Thermal tolerance in teleost fish – importance of cardiac oxygen supply, ATP production and autonomic control

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The opponent is Professor Anthony Kurt Gamperl, Department of Ocean Sciences, Memorial University of Newfoundland, Canada.

THERMAL TOLERANCE IN TELEOST FISH – IMPORTANCE OF CARDIAC OXYGEN SUPPLY, ATP PRODUCTION AND AUTONOMIC CONTROL

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Dissertation Abstract

Temperature tolerance is a key determinant of the resilience and adaptability of fish facing a warmer and more thermally variable future with climate change. Yet, the underlying physiological mechanisms determining the critical thermal maximum (CT_{max}) are poorly understood. This thesis investigated the physiological determinants of CT_{max} in teleost fish, focusing on cardiovascular function. An inability of the heart to pump and supply the body tissues with oxygenated blood could constrain whole animal tolerance to high temperatures. This has been hypothesized to be related to an oxygen limitation of the heart, which receives its oxygen supply via the venous blood (luminal circulation), and in some species also via a coronary circulation.

This hypothesis was first tested by evaluating the relationship between luminal oxygen supply, via continuous recordings of the venous oxygen tension (P_{VO2}), and *in vivo* cardiovascular performance and CT_{max} in European perch (*Perca fluviatilis*). Perch were sampled from the Baltic Sea (reference, 18°C) and the Biotest enclosure (24°C, Biotest) that is chronically warmed by cooling water effluents from a nuclear power plant. While CT_{max} was 2.2°C higher in Biotest compared to reference perch, cardiac failure (*i.e.* reduced heart rate and cardiac output) occurred at similar P_{VO2}. By artificially increasing the oxygen availability to the heart through water hyperoxia (200% air saturation), it was revealed that while heart rate still declined at high temperatures, cardiac stroke volume and cardiac output were maintained. This demonstrates that mainly stroke volume is sensitive to limitations in luminal oxygen supply. In rainbow trout (*Onchorhynchus mykiss*), coronary blood flow first increased with moderate warming, but plateaued at higher temperatures suggesting limitations to the coronary vasodilatory reserve. Ligation of the coronary artery reduced CT_{max} and impaired cardiac performance during warming, which was reflected in an elevated heart rate across temperatures, possibly to compensate for an impaired myocardial contractility and stroke volume of the oxygen deprived ventricle.

A thermal impairment of mitochondrial ATP production could also explain reductions in cardiac performance of acutely warmed fish. This hypothesis was tested by evaluating the catalytic capacities of key enzymes involved in ATP production in the perch heart. The main findings suggest that mitochondrial function is impaired at critically high temperatures by a reduced production of NADH and $FADH_2$ in the tricarboxylic acid cycle, which provides the electrons necessary for driving mitochondrial ATP production. Moreover, a temperature dependent failure of several complexes in the electron transport chain was observed, which would also limit the synthesis of ATP at high temperatures. Indications of an increase in oxidative capacity were observed in the warm acclimated Biotest perch, which may be associated with their improved cardiac thermal performance and elevated CT_{max}.

Finally, it was hypothesized that cholinergic inhibition of heart rate could improve cardiac oxygenation during warming, and that adrenergic stimulation may improve cardiac contractility at high temperatures and reduced cardiac oxygen availability. These hypotheses were tested in rainbow trout by pharmacologically blocking the cholinergic and adrenergic input to the heart. However, neither of the treatments resulted in earlier onset of cardiac failure during acute warming, or a reduced CT_{max}. This could reflect that the heart was adequately oxygenated via compensatory increases in coronary flow, and/or that an increased cardiac filling pressure served to maintain cardiac output.

Collectively, these findings provide novel insights into the causal factors underlying thermal tolerance and cardiac failure during acute warming in teleost fish *in vivo*. While whole animal thermal tolerance limits likely involve thermal failure at several levels of physiological organization, a failing heart undoubtedly plays a crucial role for the sensitivity of fish to a warmer and more thermally extreme future.

Keywords: acclimation, coronary, CT_{max}, enzyme, global warming, heart, mitochondria

Featured Papers

This thesis is based on the following four papers, which are referenced in the thesis according to their Roman numerals:

Paper I: **Ekström, A., Brijs, J., Clark, T. D., Gräns, A., Jutfelt, F. and Sandblom, E. (2016)**. Cardiac oxygen limitation during an acute thermal challenge in the European perch: effects of chronic environmental warming and experimental hyperoxia. *American Journal of Physiology – Regulatory, Integrative and Comparative Physiol*ogy. **311**, R440-449.

Paper II: **Ekström, A., Axelsson, M., Gräns, A., Brijs., and Sandblom, E. (2017)** Influence of the coronary circulation on thermal tolerance and cardiac performance during warming in rainbow trout. *American Journal of Physiology – Regulatory, Integrative and Comparative Physiol*ogy. In Press

Paper III: **Ekström, A.,Sandblom, E., Blier, P.U., Dupont Cyr, B-A., Brijs, J. and Pichaud, N. (2016)** Thermal sensitivity and phenotypic plasticity of cardiac mitochondrial metabolism in European perch, *Perca fluviatilis*. *Journal of Experimental Biology*. 220: 386-396

Paper IV: **Ekström, A., Jutfelt, F. and Sandblom, E. (2014)**. Effects of autonomic blockade on acute thermal tolerance and cardioventilatory performance in rainbow trout, *Oncorhynchus mykiss*. *Journal of Thermal Biology* **44**, 47-54.

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1. Introduction

1.1. Fish – an aquatic ectotherm

Fish belong to a paraphyletic group of aquatic gill-bearing craniate animals including more than 33 000 extant species (http://www.fishbase.org). They are commonly divided into the *Chondrichtyes* (cartilaginous fish), *Agnatha* (hagfish and lampreys), *Sarcopterygii* (lobe-finned fish) and *Actinopterygii* (ray-finned fish). The *Teleostei* (teleosts or bony fishes) belong to the ray-finned fish and comprise almost 96% of all extant fish species (Graham, 2006). All fish are ectothermic, which means that their body temperature is determined by the external thermal environment and thermoregulation is predominately achieved via behavioural means (Crockett and Londraville, 2006; Reynolds, 1977). Fish as a group display an astounding diversity and adaptability to a wide variety of thermal niches and inhabit some of the coldest (-2°C, in the Polar seas) and hottest (~45°C, in some tropical and thermally active lakes and ponds) aquatic environments on earth (Axelsson et al., 1992; Johnston et al., 1994)

1.2. Thermal tolerance limits of fish

Current climate change involves an increased global average temperatures, along with an increased prevalence of extreme weather phenomena, such as transient heat waves (Doney et al., 2012; Field et al., 2014; Ganguly et al., 2009). This may have an impact on fish populations. In fact, ocean warming has already caused a pole-ward shift in the distribution of many fish populations (Parmesan and Yohe, 2003; Perry et al., 2005), which has been correlated with species-specific thermal tolerance limits, *i.e.* the functional range of temperatures for an organism (Beitinger and Lutterschmidt, 2011; Sunday et al., 2010; Sunday et al., 2012). Individual variability in thermal tolerance is likely also subject to natural selection in fish (Clusella-Trullas et al., 2011).

The physiological mechanisms determining the thermal tolerance limits of fish are not fully understood (Beitinger and Lutterschmidt, 2011; Clark et al., 2013). One prevailing hypothesis suggests that a failure of the heart, resulting in an inability of the cardiovascular system to provide the body with oxygen and nutrients, is a primary determinant of upper thermal tolerance limits in fish (Farrell, 2009). This hypothesis builds on the idea that cardiac function may be constrained by limitations in the oxygen supply to the heart itself (Clark et al., 2008; Farrell, 2009; Lannig et al., 2004), or by a temperature-dependent impairment of aerobic metabolic processes within the myocardial cells of the heart (Iftikar and Hickey, 2013; Iftikar et al., 2014).

Another possibility that remains unexplored is whether cardiac control mechanisms influence the upper limits of cardiac and whole animal thermal tolerance in fish. Considerable knowledge gaps remain to be resolved regarding these ideas, as well as to what extent the heart may adjust to chronic exposure to elevated temperatures. This thesis addresses these problems and explores the role of the heart as an underlying determinant of the upper thermal tolerance limit in fish.

Before expanding on the specific physiological responses of fish to changes in temperature, a general description of the fish cardiorespiratory system and its role in overall oxygen transport is provided in the following sections.

1.3. Cardiovascular oxygen transport in teleost fish

All teleosts have closed cardiovascular systems that are comprised of a heart connected to arterial and venous vasculature. The heart generates the arterial blood pressure that drives blood flow to the tissues. The blood transports oxygen, which is primarily bound to haemoglobin in the red blood cells (erythrocytes) but is also physically dissolved in the plasma. The oxygen, along with various substrates, are essential for cellular aerobic production of adenosine triphosphate, ATP, which is the fuel for most biochemical and physiological processes in the cells (Berg et al., 2002; Gautheron, 1984). The blood is also responsible for the removal of cellular metabolites and metabolically produced CO_2 (Olson, 2011a; Olson and Farrell, 2006).

1.3.1. Blood flow patterns

Deoxygenated venous blood leaves the heart and enters the ventral aorta, which diverges into four pairs of afferent gill (branchial) arteries that supplies the gill arches where the blood is oxygenated in the *lamellae* of the gill filaments (Fig. 1). The gills constitute the predominant site for oxygen uptake and are also important for excretion (Johansen, 1971). Some of the oxygenated blood exits the branchial circulation via the arterio-venous pathway, while the majority of the blood flow enters the systemic circulation via efferent branchial arteries that connect to the dorsal aorta, *i.e.* the `arterio-arterial´ or `respiratory pathway´ (Nilsson and Sundin, 1998). The dorsal aorta diverges into a network of systemic arteries and arterioles, which perfuse the capillary beds at the tissues, where the exchange of O_2 and CO_2, nutrients and cellular waste products occurs. Deoxygenated blood is subsequently transported away from the tissues, back to the heart, via the venous vasculature (Olson, 2011a; Satchell, 1992).

The physical force that drives blood flow through the vasculature is the arterial blood pressure, which is a function of vascular resistance and cardiac output (the blood flow generated by the heart). Cardiac output is the product of heart rate and cardiac stroke volume, and generates the ventral aortic blood

pressure, which constitutes the pressure driving blood flow through the vasculature. Collectively, the product of cardiac output and ventral aortic blood pressure reflect the power-generation of the heart, or cardiac power output. The small arteries and arterioles (*i.e.* the resistance vessels) constitute the main site for the local regulation of tissue blood flow and changes in the diameter of these vessels have profound effects on overall vascular resistance and arterial blood pressure (see *section 1.5.4*). The relationship between oxygen consumption rate (M_{O2}) of the tissues and oxygen delivery by the cardiovascular system to the tissues is summarized by the Fick equation:

$$M_{O_2} = Q * (C_{aO_2} - C_{vO_2})$$

where Q represents cardiac output, and C_{aO2} and C_{vO2} represent the oxygen content of arterial and venous blood, respectively. Thus, $C_{aO2} - C_{vO2}$ represents the oxygen extraction at the tissues. Tissue oxygen demand is predominately met by regulating cardiac output, via the modulation of heart rate and/or stroke volume, or the extraction of oxygen at the tissues. The oxygen carrying capacity of the blood (*i.e.* Cao_2) may also regulated by splenic release or uptake of erythrocytes, or by modulation of gill ventilation and thus oxygen uptake (Farrell et al., 2009; Olson, 2011a; Smith and Jones, 1982).

1.4. The morphology and oxygen supply to the heart

The heart is enclosed in a pericardial cavity and consists of four serially-arranged chambers including the *sinus venosus*, atrium, ventricle and an outflow tract, the *bulbus arteriosus* (Fig. 1) (Icardo, 2012). The deoxygenated venous blood returns to the heart via the ducts of Cuvier, which empties into the *sinus venosus*, a highly compliant (elastic) vessel that functions as a storage conduit for venous blood filling the atrium. The atrium consists predominately of cardiomyocytes (contractile cardiac muscle cells) and its contraction facilitates the filling of the ventricle, the force-generating chamber of the heart. The ventricle contraction expels blood through the *bulbus arteriosus*, which functions as a "windkessel" that expands and stores the majority of stroke volume following ventricular contraction (systole) and blood ejection. A gradual elastic recoil of the *bulbus* serves to dampen the ventral aortic pulse pressure, which leads to a more even blood flow through the gill circulation (Icardo, 2012; Jones et al., 1993; Nilsson and Sundin, 1998; Priede, 1976).

1.4.1. Ventricular morphology and oxygen supply

Teleost ventricles exhibit substantial inter-species variability in size, shape and composition of the myocardium, which reflects species-specific differences in functional demands (Gamperl and Farrell, 2004). A fundamental difference among various teleost species relates to how the ventricular tissues receives oxygen (Farrell et al., 2012).

Spongy myocardium and luminal oxygen supply

The hearts of all fish consist of myocardial cells arranged in a trabecular mesh that span the inner wall of the ventricle. This arrangement forms numerous invaginations (*lacunae*) creating a large surface area, which allows oxygen diffusion and nutrient uptake into the spongy myocardium from the venous blood filling the lumen, and is referred to as the 'luminal circulation' (Cameron, 1975; Davie and Farrell, 1991b; Farrell et al., 2012; Tota, 1983). In about two thirds of all teleost species, the spongy myocardium is only supplied with oxygen via the luminal circulation. This is commonly referred to as a Type I heart. (Davie and Farrell, 1991b; Farrell et al., 2012; Tota, 1989). The driving force for the diffusion of oxygen into the spongy myocardium is the partial pressure of oxygen in the venous blood (P_{VO2}). However, the diffusion distance and time for diffusion are affected by the end-diastolic volume and heart rate, which are probably also important determinants of myocardial oxygenation. An increase in end-diasolic volume reduces the luminal-trabecular diffusion distance by stretching and therefore decreasing the diameter of the trabeculae, as well as promoting the mixing of blood in the ventricular lumen. A reduced heart rate also increases the retention time of the blood in the ventricle lumen, which prolongs the time available for oxygen diffusion (Farrell, 2007).

Compact myocardium and coronary oxygen supply

In the remaining one third of teleost species, the spongy myocardium is enclosed by a layer of more densely packed cardiomyocytes forming the 'compact myocardium' (Davie and Farrell, 1991b; Poupa et al., 1974; Santer, 1985). In teleosts, the proportion of the compact layer is commonly less than 50% of the total ventricle mass, but some species such as tunas and some salmonids may have an even higher proportion of compact myocardium (Clark et al., 2008; Santer, 1985). Furthermore, the amount of compact myocardium is known to differ according to sex (Davie and Thorarensen, 1996), age, body mass, and ventricular mass in salmonid species (Brijs et al., 2016; Farrell et al., 1988a; Poupa et al., 1974).

The compact myocardium receives oxygenated blood via the coronary vasculature. Hearts containing compact myocardium can be of three principle types (Types II-IV). In Type II hearts, the coronaries perfuse only the compact ventricular myocardium, as observed in salmonids. In Type III and IV hearts, the coronary vasculature also perfuses the spongy ventricular myocardium and even the atrium in Type IV hearts. Such hearts are found in elasmobranchs and a few highly active teleost species such as tunas (Davie and Farrell, 1991b; Santer, 1985; Tota, 1983; Tota et al., 1983).

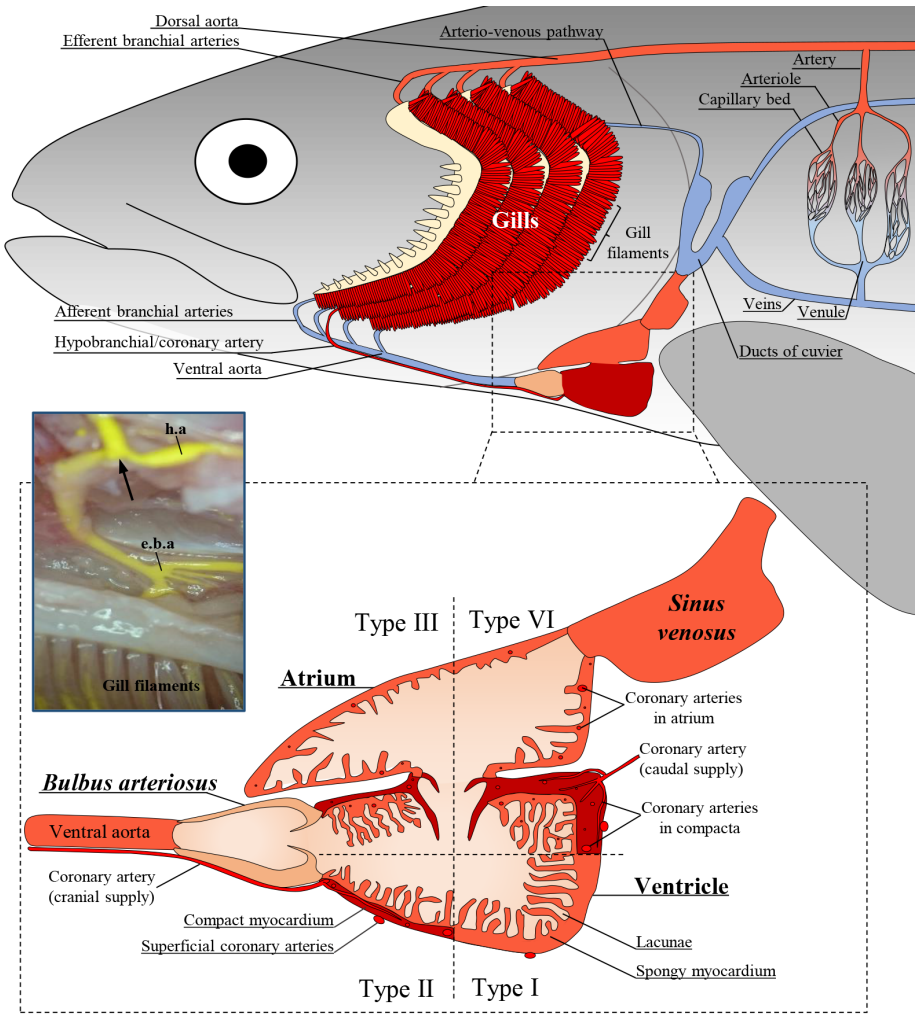

Figure 1. Schematic illustration of the cardiovascular system in a teleost fish. A) The cardiovascular system forms a closed loop comprising the heart and the vascular system. Deoxygenated blood (blue) from the tissues is returned to the heart via the venous vasculature that includes small venules and larger conducting veins that merge with the ducts of Cuvier. The blood then enters the four chambered heart that is comprised of the *sinus venosus*, atrium, ventricle and *bulbus arteriosus*. The heart pumps blood into the ventral aorta perfusing the gill circulation where gas exchange occurs. The oxygenated blood (red) can either exit into the arterio-venous pathway or the arterio-arterial pathway that enters the dorsal aorta. Conducting arteries and subsequently smaller arterioles perfuse the capillary beds where exchange of gases and nutrients with the tissues occurs. The inset shows the ventricle that can be composed of entirely spongy myocardium that obtains oxygen from the venous blood (Type 1 hearts); or various combinations of spongy and compact myocardium (Type II-IV hearts). The compact myocardium receives oxygen from a dedicated arterial coronary circulation (cranial or caudal supply). The inset picture shows a silicone cast (yellow) of the cranial supply in Northern pike (*Esox lucius*). Blood from the efferent branchial arteries (e.b.a.) from the 3rd left and right gill arches merge (arrow) and proceeds to perfuse the hypobranchial artery (h.a.) and subsequently the coronary artery. In type IV hearts the coronaries may also perfuse the atrium. Graphical illustration by Andreas Ekström and Albin Gräns, partly modified from Davie and Farrell (1991b).

The coronary artery originates from different sites in different species of fish, either from the hypobranchial artery that branches off the efferent branchial vasculature (cranial supply), or from the coracoid artery (caudal supply), which is the first vessel that branches off from the dorsal aorta (Davie and Daxboeck, 1984; Farrell et al., 2012). Salmonids have a cranial coronary supply (see Fig. 1), whereby the coronary artery penetrates the pericardium and approaches the ventricle along the dorsal surface of the *bulbus arteriosus,* before entering the compact myocardium (Davie and Daxboeck, 1984; Farrell et al., 2012; Tota, 1983; Tota et al., 1983).

Coronary blood flow in fish is determined by the dorsal aortic blood pressure and the coronary vascular resistance (for a review, see Axelsson, 1995). The fact that large increases in coronary blood flow may result in only a small (or no) increase in dorsal aortic pressure indicates that a large coronary vasodilatory reserve exists (Axelsson and Farrell, 1993; Farrell, 1987; Gamperl et al., 1995). For example, the microvasculature of the coronary system is likely is vasoconstricted via an α-adrenergic tonus, which can be released to increase coronary blood flow (Axelsson, 1995; Davie and Daxboeck, 1984; Farrell, 1987). Moreover, recent evaluations of the vasoactive responsiveness of the coronary microcirculation (*i.e.* resistance vessels) in wild steelhead trout (*Onchorhynchus mykiss*) have revealed potent coronary vasodilation in response to several endogenous agents, including adenosine, interleukin 1β, serotonin, nitric oxide and high concentrations of catecholamines (Costa et al., 2015a; Costa et al., 2015b). Coronary blood flow may also be affected by the ventricular contraction as the mechanical forces imposed by the ventricle and *bulbus arteriosus* during systole may compress the coronary vasculature. Thus, an increased time spent in systole, for example during periods of elevated heart rate, increases the coronary vascular resistance, which consequently reduces coronary blood flow (Axelsson and Farrell, 1993; Davie and Franklin, 1993; Farrell, 1987; Gamperl et al., 1995).

1.5. Cardioventilatory control mechanisms in teleosts

To maintain an adequate perfusion pressure of oxygenated blood to the tissues during varying conditions of tissue oxygen demand, the cardiovascular and ventilatory systems are constantly regulated by local, neural and hormonal control mechanisms. Neural control is predominately mediated via branchially located sensory receptors, which detect changes in blood pressure (baroreceptors), or in the levels of O_2, CO_2 and/or pH (chemoreceptors) (Olson and Farrell, 2006; Perry and Gilmour, 2002). An increased or decreased action potential firing rate mediated via these receptors conveys input to the central and/or the autonomic nervous system via afferent fibres of the branchial cranial nerves V, VII, IX and X (the vagus nerve). This may elicit a responsive stimulus, which is conveyed via efferent fibres of the cranial and spinal nerves

to the effector tissues including the heart, vasculature and ventilatory muscles (see *sections 1.5.3–1.5.5*; Fig. 2) (Nilsson, 1983; Sandblom and Axelsson, 2011; Taylor et al., 1999).

1.5.1. Cellular mechanisms of heart contraction

The contraction of the heart is initiated in a patch of pacemaker cells located between the *sinus venous* and the atrium, called the sinoatrial node (Farrell and Jones, 1992; Haverinen and Vornanen, 2007; Olson and Farrell, 2006; Santer, 1985). The pacemaker generates action potentials by the rapid depolarisation and repolarisation of the cell membrane, which is caused by an increased transmembrane flux of extracellular Na^+ and Ca^{2+}, as well as intracellular K^+ along their respective concentration gradients (Haverinen and Vornanen, 2007; Irisawa et al., 1993). The action potential is conducted through the atrial and ventricular myocardium that causes an influx of Ca^{2+} into the myocardial cells. The Ca^{2+} influx stimulates further release of Ca^{2+} from the sarcoplasmic reticulum, which increases the intracellular Ca^{2+} concentration. This leads to the binding of Ca^{2+} to troponin C and mediates the formation of actin-myosin cross-bridges, which in turn leads to the contraction of the atrium and ventricle. Following contraction, the reflux of Na^+, Ca^{2+} and K^+, as well as the active re-uptake of Ca^{2+} into the sarcoplasmic reticulum, are important for the repolarization and relaxation of myocardial cells. These processes are partly regulated via ATP-dependent ion transporters, such as Na^+/K^+-ATPase and sarcoplasmic reticulum ATPase (SERCA) (Boron and Boulpaep, 2009; Galli and Shiels, 2012).

1.5.2. Intrinsic control of cardiac contraction force

Cardiac contractility and stroke volume, are intrinsically regulated via changes in cardiac filling pressure (central venous blood pressure). Increases in filling pressure result in an increased end-diastolic volume and greater myocardial stretch, which results in an increase in cardiac contractility and stroke volume. This is called the Frank-Starling mechanism (Shiels and White, 2008). This relationship also means that stroke volume is inversely related to heart rate, as an increased contraction frequency results in a reduced diastolic filling time (Altimiras and Axelsson, 2004; Keen and Gamperl, 2012). However, such heart rate-dependent effects on stroke volume can be compensated via increases in cardiac filling pressure. Active increases in venous vascular tone are therefore important for maintaining or increasing stroke volume during conditions involving elevated cardiac output (Altimiras and Axelsson, 2004; Sandblom and Axelsson, 2007a; Sandblom et al., 2006).

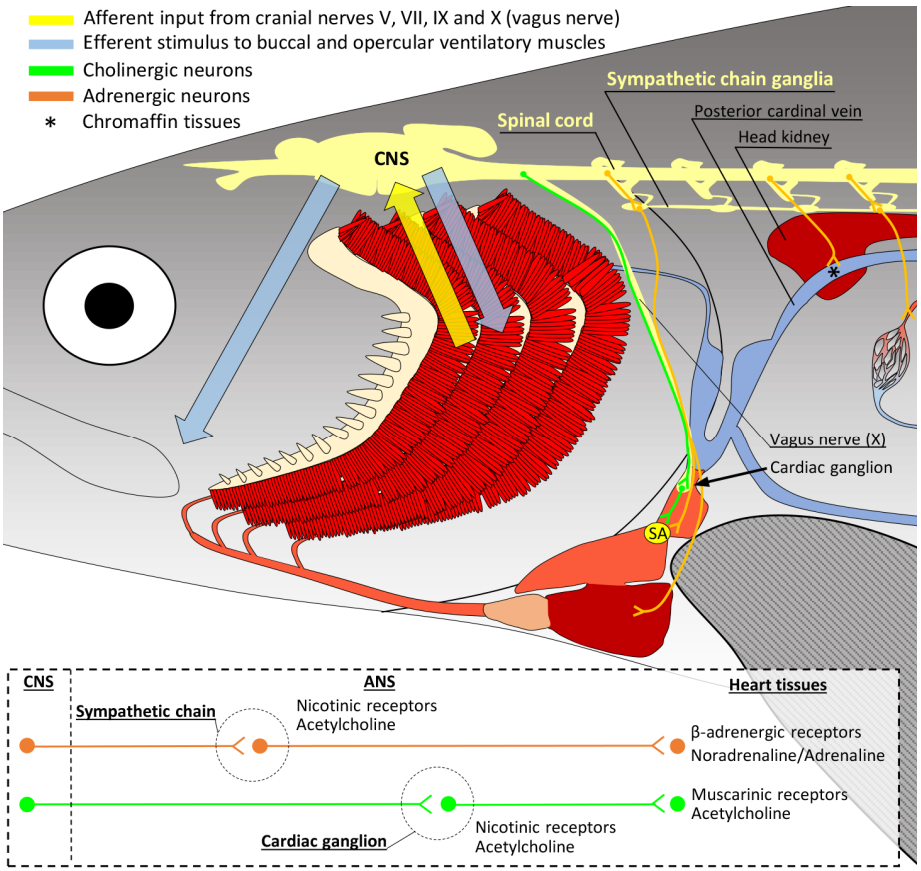

Figure 2. Schematic illustration of neural and humoral control mechanisms of the cardiovascular and ventilatory system in a teleost fish. Stimulatory input from branchially located baro-, and chemoreceptors send afferent input (yellow arrow) to the central nervous system (CNS). Efferent stimulus is relayed via efferent cranial (cholinergic) nerves to the ventilatory muscles (blue arrow), or via spinal (adrenergic, orange) and cholinergic (green) efferent neurons to the sinoatrial (SA) node, heart ventricle and the resistance vasculature. Spinal autonomic preganglionic (cholinergic) neurons innervate the chromaffin tissues within the head kidney that release circulating catecholamines into the posterior cardinal vein. Post-ganglionic adrenergic neurons release noradrenaline onto α-adrenergic receptors at the resistance vessels. The inset shows the schematic anatomical arrangement of cranial and spinal autonomic neural pathways innervating the heart. Spinal autonomic preganglionic neurons synapse and release acetylcholine onto nicotinic receptors located on postganglionic cell bodies in the sympathetic chain ganglia. Postganglionic adrenergic neurons innervate and release adrenaline or noradrenaline onto β-adrenergic receptors on the SA node and ventricular tissues. Preganglionic cholinergic neurons synapse at the cardiac ganglion, and upon release and binding of acetylcholine to postganglionic nicotinic receptors, the signal is relayed to the SA node where acetylcholine is released onto muscarinic receptors. Graphical illustration by Andreas Ekström and Albin Gräns.

1.5.3. Extrinsic control of the heart

The heart is also regulated by extrinsic neuronal and hormonal factors that act directly on cardiac tissues. The autonomic nervous system has an important bimodal stimulatory/inhibitory influence on the heart in most teleosts.

Stimulatory adrenergic nerves can reach the heart via the vagus nerve (the vago-sympathetic trunk), and/or along the coronary artery and the anterior spinal nerves, and innervate the sinoatrial pacemaker cells and the ventricular myocardium (Nilsson, 1983; Sandblom and Axelsson, 2011). The release and binding of noradrenaline and adrenaline to β-adrenoreceptors on the pacemaker cells increases ionic transmembrane conduction rates and action potential firing frequency, elevating heart rate. Receptor activation also increases the influx of Ca^{2+} into the ventricular myocardium, which stimulates cardiac contractility (Hartzell, 1988; Harvey and Belevych, 2003; Irisawa et al., 1993). Catecholamines can also be humorally released into the blood via the stimulation of chromaffin cells located within the walls of the posterior cardinal vein in the head kidney (Perry and Capaldo, 2011). Cholinergic neurons reach the heart via the vagus nerve, which travels along the ducts of Cuvier to the sinoatrial node. Release of acetylcholine leads to the stimulation of muscarinic receptors on the pacemaker cells, which primarily reduces transmembrane ion conduction rates and therefore lowers heart rate (Hartzell, 1988; Harvey and Belevych, 2003; Nilsson, 1983; Sandblom and Axelsson, 2011).

1.5.4. Vascular resistance

As in other vertebrates, the diameter of the resistance vessels in teleosts may be regulated via the local release of metabolites (*e.g.* H^+, CO_2, ATP, ADP, AMP and adenosine), or from paracrine signalling molecules (*e.g.* endothelin, prostaglandin, leukotriene, thromboxane) and gasotransmitters (*e.g.* nitric oxide, carbon monoxide and hydrogen sulphide). Such metabolites may be released from the endothelium, vascular smooth muscle or surrounding tissues. These substances can cause the contraction or dilation of vascular smooth muscle. Vascular resistance is also neurally regulated, whereby autonomic adrenergic stimulation constricts (noradrenalin acting on α-adrenoceptors) or dilates (adrenalin acting on β-adrenoceptors) vascular smooth muscle (Olson, 2011b; Sandblom and Axelsson, 2011).

1.5.5. Ventilation

Oxygen uptake at the gills may be regulated by altering gill ventilation rate and/or the force and amplitude of ventilation (*i.e.* the ventilatory stroke volume) (Rogers and Weatherley, 1983; Smith and Jones, 1982). Opercular and buccal respiratory movements are controlled via efferent stimuli generated by the respiratory rhythm generator in the brainstem, from which cranial nerves V, VII, IX and X proceed to innervate the ventilatory muscles (Taylor et al., 1999).

1.6. ATP production in the teleost heart

The fish heart is almost entirely dependent on aerobic metabolic processes for its ATP production (Driedzic et al., 1983; Sidell et al., 1987). This is accomplished by converting metabolic substrates to ATP via a series of metabolic pathways in the mitochondria (Fig. 3) (Berg et al., 2002; Gautheron, 1984).

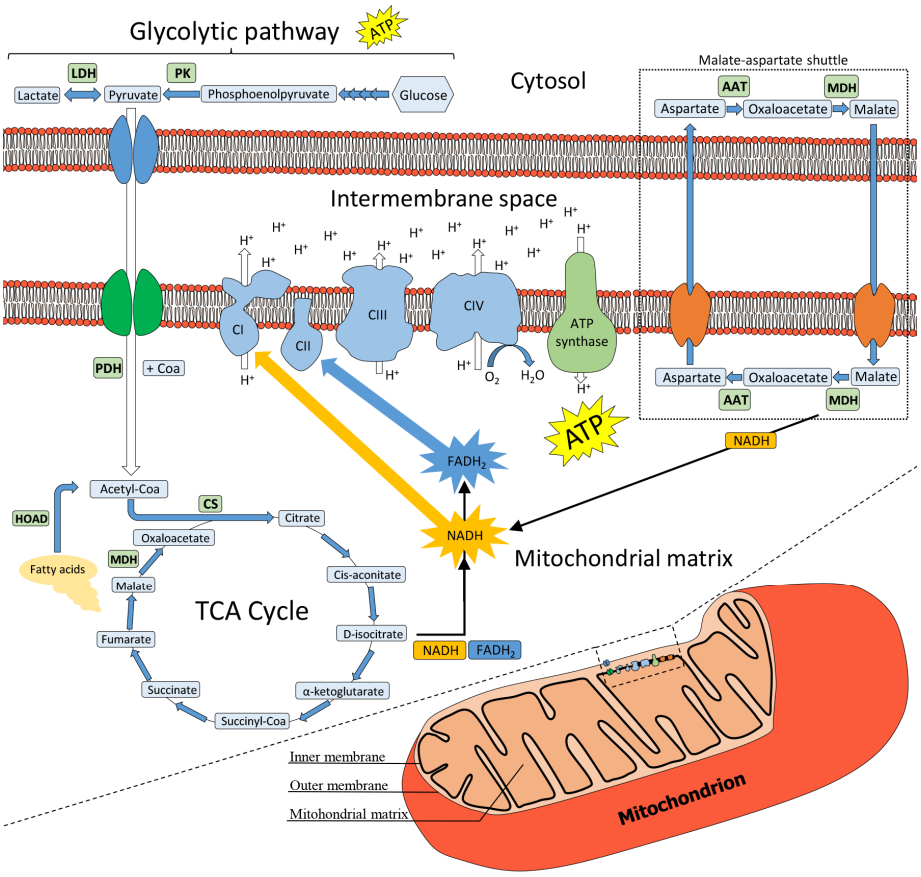

Figure 3. Schematic overview of the metabolic pathways governing ATP production in the fish heart. The glycolytic pathway converts glucose to pyruvate and results in the production of ATP. In anaerobic conditions, pyruvate is converted to lactate. If oxygen is present, pyruvate is further converted to acetyl-coA, which fuels the tricarboxylic acid (TCA) cycle. Acetyl-coA is also provided via the oxidation of fatty acids. The TCA cycle and the malate-aspartate shuttle produce NADH and $FADH_2$, which donate electrons to the electron transport system (*i.e.* complexes I-IV, CI-CIV). The transport of electrons through the system drives the extrusion of protons (H^+) across the membrane and results in the reduction of oxygen (O_2, the final electron acceptor) to water (H_2O) at CIV. The resulting electrochemical H^+ gradient drives the generation of ATP by ATP-synthase. Green boxes symbolize the enzymes catalysing the biochemical conversions of specific substrates (blue boxes) in the metabolic pathways. Abbreviations are: AAT=asparate aminotransferase; CS=citrate synthase; HOAD=hydroxy-acyl coenzyme A dehydrogenase; LDH=lactate dehydrogenase; MDH=malate dehydrogenase; PDH=pyruvate dehydrogenase; PK=pyruvate kinase.

1.6.1. The glycolytic pathway

Aerobic ATP production begins with an anaerobic (*i.e.* oxygen-independent) chain of biochemical reactions called the glycolytic pathway. This pathway is responsible for converting glucose into phosphoenolpyruvate, which is subsequently converted to pyruvate by the enzyme pyruvate kinase (PK). The pyruvate then enters one of two routes depending on the availability of oxygen in the cell. In anaerobic conditions, pyruvate is reduced to lactate by lactate dehydrogenase (LDH) (Berg et al., 2002; Gautheron, 1984).

1.6.2. The tricarboxylic acid cycle

Anaerobic glycolytic degradation of glucose harvests only a fraction (2-3 ATP) of the potential ATP yield available per molecule of glucose, whereas the presence of oxygen generates a much higher ATP yield (32 ATP). This is mediated via the formation of the reducing equivalents, nicotinamide adenine dinucleotide (NAD) and flavin adenine dinucleotide (FAD) that drive oxidative phosphorylation in the mitochondrial matrix via the tricarboxylic acid (TCA) cycle. The TCA cycle begins with the entry of acetyl-coenzyme A into the cycle, which is supplied either via a reaction between pyruvate and coenzyme-A (CoA), or catalysed by the enzyme complex pyruvate dehydrogenase (PDH), or via the oxidation of fatty acids (β-oxidation) by hydroxyacyl-Coenzyme A dehydrogenase (HOAD). Acetyl-coA and oxaloacetate are then converted into citrate by citrate synthase (CS). Oxaloacetate is supplied either from the conversion of malate by malate dehydrogenase (MDH), or via the conversion of amino acids by aspartate aminotransferase (AAT). In addition to their catalysing properties, MDH and AAT are present as cytosolic and mitochondrial isozymes and mediate the conversion and transport of malate across the mitochondrial membrane via the malate-aspartate shuttle. This results in the formation of NADH in the mitochondrial matrix, which donates electrons to the electron transport system (Hochachka et al., 1979; Safer, 1975).

1.6.3. The electron transport system

NADH and $FADH_2$ produced in the TCA cycle are oxidized and donate electrons to complex I (CI) and complex II (CII) of the electron transport system, respectively. The electrons are subsequently transported via a series of lipid-soluble carrier molecules to complex III (CIII) and finally cytochrome c oxidase (CCO or complex IV, CIV), whereupon oxygen is reduced to water. The electron transport drives the transfer of protons (H^+) across the inner membrane into the intermembrane space of the mitochondria via CI, CIII and CIV. This generates a transmembrane electrochemical proton gradient that drives the proton flux through ATP-synthase, which results in the synthesis of ATP (Berg et al., 2002; Gautheron, 1984).

1.7. Effects of environmental warming in teleosts

One of the early pioneers of fish respiratory physiology, John R. Brett, referred to temperature as the "ecological master factor" due to its influence on a wide range of physiological processes (Brett, 1971). Indeed, temperature constitutes a predominant driving force influencing the rate of biochemical and metabolic reactions and thus ATP turnover in the body. This in turn influences the energy availability for maintaining physiological homeostasis, activity, digestion, reproduction and growth, all essential traits for the survival and fitness of the animal (Aledo et al., 2010; Angilletta, 2009; Berg et al., 2002; Farrell, 2002).

1.7.1. Effects of temperature on biochemical and physiological processes

The effects of warming on biochemical or physiological processes may be explained by the thermal performance curve (Schulte, 2015). Typically, the thermal performance curve of a wide range of processes tend to consist of three phases: 1) an initial often exponential increase, 2) a plateau phase that usually encompasses the maximum response, and 3) a rapid decline when approaching critically high temperatures (Dell et al., 2011). On a cellular level (*i.e.* biochemical reaction rates), phase 3 may be attributed to a decreased catalytic capacity and/or denaturation of catalyzing enzymes (Fields, 2001). The decline in physiological processes such as whole animal aerobic metabolism and cardiovascular function may comprise several levels of biological organization, which will be discussed in later sections of this thesis. The thermal sensitivity of biochemical and physiological processes may be quantified by assessing a thermal coefficient, Q_{10}, which reflects the thermal sensitivity of a temperature-dependent process over a 10°C change in temperature. This concept was first described by a Swedish chemist, Svante Arrhenius, who also developed a method for identifying the breakpoint at which thermally dependent processes stop increasing exponentially with temperature, which is referred to as the Arrhenius breakpoint temperature (ABT) (Arrhenius, 1915). This approach has been used extensively to analyse ABT´s for biochemical reactions, as well as physiological processes such as heart rate (Anttila et al., 2014; Badr et al., 2016; Chen et al., 2015).

1.7.2. Oxygen consumption and cardiovascular responses to acute warming

The rate of aerobic ATP production is traditionally assumed to be directly proportional to whole animal oxygen consumption rate. However, a relatively large proportion of the oxygen consumed may be uncoupled from ATP production, due to a transmembrane proton leak across the inner mitochondrial membrane (Salin et al., 2015). Nevertheless, the rate of mitochondrial oxygen

consumption in response to acute warming typically increases with a Q_{10} of ~2 in fish (Blier et al., 2014; Iftikar and Hickey, 2013; Lemieux et al., 2010). This is also reflected in the routine whole animal oxygen consumption rate, which also increases with a Q_{10} of 2-3 in most species examined (Brett, 1971; Clark et al., 2008; Ege and Krogh, 1914; Fry and Hart, 1948; Gollock et al., 2006; Rodnick et al., 2004; Sandblom et al., 2016a). The increased tissue oxygen demand associated with rising temperatures is met by an increase in cardiac output, which also typically increases by a Q_{10} of ~2-3 in most fish during acute warming (Clark et al., 2008; Farrell et al., 2009; Gamperl et al., 2011; Gollock et al., 2006; Mendonca and Gamperl, 2010; Sandblom et al., 2016a). While routine metabolic and cardiovascular rates display substantial sensitivity in response to acute warming, maximum oxygen consumption rate and cardiovascular performance typically reaches an earlier plateau and may decline at higher temperatures. Therefore, the cardiorespiratory scope (*i.e.* the difference between maximum – routine values) may be reduced at higher temperatures, which has been suggested to constrain metabolically demanding activities such as locomotion, predator avoidance, prey capture, digestion, growth and reproduction (Farrell, 2002; Farrell, 2009; Fry and Hart, 1948).

The predominant mechanism for increasing cardiac output during acute warming in most fish species is via an elevation of heart rate, as stroke volume remains relatively unchanged or may even decrease which is likely due to a reduced diastolic filling time (Altimiras and Axelsson, 2004; Clark et al., 2008; Gamperl et al., 2011; Gollock et al., 2006; Keen and Gamperl, 2012). The increase in heart rate is mediated by the direct stimulatory effects of temperature on the cardiac pacemaker cells (Harper et al., 1995; Haverinen et al., 2016), as well as by changes in extrinsic cardiac control mechanisms (Ekström et al., 2016). For example, neurally and humorally released catecholamines may stimulate and increase heart rate and cardiac contractility during acute warming, which consequently increases cardiac output (Boron and Boulpaep, 2009; Currie et al., 2013; Currie et al., 2008; LeBlanc et al., 2012; LeBlanc et al., 2011; Reid et al., 1998).

1.7.3. Cardiorespiratory acclimatization and acclimation to chronic temperature change

Chronic temperature changes may result in alterations in the genotype and/or phenotype, which may improve the ability of animals to cope with alterations in their thermal environment. While irreversible genotypic and phenotypic changes may occur across generations on a population level (thermal adaptation), reversible change in the phenotype may also occur within generations on an individual level (phenotypic plasticity) (Angilletta, 2009). Furthermore, plastic phenotypic traits may be transmitted across generations (transgenerational plasticity), which is mediated via nutritional, somatic,

cytoplasmic or epigenetic factors (Munday, 2014; Veilleux et al., 2015). Phenotypic plasticity in response to chronic thermal change is typically referred to as acclimation and the capacity for thermal acclimation has been suggested to be an important determinant for the resilience of fish to changing thermal conditions with global warming (see Chevin et al., 2010; Seebacher et al., 2015). Throughout the remainder of this thesis, the term acclimation refer to reversible plastic responses of individuals to chronic temperature changes.

Warm acclimation typically reduces the thermal sensitivity of physiological processes (*i.e.* Q_{10} decreases towards 1), which is often referred to as thermal compensation (Seebacher et al., 2015). Such compensations are typically observed in routine cardiorespiratory functions, including oxygen consumption rate, heart rate and cardiac output, and may restore cardiorespiratory scope in fish, while maximum capacitites usually exhibit considerably lower plasticity with chronic warming (Ekström et al., 2016; Franklin et al., 2007; Sandblom et al., 2016b; Sandblom et al., 2014).

While the underlying mechanisms for the decline in whole animal oxygen consumption rate with warm acclimation are not fully understood, this likely relates to a reduction in aerobic metabolic processes. For example, warm acclimation in fish is known to down-regulate the expression of enzymes governing glycolysis, the TCA cycle and the electron transport system (Jayasundara et al., 2015; West et al., 1999), as well as reducing the overall mitochondrial density (Shiels et al., 2011). Furthermore, a reduced level of unsaturation of the mitochondrial membrane occurs with chronic warming (Calabretti et al., 2003; Hazel, 1995; Kraffe et al., 2007; Skalli et al., 2006), which may reduce the catalytic activity of membrane bound enzymes while simultaneously reducing mitochondrial trans-membrane proton leak. This would reduce mitochondrial oxygen utilization and improve the efficiency of the electron transport system (Brookes et al., 1998; Hulbert and Else, 1999). While warm acclimation typically results in an elevated critical thermal maximum (CT_{max}) (Beitinger et al., 2000), the underlying mehanisms for this is not fully understood and is an important focus of this thesis.

1.8. Cardiorespiratory linkages to thermal tolerance

A failure of the cardiovascular system to deliver oxygen at high temperatures has been suggested to be a primary factor determining the upper thermal tolerance limits in fish (Clark et al., 2008; Farrell, 2009; Lannig et al., 2004). This hypothesis is based on the observation that heart rate and cardiac output tend to plateau before rapidly declining when fish approach their upper thermal tolerance limit. Such responses have been observed in numerous species *in vivo* (Badr et al., 2016; Clark et al., 2008; Farrell, 2009; Gollock et al., 2006; Mendonca and Gamperl, 2010; Sandblom et al., 2016b; Seebacher et al., 2005;

Somero, 2011), as well as in anaesthetised fish when subjected to pharmacological treatment to induce a maximal heart rate stimulation (Anttila et al., 2014; Casselman et al., 2012). The underlying causal factors for heart failure at high temperatures is currently not fully understood, but has been hypothesized to be related to an impairment of oxygen supply to the heart itself (Clark et al., 2008; Farrell, 2009; Lannig et al., 2004) and/or a thermal impairment of aerobic ATP production in the mitochondria (Iftikar and Hickey, 2013; Iftikar et al., 2014). Reduced aerobic production and provision of the cardiac ATP required for sustaining cellular ion conductance rates and homeostasis could impair cardiac depolarization rate (heart rate), the contractility of myocardium (stroke volume) and thus cardiac output. It has also been hypothesized that an active cholinergic cardio-inhibition may be beneficial for cardiac oxygenation (Farrell, 2007), and that adrenergic stimulation may augment myocardial ion conductance, thus improving ventricular relaxation and contractility at high temperatures (Aho and Vornanen, 2001; Hanson et al., 2006). However, few studies have examined how autonomic control of the heart changes during warming and how this affects overall thermal tolerance.

The experiments included in this thesis address these hypotheses, and are based on the following three main research aims as presented in the next section.

1.9. Research aims

Research aim 1: Determine the relationship between cardiac oxygenation, cardiac function and whole animal thermal tolerance

It has been proposed that below a species-specific P_{VO2} threshold, luminal oxygen supply becomes insufficient and hence cardiac oxygen delivery to the tissues is impaired (Davie and Farrell, 1991b; Farrell, 2007; Farrell and Clutterham, 2003). Indeed, the P_{VO2} is inversely related to temperature during acute warming (Clark et al., 2008; Heath and Hughes, 1973; Lannig et al., 2004; Sartoris et al., 2003), and the reduction in P_{VO2} has been related to the onset of cardiac arrhythmias and reduced cardiac output in a range of teleosts (Clark et al., 2008; Heath and Hughes, 1973; Lannig et al., 2004; Schulte, 2015). However, some of these findings were acquired from species that had an alternative route of myocardial oxygenation via the coronary blood supply (salmonids). In most cases it was not possible to pinpoint a specific P_{VO2} threshold for cardiac function, either because the determinations of P_{VO2} were performed on ventral aortic blood after the heart (Heath and Hughes, 1973; Sartoris et al., 2003), or due to low samples sizes (Lannig et al., 2004). Cardiac power output provides a good approximation of cardiac oxygen demand (Driedzic et al., 1983), yet, the effects of acute warming on cardiac power output remain unexplored *in vivo*. Therefore, further experiments are required

to discern the relationship between cardiac work, myocardial oxygen demand and luminal oxygen supply, and how these factors relate to cardiovascular performance and whole animal thermal tolerance in fish.

Routine P_{VO2} at a given test temperature typically increases with warm acclimation (Farrell and Clutterham, 2003; Perry and Reid, 1994). This is probably due to a right-shift in the oxygen-hemoglobin dissocation curve, and also a reduced oxygen consumption rate following warm acclimation that can be expected to translate into a reduced tissue oxygen extraction (Farrell and Clutterham, 2003; Perry and Reid, 1994). Moreover, a reduction in heart rate and cardiac output would reduce the cardiac oxygen demand at any given temperature (Ekström et al., 2016; Sandblom et al., 2016a; Sandblom et al., 2014). Therefore, warm acclimation likely improves luminal myocardial oxygenation at high temperatures, possibly explaining the increased acute thermal tolerance following chronic warm exposure in fish (Beitinger et al., 2000; Cossins et al., 1977; Seebacher et al., 2005; Stitt et al., 2014). However, this hypothesis remains to be explored.

Most species with a coronary circulation do not seem to rely on their coronary oxygen supply for maintaining routine cardiac functions (Davie and Farrell, 1991a; Davie and Farrell, 1991b; Davie et al., 1992; Daxboeck, 1982; Gamperl et al., 1994a). However, previous *in vivo* studies on salmonids showed an increase in coronary blood flow during hypoxia (Axelsson and Farrell, 1993), and during spontaneous activity and swimming in either normoxic or hypoxic conditions (Axelsson and Farrell, 1993; Farrell, 1987; Gamperl et al., 1994b; Gamperl et al., 1995). This reveals an increased importance of the coronary oxygen supply for cardiac performance during conditions of reduced environmental oxygen availability or increased cardiac oxygen demand. Furthermore, surgical blockade of coronary blood flow by ligation of the coronary artery resulted in a reduced ability to maintain ventral aortic blood pressure in rainbow trout, likely due to an impairment of cardiac contractility (Steffensen and Farrell, 1998). Coronary ligation also reduced the maximum swimming performance in chinook salmon (Farrell and Steffensen, 1987), and prolonged the post-exercise recovery time in rainbow trout (Steffensen and Farrell, 1998).

Considering the known inverse relationship between luminal P_{VO2} and temperature, the fish heart likely becomes increasingly reliant on coronary blood flow to sustain myocardial oxygenation during warming. Surprisingly, the influence of coronary oxygen supply on cardiac and whole animal performance during acute warming is unexplored in fish.

Specific aims

In papers **I** and **II**, the primary aim was to determine the effects of an acute temperature elevation on the luminal oxygen supply in European perch (*Perca*

fluviatilis) (**I**) and coronary blood flow in rainbow trout (**II**). The relationship between luminal and coronary myocardial oxygenation, cardiac function and whole animal thermal tolerance was then evaluated. A second aim was to determine whether chronic warm acclimation provides any beneficial influence on luminal myocardial oxygenation, cardiac function and thermal tolerance limits in perch (**I**).

Research aim 2: Investigating the effects of temperature on enzymatic mitochondrial functions in the fish heart

Recent evidence implicates the cardiac mitochondria as being a principal component responsible for the failure of the heart at higher temperatures (Blier et al., 2014; Chung et al., 2017; Hilton et al., 2010; Iftikar and Hickey, 2013; Iftikar et al., 2014). For example, cardiac failure was found to coincide with the decline in the activity of CII, CIV and the electron transport system (CI and CIII), which consequently impacted the capacity for ATP production in three species of New Zealand wrasse (*Notolabrus celidotus, Notolabrus fucicola* and *Thalassoma lunare*) (Iftikar and Hickey, 2013; Iftikar et al., 2014). Furthermore, a failure of CI and CIII resulted in reduced mitochondrial respiration rates in acutely warmed wolffish, *Anarhichas lupus* (Lemieux et al., 2010). However, there is currently no evidence available to determine whether the failure of the electron transport complexes during acute warming may be directly related to an impaired provision of NADH and $FADH_2$ from the preceding metabolic pathways (see Fig. 3). For example, the TCA cycle has been hypothesized to be restricted by an acute thermal impairment of its integrated enzymatic components, the provision of substrates from the glycolytic pathway or the oxidation of amino acids and fatty acids (Blier et al., 2014; Lemieux et al., 2010; Pichaud et al., 2011). This hypothesis remains to be investigated. Furthermore, it is currently unknown whether the increase in CT_{max} following warm acclimation in fish may be directly related to a reduced thermal sensitivity of the enzymatic machinery, an increased oxidative capacity or an altered substrate utilization, all of which may augment ATP production at high temperatures. It is also unknown whether a reorganization of the lipid profile provides any benefits on cardiac ATP production and function at high temperatures in fish following chronic exposure to warmer conditions.

Specific aims

In paper **III**, the aim was to determine the thermal sensitivity of the metabolic processes governing key metabolic pathways in the perch heart. Specifically, the aim was to investigate whether any changes in upper thermal limits for cardiac function and whole animal performance in perch in paper **I** could be related to differences in cardiac ATP production. A second aim was to

determine how chronic warm acclimation in the field affected cardiac enzymatic functions and mitochondrial membrane composition in the heart of perch.

Research aim 3: Investigating the importance of the autonomic nervous system on cardiac function and whole animal thermal tolerance

The plateau in heart rate typically observed in fish at high temperatures may be attributed to an increased cholinergic inhibition of the heart during warming (Franklin et al., 2001; Lowe et al., 2005). Farrell (2007) postulated that the lowering of heart rate during hypoxia (*i.e.* 'hypoxic bradycardia') may improve myocardial oxygenation by reducing diffusion distances and increasing the time for oxygen diffusion, as diastolic filling and luminal blood retention time increases. Furthermore, a reduced heart rate and prolonged ventricular diastole would augment coronary blood flow in species with coronaries, as there is generally a reduction in coronary blood flow during systole due to the mechanical compression of the coronary vasculature during systole (Axelsson and Farrell, 1993; Farrell, 1987). Thus, an increased cholinergic tone on the heart may be an adaptive response at critically high temperatures, however, currently no studies have examined whether cholinergic cardio-inhibition provides any beneficial influence on cardiac performance and whole animal thermal tolerance in fish.

While acute warming and/or reduction in oxygen availability impair myocardial contractility of *in situ* perfused hearts and *in vitro* myocardial strip preparations (Aho and Vornanen, 2001; Driedzic and Gesser, 1994; Farrell, 1984; Hanson et al., 2006; Nielsen and Gesser, 2001), adrenergic stimulation may alleviate such negative effects (Aho and Vornanen, 2001; Hanson et al., 2006). Moreover, an increased β-adrenoreceptor density has been suggested to be associated with heightened cardiac and whole animal thermal tolerance in sockeye salmon (*Oncorhynchus nerka*) (Eliason et al., 2011). Yet, few studies have directly examined the importance and potential beneficial influence of the adrenergic branch of the autonomic nervous system on *in vivo* cardiac performance and thermal tolerance in fish.

Specific aims

The aim of paper **IV** was to determine whether pharmacological blockade of cholinergic and adrenergic control of the heart affects thermal tolerance and cardiac performance during warming in trout.

2. Methodological considerations

The following section provides a general description of experimental fish and study sites used in the thesis, as well as a discussion of the methodological and analytical approaches used to evaluate thermal tolerance and cardiorespiratory functions. For detailed descriptions of the surgical procedures and the experimental and analytical approaches, see the *Material and methods* sections of the individual papers.

2.1. Experimental animals and study sites

This thesis focuses on two teleost species: rainbow trout (*Onchorhynchus mykiss* Walbaum 1792, family *Salmonidae*) and European perch (*Perca fluviatilis* Linnaeus 1758, family *Percidae*). Both species are considered to be eurythermal, which means that they can cope with a relatively large span of environmental temperatures. The rainbow trout have a well-developed coronary circulation, whereas the perch lack coronaries and are solely reliant on the luminal circulation for cardiac oxygenation. Thus, these species are suitable models for studying the relationship between cardiac oxygenation via the coronary or luminal circulation, and how that relates to cardiac function and thermal tolerance. Rainbow trout has been extensively used for physiological studies and considerable physiological information is available for this species. The perch has not been as extensively studied with regards to its physiology, yet some recent studies have provided detailed information on both metabolic and cardiovascular function in this species (Brijs et al., 2015; Christensen et al., 2016; Sandblom et al., 2016a).

2.1.1. European perch and the Biotest enclosure

European perch are a common species inhabiting both freshwater and brackish aquatic environments in Sweden as well as throughout Europe and northern Asia (http://www.fishbase.org). In paper **I** and **III**, perch from the Biotest enclosure were used to study the effects of chronic warm-acclimation on cardiac and metabolic functions. The Biotest enclosure is located in the Baltic Sea (brackish water, ~5 ppm) ~150 km north of Stockholm, Sweden (60°25'41.2"N 18°11'20.2"E) and is a ~ 1 km^2 large man-made enclosure that was constructed by building dikes between adjacent natural islands (Fig. 4A, B).

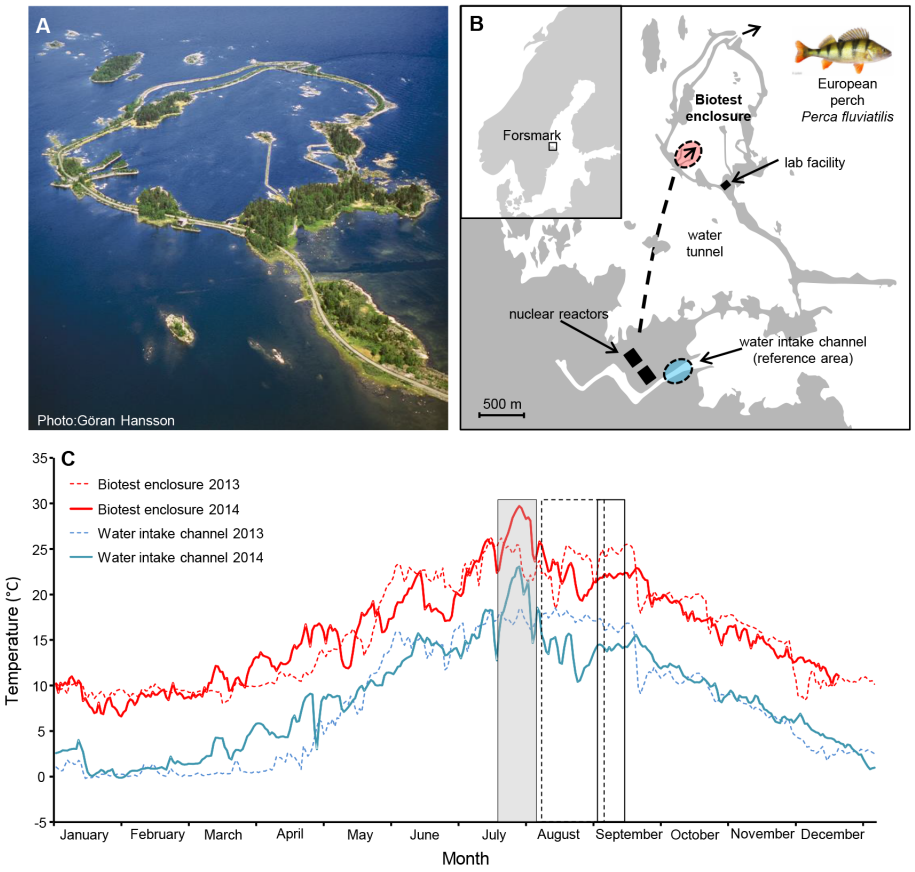

Figure 4. Overview and annual thermal profiles of the Biotest enclosure in Forsmark. Panel A shows an aerial photograph and panel B shows a schematic illustration of the Biotest enclosure where heated cooling water from the nuclear reactors is led into the enclosure via underground tunnels (dashed line). The inset I panel B shows the location of Forsmark along the Swedish Baltic Sea coast. European perch, (*Perca fluviatilis*), used in Papers **I** and **III**, were collected from the water intake channel (reference area, blue ellipse) or from the Biotest enclosure (red ellipse). Panel C illustrates the annual thermal profiles of the reference and Biotest areas during the experimental periods in 2013 (**I**, dashed line box) and 2014 (**III**, solid line box). The shaded box highlights the extreme thermal event that occurred prior to the sampling period in 2014. Figure modified from Sandblom et al. (2016) and **III**.

The Biotest enclosure is continually heated as it receives heated cooling water (~90 m^3 s^{-1}) via underground tunnels from two of the nuclear reactors at the Forsmark nuclear powerplant located on the mainland (Fig. 4B). The average water temperature in the enclosure is approximately 5-10°C higher than the adjacent Baltic Sea (Fig. 4C) (Hillebrand et al., 2010; Sandblom et al., 2016a; Sandström et al., 1995). Upon its completion in the early 1980´s, the Biotest enclosure enclosed a segment of the Baltic ecosystem and now constitutes a unique model for the studying the effects of chronic temperature elevations on aquatic fauna. The enclosure contains a number of teleost species including

roach (*Rutilus rutilus*), northern pike (*Esox lucius*), bleak (*Alburnus alburnus*) and a population of European perch (Sandström et al., 1995).

Perch used in these studies were caught by hook and line from the Biotest enclosure and from the intake channel from which water from the Baltic Sea enters the cooling system of the nuclear reactor (*i.e.* reference fish, Fig. 4A, B). The experiments where conducted in August 2013 (**I**) and September 2014 (**III**). During this time, the average temperatures of the reference and Biotest sites were 18 and 24°C (**I**), and 15.5°C and 22.5°C (**III**), respectively. Following capture, the fish were kept in holding tanks supplied with water from either the Baltic Sea or the Biotest enclosure, and the water temperature was controlled to reflect the temperature at each site. Worth noting for later reference, is the extreme thermal event that occurred in July 2014, which raised the average temperatures in the reference area and the Biotest enclosure by 3.5-5.9°C compared to 2013 (highlighted in Fig. 4C). This event may have resulted in mortality and subsequent thermal selection of perch and other species in the Biotest enclosure, which may have had implications for the experiments performed later that year (**III**).

2.1.2. Rainbow trout

In **II** and **IV**, farm-raised rainbow trout were obtained from a local fish farm (Antens laxodling AB, Alingsås, Sweden). Rainbow trout are not a naturally occurring species in Sweden and the fish used for the current experiments likely belong to the strain that was introduced to Sweden from Germany in 1893. Since its introduction, this species has been extensively used in the aquaculture industry for food production and put-and-take angling purposes (for a review, see Stanković et al., 2015). The trout were kept in holding tanks and were acclimated to 10°C for several weeks prior to the experiments.

2.2. *In vivo* recordings of cardioventilatory variables

2.2.1. Blood flow

Blood flow (cardiac output and coronary blood flow), was measured using pulsed Doppler flow probes (Iowa Doppler products, Iowa City, IA, USA, **IV**) or transit time flow probes (Transonic flow probes, Transonic Systems, Ithaca, NY, **I** and **II**). The probes were fitted around the ventral aorta or the coronary artery, respectively (see methods of papers **I, II** and **IV** and Fig. 5).

The Doppler technique allows measurement of relative changes in blood flow (reported as % of resting values in **I**) by assessing the change in frequency of the echo of the ultrasonic sound signal (20 MHz) from a transducer (a small piezoelectric crystal) that is transmitted through the vessel at a ~45° angle. The transmitting crystal is also acting as a receiver and listens to the echo of the

sound signal. When bouncing off the erythrocytes in the blood stream, the signal undergoes a change in its frequency that is detected by the transducer/receiver. The change in frequency (Doppler shift) is proportional to the blood flow velocity.

Transit time flow probes builds on a technology where a transducer/receiver (piezoelectric crystal) emits an ultrasound signal that traverses the vessel and bounces back to a second transducer via a reflector. By assessing the phase shift in transit-time (acceleration/deceleration) of the signal when passing through the vessel and its constituents (blood plasma and erythrocytes), the transducer assess the volume of fluid passing the transducer per unit of time in ml min^{-1} (http://www.transonic.com).

Doppler probes are advantageous as they are relatively inexpensive and can be custom-made for a precise fit on the vessel, whereas transit time flow probes are bulkier, only comes in certain sizes and are quite expensive. The main benefits of transit time flow probes is that they provide absolute flow values, however, they are factory calibrated and if used at different temperatures, they need to be recalibrated for an accurate determination of blood flow. This typically requires one calibration procedure for each probe. Conversely, acquiring absolute flow values using Doppler flow probes is more time consuming as the calibration process necessitates cannulating and perfusing the instrumented vessel *in situ,* that is, in the experimental animal after the completion of the experiments.

In papers **I** and **II**, transit time flow probes were used to acquire absolute values for coronary blood flow (**II**) and to enable calculation of cardiac power output (**I**). In paper **II**, a Transonic blood flow probe was placed around the coronary artery of rainbow trout downstream of where this vessel branches off from the hypobranchial artery and before it enters the pericardium. Thus, coronary blood flow was recorded without opening the pericardium (Fig. 5) which is important in order to avoid the known deleterious effects that pericardial opening may have on cardiac filling and function (Farrell et al., 1988b). A Transonic flow probe was also placed on the ventral aorta to record cardiac output in these fish. In paper **IV**, Doppler probes were used as estimating the relative changes in ventral aortic blood flow and stroke volume was deemed sufficient for assessing the effects of autonomic blockade on cardiac performance during acute warming in rainbow trout. Consequently, the measurements of these variables started from an initial common starting point in this experiment.

2.2.2. Heart rate and electrocardiogram recordings

Heart rate was determined either by analysing the pulsatile systolic traces from the flow probe signals, or by implanting electrodes (**II**) to record the electrocardiogram (ECG, Fig. 5B). The ECG of most fish is similar to the

mammalian ECG; and is comprised of a P-wave and QRS complex which represents the depolarisation of the atrium and ventricle, respectively, and a T-wave reflecting the repolarization of the ventricle (Farrell and Jones, 1992). Thus, the ECG reflects distinctive events of the cardiac cycle and provides useful information to evaluate heart rate and cardiac function (Olson and Farrell, 2006). In paper **II,** *in vivo* recordings of the ECG were used to assess cardiac activity during routine conditions and during acute warming, and to evaluate the effects of oxygen deprivation of the compact myocardium following ligation of the coronary artery on the ECG in rainbow trout. The ECG was also used to reveal cardio-pathological conditions such as cardiac arrhythmias and symptoms indicating cardiac ischemia (oxygen deprivation) and/or hyperkalemia (elevated K^+ levels), which may arise from a blockade of the coronary oxygen supply to cardiac tissues (Banka and Helfant, 1974; du Toit et al., 2001; Hill and Gettes, 1980; Munz et al., 2011; Preda and Burlacu, 2010; Sun et al., 2013). For example , acute occlusion of coronary blood flow in mammals can result in electrocardiogram alterations including elevation of the S-T segment (*i.e.* the baseline between the S and T wave), reduction in the R-wave amplitude, as well as inversion/disappearance of the T wave (Munz et al., 2011; Preda and Burlacu, 2010; Sun et al., 2013).

2.2.3. Determinations of blood pressure and cardiac power output

In the perch, the afferent branchial artery is easily accessible, and a saline-filled catheter (PE-31) was inserted at the base of the third gill arch and forwarded towards the ventral aorta. This allowed recordings of ventral aortic blood pressure in these fish (see **I** and Fig. 5), which in combination with simultaneous measurements of cardiac output allowed the first *in vivo* determination of cardiac power output in fish exposed to acute warming.

2.2.4. Opercular ventilation frequency

In paper **IV**, a water-filled catheter (PE-90) was used to determine the opercular ventilation rate in rainbow during acute warming (Fig. 5). The insertion of a catheter in the operculum represents a relatively non-invasive method that allows online recordings of ventilation rate. This method provide obvious benefits to other methods, such as, recordings of the electrical activity of respiratory muscles which require a more invasive methodology (Rogers and Weatherley, 1983), or by time-consuming visual quantifications of opercular movements (Smith and Jones, 1982).

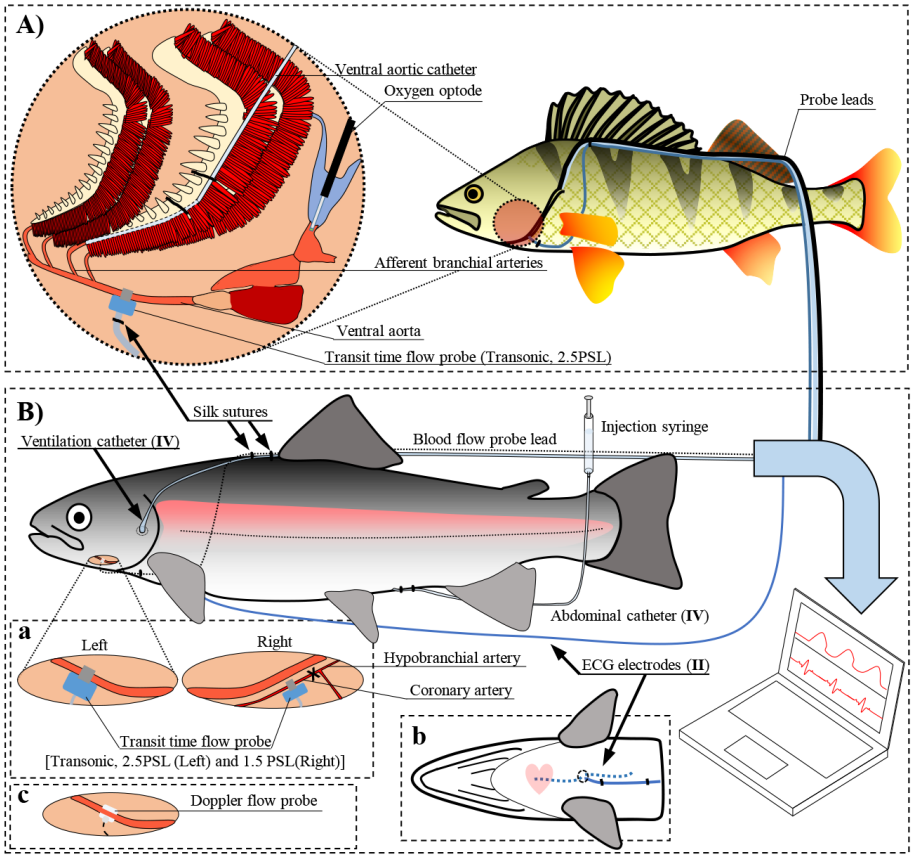

Figure 5. Illustration of the surgical instrumentation in papers I-IV. Panel A show European perch (*Perca fluviatilis*; paper **I**) instrumented with a transit-time flow probe around the ventral aorta to record cardiac output, a catheter in the afferent branchial gill artery to measure ventral aortic blood pressure and a fibre-optic oxygen optode in the ducts of Cuvier to measure the partial pressure of oxygen in the venous blood. Panel B show the instrumentation of rainbow trout (*Onchorhynchus mykiss*). In paper **II**, fish were instrumented with transit time flow probes around the ventral aorta (from the left side) and the coronary artery (right side) to record cardiac output and coronary blood flow, respectively (inset a). Trout were also equipped with sub-cutaneous electrodes for electrocardiogram (ECG, inset b) recordings during and after coronary blood flow occlusion using a vascular clamp (marked by **X**). The ECG pattern was also used to obtain the heart rate from trout where the coronary artery had either been surgically ligated (marked by **X**), or were left intact (*i.e.* sham). In paper **IV**, rainbow trout were equipped with a Doppler flow probe around the ventral aorta (inset c) to record heart rate and relative changes in cardiac output and a ventilation catheter in the operculum to record ventilation frequency and opercular ventilation pressure. A catheter in the abdominal cavity was used to inject pharmacological substances to block specific autonomic nerve functions. Graphical illustration by Andreas Ekström and Albin Gräns.

2.2.5. In vivo recordings of venous oxygen tension

In paper **I,** a modified method of that described by Farrell and Clutterham (2003) was used to record the P_{VO2} in perch. Briefly, a fibre optic oxygen optode (Firesting, Pyroscience, Aachen, Germany) was inserted into the ducts

of Cuvier pointing towards the *sinus venous* allowing recordings of the P_{VO2} of the venous blood entering the heart. The Firesting optodes relies on REDFLASH technology for the high resolution determination of oxygen consumption by quantifying the change in luminescence caused by the reaction between oxygen and the REDFLASH dye contained on the optode tip. The optodes are temperature sensitive and during the measurements an external temperature sensor was used to automatically compensate for changes in temperature (http://www.pyro-science.com). This method allowed continuous recordings of P_{VO2}, which along with the recordings of cardiovascular parameters allowed the first *in vivo* determinations of P_{VO2} thresholds for cardiac performance (*i.e.* heart rate and cardiac output) during acute warming in fish.

2.3. *In vitro* determinations of cardiac mitochondrial enzymatic function and lipid composition during warming

2.3.1. Enzymatic catalytic capacity

In paper **III,** the effects of acute and chronic temperature changes on the thermal sensitivity of key enzymes governing aerobic and anaerobic ATP production in the heart were investigated. Ventricular tissues were collected from reference and Biotest perch during the summer of 2014. The catalytic capacity of PK, LDH, PDH, CS MDH, AAT HOAD, CI, CIII, the ETS (CI+CIII) and CIV were determined in ventricular homogenates using a spectrophotometric assay technique at the approximate acclimation temperatures of the reference and Biotest populations (16 and 23°C, respectively), at the CT_{max} of each population as determined from paper **I** and previous determinations (Sandblom et al., 2016a) (30 and 32.5°C, respectively) and at a thermally extreme temperature (36°C). This method estimates the catalytic rate of enzymes over time by continuous recordings of the concentration dependent change in light absorbance resulting from the synthesis or consumption of metabolic products and substrates, respectively. Estimating catalytic rates offers several advantages over, for example, gene expression by qPCR or protein level quantifications by Western blot, when evaluating acute and chronic effects of increasing temperature on metabolic processes. While determining quantitative changes of the enzymatic components governing metabolic pathways offer indirect indications of metabolic capacity in the cell, the current method directly estimates the functional capacity and thermal sensitivity of these processes (Newsholme and Crabtree, 1986). It is however important to bear in mind that *in vitro* assessments of enzymatic activities may not completely reflect the actual *in vivo* metabolic flux of living tissues (Johnston, 1977; Suarez et al., 1997; West et al., 1993).

2.3.2. Mitochondrial membrane composition

In Paper **III**, the mitochondrial membrane composition, *i.e.* the lipid profile, of the perch heart was characterized for reference and Biotest perch by quantifying the proportions of saturated, monounsaturated and polyunsaturated (including omega-3 and omega-6) fatty acids. Briefly, the proportions of fatty acids were distinguished by first separating the individual fatty acids by direct transesterification, yielding fatty acid methyl esters (FAMEs), which were subsequently identified and quantified using gas chromatography.

2.4. Experimental protocols

2.4.1. Thermal challenge and determination of the upper critical thermal maximum

In papers **I, II** and **IV**, fish were exposed to an acute thermal challenge, starting at the acclimation temperature for rainbow trout (10°C, **II** and **IV**), or at an intermediate temperature for reference and Biotest perch (19°C, **I**). In practice, two methods are generally used to determine the upper thermal limit of fish. The incipient upper lethal temperature (IULT), developed by Frederick Fry and colleagues, is used to determine the temperature at which 50% of the individuals in a sample of fish die after an acute transition from their acclimation temperature to a range of test temperatures (Fry et al., 1942). This method is thus a static and lethal method, which requires rather large sample sizes and cannot be used to record the continuous effects of temperature on physiological parameters. In contrast, the critical thermal methodology (CTM) is a dynamic method where the environmental temperature is gradually increased to the point at which locomotory functions starts to deteriorate and the animal loses its ability to escape from unfavourable (*e.g.* thermally extreme) conditions (Cowles and Bogert, 1944). This method is beneficial as it is nonlethal, require smaller sample sizes and is suitable when recording the effects of warming on physiological parameters (Beitinger and Lutterschmidt, 2011).

In, **I, II** and **IV**, the water temperature was gradually increased in 1°C steps at a rate of 2-3°C h^{-1} and CT_{max} was determined as the temperature where the fish were unable to maintain an upright body position (*i.e.* loss of righting) for >3 seconds. The heating rate was chosen to allow sufficient time for the deep muscle tissues of the fish to thermally equilibrate with the surrounding water, as previously determined in perch (Sandblom et al., 2016a).

2.4.2. Pharmacological blockade of autonomic tone

In paper **IV**, the cholinergic and adrenergic effect on the heart were blocked by intra-abdominal injections of atropine sulphate and sotalol hydrochloride, respectively. Atropine sulphate is a competitive antagonist that prevents the binding of acetylcholine to muscarinic receptors, while sotalol hydrochloride is a competitive β-adrenoreceptor blocker, which prevents binding of noradrenaline and adrenaline to the receptor.

2.4.3. Manipulations of myocardial oxygen supply

To determine the importance of coronary oxygen supply to the heart in trout (**II**), the coronary blood flow was blocked, either by placing a surgical suture (irreversible) or a vascular clamp (reversible) around the coronary artery. In **I**, the luminal venous oxygen tension in perch was manipulated during warming by exposing the fish to water hyperoxia (*i.e.* 200% air saturation), which was achieved by bubbling the water in the experimental chamber with pure oxygen.

3. Results and Discussion

3.1. Effects of warming and cardiac oxygen supply on *in vivo* cardiovascular function and thermal tolerance

3.1.1. Importance of luminal oxygen supply in European perch

The recordings of P_{VO2} in European perch (**I**), revealed that the routine P_{VO2} of reference fish at 19°C was similar to previously reported values in salmonids at 6-15°C (Clark et al., 2008; Farrell and Clutterham, 2003), and in cod at 10°C (Lannig et al., 2004). However, the P_{VO2} in Biotest perch was significantly elevated compared to the reference fish across test temperatures. This likely reflects a right-shift in the oxygen-hemoglobin dissociation curve (Perry and Reid, 1994), and also a thermal compensation of cellular metabolism, oxygen consumption rate and thus tissue oxygen extraction as can be expected with warm acclimation (Sandblom et al., 2016; Sandblom et al., 2014). Furthermore, the reduced tissue oxygen demand alleviates the demand of the cardiovascular system to transport oxygen to the tissues, and was here reflected in a reduced heart rate and cardiac output in Biotest perch compared to reference perch across test temperatures (**I**). This also resulted in a significantly reduced routine cardiac power output in Biotest perch. Ambient hyperoxia (200% air saturation) significantly increased the resting P_{VO2} in perch, which likely reflects an increased oxygen uptake at the gills and over the body surface (Farrell et al., 2014; Glover et al., 2013).

The general thermal performance curves for cardiorespiratory variables observed in all treatment groups in perch (and also in trout, papers **II** and **IV**), were consistent with the typical patterns observed in other species of fish exposed to acute warming, with an initial increase followed by a more or less prolonged plateau phase, and a subsequent rapid decline when approaching critically high temperatures (Farrell, 2009; Gamperl et al., 2011; Gehrke and Fielder, 1988; Gollock et al., 2006; Steinhausen et al., 2008). The simultaneous recordings of ventral aortic blood pressure and cardiac output in perch allowed, to my knowledge, the first determination of cardiac power output in a eurythermal fish during acute warming. The progressive increase in cardiac output and ventral aortic blood pressure resulted in an approximate doubling of cardiac power output with a 10°C increase in temperature. Thus, in addition to the expected general effects of temperature on cardiac cellular metabolism (see *section 3.2*, **III**), this increase in cardiac workload reveals that the cardiac oxygen demand should increase substantially with acute warming. However, as we will see, the supply of oxygen may limit cardiac performance at increasing temperatures in perch (**I**), and also in trout (**II**) as will discussed later.

As expected, the P_{VO2} decreased progressively with warming in both reference and Biotest perch (**I**), likely due to an increased oxygen extraction at the tissues with increasing temperature (Clark, 2008 #1068). However, in the hyperoxic fish, the P_{VO2} remained significantly elevated until reaching 28°C, at which a sharp drop in P_{VO2} was observed. The explanation for this observation is unknown but it may relate to a sudden increase in tissue metabolism and oxygen extraction, as observed previously at extreme temperatures in Biotest perch (Brijs et al., 2015). This may be due to an increased leak across the inner mitochondrial membrane and thus an uncoupling of mitochondrial respiration (Chung et al., 2017; Chung and Schulte, 2015; Iftikar and Hickey, 2013; Iftikar et al., 2014), and/or a reduced cutaneous oxygen uptake (Farrell et al., 2014), but further studies are required to substantiate this.

The P_{VO2} thresholds where heart rate and cardiac output declined at high temperature where not statistically different between populations (2.3 and 2.6 *vs* 4.0 and 3.9 in reference and Biotest perch, respectively). However, the temperature where the peaks occurred was significantly higher in Biotest fish (**I**). There was also a tendency for a reduced cardiac power output during warming in Biotest perch. Thus, their improved capacity to maintain heart rate and cardiac output at higher temperatures likely reflected a combined effect of reduced cardiac oxygen demand, as well as a higher P_{VO2} due to lower tissue oxygen extraction across temperatures. This may also be related to an increased oxidative efficiency in the hearts of Biotest perch during acute warming (**III)**, as will be discussed more in *section 3.2*. Interestingly, the drastic decline in heart rate in hyperoxic reference fish occurred at a P_{VO2} threshold value (5.2±0.7 kPa) that was significantly higher than the normoxic reference fish (**I**). This observation contrasts with previous hypotheses suggesting that the decline in heart rate at high temperatures can be explained by a limitation in cardiac oxygen availability in acutely warmed fish (Clark et al., 2008; Lannig et al., 2004). In fact, the current data suggests that heart rate is limited by elevated temperature *per se* and not oxygen availability. Although the underlying mechanism of this heart rate limitation is presently unknown, it could be related to a thermally induced impairment of Na^+ conductance and action potential generation in the cardiomyocytes during warming. This may in turn be attributed to an impaired Na^+ channel function at high temperatures (Lennard and Huddart, 1991; Vornanen et al., 2014). However, warm acclimation may alleviate such negative effects (Haverinen et al., 2016; Haverinen and Vornanen, 2009), and may explain the maintenance of heart rate at higher temperatures in the Biotest perch. However, the thermal impairment of heart rate may also relate to a deterioration of mitochondrial ATP production (Chung et al., 2017; Iftikar and Hickey, 2013; Iftikar et al., 2014; Lemieux et al., 2010), which in turn may be due to a thermal failure of

enzymatic components governing ATP production in the heart. This was examined in paper **III** and is further discussed in *section 3.2*.

In contrast to the response of both normoxic reference and Biotest fish, the decline in heart rate approximately 3°C prior to CT_{max} in the hyperoxic fish was compensated for by an increased stroke volume, which maintained and even increased cardiac output at high temperatures in these fish (**I**). While an increased stroke volume may be expected considering the positive relationship between temperature and cardiac diastolic filling pressure and filling time as heart rate declined (Shiels et al., 2002;Clark et al., 2008), normoxic fish did clearly not have the capacity to increase stroke volume. Thus, the current *in vivo* data confirm previous findings from *in situ* perfused hearts and *in vitro* myocardial strip preparations, demonstrating that myocardial tissues rely on myocardial oxygen availability for providing adequate amounts of ATP to sustain or increase cardiac contractility (Driedzic and Gesser, 1994; Farrell, 1984). Indeed, an impaired aerobic ATP production would ultimately constrain the activity of the ATP-dependent SERCA pumps (Landeira-Fernandez et al., 2004), which would impair Ca^{2+} re-uptake by the SR and thus the diastolic relaxation and filling of the ventricle (Galli and Shiels, 2012; Periasamy and Janssen, 2008). Furthermore, ischemia in all body tissues including the heart typically results in an accumulation of anaerobic end-products, which renders the extracellular environment acidic and hyperkalemic, further impairing myocardial conductivity, rhythmicity and contractility of the heart (Chapman and Rodrigo, 1987; Driedzic and Gesser, 1994; Farrell, 1984; Hove-Madsen and Gesser, 1989; Kalinin and Gesser, 2002; Nielsen and Gesser, 2001).

The decline in cardiac output ultimately coincided with the CT_{max} in all treatment groups. However, whether the decline in cardiovascular performance is the principal cause of overall organismal failure at CT_{max} cannot be conclusively determined from the present data as discussed in *section 3.4*. At CT_{max}, there were no differences in P_{VO2} or any of the cardiovascular variables between the two normoxic groups (**I**). However, the Biotest perch displayed an elevated CT_{max} compared to reference perch (32.0±0.3°C *vs* 29.8±0.2°C), which was expected considering the known relationship between increasing acclimation temperature and CT_{max} (Beitinger et al., 2000; Sandblom et al., 2016a). Interestingly, stroke volume and cardiac output were higher in hyperoxic fish compared to both normoxic groups at CT_{max}, which coincided with higher P_{VO2}. The hyperoxia treatment increased the CT_{max} of the reference fish to 30.9±0.3°C, which is in accordance with the increased CT_{max} observed in goldfish (*Carassius carassius*) exposed to hyperoxia (Weatherley, 1970). These findings do, however, contrast with previous observations in Biotest perch (also acclimated to 24°C), for which an identical hyperoxia treatment did not increase CT_{max} further (Brijs et al., 2015).

It is possible that the Biotest fish used in that study had reached their upper ceiling of acute thermal tolerance, which could not be improved further with hyperoxia. The finding that hyperoxia did not increase CT_{max} of reference fish to the same level as normoxic Biotest fish, despite the significantly improved cardiovascular performance in the latter, suggests that factors other than cardiac oxygen availability at least in part explains the increased CT_{max} of these fish (see *section 3.4*).

The calculated P_{VO2} threshold values at which heart rate and cardiac output declined during warming in normoxic reference perch is in good agreement with previously reported values in 10°C acclimated cod (3.4 kPa; Lannig et al., 2004). However, it is higher than the P_{VO2} threshold values reported for salmonids species acclimated to 6-15°C (0.8-2.0 kPa; Clark et al., 2008; Davie and Farrell, 1991b; Farrell and Clutterham, 2003; Steffensen and Farrell, 1998). An intriguing possibility is that these differences reflect the fact that the salmonid heart receive additional oxygen via the coronaries. Indeed, as we will see in the next section, coronary blood flow plays an important role in maintaining cardiac oxygenation and performance during warming, which may affect the upper thermal tolerance limit of salmonids (**II**).

3.1.2. Importance of coronary oxygen supply during warming in rainbow trout

In paper **II**, it was hypothesized that coronary blood flow in trout would increase to improve myocardial oxygenation during acute warming. Similar to observations during other metabolically demanding activities such as spontaneous activity and sustained swimming (Axelsson and Farrell, 1993; Gamperl et al., 1995), coronary blood flow increased during acute warming from an initial value of 0.47±0.07 ml min^{-1} g^{-1} ventricle (representing 4% of cardiac output) at 10°C, to 0.72±0.09 ml min^{-1} g^{-1} ventricle at 14°C. However, between 14 and 18°C, there was no further increase indicating that a plateau was reached (**II**). Acute warming over the thermal range evaluated in paper **II** typically only elicits minor changes in dorsal aortic pressure (Clark et al., 2008; Gamperl et al., 2011; Heath and Hughes, 1973; Sandblom and Axelsson, 2007b), which represents the coronary perfusion pressure (see Axelsson, 1995). Thus, the plateau in coronary blood flow likely represents a point at which the coronaries were maximally dilated. However, it is also possible that the continuous increase in heart rate, and thus an increased time spent in cardiac systole with elevated temperatures (**II**), may have mechanically compressed the coronary vasculature and increased the coronary vascular resistance and counteracted any coronary vasodilation (Farrell, 1987). Interestingly, the plateau in coronary blood flow occurred despite that cardiac output (and likely cardiac power output as observed in perch in paper **I**)

increased continuously with temperature (**II**). This indicates an increasing mismatch between cardiac oxygen demand and cardiac oxygen supply via the coronaries during warming in trout, which will have increasingly severe implications for cardiac performance as the temperature increases. This would likely also cause a further reduction in P_{VO2}.

Occlusion of the coronary artery in anaesthetized rainbow trout resulted in reversible alterations of the ECG including S-T elevation, a disappearance of the T-wave and a reduced R-wave amplitude (**II**). While the causal mechanisms for these responses were not investigated further, they are similar to what is typically observed in mammals following brief coronary occlusion and is indicative of myocardial ischemia and transmembrane ion imbalances such as extracellular hyperkalemia (Banka and Helfant, 1974; du Toit et al., 2001; Hill and Gettes, 1980; Munz et al., 2011; Preda and Burlacu, 2010; Rodriguez et al., 2006; Sun et al., 2013). These effects of acute occlusion in anaesthetized fish may also explain the significant increase in routine heart rate in un-anaesthetized trout following chronic coronary ligation (**II**). The elevated heart rate following chronic coronary ligation is consistent with the findings of Farrell and Steffensen (1998) in moderately swimming rainbow trout (0.5 body lengths s^{-1}). This also coincided with a reduced ventral aortic blood pressure during exercise and indicates an impaired cardiac contractility and stroke volume, possibly as a consequence of an ischemic and hyperkalemic ventricular myocardium. Indeed, as 41% of the trout ventricle (the compact layer) were rendered anoxic following ligation, this likely constrained the ventricular contractile force development in the trout in paper **II**. Thus, the increased heart rate observed *in vivo* may have served to maintain systemic blood pressure following coronary ligation, which could be due to a release of cholinergic tone on the heart.

Acute warming significantly increased heart rate in both coronary ligated and sham-treated fish until reaching maximum values (120±20 and 115±29 beats min^{-1}) at 20 and 23°C, respectively, and thereafter plateaued (**II**). Thus, both groups reached heart rates that were close to the upper limit for heart rate of 120 beats min^{-1} in adult teleosts, as suggested by Farrell (1991). The reason why this occurred at a lower temperature in ligated fish was probably due to the elevated resting heart rate in this group, which left a smaller scope for increasing heart rate during warming. This was also evident in the significantly lower ABT for heart rate in ligated compared to sham treated fish (24.6±0.4 *vs* 23.0±0.6°C, respectively). While cardiac output was not measured in paper **II**, an interesting possibility is that the increase in heart rate in the ligated fish could not fully compensate for an impaired stroke volume, which may have led to compromised cardiac output and a compensatory increase in tissue oxygen extraction during warming. This would have reduced the P_{VO2} and compromised luminal oxygen supply and aerobic ATP production of the

myocardial cells further. Additionally, an elevated heart rate would also have reduced diastolic retention time and myocardial oxygenation via the luminal circulation. Such a chain of events may indeed have impaired cardiac contractility and stroke volume further, as observed in the perch heart when luminal oxygen availability decreased (**I**), while also impairing sinoatrial ion conductance and action potential generation in the pacemaker cells. This could explain the lower ABT for heart rate in ligated fish. Not surprisingly, the earlier onset of cardiac decline in ligated fish during warming was associated with a significantly reduced CT_{max} (26.3±0.3 *vs* 25.3±0.2°C, **II**).

3.2. Effects of warming on cardiac ATP production

3.2.1. Acute responses

Whereas oxygen availability is crucial to maintain cardiac contractility (**I** and **II**), a thermal impairment of mitochondrial function could explain the reduction in heart rate in acutely warmed fish (**I, II** and **IV**) (Iftikar and Hickey, 2013; Iftikar et al., 2014). In European perch, the activity of most of the cardiac metabolic enzymes governing aerobic and anaerobic metabolism increase with warming (**III**). This is consistent with previous observations of increased enzymatic activities and mitochondrial respiration rate in fish hearts during acute warming (Blier et al., 2014; Chung et al., 2017; Iftikar and Hickey, 2013; Lemieux et al., 2010). However, at temperatures exceeding 30°C, the catalytic capacity to oxidize pyruvate to acetyl-CoA by PDH, and further conversion of acetyl-CoA to citrate by CS was significantly reduced in the heart of both reference and Biotest perch (**III**). The concomitant elevation in AAT activity (**III**), along with the strong correlations between MDH, AAT and CI at higher temperatures (32.5°C), indicates an increased reliance on the malate-aspartate shuttle to produce and provide CI with NADH. This may partially compensate for a reduced TCA cycle activity, yet most likely resulting in a lowered provision of NADH to CI.

The current findings indicate a reduced oxidative capacity above 30°C, which may constitute a bottleneck for the production of NADH and $FADH_2$ in the TCA cycle. Thus, previous indications of a thermally induced impairment of mitochondrial respiration and ATP production (Blier et al., 2014; Iftikar and Hickey, 2013; Lemieux et al., 2010) may indeed be related to a limited provision of the electron donors produced in the TCA cycle (Blier et al., 2014; Pichaud et al., 2011). The current observations could therefore constitute a contributing explanatory mechanism for the temperature dependent decline of heart rate reported in New Zealand wrasse (Iftikar and Hickey, 2013; Iftikar et al., 2014), and also in perch (**I**) and trout (**II, IV**) at critically high temperatures. Indeed, a limitation of myocardial ATP availability may constrain Na^+/K^+-ATPase activity, thus disrupting the maintenance of the

electrochemical Na^+ and K^+ gradients across the sarcolemma. In fact, it is possible that such a sequence of events could explain the heat dependent reduction in Na^+ flux, action potential generation and ultimately heart rate as previously reported in fish (Lennard and Huddart, 1991; Vornanen et al., 2014). Even so, the deterioration of the substrate oxidation system did not fully coincide with the deterioration of heart rate and consequently cardiac output as observed *in vivo* in either reference (between 28 and 29°C) or Biotest perch (between 30-33°C) in paper **I** and as previously reported by Sandblom and colleagues (Sandblom et al., 2016a). These discrepancies may reflect differences in the thermal history of the perch used in these studies. During the summer before the sampling of the perch in paper **III** (2014), a thermal event occurred that elevated the mean peak temperature in both the reference area and the Biotest enclosure with 3.5-5.9°C compared to the previous year of sampling [*i.e.* 2013; **I** and (Sandblom et al., 2016a); see *section 2.1*]. Thus, it is likely that the perch used in paper **III** represents a thermally selected sub-sample of fish, having a higher upper thermal tolerance limit compared to the perch used in the studies conducted during 2013.

While the increased activities of PK and LDH (**III**) could potentiate the anaerobic ATP production in both populations of perch at critically high temperatures, such processes lower internal cardiac pH which is known to impair enzymatic activity, heart rate and cardiac contractility in fish (Clark et al., 2008; Driedzic and Gesser, 1994; Farrell et al., 1983). This may have contributed to the decline in heart rate, as well as exacerbated the aerobic limitation of cardiac contractility observed in perch at critically high temperatures *in vivo* (**I**).

3.2.2. Chronic responses

Warm acclimated Biotest perch exhibited a significantly reduced anaerobic (LDH, **III**) and aerobic metabolic activity (PDH, CS, MDH AAT and CI, **III**). This reduction may have been related to a down-regulated expression of these enzymes, as observed in other studies following warm acclimation or adaptation (Jayasundara et al., 2015; West et al., 1999). Moreover, as CS is considered a reliable marker of mitochondrial content (Larsen et al., 2012), the lower activity of CS in Biotest compared to reference perch indicate a reduced mitochondrial content following warm acclimation in these perch. This would also be consistent with the known inverse relationship between mitochondrial density and environmental habitat temperature in fish (Shiels et al., 2011).

Considering the positive relationship between membrane unsaturation, metabolic activity of membrane-bound enzymes and transmembrane proton leak rates (Brookes et al., 1998; Hulbert and Else, 1999), the reduced cardiac metabolic activity in Biotest perch may be related to the lipid composition of the mitochondrial membrane (**III**). Cardiac tissues of Biotest perch exhibited

a reduced degree of unsaturation of the mitochondrial membrane, as indicated by a lower proportions of omega-3 and the general unsaturation index. This is also consistent with previous observations in fish exposed to chronic warming (Calabretti et al., 2003; Cossins et al., 1977; Hazel, 1995; Kraffe et al., 2007; Skalli et al., 2006). Preliminary observations in Biotest perch from our research group (Pichaud et al., unpublished observation) have demonstrated a reduced proton leak in these fish, which would favor oxygen utilization and thus efficiency of the cardiac mitohondria. Collectively, these changes would reduce myoardial oxygen demand at any given temperature (**I**).

The significantly increased activity of HOAD and CIV (**III**) in the Biotest fish at temperature exceeding 32.5°C suggest an increased oxidative capacity which may have improved the aerobic ATP production in these fish. This is also indirectly implied by the reduced activity of LDH, which indicates a reduced dependence on anaerobic metabolism in Biotest perch (**III**). These mechanisms may explain that the Biotest fish maintain heart rate and cardiac output to higher temperatures compared to reference fish, which coincided with an increased CT_{max} (**I**).

Regardless of any potential compensatory mechanisms which may aid in maintaining the provision of electrons to the electron transport chain, the deteriorating catalytic capacity of CI, CI+CIII and CIV at higher temperatures (between 32.5 and 36°C) would constrain aerobic ATP production in both populations of perch (**III**). This corroborates previous findings in fish (Iftikar and Hickey, 2013; Iftikar et al., 2014; Lemieux et al., 2010). Therefore, the upper thermal ceiling of cardiac functions, and possibly whole animal performance, may be related to a thermal impairment of aerobic metabolism.

3.3. Importance of autonomic control on thermal tolerance and cardiovascular performance during warming

3.3.1. Influence of cholinergic cardio-inhibition during warming

The current experiments suggests that the activity in the cholinergic branch of the autonomic nervous system reaching the heart increases during acute warming in both perch (**I**) and rainbow trout (**II** and **IV**). In trout, there was a progressive increase in the numerical difference of heart rate between control and atropinized fish during warming indicating an increasing cholinergic cardiac tonus, consistent with findings in Antarctic fishes (Franklin et al., 2001; Lowe et al., 2005), and in rainbow trout subjected to an acute warming challenge (Ekström et al., 2016).

Additionally, the divergent heart rate pattern that started to develop at 22°C between normoxic and hyperoxic reference perch likely reflects a cholinergic

inhibition of heart rate in the normoxic fish (**I**). This may reflect a reflex bradycardia which is known to occur with both external and internal hypoxia in fish, mediated via branchially located chemoreceptors (Farrell, 2007; Reid and Perry, 2003; Sundin et al., 1999; Taylor, 1985). Whether this provides any beneficial influence on the upper thermal limits for cardiac function and whole animal thermal tolerance was explored in **IV**, and discussed next.

In paper **IV**, it was hypothesized that if cholinergic inhibition of heart rate favors luminal oxygen diffusion in fish, a pharmacological blockade of cholinergic tone on the heart with atropine would be expected to impair myocardial oxygenation, contractility and possibly thermal tolerance during acute warming. However, no significant differences were observed in regards to cardiac output or CT_{max} between control and atropinized fish (25.9±0.2 and 25.9±0.3°C, respectively; **IV**). This finding was unexpected and implies that cholinergic input on the heart did not have any beneficial influence on these parameters. Considering the now known positive relationship between acute warming and coronary blood flow in trout (**II)**, it is possible that coronary blood flow increased to compensate for any reduction in luminal oxygenation following the atropine treatment. Thus, an increased supply of oxygen via the coronaries may have been sufficient to maintain cardiac oxygenation in these fish.

The findings in paper **I** and **II** indicates that a limitation of the cardiac oxygen supply primarily constrain myocardial contractility during warming. As adrenergic stimulation may improve cardiac contractility and thermal tolerance (Aho and Vornanen, 2001; Graham and Farrell, 1989; Hanson et al., 2006), it was hypothesized that pharmacological blockade of the β-adrenergic stimulation to the heart with sotalol would translate to impaired cardiac performance during warming and a reduced overall thermal tolerance in rainbow trout. In contrast, however, the upper thermal limit for heart rate (but not cardiac output) was significantly increased in the sotalol treated fish as compared to control fish, and no differences in CT_{max} were observed (26.0±0.3 *vs* 25.9±0.2°C). These findings were also unexpected and may reflect that an increased filling pressure served to maintain stroke volume and cardiac output during the thermally induced elevation in heart rate (Clark et al., 2008). Again, it is also likely that the supply of ATP needed to maintain cardiac contractility was improved by an increased cardiac oxygenation via the coronaries (**II**).

Collectively, the current findings may reflect compensatory changes in coronary flow maintaining cardiac function during warming, which masked the effects of the pharmacological treatments. Thus, an interesting avenue for future research would be to repeat the current experiments in coronary ligated trout or in a fish species that lack a coronary oxygen supply to the heart.

3.4. Does oxygen-dependent cardiac failure set thermal tolerance limits of teleost fish?

While the thermal physiology of ectothermic animals have been studied for well over a century (Carter, 1887; Davenport and Castle, 1895; Davy, 1863), the physiological mechanisms governing the upper limits for thermal tolerance in fish are still not fully understood (Beitinger and Lutterschmidt, 2011; Clark et al., 2013). The findings of this thesis provide further evidence that cardiac failure constraining oxygen delivery to vital somatic tissues, such as the central nervous system is an important mechanism determining acute heat tolerance limits of fish. Failure of the heart coincided with loss of righting and CT_{max} in both perch and trout (**I, II** and **IV**). While this may reflect an oxygen deprivation of the skeletal muscles and/or neural tissues governing locomotory functions, it may still be questioned whether heart failure was the only principal factor resulting in CT_{max}.

Interestingly, several lines of evidence suggest that CT_{max} is mainly due to a direct thermal impairment of central nervous functions. For example, Friedlander and colleagues (1976) found that heating of the whole animal, or only the cerebellum, induced similar behavioral disturbances and loss of righting at similar temperatures in goldfish. This was related to declining interneuron activity in the cerebellum, which may in turn be attributed to the increased permeability and reduced stability of excitable neural membranes in ectotherms at high temperatures (Bowler, 1963; Cossins et al., 1977). Moreover, recent findings in killifish (*Fundulus heteroclitus*) suggest that the functional failure of neural tissues may be specifically related to failing mitochondrial function in neurons at high temperatures (Chung et al. 2017). Such deterioration of neural conduction pathways may have adverse effects on both central and autonomic neural pathways regulating the coordination and activity of skeletal and cardiac muscle. Indeed, Ern at al. (2015) recently demonstrated that motor-neuron action potential conduction deteriorated at high temperature close to the upper thermal limits of European crayfish (*Astacus astacus)*, which also coincided with a decline in heart rate and ventilation rate. Thus, it is possible that the decline in heart rate observed in both perch and trout (**I, II** and **IV**), as well as ventilation rate in trout (**IV)**, at high temperatures could be a consequence of a failed conductance in the cranial and spinal nerves controlling the heart and respiratory muscles. However, there was a trend (P=0.06) indicating that the decline in ventilation rate occurred at higher temperature (~0.9°C) than the decline in heart rate (**IV**). While speculative, these findings suggest that cardiac failure precedes neural failure in trout.

Similar to the observation in cardiac tissue from Biotest perch (**III**), warm acclimation reduced the degree of unsaturation of neural membranes, which improves neural signal transduction at high temperatures (Cossins et al., 1977). Thus, the increased CT_{max} in Biotest fish may reflect an improved neural function (Cossins et al., 1977; Lagerspetz, 1974), which in combination with improved cardiovascular function at high temperatures (**I**) increased the thermal tolerance of these fish.

Collectively, it is reasonable to conclude that the underlying determinants of acute thermal tolerance of fish and other ectotherms likely involve several levels of physiological organization, including cardiorespiratory and neural functions.

4. Main findings and future perspectives

This thesis provides important novel insights into the physiological determinants of the upper temperature limits for *in vivo* cardiac function and whole animal thermal tolerance in teleost fish.

In contrast to the hypothesis suggesting that the decline in heart rate of fish at high temperatures is due to an oxygen limitation, the current findings show that the decline is instead due to the direct effects of temperature, possibly on the metabolic machinery of the heart. Indeed, several enzymatic components that govern ATP production in the heart, such as citrate synthase and several complexes in the electron transport chain, were impaired at critically high temperatures. Interestingly, the maintenance of cardiac contractility and stroke volume at high temperatures was dependent on oxygen availability. This was demonstrated in the perch by experimentally enhancing cardiac oxygen availability via hyperoxia, which improved cardiac stroke volume and output at high temperatures. The importance of cardiac oxygen supply was further demonstrated in rainbow trout, as a blockade of the coronary blood flow impaired cardiac stroke volume and resulted in a reduced upper limit for cardiac performance during warming. While an increased cholinergic tone on the heart during warming was hypothesized to improve cardiac oxygenation and function, a pharmacological blockade of this input did not affect cardiac or whole animal thermal tolerance in trout. Furthermore, cardiac contractility and stroke volume were hypothesized to be improved by adrenergic stimulation at high temperatures, yet when the adrenergic input to the heart was pharmacologically abolished, neither cardiac function nor thermal tolerance in trout was affected.

Collectively, these findings clearly demonstrate that direct temperature effects on the cardiac metabolic machinery, as well as a limitation of oxygen supply to cardiac tissues constrain cardiac functions at high temperature. My findings also highlight that the coronary circulation plays a vital role for oxygenating the heart during warming in fish, as the luminal oxygen supply is constrained at high temperatures. Thus, the coronaries may constitute an essential anatomical feature in some fish species, as they aid to maintain cardiac oxygenation and function when exposed to a warming environment. An interesting prospect for future investigations would be to more closely discern the relationship and/or interactions between the luminal and coronary oxygen supply to the heart. For example, simultaneous quantifications of P_{VO2}, coronary blood flow and cardiac performance would aid to provide a more detailed understanding of the functional significance of the coronary circulation during warming. Furthermore, unravelling the autonomic influence on heart function during warming in species that lack a coronary supply to heart would be beneficial, as the effects of the pharmacological treatments

used on trout may have been masked by compensatory increases in coronary blood flow.

The current findings also highlight that chronic warm acclimation in perch reduces the oxygen demand of the heart, increases the oxidative capacity of the mitochondria and restructures the lipid profile of the heart. These modifications were associated with an increased cardiac function and thermal tolerance in this species. However, the physiological mechanisms underlying the increase in aerobic capacity for ATP production requires further investigation. For example, a reduced saturation of the mitochondrial membrane would likely reduce the transmembrane proton leak and therefore improve mitochondrial respiration, but this hypothesis remains to be explored.

The mechanisms determining the upper limits for whole animal thermal tolerance in fish and other ectotherms most likely include the impairment of multiple physiological functions. Indeed, while a failing heart undoubtedly plays a crucial role in this relationship, the implications of a thermally impaired central nervous system cannot be underestimated. Future studies should therefore be focused towards a multifaceted approach in which the interaction and relative contribution of both cardiorespiratory and neural functions are evaluated in response to both acute and chronic elevations in temperature. In doing so, several levels of physiological organization should be taken into account, ranging from a cellular and metabolic level to the whole animal. Such an approach would allow a more complete understanding of the mechanistic determinants of thermal tolerance. This may also provide a more integrated picture of how fish may have to adjust their physiology to cope with a more thermally variable and warmer future.

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