ratios shown for *O. catharus* (black bars) and *C. japonica* (grey bars) at 20 °C, 25 °C and 30 °C. Means sharing the same letter of the same case are not significantly different from one another at $p \leq 0.05$. 
The addition of the uncoupling agent FCCP did not lead to any significant changes within and between species except at 20 °C (figure 5.3A, B). This suggests that OXP-I, II matches electron transport flux (ETS). Comparison of the ETS flux relative to the atracyloside induced Leak-I, II states indicate that the phosphorylation system was only limiting for \textit{O. catharus} at 20 °C \((p \leq 0.05, \text{table 5.1; ETS/Leak-I, II})\), whereas this system was limiting at all temperatures in \textit{C. japonica}. Between species, flux due to CCO was significantly higher at all temperatures in \textit{O. catharus} where the highest flux occurred at 30 °C \((p \leq 0.05, \text{figure 5.3A})\). Although CCO flux was consistently higher relative to OXP-I, OXP-I, II and ETS flux rates for both species, the ratio of CCO/ETS and OXP-I was considerably higher in \textit{O. catharus} at 30 °C \((p \leq 0.05, \text{table 5.1})\) and increased with temperature, while this ratio decreased with temperature for \textit{C. japonica}.

5.3.2.1 Complex I flux stability

\textbf{Figure 5.5:} Mean CI respiration (OXP-I, with the addition of ADP to CI substrates) after 5, 10, 15, 20, 25, 30 minutes and CI, CII respiration (OXP-I, II, with the addition of succinate after 30 minutes CI respiring) expressed as a percentage of initial CI respiration (at 0 minutes) in \textit{O. catharus} (coloured black circles) and \textit{C. japonica} (clear white circles). Means sharing the same letter of the same case are not significantly different from one another at \(p \leq 0.05\).
Incubation of phosphorylating fibres at 30 °C with CI substrates showed that for *O. catharus* OXP-I flux declined by 14% after only five minutes, and lost 56% CI derived flux over 30 minutes (figure 5.5). Flux values with the addition of succinate at the end of the CI assay were significantly similar to OXP-I respiration at the beginning of the assay, indicating that CII was still relatively intact (*p* ≥ 0.05, figure 5.5). *C. japonica* maintained its integrity for a longer period, where flux was significantly depressed from initial OXP-I flux only after 15 minutes of ADP addition, and was depressed by 21.1% at 30 minutes. This species also indicated an intact CII, as flux values were similar to those of OXP-I respiration at the beginning of the assay (figure 5.5).

### 5.3.3 Enzyme and metabolites

Table 5.2: Enzyme activities and metabolite concentrations in the heart and haemolymph of control and temperature exposed (experimental) *O. catharus* and *C. japonica*.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Enzyme Activity/ [Metabolite]</th>
<th><em>O. catharus</em></th>
<th><em>C. japonica</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>Experimental</td>
</tr>
<tr>
<td>Heart</td>
<td>Citrate Synthase (U/mg tissue)</td>
<td>7.34 ± 1.45 A</td>
<td>7.92 ± 0.46 A</td>
</tr>
<tr>
<td>Heart</td>
<td>Lactate Dehydrogenase (U/mg tissue)</td>
<td>5.31 ± 0.57 A</td>
<td>5.89 ± 0.82 A</td>
</tr>
<tr>
<td>Haemolymph</td>
<td>Lactate Dehydrogenase (U/µl haemolymph)</td>
<td>0.013 ± 0.003 A</td>
<td>0.021 ± 0.004 A</td>
</tr>
<tr>
<td>Haemolymph</td>
<td>Lactate (µM)</td>
<td>104.71 ± 9.62 A</td>
<td>132.64 ± 17.24 A</td>
</tr>
</tbody>
</table>

Values are means ± s.e.m. (*N* = 6 for each species at each temperature).

Within a species, means sharing the same letter of the same case are not significantly different from one another (*p* ≤ 0.05)

* Significant difference between species at each temperature (*p* ≤ 0.05)

Since there were no significant differences between species, in the activity of the mitochondrial mass marker citrate synthase (CS) (*p* ≥ 0.05), mitochondrial respiratory flux
was not corrected for CS (table 5.2). Similarly, lactate dehydrogenase (LDH) activity in the heart showed no changes with temperature exposure within and between species \((p \geq 0.05)\), however activity was much higher in the heart than that in the haemolymph (table 5.2). LDH activity in the haemolymph of \(C. japonica\) was significantly higher \((p \leq 0.05)\) than in \(O. catharus\) but no significant changes occurred with temperature exposure in both species. With temperature exposure, lactate concentration in the haemolymph also remained constant in \(O. catharus\) and was comparable to control lactate levels in the haemolymph of \(C. japonica\). However, haemolymph from temperature exposed crabs of both species exhibited a significantly higher concentration of lactate \((p \leq 0.05)\;\text{table 5.2}).

5.4 Discussion
Temperature is a governing factor in species survival, and this is now becoming more pertinent with the potential for increased global oceanic temperatures (Hochachka and Somero, 1984; Pörtner and Knust, 2007). Here we show clear differences in thermal tolerance capacities of cardiac and mitochondrial function in two paddle crab species. These differences may provide mechanistic explanations and predictive value for each species’ current and future coastal distributions around New Zealand.

5.4.1 Cardiac function in \(O. catharus\) and \(C. japonica\)
The observed increase in heart rate with elevated temperature (figure 5.2A) was expected for these ectotherms (Lillywhite et al., 1999) and this has been reported for other species of crabs (Ahsanullah and Newell, 1971; De Wachter and McMahon, 1996). It was noted that the \(Q_{10}\) values lie within the upper values of those recorded for ectotherm heart rates (1.5 - 4) (Ahsanullah and Newell, 1971; Burton et al., 1980; Reiber and Birchard, 1993; Zainal et al., 1992). Given the greater response to increasing temperature, data from this study suggests that \(O. catharus\) is more stenothermal, than \(C. japonica\) (figure 5.2A insert). This fits the concept that high \(Q_{10}\) values reflect a stenothermal species (De Wachter and McMahon, 1996).

Each species showed different strategies to accommodate elevated temperatures. \(C. japonica\) showed greater plasticity in heartbeat duration, or contraction rate with increasing temperatures, while \(O. catharus\) was more inclined to increase heart rate, and already had a shorter more rapid contraction at 19 °C (figure 5.2A, B). Given that \(O. catharus\) was
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moribund at 30 °C, and *C. japonica* was not, one could assume that the strategy adopted by *O. catharus* is less effective, and a lack of contractile plasticity in *O. catharus* may indicate a limit for this species at elevated temperatures.

Although beat duration, or contraction rate, became quicker with temperature (figure 5.2B), it remains unclear if elevated heart rate increased cardiac output, as stroke volumes were not determined. Elevated temperature has been shown to increase cardiac output while decreasing stroke volume in *Cancer magister* (De Wachter and McMahon, 1996). The similar cardio-somatic indices for both species suggests that cardiac pumping requirements may be similar for each species, and at maximum temperatures both crab species showed similar contraction rates. The more dramatic increase in heart beat duration with equivalent contraction rate in *O. catharus* suggests that greater demands are being placed on this species’ heart, and that these demands are not being met. These species differences may result from factors intrinsic (excitation contraction coupling, mitochondrial function) and extrinsic (cardioregulatory nerves, humoral neurohormones (Wachter and Wilkens, 1996) to the heart and warrant further examination (figure 5.2A).

5.4.2 The effects of temperature on mitochondrial respiration

Although the respiratory rates were found to be similar across species, particular differences were apparent between species that were dependent on, and independent of temperature. The most obvious difference between species was Leak-I respirational flux. This was highest for *O. catharus* at all temperatures (figure 5.3A). Leak-I suggests greater inner membrane permeability in this species and the lower RCR values with increasing temperature (figure 5.4A) indicate a probable loss of membrane potential and uncoupling from the OXP. This was also observed in fish heart fibres respiring at elevated temperatures (Hilton et al., 2010). A species difference in OXP coupling was further shown after uncoupling with the proton ionophore FCCP, which disrupts the proton gradient and uncouples the ETS from ATP production (Dahlhoff and Somero, 1993; Lehninger et al., 1995). In *O. catharus* a significant increase in flux (ETS/Leak-I, II, table 5.1) was only evident at 20 and 25 °C, while *C. japonica* increased flux at all temperatures. This further shows a greater integrity of inner mitochondrial membranes in *C. japonica*.
Although both species significantly lowered RCR values with CI and combined CI and CII substrates as temperature increased, *C. japonica* always maintained higher RCR values compared to *O. catharus* at each temperature (figure 5.4A, B). This suggests more tightly coupled mitochondria in *O. catharus* (Johnston et al., 1994). Indeed even at 25 °C the Leak-I rate accounts for ~80% of OXP respiration on CI substrates, and at 30 °C this flux is equivalent (figure 5.3B). Elevated temperature-induced drops in the RCR has been shown in the Atlantic wolfish *Anarhichas lupus* (Lemieux et al., 2010b) and three species of New Zealand triplefin (Hilton et al., 2010). In the latter study, decreased mitochondrial RCR values determined with permeabilised ventricle fibres were less pronounced with increasing temperature in a eurythermal rockpool species, and this was assumed to indicate a greater thermostability.

Increased Leak-I drives the RCR-I indices down, and this has been interpreted to suggest a lesser phosphorylation capacity (Brand, 1990). In this study it may also reflect an apparent loss of efficiency for ATP synthesis in *O. catharus* heart muscle relative to *C. japonica*. However, in mammalian pathological models, “leaky” mitochondria have been shown to still drive phosphorylation adequately. This is assumed to occur as proton conductance is non-Ohmic (Rolfe and Brand, 1997), whereby mitochondrial membrane potentials need not be particularly high to maintain phosphorylation (Benard et al., 2010).

Additionally, it was shown that CI loses function over time at 30 °C in both species, yet it does so at a greater rate in *O. catharus* (figure 5.5). Infusion of succinate then shows that CII flux remains intact at elevated temperatures. CI is a multimeric protein composed of up to 40 to 47 subunits in mammals (Papa et al., 2008) where structural similarities between yeast and mammalian CI complexes would suggest that super structures may be similar for ectotherms (Liò, 2005). Mammalian CI is prone to ischemic damage (Mittal et al., 2009; Papa et al., 2008), most likely resulting from reactive oxygen species (ROS) (Murphy, 2009). CI dysfunction at elevated temperatures in ectotherms could partly evolve from ROS release once CI is damaged. Although these data indicate that CI may also be labile in ectotherms, decreased CI flux can result from increased proton leakage that decreases membrane potentials. Mitochondrial uncoupling agents such as FCCP can depress respiration in ectotherms (Hilton et al., 2010) potentially because electrogenically imported substrates require a membrane potential (Andrei and Vera, 2001), and proline import is ultimately
dependent on the membrane potential (Guignard et al., 1984). Therefore CI may fail due to impaired substrate supply as a result of depressed membrane potentials and/or CI dysfunction due to ROS release at elevated temperatures (figure 5.5).

CII function remains stable at high temperature (figure 5.5), potentially as this complex is more robust and/or because CII is fuelled by succinate, where the import of succinate is not electrogenic (Pon et al., 1989). Problematically perhaps, CII is also less coupled to OXP, as it does not contribute directly to the proton gradient (note it does through CIII and CIV, and this explains why RCR-II is lower than RCR-I (figure 5.4A, B)), and theoretically succinate electrons produce only 2/3rds of the ATP of CI derived electrons. Consequently a loss in CI function should impact OXP efficiencies and elevate the cellular redox state (NADH/NAD). In mammals, an elevated redox state can also promote apoptosis (Ueda et al., 2002).

*O. catharus* had higher CCO flux at all temperatures compared to *C. japonica* and was significantly higher at 30 °C compared to flux observed at 20 °C and 25 °C (figure 5.3A, B), which indicates elevated concentrations of CCO, or different kinetics in *O. catharus*. Interestingly a scope to increase CCO flux was apparent in the more stenothermal *O. catharus* than in *C. japonica* and this is surprising as previous work exploring permeabilised triplefin fish heart fibres showed that a greater CCO capacity was present in a eurythermal rockpool species (Hilton et al., 2010). Moreover the activities of CCO in *C. japonica* suggest that CCO flux limits respiratory flux (figure 5.3B, table 5.1, i.e. it may restrict flux), as the relative contribution of CCO to respirational flux (CCO/OXP, CCO/ETS) increases in *O. catharus*, while it decreases in *C. japonica* (table 5.1). Paradoxically this should restrict respirational flux in *C. japonica* by limiting the flux through the ETS (Gnaiger et al., 1998). At least in mammals CCO is regulated by ATP (Beauvoit and Rigoulet, 2001), nitric oxide (Antunes et al., 2004), hydrogen sulphide (Kabil and Banerjee, 2010), carbon monoxide (Zuckerbraun et al., 2007) and oxygen (Chandel et al., 1996). As such species differences may reflect regulatory differences in *vivo*, but may also reflect thermally mediated alterations in membrane fluidities (Lemieux et al., 2008). Indeed these are the first data comparing CCO flux relative to ETS flux for crustaceans and the data presented here show clear differences between species.
5.4.3 Enzymatic and metabolite activities

Citrate synthase (CS) introduces acetate into the citric acid cycle and provides a useful measure of overall aerobic flux and mitochondrial mass within tissues (Hickey and Clements, 2003; Moyes, 1996). CS activity did not differ between control vs. temperature exposed crabs for either species, nor between species (table 5.2). This suggests that each species may have similar mitochondrial masses per gram of heart tissue and that the respirational differences discussed above result from changes within the mitochondria. Although lactate dehydrogenase (LDH) activity in the heart was similar in both species, higher haemolymph lactate values were measured in *C. japonica* and the relative increase in lactate between control and temperature exposed was almost 20% greater in *C. japonica*. Assuming lactate transport is equivalent in each species, these data suggest a greater capacity to produce lactate in *C. japonica* and therefore supply ATP by anaerobic glycolysis (table 5.2, (Lallier and Walsh, 1991).

LDH activity in serum is commonly used as an indicator of cardiac infarction and cell lysis, and clear differences were observed between species. Although *C. japonica* had greater LDH activity in the haemolymph in control and post-heat stress (table 5.2), LDH was 12.5% greater in temperature exposed *O. catharus* haemolymph compared to control crabs of the same species. This perhaps indicates a greater relative cellular disruption occurred in *O. catharus* exposed to thermal challenge.

5.4.4 Conclusion

Based on this study, the distributions of the native crab *O. catharus* and the invasive species *C. japonica* within New Zealand shores may be defined by thermal tolerance. The data presented here provides evidence that heart and mitochondrial function/integrity may in part account for these limitations. These data further suggest that mitochondrial dysfunction may precede heart failure in *O. catharus*. If heart mitochondria do define species robustness to thermal stress, *C. japonica* appears to be the more eurythermal species and should have a competitive advantage over *O. catharus* in context of climate change mediated increases in ocean temperature.
6. CHAPTER 6 - GENERAL DISCUSSION & CONCLUSIONS

The primary aim of this thesis was to understand if cardiac mitochondria contribute to heat stress induced HF in marine ectotherms. The previous chapters have attempted to resolve the cardiac mitochondrial mechanisms that are compromised prior to HF in a temperate teleost. Additionally, this thesis also aimed to test whether the point of cardiac mitochondrial failure, $T_{mt}$, was similar relative to $T_{HF}$, in other teleosts from different thermal habitats, and how plastic $T_{mt}$ is with acclimation. This was considered important for predicting species capacities if oceans are to become warmer.

6.1 Heat stress driven HF in ectotherms

The cause of HF within ectotherms as environmental temperatures rise has been generally attributed to a lack of adequate oxygen supply (Clark et al., 2008; Pörtner and Farrell, 2008; Pörtner and Knust, 2007; Pörtner et al., 2004). Some of these studies have deduced that oxygen limitation at warm temperatures commences at temperatures below that at which the heart ceases to function adequately (Frederich and Pörtner, 2000; Pörtner, 2001; Pörtner and Farrell, 2008; Pörtner and Knust, 2007; Pörtner et al., 2004; Zielinski and Pörtner, 2000). Specifically, the decrease in arterial $pO_2$ in fish exposed to increasing temperatures is postulated to result from limits on oxygen delivery to the gills, and/or the diffusion of oxygen across the gills into the blood (Farrell et al., 2009). However, previous studies have shown that the arterial $pO_2$ can remain unchanged as fish were exposed to progressively increasing temperatures (Sartoris et al., 2003; Steinhausen et al., 2008), and even under conditions when oxygen delivery to tissues were compromised (Clark et al., 2008; Farrell, 2007). Results from Chapter 2 show that when Notolabrus celidotus was exposed to warming waters, oxygen saturation of haemoglobin within and around the heart was not oxygen restricted (figure 2.4).

Cardiac output (CO) is the product of heart rate and stroke volume, and in fishes facing situations of high oxygen demand (e.g. heat stress), CO increases are largely met through increased stroke volumes (Farrell and Jones, 1992; Franklin and Davie, 1992; Willmer et al., 2005). A clear heart rate response to temperature was not observed in N. celidotus because a minimal increase was observed until $T_{HF}$ at 27.81 ± 0.39 °C (figure 2.3). Likewise, heart rate modulation was also less obvious in the tropical wrasse Thalassoma lunare, and the cold-temperate wrasse N. fucicola, until a $T_{HF}$ of 32.8 ± 0.3 °C, and 21.7 ± 0.3 °C, respectively.
(figure 3.1). This suggested that much of the change in heart function results from stroke volume modulation.

Given the greater dependence on stroke volume modulation in many fishes, a sudden loss of heart function and the occurrence of HF after maintaining a relatively constant heart rate with increasing temperature may perhaps be explained by Laplace’s law. In its simplest form when assuming that the heart is a sphere, this law states that $T = (P \times R)/M$, where the tension ($T$) that can be developed by a heart with a ventricle wall of defined thickness ($M$), is related to pressure ($P$), and importantly the radius ($R$) of the lumen. Pressure development within the heart is therefore disproportionately influenced by ventricular radius. As this increases, a disproportionate amount of tension and contractile work must occur in order to maintain the same ejection pressures as the heart pumps more blood with increasing stroke volume.

To maintain myocardial pressure, fish myocytes can extend up to 40% to accommodate large blood volumes in conditions that require increased cardiac output (Shields et al., 2006), such as during heat stress. The increased stretch of cardiac myofibrils is contended to expose a larger number of myosin ATP binding sites, which elevates ATP hydrolysis and force production. Thus stretching of the cardiomyocyte provides a molecular explanation for Starling’s law of the heart, where the force of contraction increases proportionally to diastolic stretch, and this elevates ATP demand (Saks et al., 2006). Therefore, additional to thermodynamic mediated increases in ATP turnover with increasing temperatures, physical constraints enforced by Laplace’s and Starling’s laws likely drive the heat stressed fish heart to demand more ATP. The ability for cardiac mitochondria to sustain continual ATP supply under heat stress becomes paramount, yet as shown in this thesis the mitochondria of wrasses are shown to become less efficient at synthesising ATP with rising temperatures.

In contrast, the paddle crabs *Ovalipes catharus* and *Charybdis japonica* increased their heart rates with elevated temperature (figure 5.1 A), and this expected increase has previously been reported for other species of crabs (Ahsanullah and Newell, 1971; De Wachter and McMahon, 1996). Furthermore, each species utilised varying strategies to accommodate elevated temperatures. *C. japonica* showed greater plasticity in heartbeat duration and beat rate with increasing temperatures, whilst *O. catharus* was more inclined to increase beat rate, and already had a shorter more rapid contraction at 19 °C (figure 5.1 A, B). Given that *O.
catharus was moribund at 30 °C, and C. japonica was not, one could assume that the strategy adopted by O. catharus is less effective with rising temperatures. The lesser contractile plasticity and inability of O. catharus heart function to adapt may in part promote its replacement by the invasive C. japonica as ocean temperatures increase.

6.2 Is the hot heart limited by mitochondria? (Chapter 2)

The results of this chapter are first to provide evidence that mitochondrial dysfunction with increasing environmental temperature is a contributing factor to HF in fish. The contribution of mitochondrial dysfunction to HF has been discounted by some researchers as respiration rates (or oxygen flux) at T\text{max} appeared to be robust at temperatures well above the T\text{HF} of many ectotherms (Somero, 2002; Somero et al., 1996).

The main findings of this study showed that several mitochondrial components were compromised before the T\text{HF} (above 27.5 °C) of N. celidotus, and most importantly at saturating oxygen levels. Membrane leakiness (indicated by leak-I) increased by 25 °C (figure 2.6 A) and significantly depressed RCRs from this temperature upwards (figure 2.7 A), suggesting increased oxygen flux rates are required to maintain mitochondrial membrane potentials. Additionally the depressed RCR indices predict decreased phosphorylation efficiencies and capacities (Brand, 1990; Iftikar et al., 2010; Seebacher et al., 2010). The integrity of the outer mitochondrial membrane was also compromised above 20 °C as respiration following cytochrome c addition increased before T\text{HF} (figure 2.7 B). The loss of outer mitochondrial permeability leads to the release and depletion of cytochrome c from mitochondria (Borutaite and Brown, 2003; Hand and Menze, 2008). A substantial loss of cytochrome c depresses OXP flux, potentially affecting superoxide scavenging (Mailer, 1990) and may promote intrinsic apoptosis.

Although the affinity of primary CI substrates did not change at T\text{HF} (figure 2.9 A), V\text{max} rates (figure 2.9 B) and the capacity for the ETS to uncouple (indicated by the RCR, UCR, figure. 2.7 A; FCR, Table 2.3) were altered by 25 °C. Most importantly, the capacity to efficiently produce ATP in the heart was limited at 25 °C (figure 2.10), which is below the acute T\text{HF} for N. celidotus. Decreases in the mitochondrial ATP production and in the transfer of energy through the phospho-transfer kinases contributes to HF in mammalian cardiac muscle (Ventura-Clapier et al., 2004). This chapter directly tested ATP production from heart
mitochondrial fibres with increasing temperature and found that ATP production at 25 °C was lower than synthesis rates at 20 °C (figure 2.10 A). ATP demands will likely increase with rising temperatures due to increased demands on cardiac output and simple thermodynamic effects on ATPases in general. These data are the first to directly show that the fish heart is limited by depressed mitochondrial ATP production as $T_{HF}$ approaches.

The deprivation of energy plays a major role in HF in the mammalian model. In this model, metabolism of the heart is determined by substrate utilisation, oxidative phosphorylation and ATP transfer/utilisation of the mitochondria (Neubauer, 2007). Inadequacy or a break down in any or all of these components will contribute to HF. Notably all mitochondrial components were studied at maximum oxygen saturation. Therefore, the changes observed were a direct impact of elevated temperature stress. Heat stress can further limit contractile function of the heart due to inadequate ATP production leading to mechanical failure (Neubauer, 2007). However, further investigation of ATP transfer/utilisation of cardiac mitochondria is needed, as the balance between ATP consumption and production remains unmeasured. Given the depression in ATP production and RCRs, and potential apoptotic signals from cytochrome $c$ and NADH, a general theme of altered mitochondrial function and stability prior to $T_{HF}$ was apparent. A top-down study in a non-model fish species has successfully provided evidence that the mitochondrial dysfunction may well cause HF as environmental temperatures increase.

6.3 Species limitations in a changing climate (Chapter 3)

An understanding of differences in thermal tolerances among related taxa with varying thermal niches would provide information on how the effects of ocean warming on temperature-related geographic distributions may change for a given species (Pörtner, 2002). This chapter compares the cold temperate $N. fucicola$, temperate $N. celidotus$, and tropical $T. lunare$, and shows that while mechanisms differ, some form of cardiac mitochondrial dysfunction also occurs prior to HF. Importantly, mitochondria and heart function in species with narrow thermal niches, in particular, $T. lunare$ and $N. fucicola$, were more susceptible to heat stress.

The thermal scope of cardiac function was lowest in the tropical wrasse $T. lunare$ (figure 3.1). Plasma metabolite profiles in this species shifted the most with the greatest relative succinate
accumulation in heat stressed fish, indicating an inhibition of mitochondria and anaerobiosis (figure 3.2, table 3.2). The RCR-II also altered substantially just above $T_{\text{tank}}$ for $T_{\text{lunare}}$ and $N. \text{celidotus}$ indicating that OXP was compromised below $T_{\text{HF}}$ (figure 2.4 D). The RCR-II was determined from the OXP-I, II state, and this is more likely to closely reflect mitochondrial function and OXP efficiencies in vivo. Part of CII is succinate dehydrogenase which is reported to be sensitive to heat stress in ectotherm mitochondria (O'Brien et al., 1991). This perhaps explains the more prominent inflexion in the RCR-II and decreased responsiveness of CII mediated flux with increasing temperature in $T. \text{lunare}$, relative to CI flux (figure 3.5 B, C). Only in this maximally fuelled state was this inflexion apparent. Heart mitochondria from the tropical $T. \text{lunare}$, and to a lesser extent the temperate $N. \text{celidotus}$, were also less able to chemically uncouple with increasing assay temperatures prior to $T_{\text{HF}}$ (figure 3.6 A, B). This indicates a loss in ETS reserve capacity, and approach of maximal capacity to generate mitochondrial membrane potentials, with increasing temperatures in these fish.

While $N. \text{fucicola}$ showed more stable cardiac mitochondria in the in situ assays across temperatures, mitochondria of this species were the most perturbed following in vivo acute heat stress (figure 3.3). It is known that in vitro mild heat stress will depress OXP coupling, which can rectify on cooling (Žūkienė et al., 2010). However, extreme heat stress will cause irreversible changes in mitochondrial inner membrane integrity in the mammalian heart (Brand and Nicholls, 2011; Žūkienė et al., 2010). This causes increased inner mitochondrial membrane proton leakage, and thereby decreases OXP efficiency. Here it resulted in a significant lowering of the RCR-I in $N. \text{fucicola}$ when assayed in vitro (figure 3.3 A), and sustained depression of ETS and CCO fluxes (figure 3.3 C, D). Additionally, with increasing in situ assay temperatures, CI respiration in $N. \text{fucicola}$ showed a diminished response (figure 3.5 A). This indicates that CI is more thermo-labile in this cold temperate species.

Overall, this chapter found that $T_{\text{mt}}$ also occurs below $T_{\text{HF}}$ for both, $N. \text{fucicola}$ and $T. \text{lunare}$, which generally inhabit more stable thermal niches. Importantly, these altered cardiac mitochondrial conditions manifest at saturating oxygen concentrations indicating again that cardiac mitochondria can become impaired without invoking oxygen limitation. These data provide support that heat stress can mediate cardiac mitochondrial insufficiency in $N.$
fucicola, N. celidotus and T. lunare, and therefore, cardiac mitochondria can provide insight at least within wrasse species as environmental temperatures increase.

6.4 Thermal acclimation of cardiac mitochondria (Chapter 4)

Ectotherm species occupy thermal environments that fluctuate with season. Acclimating to average seasonal temperatures requires plasticity in thermal tolerance by altering biochemical and metabolic processes so that performance can be optimised or at least maintained (Niehaus et al., 2012; Rantin et al., 2007). This chapter provided insights to the impacts of acclimation to seasonal variations in temperature on cardiac mitochondrial function in N. celidotus.

The acclimation capacity of cardiac mitochondria was tested in fish acclimated to average winter and summer sea surface temperatures of 15 °C (cold acclimated, CA) and 21 °C (warm acclimated, WA), respectively. The most important observations were that WA compromises cardiac mitochondria from N. celidotus more than those from CA fish, and mitochondrial dysfunction occurred below temperatures where the heart fails in WA fish. Our data also revealed the ability of CA fish to maintain a broader cardiac scope, compared to WA fish. WA fish also appeared to be closer to their aerobic limits at their summer temperatures.

As assay temperatures increased, a compromised OXP was demonstrated by a decreased capacity to increase OXP-I, along with significantly depressed RCR-I values at 20 °C (figure 4.3 B, D), and an altered RCR-II at T_HF (figure 4.4. C). On comparison, acclimating N. celidotus to winter temperatures appears to protect heart mitochondrial CI from heat induced stress as demonstrated by robust OXP-I rates and RCR-I values even as assay temperatures became warmer (figure 4.3 B-D). Although Leak-I, II rates remained comparable in both groups (figure 4.4 B), the inability of WA N. celidotus to uncouple heart mitochondria with increasing assay temperature may lower OXP capacities by 25 °C, which is prior to HF in these fish (figure 4.4 C). In contrast, the more coupled mitochondria observed in CA fish decreases mitochondrial proton leakage and therefore, prevents energy loss at low temperatures (figure 4.5 A). CI and CII respiration in WA fish showed a diminished response as assay temperatures increased (figure 4.5 A, B), indicating decreased cardiac mitochondrial robustness in these fish. Therefore, WA fish are already facing energetic costs at their summer temperatures. The inability of WA fish cardiac mitochondria to overcome thermal
stress may become a greater challenge for *N. celidotus* as oceans become warmer. This opens an area for future inquiry about the governing mechanisms that underlie the variable capacities of eurytherms to acclimate as they face global warming.

### 6.5 A mitochondrial advantage for invasive species in a warming ocean (Chapter 5)

As oceans become warmer, disruption of thermal habitats may outstrip some species capacities to adapt to temperature stress, and as a result may decrease the ranges of some native species (Helmuth et al., 2002) while increasing the ranges of invasive eurythermal species (Walther et al., 2002). This chapter illustrates that the distributions within New Zealand shores of the native crab, *O. catharus*, and the invasive species, *C. japonica*, could be determined by thermal tolerance. Further evidence was provided that heart and mitochondrial function/integrity may in part account for different thermal tolerance capacities.

Although the respiratory rates were similar across species, particular differences were apparent between species that were dependent on temperature. The most obvious difference between species was that Leak-I that was highest for *O. catharus* at all temperatures measured (figure 5.3 A). Leak-I suggests greater inner membrane permeability in this species and the lower RCR values with increasing temperature (figure 5.4 A) indicate a probable loss of membrane potential and uncoupling from OXP. Although, both species significantly lowered RCR-I and RCR-II as temperature increased, *C. japonica* always maintained higher RCR values, compared to *O. catharus*, at each temperature (figure 5.4 A, B). This suggests greater thermostability in the cardiac mitochondria of *C. japonica* (Johnston et al., 1994).

Additionally, CI loses function over time at 30 °C in both species, yet it does so at a greater rate in *O. catharus* (figure 5.5). Mammalian CI is prone to oxidative damage (Murphy, 2009; Papa et al., 2008), most likely resulting from reactive species (Murphy, 2009). Although these data indicate that CI may be labile in ectotherms, decreased CI flux can also result from increased proton leakage that decreases membrane potentials, which are also required for substrate uptake. Mitochondrial uncoupling agents such as FCCP can depress respiration in ectotherms (Hilton et al., 2010) potentially because electrogenically imported substrates require a membrane potential (Andrei and Vera, 2001), and proline import is ultimately dependent on the membrane potential (Guignard et al., 1984). Therefore CI in *O. catharus*
may fail due to impaired substrate supply as a result of depressed membrane potentials (figure 5.5). Furthermore, LDH activity in serum is commonly used as an indicator of cardiac infarction and cell lysis, and clear differences were observed between species. Although *C. japonica* had greater LDH activity in the haemolymph in control and post-heat stress (Table 5.2), LDH was 12.5% greater in temperature exposed *O. catharus* haemolymph compared to control crabs of the same species. This perhaps indicates a greater relative cellular disruption occurred in *O. catharus* exposed to thermal challenge.

The differences in mitochondrial function between *O. catharus* and *C. japonica* also provide mechanistic explanations and predictive value for each species’ current and future coastal distributions around New Zealand. Data from this chapter suggests that mitochondrial dysfunction may precede HF in *O. catharus*. If heart mitochondria do define species robustness to thermal stress, *C. japonica* appeared to be the more eurythermal species and should have a competitive advantage over *O. catharus* in context of climate change mediated increases in sea temperature.

### 6.6 Limitations of the thesis

The primary limitation of this thesis was the lack of ATP production measurements in Chapter 3, Chapter 4 and Chapter 5. The sensors and assays to detect ATP production were only developed after these studies had taken place and to reassess ATP production in these studies were beyond the financial capacity of this project. Likewise, the assay and sensors to detect ROS were initially unavailable and not developed when the study with crabs in Chapter 5 was carried out.

Furthermore additional measures may have provided information about the integrity of heart mitochondria and oxidative phosphorylation in the context of increasing temperature. In all the chapters, a measure of the activities of individual reactions of the ETS (Complexes I-IV and ATP synthase) with increasing temperature would have provided information about the thermal sensitivity of the components of the ETS (see Lemieux et al. (2010a) for methodology). These activities are measured in isolated mitochondria and this proved to be a challenge due to the extremely small mass of *N. celidotus* and *T. lunare* hearts. A large sample (~0.5-1g) is needed for a pure sample of isolated mitochondria and the average heart size in *N. celidotus* was ~15 mg. Additionally, although not the focus of this thesis, the ability
to non-invasively measure stroke volume of the fish heart would have greatly helped to determine the cardiac output during the cardiac function experiments.

In retrospect, another important contribution in this study would have been to investigate whether species living in the more extreme habitats, i.e. the tropical *T. lunare* and cold-temperate *N. fucicola*, were able to acclimate to their winter minimum and summer maximum temperatures and if this shifted their heart mitochondrial capacity. When *N. celidotus* was acclimated to its extreme temperatures, this was conducted at the established acclimation facilities that were in place at Leigh marine laboratory (Chapter 4). A similar system was unavailable at Moreton Bay Research Station and Portobello Marine Laboratory where experiments were conducted for *T. lunare* and *N. fucicola*, respectively. Additionally transporting these fish to Leigh Marine Laboratory would not have been feasible due to the large financial cost and animal survivability during transportation.

### 6.7 Future directions

Global climate change models predict that over the next 100 years tropical ocean temperatures will rise by 3 °C (Solomon et al., 2007). However, the pH of oceans is predicted to also drop by approximately 0.3-0.4 units due to rising CO₂ levels (Pörtner, 2008). Most climate change research examining the physiological changes in fish species has focused primarily on the effects of increasing water temperatures on tolerance mechanisms. Additionally, there has also been a large influx of data on the effects of ocean acidification on tropical marine fish. However, the synergistic effects of high water temperatures and CO₂ concentrations that marine fish are increasingly likely to encounter with the progression of climate change has yet to be examined at the individual species level.

Until recently, most physiological research on the impacts of climate change on fish species has mainly focused on characterising the ‘aerobic scope’, the aerobic performance across a range of temperatures. Fish can optimally perform at a specific range of temperatures outside of which survival is limited which dictates species distributions. At the time of this thesis, the specific biochemical and molecular mechanisms governing aerobic scope in fish remains to be investigated. Specifically, the thermal limitation on fishes performing at their maximal aerobic capacity needs to be investigated. In fishes, temperature is shown to influence the storage and utilisation of ATP required for burst activity, and the production and removal of
metabolic end products such as lactate that are released from burst activity (Kieffer, 2000). Therefore, cardiac mitochondria can metabolically limit fishes experiencing burst activity with increases in environmental temperature. Moreover, further investigation is also needed to elucidate whether fish performance under the stress of increased water temperatures and CO$_2$ is limited at the mitochondrial level. It is currently unclear if mitochondria can functionally remodel to maintain ATP production and therefore, cope with the effects of increased temperatures and elevated environmental CO$_2$. 
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