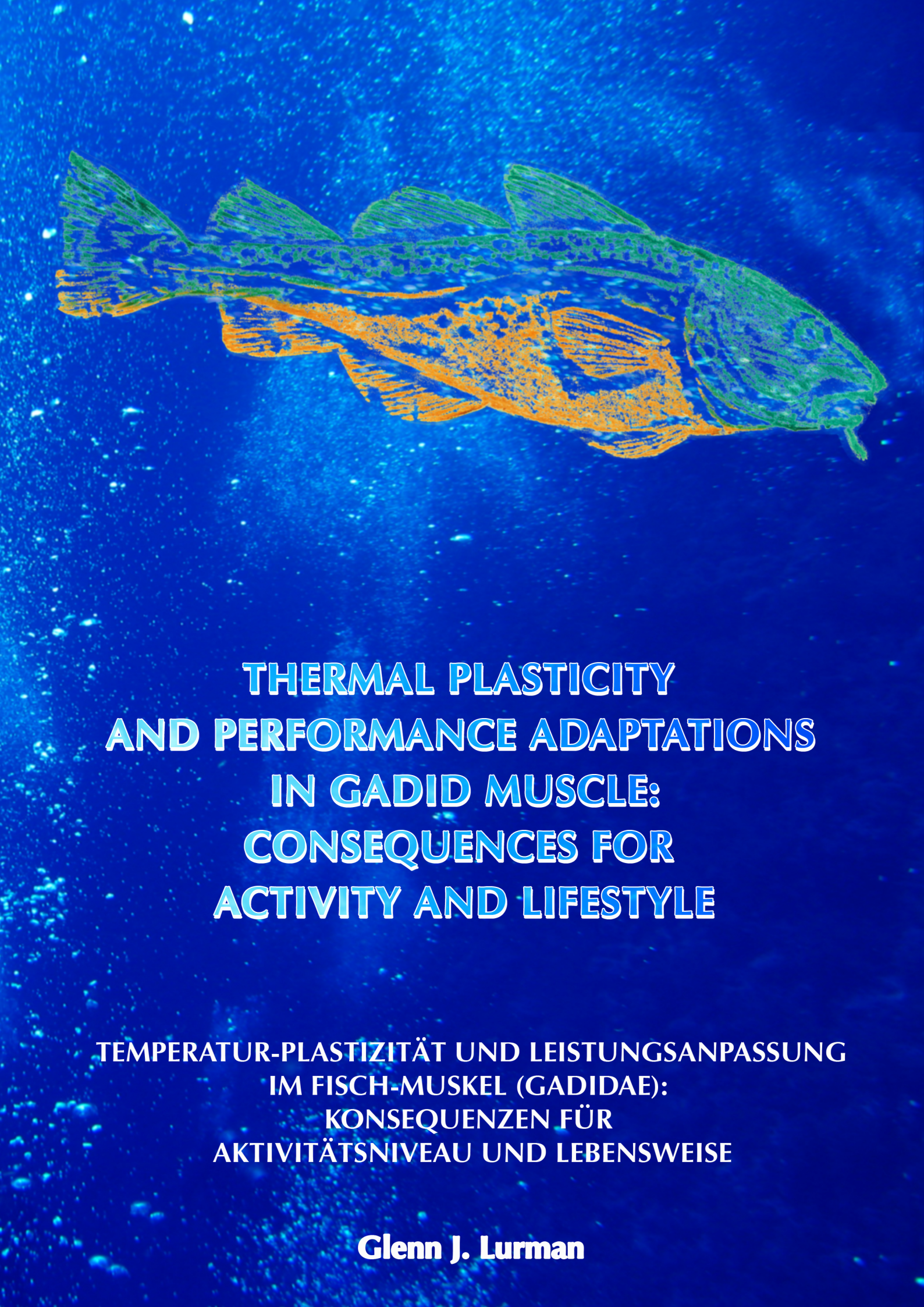

THERMAL PLASTICITY AND PERFORMANCE ADAPTATIONS IN GADID MUSCLE: CONSEQUENCES FOR ACTIVITY AND LIFESTYLE

TEMPERATUR-PLASTIZITÄT UND LEISTUNGSANPASSUNG IM FISCH-MUSKEL (GADIDAE): KONSEQUENZEN FÜR AKTIVITÄTSNIVEAU UND LEBENSWEISE

Glenn J. Lurman

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Temperatur-Plastizität und Leistungsanpassung im Fisch-Muskel (Gadidae): Konsequenzen für Aktivitätsniveau und Lebensweise

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Human understanding when it has once adopted and opinion… draws all things to support and agree with it. And though there may be a greater number and weight of instances to be found on the other side, yet these it either neglects and despises… in order that by this great and pernicious predetermination the authority of its former conclusions may remain inviolate.

Sir Francis Bacon, Nova Organum, 1620

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Dedication

To my family,

Daniela, Jasinta and Cléo.

For the unerring patience, love and support.

Contents

Summary

A recently developed model of thermal tolerance proposed that a hierarchy of biological functioning exists, each level of which displays different thermal tolerances. As the hierarchy is ascended from a molecular and biochemical level, through cellular, organ, systemic, whole animal and ultimately ecological levels, the windows of thermal tolerance narrow. The current study looked at the thermal plasticity and adaptational mechanisms at selected biological levels of cod populations from the North East Arctic (NEAC), the North Sea (NSC) and Newfoundland (NFC), to explore their potential impacts on activity and lifestyle.

NEAC and NSC were acclimated to 10 °C and 4 °C for more than 1 year. At a cellular level, this resulted in mitochondrial proliferation in red skeletal muscle. This was hypothesized to have down-stream effects on oxygen supply and demand in muscle tissues. An increase in the cardiac myoglobin expression was seen concomitant with mitochondrial proliferation. This was most likely a response to the increased myocyte oxygen demand. The myosin heavy chain enzyme activity in cardiac myocytes decreased with reduced assay temperature, whereas the acclimation temperature did not influence the activity at a given temperature. This indicated that myosin heavy chain isoform switiching seen in many other fishes was not active between 4 °C and 10 °C.

In situ cardiac performance was examined in NFC acclimated to 10 °C, 4 °C and 0 °C to see whether any of the above examined cellular adaptations affected cardiac performance. There was a continuous decrease in cardiac performance, namely heart rate, maximal cardiac output and maximal power output with decreasing acclimation temperature. Stroke volume was unaltered by acclimation temperature. An acute temperature reduction from 10 °C to 4 °C also resulted in cardiac performance comparable to the 4 °C acclimated hearts, while an acute increase from 4 °C to 10 °C resulted in significantly higher cardiac performance in comparison to the 10 °C acclimated hearts at 10 °C, possibly due to changes in the electrophysiological properties of the heart. An extra group of 0 °C acclimated cod revealed greater thermal sensitivity of cardiac performance at 0 °C compared to 4 °C acclimated hearts acutely exposed to 0 °C. Resting and maximal adrenaline doses (5 and 200 nM respectively) were found to have no impact on cardiac performance at any of the temperatures examined.

It was also hypothesized that mitochondrial proliferation in the cold would affect swimming performance. Therefore NEAC and NSC were acclimated to 10 °C and 4 °C. The standard and active metabolic rates, kick and glide duration and critical swimming speed were all reduced at 4 °C compared to 10 °C. During acute thermal challenge 4 °C acclimated cod were warmed to 10 °C and 10 °C acclimated cod were cooled to 4 °C. No differences were seen in any of the aforementioned physiological parameters between the populations at a given experimental temperature, regardless of acclimation temperature. Differences were seen in swimming efficiency measured as oxygen consumption increment, where 10 °C acclimated cod were more efficient than 4 °C acclimated cod at both experimental temperatures of 4 °C and 10 °C. This was also reflected in the net and factorial aerobic scopes which were consistently higher in 10 °C acclimated cod from both populations.

A unique ^{31}P-NMR spectroscopic technique in combination with a swim tunnel was developed to examine the metabolic processes during swimming and ultimately the cause of fatigue in NEAC. The onset of kick and glide bursts coincided with a significant accumulation of inorganic phosphate, and a drop in the intracellular pH and phosphocreatine. This indicated that kick and glide swimming was fuelled by anaerobic metabolism and that the traditionally defined critical swimming speed is the point of complete fatigue. Fatigue was the result of a significant drop in the Gibbs free energy of ATP hydrolysis. Consequently, ATP hydrolysis was unable to support cellular processes important to muscular contraction, i.e. ion transport and myosin heavy chain ATPase activity.

The mitochondrial proliferation and increased myoglobin expression, in combination with the reduced kick and glide duration and a lower swimming efficiency all point to a metabolic shift from anaerobic pathways at 10 °C, towards a greater dependence on aerobic metabolism at 4 °C. All Atlantic cod populations were able to maintain cellular and biochemical, physiological and systemic functioning between 4 °C and 10 °C. Although biochemical, physiological and whole animal performance were reduced at 4 °C, acclimation temperature had no effect. The thermal flexibility of the examined functions at the different levels of organisation observed in the current study between 4 °C and 10 °C is evidently a requirement of living in the thermally unstable environment in which Atlantic cod find themselves.

Zusammenfassung

Ein kürzlich entwickeltes Modell zur Temperaturtoleranz postulierte, dass eine Hierarchie der biologischen Funktionen besteht, dessen Ebenen sich in ihrer Temperaturtoleranz unterscheiden. Mit aufsteigender Hierarchie von der molekularen und biochemischen Ebene über die zelluläre, organische, systemische, Ganztier und schließlich hin zur ökologischen Ebene, verengen sich die Fenster der Temperaturtoleranz. Die vorliegende Arbeit beschäftigt sich mit der Temperaturplastizität und den adaptiven Mechanismen ausgewählter biologischer Ebenen des Kabeljau (*Gadus morhua* L.) von Populationen der nordöstlichen Arktis (NEAC), der Nordsee (NSC) und vor Neufundland (NFC), um die potenziellen Einflüsse auf die Aktivität und/oder das Lebensziel zu untersuchen.

NEAC und NSC wurden ein Jahr bei 10 °C und 4 °C akklimatisiert (*acclimated*). Auf der zellulären Ebene hatte dies eine mitochondriale Proliferation im roten Skelettmuskel zur Folge. Es wurde angenommen, dass dies einen Einfluss auf die Sauerstoffversorgung und den Bedarf im Muskelgewebe hat. Eine Expressionszunahme des kardialen Myoglobins in Verbindung mit der mitochondrialen Proliferation ist höchstwahrscheinlich eine Folge eines höheren Sauerstoffbedarfs der Myozyten. Die Enzymaktivität der schweren Myosin-Ketten in den kardialen Myozyten nahm mit abnehmender experimenteller Temperatur ab, wohingegen die Akklimatisierungstemperatur bei vorgegebener Temperatur keinen Einfluss auf die Aktivität hatte. Dies wies darauf hin, dass die Isoformen der schweren Myosin-Ketten zwischen 4 °C und 10 °C nicht umgeschaltet werden, wie es bei vielen anderen Fischen beobachtet werden kann.

Die *in situ* Herzleistung wurde am NFC untersucht, der an 10 °C, 4 °C oder 0 °C akklimatisiert wurde, um festzustellen, ob die oben geprüften zellulären Adaptationen die Herzleistung beeinflussen würden. Mit abnehmender Akklimatisierungstemperatur nahm die Herzleistung, gemessen als Herzfrequenz, maximales Herzminutenvolumen und maximale Leistungskraft, stetig ab. Das Herzschlagvolumen blieb unverändert durch die Akklimatisierungstemperatur. Auch eine akute Temperaturänderung von 10 °C auf 4 °C resultierte in einer Herzleistung, die mit der an 4 °C akklimatisierten und gemessenen Herzen vergleichbar war. Ein Anstieg der Temperatur von 4 °C auf 10 °C verursachte hingegen einen signifikanten Anstieg der Herzfunktion im Vergleich zu 10 °C akklimatisierten und gemessenen Herzen. Dies ist möglicherweise auf

Veränderungen der elektrophysiologischen Eigenschaften des Herzens zurückzuführen. Eine weitere bei 0°C akklimatisierte Kabeljaugruppe zeigte eine höhere Temperatursensitivität der Herzfunktionen bei 0°C im Vergleich zu 4°C akklimatisierten Herzen, die akut 0°C ausgesetzt wurden. Ruhe und maximale Adrenalindosen (5 bzw. 200 nM) hatten unabhängig von den untersuchten Temperaturen keinen Einfluss auf die Herzleistung.

Eine weitere Hypothese war, dass die kältebedingte, mitochondriale Proliferation einen Einfluss auf die Schwimmperformanz hat. NEAC und NSC wurden bei 10 °C und 4 °C akklimatisiert, um diese Hypothese zu prüfen. Die Ruhe- und Aktivstoffwechselrate, die Dauer des Stoß- und Gleitschwimmens („kick and glide“) und die kritische Schwimmgeschwindigkeit waren bei 4 °C im Vergleich zu 10 °C reduziert. Bei akuter Temperaturänderung wurde an 4 °C akklimatisierte NEAC und NSC auf 10°C erwärmt und an 10 °C akklimatisierte Kabeljau auf 4 °C abgekühlt. In keinen der untersuchten physiologischen Parametern wurden Unterschiede zwischen den Populationen bei gegebener Temperatur oder bei der entsprechenden Akklimatisierungstemperatur festgestellt. Es gab geringe Unterschiede in der Schwimmeffizienz, gemessen als Zunahme im Sauerstoffverbrauch. Bei beiden experimentellen Temperaturen von 4°C und 10°C schwammen die an 10 °C akklimatisierten Kabeljau effizienter als die an 4°C akklimatisierten Kabeljau. Dieser Befund spiegelte sich auch in einem höheren netto und faktoriellen aeroben Bereich der an 10°C akklimatisierten Kabeljau beider Populationen wider.

Es wurde eine einzigartige ^{31}P-NMR spektroskopische Technik in Kombination mit einem Schwimmtunnel entwickelt, um metabolische Prozesse während des Schwimmens und Ursachen der einsetzenden Erschöpfung zu untersuchen. Der Beginn des Stoß- und Gleitschwimmens war mit einer signifikanten anorganischen Phosphatakkumulation, einem Abfall im intrazelluären pH und im Phosphokreatin verbunden. Dies zeigt, dass das Stoß- und Gleitschwimmen ausschließlich durch anaeroben Metabolismus gedeckt wird und dass die traditionell definierte kritische Schwimmgeschwindigkeit den Zeitpunkt vollständiger Erschöpfung darstellt. Die Erschöpfung war die Folge einer signifikanten Abnahme der Gibbs freien Energie der ATP Hydrolyse. Die für die muskuläre Kontraktion wichtigen zellulären Prozesse, wie der Ionentransport und die ATPase Aktivität der schweren Myosin-Ketten, konnten demzufolge nicht durch die ATP Hydrolyse aufrechterhalten werden.

Die mitochondriale Proliferation und höhere Myoglobinexpression in Verbindung mit der reduzierten Stoß- und Gleitschwimmdauer und einer geringeren Schwimmeffizienz bei 4 °C zeigen eine metabolische Verschiebung von anaeroben Stoffwechselwegen bei 10 °C hin zu einer größeren Abhängigkeit von aeroben Stoffwechselwegen bei 4 °C. Alle analysierten Kabeljaupopulationen konnten die zellulären und biochemischen, physiologischen und systemischen Funktionen zwischen 4 °C und 10 °C aufrechterhalten. Obwohl die biochemischen und physiologischen Leistungen bei 4 °C herabgesetzt waren, hatte die Akklimatisierungstemperatur keinen Einfluss. Die thermische Flexibilität der untersuchten Funktionen auf den verschiedenen Ebenen zwischen 4 °C und 10 °C ist offenbar eine Anforderung an die unbeständigen Temperaturbedingungen im natürlichen Lebensraum des Atlantischen Kabeljau.

1 Introduction

1.1 Thermal tolerance

Temperature has been said to be the ecological master factor (Brett, 1971). As such it is the primary factor determining the biogeographic distribution of aquatic animals (Varley, 1967). This is especially true for ectotherms such as fishes, where changes in environmental temperatures have been related to alterations in fish stocks and migration patterns (Farrell, 1996; Dippner, 1997; Klyashtorin, 1998). Consequently, fish have been subject to a considerable body of work in relation to temperature. This is best demonstrated by the development of various temperature tolerance models (Fry, 1948; Varley, 1967; Brett, 1971; Fry 1971; Elliot, 1981; Beitenger et al., 2000). At either end of these models invariably lie the thermal limits. More recently, critical temperatures were defined as those at which an animal switches to anaerobic metabolism. Evidence for this was found in both marine invertebrates (Zielinski & Pörtner, 1996; Sommer & Pörtner, 1999; Frederich & Pörtner, 2000; Melzner et al., 2006) and fish (van Dijk et al., 1999; Mark et al., 2002; Sartoris et al., 2003). Expanding on this, a model of oxygen and capacity limited thermal tolerance based on Shelford's (1931) law of tolerance was recently proposed (Pörtner, 2001 & 2002). This model (figure 1.1), defines *pejus* temperatures as those at which an

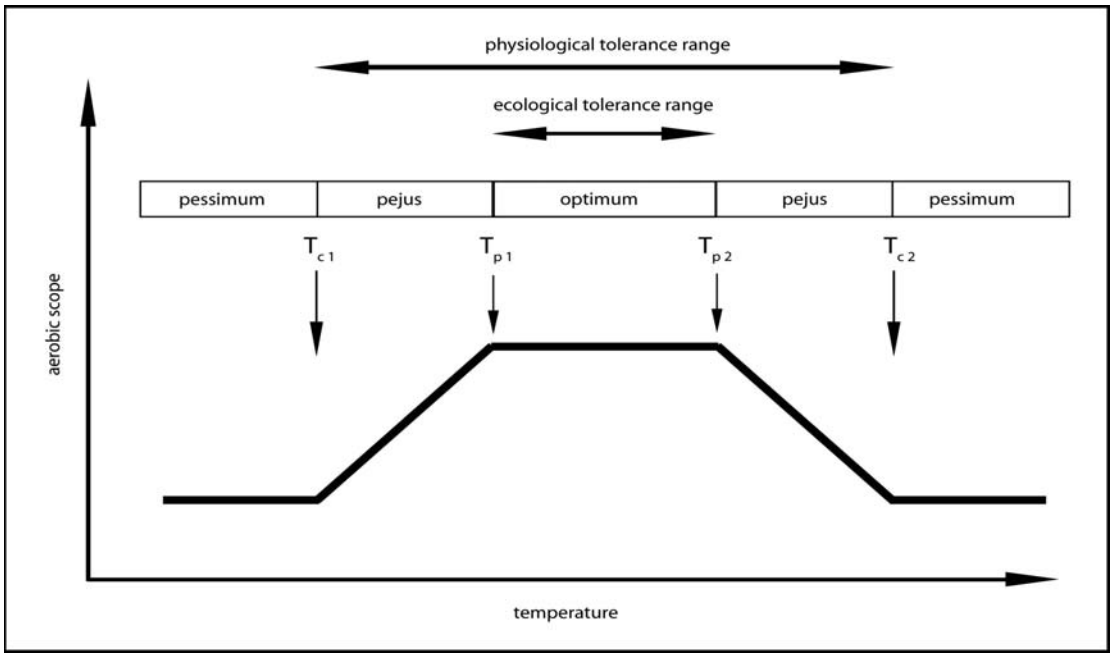

Figure 1.1. Oxygen limitation of thermal tolerance model. Modified from Frederich & Pörtner (2000) by Melzner (2007). See text for explanations.

animal experiences a reduction in the aerobic scope. The aerobic scope is the difference between the basal or standard metabolic rate (SMR; the energy requirements of a resting non-feed animal) and the active metabolic rate (AMR; the maximal aerobic energy requirements), see figure 1.2.

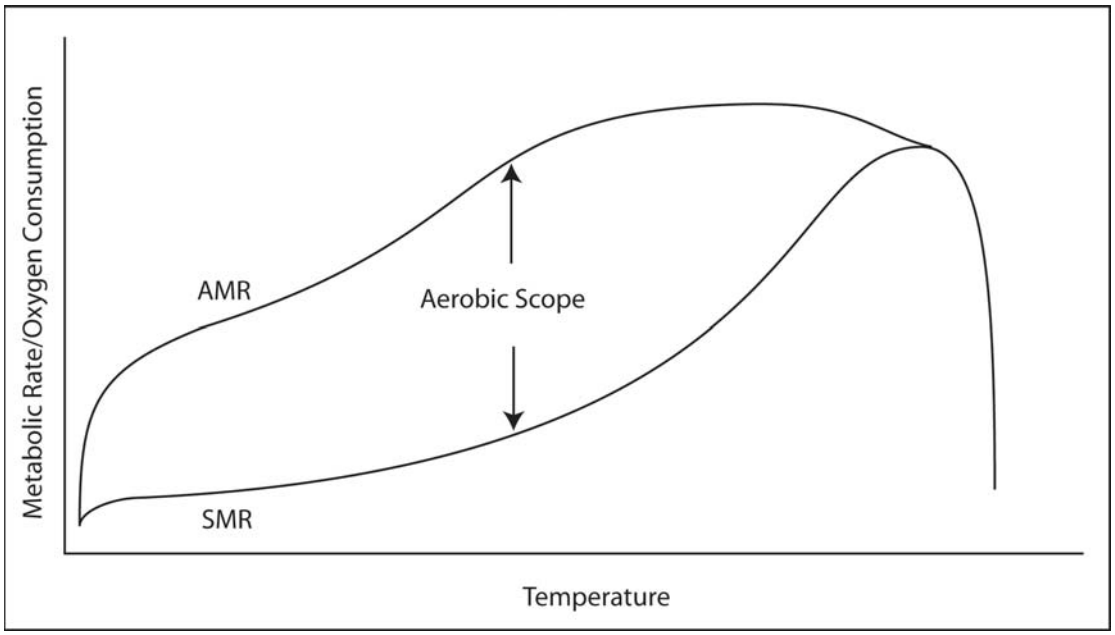

Figure 1.2. A model showing the influence of temperature on oxygen consumption and aerobic scope. Lines labelled AMR and SMR represent the active and standard metabolic rates respectively. The difference between the AMR and SMR is the aerobic scope.

According to Pörtner's (2001) model, a reduction in the aerobic scope is the product of a mismatch between oxygen supply capacity and oxygen demand. Over a range of optimal temperatures, any increase in oxygen demand is compensated for by a corresponding increase in oxygen supply. At some point (beyond the *pejus* temperatures, Tp_1 and Tp_2) the oxygen demand outstrips the oxygen supply. As the temperature continues to increase or decrease towards the critical temperature (Tc) the aerobic scope decreases until it is virtually eliminated. Any short-fall in energetic requirements beyond the critical temperatures are met by anaerobiosis. However, this is time limited and when left long enough will lead to death.

What are the physiological mechanisms that lead to the mismatch between oxygen supply and demand, thus setting thermal limits? A body of data exists which suggests that the oxygen supply to mitochondria is compromised by the ventilatory and/or circulatory systems. This was seen in various marine invertebrates (Frederich &

Pörtner, 2000; Peck et al., 2002; Melzner et al., 2007) and fishes (Hardewig et al., 1999; Mark et al., 2002; Lannig et al., 2004). At some point, the oxygen demand outstrips oxygen supply due to limited circulatory and/or ventilatory capacity at the upper end of the thermal spectrum. During cooling, the mitochondrial aerobic capacity is limited, therefore limiting energy supply to, and consequently performance of the circulatory and/or ventilatory systems (Pörtner et al., 2000). Because the maximum oxygen consumption by mitochondria is dependent on sufficient oxygen supply by the ventilatory and circulatory systems, the whole animal aerobic scope is reflected in mitochondrial aerobic scope (Pörtner, 2002).

Further evidence for this can be seen in temperate eurythermal fishes where acclimatization/acclimation to warm and cold temperatures will cause a shift in the mitochondrial capacity that is mirrored by the aerobic capacity. Acclimatization/acclimation to the cold leads to a hallmark increase in the mitochondrial density (Guderley & St. Pierre, 1996; Johnston et al., 1998; St. Pierre et al., 1998). It was hypothesized that this is because as temperature decreases mitochondria become less efficient due to an increase in the proton leak rate and a decrease in the ATP synthesis capacity (Pörtner et al., 2000). Because the mitochondria are less efficient, more are needed, leading to mitochondrial proliferation in the cold.

For example, in Atlantic cod from the North East Arctic (NEAC), both white muscle and liver citrate synthase and cytochrome-*c*-oxidase (markers of mitochondrial capacity) are higher at 4 °C than in Atlantic cod from the North Sea (NSC) at 4 °C (Lannig et al., 2003; Lucassen et al., 2006). This increase in mitochondria is thought to have caused a 1.4 fold increase in the basal metabolic demands, i.e. the standard metabolic rate, seen in 4 °C acclimated NEAC when compared to NSC acclimated to 4 °C (Fischer, 2002), a phenomenon known as metabolic cold adaptation. Metabolic cold adaptation (MCA) was first described in goldfish (*Carassius auratus*) by Krogh (1914), and since then in both worms *Arenicola marina* (Sommer & Pörtner, 1999) and as previously mentioned, in Atlantic cod (Fischer, 2002). Early evidence was also found for MCA in cold adapted Antarctic fishes (Wohlschlag, 1960), however this phenomenon has since been disproved in Antarctic fishes (Holeton, 1974; Clark & Johnston, 1999).

Why should MCA be evident in cold acclimatized/acclimated temperate eurythermal fishes that have mitochondrial volume densities of 29-33% (Johnston et al., 1998) and not cold adapted stenothermal Antarctic fishes that have similar

mitochondrial volume densities? Cold adapted stenothermal fishes, while indeed having high mitochondrial volume densities, also have key metabolic enzymes that have higher Arrhenius activation energies, thus reducing their metabolic capacity (Pörtner et al., 2005). Cold acclimatized/acclimated eurytherms do have higher SMRs as the activation energy is lower and consequently have a higher mitochondrial oxygen demand (Pörtner et al., 2000; Pörtner, 2002). More importantly perhaps, the effect of temperature on proton leak is different in different fishes. For example, cold-acclimated North Sea eelpout (*Zoarces vivparus*), show increased expression of UCP2 (uncoupling protein) and a higher state IV+ mitochondrial respiration rate compared to warm acclimated eelpout (Lannig et al., 2005; Mark et al., 2006). In the deep-sea Antarctic eelpout (*Pachycara brachycephalum*), a fish adapted to the cold, state IV+ mitochondrial respiration rates were lower in cold acclimated fish compared to warm acclimated fish (Lannig et al., 2005), indicating that adaptation to the cold results in a compensated proton leak rate while acclimation to the cold does not.

An increase in the SMR during cold acclimatization/acclimation in eurythermal fishes leads to two questions a) is AMR, and ultimately aerobic scope compensated in cold acclimated eurytherms, and b) how do any changes in SMR and AMR impact on aerobic performance? A meta-analysis of published data (Bushnell et al., 1994; Schurmann & Steffensen, 1997) reveals SMR was significantly higher in cold adapted Atlantic cod from Greenland compared to NSC at the same temperature. Furthermore, the AMR was slightly (but not significantly) lower. Yet the Greenland cod had a similar critical swimming speed (U_{crit}), indicating they were able to swim more efficiently (see table 1). However, it should be noted that the NSC were significantly larger and a) U_{crit} is known to scale inversely with increasing body size (Wardle, 1975), while b) the SMR of fish increases to the power of 0.8 with increasing size (Saunders, 1963). The smaller size may therefore explain both the higher U_{crit} and significantly higher SMR.

Table 1. A comparison of metabolic rates from Atlantic cod (*Gadus morhua*) sampled in the North Sea and southern Greenland. Data (mean ± SEM) from [1]Schurmann & Steffensen (1997) and [2]Bushnell et al. (1994). An asterisk indicates a significant difference.

	North Sea [1]	Greenland [2]
Temperature (°C)	5	4
Mass (g)	243.3 ± 30.5*	152 ± 14*
SMR ($mgO_2\ kg^{-1}\ hr^{-1}$)	47.5 ± 3.6*	66.2 ± 5.3*
AMR ($mgO_2\ kg^{-1}\ hr^{-1}$)	146.6 ± 11.0	137.3 ± 6.1
U_{crit}	1.6 ± 0.5	2.24 ± 0.21

It needs also to be mentioned that a potential trade-off for the increased mitochondrial volume density typically seen as a result of cold acclimation is a reduction in the number of myofibrils due to space and diffusion constraints, the so called optimal fibre number hypothesis (Johnston et al., 2003, 2004, 2005). A consequence of this functional trade-off is a reduction in the contractile ability of the muscle (Rome, 1990 & 1995). Because the power generated by the same muscle mass is reduced, more oxidative muscle fibres are needed to produce the same amount of power, so the proportion of red muscle is increased (Egginton & Sidell, 1989), and/or the white muscle fibres are recruited at lower swimming speeds. This phenomenon is known as a compression of recruitment order (Rome et al, 1984; Rome 1990, 1995). Ultimately this will impact upon swimming performance too.

1.2 Swimming performance

A fish's swimming ability is an excellent measure of its' ecological fitness. Swimming ability plays a vital role in survival. Sufficient swimming performance is needed during migration, e.g. European eels (*Anguilla anguilla*) migrating 5-6000km to the Sargasso sea (van Ginneken et al., 2005), salmonids (*Oncorhynchus* sp.) migrating up rivers in north west America (Farrell, 1996) and Atlantic cod migrating from the Barents Sea to Lofoten each year to spawn (Godø & Michalsen, 2000). Furthermore, a fish's swimming ability will determine whether it can escape from predators or capture prey (Beddow et al., 1995), and with the advent of fishing, a fish's swimming ability determines whether it can 'out run' a trawl (Winger et al., 2000).

A number of tagging/tracking experiments in the field have revealed interesting insights into the effects of temperature on fish swimming and behaviour. In the Arctic,

NEAC typically inhabit "Atlantic" water near the Arctic polar front that has a temperature warmer than, or equal to 2°C (Woodhead & Woodhead 1959). Nevertheless, NEAC were found to plunge into cold, Arctic waters (i.e. less than 1 °C) for short periods before returning to the warmer Atlantic water. Similar behaviour has recently been described in tuna (*Thunnus thynnus*) diving to cold deep water to catch prey (Wilson et al., 2005; Galli et al., 2007). In the lab, the effects of temperature on swimming performance have received much historical, and still recent attention (Fry, 1948; Brett 1967; Beamish, 1978; Taylor et al., 1996b; Schurmann & Steffensen, 1997; Seebacher et al., 2005), primarily due to a) the ease with which it can be observed and measured, b) it's relevance to survival and propagation and c) it is an integrative measure of various metabolic processes and overall fitness.

Three main swimming tests have been developed for fish (for a complete review see Hammer, 1995). The sustained swimming speed test is designed to determine the maximal speed a fish can maintain for more than 200 min, i.e. it tests a fish's stamina. The burst swim test is designed to determine the maximum burst speed of a fish over a short distance, e.g. a few meters. The critical swimming speed test is designed to determine the maximal swimming speed using step-wise speed increments. During each test, different gaits are employed. During sustained swimming, Atlantic cod use subcarangiform swimming, which is primarily aerobic. Subcarangiform swimming uses undulatory wave propogation along the length of the body, posterior to the (for cod, first) dorsal fin, such that one wave occurs along this length. Amplitude increases towards the tail (see Webb, 1993 for a full discussion of different swimming styles). Burst swimming, which is anaerobic, typically involves one powerful kick and is over within a second. During a critical swimming speed test, swimming below the critical swimming speed involves subcarangifom at lower speeds, e.g. 1-2 BL s^{-1} for a 20-30 cm cod, and a 'kick and glide' style at higher speeds (Videler, 1981). Critical swimming is the sum of both aerobic and anaerobic metabolism.

1.3 Cardiac performance

Cardiac performance plays a pivotal role in supporting aerobic swimming and is therefore regarded as the limiting factor for maximum exercise (Farrell, 1996; Taylor et al., 1996c). When superimposed on the temperature-performance curve for swimming performance and metabolic scope of sockeye salmon (*Oncorhynchus nerka*), the temperature-performance curve for cardiac scope displays an almost identical thermal

function (figure 1.3). This further supports the previous supposition that, oxygen supply, via the cardiorespiratory system is of critical importance in setting aerobic limits, be they during exercise or during thermal stress. As a corollary, we also find that animals with a larger cardiac scope, have a larger aerobic scope and consequently a larger thermal tolerance window (Pörtner, 2002).

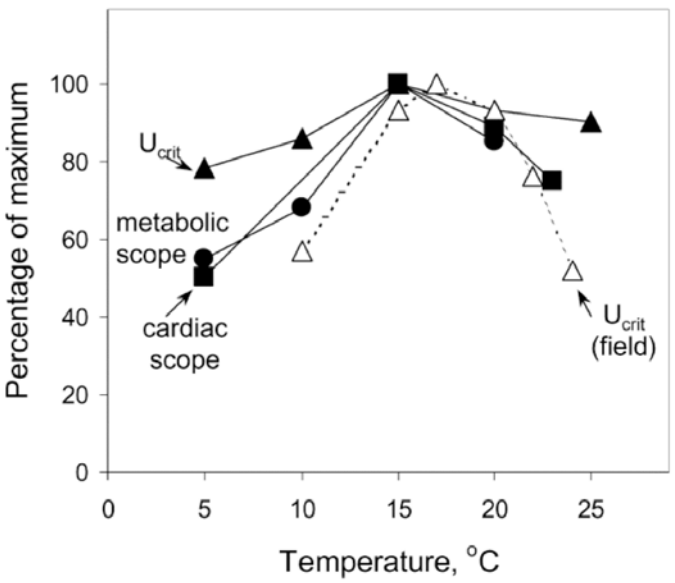

Figure 1.3. The effect of temperature on maximum critical swimming speed, metabolic aerobic scope and cardiac scope in adult sockeye salmon (*Oncorhynchus nerka*). Data are from laboratory experiments (solid lines) and field measurements (dotted lines). From Farrell (2002).

Maximum cardiac performance is limited by an insufficient oxygen supply (Farrell, 1996 & 2002). This has previously been observed during hypoxia (Farrell et al., 1985; Davie & Farrell, 1991; Driedzic & Gesser, 1994; Steffensen & Farrell, 1998). The fish heart is the last organ to receive blood (figure 1.4). Because the blood has already served the rest of the body, the venous oxygen partial pressure (Po_2) is low. In resting cod at 10 °C it is typically between 45 and 55 mm Hg, but it can be as low as 30 mm Hg (Lannig et al., 2004). During acute warming it is reduced to a critical threshold at which point the heart fails. This threshold is highly variable i.e. from 5 to 40 mm Hg (Lannig et al., 2004). Furthermore, a low threshold of 16 mm Hg appears evident during swimming in rainbow trout (Kiceniuk & Jones, 1977). What is perhaps more interesting is that temperature also has a significant impact on the venous Po_2 during swimming. The venous Po_2 in trout acclimated to 6-10 °C dropped from ~35 mm Hg at rest to a critical threshold of 15 mm Hg at about 85% of the critical swimming speed, while trout acclimated to 13-15 °C had a higher resting venous Po_2 of ~47 mm Hg and this dropped to 29 mm Hg at the critical swimming speed (Farrell & Clutterham, 2003).

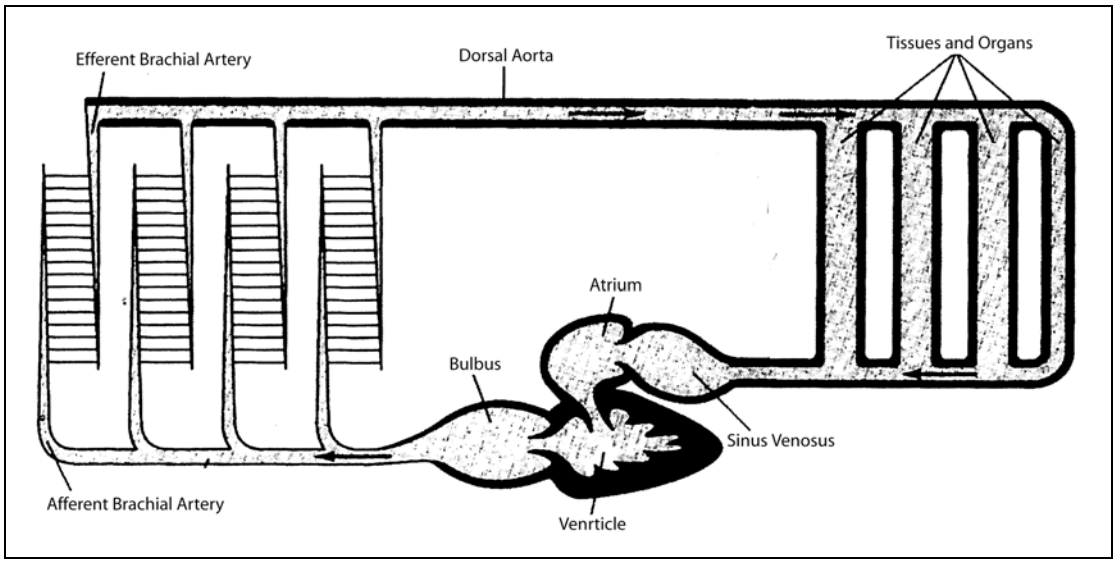

Figure 1.4. Schematic diagram of the fish cardio-vascular system. From Randall et al. (1997).

While the effects of acute temperature increase on cardiac performance have been comparatively well studied, the effects of acute temperature decrease are not nearly as well understood. Only one study has looked at cardiac performance in relation to decreasing temperature (Graham & Farrell, 1985). After acclimation of sea raven (*Hemitripterus americanus*) to 3 °C, the *in situ* heart rate and thus, power output of sea raven hearts were lower than those from 13 °C acclimated hearts, while stroke volume was unaltered. The changes seen during an acute thermal challenge, i.e. temperature reversal in the two groups, largely reflected those seen after acclimation, i.e. significant changes in heart rate and power output but not stroke work.

The ectothermic nature of fish, and their need to survive over a range of temperatures has led to a number of adaptations that facilitate relative thermal independence of cardiac performance. Cardiac hypertrophy increases stroke volume (Graham & Farrell, 1989; Kolok & Farrell, 1994; Farrell, 1996), electrophysiological changes to the pace-maker involving changes in membrane potentials compensate heart rate (Vornanen et al., 2002a & 2002b), and calcium handling, i.e. the contractile force generated is also known to be responsive to temperature in cardio-myocytes (Tiitu & Vornanen, 2001). All these processes counteract thermal effects compensating cardiac output.

Circulating catecholamines, and in particular adrenaline may also serve a vital role in maintaining cardiac performance during cold acclimation and acute thermal challenge (Taylor et al., 1996c). The salmonid heart is significantly more responsive to

adrenaline at low temperatures than high (Farrell et al., 1996; Shiels et al., 2003). What's more, adrenaline is known to serve a cardio-protective function in salmonids, under various conditions of stress, e.g. hyperkalemia, hypoxia, acidosis, and thermal challenge (Driedzic & Gesser, 1994; Mercier et al., 2002; Hanson et al., 2006). The role of adrenaline during thermal acclimation in other species, namely Atlantic cod is perhaps more enigmatic. While assorted studies have looked at the effects of adrenaline on vascular system in Atlantic cod (Axelsson & Nilsson 1986; Axelsson, 1988), little is understood of the relationship between cardiac performance, adrenaline, and temperature in Atlantic cod.

1.4 The heart

Within the heart there are two types of tissue, the spongiosa and the compacta (Axelson et al, 1998). The spongiosa is composed of trabeculated spongy muscle. The trabecular nature serves to increase the muscle surface area exposed to blood, thus increasing the oxygen extraction efficiency and avoiding the need for a coronary circulation (i.e. vascular system within the heart itself) to distribute the oxygen (Santer, 1985; Davie & Farrell, 1991; Axelson et al, 1998). Consequently, fish hearts with only a spongiosa derive all their oxygen and nutritional requirements from the venous blood (figure 1.4).

In some fishes, a compact layer (compacta) surrounds the spongiosa and is supplied by a coronary circulation system that delivers freshly oxygenated blood directly from the gills (Tota, 1983; Davie & Farrell, 1991; Axelson et al, 1998). Life style is thought to have a strong influence on the anatomy of the fish heart. As a rule of thumb, the more active the fish species, the more likely it is to have a vascularised compact layer (Davie & Farrell, 1991). For example, salmonids and scombrids are highly active species. Both have a well-developed compact layer that is perfused by a coronary circulation. It is also interesting to note that neither of these fishes is very hypoxia tolerant due to their high oxygen demands. Atlantic cod lie towards the other end of the spectrum, being less active than tuna and trout, meaning they have a heart that is made up solely of trabeculated spongiosa (Santer et al., 1983; Santer, 1985).

In fishes with hearts consisting solely of spongiosa, oxygen diffusion to all parts of the organ is one of the main factors limiting performance (Davie & Farrell, 1991). Further to this, temperature is known to have a significant affect on oxygen diffusion through tissues, slowing it down as temperature decreases (Sidell, 1983; Egginton &

Sidell, 1989). This is partially offset by an increase in the oxygen solubility with decreasing temperature, so that a decrease from 25 to 5 °C will result in a net 30% decrease in diffusivity within cells (Egginton & Sidell, 1989). The increased oxygen demand caused by mitochondrial proliferation during cold acclimation (as seen in NSC by Bremer, 2007) would seem to further aggravate this problem.

A number of measures have evolved to facilitate oxygen diffusion through various tissues during decreased temperature (for a comprehensive review, see Sidell, 1983), for example, myoglobin, which also combines to make the 4-subunit homodimer haemoglobin molecule in the blood, is present in muscle cells. Myoglobin has an oxygen binding affinity between that of haemoglobin and cytochrome-*c*-oxidase and acts as an intracellular carrier molecule facilitating oxygen diffusion and as an intracellular oxygen store (Egginton & Sidell, 1989). While much research has focused on the response of myoglobin to hypoxia (van Bui & Banchero, 1980; Driedzic, 1983; Driedzic et al., 1982; Silverman et al., 1997; Fraser et al 2006; Roesner et al., 2006), to date only two of studies have looked at myoglobin in relation to temperature, despite the significance of oxygen diffusion and its' influence on heart performance (Rosenmann & Morrison, 1965; van Bui & Banchero, 1980).

Data from a number of different fish species with different lifestyles, e.g. rainbow trout, European eel, and lingcod (*Ophiodon elongates*) indicates that the threshold for the effective extraction of oxygen from the venous blood by cardiac myocytes is between 6 and 16 mm Hg (Davie & Farrell, 1991). The oxygen partial pressure causing 50% saturation (P_{50}) of Atlantic cod myoglobin is unknown, however for coho salmon (*Oncorhynchus kisutch*) the P_{50} was 1.0 mm Hg at 12 °C (Nichols & Weber, 1989). This is similar to the P_{50} of 1.4 mm Hg seen with mackerel (*Scomber japonicus*) myoglobin at 20 °C (Marcinek et al., 2001). It is also interesting to note that the P_{50} decreases with declining temperature meaning the affinity increases (Nichols & Weber, 1989; Marcinek et al., 2001). These P_{50} values lie below the lowest venous Po_2 and indicate that even at the lowest venous Po_2, myoglobin should still be well oxygenated. Within (mammalian) myocytes, localised drops in Po_2 around mitochondria are only in the order of several hundredths of a mm Hg (Clark Jr et al., 1987). On the face of it, it appears that oxygen diffusion via myoglobin is unaffected even at the point when venous Po_2 is at its' lowest. But the importance of myoglobin with respect to cardiac performance is emphasized by the fact that when myoglobin is biochemically (Bailey & Driedzic, 1986) or adaptationally (Acierno et al., 1997) knocked-out, a significant reduction is seen in cardiac performance.

1.5 Atlantic cod

1.5.1 Distribution of Atlantic cod

Atlantic cod is a widely distributed benthopelagic temperate water fish. It inhabits the continental shelf waters of the North West Atlantic, from northern USA along the eastern coast of Canada and the Gulf of St. Lawrence, around southern Greenland, Iceland, the Faroe Islands, and in the eastern North Atlantic from the Bay of Biscay in the South up through the Irish, North and Baltic Seas to the Barents Sea and Spitzbergen/Svålbard in the North (figure 1.5; Esmark & Jensen, 2004). A number of different studies have found the fish in these varied locations are phenotypically (Sick, 1961; Fyhn et al., 1994) and genetically (Mork et al., 1985; Pogson et al., 1995; Nielsen et al., 2001; Pogson et al., 2001; Jónsdóttir et al., 2003) distinct populations. Depending on the population, cod are found in waters between 1 and 24 °C (Jobling, 1988). Their preferred or optimal temperature is, however, subject to much debate.

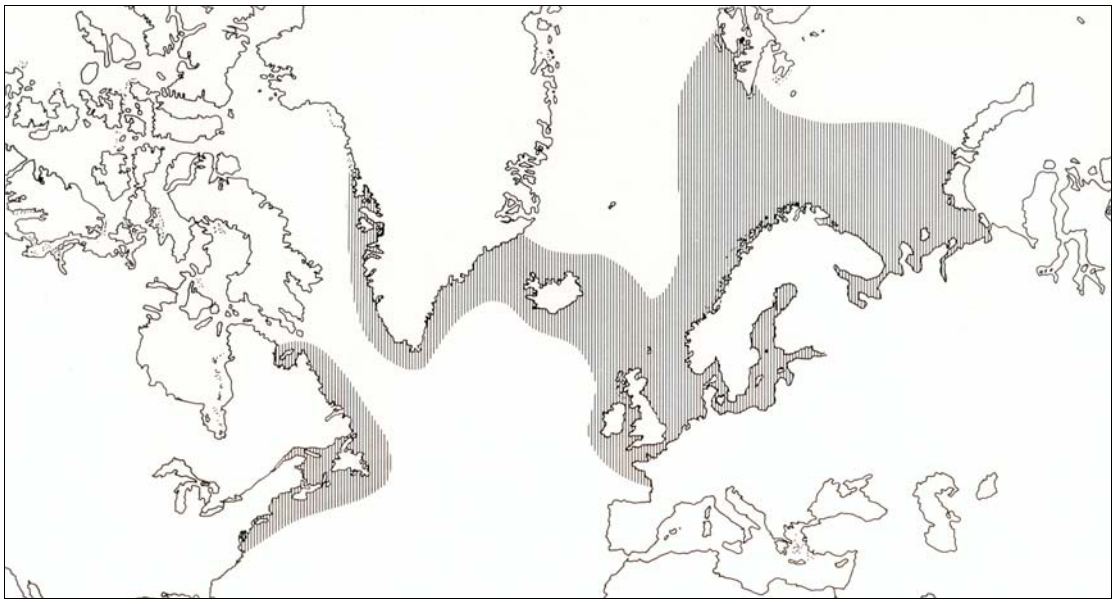

Figure 1.5. Map showing the distribution of Atlantic cod in the North Atlantic. From Meyer et al. (1974).

Due to the economic importance of cod, much effort has gone into determining their preferred or optimal temperature, principally with respect to growth (Jobling, 1981; Michalsen et al., 1998; Björnsson et al., 2001; Pörtner et al., 2001). Data on the preferred temperature of cod generally come from two main groups, from the Canadian Atlantic or from the North East Atlantic. The preferred temperatures are reported as being 4-7 °C for Canadian Atlantic cod (Clark & Green, 1991; Despatie et al., 2001)

and 13 - 14 °C for North East Arcitic cod (Tat'yankin, 1972; Bøhle, 1974; Tat'yankin, 1974; Jobling, 1981; Brown et al., 1989; Schurmann & Steffensen 1992; Pörtner et al., 2001). While these vastly different thermal preferences may well be a result of population differences, a comparison of the techniques used to measure preferred temperature reveals that these differences may in fact be artefacts. Nevertheless, thermal preference positively correlates with haemoglobin phenotype, where Atlantic cod that come from the northern North Atlantic, where it is colder, have the HbI-2/2 phenotype, which has a higher oxygen binding affinity at lower temperatures. Atlantic cod from the southern North East Atlantic, HbI-1/1 has a higher oxygen binding affinity at higher temperatures (Karpov & Novikov, 1980; Petersen & Steffensen, 2003; Brix et al., 2004).

1.5.2 Ecology of different cod populations

Atlantic cod from the Barents Sea or North East Arctic, i.e. NEAC generally experience relatively stable temperatures. During winter, temperatures range between 0 - 3 °C in the Barents Sea and as previously mentioned, NEAC are known to make short forays into colder Arctic waters (Woodhead & Woodhead, 1959). In spring the NEAC undertake a ~1000km round trip migration to their spawning ground near Lofoten where water temperatures can climb as high as 9 °C (Bergstad et al., 1987; Godø & Michalsen, 2000).

Little hard scientific evidence exists regarding the temperatures experience by North Sea cod, particularly those in the German bight around Helgoland. Sea surface temperature data from the German Federal Office for Shipping and Hydrography (http://www.bsh.de/aktdat/bm/DBWasserMess.htm) shows that in winter, temperatures may sink to 3 - 4 °C and in summer they can get as high as 20 °C. Due to the shallow nature of the North Sea, particularly around Helgoland, and the strong mixing of the water due to tidal currents, we may suppose that the surface temperatures reflect the entire body of water. Anecdotal evidence also suggests that NSC around Helgoland are non-migratory, and consequently endure the full spectrum of temperatures.

Wild Canadian cod from Newfoundland (NFC) live in waters that typically range between 0.7 and 8 °C (Lear, 1984; Clark & Green, 1991). Newfoundland cod are migratory, wintering-over off-shore in deeper, warmer oceanic waters before following the warm channels of oceanic warm water to spawning grounds in early spring, and then migrating onwards to Newfoundland coastal waters in summer (Rose, 1993). That said, Atlantic cod kept in sea cages off the Newfoundland coast can experience

temperatures up to 20 °C in the summer month as the cages are relatively close to the surface. Furthermore, temperatures in the cages can vary as much as 10 °C in a single day during summer (Gollock et al., 2006).

1.6 Central questions

We can see from the current data above that a series of links can be made between increasing levels of complexity. Oxygen supply/diffusion may influence cardiac performance, which may in turn influence swimming performance and ultimately the survival of the species. Consequently, an integrative approach within the framework of the thermal tolerance model presented by Pörtner (2001 & 2002) was deemed necessary to explore these links and the influence that temperature may have on them.

1) Are Atlantic cod subject to mitochondrial proliferation after cold acclimation?

   Mitochondrial proliferation is a phenomenon common to all marine ectotherms studied to date and previously seen in both NEAC and NSC white muscle.

2) What influence does mitochondrial proliferation have on cardiac myoglobin expression?

   Mitochondrial proliferation may decrease the venous blood oxygen content because the oxygen extraction by other tissues may be greater, and the myocyte oxygen demand may increase after cold acclimation. As myoglobin is responsive to hypoxia, I wanted to see whether cold acclimation would alter myoglobin expression.

3) What effect does long-term thermal acclimation and acute thermal challenge have on cardiac performance?

   Changes in oxygen supply and demand due to mitochondrial proliferation may influence cardiac performance following long-term acclimation and during an acute thermal challenge. It was also necessary to see whether adrenaline might enhance cardiac performance following acclimation and acute thermal challenge.

4) What effect does long-term thermal acclimation and acute thermal challenge have on swimming performance?

   Mitochondrial proliferation as a result of cold acclimation will lead to a reduction in the fibre number within the cell and therefore a reduction in the power generating capabilities of the myocytes. Similarly, mitochondrial proliferation results in a metabolic shift towards more aerobic pathways. Combined, these effects may significantly alter swimming performance.

5) What are the metabolic processes that underpin critical swimming?

I examined the relationship between the different swimming styles used by cod and the metabolic processes that fuel such styles. In this way it was possible to elucidate limiting factors and what ultimately leads to fatigue in cod during a critical swimming speed test.

6) How might MCA previously observed in NEAC affect the swimming performance?

A higher mitochondrial volume density in the NEAC at 4°C may lead to a higher SMR. If AMR is not changed there will be a reduction in the aerobic scope that may impact on the swimming performance.

2 Materials and Methods

Although the current section aims at giving a comprehensive outline of the experimental procedures employed in all aspects of the current study, more detailed information can be found in the relevant publications.

2.1 Experimental cod

Three different populations or stocks of Atlantic cod were used in the current dissertation, North East Arctic cod (NEAC), North Sea cod (NSC) and Newfoundland cod (NFC). The two different groups of NEAC and NSC were obtained, the NEAC from two different experimental aquaculture facilities, the High Technology Centre in Bergen, Norway and from the Institute for Marine Research in Tromsø, Norway (see table 2.1). They were collected at different times and kept at the AWI for different periods before the experiments were carried out. All NSC were caught in cages around Helgoland at two different times (see table 2.1), were shipped to and kept at the AWI for different periods before experiments were carried out.

Table 2.1. Details of the origins of the various Atlantic cod groups and the acclimation periods used in the current and additional studies. "Acclim begin" refers to the beginning of the acclimation period and "Acclim end" refers to the end of the acclimation period when the animals were either killed or, in the case of the swim tunnel experiments, the experiments began.

Stock	Origin	Type	Acclim begin	Acclim end	Experimenter	Experiment
NEAC	Bergen	Aquaculture	Feb 01	Jul 03 Jul 03 Jun 05 Jun 05	Lucassen Lurman Lurman Polleichtner	CS & COX activity Myoglobin & MHC Microscopy Mitochondrial Vo_2
NEAC	Tromsø	Aquaculture	Apr 04	Nov 05	Lurman	Swim tunnel
NSC	Helgoland	Wild	Apr 03	Jul 03 Jul 03	Lucassen Lurman	CS & COX activity Myoglobin & MHC
NSC	Helgoland	Wild	Aug 04	Nov 05 Jun 05 Jun 05	Lurman Lurman Polleichtner	Swim tunnel Microscopy Mitochondrial Vo_2
NFC	Newfoundland	Aquaculture	Mar 06	May 06	Lurman	*In situ* heart prep

Throughout the acclimation period, NEAC and NSC were kept in the AWI aquaria and fed a diet of mussels (*Mytilus edulis*) and live crangon (*Crangon crangon*) two to three times weekly. NFC were fed commercial pellets daily. Feeding was stopped at least 5 days before any experiment started.

2.2 Exercise experiments

2.2.1 Swim tunnel

The swim tunnel consisted of 3 major parts (figure 1, publication III). A Perspex® pipe was fed through a 40 cm inner diameter Bruker Biospec 47/40 NMR magnet operating at 4.7 T. This pipe was attached to a circulated seawater system. When "closed," a 256 L volume of seawater was hermetically sealed to measure oxygen consumption. When "open," a supplemental 444 L volume with a reservoir of constantly aerated seawater was used to flush the system. A digital motion camera system connected to a computer was located downstream of the fish and used for observing the fish.

2.2.2 Surgical procedure

NEAC and NSC acclimated to 10 or 4°C were initially anesthetised (0.05 mg/L MS 222 in seawater) until respiratory movements stopped, typically 5 minutes. Fish were then transferred to an operating table where the gills were irrigated with 0.02 mg/L MS 222 in seawater. While anesthetised, an inductive ^{31}P-NMR coil measuring 3 x 3 x 0.2 cm was sewn to the side of the body, 15 cm distal to the end of the caudal fin with two sutures on the leading edge. The trailing edge was left free to move. Fish were then placed in a tubular shaped cage (15 cm diameter by 60 cm length) within the chamber. Here, the receive coil was positioned to allow optimal signal transduction while at the same time the fish was observed as it recovered from anaesthesia.

2.2.3 Exercise protocol

All fish were swum twice, once at the acclimation temperature and once at the non-acclimation temperature (figure 2, publication III gives a visual representation of the protocol). To avoid possible training effects biasing the groups, approximately half of the fish were swum at their acclimation temperature first and the other half at their non-acclimation temperature first. Those fish swum at their non-acclimation temperature first were warmed or cooled after the surgery recovery period in two 3 °C steps, each 4 hours in duration, to the required temperature, i.e. 4 or 10 °C. Fish were then left overnight with minimal flow i.e. 3.3 m^3 hr^{-1}. This typically equalled 0.15-0.19

BL (body lengths) s^{-1}. The routine metabolic rate was determined in fish swimming with minimal flow. The flow was then increased in 1 m^3 hr^{-1} (approx. 0.05 BL s^{-1}) steps with each step lasting 30 minutes. At sufficiently high water flows, fish would rest on the rear grid of the cage. U_{crit} was defined as the time when the fish was no longer able to move from the grid (as per Nelson et al., 1994), and calculated according to the formula given in Brett (1964).

$$U_{crit} = u_i + \left(\frac{t_i}{t_{ii} \times u_{ii}} \right)$$

Where u_i is the highest velocity in BL s^{-1}, u_{ii} is the velocity increment, t_i is the time in minutes that the fish swam at the fatiguing velocity, and t_{ii} is the prescribed swimming period, i.e. 30 minutes. Following U_{crit}, the water flow was reduced to the minimum of 3.3 m^3 hr^{-1} to allow recovery.

After four hours of post exercise recovery, fish were again either warmed or cooled in the same stepwise manner to the appropriate temperature and left at minimal flow overnight until they were swum again according to the same protocol outlined above on the third day. Fish were then allowed a further four hours to recover, and if necessary, warmed or cooled back to their acclimation temperature in the same stepwise manner outlined above, before they were taken out, the inductive coil removed and placed back in the aquarium. Water ammonium and nitrite contents were checked every 12 hours and water was changed when necessary.

Tail beat frequency was measured manually by counting the number of tail beats in a 30 second period using the digital camera system. This was repeated 8 times at each of the 30 minute swimming stages. The mean of these 8 was then taken as the tail-beat frequency. Eight 30 second sampling periods were not always possible at U_{crit}, so the mean was taken of as many sampling periods as were permitted, minimum 3. The time between the first kick and U_{crit} is defined as the kick and glide duration.

2.2.4 Oxygen measurement

Oxygen was measured constantly at a sampling rate of 0.5 Hz using Fibox optodes (Presens, Germany) with the temperature compensation entered manually. Oxygen consumption was calculated from the slope of the drop in water oxygen content, which was monitored over 20 minute measurement periods at each speed. After the initial 20 minute measurement period the circulation was opened for a 10

minute flush/re-oxygenation. At the end of each experiment when the fish had been removed, a "blank" respiration run was performed to quantify any background microbial respiration. This was then subtracted from the fish's oxygen consumption rate. Oxygen consumption rates were corrected for possible minor allometric size effects using the mass exponent of 0.8 (Saunders, 1963):

$$\dot{M}o_2 = \left(\frac{1}{M}\right)^{0.8} \times \dot{M}o_2 m$$

where $\dot{M}o_2$ is the standardised oxygen consumption rate in mg O_2 kg^{-1} hr^{-1}, M is the mass of the fish in kg, and $\dot{M}o_2 m$ is the measured oxygen consumption.

2.2.5 Respiration data modelling

Active metabolic rate was taken to be the maximal oxygen consumption rate measured during swimming. Non-linear regression was used to fit a curve to the data on a plot of respiration as a function of swimming speed for each individual at each test temperature. The curve was described by the equation below:

$$\dot{M}o_2 = SMR \times \exp^{(k \times U)}$$

Where k is a constant and U is a given speed in BL s^{-1}. The best fitting curve allowed an estimation of the standard metabolic rate for each fish at each temperature.

2.2.6 ^{31}P-NMR spectroscopy

In vivo ^{31}P-NMR spectra included primarily white muscle with a minor contribution from red muscle. Spectra were collected using a 200 µs bp32 pulse with a flip angle of 45°, sweep width was 5000 Hz at 4k, repetition time was 0.8s. 256 scans were collected resulting in a total acquisition time of around 3 minutes. *In vivo* ^{31}P-NMR spectra were recorded over the whole 30 minute time period for each swimming speed.

^{31}P-NMR spectra were acquired using Paravision 3.0 (Bruker, Germany). Spectra were processed in Topspin 1.5 (Bruker, Germany) first by fast Fourier transformation, then filtered with line broadening in the range of the half width of the PCr signal. Phase and baseline were corrected using a specially adapted automatic

correction routine (R.-M. Wittig, AWI, Germany). Typically, six to seven spectra were collected per swimming speed, i.e. 30 minute period. The best six spectra were then added for each swimming speed. Metabolite concentrations were determined by operator defined integration limits using the standard integration routine in Topspin 1.5 (Bruker, Germany).

The control PCr integral was converted into μmol g^{-1} using the intracellular concentration of 27.3 μmol g^{-1} for resting Atlantic cod reported by Sartoris et al. (2003). All other concentrations, i.e. subsequent PCr measurements, Pi and ATP, were then calculated relative to this. The intracellular pH was calculated from the Pi chemical shift using the temperature compensated formula given in Bock et al. (2001). Gibbs free energy change of ATP hydrolysis ($dG/d\xi_{ATP}$) was estimated for NMR visible metabolites as described earlier (Pörtner et al., 1996; van Dijk et al., 1999; Satoris et al., 2003), except that the creatine concentration was estimated using the following equation:

$$Cr = \left(\frac{PCr}{0.65}\right) - PCr$$

Where Cr is the estimated creatine concentration in μmol g^{-1}, PCr is phosphocreatine concentration in μmol g^{-1} and 0.65 is the ratio of PCr to Cr measured in resting fish white muscle (Hardewig et al., 1998).

2.3 *In situ* heart performance

2.3.1 *In situ* heart preparation

NFC acclimated to 10, 4 or 0 °C were anaesthetized (MS 222, 0.1 gL^{-1} in seawater) until ventilatory movements ceased. Fish were weighed and placed on the operating table on a wetted sponge in a supine position. The gills were irrigated with chilled (2 - 4 °C) oxygenated seawater containing 0.05 gL^{-1} MS 222. Before surgery, 1.0 ml of heparin (2500 i.u. ml^{-1} saline solution) was injected into the caudal vein to prevent blood clots in the heart.

The peritoneal cavity was exposed with a midline incision and by cutting through the abdominal muscle in a ventral-dorsal line. Blood flow to the stomach, intestines and other abdominal organs was stopped by tying off the gastrointestinal tract, inferior to the liver, with umbilical tape. The abdominal and digestive organs

were carefully removed in order to obtain proper placement of the input cannula whilst keeping the liver intact.

The right hepatic vein was selected for cannulation while the other was tied off with silk sutures. A small incision was made in the hepatic vein and the steel cannula (0.9 mm outer diameter.) inserted into the hepatic vein. At this point the 1st and 2nd gill arches were cut on each side of the fish to prevent excessive pressure development by the heart. At the same time as the gill arches were cut, perfusion of the heart with ice-cold (2 - 4 °C) 100% oxygenated saline was begun. The level of saline in the perfusion bottle was kept at approximately the same height as the heart in order to obtain basal cardiac output and prevent cardiac stretch.

The lower jaw and operculum were removed and the isthmus posterior to the 2nd gill arches was completely cut away to expose the ventral aorta which was dissected free of any surrounding tissue. The 3rd and 4th gill arches were then cut in half and clamped with cable ties (10 cm) to avoid leakage. The steel output cannula (0.8 mm outer diameter) was inserted into the ventral aorta and tied in place with silk sutures.

After placement of the output cannula, the ducti Cuvier were tied off with a silk suture passed at an angle from the corner of the opercular cavity to the muscle of the abdominal wall. The suture was then passed under the liver, through the oesophagus and back into the buccal cavity, thus occluding these veins and crushing the cardiac branches of the vagus nerve. The fish was then bisected just below the pectoral fins. After surgery, the *in situ* heart preparation was placed in the double-jacketed chamber.

Shortly before use, 250 µL of 0.1 µM adrenaline bitartate salt dissolved in dH_2O was added to 500 mL of the cod ringer to give and end "resting" concentration of 5 nM adrenaline (AD). Alternatively, 250 µL of 2 µM was added to obtain a final concentration 200 nM AD. These doses were refreshed every 20 min to avoid photo-degradation of AD.

After mounting the *in situ* preparation in the experimental chamber the heart was allowed to recover from surgery for about 5-10 minutes with an output pressure (P_{OUT}) of 20 cm H_20 and a P_{IN} required to maintain 16, 10 or 8 mL min^{-1} kg^{-1} at 10, 4 and 0 °C. These values were taken as reasonable estimates of in vivo resting cardiac output from published values (Weber, et al 1998). The P_{IN} and P_{OUT} loadings on the heart were set by attaching the in and output cannula to adjustable in and output pressure-heads. After this initial setting, output pressure was increased to a physiological output pressure of 50 cm H_2O. The heart was then allowed to stabilize

for a further 20 min at resting cardiac output before the temperature was changed in those heart exposed to an acute thermal challenge. See figure 1 in publication II for a schematic representation of the experimental protocol.

2.3.2 Cardiac performance tests

Firstly, a maximum cardiac output test was performed at 5 nM AD. Input pressure was increased in steps from resting to 4.0, 5.0, 5.5 and finally 6.0 cm H_2O. Each increase in P_{IN} was maintained for approximately 30 seconds. During the maximum cardiac output test output pressure was maintained at 50 cm H_20. After this trial, P_{IN} was left at 6 cm H_2O and a maximum power output test was performed where P_{OUT} was decreased to 20 cm H_2O and then increased in 10 cm H_2O steps up to 80 cm H_2O. Following this, the hearts were allowed to recover under resting conditions for a further 20 minutes. After recovery, the cod ringer AD concentration was changed from 5 nM to 200 nM. The heart was given a further 3 minutes before resting parameters were recorded and the maximum cardiac output and power output tests were carried out as described above.

2.3.3 Data collection and analysis

Cardiac output was measured using a model T206 small animal blood flow meter in conjunction with a pre-calibrated in-line flow probe (2N, Transonic Systems Inc. Ithaca, NY). A Gould statham pressure transducer (Model P23 ID, Oxnard, CA) was used to measure P_{OUT}, and P_{IN} was measured using a Grass pressure transducer (Model PT300, Warwick, RI, USA). Before the start of each experiment, the pressure transducers were calibrated against a static column of water, where zero pressure (0 cm H_2O) is set at the saline level in the experimental bath. Pressure and flow signals were amplified and filtered using a Model MP100A-CE data acquisition system (BIOPAC Systems Inc., Santa Barbara, CA). The acquired signals were then analysed and stored using Acknowledge 3.7 Software (BIOPAC Systems Inc., Santa Barbara, CA).

Cardiac performance was continuously measured throughout the experiment by measuring cardiac output (CO, mL min^{-1} kg^{-1}), P_{IN}, and P_{OUT}. Before every maximum cardiac output test, P_{IN} was measured in order to determine the P_{IN} required to obtain resting cardiac output. Cardiac output, heart rate (HR), stroke volume, (SV) and P_{IN} were measured/calculated at each step of the maximum cardiac output and power output tests. Heart rate was calculated by counting the number of systolic peaks during a 20-30 second interval. Cardiac output (ml min^{-1} kg^{-1}) and stroke volume (ml kg^{-1}) were calculated as:

Cardiac output (ml min^{-1} kg^{-1}): cardiac output (ml min^{-1})/kg animal weight.

Stroke volume (ml kg^{-1}) was calculated by the relationship: CO/HR

Myocardial power output (mWg^{-1} ventricle) was calculated as:

Power output (PO): (CO x (P_{OUT} - P_{IN}) x a)/ventricular mass.

Where P_{OUT} and P_{IN} are output and input pressures (cm H_2O) respectively, and a = 0.0016 mW min ml^{-1} cm H_2O^{-1} is a conversion factor to mW.

2.4 Red skeletal muscle stereology

2.4.1 Tissue sampling

NEAC and NSC acclimated to 10 or 4 °C were anaesthetized (MS 222, 0.3 gL^{-1} in seawater) until ventilatory movements ceased. Fish were weighed, total length recorded and placed on a tray with ice on its' side before a 2 mL blood sample was taken using a 20 gauge (0.9 mm internal diameter) needle. Fish were then laid on ice in a supine position. The peritoneal cavity and heart were exposed with a midline incision and by cutting through the abdominal muscle in a ventral-dorsal line. The heart was removed, perfused with the same Ringer solution outlined above to remove any blood, divided into the atrium and ventricle, each of which was then weighed and frozen in liquid nitrogen. The liver was removed and a portion frozen in nitrogen before the majority was prepared for the determination of mitochondrial respiration rates (see Polleichtner, 2006). Other organs, namely gill, spleen, white dorsal muscle were similarly removed and frozen in liquid nitrogen. The length from the operculum to where the caudal fin rays enter the musculature was then divided into four to give four different red muscle sampling positions down the fish. A random number indicated how far (in mm) from behind the operculum the first sample was taken. A second random number is added to the first number to get the step length. This procedure ensures randomisation of the sampling. Traverse sections were cut so that they were 1 cm wide and 3 cm high to ensure capture of the red muscle.

2.4.2 Tissue fixation

The biopsies were primary fixed in phosphate buffered 4% formaldehyde/1% glutaraldehyde for estimation of the mitochondrial volume density. To estimate the volume it was necessary to introduce isotropy in the randomised sampling strategy. To do this, the isector was used (Nyengaard and Gundersen, 1992). The isector makes it

possible to make isotropic, uniform random sampling (IUR) by embedding the muscle sample in 5% agar to make a sphere with a diameter of 4 mm in a rubber mould. Samples were then secondary fixed in 4% osmium tetroxide and dehydrated in increasing concentrations of alcohol. The spheres then were rolled across a table, stopped and embedded in an epoxy resin (Epon 828). Ultrathin sections were then cut using an ultramictotome and placed on copper grids before staining with uranyl acetate and lead citrate for transmission electron microscopy.

2.4.3 Stereology

The tissue was processed and photos were taken randomly with a superimposed grid in an electron microscope. The grid was designed so for every tissue point there were 9 mitochondria points. The fraction of mitochondria (%) was calculated as.

Mitochondrial Fraction (%) = (Q/9)/p*100

Where Q is the number of points that hit the mitochondria and p is the number hitting the muscle cells.

2.5 Myosin heavy chain (MHC) ATPase activity

Both NEAC and NSC acclimated to 10 °C or 4 °C were anaesthetized with MS 222 (0.3 g/L seawater), then killed by cervical dislocation. Fish were weighed and total length was recorded. They were then placed on a tray of ice in a supine position. The peritoneal cavity and heart were exposed with a midline incision and by cutting through the abdominal muscle in a ventral-dorsal line. The liver was removed and frozen in nitrogen along with white dorsal muscle for later determination of CS and COX enzyme activity (see Lucassen et al., 2006). Other organs, namely the heart, gill, and spleen, were similarly removed and frozen in liquid nitrogen.

2.5.1 Myofibril isolation

The method for preparation of myofibrils for measuring ATPase activity was adapted from Tiitu & Vornanen (2002). Whole hearts (atrium and ventricle) from 10 or 4 °C acclimated NEAC or NSC were ground with a RNase free mortar and pestle in liquid nitrogen. Between 50 and 80 mg of heart tissue was then homogenised 3 x 10 sec in 20 volumes of ice-cold homogenisation buffer (100 mM KCl, 250 mM sucrose, 1 mM dithiothretol and 50 mM imidazole, pH 7.4 at 4 °C). The remaining tissue was frozen and stored for the myoglobin RNase protection assay (see section 2.7). The

homogenate was then centrifuged at 10,000 *g* for 10 min at 4 °C. The supernatant was removed and frozen at -20 °C for subsequent myoglobin western blotting (see section 2.6). Pellets were re-suspended in homogenisation buffer with 1% triton X-100 before being centrifuged again at 10,000 *g* for 10 min. Pellets were then re-suspended and washed four times with a wash buffer (50 mM KCl, 1 mM dithiothretol, and 45 mM imidazole, pH 7.4 at 4°C), while centrifuging at 600 *g* between washes. The pellets were finally re-suspended in an ATPase buffer (50 mM KCl, 5mM $MgSO_4 \cdot 7H_2O$, 5 mM EGTA and 45 mM imidazole, pH 7.0 at assay temperature). The final protein concentration was determined as per Bradford (1976).

2.5.2 MHC ATPase activity

The myosin ATPase reaction took place in the same reaction mixture outlined by Johnson et al. (1972). The re-suspended pellet was diluted to an empirically determined optimal concentration of 150 µg/mL in ATPase buffer, of which 99 µL were added to a 0.5 mL cuvette, together with 1 µL of 100 mM $CaCl_2$ and 100 µL of 2 mM ATP to initiate the reaction. The activity was determined spectrophotometrically after exactly 10 min of incubation at 4 and 10 °C using the malachite green method outlined by Henkel et al. (1988). MHC ATPase activity was calibrated against a phosphate standard curve, where, instead of 99 µL of myofibrillar protein, known amounts of KH_2PO_4 were incubated for the same length of time. All measurements were made in duplicate.

2.6 Myoglobin western blotting

The protein concentration of the supernatant from the homogenised heart tissue (section 2.5.1) was determined as per Bradford (1976). Protein samples (2.5 µg) were denatured at 96 °C for 10 min and then separated by polyacrylamide gel electrophoresis (17% polyacrylamide) under denaturing conditions (Laemmli, 1970). Protein was then transferred to a PVDF membrane, and a monoclonal mouse anti-human myoglobin antibody (M7773, Sigma, Traunstein, Germany) diluted 1:1000 was used for immunodetection. Antibody binding was visualized by the ECL-system (Amersham Biosciences, Freiburg, Germany) using goat anti-mouse antibody conjugated to horseradish peroxidase (dilution 1:2500; Amersham Biosciences, Freiburg, Germany) as secondary antibody. Chemiluminescence was detected and quantified with a cooled CCD-camera system (LAS-1000; Fuji, Tokyo, Japan) and the AIDA software package (Raytest, Straubenhardt, Germany).

2.7 Myoglobin mRNA characterisation and quantification

2.7.1 RNA isolation

Whole RNA was extracted from approximately 150-200 mg of frozen cardiac tissue (ventricle and atrium) using the Peqlab Gold-Trifast RNA extraction kit (Peqlab, Erlangen, Germany) and quantified spectrophotometrically. Cardiac mRNA was purified using the Qiagen Oligotex kit (Qiagen, Hilden, Germany).

2.7.2 Myoglobin characterisation

Fragments of the myoglobin gene were isolated by means of reverse transcription PCR. Primers were designed using MacVector 7.0 (Accelrys, UK) using highly conserved regions of published sequences from numerous fish species. Reverse transcription was performed with Superscript RT II (Invitrogen, Karlsruhe, Germany) and the reverse primer Mb B3 using 0.2 µg of extracted mRNA as a template. PCR amplification of the resulting single strand cDNA was accomplished using the forward primer 1 (Mb F2) and primer Mb-B3, resulting in a 363-nucleotide fragment, which was then cloned using the TOPO-TA cloning kit (Invitrogen, Karlsruhe, Germany), and the subsequent plasmids were sequenced by MWG Biotech (Ebersberg, Germany). Another fragment within this 363 bp fragment was constructed with primers Mb-F1 and Mb-B2, to obtain a cod specific probe for the RNase protection assay (see below). The resultant 300 bp fragment was similarly cloned and sequenced. This 300 bp fragment was then used for designing primers for both 3′ and 5′ RACE to gain the entire myoglobin sequence (see table 1, publication I for a list of primer sequences). RLM-RACE was performed using the First Choice RLM-RACE kit from Ambion (Austin, USA). Since the outer PCR had already produced specific fragments, a nested PCR, usually necessary, could be avoided. Fragments were then cloned using the TOPO-TA cloning kit, and the subsequent plasmids were sequenced by MWG Biotech (Ebersberg, Germany). The cDNA sequence of *G. morhua* myoglobin was submitted to Genbank and can be obtained with following accession number: EF121552.

2.7.3 Sequence analysis and molecular modelling

Alignment of amino acid sequences of Atlantic cod myoglobin with those of other teleosts was performed using the ClustalW tool in MacVector (Acclerys, UK). The following sequences were used: *Notothenia coriiceps* (U71058), *Chaenocephalus aceratus* (U71153); *Chionodraco rastospinosus* (U71059), *Danio rerio* (AY 337025), *Cyprinus carpio* (DQ338464), *Thunnus obesus* (AB104433), *T. thynnus* (AF291831), *T.*

albacares (AF291838), *Auxis rochei* (AB154433), *Katsuwonus pelamis* (AF291837), *Tetraodon nigroviridis* (AJ628044), *Scomber japonicus* (AF291835), and *Physeter catodon* (sperm whale: AB 27114) as an outgroup. An homology three-dimensional model of cod myoglobin was constructed based on the amino acid sequence of the protein and the published X-ray structure of yellowfin tuna myoglobin (Birnbaum et al., 1994) utilizing Swiss-model (36.0003) program (Schwede et al., 2003). The structure was visualized with Swiss-PdbViewer (http://www.expasy.org/spdbv/) and POV-Ray (v.3.6; Persistence of Vision Raytracer).

2.7.4 Myoglobin mRNA quantification

Ribonuclease or RNase protection assays (RPA) were performed with the RPA-III kit from Ambion (Austin, USA). Total RNA (5 µg) was simultaneously hybridized at 42 °C to antisense probes for myoglobin mRNA and 18S-rRNA (18-S MEGAshort). Anti-sense RNA probes were synthesized by *in vitro* transcription with T7 or T3 RNA Polymerase (Ambion, Austin, USA) with the plasmid containing the 300 bp myoglobin cDNA fragment described above. For 18S-rRNA, a commercial plasmid containing a highly conserved 80 bp fragment (T7-MEGAshortscript RNA 18S, Ambion, Austin, USA) was used. All probes were labelled with α-^{32}P uridine 5´-triphosphate (Amersham Biosciences, Freiburg, Germany). To equalize protected fragment intensities, a specific radioactivity of 45 Ci/mmol was used for myoglobin, and 0.1 Ci/mmol for 18S-rRNA. Protected fragments were separated by denaturing PAGE (8 M urea, 5% acryl amide gel with 1xTBE running buffer). Probes were prepared and checked for specificity as per Mark et al., (2006). After drying the gel, radioactivity was detected and quantified with a phosphorous storage image system (FLA-5000; Fuji, Tokyo, Japan) and AIDA software (Raytest, Straubenhardt, Germany).

2.8 Statistical Analysis

Appropriate statistical analysis and data modelling was accomplished using the statistical software Instat 3.0, Prism 4.0, SigmaPlot 10.0 and SigmaStat 3.5. See publications for more details. All differences were considered significant when $p < 0.05$. Values are given as the mean ± one standard error of the mean (SEM) if not stated otherwise.

3 Publications

A list of publications and declarations of my contribution to them.

I **Lurman, G.J.**, Koschnick, N., Pörtner, H.-O. & Lucassen, M., (2007) Molecular characterisation and expression of Atlantic cod (*Gadus morhua*) myoglobin from two populations held at two different temperatures. **Comparative Biochemistry and Physiology, Part A: Molecular and Integrative Physiology, 148**, 681-689.

Experiments were designed and executed by myself with help from the second and fourth co-authors. The myoglobin sequence analysis and molecular modelling were carried out primarily by the fourth co-author. The resultant manuscript was written by myself and the fourth co-author, then revised together with the second, third and fourth co-authors.

II **Lurman, G.J.**, Petersen, L., Pörtner, H.-O. & Garmperl, A. K., Atlantic cod (*Gadus morhua* L.) *in situ* cardiac performance: Long-term acclimation, acute thermal challenge and the role of adrenaline. Submitted to **The Journal of Experimental Biology.**

Experiments were designed and executed by myself with help from the second and fourth co-authors. The "*in situ* heart prep" technique had previously been developed by the second and fourth co-authors. The resultant manuscript was written by myself and revised together with the second, third and fourth co-authors.

III **Lurman, G.J.**, Bock, C.H. & Pörtner, H.-O., Effects of long-term and acute thermal acclimation on swimming performance in two populations of Atlantic cod (*Gadus morhua*). Submitted to **Journal of Experimental Zoology.**

Experiments were designed and executed by myself with help from the second co-author. The resultant manuscript was written by myself and revised together with the second and third co-authors.

IV **Lurman, G.J.**, Bock, C.H. & Pörtner, H.-O., (2007) An examination of the metabolic processes under-pinning critical swimming in Atlantic cod (*Gadus morhua* L.) using *in vivo* ^{31}P-NMR spectroscopy, **The Journal of Experimental Biology, 210**, 3749-3756.

Experiments were designed and executed by myself with help from the second co-author. ^{31}P-NMR spectroscopy techniques were previously developed by the second co-author (see supplemental publication V below). The resultant manuscript was written by myself and revised together with the second and third co-authors.

Supplemental Publication

V Bock, C., **Lurman, G. J.**, Wittig, R. M., Webber, D. M. and Pörtner, H. O. (2008). Muscle bioenergetics of speeding fish: *in vivo* MRS studies in a 4.7 T MR scanner with an integrated swim tunnel. ***Concepts in Magnetic Resonance Part B: Magnetic Resonance Engineering,*** In Press.

Experiments were designed and executed by myself, the first, fourth and fifth co-authors. ^{31}P-NMR spectroscopy techniques were developed by the first author. The tail-beat pressure measurement technique was developed by the fourth co-author. Spectroscopy and tail-beat data was analysed by the third author. The resultant manuscript was written by the first author and revised together with myself and the fifth co-author.

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Molecular characterisation and expression of Atlantic cod (*Gadus morhua*) myoglobin from two populations held at two different acclimation temperatures

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Abstract

Much previous research has demonstrated the plasticity of myoglobin concentrations in both cardiac and skeletal myocytes in response to hypoxia and training. No study has yet looked at the effect of thermal acclimation on myoglobin in fish. Atlantic cod (*Gadus morhua*) from two different populations, i.e. the North Sea and the North East Arctic, were acclimated to 10 and 4 °C. Both the myoglobin mRNA and myoglobin protein in cod hearts increased significantly by up to 3.7 and 2.3 fold respectively as a result of acclimation to 4 °C. These increments were largest in the Arctic population, which in earlier studies have been shown to possess cold compensated metabolic demands at low temperatures. These metabolic demands associated with higher mitochondrial capacities may have driven the increase in cardiac myoglobin concentrations, in order to support diffusive oxygen supply. At the same time the increase in myoglobin levels may serve further functions during cold acclimation, for example, protection of the cell against reactive oxygen species, and scavenging nitric oxide, thereby contributing to the regulation of mitochondrial volume density.

Keywords: Myoglobin; Atlantic cod; *Gadus morhua*; mRNA; Protein; Expression; Temperature

1. Introduction

Oxygen availability is thought to play a major role in influencing thermal tolerance in fish (Fry, 1971; Pörtner and Knust, 2007) in line with a key role of oxygen uptake and delivery in setting the thermal tolerance of water breathers (Pörtner, 2001). In sockeye salmon (*Oncorhynchus nerka*), cardiac performance has been shown to display the same thermal optimum as swimming performance (Farrell, 2002), suggesting that the two are linked. More recent evidence further suggests that it is initially the heart that limits thermal tolerance (Mark et al., 2002; Lannig et al., 2003; Sartoris et al., 2003). As the fish heart is the last organ to receive oxygenated blood, the principle reason for collapse of cardiac function is oxygen deprivation (Wang and Overgaard, 2007). Efficient oxygen extraction from blood within the cardiac tissue is in part facilitated by the high oxygen affinity of myoglobin (Marcinek et al., 2001).

Biochemical and molecular studies have found that the concentrations of the intracellular oxygen carrier myoglobin are adaptively expressed according to mitochondrial oxygen demand (Millikan, 1939; Williams and Neufer, 1996; Wittenberg and Wittenberg, 2007), with elevated demand causing an increase in concentration. Two lines of comparative evidence also support this conclusion, firstly, studies across different fish species with different metabolic rates, and secondly within species comparisons examining the influence of exercise/migration on myoglobin concentrations. A number of studies have examined the relationship between metabolic rate and myoglobin. For example, mammals that have high metabolic rates also have high myoglobin concentrations (O'Brien et al., 1992). Red skeletal muscle myoglobin concentrations in various fishes also correlate with lifestyles, such that highly active scombrid tuna fishes have a much higher red muscle myoglobin content than sedentary red-blooded notothenioid fishes (Cashon et al., 1997). In fact a number of notothenioid

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Table 1
Fish morphology

	NEAC 10 °C	NSC 10 °C	NEAC 4 °C	NSC 4 °C
N	6	5	5	6
Length (cm)	33.0±0.56	37.5±1.9	32.9±1.3	36.7±1.8
Mass (g)	151±48	232±82	156±46	241±77
Condition factor	0.81±0.08	0.85±0.06	0.83±0.03	0.92±0.03
HSI (%)	1.63±0.34	1.26±0.23	3.26±0.61*	2.92±0.82

Mean±SEM, asterisk indicates significant difference.

species, namely the icefishes, express no functional myoglobin at all (Moylan and Sidell, 2000).

Additionally, citrate synthase (a marker of mitochondrial enzymatic capacity) mRNA was also observed to correlate with myoglobin mRNA (Terrados et al., 1990). Previous studies have already found that 4 °C acclimated North Eastern Arctic cod (NEAC), and to a lesser degree North Sea cod (NSC) have higher citrate synthase and cytochrome *c* oxidase mRNA and enzyme activities in skeletal muscle (Lannig et al., 2003; Lucassen et al., 2006), and a greater mitochondrial volume density (Lurman, Bennedsen, Nyengaard & Pörtner, unpublished) than their 10 °C acclimated counterparts.

As mentioned above the second factor that can affect myoglobin expression is exercise. Love et al. (1977) were able to show that Scottish North Sea cod red skeletal muscle myoglobin concentration increased as a result of swim training. Similar results were obtained by training zebrafish (*Danio rerio*; van der Meulen et al., 2006). Indeed, naturally occurring variation in the myoglobin concentration has been previously documented in Atlantic cod, where the red skeletal muscle concentration was higher in migratory NEAC when compared to non-migratory Faroe Island cod (Love et al., 1974, 1975). Both were subsequently shown to be genetically distinct populations of Atlantic cod (Nielsen et al., 2001). Furthermore, exercise was also observed to stimulate mitochondrial biogenesis (Hood, 2001), thus lending more weight to the argument that the regulation of myoglobin content and mitochondrial capacities are closely coordinated.

In this study we examined the cardiac myoglobin expression in two different cod populations, both acclimated to two different temperatures. Thus, our principle question was, if elevated mitochondrial enzymatic activity and volume density during cold acclimation has previously been seen, does this increase in oxygen demand cause the myoglobin expression to also be elevated? We also wanted to see whether the response differed between different cod populations and more generally, compare Atlantic cod myoglobin on a molecular scale, i.e. gene and protein sequence, with other species.

2. Materials and methods

2.1. Animals

Two populations of Atlantic cod (*Gadus morhua*) were used in this experiment. Atlantic cod from the North Sea (NSC) were caught off Helgoland in spring 2003 using traps. North East Arctic cod (NEAC) were obtained from the University of Bergen, also in 2003. Cod were held for a minimum of 2 months at 10 or 4±0.2 °C in 2.6 m^3 circular tanks with filtered and ozone treated re-circulated seawater. Water quality was regularly checked and water changed when necessary. Fish were fed frozen mussels (*Cerastoderma edule*) and live common shrimp (*Crangon crangon*) twice a week.

2.2. RNA isolation

Fish were anaesthetized with MS-222 (0.3 g/L seawater), then killed by cervical dislocation. Cardiac tissues samples, i.e. both ventricle and atrium were excised and placed in sterile, RNase free plastic tubes, then frozen immediately in liquid nitrogen. Total body weight, length and liver weight were determined and the hepatosomatic index and condition factor were subsequently calculated (Table 1).

Whole RNA was extracted from approximately 150–200 mg of cardiac tissue (equivalent amounts of atrium and ventricle) using the Peqlab Gold-Trifast RNA extraction kit (Peqlab, Erlangen, Germany) as per the manufacturer's instructions. Total RNA was determined spectrophotometrically and quality was checked on a 1% TBE agarose gel. Cardiac mRNA was purified using the Qiagen Oligotex kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions from 300 μg of total RNA.

2.3. Myoglobin characterisation

Fragments of the myoglobin gene were isolated by means of reverse transcription PCR. Primers were designed using MacVector 7.0 (Accelrys, UK) using highly conserved regions of published sequences from numerous fish species. Reverse transcription was performed with Superscript RT II (Invitrogen, Germany) and the reverse primer Mb B3 using 0.2 μg of extracted mRNA as a template. PCR amplification of the resulting single strand cDNA was accomplished using the forward primer 1 (Mb F2) and primer Mb-B3, resulting in a 363-nucleotide fragment which was then cloned using the TOPO-TA cloning kit and the subsequent plasmids were sequenced by MWG Biotech (Ebersberg, Germany). Another fragment within this 363 bp fragment was constructed with primers Mb-F1 and Mb-B2, to obtain a cod specific probe for the RNase protection assay (Lurman, Bennedsen, Nyengaard & Pörtner, unpublished). The resultant 300 nucleotide fragment was similarly cloned and sequenced. This 300 bp fragment was then used for designing primers for both 3′ and 5′ RACE to gain the entire myoglobin sequence (see Table 2 for a list of primer

Table 2
Primer sequences

Primer	Sequence	Position	Size
Mb F2	5′ GTKCTGAAGTGYTGGGGTCCAG 3'	68–89	363 bp
Mb B3	5′ CATCACGTTCCTCAGGGCWGTC 3′	430–409	
Mb-P-F1	5′ ACTACAACACACACGGCGGGCT 3′	99–120	300 bp
Mb-P-B1	5′ CATCAAGCCCCGCCTTCTCC 3'	398–379	
Mb-3R-F2	CCCGTTTATTCACAGAGCACC	187–207	
Mb-5R-B16	CAGCTTCTTCAGGACCGTCGC	247–227	

The positions refer to the final gene sequence.

Fig. 1. Primary sequence alignment of teleost myoglobin. The primary sequences of fish sequences (see Materials and methods section) and sperm whale are shown together with the predicted secondary structure including the seven α helices and the d-loop. Important residues are highlighted (see text).

sequences). RLM-RACE was performed using the First Choice RLM-RACE kit from Ambion (Austin, USA). Since the outer PCR produced already specific fragments, a nested PCR, usually necessary, could be avoided. Fragments were then cloned using the TOPO-TA cloning kit and the subsequent plasmids were sequenced by MWG Biotech (Ebersberg, Germany). The cDNA sequence of *G. morhua* myoglobin has been submitted to Genbank and can be obtained with the following accession number: EF121552.

2.4. Sequence analysis and molecular modelling

Alignment of amino acid sequences of cod myoglobin with those of other teleosts was performed using the ClustalW tool in MacVector (Acclerys, UK). The following sequences were used: *Notothenia coriiceps* (U71058), *Chaenocephalus aceratus* (U71153); *Chionodraco rastospinosus* (U71059), *Danio rerio* (AY337025), *Cyprinus carpio* (DQ338464), *Thunnus obesus* (AB104433), *T. thynnus* (AF291831), *T. albacares*

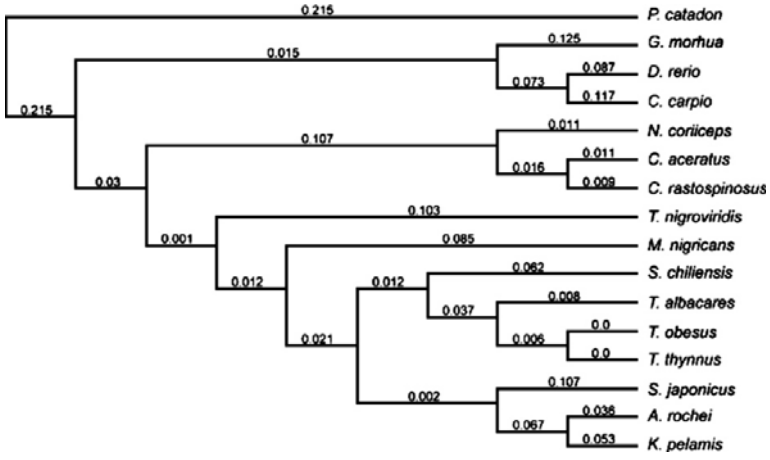

Fig. 2. Phylogenetic reconstruction of teleost myoglobin deduced protein sequences. The tree was constructed using the fish sequences listed in the Materials and methods section with the sperm whale protein as an outgroup. Method: Neighbour joining, best tree; distance: uncorrected "*p*". Gaps were distributed proportionally.

(AF291838), *Auxis rochei* (AB154433), *Katsuwonus pelamis* (AF291837), *Tetraodon nigroviridis* (AJ628044), *Scomber japonicus* (AF291835), and *Physeter catodon* (sperm whale: AB27114) as an outgroup. An homology three-dimensional model of cod myoglobin was constructed based on the amino acid sequence of the protein and the published X-ray structure of yellowfin tuna myoglobin (Birnbaum et al., 1994) utilizing Swiss-model (36.0003) program (Schwede et al., 2003). The structure was visualized with Swiss-PdbViewer (http://www.expasy.org/spdbv/) and POV-Ray (v.3.6; Persistence of Vision Raytracer).

2.5. mRNA quantification

Ribonuclease or RNase protection assays (RPA) were performed with the RPA-III kit from Ambion (Austin, USA). Total RNA (5 μg) was simultaneously hybridized at 42 °C to antisense probes for myoglobin mRNA and 18S-rRNA (18-S MEGAshort). Probes were synthesized by *in vitro* transcription with T7 or T3 RNA Polymerase (Ambion, Austin, USA) with the plasmid containing the myoglobin cDNA fragment described above. For 18S-rRNA, a commercial plasmid containing a highly conserved 80 bp fragment (T7-MEGAshort-script RNA 18S, Ambion, Austin, USA) was used. All probes were labelled with α-^{32}P uridine 5′-triphosphate (Amersham Biosciences, Freiburg, Germany). To equalize protected fragment intensities, a specific radioactivity of 45 Ci/mmol was used for myoglobin, and 0.1 Ci/mmol for 18S-rRNA. Probes were prepared and checked for specificity as per Mark et al. (2006). After drying the gel, radioactivity was detected and quantified with a phosphorous storage image system (FLA-5000; Fuji, Tokyo, Japan) and AIDA software (Raytest, Straubenhardt, Germany).

2.6. Western blotting

Approximately 70 mg of frozen cardiac tissue was homogenised using an Ultra Turrax in 1.5 mL homogenisation buffer (50 mM imidazole pH 7.4 at 4 °C, 100 mM KCl, 1 mM DTT and 250 mM sucrose). Samples were then centrifuged at 10,000 *g* and 4 °C for 10 min. The resulting supernatant contained myoglobin. Total protein was measured as per Bradford (1976) using a bovine serum albumin standard. Protein samples (2.5 μg) were separated by polyacrylamide gel electrophoresis under denaturing conditions (Laemmli, 1970), using a vertical mini-slab apparatus (Bio-Rad, Munich, Germany) and a 17% polyacrylamide gel. Samples were denatured at 96 °C for 10 min. Western blots were performed as per Mark et al. (2006). A monoclonal mouse anti-human myoglobin antibody (M7773, Sigma, Traunstein, Germany) diluted 1:1000 was used for immunodetection. Antibody binding was visualized by the ECL-system (Amersham Biosciences, Freiburg, Germany). Chemiluminescence was detected and quantified with a cooled CCD-camera system (LAS-1000; Fuji, Tokyo, Japan) and the AIDA software package (Raytest, Straubenhardt, Germany). For quantification, a protein concentration was used in a range, where the signal changed linearly with antibody binding.

2.7. Statistics

Data were checked for outliers using Nalimov's test (Noack, 1980). Two-way ANOVAs were used to test for significant effects of population and temperature. Bonferroni post-tests were used to identify significant differences between individual groups. All data analyses and statistical tests were performed

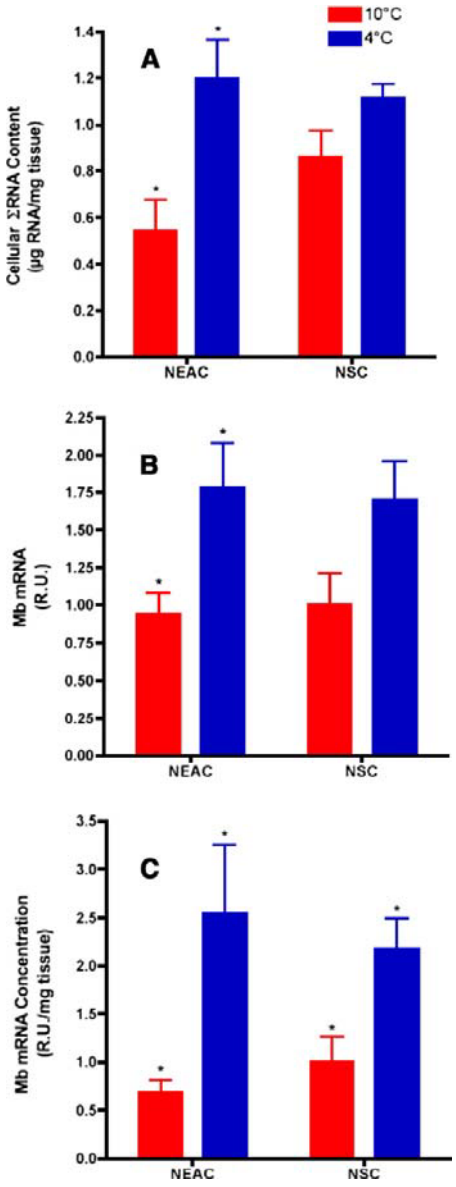

Fig. 3. A = Extracted cardiac RNA concentration, B = relative myoglobin mRNA content, C = relative myoglobin mRNA per mg tissue. R.U = relative units. Asterisks indicate significant differences between acclimation temperatures, within a population.

using SigmaPlot 8.0 and SigmaStat 3.0. All values are given as mean±standard error of the mean (SEM).

3. Results

3.1. Myoglobin characterisation

Using RT-PCR we were able to identify a 363 nucleotide fragment of Atlantic cod myoglobin mRNA. This fragment was then used to construct primers for the isolation of the entire mRNA sequence. The sequence of 625 nucleotides contains an open reading coding for a 146 amino acid polypeptide. This polypeptide showed highest similarity to other teleost myoglobins with about 72% identity (87 % similarity) to scombrid proteins (Fig. 1). The sequence divergence is therefore similar to what was observed earlier among fish and significantly higher to reptilian and avian proteins (Madden et al., 2004). Phylogenetic reconstruction revealed some deviation from established phylogenetic relationships (Fig. 2). Although all scrombrid, notothenioid and cypriniform myoglobins were contained in their respective groups, and both Scrombroidei and Notothenioidei myoglobins grouped into Perciformes, the gadid myoglobin seemed to be more closely related to cypriniform myoglobin than to the perciforms. Therefore, convergent evolution after separation of the phyla might have co-defined protein structure.

3.2. Myoglobin expression

A significant effect of temperature, but not population was seen on total RNA levels. Total RNA from 4 °C acclimated NEAC was significantly elevated by a factor of approximately 2.1 and 1.4 above that of NEAC and NSC acclimated to 10 °C respectively (Fig. 3A). However, NSC acclimated to 4 °C showed no significant difference compared to any of the groups, although RNA content also tended to be elevated.

Similarly, a significant effect of temperature, but not population was seen on myoglobin mRNA. Myoglobin mRNA from 4 °C acclimated NEAC was significantly elevated by factors of approximately 1.8 and 1.75 above those of 10 °C acclimated NEAC and NSC respectively, when normalized to the 18S-rRNA signal (Fig. 3B). Although not significantly different, myoglobin mRNA in 4°C acclimated NSC was also elevated by a similar factor.

To account for the differences in total RNA levels, myoglobin mRNA was expressed in relative units (R.U.) per mg of tissue (Fig. 3C). Myoglobin mRNA in the 4 °C acclimated NEAC was strongly elevated by a factor of approximately 3.7 and 2.5 above the 10 °C acclimated NEAC and NSC respectively. Myoglobin mRNA from cold acclimated NSC was only slightly less elevated, i.e. by a factor of 3.3 and 2.2 above the 10 °C acclimated NEAC and NSC respectively.

A similar increment became visible for the myoglobin protein levels as for RNA. Although neither temperature nor population had a significant effect on the extracted total protein and it differed little between the groups, (Fig. 4C), the myoglobin protein content was found to be significantly increased upon cold acclimation with a 2.3 fold increase compared to 10 °C acclimated NEAC and NSC when expressed on a tissue wet weight basis (Fig. 4A). The 4 °C acclimated NSC had an elevated myoglobin amount by only 1.7 fold ($p=0.08$) compared to both 10 °C acclimated populations. The specific myoglobin protein content per mg protein measured using western blotting tended to be elevated approximately 1.5 fold in both 4 °C acclimated groups, although this was not quite significant ($p=0.06$). No significant difference was seen between populations (Fig. 4B).

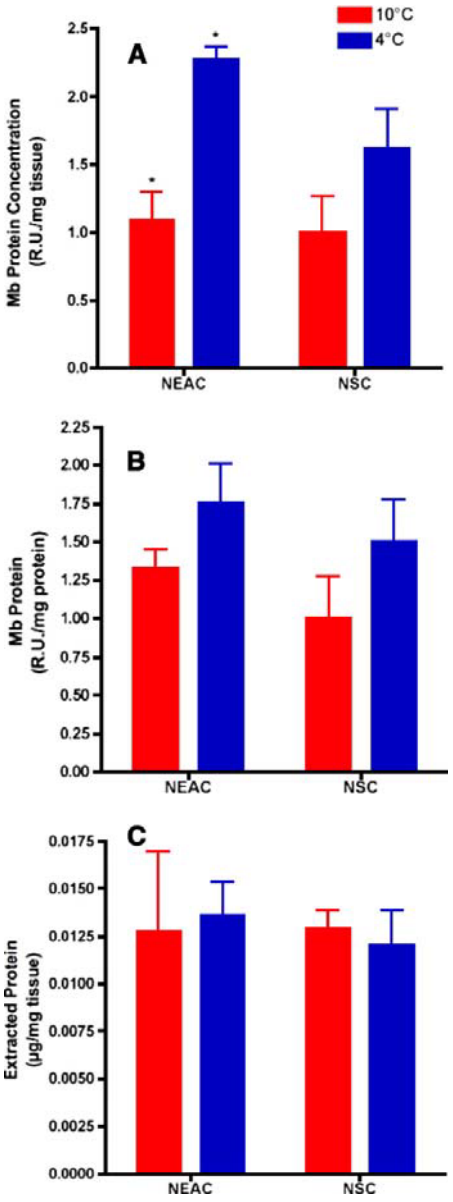

Fig. 4. A = Relative myoglobin protein per mg tissue. R.U. = relative units, B = relative myoglobin protein content, C = extracted cardiac protein concentration. Asterisks indicate significant differences between acclimation temperatures, within a population.

4. Discussion

While much research has looked at myoglobin and how it responds to hypoxia; (Driedzic, 1983; Driedzic et al., 1982; Fraser et al., 2006; Roesner et al., 2006; Silverman et al., 1997; van Bui and Banchero, 1980), to our knowledge, only a handful of other studies have previously looked directly at the effect of temperature on myoglobin in mammals (van Bui and Banchero, 1980; Rosenmann and Morrison, 1965). Our's is the first study looking directly at the effects of temperature on myoglobin expression in ectothermic fish.

4.1. Myoglobin characterisation

It can be seen from the sequence homology of the Atlantic cod myoglobin gene that it is well conserved, as are all myoglobin genes. Substitutions were not seen at vital positions, for example both haem binding histidines (Fig. 1). In the present study myoglobin concentrations were shown to relate to acclimation temperature, pointing to an important role of myoglobin function under different temperature regimes. This role is most likely in improving oxygen supply to mitochondria at different temperatures, and fits well with the concept of oxygen and capacity limited thermal tolerance (Pörtner, 2001).

Structural adaptations related to temperature may have taken place to further improve protein functioning at the comparatively low environmental temperatures of eurythermal cod. Based on the analysis of orthologues from closely related species with different climatic background, it has been proposed that minor changes in sequence are sufficient to adapt kinetic properties to different habitat temperatures. Structural flexibility seems to be increased by amino acid exchanges in cold-adapted proteins, and seems to be conserved for optimized function at the respective physiological temperature (Somero, 1997; Fields and Somero, 1997). Earlier studies on scombrid proteins have already identified important amino acids, which influence structural stability of fish myoglobin (Ueki and Ochiai, 2006). Proline 13 was considered to be an important substitution allowing proper functioning at low temperature. Cod and all notothenioids have a proline at this position. Furthermore, two amino acids are missing in the cod d-loop which is known to affect the flexibility of the myoglobin protein (Madden et al., 2004), thus strengthening the view that

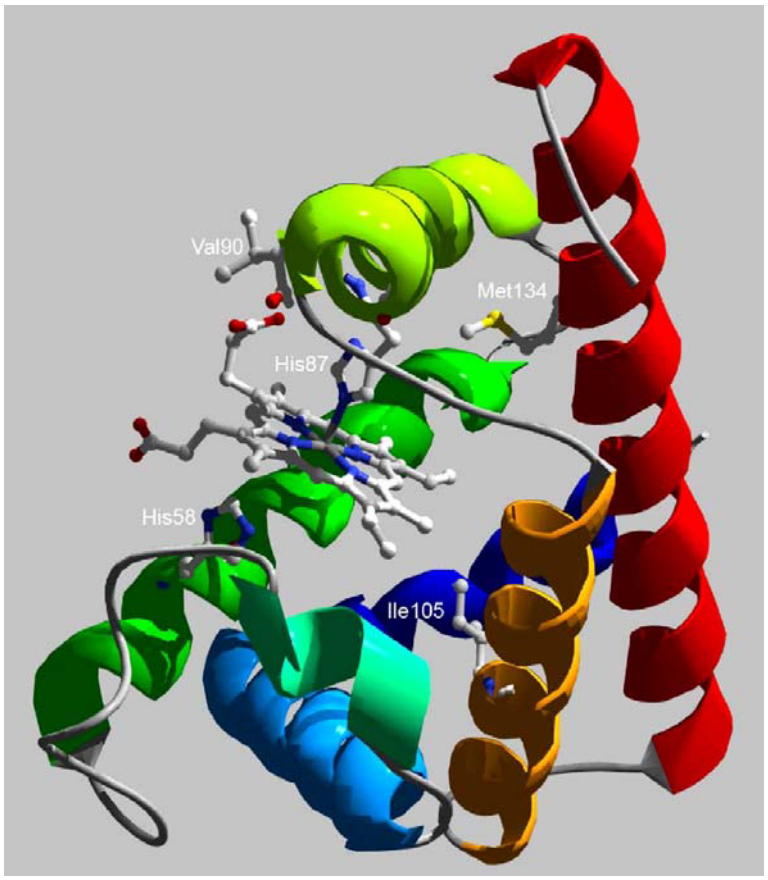

Fig. 5. Three-dimensional model of the Atlantic cod (*Gadus morhua*) myoglobin protein based on homologous protein structures using the Swiss-model program, with the haeme pocket and interesting substitutions marked (see text).

Table 3
Theoretical p*I* and number of myoglobin histidine residues from the sperm whale (*Physeter catodon*) and from different fish species in relation to their habitat/body temperature

Species	p*I*	His	Temperature	
Chaenocephalus aceratus	6.25	8	(−1.8)–(+1) °C	Cold stenotherm
Chionodraco rastospinosus	6.25	8	(−1.8)–(+1) °C	Cold stenotherm
Notothenia coriiceps	6.16	7	(−1.8)–(+1) °C	Cold stenotherm
Gadus morhua	7.42	7	0–16 °C	Cold eurytherm
Tetraodon nigroviridis	8.25	5	24–28 °C	Warm stenotherm
Danio rerio	8.25	5	18–24 °C	Warm stenotherm
Cyprinus carpio	8.97	6	3–32 °C	Extreme eurytherm
Scomber japonicus	8.70	5	10–27 °C	Warm eurytherm
Thunnus albacares	8.98	6	15–31 °C	Warm homeotherm
Thunnus thunnus	8.98	6	18–20 °C	Warm homeotherm
Physeter catodon	8.60	12	37 °C	Endotherm

Habitat temperatures derived primarily from www.fishbase.org.

structural flexibility of this region is necessary to maintain function at physiological temperatures.

Since the phylogenetic reconstruction (Fig. 2) clearly separated the cold-adapted notothenioid myoglobins from cod myoglobin, amino acid substitutions that are conserved in both, but not in warm-water fishes, are presumably important for thermal adaptation of the protein. Four such potentially important substitutions were identified: Thr 31 replaced Lys31, Arg73Lys, Ile105Leu, Met134Ile/Leu. Three-dimensional modelling of the myoglobin primary sequence using the yellowfin tuna (*Thunnus albacares*; Birnbaum et al., 1994) indicated that Thr31 and Arg73 are located on the surface of the protein (B-helix and EF loop, respectively), thereby influencing the interaction of myoglobin with the solvent or other proteins. Ile105 and Met134 are located in the hydrophobic centre (Fig. 5), the later in close proximity to the haem group. Met134 may be an especially promising target for increasing the structural flexibility within the hydrophobic core of the protein thereby influencing the kinetic properties of the prosthetic haem group.

The substitution of Lys31 by Thr increases the overall acidity of the normally basic myoglobin (Stewart et al., 2004). An analysis of the theoretical p*I* of entire protein revealed that the myoglobins from cold-adapted notothenioids is about three pH units more acidic than the p*I* from heterothermal scombrids. The p*I* of myoglobin from the eurythermal cod and cyprinids is intermediate (Table 3). Since it is well known that oxygen binding is strongly pH dependent in all haem binding proteins, this overall acidification of the cold adapted myoglobins should have consequences for the transport properties of the protein. Despite this, oxygen affinity and dissociation rates were similar in *N. coriiceps*, yellowfin tuna and zebrafish at all measured temperatures, and only the autoxidation rates seemed to correlate with environmental temperature (Madden et al., 2004).

4.2. Myoglobin expression

The results of the current study reflect what has been previously seen in various other animals, where the total RNA was observed to increase as a function of decreasing temperature in both fish (Storch et al., 2005) and scallops (Storch et al., 2003), while the total protein concentration remained stable. Total RNA represents the translation machinery and the RNA within these complexes is quite stable despite changes in temperature (Houlihan, 1991). Therefore, higher levels of RNA in the cold reflect a mechanism of cold compensation of the translation machinery at uncompensated protein levels, i.e. that translational efficiency is lower in cold acclimated animals (Houlihan, 1991). On top of the compensatory increase in total RNA a specific cold compensation of myoglobin message resulted in augmented protein levels in both populations.

Although no significant differences were seen between either of the cod populations with respect to total RNA, myoglobin mRNA, total protein or myoglobin protein expression, it is noteworthy that the effects of temperature were more pronounced in NEAC than in NSC. This again reflects earlier findings in these cod where mitochondrial enzymatic capacities and densities were significantly higher in NEAC (Lannig et al., 2003; Lucassen et al., 2006; Lurman, Bennedsen, Nyengaard & Pörtner, unpublished). This supposition is further supported by more recent work in our group that has also found significantly higher CS activity in cardiac tissue from 4 °C acclimated NSC compared to 16 °C acclimated NSC (Bremer, 2007).

During cold adaptation and cold acclimation in fishes, a suite of compensatory mechanisms are typically employed, such as a reduction in metabolic rate (Johnston et al., 1991), an increase in the number of intracellular lipid droplets (Hubley et al., 1997; Londraville and Sidell, 1990a,b; Sidell, 1998), and a reduction in diffusion path length due to mitochondrial proliferation (Hubley et al., 1997; Londraville and Sidell, 1990a,b), which together with an increase in the water PO_2 will overcome the slowed oxygen diffusion at low temperature. In contrast, the myoglobin concentration is typically thought to be static and unresponsive to temperature (Driedzic pers. com.).

Various studies have suggested that the myoglobin concentration a) is driven by mitochondrial oxygen demand (Wittenberg and Wittenberg, 2007; Yan et al., 2001) and b) correlates with mitochondrial volume density and among other enzymes, citrate synthase activity (Kanatous et al., 1999; Terrados et al., 1990). Previous work has found that white muscle CS and COX (Lannig et al., 2003; Lucassen et al., 2006) increased in Atlantic cod during cold acclimation. More recent work in our lab found red muscle mitochondrial volume density was also significantly elevated at 4°C (Lurman, Bennedsen, Nyengaard & Pörtner, unpublished). This was most apparent in the NEAC, where a five-fold increase in white muscle CS activity was observed and red muscle mitochondrial volume density increased by 40%. Thus, the increased metabolic demands seen in both red and white muscle, and indeed in the whole animal (Fischer, 2003), appear to cause higher demands for myoglobin as observed in the current study.

Yet it is worth mentioning here that research examining the role of myoglobin and its effect on cardiac function has revealed some interesting insights into the need for myoglobin, at least with regards to oxygen supply. Early experiments found that myoglobin, when rendered non-functional using hydroxylamine, did not reduce maximum cardiac performance in sculpin (*Myoxocephalus octodecimspinosus*) under normoxic conditions (Canty and Driedzic, 1987). Comparative experiments

looking at the importance of myoglobin in nature's own knock-outs, the notothenioid icefishes, found that hearts from icefishes that do not express myoglobin had a lower performance limit than those that did and that poisoning of myoglobin function impaired cardiac performance in the latter (Acierno et al., 1997). Subsequent work in mice using engineered myoglobin knock out mice found that through a series of compensatory mechanisms, the knock-outs hearts were able to perform equally well (Gödecke et al., 1999). Similar compensatory mechanisms like an enhanced mitochondrial density for improved oxygen diffusion are found in the myoglobinless icefish hearts (Londraville and Sidell, 1990a,b). In addition to an important role of myoglobin with respect to facilitated diffusion and buffering of cellular oxygen other auxiliary roles may exist.

Mitochondria are major generators of cellular ROS (Boveris and Chance, 1973). A major target of ROS effects is lipid (Gieseg et al., 2000). As previously mentioned, during cold acclimation both the mitochondrial density and intracellular lipid content increase. Thus we can expect a higher rate of ROS production in Atlantic cod acclimated to lower temperatures. As myoglobin was recently found to act as an ROS scavenger, protecting against oxidative stress (Flögel et al., 2001; Flögel et al., 2004), one beneficial side effect of increased myoglobin during cold-acclimation may be decreasing ROS production and effects. It is also worth noting that the role of myoglobin as an antioxidant is not so clear-cut. A body of literature exists (see Svistunenko, 2005 for a general review) that demonstrates the pro-oxidant effects of myoglobin when combined with hydrogen peroxide, but the vast majority of these deal with *in vitro* systems using high peroxide concentrations, and the significance of this *in vivo* is yet to be clarified.

More recent research has also found other roles for myoglobin, particularly with respect to nitric oxide (NO). Myoglobin acts as an effective NO scavenger in the heart thus preventing the inhibition of COX (Brunori, 2001; Flögel et al., 2001), and thereby protecting mitochondrial capacity (Wittenberg and Wittenberg, 2003). NO is also known to be a regulatory signal in mitochondrial biogenesis (Nisoli et al., 2003; Clementi and Nisoli, 2005). Thus, by acting as an NO scavenger, myoglobin content, mitochondrial density and activity may be functionally intertwined. The cold compensation of oxygen delivery and storage in the cold may have beneficial effects through protection against ROS and against disturbance of mitochondrial function. In conclusion, myoglobin may support cold compensation of the metabolic machinery by improved oxygen delivery to mitochondria and the protection of mitochondrial functioning as well as cellular integrity.

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Atlantic Cod (*Gadus morhua* L.) *in situ* Cardiac Performance: Long-Term Acclimation, Acute Thermal Challenge and the Role of Adrenaline.

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Running head: Cod cardiac performance in the cold

Keywords: Atlantic cod, *Gadus morhua*, cardiac performance, heart rate, power output, cardiac output, temperature, adrenaline

Abstract:

Cardiac performance is a primary factor influencing swimming performance in teleost fish. Using the *in situ* perfused heart set-up we examined cardiac performance in cod acclimated to 10, 4 and 0 °C. Only small differences in maximal cardiac output (CO) and the maximal cardiac power (PO) were seen between 10 °C and 4 °C acclimated cod, however the reduction was significant at 0 °C. Changes were due primarily to significant chronotropic rather than inotropic effects. Acute temperature reversal produced similar cardiac performance in 10 °C acclimated cod cooled to 4 °C when compared to 4 °C acclimated cod at 4 °C. However, CO was significantly higher in 4 °C acclimated cod warmed to 10 °C when compared to 10 °C acclimated cod at 10 °C, a result of subtle chronotropic and inotropic increases. This may be due to alterations in the electrophysiological state after warming. Cardiac scope was lowest in cod acclimated to 0 °C, yet acute cooling from 4 °C to 0 °C did not alter cardiac scope. Unlike most teleost hearts, the Atlantic cod heart proved to be indifferent to direct adrenergic stimulation at rest and during maximal output, indicating that circulating adrenaline must regulate cardiac performance by altering venous return in concert with other catecholamines.

Introduction:

Temperature is a critical environmental factor that influences all life functions through changes in the rates of biochemical and physiological processes, and affects the stability of biological molecules. Consequently, the thermal tolerance range of aquatic organisms has been studied for decades (e.g. Brett, 1971; Fry, 1971; Beitenger et al., 2000; Pörtner, 2001), and there is accumulating evidence that the thermal tolerance of marine organisms (including fishes) is limited by blood oxygen transport and aerobic scope, and that at the limits of acclimation capacity, temperature dependent constraints on these physiological processes translate into alterations in population dynamics and biogeography (Pörtner et al., 2001; Pörtner 2002; Pörtner and Knust, 2007). For an estimate of thermal tolerance under field conditions, the influence of acclimatization on physiological mechanisms/processes and thermal tolerance needs therefore to be considered (e.g. see Sokolova and Pörtner, 2003; Stillman, 2003). Further, a recent study (Seebacher et al., 2005) reports that even archetypical stenothermal fish (e.g. the Antarctic fish *Pagothenia borchgrevinki*) display considerable plasticity in cardiovascular and metabolic control, and swimming performance as a result of temperature acclimation.

Given the economic importance of Atlantic cod fisheries world-wide, the collapse of wild Atlantic cod populations over the past two decades, and emerging cod aquaculture industries in several countries (e.g. Canada, Norway, Scotland, United States), it is not surprising that the thermal biology of this species has received considerable attention. Based on measurements of venous P_{O_2}, cardiac performance/blood flow and plasma cortisol concentrations during acute temperature challenges (1.5 - 2 °C h^{-1}), the upper and lower pejus ('getting worse') and upper critical temperatures for Atlantic cod acclimated to 10 °C are estimated to be 2 (Tp low), 7 (Tp high) and 16 °C (Tc high a.k.a. CTM), respectively (Sartoris et al., 2003; Lannig et al., 2004; Gollock et al, 2006). These thermal limits shift with acclimation. The following picture characterizes current understanding: 1) the maximum aerobic scope for European Atlantic cod acclimated to a wide range of temperatures (2 - 15 °C) is in the range of 13 - 15 °C (Claireaux et al., 2000); 2) cardiovascular responses play an important role in exercise and temperature changes (Webber et al., 1998; Lannig et al. 2004; Gollock et al., 2006); and 3) the optimal temperature for growth (Björnsson et al., 2001) and temperature preference (Lafrance et al., 2005), both

parameters that are linked with maximum aerobic scope (Jobling et al., 1981; Claireaux et al, 2000), are significantly influenced by ontogeny.

Based on the above, it is clear that further research is still needed on how acclimation temperature/thermal history influences the temperature limits of fishes, and the physiological processes that determine them. Furthermore, the role of cardiocirculation in thermal physiology of cod requires further study. Thus, in this study, we used the *in situ* cod heart to examine how acclimation to 10, 4 and 0 °C, and acute temperature changes (10 - 4 °C, 4 - 10 °C, and 4 - 0 °C), influence maximum cardiac performance. There were several reasons for examining *in situ* cardiac performance. *In situ* values for maximum cardiac performance are, in general, very comparable to measurements made *in vivo* (Claireaux et al., 2005; Mendonca et al., in press). Further, maximum cardiac performance/scope shares the same temperature optimum as aerobic scope, and is linked to both maximum metabolic rate and swimming performance in salmonids (Farrell, 1996; Farrell, 2002; Claireaux et al., 2005). In our experiments, we chose temperatures below 10 °C because fish that inhabit the continental shelf off Atlantic Canada typically face water temperatures between 0.7 and 8 °C (Lear, 1984; Clark & Green, 1991). These temperatures span those used by Claireaux et al. (2000) to examine the influence of acclimation temperature on cod aerobic scope, and thus will allow for a comparison with values of cardiac scope generated in this study. This approach complements previous work as there are currently no data on cod cardiac performance below 5 °C. To our knowledge only one study (Axelsson et al., unpubl; data presented in Axelsson, 2005) has in fact examined maximum cardiac performance/cardiac scope in a non-Antarctic teleost at 0 °C.

Finally, our study was also designed to address the role of catecholamines during temperature dependent cardiac performance. Data on 10 °C acclimated Atlantic cod (Axelsson, 1988; Gamperl and Genge, unpubl) suggests that adrenaline is not required for basal or maximum cardiac performance. This result is in contrast to the data for the vast majority of teleosts (Axelsson et al., 1987; Axelsson et al., 1998), and with findings that suggest that adrenergic sensitivity is a critical compensatory mechanism that allows the fish myocardium to maintain contractility during acute exposure to cold temperatures (Keen et al, 1993; Shiels et al., 2003). However, the apparent lack of myocardial responsiveness to adrenaline in cod may be due to the use of a single experimental temperature (10 °C). For example, research on other teleosts

indicates that acclimation to 'warm' temperatures or those within a fish's optimal thermal range may reduce myocardial adrenergic sensitivity and/or adrenoreceptor density (Graham and Farrell, 1989; Keen et al., 1993, Farrell et al., 1996; Farrell et al., 2007). Thus, by examining the interactive effects of adrenergic stimulation and cold (<10 °C) temperatures on maximum cardiac performance, we were able to further evaluate what role circulating catecholamines play in supporting cod cardiac performance, and indeed, whether this species differs from other teleosts in this regard.

Methods:

Experimental Animals

The Atlantic cod *Gadus morhua* L. used were transported from a sea-cage facility at Northwest Cove (Hermitage Bay, Newfoundland, Canada) to the Aquaculture Research Development Facility (ARDF) at the Ocean Sciences Centre in St John's, Newfoundland in March 2006. These fish were maintained in 3000 L tanks in the ARDF supplied with aerated seawater at 10, 4 and 0 °C for at least 6 weeks prior to experimentation. The fish were fed a commercial cod diet daily, and maintained under an ambient photoperiod.

Surgical Procedure

Fish were anaesthetized in tricaine methane sulphonate (MS 222, 0.1 g L^{-1}) in seawater until ventilatory movements ceased. Fish were weighed and measured (see table 1) and placed on the operating table on a wetted sponge in a supine position. The gills were irrigated with chilled (2 - 4 °C) oxygenated seawater containing 0.05 g L^{-1} MS 222. Before surgery, 1.0 ml of heparin (2500 i.u. ml^{-1} saline solution) was injected into the caudal vein to prevent blood clots in the heart.

The peritoneal cavity was exposed with a midline incision and by cutting through the abdominal muscle in a ventral-dorsal line. Blood flow to the stomach, intestines and other abdominal organs was stopped by tying off the gastrointestinal tract, inferior to the liver, with umbilical tape. The abdominal and digestive organs were carefully removed in order to obtain proper placement of the input cannula whilst keeping the liver intact.

The right hepatic vein was selected for cannulation while the other was tied off with silk sutures. A small incision was made in the hepatic vein and the steel

cannula (0.9 mm outer diameter) inserted into the hepatic vein. At this point the 1st and 2nd gill arches were cut on each side of the fish to prevent excessive pressure development by the heart. At the same time as the gill arches were cut, perfusion of the heart with ice-cold (2 - 4 °C) 100% oxygenated saline was begun. The level of saline in the perfusion bottle was kept at approximately the same height as the heart in order to obtain basal cardiac output and prevent cardiac stretch.

The lower jaw and operculum were removed and the isthmus posterior to the 2nd gill arches was completely cut away to expose the ventral aorta which was dissected free of any surrounding tissue. The 3rd and 4th gill arches were then cut in half and clamped with cable ties (10 cm) to avoid leakage. The steel output cannula (0.8 mm outer diameter) was inserted into the ventral aorta and tied in place with silk sutures.

After placement of the output cannula, the ducts of Cuvier were tied off with a silk suture passed at an angle from the corner of the opercular cavity to the muscle of the abdominal wall. The suture was then passed under the liver, through the oesophagus and back into the buccal cavity, thus occluding these veins and crushing the cardiac branches of the vagus nerve. This ensured that any flow to the heart was from the input cannula and that any nervous stimulation of the heart during the experiment was inhibited. A noticeable pectoral fin twitch and transitory cardiac arrest indicated that the cardiac branches of the vagus nerve had indeed been crushed.

The fish was then bisected just below the pectoral fins. After surgery, the *in situ* heart preparation was placed in the double-jacketed chamber. This chamber was attached to a cryostat, thus allowing manipulation of the temperature.

The cod ringer perfusate contained: [g L^{-1}] 10.5 NaCl; 0.49 $MgSO_4*7H_2O$; 0.37 KCl; 0.34 $CaCl_2*2H_2O$; 0.14 $NaH_2*PO_4*H_2O$; 1.84 sodium TES base; 0.59 TES acid; 1.0 glucose, pH 7.67 at 20 °C. Shortly before use, 250 µL of 0.1 µM adrenaline bitartate salt (AD) dissolved in dH_2O was added to 500 mL of the cod ringer to give an end concentration of 5 nM AD, equal to a resting concentration (Wahlqvist, 1980; Wahlqvist & Axelsson, 1980). Alternatively, 250 µL of 2 µM was added to obtain a final concentration 200 nM AD, equal to a maximal *in vivo* concentration previously seen in stressed fish (Wahlqvist, 1980; Wahlqvist & Axelsson, 1980). These doses were refreshed every 20 min to avoid photo-degradation of AD. These two doses were used based on published values for resting and stressed Atlantic cod (Axelson & Nilsson; 1986; Wahlqvist 1980).

After mounting the *in situ* preparation in the experimental chamber the heart was allowed to recover from surgery at the acclimation temperature for about 5-10 minutes with an output pressure (P_{OUT}) of 20 cm H_20 (1 cm H_2O = 0.091 kPa) and an input pressure (P_{IN}) required to maintain 16, 10 or 8 mL min^{-1} kg^{-1} at 10, 4 and 0 °C. These values were taken as reasonable estimates of *in vivo* resting cardiac output from published values (Webber et al., 1998). The P_{IN} and P_{OUT} loadings on the heart were set by attaching the in and output cannula to adjustable in and output pressure-heads. After this initial setting, output pressure was increased to a physiological output pressure for cod of 50 cm H_2O (Axelsson and Nilsson, 1986; Pettersson & Nilsson 1980). The heart was then allowed to stabilize for a further 20 min at resting cardiac output before the temperature was changed in those heart exposed to an acute thermal challenge. To mitigate biases resulting from possible deterioration in the hearts due to the 1 hr period necessary for changing the temperature, we also maintained the hearts not exposed to an acute thermal challenge for 1 hr under resting conditions without changing the temperature before proceeding with the cardiac performance tests.

Cardiac Performance Tests

Firstly, a maximum cardiac output test was performed at 5 nM AD. Input pressure was increased in steps from resting to 4.0, 5.0, 5.5 and finally 6.0 cm H_2O (figure 1). Each increase in P_{IN} was maintained for approximately 30 seconds. During the maximum cardiac output test output pressure was maintained at 50 cm H_20. After this trial, P_{IN} was left at 6 cm H_2O and a maximum power output test was performed where P_{OUT} was decreased to 20 cm H_2O and then increased in 10 cm H_2O steps up to 80 cm H_2O (figure 1). Following this, the hearts were allowed to recover under resting conditions for a further 20 minutes.

After recovery, the cod ringer AD concentration was changed from 5 nM to 200 nM. The heart was given a further 3 minutes before resting parameters were recorded and the maximum cardiac output and power output tests were carried out as described above.

Data collection and analysis

Cardiac output was measured using a model T206 small animal blood flow meter in conjunction with a pre-calibrated in-line flow probe (2N, Transonic Systems Inc. Ithaca, NY). A Gould statham pressure transducer (Model P23 ID, Oxnard, CA)

was used to measure P_{OUT}, and P_{IN} was measured using a Grass pressure transducer (Model PT300, Warwick, RI, USA). Before the start of each experiment, the pressure transducers were calibrated against a static column of water, where zero pressure (0 cm H_2O) is set at the saline level in the experimental bath. Pressure and flow signals were amplified and filtered using a Model MP100A-CE data acquisition system (BIOPAC Systems Inc., Santa Barbara, CA). The acquired signals were then analysed and stored using Acknowledge 3.7 Software (BIOPAC Systems Inc., Santa Barbara, CA).

Cardiovascular performance was continuously measured throughout the experiment by measuring cardiac output (CO), P_{IN}, and P_{OUT}. Before every maximum cardiac output test, P_{IN} was measured in order to determine the P_{IN} required to obtain resting cardiac output. Cardiac output, heart rate (HR), stroke volume, (SV) and P_{IN} were measured/calculated at each step of the maximum cardiac output and power output tests. Heart rate was calculated by measuring the number of systolic peaks during a 20 - 30 second interval. Cardiac output (ml min^{-1} kg^{-1}) and stroke volume (ml kg^{-1}) were calculated as:

Cardiac output (ml min^{-1} kg^{-1}): cardiac output (ml min^{-1})/kg animal weight.
Stroke volume (ml kg^{-1}): (CO/HR)/animal weight in kg.
Cardiac power output (PO, mWg^{-1} ventricle): (CO x (P_{OUT}-P_{IN}) x a)/ventricular mass in grams.
Where P_{OUT} and P_{IN} are output and input pressures (cm H_2O) respectively, and a = 0.0016 mW min ml^{-1} cm H_2O^{-1} is a conversion factor to mW.

Subsequent statistical analysis and graphing was performed using SigmaStat 3.5 and Sigma Plot 10. Two-way repeated measures ANOVAs were used to test for significant effects of temperature and adrenaline concentration, and Holm-Sidak post tests were then used to determine differences between individual groups. Significance = $p < 0.05$. Values are given as mean ± s.e.m.

Results:

Cardiac hypertrophy, i.e. an increase in heart size is typical of cold-acclimation (Driedzic et al., 1996; Farrell, 1996; Axelsson et al., 1998), however, no cardiac hypertrophy was seen as a result of cold acclimation, i.e. thermal acclimation had no significant effect on the ventricular or atrial mass and therefore, relative ventricular and atrial weights were also similar between groups (table 1).

Adrenergic Effects

Under resting conditions, no significant differences were seen as a result of adrenergic stimulation at 5 or 200 nM AD in any of the parameters measured, i.e. heart rate, stroke volume, cardiac output, power output (table 2). Furthermore, this non-responsiveness to AD was consistent across temperatures and also during both the maximum cardiac output (table 2, and figures 2 and 5) and maximum power output tests (table 2, and figures 4 and 5). Similarly, there was no evidence of increased end systolic volume as a result of adrenaline (figure 5).

Temperature Effects

Under resting conditions the hearts at 10 °C (when acclimated or acutely challenged) required a slightly positive input pressure to maintain the required resting cardiac output of ~16 mL min^{-1} kg^{-1} (table 2). This was ~0.5 to 1 cm H_2O higher than the negative input pressures required by the hearts at 4 °C (again when acclimated or acutely challenged). The 0 °C acclimated hearts required a still lower mean input pressure of -1.14 cm H_20 to maintain the required 8.9 mL min^{-1} kg^{-1}.

Temperature had a significant effect on heart rate during the maximum cardiac output test after long-term acclimation. Heart rate dropped by 37% as acclimation and test temperature dropped from 10 to 0 °C (figure 2A). Q_{10}s of 0.46, 1.35 and 1.45 were calculated for HR between the 10 and 4 °C, 4 and 0 °C, and the 10 and 0 °C acclimated groups, tested at their respective temperatures. In stark contrast, stroke volume was completely unaffected by temperature, remaining between 1.2 and 1.4 mL kg^{-1} at all temperatures (figure 2B). Nevertheless, the drop in heart rate did significantly affect cardiac output, which was 26% lower at 0 °C compared to 10 °C (figure 2C). The maximum power test found the maximum power output was generated between 50 and 60 cm H_2O (figure 3A) while the maximum cardiac output

was generated between 20 and 30 cm H_2O (figure 3B), and that the maximum power decreased 27% from 10 to 0 °C (figure 4), though not significantly.

During an acute thermal challenge the heart rate was again significantly reduced as temperature decreased (table 2 and figure 2A). The 10 °C acclimated hearts had a similar heart rate at 4 °C to hearts acclimated to 4 °C, and the 4 °C acclimated hearts had a similar heart rate at 10 °C compared to hearts acclimated to 10°C. Furthermore, when the 4 °C acclimated hearts were cooled to 0 °C, they had a heart rate comparable to that of the 0 °C acclimated hearts.

Stroke volume was largely unaffected by an acute thermal challenge (figure 2B). However, the combined effects of the subtle changes in the heart rate and stroke volume after acute warming of the 4 °C acclimated hearts to 10 °C was that the maximum cardiac output for this group was significantly higher at 61 mL min^{-1} kg^{-1} when compared to 45 mL min^{-1} kg^{-1} for the 10 °C acclimated hearts at 10 °C, as was the maximum power output (10.9 and 7.2 mW g^{-1} respectively).

Discussion:

Cardiac Performance After Long-term & Acute Thermal Challenge

Cardiac hypertrophy is often cited as an indicator of cold-acclimation/adaptation (Driedzic et al., 1996; Farrell, 1996; Axelsson et al., 1998). However, ours is not the only case where no increase in RAM or RVM was seen after cold-acclimation. For example, Foster et al. (1993) saw no evidence for myocyte hyperplasia or hypertrophy in juvenile cod, as evidenced by constant DNA to tissue wet weight and DNA to protein ratios. Although heart sizes were not given, we may infer that there was no change in relative heart mass. Similarly, various other fishes, namely the white (*Morone americana*) and yellow perch (*Perca flavescens*) exhibit no changes in heart size during cold-acclimation (Sephton & Driedzic, 1991).

The heart rate and stroke volume under resting conditions and during maximal exertion in the current study compare well with previously published *in vivo* data from the same cod population. A recent study of 10 °C acclimated Newfoundland cod found resting HR and SV at 10 °C to be 36.3 bpm and 0.6 mL kg^{-1} respectively, (Gollock et al., 2006). This compares to 41.4 ± 1.0 bpm, 0.39 ± 0.1 mL kg^{-1} measured here at 10 °C (see also table 2). Although the HR in the current study is slightly higher by comparison, others have previously reported a similarly high HR (41 bpm), concomitant with an even higher CO (29 mL min^{-1} kg^{-1}) in instrumented cod at 10°C (Pettersson & Nilsson, 1980), and Claireaux et al. (1995) reported a HR of 42 bpm in 5 °C acclimated, free-ranging, telemetry tagged cod at 10 °C. During an acute temperature decrease from 10 to 6.4 °C, HR fell to 20 bpm. Webber et al. (1998) found HR to be 28 bpm, and CO to be 12 mL min^{-1} kg^{-1} at 10 °C and these dropped to 16 bpm and to 9 mL min^{-1} kg^{-1} respectively in the same 10 °C acclimated cod acute thermal challenge at 5°C. During maximal swimming, Webber et al. (1998) also found that the HR increased to 28 and 40 bpm at 5 and 10 °C respectively, and CO also increased to 31 and 35 mL min^{-1} kg^{-1} at the same temperatures. Wardle & Kanwisher (1974) reported a peak HR of approximately 50 bpm in burst swimming 9 °C acclimated cod. Again, this is very similar to what was observed in the current study.

The maximal CO in 10 °C acclimated cod observed in the current study of 43 ±1.9 and 45 ± 1.9 mL min^{-1} kg^{-1} (5 nM and 200 nM AD respectively) was similar to the CO seen by Gollock et al. (2006) of ~50 mL min^{-1} kg^{-1} at 19 °C, shortly before the

CTM. This indicates that cardiac output for 10 °C acclimated cod has an upper limit of approximately 50 mL min^{-1} kg^{-1} independent of acute temperature. As the acute temperature increases, so too does the resting cardiac output. Eventually a point is reached (19 °C) where the cardiac scope has been effectively reduced to zero and the resulting increased demand placed on the heart upon further temperature increases (e.g. to 22 °C) results in cardiac collapse at the CTM.

At the other end of the spectrum, Woodhead & Woodhead (1959) reported earlier that North East Arctic cod generally avoided long periods in water bodies colder than 2 °C and suggested that cod "lose their ability to survive" at temperatures below 2 °C. In the current study, severe lethargy was observed in 0 °C acclimated fish throughout the acclimation period, and was concomitant with a very low food intake. Extrapolation of principles from Brett's thermal relationships in trout (Brett, 1971) led us to expect that the 0 °C acclimated cod would have a very low HR, and CO due to the low metabolic demand. The robust nature of cardiac performance at 0 °C, especially during maximal exertion observed in the current study is indeed surprising, in the light of these earlier findings.

Oxygen consumption and thus, aerobic scope previously displayed a negative exponential relationship with temperature in Newfoundland cod (Claireaux et al., 2000), indicating that the lower the temperature, the steeper the decline in oxygen consumption. The current findings are similar, where the reduction in cardiac scope in acclimated cod was most pronounced between 0 and 4 °C (figure 6). However, after an acute drop to 0 °C, cardiac scope was unchanged (figure 6). Thus, during acclimation to extremely low temperatures, i.e. (0 °C), cardiac performance was uncompensated, but during an acute drop to such a low temperature, cardiac performance is maintained. This potentially explains how cod are able to make short forays into waters at -1 to 0 °C to catch prey (Woodhead & Woodhead, 1959), and yet do not acclimate well to the extreme cold (Brown et al., 1989).

Changes in CO and as a consequence, PO, seen in the current experiments, were due almost solely to changes in HR. This mechanism reflects that previously seen *in vivo* in Atlantic cod (Gollock et al., 2006) during warming and various other fishes following temperature changes (Cech Jr et al., 1976; Randall, 1968; Sandblom & Axelsson, 2007; Watters & Smith, 1973). During acute warming in Atlantic cod from Newfoundland, both the CO and HR increased while the SV remained unchanged until only a few degrees before the upper CTM (Gollock et al., 2006). At

the CTM a significant increase in SV was seen. This was also recently documented in rainbow trout (Sandblom & Axelsson, 2007). It has been proposed that the increase in SV at this physiological extreme compensates for the increased end systolic volume due to a high HR (Sandblom et al., 2005; Sandblom & Axelsson, 2007). In cod however, adrenaline had no effect on the end systolic volume (figure 5).

In addition to a slightly elevated HR, the 4 °C acclimated cod warmed to 10 °C also had a slightly elevated SV compared to 10 °C acclimated cod at 10 °C. While neither of these differences were significant, their combined effect led to significantly elevated cardiac and power outputs. Marked differences in the electrophysiological properties of the myocytes may account for this. Haverinen & Vornanen (2004) found that rainbow trout acclimated to 4 °C increased their myocyte intracellular Na current density and there was a negative shift in the steady-state activation potential, meaning the stimulus threshold was probably decreased. Moreover, these patterns were much more pronounced in 4 °C acclimated myocytes acutely exposed to 11 °C. This would suggest that during acute warming, it is a) easier to stimulate the cold acclimated hearts to contract and b) the contractions are stronger. The product of which was the over-compensation of maximum cardiac output and maximum power output seen during acute warming in the current study. This may indicate functional trade-offs in the thermal acclimation of the cod heart.

Although it has previously been observed that cod are highly responsive to subtle temperature changes (Claireaux et al., 1995; Rose, 1993; Petersen & Steffensen, 2003), the ecology of Newfoundland cod would dictate that a certain degree of thermal flexibility be maintained with respect to cardiac performance and support the eurythermy of these fish. Figure 1 from Gollock et al. (2006) clearly showed that these cod can experience daily temperature fluctuations of up to 15 °C during summer.

Cardiac Performance and Adrenaline

The 5 and 200 nM AD concentrations used in this study were decided upon after consulting published values. For example, resting *in vivo* plasma AD concentrations of 20-40 nM and up to 300 nM during stress have been previously reported in cod (Wahlqvist 1980; Wahlqvist & Axelsson 1980). To ensure that 5 nM AD was not already causing maximal stimulation, a pilot study with three cod at 7 °C compared effects of no AD with both 5 and 200 nM AD and found little difference

between them (data not shown). Previous experimental work has similarly found that AD has little effect on cod cardiac performance (Gamperl et al. unpublished).

In contrast to the data for the vast majority of teleosts (Axelsson et al., 1987; Axelsson et al., 1998), the lack of adrenergic stimulation seen in the current data confirms previous *in vivo* findings that AD does not alter resting or maximal cardiac performance in cod (Axelsson, 1988; Gamperl and Genge, unpubl). It also extend this finding from one temperature, i.e. 10 °C, down to 4 and 0 °C. The indifference of the Atlantic cod heart to AD begs two questions: firstly, if AD has no direct affect on the heart, how is cardiac performance regulated under differing physiological circumstances? Early work by Holmgren (1977) found evidence for the presense of a vagal inhibitory cholinergic nerve supply to the cod heart, acting on muscarinic receptors. Further work by Axelsson & Nilsson (1986) also found evidence that the cardio-regulatory mechanism was non-adrenergic, i.e. cholinergic vagal cardio-inhibitory tonus. Indeed the inhibitory cholinergic vagal tone on the cod heart was significantly greater than the effect of adrenergic stimulation (Axelsson, 1988). Consequently, alteration/removal of vagal tone has a much greater influence on HR and ultimately CO at rest and during exercise (Axelsson, 1988).

The second question is, what is the role of circulating adrenaline if it does not stimulate the heart? There are two answers to this. Firstly, AD may impart a cardio-protective function under "extreme" conditions preserving normal cardiac performance, as has previously been seen during acidosis, hyperkalemia and hypoxia in rainbow trout (Gesser et al., 1982; Hanson et al., 2006). This would also explain why large plasma AD concentrations, similar to those used in the current experiment were only measured *in vivo* after repeated burst swimming or 'stress' induced by handling, but not after moderate swimming (Axelsson & Nilsson, 1986). Whether temperature is enough of a stressor remains to be elucidated by measuring *in vivo* blood catechoamines during acclimation and acute changes in temperature.

Secondly, AD may affect cardiac performance indirectly by regulating blood and ultimately filling pressure. In contrast to the heart, systemic blood pressure in Atlantic cod is maintained by adrenergic tone (Axelsson & Nilsson 1986). Adrenaline has potent affects on Atlantic cod vasculature, for example, opening the brachial gill vessels and closing systemic vessels (Wahlqvist, 1980, Wahlqvist & Axelsson, 1980). Pettersen & Nilsson (1980) found systemic vasoconstriction was α-adrenoceptor mediated, and AD stimulation caused a 130% increase in systemic vascular

resistance, which lead to 82% increase in dorsal aortic pressure, i.e. systemic vascular resistance is affected far more by the AD than the heart. (Pettersen & Nilsson, 1980). "The increase in blood pressure can… be explained by an increase in CO, which in turn, may be affected by an increase in the venous return of blood during exercise." (Axelsson & Nilsson, 1986). This scenario was already observed in the sea bass (*Dicentrarchus labrax*), where α-adrenoceptor mediated increases in the systemic venous pressure increased the venous return. This increased the cardiac output. And similar to the current experiments, the increased CO in sea bass was a product of the increased HR, while SV remained unaltered (Sandblom et al., 2005).

Conclusions

In general, cardiac performance was reduced as the temperature was reduced, primarily due to the thermal dependence of the HR. Atlantic cod *in vivo* data have also shown that alterations in HR were found to be the primary affecter of CO during acute thermal challenge until shortly before the CTM is reached, when SV is increased to overcome the increased end systolic volume. However, temperature effects on cardiac performance after long-term acclimation were different to those of acute thermal challenge, particularly when 4 °C acclimated cod were warmed to 10 °C. This led to significant increases in CO and PO. This may be due to changes in the electrophysiological state of the heart during warming. Acute cooling of the heart from 4 to 0 °C did not impact cardiac scope, while acclimation to 0 °C led to a reduction in cardiac scope. Adrenaline did not affect cardiac performance directly. No alterations in HR, SV and consequently CO were seen. However, AD may affect cardiac performance in indirect ways. Firstly, by increasing α-adrenoceptor mediated vasoconstriction, resulting in an increase in the venous return and filling pressure, thus affecting HR, as has been seen *in vivo* cod, and secondly, by preserving cardiac performance only under other extreme situations, i.e. hyperkalemia, hypoxia, acidosis, etc.

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Figures:

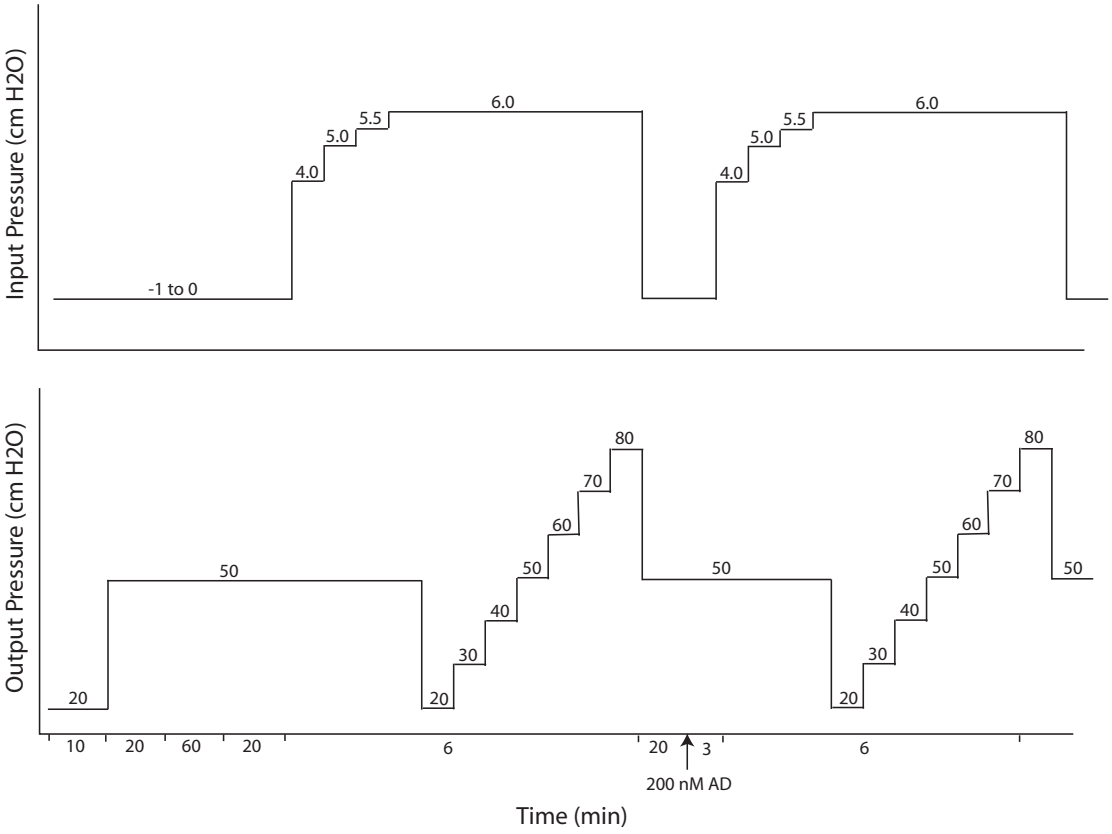

Fig. 1: Experimental protocol of the effect of temperature on cardiac performance. After the *in situ* heart preparation was placed in the experimental chamber at the acclimation temperature and initially allowed to recover at a sub-physiological output pressure (20 cm H_20) for 10 min, the output pressure head was increased to a physiological value of 50 cm H_2O for another 20 min. Resting parameters were recorded every 5 minutes. A subsequent hour was then used to change the temperature for the acutely challenged hearts. Then a second 20 min resting period at the desired temperature preceded the maximum cardiac output test, which was performed by increasing input pressure in 4 steps starting at 4.0, 5.0, 5.5 and finally 6.0 cm H_2O. For the subsequent maximum power output test the P_{IN} was left at 6 cm H_2O and the P_{OUT} dropped to 20 cm H_2O before being increased in 10 cm H_2O steps to 80 cm H_2O. The heart was allowed to recover for 20 min. The heart was then dosed with 200 nM AD for 3 min after which the resting parameters were recorded and a second maximum cardiac output and maximum power output test was performed.

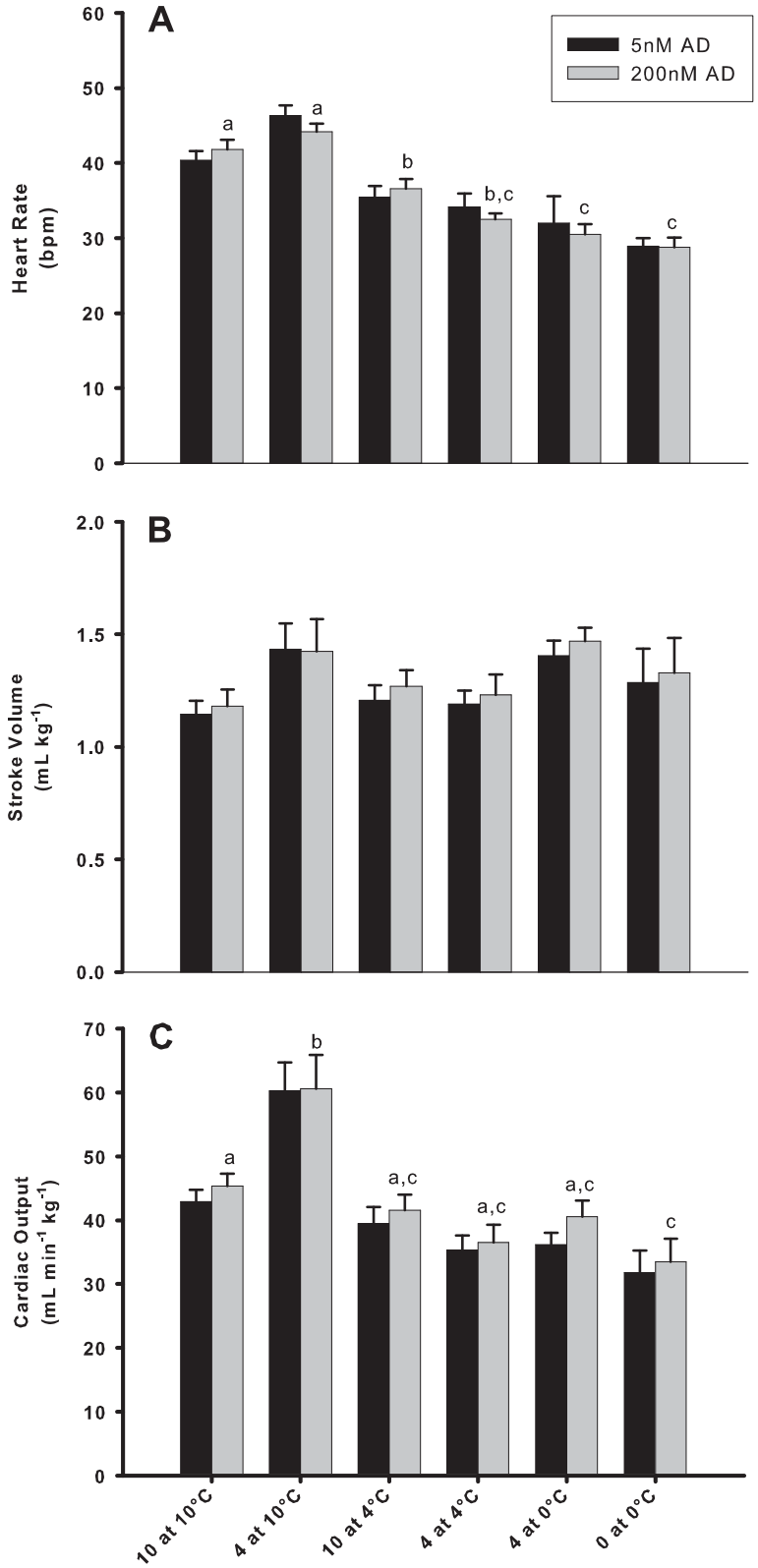

Figure 2: Maximum cardiac output test. P_{IN} = 6 cm H_2O, P_{OUT} = 20 cm H_2O, A = heart rate, B = stroke volume, and C = cardiac output. The horizontal axis indicates the acclimation temperature and the final test temperature. Dissimilar letters indicate a significant difference.

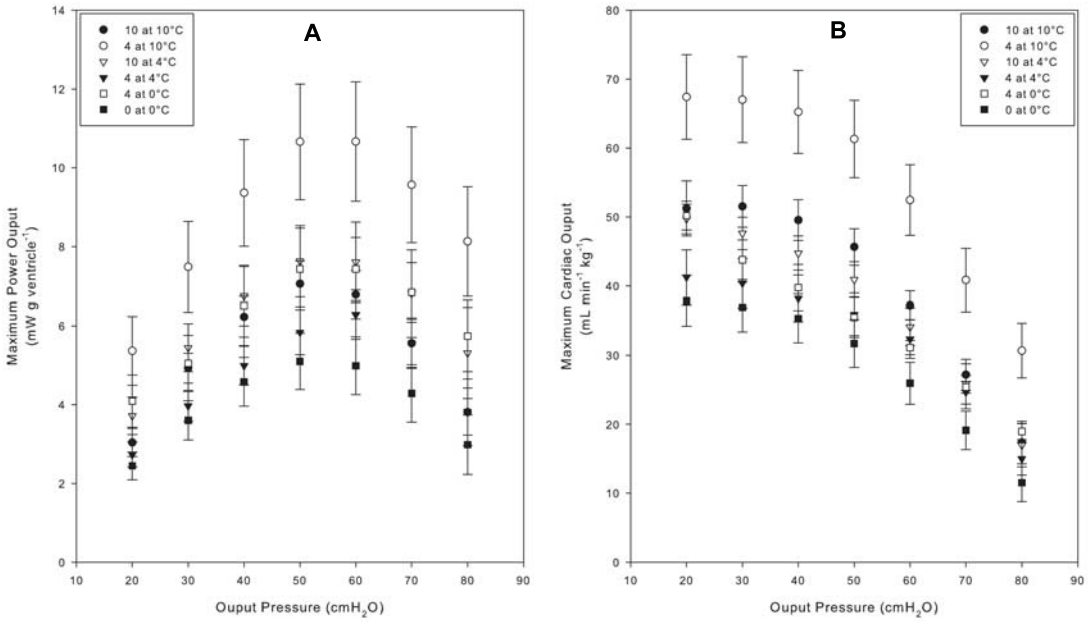

Figure 3: Maximum power output test. For clarity, only the 200 nM AD data are depicted. A = power output, B = cardiac output.

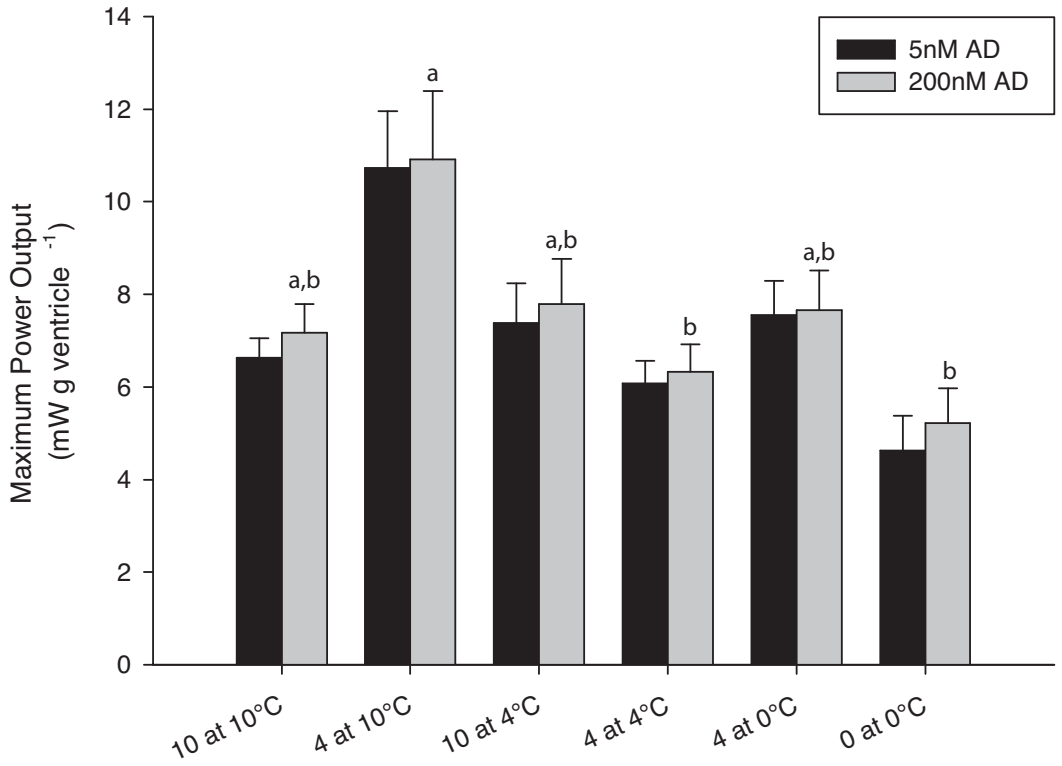

Figure 4: Maximum power output test. P_{IN} = 6 cm H_2O, P_{OUT} = 50 - 60 cm H_2O. The horizontal axis indicates the acclimation temperature and the test temperature. Dissimilar letters indicate a significant difference.

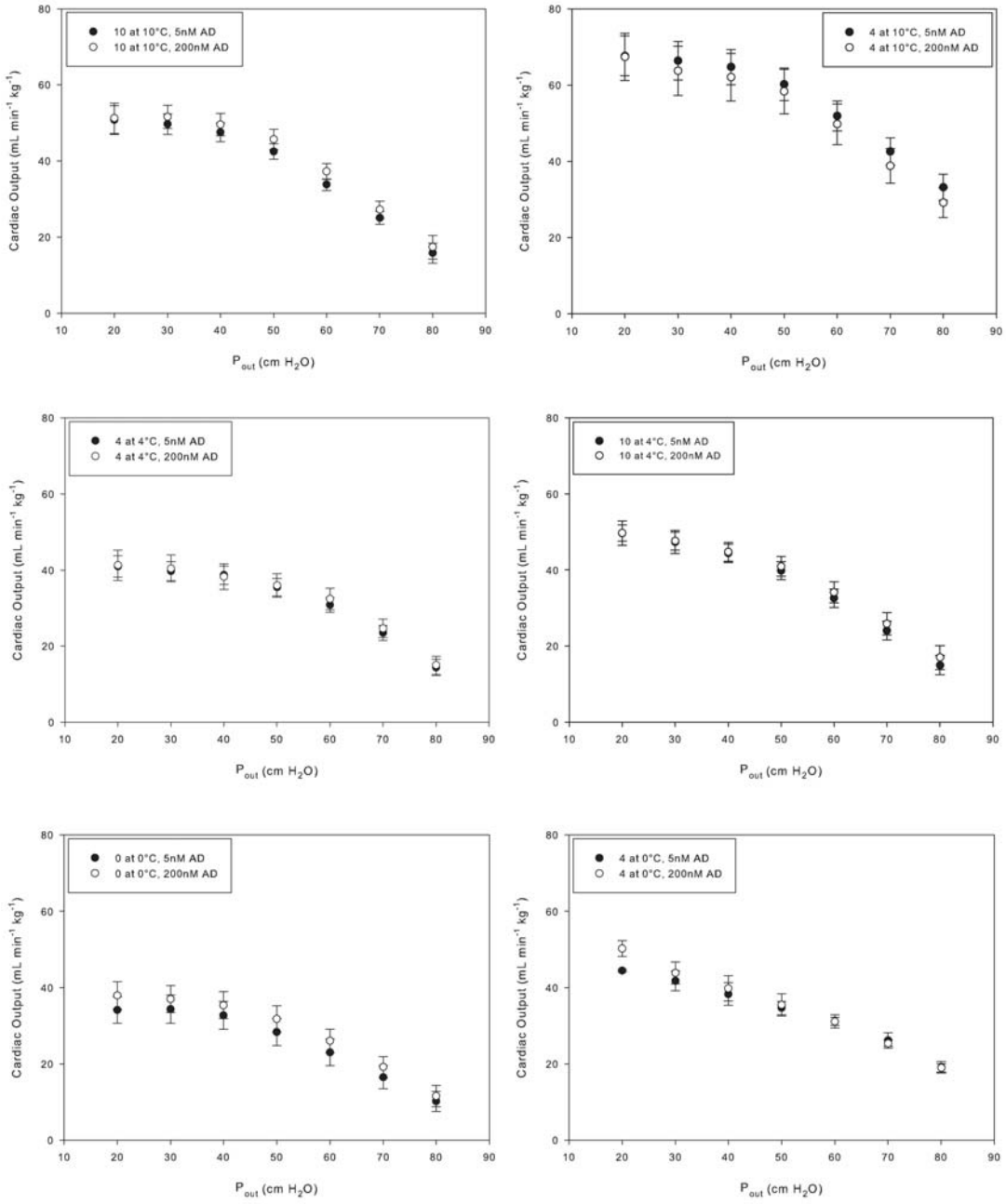

Figure 5. Cardiac output as a function of increasing output pressure at different experimental temperatures.

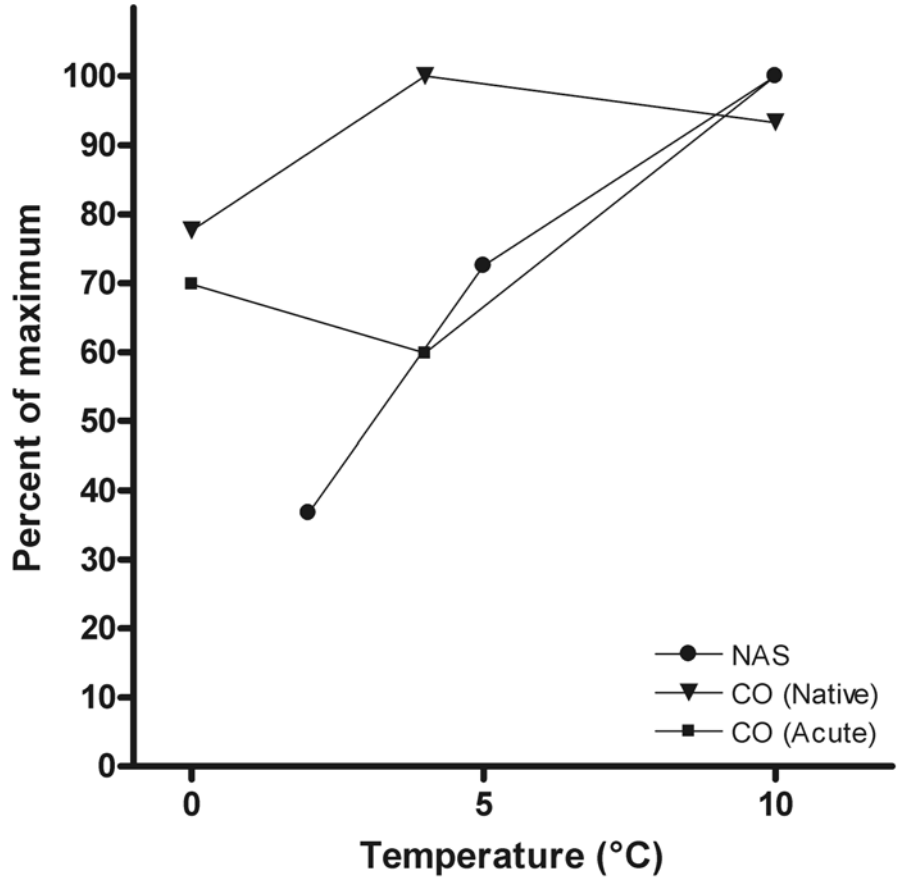

Figure 6. Aerobic and cardiac scope as a function of temperature. CO native indicates cardiac output of cod acclimated to and tested at the same temperature as that to which they were acclimated, i.e. 10°C acclimated cod tested at 10°C, 4°C at 4°C and 0°C at 0°C. CO acute indicates cardiac output of cod acclimated to and tested at different temperatures, i.e. acclimated to 10°C and tested at 4°C, 4°C at 10°C and 4°C at 0°C. NAS indicates net aerobic scope calculated from Claireaux et al. (2000).

Tables:

Table 1: Morphometric data from all experimental animals. Relative ventricular and atrial mass as a percentage of total body weight. All values are mean ± SEM.

Accl Temp	10	10	4	4	4	0
Test Temp	10	4	10	4	0	0
Weight (kg)	0.60 ± 0.03	0.54 ± 0.03	0.054 ± 0.03	0.59 ± 0.02	0.49 ± 0.10	0.56 ± 0.17
Length (cm)	41.6 ± 0.6	40.5 ± 0.8	40.3 ± 0.5	40.8 ± 0.4	40.8 ± 1.7	39.9 ± 1.2
Ventricle (g)	0.49 ± 0.02	0.46 ± 0.03	0.49 ± 0.04	0.47 ± 0.02	0.40 ± 0.04	0.48 ± 0.03
Atrium (g)	0.154 ± 0.011	0.123 ± 0.008	0.133 ± 0.008	0.133 ± 0.008	0.123 ± 0.017	0.117 ± 0.006
RVM (%)	0.083 ± 0.014	0.080 ± 0.012	0.091 ± 0.012	0.083 ± 0.010	0.081 ± 0.009	0.089 ± 0.013
RAM (%)	0.026 ± 0.003	0.023 ± 0.004	0.025 ± 0.003	0.023 ± 0.002	0.025 ± 0.006	0.022 ± 0.004
N	9	10	9	8	4	7

Table 2. Input pressure (P_{IN}) in cm H_2O, heart rate (HR) in bpm, stroke volume (SV) in mL kg^{-1}, cardiac output (CO) in mL min^{-1} kg^{-1}, and power output (PO) in mW g $ventrical^{-1}$ under resting conditions at 5 or 200 nM adrenaline. Mean ± SEM, no significant differences were found.

Acclim Temp	10		4		10		4		4		0	
Test Temp	10		10		4		4		0		0	
AD	5nM	200nM	5nM	200nM	5nM	200nM	5nM	200nM	5nM	200nM	5nM	200nM
P_{IN}	0.04 ± 0.20	-0.08 ± 0.18	0.04 ± 0.28	0.02 ± 0.45	-0.37 ± 0.22	-0.40 ± 0.25	-0.86 ± 0.40	-0.74 ± 0.34	-0.07 ± 0.31	-0.01 ± 0.34	-1.14 ± 0.50	-0.92 ± 0.47
HR	41.4 ± 1.0	42.6 ± 1.5	45.2 ± 1.3	43.6 ± 2.6	34.3 ± 1.5	34.9 ± 4.1	33.2 ± 1.5	32.3 ± 1.2	27.0 ± 2.9	26.6 ± 1.8	24.9 ± 0.5	24.7 ± 1.0
SV	0.39 ± 0.01	0.42 ± 0.02	0.34 ± 0.02	0.41 ± 0.03	0.32 ± 0.01	0.37 ± 0.2	0.31 ± 0.02	0.39 ± 0.02	0.34 ± 0.04	0.34 ± 0.03	0.33 ± 0.01	0.36 ± 0.03
CO	16.3 ± 0.1	18.0 ± 0.7	15.6 ± 2.2	17.5 ± 3.6	10.8 ± 0.6	12.9 ± 0.7	10.2 ± 0.1	12.5 ± 0.9	8.8 ± 0.1	9.1 ± 0.7	8.3 ± 0.1	8.9 ± 0.9
PO	2.78 ± 0.38	3.01 ± 0.21	2.88 ± 0.29	3.35 ± 0.46	2.08 ± 0.15	2.48 ± 0.18	1.83 ± 0.10	2.21 ± 0.10	1.51 ± 0.17	1.57 ± 0.24	1.19 ± 0.06	1.24 ± 0.10

Effects of long-term acclimation and acute thermal challenge on swimming performance in two populations of Atlantic cod (*Gadus morhua*).

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Running Title: Temperature and swimming performance in cod

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Abstract

Swimming performance and oxygen consumption parameters from North East Arctic cod (NEAC) and North Sea cod (NSC) were measured in a Brett-type swim tunnel after long-term acclimation to 4 and 10 °C, and during acute thermal challenge, i.e. temperature reversal. Despite the very long acclimation period (>1 year), no significant differences were seen in the standard (SMR) or active metabolic rates (AMR), however, differences were seen in their acclimation abilities (Q_{10}s), which are argued to be a product of muscle mitochondrial density. Both net and factorial aerobic scopes had no significant effect on critical swimming speeds (U_{crit}s). Nevertheless, a significant temperature effect was seen on SMR, AMR, aerobic scopes and U_{crit}s, with all generally being higher in all cod at 10 °C (except the factorial aerobic scope which showed the opposite trend) regardless of acclimation temperature or population. Evidently both populations experience enough selection pressure within their respective environments to cause them to retain swimming performance over a range of fluctuating temperatures.

Introduction

Temperature is a primary affecter of swimming performance in fish and therefore a crucial parameter in thermally variable environments. It has often been reported that temperate eurythermal fish species swim faster as temperature increases up to their thermal optimum (Brett, 1967; Sisson & Sidell, 1987; Schurmann & Steffensen, 1997). In addition to kinetic stimulation by temperature, muscle contraction becomes more effective as temperature increases (Rome 1995; Rome & Sosnicki, 1991). One compensatory mechanism which fish such as carp (*Cyprinus carpio*) and scup (*Stenotomus chrysops*) employ during cold acclimation is to recruit more red muscle at lower swimming speeds (Rome, 1995). One of the long-term results of this strategy is a compensatory increase in the relative red muscle mass (Johnston & Lucking, 1978; Eggington & Sidell, 1989), suggesting that the maximum swimming speed may be lowered due to a limitation of aerobic metabolism.

In addition, muscle mitochondrial density is often increased in cold adapted temperate eurytherms such as NEAC (e.g. Lucassen et al., 2006), with one potential knock-on effect being an increased standard metabolic rate (Fischer, 2003). Scant evidence suggests that up-regulation of the active metabolic rate does at least partially occur in cold acclimated Atlantic cod. Schurmann & Steffensen (1997) reported that between 5 and 10 °C both the AMR, and as a result the net aerobic scope were both partially compensated.

However, a meta-analysis by Pörtner (2002) of data from Schurmann & Steffensen (1997) and another study by Bushnell et al. (1994) found that although cold adapted Atlantic cod from Greenland had a higher SMR, the AMR was in fact slightly lower when compared to cold acclimated Atlantic cod from the North Sea (NSC). Consequently the aerobic scope was reduced, yet the critical swimming speed was slightly higher in the Greenland cod. Thus, the oxygen consumption increment during swimming was reduced in Greenland cod, that is to say, the cold adapted cod swam more efficiently than cold acclimated cod

It must be noted that the measurements were made in different seasons. The oxygen consumption measurements of the Greenland cod were made in summer under 24hr light conditions. Summer in Greenland is a season of high productivity and the main feeding time for Atlantic cod (Brander et al., 2001; Hansen et al, 2003). The oxygen consumption measurements from the NSC were made in spring and autumn, under 12:12hr light:dark conditions. Spring and autumn generally lie outside the time of maximal feeding for cod (Kiorboe & Nielsen, 1994; Brander et al, 2001; Beaugrand et al., 2003). Although no study has looked directly at the seasonality of metabolic rates in Atlantic cod it seems perfectly reasonably to postulate these uncontrolled variables may have influenced the metabolic rates (see Beamish, 1964 and Lapointe et al., 2006).

To build on the aforementioned data and resolve the outstanding issues, we undertook a series of experiments in which oxygen consumption rates and critical swimming speeds for NEAC and NSC were compared at 4 and 10 °C. We hypothesised that at 4 °C, SMR would be higher in NEAC than in NSC, while AMR would not be completely cold compensated, i.e. similarly reduced for both NEAC and NSC at 4 °C. Thus, aerobic scope would also be reduced, but the critical swimming speed would not be significantly different due to increased efficiency in NEAC.

Furthermore, few studies have looked at longer-term acclimation of fish. Typically, acclimation periods range between four weeks and two months. This study examined the effect of longer-term acclimation, i.e. >1 year, in both Atlantic cod populations. As cod are known to experience a range of temperatures over the short term, for example during diurnal migrations (Woodhead & Woodhead, 1959; Claireaux et al., 1995), we also examined the effects of acute thermal challenges on the same acclimated cod populations.

Methods:

Animals

A total of 24 NSC (*Gadus morhua*) were caught using traps during August 2004 from around Helgoland in the German Bight. Seawater temperature was 18 °C. Fish were transported to the AWI in Bremerhaven aboard the RV Uthörn. Initially, the fish were maintained in a re-circulated 16 °C aquarium for 2 weeks to recover from handling and transportation stress and then moved to a 10 °C aquarium. After two additional weeks, half of the fish were moved to a 4 °C aquarium. First generation cultured NEAC (*Gadus morhua*; a generous gift from Dr. M. Delghandi's at the IMR in Tromsø, Norway) were air freighted over a 24hr period to the AWI in September 2004. Upon arrival the fish were held at 4 °C for a minimum of 2 weeks before half were moved to the 10 °C aquarium. All fish were then kept at their respective acclimation temperatures until the experiments began in October 2005.

All fish were fed to satiation twice a week with a mixture of mussels (*Mytilus edulis*) and/or live common shrimp (*Crangon crangon*). Feeding was stopped a minimum of 5, maximum 7 days before experimentation.

Experiments were carried out within German animal care legislature. Mortality during the entire holding period, i.e. from Aug 2004 – Feb 2006 was approximately 25%. No fish died during the course of the experiments, however, one did die a week after experimentation.

Experimental Setup

The swim tunnel consisted of 3 major parts (figure 1). A Perspex pipe was fed through a 40 cm inner diameter Bruker Biospec 47/40 NMR magnet operating at 4.7 T (see Lurman et al., 2007 for more details). This pipe was attached to a seawater circulation system. When "closed," a 256 L volume of seawater was hermetically sealed to measure oxygen consumption. When "open," a supplemental 444 L volume with a reservoir of constantly

aerated seawater was used to flush the system. Both circulations were temperature controlled to within ± 0.3 °C. Switching between the two circulations was accomplished via three large taps. The gas-tightness of the closed system was checked periodically by bubbling the seawater with nitrogen gas overnight, then monitoring the seawater oxygen content over a 12 hour period. On no occasion did the oxygen content increase. A digital motion camera system connected to a computer was located downstream of the fish and used for observing the fish. All swimming speeds were corrected for solid blocking effects using the procedure outlined by Nelson et al. (1994).

Surgical Procedure

As part of an accompanying study using ^{31}P-NMR to examine metabolic status during swimming (Lurman et al., 2007), fish were initially anesthetised with 0.05 mg/L MS 222 in seawater for 5 minutes. Fish were then transferred to an operating table where the gills were irrigated with 0.02 mg/L MS 222 in seawater. While anesthetised, an inductive ^{31}P-NMR coil measuring 3 x 3 x 0.2 cm was sewn to the side of the body, 15 cm distal to the end of the caudal fin with two sutures on the leading edge. The trailing edge was left free to move. This took less than 15 minutes. Fish were then placed in a tubular shaped cage (15 cm diameter by 60 cm length) within the chamber. Here, the receive coil was positioned to allow optimal signal transduction while at the same time the fish was observed as it recovered from anaesthesia. Recovery usually took approximately 15 min. Before the fish had completely recovered, the cage was pulled by means of a cord into the centre of the magnet and the fish allowed to recover for at least another 2 hours.

Exercise Protocol

All fish were swum twice, once at the acclimation temperature and once at the non-acclimation temperature (figure 2 gives a visual representation of the protocol). To avoid

possible training affects biasing the groups, approximately half of the fish were swum at their acclimation temperature first and the other half at their non-acclimation temperature first. Those fish swum at their non-acclimation temperature first were warmed or cooled after the surgery recovery period in two 3 °C steps, each lasting 4 hours, to the required temperature, i.e. 4 or 10 °C. Fish were then left overnight with minimal flow i.e. 3.3 m^3 hr^{-1}. This typically equalled 0.15-0.19 BL (body lengths) s^{-1}. The routine metabolic rate was determined in fish swimming with minimal flow. The flow was then increased in 1 m^3 hr^{-1} (approx. 0.05 BL s^{-1}) steps with each step lasting 30 minutes. At sufficiently high water flows, fish would rest on the rear grid of the cage. U_{crit} was defined as the time when the fish was no longer able to move from the grid (as per Nelson et al., 1994), and calculated according to the formula given by Brett (1964).

$$U_{crit} = u_i + \left(\frac{t_i}{t_{ii} \times u_{ii}} \right)$$

Where u_i is the highest velocity in BL s^{-1}, u_{ii} is the velocity increment, t_i is the time in minutes that the fish swam at the fatiguing velocity, and t_{ii} is the prescribed swimming period, i.e. 30 minutes. Following U_{crit}, the water flow was reduced to the minimum of 3.3 m^3 hr^{-1} to allow recovery.

After four hours of post exercise recovery, fish were again either warmed or cooled in the same stepwise manner to the appropriate temperature and left at minimal flow overnight until they were swum again according to the same protocol outlined above on the third day. Fish were then allowed a further four hours to recover, and if necessary, warmed or cooled back to their acclimation temperature in the same stepwise manner outlined above, before they were taken out, the inductive coil removed and placed back in the aquarium. Water ammonium and nitrite contents were checked every 12 hours and water was changed when necessary.

Tail-beat Frequency

Tail beat frequency was measured manually by counting the number of tail beats in a 30 second period using the digital camera system. This was repeated 8 times at each of the 30 minute swimming stages. The mean of these 8 was then taken as the tail-beat frequency. Eight 30 second sampling periods were not always possible at U_{crit}, so the mean was taken of as many sampling periods as were permitted, minimum 3.

Oxygen Measurement

Oxygen was measured constantly at a sampling rate of 0.5 Hz using Fibox optodes (Presens, Germany) with the temperature compensation entered manually. Optodes were zeroed chemically with sodium dithionite in seawater, and 100% air saturation was calibrated by placing the optode in the open swim-tunnel circulation. This was checked periodically against a MultiLine P4 CellOx 325 oxymeter (WTW, Germany).

Oxygen consumption was calculated from the slope of the drop in water oxygen content which was monitored over 20 minute measurement periods at each speed. After the initial 20 minute measurement period the circulation was opened for a 10 minute flush/re-oxygenation. At the end of each experiment when the fish had been removed, a "blank" respiration run was performed to quantify any background microbial respiration. This was then subtracted from the fish's oxygen consumption rate. Oxygen consumption rates were corrected for possible minor allometric size effects using the mass exponent of 0.8 (Saunders, 1963):

$$\dot{M}o_2 = \left(\frac{1}{M}\right)^{0.8} \times \dot{M}o_2 m$$

where $\dot{M}o_2$ is the standardised oxygen consumption rate in mg O_2 kg^{-1} hr^{-1}, M is the mass of the fish in kg, and $\dot{M}o_2 m$ is the measured oxygen consumption. Standardized respiration data were not transformed before analysis. AMR was taken to be the maximal oxygen

consumption rate measured during swimming. Non-linear regression was used to fit a curve to the data on a plot of respiration as a function of swimming speed for each individual at each test temperature. The curve was described by the equation below:

$$\dot{M}o_2 = SMR \times \exp^{(k \times U)}$$

Where k is a constant and U is the speed (in BL s^{-1}) at a given temperature. The best fitting curve allowed an estimation of the SMR for each fish at each temperature. Statistically significant differences in oxygen consumption were unaffected by allometric adjustment.

Statistical Analysis and Modelling

All data analyses and modelling were performed using SigmaStat 3.5 and Prism 4.0 software. The length was subjected to non-parametric Kruskal-Wallis tests for significance and *post hoc* Dunn's tests to distinguish between groups. Differences between mass and condition factor were tested using ANOVAs, while differences between U_{crit}, SMR, AMR, net and factorial aerobic scopes were tested using repeated measures two-way ANOVAs with Holm-Sidak post-tests to test for significance between groups and treatments. Data are presented throughout as means ± the 95% confidence interval, unless otherwise indicated. Differences were considered significant when $p \leq 0.05$. An asterisk indicates a significant difference within the group.

Results

The NEAC from both acclimation temperatures tended to be larger (table 1). A significant difference in body length was seen between both 10 °C acclimated populations. Both NSC populations had significantly higher condition factors within their acclimation groups than the NEAC. The 4 °C acclimated NSC also had higher condition factors than both 10°C acclimated groups (table 1).

Similar to Soofiani & Priede (1985), high variability was seen in oxygen consumption measurements in all four groups at minimal flow (see figure 3). This was because a high proportion of the fish only swam intermittently and/or struggled occasionally. Obvious oxygen consumption rate outliers at low speeds where struggling was jointly observed were excluded. Other fish, particularly from the NEAC population, simply refused to swim at any speed and "wedged" themselves into the chamber, or they struggled constantly. The data from these animals were omitted from the analyses completely. Higher variability was seen in the oxygen consumption rate measurements for both 10 °C acclimated populations when compared to the measurements for the 4 °C cod.

The SMRs was significantly higher at 10 °C, regardless of acclimation temperature or population (figure 4A). No significant differences were seen in the SMRs between either of the populations, at a given experimental temperature (figure 4A). However, thermal compensation ability appeared to differ between populations. Long-term acclimation to 10 °C resulted in higher Q_{10}s in NEAC when compared to the NSC (figure 4B and 4C). The Q_{10}s for acute warming and cooling from 10 to 4 °C were similar for both populations.

Similar to SMRs, AMRs were also higher when measured at 10 °C than at 4 °C, regardless of acclimation temperature or population (figure 5A). The long-term acclimation comparison (i.e. within population, between acclimation groups) revealed remarkably similar Q_{10}s of 2.0 and 1.9 for the NEAC and NSC respectively. Q_{10}s for acute temperature changes

were somewhat lower, i.e. between 1.5 and 2.0 indicating partial compensation. (figure 5B and 5C).

The net and factorial aerobic scopes for all groups at their experimental temperatures are presented in table 2. The net aerobic scope was generally higher for all cod at 10 °C, especially the 10 °C acclimated cod at 10 °C, although this was not significant. The exception was the net aerobic scope for the 4 °C acclimated NSC, which was approximately equal at both 4 and 10 °C. No significant differences in net aerobic scope were seen as a result of population. The factorial aerobic scope showed the opposite trend and was significantly reduced at 10 °C, particularly in the 4 °C acclimated cod. Yet the factorial aerobic scope was significantly higher in both 10 °C acclimated groups at 4 and 10 °C.

Critical swimming speeds were also consistently higher at 10 °C, regardless of acclimation temperature (figure 6). The highest mean U_{crit} was 0.92 BL s^{-1} for the 10 °C acclimated NSC at 10 °C, and the lowest U_{crit} was 0.69 BL s^{-1} for the 4 °C acclimated NSC. No differences were found between populations at their experimental temperatures.

Discussion

The standard metabolic rates extrapolated from the swimming data in the current study agree well with previous empirical measurements of SMR given in the literature (e.g. Nelson et al., 1994; Claireaux et al., 2000; Reidy et al., 2000; Lapointe et al., 2006). Furthermore, the SMRs are also below some of those measured in juvenile Atlantic cod (Soofiani & Hawkins, 1982; Bushnell et al., 1994; Steffensen et al., 1994) as would be predicted by the allometric relationship for oxygen consumption (Saunders, 1963). This implies that the attached ^{31}P-NMR inductive coil had minimal effect on the fish under resting conditions. Similarly, the large exponent observed for the oxygen consumption rate as a function of swimming speed indicates that all fish used in the current study were not stressed, and had sufficient aerobic scope remaining for swimming.

Thermal Effects on Physiology and Swimming Performance

In contrast to our original hypothesis, SMR was not significantly different between NEAC and NSC neither when measured at 4 °C nor when measured at 10 °C, apparently dispelling any claim of metabolic cold adaptation. However, subsequent calculation and analysis of Q_{10}s (figures 4B and 4C) for both populations reveals subtle differences in their long-term compensatory abilities, with the NEAC showing a complete lack of compensation (Q_{10} = 2.5), while the NSC were able to at least partially compensate (Q_{10} = 1.3). This seems surprising given the extended acclimation period (>1 year). The reason underlying this reduced compensatory ability may be attributed to the reduced ability to regulate mitochondrial density or capacity in NEAC. A well-documented and common response of fish during cold acclimation and adaptation is to increase cellular mitochondrial density and/or the cristae density therein, to compensate for the thermodynamic effects of cooling (Guderley & Blier, 1988; Eggington & Sidell, 1989; St Pierre et al., 1998; Bouchard &

Guderley, 2003). This has previously been documented in 4 °C acclimated NEAC compared to NSC (Lannig et al., 2003; Lucassen et al., 2006).

The AMR displayed the same trends as the SMR, although not as pronounced. Again no significant differences were seen between NEAC and NSC at their respective temperatures. Schurmann & Steffensen (1997) reported only minimal compensation (Q_{10} = 1.8) of AMR of NSC between 5 and 10 °C, which is similar to what we observed in both NEAC (Q_{10} = 2.0) and NSC (Q_{10} = 1.9). It is also interesting to note the minimal thermal compensation of AMR in both populations during acute thermal challenge. Thus, it appears that below the thermal optimum (Colosimo et al., 2003; Fischer, 2003) cold compensation extends only minimally, if at all to the level of AMR.

Aerobic scope was also not compensated and did not significantly differ between any of the populations at their respective temperatures, although a temperature effect was seen, with net aerobic scope being elevated at 10 °C, particularly in the 10 °C acclimated cod. The increased net aerobic scope meant that the fish had more 'energy' available to them and as a result had a higher U_{crit}. The factorial aerobic scope showed the opposite trend and decreased significantly with increasing temperature particularly for the 4 °C acclimated cod. This reflected the higher basal costs, i.e. higher SMRs seen in 4 °C acclimated fish at both temperatures. In juvenile cod, Schurmann & Steffensen (1997) demonstrated an increase in net aerobic scope from 5 to 10 °C where it was maximal, but it then decreased again at 15 °C.

A compensation mechanism appears to be at work on U_{crit} (Q_{10}s of 1.3 and 1.6 for NEAC and NSC respectively), although there was still a definite degree of thermal dependence evident in our study with U_{crit} consistently higher at 10 °C, particularly for the 10 °C acclimated fish. This may be due to two reasons, 1) thermodynamic effects on mitochondrial enzyme capacity (Tyler & Sidell, 1984; Guderley & Blier, 1988; Eggington & Sidell, 1989; Guderley & St. Pierre, 2002) resulting in increased net aerobic scope and an improved ability to fuel swimming at warmer temperatures, and/or 2) the muscular

contraction rate increases with increasing temperature (Rome, 1995; Johnston & Ball, 1996) as the myosin ATPases driving contraction (Rome, 1995) are also affected thermodynamically, and as a result the maximal tail-beat frequency was higher at 10 °C (table 3).

As a general trend, the oxygen consumption increment as a function of swimming speed was consistently lower at 10 °C indicating that the fish swam more efficiently at this temperature, regardless of acclimation temperature, (figure 3). At present we can only speculate how this may be achieved, for example, by improved transmission of power along the body to the caudal fin (Johnston & Temple, 2002), alterations in myosin ATPase activity or cross-bridge cycling (Rome, 1995; Wakeling et al., 2000), and/or reduced muscle relaxation time (Rome & Swank, 2001), which resulted in a increased maximal tail-beat frequency at 10 °C (table 3), while the reduced swimming efficiency resulted in a reduced U_{crit} (figure 6). Differences in swimming efficiency were clearly not a product of the shape of the fish, as indicated by the similar morphometric measurements of all fish (table 1).

Why do both acute thermal challenge and long-term acclimation elicit similar swimming performances in both NEAC and NSC? The answer to this may lie in the different ecologies of these two populations. The NSC are a eurythermal species which experiences large seasonal fluctuations, 3 - 20 °C (German Federal Office for Shipping and Hydrography, http://www.bsh.de/aktdat/bm/DBWasserMess.htm). Similarly, as the NEAC grow older and migrate to spawning grounds near Lofoton, Norway, the seasonal variation experienced also increases. In autumn it may get up to 9 °C (Ottersen et al., 1998; Godø & Michalsen, 2000). Although little is known about the temperature range these two populations experience on a diel basis, there is evidently sufficient selection pressure in their respective environments to ensure both populations retain swimming performance within the temperature range examined in the current study.

Conclusions

Long-term cold acclimation of NEAC and NSC for more than 1 year did not produce significant differences in the SMR or AMR. However, differences were seen in their acclimation abilities (Q_{10}s), which were interpreted to be a product of the capacity to modulate mitochondrial densities and capacities. Both the net and factorial aerobic scopes had no significant effect on the U_{crit}s. A significant temperature affect was seen on the SMR, AMR, aerobic scopes and U_{crit}s. All were generally higher in all cod at 10 °C (except the factorial aerobic scope which showed the opposite trend) regardless of acclimation temperature or population.

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Figure Captions

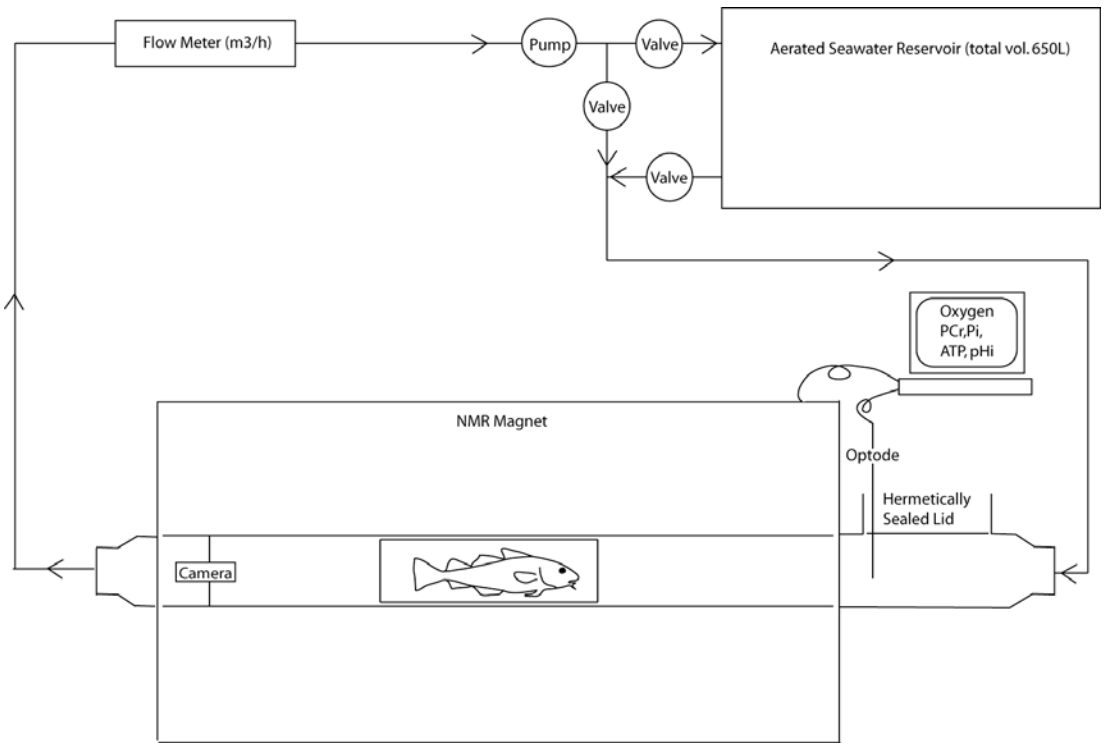

Figure 1. The NMR/swim tunnel apparatus. Arrows indicate direction of water flow.

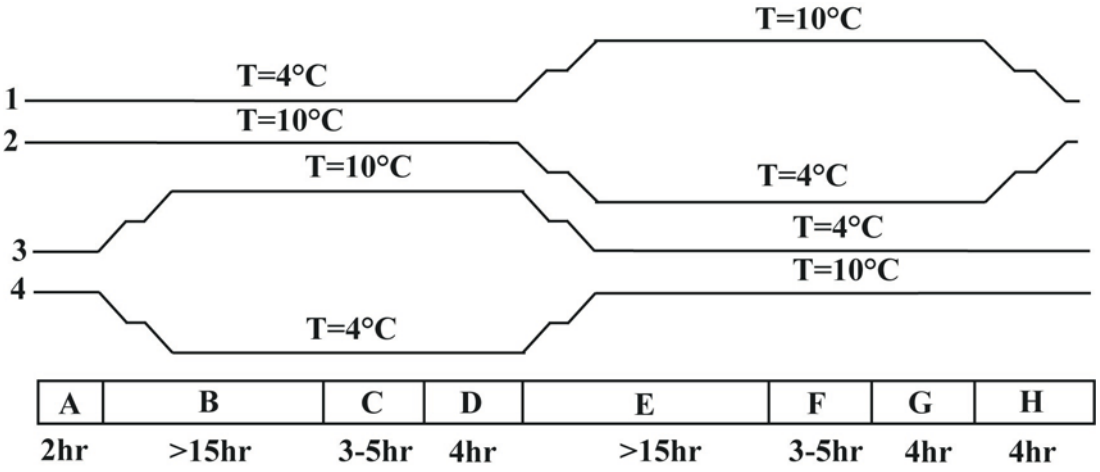

Figure 2. The four different experimental protocols showing temperature change as a function of time. Not to scale.

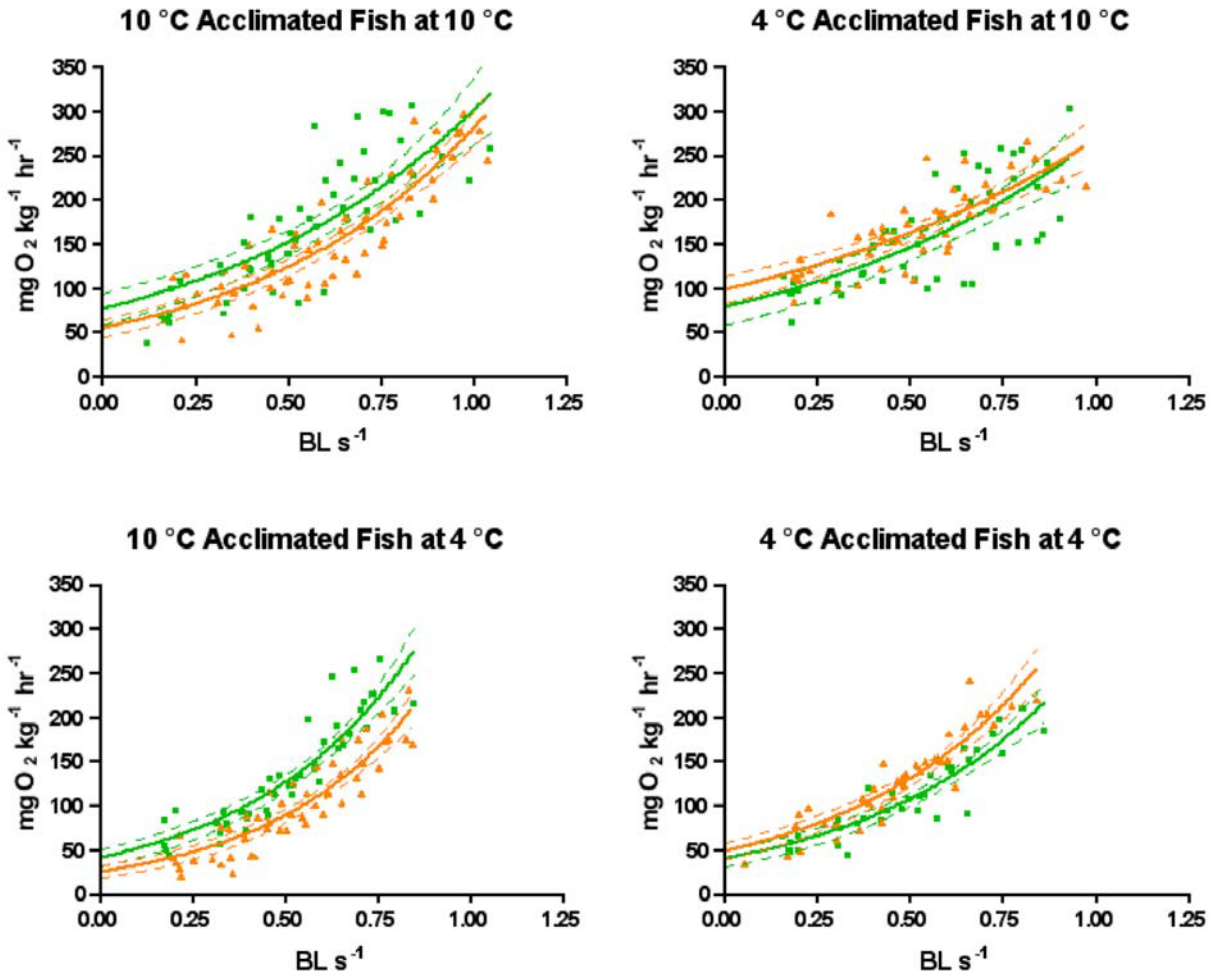

Figure 3. Oxygen consumption rates as a function of swimming speed for 10 °C acclimated cod at 10 °C (A) and 4 °C (C) and 4 °C acclimated cod at 10 °C (B) and 4 °C (D). Brown triangles represent NSC and green squares represent NEAC.

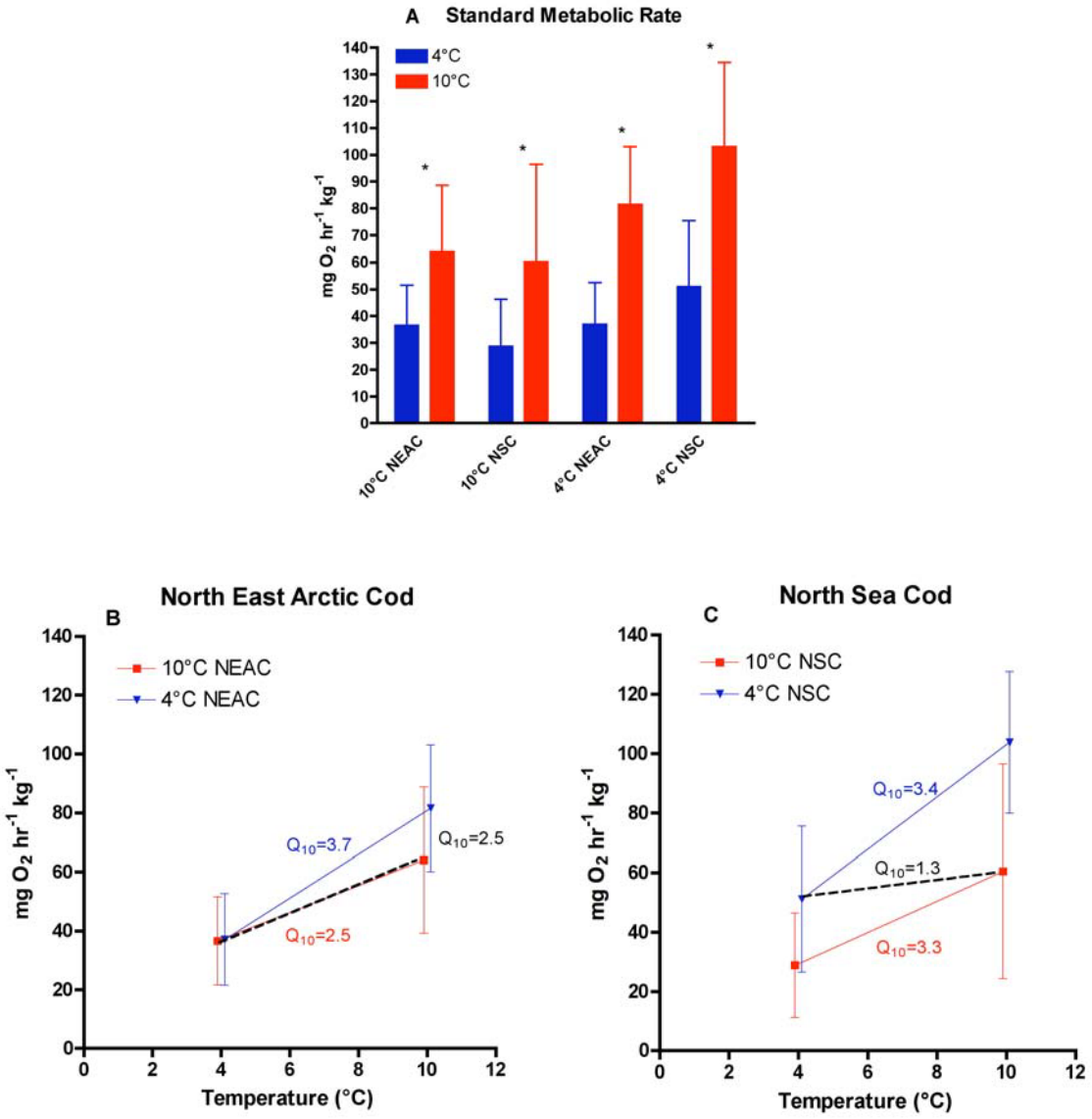

Figure 4. The standard metabolic rates of North East Arctic cod and North Sea cod. Figures 4A show SMR for NEAC and NSC at their respective test temperatures. An asterisk indicates within group significant difference. On figures 4B and 4C squares = 10 °C acclimated cod and triangles = 4 °C acclimated cod. Red and blue Q_{10}s were calculated from SMRs of fish within in their acclimation group, i.e. at their acclimation temperatures and acute temperatures. Black Q_{10}s are calculated from SMRs of fish at their acclimation temperatures only. The triangles were moved 0.1 °C to the left and the squares 0.1 °C to the right to aid viewing. Error bars represent 95% confidence interval.

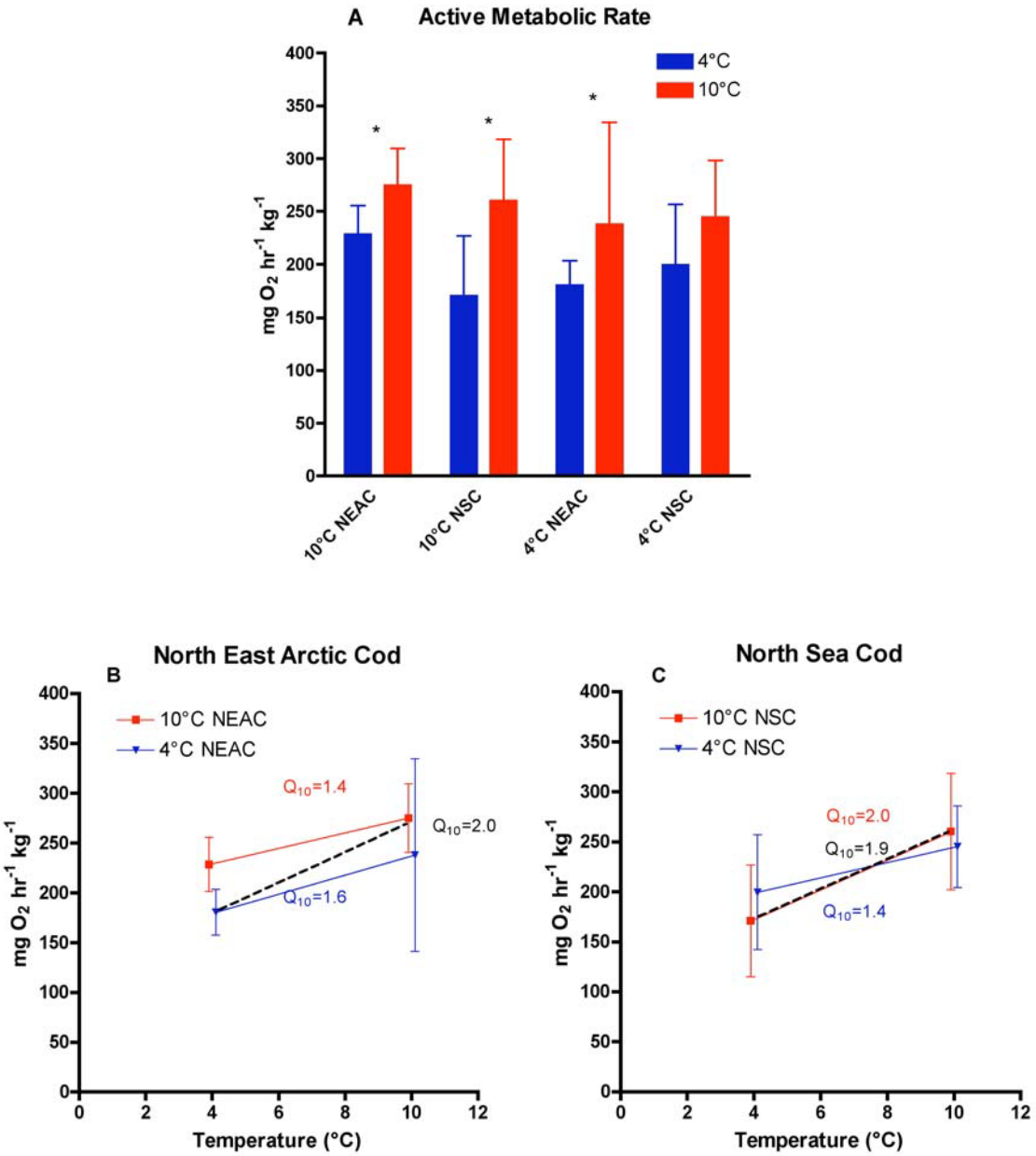

Figure 5. The active metabolic rates of North East Arctic and North Sea cod. Figure 5A shows AMRs for NEAC and NSC at their respective test temperatures. An asterisk indicates within group significant difference. On figures 5B and 5C squares = 10 °C acclimated cod and triangles = 4 °C acclimated cod. Red and blue Q_{10}s were calculated from AMRs of fish within in their acclimation group, i.e. at their acclimation temperatures and acute temperatures. Black Q_{10}s are calculated from AMRs of fish at their acclimation temperatures only. The triangles were moved 0.1 °C to the left and the squares 0.1 °C to the right to aid viewing. Error bars represent 95% confidence interval.

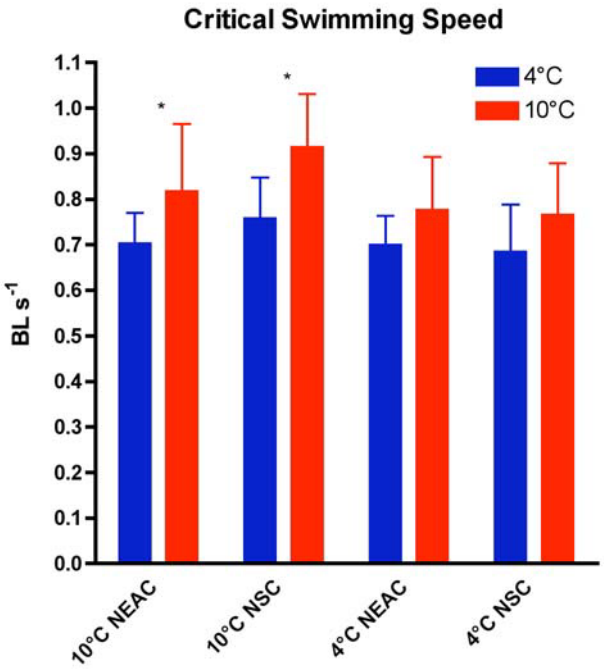

Figure 6. Critical swimming speeds of 10 and 4°C acclimated North East Arctic (NEAC) and North Sea (NSC) cod. Error bars represent 95% confidence interval. An asterisk indicates a significant within group difference.

Tables:

Table 1. Morphometric data for the 10 and 4°C acclimated North East Arctic (NEAC) and North Sea (NSC) cod. Dissimilar letters indicate significant difference.

Population	NEAC	NSC	NEAC	NSC
Acclimation Temperature	10 °C	10 °C	4 °C	4 °C
Weight (kg)	0.959 ± 0.18	0.712 ± 0.23	1.10 ± 0.54	0.899 ± 0.21
Length (cm)	49.3 ± 1.79 [a]	43.5 ± 2.53 [b]	47.2 ± 8.05 [a,b]	43.6 ± 3.97 [a,b]
Height (cm)	8.81 ± 0.79	9.15 ± 1.30	7.61 ± 1.21	9.09 ± 1.06
Width (cm)	6.16 ± 0.62	6.65 ± 1.23	5.35 ± 0.68	5.92 ± 0.54
Condition Factor	0.799 ± 0.12 [a]	0.843 ± 0.12[a,b]	0.989 ± 0.11[b,c]	1.08 ± 0.10 [c]
N	5	5	5	5

Table 2. Net aerobic scope (NAS) and factorial aerobic scope (FAS) for each group at each test temperature.

Temp	10 °C NEAC	10 °C NSC	4 °C NEAC	4 °C NSC
NAS at 4°C ($mgO_2\ hr^{-1}\ kg^{-1}$)	192 ± 24.3	142 ± 37.3	143 ± 23.4	149 ± 45.1
NAS at 10°C ($mgO_2\ hr^{-1}\ kg^{-1}$)	211 ± 45.6	200 ±50.9	156 ± 84.2	142 ± 50.2
FAS at 4°C	6.69 ± 1.70	6.77 ± 2.59	5.6 ± 2.75	4.79 ± 3.31
FAS at 10°C	4.60 ± 1.69	5.44 ± 3.35	2.90 ± 0.99	2.50 ± 0.82

Table 3. Maximal tail-beat frequency in beats per minute (BPM_{max}). An asterisk indicates significantly different from 4 °C swim temperature.

Temp	10 °C NEAC	10 °C NSC	4 °C NEAC	4 °C NSC
BPM_{max} at 4°C	76.4 ± 14.9	91.3 ± 13.4	101.6 ± 7.8	92.3 ± 11.6
BPM_{max} at 10°C	98.5 ± 14.1*	114 ± 14.9*	109.6 ± 6.1	109.5 ± 10.8

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An examination of the metabolic processes underpinning critical swimming in Atlantic cod (*Gadus morhua* L.) using *in vivo* ^{31}P-NMR spectroscopy

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Summary

Traditionally, critical swimming speed has been defined as the speed when a fish can no longer propel itself forward, and is exhausted. To gain a better understanding of the metabolic processes at work during a U_{crit} swim test, and that lead to fatigue, we developed a method using *in vivo* ^{31}P-NMR spectroscopy in combination with a Brett-type swim tunnel. Our data showed that a metabolic transition point is reached when the fish change from using steady state aerobic metabolism to non-steady state anaerobic metabolism, as indicated by a significant increase in inorganic phosphate levels from 0.3±0.3 to 9.5±3.4 μmol g^{-1}, and a drop in intracellular pH from 7.48±0.03 to 6.81±0.05 in muscle. This coincides with the point when the fish change gait from subcarangiform swimming to kick-and-glide bursts. As the number of kicks increased, so too did the Pi concentration, and the pH_i dropped. Both changes were maximal at U_{crit}. A significant drop in Gibbs free energy change of ATP hydrolysis from –55.6±1.4 to –49.8±0.7 kJ mol^{-1} is argued to have been involved in fatigue. This confirms earlier findings that the traditional definition of U_{crit}, unlike other critical points that are typically marked by a transition from aerobic to anaerobic metabolism, is the point of complete exhaustion of both aerobic and anaerobic resources.

Key words: Atlantic cod, *Gadus morhua*, critical swimming speed, *in vivo* ^{31}P-NMR spectroscopy, high-energy phosphates, Gibb's free energy, intracellular pH.

Introduction

The critical swimming speed (U_{crit}) test was originally introduced in a study looking at the fitness of salmon (*Oncorhynchus* sp.) in relation to temperature (Brett, 1964). Brett defined the U_{crit} as the swimming velocity when the fish could no longer propel itself forward off the downstream grid due to exhaustion. The U_{crit} test provides an easy way of directly assessing fitness in fish, and by proxy, the sum of both the aerobic and anaerobic metabolic scopes. More specific tests, such as the burst swimming test and the endurance swimming test, have been developed to separately examine anaerobic and aerobic scope, respectively. Since its inception, however, many experiments have used the critical swimming speed test to assess the fitness of various fishes under different conditions, e.g. after feeding, during hypoxia, in polluted waters (for a complete review, see Hammer, 1995).

A great body of literature exists on the different swimming modes in fish (Schultz and Webb, 2002; Webb, 2002; Jayne and Lauder, 1994; Videler, 1993; Videler, 1981; Beamish, 1978; Brett, 1964) and the metabolic processes fuelling muscle contraction (Jones, 1982). It has been clearly demonstrated that red muscle is oxidative (Johnston, 1977) and produces the slow contractions during subcarangiform swimming (Jayne and Lauder, 1994), while white muscle is glycolytic and responsible for fast twitch contractions (Johnston, 1977), which produce tail kicks during burst swimming (Jayne and Lauder, 1994). A handful of studies have also found white muscle to be recruited during subcarangiform swimming (Rome et al., 1984; Jones, 1982; Greer-Walker and Pull, 1973). Yet to date, few studies have looked at the metabolic processes underpinning the continuum, i.e. from subcarangiform swimming through the gait transition to kick-and-glide bursts to exhaustion.

Although an increase in inorganic phosphate and an acidification of the intracellular milieu is known to be involved in muscular fatigue, the exact processes leading to muscular fatigue are ill-defined. Allen and Westerblad (Allen and Westerblad, 2001) contend that it stems from excess inorganic phosphate altering intracellular concentration of Ca^{2+} and/or the Ca^{2+} sensitivity of the myofilaments. Building on previous work (Hibberd et al., 1985), Debold et al. add that inorganic phosphate and H^+ also reduce the force generated by cross-bridge cycling (Debold et al., 2004). Others have argued that a drop in the Gibbs free energy of ATP hydrolysis ($dG/d\xi_{ATP}$) below a certain threshold results in fatigue (Hardewig et al., 1998).

Nevertheless, the anaerobic products of kick-and-glide bursts leave a 'fingerprint' on the fish's metabolic state, and a small number of studies have examined the relationship between swimming gait and metabolic processes. For example, it was concluded that elevated post-exercise oxygen consumption (EPOC) measured in salmon was a product of the anaerobiosis that fuels swimming shortly before U_{crit} (Lee et al., 2003). Furthermore, a clear relationship was demonstrated between

anaerobic markers (plasma and tissue lactate levels, tissue glycogen and EPOC), and kick-and-glide swimming in smallmouth bass (*Micropterus dolomieu* Lacepedé) (Peake and Farrell, 2004). Thus, Brett's traditional definition of U_{crit} is the speed that causes complete fatigue, i.e. when energy demand has exceeded both the aerobic and anaerobic supply (Brett, 1964).

Traditionally, metabolic critical points such as critical temperatures and critical oxygen tensions, are points marked by a transition from steady state aerobic metabolism to non-steady state anaerobic metabolism (Pörtner, 2002; Pörtner et al., 1985; Claireaux and Dutil, 1992). The traditional definition of U_{crit} does not correspond with these 'critical' parameters. This was initially noted (Brett, 1964) and further discussed (Beamish, 1978) in relation to swimming duration. Subsequent research has expanded upon this; for example, the metabolic cost of swimming in common brief squid (*Loliguncula brevis* Blainville) was investigated using the equivalent of a U_{crit} test, and it was found that anaerobiosis had commenced long before the squid could no longer swim (Finke et al., 1996). Pilot studies in our laboratory have found similar results using Atlantic cod (Pörtner et al., 2002). While Brett's U_{crit} test gives information about the swimming performance, little is known about the underlying metabolic processes, and in particular, what causes fatigue during exhaustive exercise.

The use of *in vivo* ^{31}P-NMR spectroscopy has proved to be an exceptional method for non-invasively monitoring the metabolic status of high-energy phosphates like PCr (phosphocreatine) and ATP, as well as the formation of metabolic products such as intracellular phosphate (Pi), and intracellular changes in pH in muscle (Gadian, 1982). Furthermore, it has been extensively used for mammalian muscle (Cozzone and Bendaham, 1994). A number of pioneering studies used ^{31}P-MRS in freshwater fishes (for reviews, see van den Thillart and van Waarde, 1996; van der Linden et al., 2004), and these methods have been further developed for marine fishes (Bock et al., 2001; Bock et al., 2002b; Sartoris et al., 2003). Early studies were carried out exclusively on restrained or resting animals. However, a technique has recently been developed for non-invasive studies of unrestrained fish under resting conditions (for reviews, see Bock et al., 2002b; Pörtner et al., 2004). Furthermore, recent preliminary trials within our laboratory have successfully investigated the use of *in vivo* ^{31}P-NMR in Atlantic cod during swimming (Bock et al., 2002a; Pörtner et al., 2002).

In this study we report on an online analysis of metabolic processes by *in vivo* ^{31}P-NMR spectroscopy during U_{crit} tests. The resulting data were used to examine three questions: (1) at what point in the U_{crit} test do cod go from using steady state aerobic metabolism to time-limited, non-steady state anaerobic metabolism to fuel swimming and how does this tie in with the different swimming gaits; (2) how does the traditional definition of U_{crit} compare with these underlying metabolic processes; (3) what are the metabolic processes that potentially cause fatigue in swimming Atlantic cod?

Materials and methods

Animals

First generation cultured North Eastern Arctic cod *Gadus morhua* L. (a generous gift from Dr M. Delghandi at the Institute of Marine Research, Tromsø, Norway) were air freighted over a 24 h period to the Alfred Wegener Institut (AWI) in September 2004. Upon arrival, fish were held at 4°C for a minimum of 2 weeks before being moved to the 10°C aquarium.

All fish were fed to satiation twice a week with a mixture of mussels (*Mytilus edulis* L.) and live common shrimp (*Crangon crangon* L.). Feeding was stopped a minimum of 5 days, maximum 7, before experimentation.

All experiments were carried out within German animal care legislature. Mortality during the entire holding period, i.e. from August 2004 to February 2006, was approximately 25%. No fish died during the course of the experiments; however, one died a week after experimentation.

Experimental setup

The swim tunnel consisted of three major parts (Fig. 1). A Perspex^{TM} pipe was fed through a 40 cm inner diameter Bruker Biospec 47/40 (Ettlingen, Germany) operating at 4.7 T. This pipe was attached to a circulation system. When 'closed,' a 256 l volume of seawater was hermetically sealed to measure oxygen consumption. When 'open,' a supplemental 444 l volume with a reservoir of constantly aerated seawater was used to flush the system. Switching between the two circulations was accomplished *via* three large taps. The gas-tightness of the closed system was checked periodically by bubbling the seawater with nitrogen gas overnight, then monitoring the seawater oxygen content over a 12 h period. On no occasion during this test did the oxygen content increase. A digital motion camera system connected to a computer was used for observing the fish. Both circulations were temperature controlled to within ±0.3°C. All swimming speeds were corrected for solid blocking effects using the procedure outlined by Nelson et al. (Nelson et al., 1994).

Surgical procedure

Fish were initially anesthetised with 0.05 mg l^{-1} MS222 in seawater for approximately 5 min, then weighed and the standard length (*SL*) was measured. Fish were then transferred to an operating table where the gills were irrigated with 0.02 mg l^{-1} MS222 in seawater. While anesthetised, a seawater-tight inductive ^{31}P-NMR coil (Bock et al., 2002a), measuring 3 cm×3 cm×0.2 cm, was sewn to the left side of the body, 15 cm distal to the end of the caudal fin with two sutures on the leading edge. The trailing edge was left free to move. This took less than 15 min. Fish were then placed in a tubular shaped cage (15 cm diameter × 60 cm length) within the chamber. Here, the receive coil was positioned to allow optimal signal transduction, while at the same time the fish was observed as it recovered from anaesthesia. Complete recovery usually took approximately 15 min. The cage was then pulled by means of a cord into the centre of the magnet and the fish allowed to recover for at least another 4 h.

Exercise protocol

As part of a larger project looking at the effects of thermal acclimation and acute temperature change on swimming performance (G.J.L., C.H.B. and H.-O.P., manuscript in preparation), all fish were swum twice, once at the acclimation temperature 10°C and once at the non-acclimation temperature of 4°C; however, data for this study

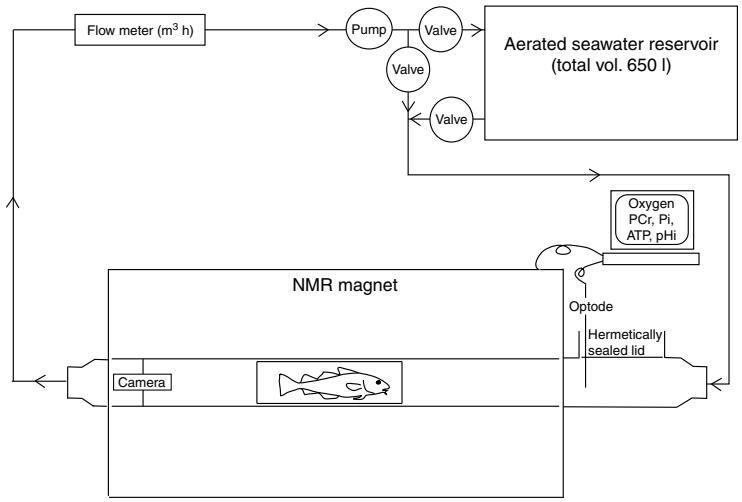

Fig. 1. The NMR/swim tunnel apparatus. Arrows indicate direction of water flow.

were only used from the 4°C swim. Fish were allowed to recover from surgery for a minimum of 4 h before they were cooled in a stepwise manner (2× 3°C steps per 2 h) to 4°C and left at minimal flow, i.e. 1 m^3 h^{-1} (typically 0.15–0.19 *BL* s^{-1}) overnight. The routine metabolic rate was determined in fish swimming with minimal flow. The flow was then increased in approximately 1 m^3 h^{-1} (approx. 0.05 *BL* s^{-1}) stepwise manner with each step lasting 30 min so that sufficient ^{31}P-NMR spectra could be recorded (see below). At sufficiently high water flows, fish would rest on the rear grid of the cage. When two of these successive pauses lasted more than 20 s, a short 9 V electric current was manually applied to a grid downstream. The traditional U_{crit} was defined as the time when the fish was no longer able to move from the grid (as per Nelson et al., 1994), and calculated according to the formula (Brett, 1964):

$$U_{crit} = u_i + \left(\frac{t_i}{t_{ii} \times u_{ii}}\right), \quad (1)$$

where u_i is the highest velocity in *BL* s^{-1}, u_{ii} is the velocity increment, t_i is the time in min that the fish swam at the fatiguing velocity, and t_{ii} is the prescribed swimming period, i.e. 30 min. Following that, the water flow was reduced to a minimum of 3.3 m^3 h^{-1} and the fish were allowed 4 h to recover. They were then warmed to their acclimation temperature in the same stepwise manner outlined above and left overnight before being swum again in the same manner, before they were taken out, the inductive coil removed and placed back in the aquarium. Water ammonium and nitrite content within the swim tunnel were checked every 12 h and water changed when necessary.

Tail-beat frequency measurement

Tail-beat frequency was measured manually by counting the number of tail beats in a 30 s period using the digital camera system. This was repeated eight times at each of the 30 min swimming stages. The mean of these eight was then taken as the tail-beat frequency. Eight 30 s sampling periods were not always possible at U_{crit}, so the mean was taken of as many sampling periods as were permitted, minimum three. The time of the first kick was also recorded.

Oxygen measurement

Oxygen was measured constantly at a sampling rate of 0.5 Hz using Fibox optodes (Presens, Regensburg, Germany) with the temperature compensation entered manually. Optodes were zeroed chemically with sodium dithionite in seawater, and 100% was calibrated by placing the optode in the open swim-tunnel circulation. This was checked periodically against a MultiLine P4 CellOx 325 oxymeter (WTW, Weilheim, Germany) calibrated to fully air saturated seawater.

Oxygen consumption was calculated from the slope of the drop in water oxygen content, which was monitored over a 20 min measurement period at each speed. At no point did the seawater oxygen content drop below 80%. After the initial 20 min measurement period the circulation was opened for a 10 min flush/re-oxygenation. At the end of each experiment when the fish had been removed, a 'blank' oxygen consumption run was performed to quantify any background microbial oxygen consumption. This was then subtracted from the fish's respiration rate. Oxygen consumption rates were corrected for any allometric size effects using the mass exponent of 0.8 (Saunders, 1963):

$$\dot{M}_{O_2} = \left(\frac{1}{M}\right)^{0.8} \times \dot{M}_{O_2 m}, \quad (2)$$

where $\dot{M}_{O_2}$ is the standardised oxygen consumption rate in mg O_2 kg^{-1} h^{-1}, M is the mass of the fish in kg, and $\dot{M}_{O2m}$ is the measured oxygen consumption.

^{31}P-NMR spectroscopy

In vivo ^{31}P-NMR spectra included primarily white muscle with a minor contribution from red muscle. Spectra were collected using a 200 μs bp32 pulse with a flip angle of 45°, sweep width was 5000 Hz at 4 k, and repetition time was 0.8 s. 256 scans were collected resulting in a total acquisition time of around 3 min. *In vivo* ^{31}P-NMR spectra were recorded over the whole 30 min time period for each swimming speed.

Statistical analysis, data processing and modeling

^{31}P-NMR spectra were acquired using Paravision 3.0 (Bruker, Ettlingen, Germany). Spectra were processed in Topspin 1.5 (Bruker) first by fast Fourier transformation, then filtered with line broadening in the range of the half width of the PCr signal. Phase and baseline were corrected using a

specially adapted automatic correction routine (R.-M. Wittig, AWI, Germany). Typically, 6–7 spectra were collected per swimming speed, i.e. per 30 min period. The best six spectra were then added for each swimming speed. Metabolite concentrations were determined by operator defined integration limits using the standard integration routine in Topspin 1.5 (Bruker).

The control PCr integral was converted into μmol g^{-1} using the intracellular concentration of 27.3 μmol g^{-1} for resting Atlantic cod (Sartoris et al., 2003). All other concentrations, i.e. subsequent PCr measurements, Pi and ATP, were then calculated relative to this. The intracellular pH was calculated from the Pi chemical shift using the temperature compensated formula given elsewhere (Bock et al., 2001). Gibbs free energy change of ATP hydrolysis ($\Delta G/d\xi_{ATP}$) was estimated for NMR visible metabolites as described earlier (Pörtner et al., 1996; Satoris et al., 2003; van Dijk et al., 1999), except that creatine concentration was estimated using the following equation:

$$Cr = \left(\frac{PCr}{0.65}\right) - PCr, \quad (3)$$

where Cr is the estimated creatine concentration in μmol g^{-1}, PCr is phosphocreatine concentration in μmol g^{-1} and 0.65 is the ratio of PCr:Cr measured in resting fish white muscle (Hardewig et al., 1998).

All statistical analysis and modelling was performed using Graphpad Instat 3.0 and Prism 4.0 software (Graphpad Software, San Diego, CA, USA). For comparative purposes, significant differences were tested using ANOVA with Tukey's post-tests. Differences were considered significant when $P \leq 0.05$. Data are presented throughout as mean ± standard deviation (s.d.).

Results

The mean critical swimming speed for all fish was 0.71±0.06 *BL* s^{-1}. Neither the effects of the inductive coil nor the receive coil on swimming performance were directly quantified; however, a decrease of approximately 25–30% in the U_{crit} was seen compared to most values in the literature (Table 1). Because each fish swam at different speeds and had a different critical swimming speed, the relative speeds are given as the % U_{crit}, with the variability shown as horizontal error bars in Figs 2–5.

The oxygen consumption rate increased exponentially and significantly as a function of swimming speed up to the active metabolic rate (208±21.4 mg O_2 h^{-1} kg^{-1}; Fig. 2A). The standard metabolic rate (SMR) was estimated using non-linear regression of the oxygen consumption data at various speeds and extrapolating back to a swimming speed of 0 *BL* s^{-1}. The mean SMR for all fish was 36.3±10.7 mg O_2 h^{-1} kg^{-1}.

The tail-beat frequency increased in a linear fashion up to the point when kicking was initiated and then levelled off (Fig. 2B). Any increase in swimming speed attained thereafter must have been accomplished by an increase in amplitude or the frequency of the kick-and-glide bursts. Kicking started before the traditional U_{crit} was reached (at 89±2.5% U_{crit}). Once initiated, the number of kicks increased in frequency until U_{crit}, when the

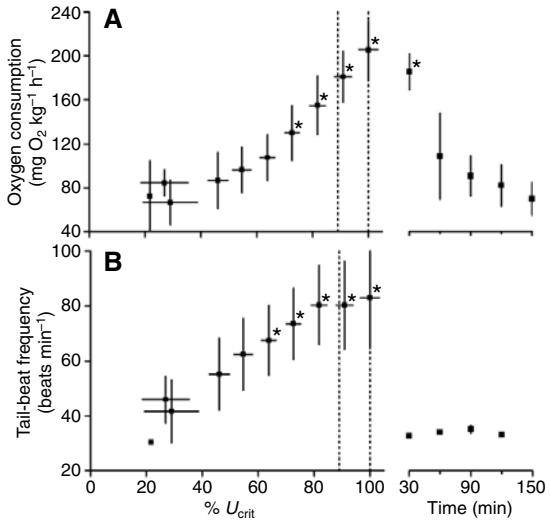

Fig. 2. Oxygen consumption rate (A) and tail-beat frequency (B) during the critical swimming speed test (left; speed as % U_{crit}) and the first 150 min of recovery [right; time after U_{crit} (min)]. The right broken vertical line indicates the traditional U_{crit} value; the left broken vertical line at 89% represents the mean time when kicking was initiated. Values are means ± s.d., N=3–6. Horizontal error bars represent s.d. of the mean speed. Asterisks indicated significant difference from control values.

fish were kicking exclusively for several minutes before they stopped swimming altogether. This paralleled the initial decrease in the PCr signal, an increase in the Pi signal, and a resultant decrease in pH_i (see below).

Difficulties were encountered in collecting sufficient ^{31}P-NMR spectra from the moving animals. For optimal signal transduction, both the inductive and receive coils had to be parallel, with a minimal distance between them. Therefore, unless the fish was cooperative and swam near the coil, only a very weak signal could be detected. These difficulties were further exacerbated at higher speeds, in particular at U_{crit}, when fish were kicking and gliding. Thus, it was possible to collect spectra from only three of the six fish at U_{crit}. The loss of signal strength and the broadening of spectral bands due to swimming movements could have been mitigated by restricting the movements of the fish; however, this would have reduced the swimming performance.

Using *in vivo* ^{31}P-NMR we were able to show a significant increase in the relative proportion of the inorganic phosphate signal above resting levels as the fish approached the critical swimming speed (Fig. 3A). Under minimal flow, the Pi signal was often below the detection limit. As the critical swimming speed was approached, the Pi signal began to intensify, until it was maximally and significantly elevated at the traditional U_{crit} (9.5±3.4 μmol g^{-1}). Thereafter it decreased and was again basal at 2 h post exhaustive exercise.

The increase in Pi was coupled with a stoichiometric decrease in the relative proportion of the PCr from 27.3 μmol g^{-1} at

Table 1. *Critical swimming speeds from Atlantic cod (Gadus morhua)*

Study	Origin	U_{crit} (*BL* s^{-1})	*N*	Temperature (°C)	Length (cm)	Mass (kg)
Present study	NEAC	0.71±0.06	6	4	45.5±4.12	0.818±0.190
(Pörtner et al., 2002)	NEAC	0.74±0.03	5	10	46.0±4.45	0.719±0.284
(Reidy et al., 2000)	Scotian Shelf	1.11±0.15	25	5	52.6±6.05	1.42±0.55
(Reidy et al., 1995)	Scotian Shelf	0.99±0.14	8	5	48.5±6.53	0.818–1.68
(Nelson et al., 1994)	Bras d'Or	1.03±0.09	5	2	46.1±1.50	1.00±0.11
	Scotian Shelf	0.95±0.07	6	2	48.8±3.42	1.10±0.19

Values are means ± s.d., *N*=number of animals.

minimal flow, to 16.7±6.2 μmol g^{-1} at U_{crit} (Fig. 3B). A possible slight overshoot in the relative PCr proportion was apparent during recovery. The free ATP at control was 4.5±0.9 μmol g^{-1} and did not change significantly throughout the course of the swimming bout, i.e. 3.8±1.4 μmol g^{-1} at U_{crit}, or recovery, although a slight dip to 2.2±3.0 μmol g^{-1} was seen 90 min post U_{crit}, at approximately the same time as the aforementioned PCr overshoot (Fig. 3C).

The pH_i decreased significantly as the fish approached U_{crit} (Fig. 4). At minimal flow pH_i was 7.48±0.03. The pH_i started to drop at approximately the same time as the Pi concentration began to increase (see below), i.e. between 72±2.6 and 91±3.0% U_{crit}. At 91±3.0% U_{crit} the increase became significant. The pH_i was minimal, 6.81±0.05, at U_{crit} and began to increase back to resting conditions during recovery.

As previously mentioned, under minimal flow conditions the Pi signal was extremely small, sometimes undetectable, as also observed by other authors (Bock et al., 2002b; Sartoris et al., 2003), which reflects the resting condition of the unrestrained fish. However, this made determination of resting intracellular pH difficult. Some of the variation in pH_i at higher speeds, particularly near U_{crit}, where a very clear Pi signal could be discerned, may be a result of the temporal resolution. As the acquisition of each spectrum took approximately 3.0 min, the pH_i may have changed in this time. Furthermore, six of these spectra were then summed for each swimming speed, potentially broadening the Pi signal. In addition, the kicking at U_{crit} would have caused turbulences in the water that may have led to magnetic inhomogeneities, resulting in broadening of all signals. As we predominantly saw a broadening of the Pi signal alone, we believe the former explanation to be correct.

At minimal flow the $\Delta G/d\xi_{ATP}$ was –55.6±1.4 kJ mol^{-1} and had significantly decreased to –49.8±0.7 kJ mol^{-1} at the traditional U_{crit} (Fig. 5). The drop in $\Delta G/d\xi_{ATP}$ was not a linear function of the speed or time before U_{crit}. The largest drops in $\Delta G/d\xi_{ATP}$ of 2.96 kJ mol^{-1} and 4.04 kJ mol^{-1} were seen between 82±2.8% and 91±3.0% U_{crit} and 91±3.0% and U_{crit}, respectively. Non-linear regression and extrapolation to 0% U_{crit}, i.e. resting conditions (Fig. 5), gave a resting $\Delta G/d\xi_{ATP}$ value of –57.3±1.5 kJ mol^{-1}.

Discussion

The primary findings of the present experimental series clearly show that as the fish approached U_{crit} a transition point in metabolism was seen. This transition from steady state aerobic metabolism to anaerobic metabolism occurred at the same time as the fish changed gait from subcarangiform swimming to kick-and-glide. Furthermore, insight was provided into fatigue processes and it was evident that the drop in $\Delta G/d\xi_{ATP}$ below a threshold appears to be ultimately responsible for fatigue.

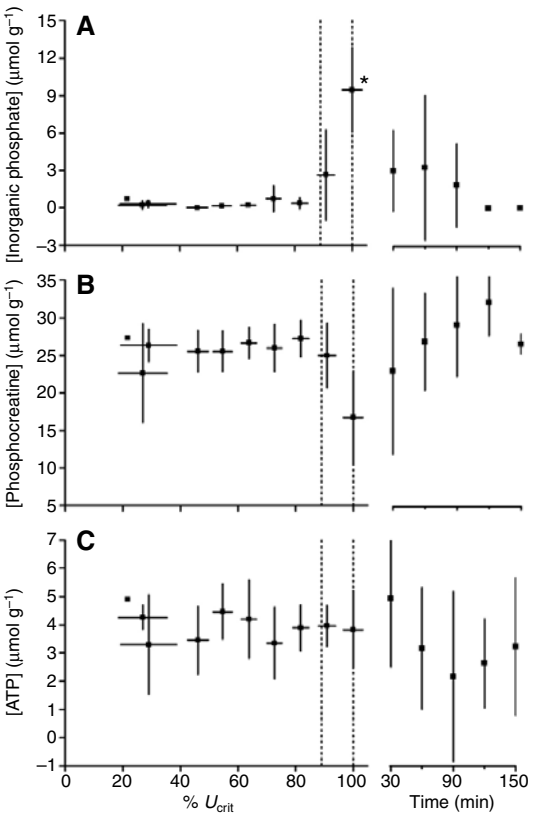

Fig. 3. Inorganic phosphate (A), phosphocreatine (B) and ATP (C) concentrations during the critical swimming speed test (left; speed as % U_{crit}) and the first 150 min of recovery [right; time after U_{crit} (min)]. The right broken vertical line indicates the traditional U_{crit} value; the left broken vertical line at 89% represents the mean time when kicking was initiated. Values are means ± s.d., *N*=3–6. Horizontal error bars represent s.d. of the mean speed. Asterisks indicated significant difference from control values.

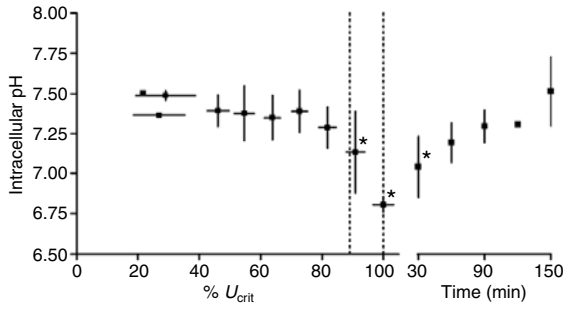

Fig. 4. Intracellular pH values during the critical swimming speed test (left; speed as % U_{crit}) and the first 150 min of recovery [right; time after U_{crit} (min)]. The right broken vertical line indicates the traditional U_{crit} value; the left broken vertical line at 89% represents the mean time when kicking was initiated. Values are means ± s.d., N=3–6. Horizontal error bars represent s.d. of the mean speed. Asterisks indicated significant difference from control values.

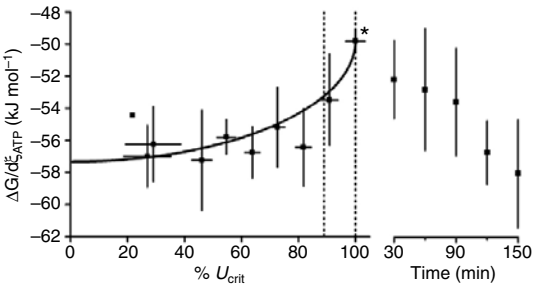

Fig. 5. The time course of the Gibbs free energy change of ATP hydrolysis during the critical swimming speed test (left; speed as % U_{crit}) and the first 150 min of recovery [right; time after U_{crit} (min)]. The right broken vertical line indicates the traditional U_{crit} value; the left broken vertical line at –89% represents the mean time when kicking was initiated. Values are means ± s.d., N=3–6. Horizontal error bars represent s.d. of the mean speed. Asterisks indicated significant difference from control values.

It is worth noting that both the inductive coil and the receive coil produced micro-turbulences. As a result, the flow in the swim tunnel behind the receive coil was turbulent, and thus swimming conditions were not optimal. When compared to literature values of similarly sized cod (Table 1), an approximate decrease of 30% in U_{crit} was observed in the current study, similar to that seen by Pörtner et al. (2002), using a similar set-up. Furthermore, fish in the present study were 10°C acclimated but swum at 4°C. Parallel work found that an acute thermal change from 10°C to 4°C reduced swimming performance by approximately 10–15%, when compared to the performance at the acclimation temperature of 10°C (G.J.L., C.H.B. and H.-O.P., manuscript in preparation).

Energetics of U_{crit}

Several previous studies have used excessive post-exercise oxygen consumption and blood parameters as measures of anaerobic metabolism (Peake and Farrell, 2004; Lee et al., 2003; Nelson et al., 1994; Brett, 1964), assuming that any oxygen debt accumulated during the swimming trial was re-paid during recovery. The online measurement of metabolic processes underpinning the entire swimming trial, i.e. from slow swimming through the transition to kick-and-glide to complete fatigue, confirm previous findings that U_{crit} is indeed the point of complete fatigue, when both the aerobic *and* anaerobic resources have been fully expended.

In the current study, kicking started at 89±2.5% U_{crit}, just as the tail-beat frequency began to plateau (Fig. 2B). At the same time Pi began to increase and the pH_i started to acidify. These kicks avoided any increase in the tail-beat frequency required as speed increased. Although it is possible that the fish were able to compensate by increasing the tail-beat amplitude at the same frequency, we primarily observed that once initiated, the number of kicks increased in frequency until close to exhaustion (i.e. U_{crit}), when the cod were kicking exclusively. This strategy indicated the involvement of white muscle fibres (Jones, 1982) and came at an additional cost fuelled anaerobically on top of enhanced aerobic metabolic rate.

Using electromyography, Rome et al. (Rome et al., 1984) previously found that as swimming velocity increased, mirror carp (*Cyprinus carpio* L.) increasingly recruited more white muscle to increase the power production before kicking was initiated. Similar findings have also been seen with other fish species (Jayne and Lauder, 1994; Rome et al., 1992). Although the precise time when kicking was first initiated was not given, a significant increase in lactate and drop in PCr was seen at 70% U_{crit} in rainbow trout (*Onchorhynchus mykiss* Walbaum) using ^{31}P-NMR (Burgetz et al., 1998). This continued to rise at 80% and was maximal at U_{crit}. A more invasive study (Nelson et al., 1994) looked at, among other parameters, lactate production in Atlantic cod as a proxy for anaerobic metabolism, and found that it had already significantly increased at approximately 80% U_{crit}. The reasons why this switch to anaerobic metabolism occurred later in the present study, i.e. at 89% U_{crit}, are discussed below.

^{31}P-NMR and energetic status

To our knowledge, only our previous study with Atlantic cod (Pörtner et al., 2002) and one other study with rainbow trout (Burgetz et al., 1998) have looked at exercised fish using ^{31}P-NMR spectroscopy. In the latter study, where the trout were swum to 70, 80 or 100% U_{crit} and then transferred to the NMR magnet, the exactitude of the measurements in relation to swimming speeds was limited due to the potential stress incurred during transfer. An artefactual decrease in PCr and an increase in Pi is almost invariably seen in studies after the initial transfer of animals to the NMR magnet (e.g. Sartoris et al., 2003). However, because the trout were restrained, the spectra were of a higher quality.

A previous study (Burgetz et al., 1998) found that PCr had dropped to approximately one half of the 30 μmol g^{-1} control values at 70% U_{crit}. This depletion continued and was maximal at U_{crit} where PCr was approximately 4 μmol g^{-1}. The severe reduction in the PCr concentration, particularly at 70% U_{crit}, is much larger than in our study. This may be due to (i) a stress related artefact, as discussed above, or (ii) species differences between athletic trout and lethargic Atlantic cod.

In the same study (Burgetz et al., 1998), biochemical analysis was used to determine lactate concentrations in the same tissue. A strong correlation (r^2=0.83) was found between the tissue lactate concentration and the pH_i calculated from the Pi shift. At 70% U_{crit}, tissue lactate concentration had already increased significantly (from ~13 μmol g^{-1} tissue to ~21 μmol g^{-1}), and was maximal at U_{crit} (~47 μmol g^{-1}). In the current study pH_i had dropped significantly at 91±3.0% U_{crit}. The disparity between the time of onset and degree of intracellular acidification may be attributable to the differences between the two species. For example, the maximum plasma lactate concentration after exhaustive exercise was found to be ~7 mmol l^{-1} in Scotian Shelf cod and ~10 mmol l^{-1} in Bras d'Or cod (Nelson et al., 1994). In comparison, other authors (Jain and Farrell, 2003) found that plasma lactate levels could get as high as ~20 mmol l^{-1} in rainbow trout post exhaustive exercise. We conclude from the cited evidence that Atlantic cod are physiologically unable to exert themselves to the same degree as trout.

Additionally, the general level of fitness of our fish may have further reduced their swimming capacity as they had been kept in our aquarium for more than 1 year and there was only a slight current against which they could exercise, possibly making them rather lethargic in comparison to freshly caught, wild fish (Soofiani and Preide, 1985; Webb, 1971; Bams, 1967; Bainbridge, 1962; Brett, 1958). The critical swimming speed indicating onset of anaerobic metabolism may not be fixed but be found at variable velocities in relation to U_{crit}. In cod it appears to be tied to the onset of kick-and-glide swimming.

Fatigue

One of the aims of the current study was to observe exactly when the pH_i decreased, i.e. at or before U_{crit}, as this would be an important step in determining the transition to non-steady state anaerobic metabolism. It has previously been hypothesised that the accumulation of physiological concentrations of Pi (e.g. 30 μmol g^{-1}) is responsible for a decrease in the power generated in the muscle as Pi moves into the sarcoplasmic reticulum and precipitates Ca^{2+} (Allen and Westerblad, 2001), and reduces steady state tension by reducing/preventing cross-bridge attachment (Hibberd et al., 1985). The mean Pi concentration at U_{crit} was significantly elevated at 9.5±3.4 μmol g^{-1}, but we expect the effect of Pi on fibre contraction would have been reduced, as levels are 30% of those cited above. Two further points supporting the reduced importance of Pi and pH_i in exercise induced fatigue must be noted. Firstly, increased intracellular Pi in fact increased force re-development during rapid contraction–relaxation cycles (Hibberd et al., 1985), which are typical of kick-and-glide bursts seen here. Secondly, a drop in pH_i led to increased excitability of working skinned muscle fibres from rats (Pedersen et al., 2004).

More important were the changes observed in tissue energetics. The stoichiometric relationship between the Pi increase and the PCr decrease indicates that these cellular energetic stores were being depleted at U_{crit} to buffer cellular ATP concentrations. The consequent drop in pH_i indicated that anaerobic metabolism was also being used to maintain ATP concentrations. Both Pörtner et al. (Pörtner et al., 1996) and Hardewig et al. (Hardewig et al., 1998) have argued that as the cytosol becomes more acidic and, more importantly, as the phosphagen (PCr in case of fish) is lysed to Pi and Cr, there is a drop in $\Delta G/d\xi_{ATP}$. For two species of Zoarcid eelpouts, the free energy of ATP hydrolysis was observed to drop from –60 kJ mol^{-1} to approximately –46 kJ mol^{-1} and for rainbow trout, $\Delta G/d\xi_{ATP}$ dropped from –60 to –47 kJ mol^{-1} after exhaustive exercise (Hardewig et al., 1998).

Various studies have looked at the effects of a drop in free energy values and the detrimental effects on cellular ion transporters (Jansen et al., 2003; Kammermeier et al., 1982). Hardewig et al. (Hardewig et al., 1998) argued that below a threshold of approximately –52 kJ mol^{-1}, cellular processes such as Ca^{2+}-ATPases, essential to muscle function, can no longer derive enough energy to be maintained. We suggest that the muscular fatigue observed in our fish was predominantly due to a drop in $\Delta G/d\xi_{ATP}$ to –49.8±0.7 kJ mol^{-1} at U_{crit}, which was below a certain threshold, potentially –52 kJ mol^{-1} (Hardewig et al., 1998), required by transporters to maintain ion gradients and fuel the muscular machinery.

The control $\Delta G/d\xi_{ATP}$ values in the current study (–55.6±1.4 kJ mol^{-1}) lie below those reported for resting eelpout (Hardewig et al., 1998) and –61 kJ mol^{-1} for resting Atlantic cod (Sartoris et al., 2003). It seems reasonable to conclude that this is because resting values in the two aforementioned studies were obtained from inactive fish, whereas our values were obtained at minimal flow. A relatively linear decrease in $\Delta G/d\xi_{ATP}$ with increasing swimming speed was previously shown at moderate speeds in squid (Pörtner et al., 1996). Consequently, the slow swimming of our fish during control conditions would have led to a shift in steady state energy status, thus reducing $\Delta G/d\xi_{ATP}$ in comparison to values from inactive fish. Extrapolation of our values back to resting (Fig. 5) gave us a base value of –57.3±1.5 kJ mol^{-1} (r^2=0.90).

Conclusions

Through the use of *in vivo* ^{31}P-NMR spectroscopy combined with a Brett-type swim tunnel, we were able to show that as Atlantic cod swimming speed increased, and gait was changed from subcarangiform swimming to kick-and-glide swimming prior to traditional U_{crit}, i.e. at 89±2.5% U_{crit}, a graded decrease in intracellular pH was observed while the oxygen consumption rate continued to increase exponentially. At the same time, phosphocreatine levels fell and this was accompanied by a significant increase in inorganic phosphate. All these changes were maximal at the traditional U_{crit} (i.e. exhaustion), when the fish were kicking exclusively. These changes were subsequently restored during recovery. The Gibbs free energy change of ATP hydrolysis was also minimal at the traditional U_{crit} (i.e. down from –55.6±1.4 at control to –49.8±0.7 kJ mol^{-1}), and this was argued to be the leading cause for muscular fatigue leading to exhaustion of the cod. Thus, a transition from steady state aerobic metabolism to non-steady state anaerobic metabolism led to a complete exhaustion of aerobic and anaerobic resources at the traditional critical swimming speed.

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Muscle Bioenergetics of Speeding Fish: In Vivo MRS Studies in a 4.7 T Magnetic Resonance Scanner with an Integrated Swim Tunnel

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ABSTRACT: Energetic studies on exercising animals are usually limited to oxygen consumption measurements in respirometers followed by invasive tissue sampling and analysis of metabolites. Noninvasive studies of exercising animals like through the use of ^{31}P NMR are typically restricted to "stop and go" measurements. Furthermore, magnetic resonance studies of marine animals are hampered by sea water, a highly electric conductive and dielectric medium, resulting in heavy loading and strong RF loss. In this work, we present a set-up for online determination of muscle bioenergetics in swimming marine fish, Atlantic cod (*Gadus morhua*), using in vivo ^{31}P NMR spectroscopy, which overcome these limitations. Special hardware and RF coils were developed for this purpose. A birdcage resonator adapted to high loadings was used for signal excitation. An insulated inductive coil (2 cm diameter) was fixed onto the surface of the fish tail and placed opposite a watertight, passively decoupled 9×6 cm^2 elliptic and curved surface coil for signal recordings. This arrangement led to enhanced penetration of the RF signal and an almost 10-fold increase in *S/N* ratio compared to the exclusive use of the elliptic surface coil. Monitoring of tail beat allowed to set trigger values resulted in an improved quality of in vivo ^{31}P NMR spectra of swimming fish. We report the first successful MRS experiments recording simultaneously tissue energetic and acid–base parameters on swimming cod depending on tail beat frequency and amplitude to determine critical swimming speeds. Concepts Magn Reson Part B (Magn Reson Engineering) 00B: 000–000, 2007

KEY WORDS: ^{31}P NMR; MRS; MRI; RF coils; trigger; gating; swim tunnel; conductivity; exercise; Atlantic cod; *Gadus morhua*; energy metabolism

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INTRODUCTION

Muscle physiology and energy metabolism under basal and exercise conditions have received significant attention, both for an understanding of the factors enabling and limiting exercise and, more recently, for an understanding of the role of temperature adaptation and acclimation shaping exercise performance in marine animals from various climate regimes (*1–3*). The function and organization of muscle tissue from ectothermic animals living at low or polar tempera-

tures is especially interesting, and studies revealed unique features of adaptation for living and swimming in cold waters at all organismic levels. Briefly, cold adaptation characteristics include a specialized muscle anatomy with an increased mitochondrial proliferation (for a review, see *4*), antifreeze proteins (for a review, see *5*), modified membrane structures and functions using unsaturated phospholipids (e.g., *6*), and specialized modes of energy metabolism including increased lipid β-oxidation rather than glycolysis (*3*). An increase of lipid content will facilitate oxygen diffusion in muscle (*7*). Some of these features are also expressed in cold acclimated temperate fish-like crucian carp that displays increased mitochondrial densities in muscle tissue of up to 45% (*8*).

Little is known about the functional consequences of these mechanisms for example during exercise (*9*; for reviews, see *3*, *4*). An understanding of these special adaptations is becoming even more important in the framework of climate change, questioning how animals specialized on limited climate and temperature regimes can cope with rising temperatures? A model explaining the oxygen limitation of thermal tolerance has recently been developed and tested in an ecological context with the goal to explain these phenomena (*1–3*, *10*). These analyses revealed a key role for the thermal sensitivity of aerobic scope and the mechanisms shaping the thermal window of aerobic scope. In the context of exercise physiology, aerobic scope is defined as the difference between basal and maximal or active metabolism (*11*, *12*). Classical oxygen consumption measurements of aerobic scope are often hampered by the experimental set-up so that reliable results are difficult to obtain within a typical experimental time frame (*12*). Any invasive studies for measurements of additional parameters like circulatory performance, blood-gas transport, or muscle perfusion in marine animals will complicate the situation further due to lower performance and increased recovery rates after surgery. For example, rainbow trout needed more or less 48 h to reestablish control values after surgery and implantation of oxygen probes into gill arteries (*13*). Noninvasive studies like ^{31}P NMR analyses on exercising animals have previously been restricted to ''stop and go'' measurements (*14*). To overcome this dilemma, we developed a minimally invasive set-up that measured several parameters correlated with aerobic scope during exercise of free-swimming fish.

The aim of this work was to develop an experimental set-up for the online determination of aerobic scope, oxygen consumption, and swimming performance, combined with studies of muscle energy metabolism and acid–base regulation by in vivo ^{31}P NMR spectroscopy of fish. For this purpose, a Brett type swim tunnel respirometer was integrated into a 4.7 T MRI scanner (Bruker Biospec, Ettlingen, Germany).

Magnetic resonance (MR) measurements in electric conductive, dielectric media are complicated mainly by inductive losses. These losses originate from eddy currents induced within the sample by the oscillating field B_1. The electric conductivity decreases the signal-to-noise ratio (*S/N*); the loading in a coil rises. Also, pulse length or power to produce a given pulse angle increases, whereas the irradiation of radio frequencies (RFs) is attenuated due to the skin effect. These strong effects might even be intensified when an electric conductive medium like sea water is flowing through an NMR probe. Principally, the induction of eddy currents must be avoided. In the case of surrounding sea water, this is usually only possible if the sensitive volume inside a probe is divided into specific parts by use of electrical insulators (*15*). However, this is not applicable to in vivo NMR studies, due to the limited space available inside an NMR probe, especially in the case of freely moving animals. Our approach was therefore mainly to adapt and optimize the RF-hardware with respect to the specific requirements for swimming marine fish in a horizontal MR scanner.

MATERIALS AND METHODS

Animals

Two groups of Atlantic cod (*Gadus morhua*) from the North East Arctic and the North Sea, with a body length between 35 and 52 cm and 0.5–1.1 kg body weight were used for a comparative NMR study (data and results will be presented in an accompanying publication, *16*). Briefly, North Sea cod were caught in the German Bight by bottom trawls or bow nets on the White Bank near Helgoland. They were transported by RV ''Heinke'' or RC ''Uthörn'' to Bremerhaven and kept in natural sea water of the aquarium system of the Alfred Wegener Institute at temperatures around 10°C. Animals were fed frozen mussels, *Mytilus edulis*, or frozen shrimps, *Crangon crangon* twice a week. Feeding was stopped at least 1 week prior to experimentation. Cultured North Eastern Arctic cod were a generous gift from Dr. M. Delghandi at the IMR in Tromsø and transported to the AWI by air. Animals from both groups were kept in aquarium tanks with a volume of 1 m^3 under identical conditions (see accompanying paper for more details, *16*).

Surgery

All animals were anesthetized with 0.08 g/l MS222 and body weights and lengths were measured. During surgery gills were irrigated continuously with aerated sea water containing 0.04 g/l MS222. An inductive coil (2 cm inner diameter, tuned to a fixed frequency of 81 MHz, covered and sealed in plastic, with a final size of a 4.5×4.5 cm^2) was sewn with two sutures to the skin just prior to the tail on one side of the fish. During an initial set of experiments, the caudal fin was punctured and fitted with a differential pressure transducer, which was fixed with suture onto the skin for online monitoring of tail beat pressures and frequency (*17–19*). The complete surgical procedure lasted no longer than 20 min. All animals survived the experimental procedure.

Swim Tunnel Set-Up

A ''Brett-type'' swim tunnel respirometer was constructed and fed through the 40 cm inner diameter of a Bruker Biospec 47/40 DBX system, operated at 4.7 T (Fig. 1). Briefly, a 2 m Perspex pipe (wall thickness 1 cm, inner diameter 17.5 cm) was fed through the MR scanner. Special custom-made adapters with an outside rubber seal and plastic clip collars provided fitting to standard PVC tubes of 10 cm inner diameter. In all other cases, union nuts with compression rings were used as connectors. An animal handling chamber was placed directly in front of the magnet for the preparation of the animal and the MR set-up. Both ends of the circular tube ended in a round Perspex reservoir with a maximum volume of around 450 l. The reservoir was connected to a separate filter and cooling system consisting of a heat exchanger (Calorplast Wärmetechnik GmbH, Germany) and a cooler with a maximum cooling rate of 12.5 kW. A protein skimmer combined with a trickle filter was connected to the sea water reservoir. An additional cooling coil was inserted into the tube system and connected to a cryostat (Lauda, Germany) to provide constant temperatures between 0 and 20°C during closed system operations. Temperature stability was around ±0.5°C in both open and closed system operation. A circulation pump (Jesco Dosiertechnik, Germany) with a maximum output of 30 m^3/h was placed directly after the reservoir. Three valves and a bypass allowed switching from an open to a closed system for respiration studies. A magnetic flow meter (Badger Meter, Isitec, Bremerhaven, Germany) was placed outside and in parallel to the Perspex tube to determine flow. Flow velocities were calibrated against an ultrasonic flow meter.

A round Perspex chamber closed on both sides with nylon grids contained the individual fish (maximum size of 50 cm body length and 1.2 kg body weight). It was connected to Kevlar threads at both ends and placed into the handling chamber (Fig. 1). The Kevlar threads were used to pull the chamber into the correct position in the center of the MR scanner. An integrated Perspex holder inside the chamber was used to fix the watertight surface coil relative to the tail muscle of the free moving fish and in parallel to the inductive coil [see Figs. 2(D,E)]. Velcro strips were used for fixation allowing easy repositioning and removal of the coil.

The temperature sensor, oxygen optodes, and cable of the water-sealed surface coil were fed through a seal in the handling chamber and connected to their specific instruments. The complete system except for the Perspex section inside the scanner was isolated with Arma Flex (Armacell International GmbH, Münster, Germany).

An underwater camera surrounded by a ring of blue LEDs was placed directly behind the animal chamber inside the Perspex tube for video observation of the swimming fish (Isitek, Bremerhaven, Germany). The camera holder and thus focus was adjustable relative to the animal chamber from outside via a plastic rod. The camera system was connected via a frame grabber to a PC equipped with video tracking software (MeDea AV, Erlangen, Germany). Analysis of differential pressure recordings as well as video sequences from the swimming behavior at different swimming speeds allowed the determination of tail beat frequencies and the onset of ''kick and glide'' bursts at critical swimming speeds.

All exercise studies were carried out with North Sea and North Eastern Arctic cod at 4 or 10°C, respectively. Both coil and fish blocking effects were calculated as described by Nelson et al. (*20*).

Oxygen Consumption Measurements

An optical fiber system (''oxygen optodes", Fibox, Presens, Germany) was used inside the MR system for oxygen consumption measurements. Briefly, a 5-m long optical fiber was fed into the animal handling chamber via a watertight seal and placed in the current of the swim tunnel. An oxygen-sensitive dye at the fiber's tip detected changes in water oxygen concentrations. Optodes were calibrated at 0% air saturation with sodium dithionite and at 100% with air-saturated sea water. Temperature compensation of recordings was carried out manually. For oxygen consumption measurements, the complete sea water set-up system without an animal inside was tested by closure with the two valves in front of the reservoir.

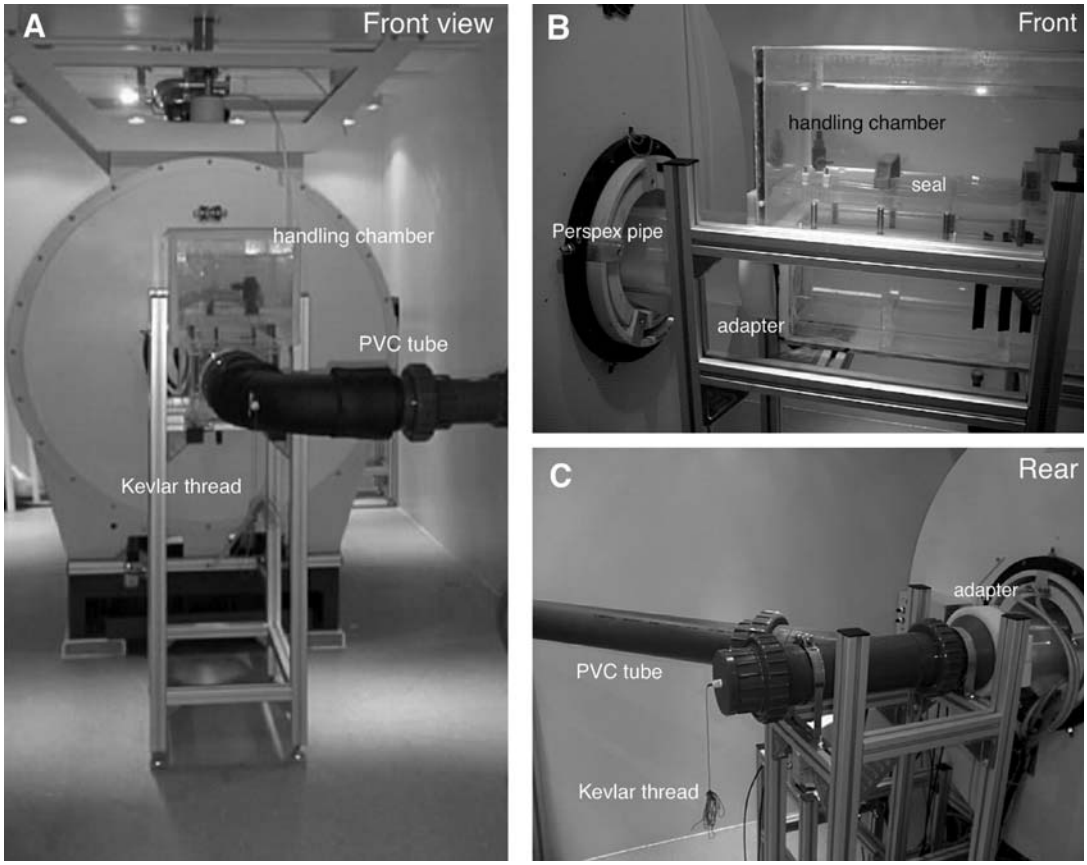

Figure 1 (A) Photographic view of the swim tunnel feeding through the MRI scanner, with (B) front and (C) rear view, showing the Perspex pipe with the handling chamber prior the insulation with Armaflex. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Any decrease in water oxygen concentration during swimming performance of the animals could then be attributed to the oxygen consumption of the animal. Usually, the system was reopened and flushed after at about 30 min of swimming or a decrease by less than 20% of water air saturation during exercise to prevent any possible oxygen deprivation effects. The calibration of the system was repeated daily, to account for possible effects of bacterial respiration. The complete water volume was exchanged after each swimming trial.

NMR Hardware

The negative effects of highly conductive sea water in a swim tunnel could only be marginally reduced, as the animals could not be restrained. The sea water volume around the animals could only be limited to a certain degree in a way that swimming performance of the animal was not affected. The main contribution of a conducting sample like sea water is the inductive loss from eddy currents induced by the oscillating field B_1. The signal-to-noise ratio (S/N) is basically proportional to the negative square root of the conductivity of the sample (*15*). The pulse length is also depending on the inductive losses resulting in a prolongation of the pulse or the need of higher power levels with increasing conductivity (*15*). Furthermore, the high dielectric permittivity increases the self-capacitance of the probe resulting in substantial changes of the matching and tuning properties. Our strategy for developing a usable NMR set-up there-

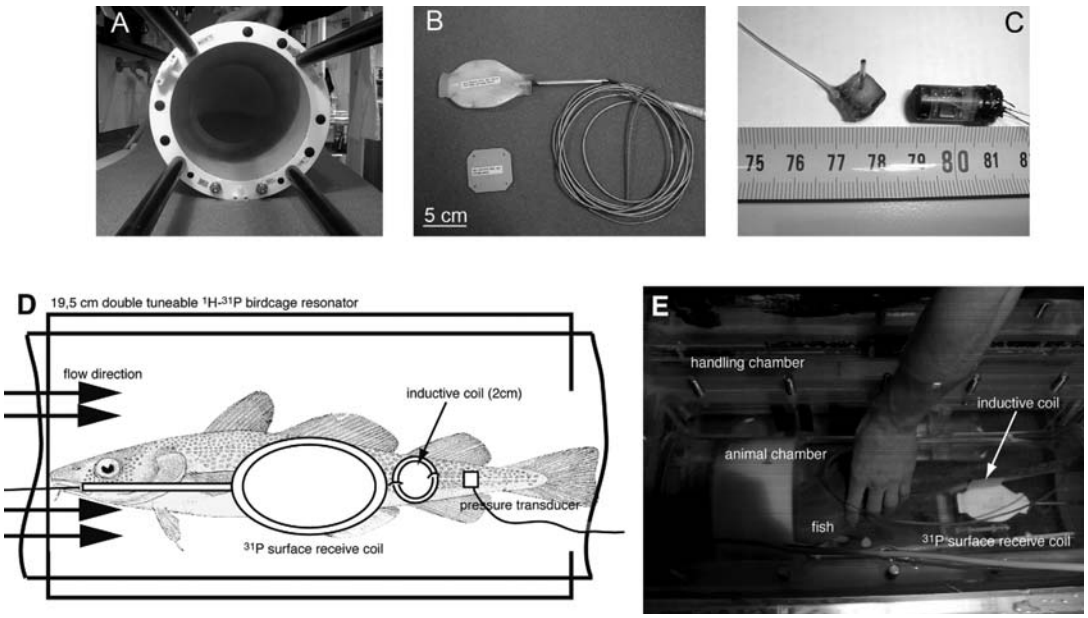

Figure 2 (A) View through the 19.5 cm diameter seawater-adapted double tunable birdcage resonator used for the swim tunnel experiments. (B) Photograph of an inductive surface coil (left) and the watertight transmit/receive curved ellipsoid ^{31}P surface coil before mounting (for more information see text). (C) Pressure transducer used for the gating experiments. (D) Schematic view of the experimental set-up illustrating all hardware components in combination. The arrows are indicating the flow direction. Figure 2(E) presents the in vivo situation at the beginning of the experiment. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

fore relied mainly on the optimization of the specific NMR hardware. We basically divided the NMR excitation and perception signals in three parts:

1. A commercial double tunable ^{1}H–^{31}P-birdcage resonator [inner diameter 19.5 cm, Fig. 2(A)] adapted for very high loadings was used for signal excitation, specially developed and optimized for our purposes (Bruker-Biospin, Ettlingen, Germany). The inner Perspex tube of the swim tunnel fitted symmetrically inside the probe. The conductivity-induced changes of the matching and tuning properties were compensated with capacitors of broad capacitance range in the electrical circuit (property of Bruker), resulting in a reflection of less than 2% on both channels with a completely filled swim tunnel. Furthermore, both channels were optimized for a maximum RF-power of 2000 W to compensate for pulse length prolongations. The probe can be actively decoupled on both channels for use in cross-coiled experiments in combination with surface coils to increase the *S/N* ratio. RF amplifiers used were a 2 kW ^{1}H-amplifier operating at 200 MHz, and a 1 kW broadband amplifier for ^{31}P NMR spectroscopy, respectively.
2. Inductive coils have the advantage that cable connections to the preamplifier can be avoided. Fixing the coil directly to the fish prevented further interference. Watertight 2 cm inner diameter inductive coils (^{1}H or ^{31}P) sealed in Teflon were developed for use in seawater [resulting size of around 4.5 cm length, see Fig. 2(B)]. Briefly, solenoid microcoils were fabricated from copper and fixed tuned and matched on sea water and their specific resonance frequencies (200 and 81 MHz, respectively). Subsequently, the coil was placed in two Teflon plates and sealed. Small holes in each corner of the Teflon allowed the coil to be sutured to one side of the fish's tail. Exemplary, Fig. 2(B) presents a ^{31}P inductive coil. The specific coil, for ^{1}H- or ^{31}P NMR,

respectively, was then sutured with two small sutures onto one side of the animal's tail with minimal effects on the swimming performance of the fish [see Figs. 2(D,E)].

3. A watertight transmit/receive curved ellipsoid ^{31}P surface coil [inner diameters: 6 and 4 cm, respectively; outer diameter including plastic seal: 9 and 6 cm, Fig. 2(B)] developed for the swim tunnel application (Bruker-Biospin, Ettlingen, Germany) was placed onto the inner wall of the swim tunnel opposite the inductive coil. The coil was shaped to match the curvature of the fish, thereby improving excitation and reception [see Figs. 2(D,E)]. Match and tune capacitors of the coil were preset to the conductivity of seawater. Matching and tuning were individually optimized for the ^{31}P channel using the active decoupling unit (Bruker, Ettlingen, Germany). The coil could be switched to a fixed matched and tuned ^{1}H channel usable for shimming procedures only. One watertight cable was fed through a watertight adapter and connected to preamplifier and active decoupling unit of the MR scanner, respectively.

   In vivo ^{31}P NMR spectra were recorded during the swimming trails consecutively. Acquisition parameters were as follows: acquisition size 4 K, sweep width 5,000 Hz, flip angle 45°, bp32 pulse, pulse length 200 μs, repetition delay 0.8 s, number of scans 256, resulting acquisition time 3.25 min. Despite of the short repetition time, no substantial relaxation effects could be observed similar to previous studies (*21*, *22*) due to the fact that the T_1 values of metabolites are greatly reduced in marine organisms. The repetition time was optimized comparing *S/N* ratio of ^{31}P NMR spectra from a phosphate solution at different repetition times. Individual FIDs of every swimming speed were added for subsequent quantification of relative metabolite concentrations and the calculation of intracellular pH changes. Resulting spectra were processed using TopSpin 2.0 (Bruker, Ettlingen, Germany) with a zero filling of 8 K and a line broadening factor of 15 Hz. Peak position and signal integrals were analyzed using a specially adapted automatic fit routine as described in (*21*, *22*). Metabolite concentrations from peak integrals were calculated as described in (*23*).
4. A separate set of experiments was performed with swimming cod equipped with a differential pressure transducer for better characterization of the swimming performance inside the MR integrated swim tunnel. Briefly, a differential pressure transducer [Fig. 2(C)] was fixed to the caudal fin of the fish and used to monitor tail beat pressures for the quantification of tail beat amplitude and frequency (for more information, see *17*). Voltage thresholds set within the pressure pulses were used to gate MR measurements via a trigger box (Isitec, Bremerhaven, Germany), which was connected to the operator console of the MR imaging system and sending TTL pulses to the console via the EKG port of the spectrometer. The trigger pulse length and time before the onset of specific MR acquisition pulses could be defined such that MR spectra were sampled during tail beat pressure maxima, minima or pressure increments and decrements, respectively. The triggering allowed the gated acquisition of NMR spectra during maximum tail beat amplitude and minimum when the tail returned to his start position. The trigger box was connected to a PowerLab system (AD Instruments, Australia) for online monitoring of the pressure pulse and the correct setting of the trigger pulse. The trigger box was equipped with additional digital filters and a baseline compensation for the correction and smoothing of the tail beat pressure recordings.

RESULTS AND DISCUSSION

The aim of this work was the development of a swim tunnel system integrated into a conventional MRI scanner for exercise studies in fish including analyses of oxygen consumption, work load, and muscle energy metabolism. Preliminary results of this work were presented by Pörtner et al. (*18*). A photographic view of the complete experimental set-up integrated to the magnet is shown in Figs. 1(A–C). Figure 2 presents the resonator [Fig. 2(A)], the surface coils [Fig. 2(B)], and the pressure transducer [Fig. 2(C)] used for the NMR studies to give an impression of the physical dimensions. A schematic depiction of the three different RF parts relative to each other together with the pressure transducer at the tail end of the fish is shown in Fig. 2(D). After surgery, the animal was placed into the chamber inside the animal handling chamber [Fig. 2(E)] and left there overnight for recovery at low flow, i.e. 1–2.5 m^3/h. During this time, the fish usually started to swim freely against

the current inside the animal chamber. No movement restrictions were visible during this initial period of swimming for any fish. After recovery and confirmation of the right orientation of the coils relative to the fish and to each other, the animal chamber was pulled into the center of the magnet using the Kevlar threads. The exact positioning of animal chamber and coils relative to the resonator was confirmed by the camera system. Furthermore, standard gradient echo pilot scans in all three directions were performed prior to each swimming trial.

The animal handling chamber was closed and the resonator as well as the surface coil were matched and tuned. An automatic shimming routine (Bruker, Ettlingen, Germany) was used to provide field homogeneity. Indeed, since the swim tunnel resembled an ideal water-filled cylinder, line widths of 10 Hz of the water signal were reached easily as already described by Bock et al. (*21*, *22*).

All parts of the NMR set-up were tested and optimized prior to exercise studies using a square bottle filled with 0.8 M $NaHPO_3^-$ as a phantom. Figure 3 presents ^{31}P NMR spectra from the phantom under different coil set-ups and positions of the sample relative to the surface receiver coil. Sagittal MR images over the entire animal chamber were obtained with a classical gradient echo sequence to locate the test set-up. Despite of the apparent magnetic field inhomogeneity over the entire field of view, position of phantom and coils could be determined in the images (Fig. 3, left). Interestingly, water flow had no influence on spectroscopic data, whereas blurring made it nearly impossible to get any information from MR images (data not shown). Therefore, water current was set to zero during imaging.

No signal could be detected using the resonator alone for ^{31}P NMR spectroscopy (data not shown). This result is in line with the excitation profiles we observed using a standard gradient echo sequence (e.g., see bright contrast at the margin of the MR images, Fig. 3). Transverse MR images through the swim tunnel acquired with the birdcage resonator showed a very inhomogeneous excitation profile with highlighted areas near the edge of the swim tunnel indicating a strong skin effect. The skin effect also accounts for the signal loss when using the resonator for spectroscopy only. The RF excitation pulse from the resonator is reduced on its way through the seawater, such that the phantom could not be excited at all within the full power range of 2 kW of the RF amplifier (data not shown). Indeed, only the combination of resonator and elliptic surface receiver coil, operating in cross-coil operation mode, resulted in ^{31}P NMR spectra with a sufficient signal-to-noise ratio (S/N = 100) after 128 acquisition pulses [resulting scan time 1.45 min, Fig. 3(A)]. Since the fish has to swim unrestricted with enough clearance to perform reliable exercise studies, the phantom was positioned 5 cm away from the receiver coil [Fig. 3(B)]. Placing a block between phantom and coil caused the ^{31}P NMR signal to almost vanish resulting again in a fairly bad S/N ratio [see spectrum in Fig. 3(B)]. Nevertheless, addition of the inductive coil onto the phantom increased the S/N ratio to suitable values [around 30–40; Fig. 3(C)].

These results clearly demonstrate that only the combination of all three coils yielded sufficient S/N ratios on reasonable time scales and giving the animals enough free space for the exercise trials. Eddy currents and skin effect decrease RF power to an extent that the complete signal is almost lost on its way through the seawater. Nevertheless, the distance between inductive and receiver coils was suitable to prevent signal loss and helped to transport the RF signal through the seawater and on its way back. Overall, an increase in S/N ratio by about one order of magnitude could be reached with the three coil set-up in comparison to just using the ^{31}P surface receive coil.

Figure 4(A) depicts typical *in vivo* ^{31}P NMR spectra from swimming cod at different water currents. The presented spectra are the results of 9–13 individual spectra (of 256 scans resulting in 3.25 min) accumulated over the entire swimming trial (normally around 30 min) and depending on the oxygen consumption of the animals. At high swimming speeds, a decrease of around 20% of water air saturation could be observed already, resulting in shorter swimming periods for the animals (see Materials and Methods section). All spectra display clearly the most prominent phosphorus signals from muscle tissue, namely phosphocreatine (PCr) and the three signals of adenosine triphosphate (ATP) [Fig. 4(A)].

Interestingly, the line width of the signals in individual spectra decreased and the S/N ratio increased with increasing swimming speed despite the faster movement of the fish relative to the coil [Figs. 4(A,C), e.g. comparison of spectra at 12.6 and 6.3 m^3/h, respectively]. At very low swimming speeds, animals moved back and forth in the tube and thus regularly changed position of the inductive relative to the receiver coil. At swimming speeds from 0.4 up to 1.2 body length/s (corresponding to flow rates of around 7 to a maximum of 14.0 m^3/h) depending on the size of the fish, the animal kept its position more or less stable during swimming, resulting in an equally stable position of the inductive relative to the receiver coil. Not until critical swimming speeds

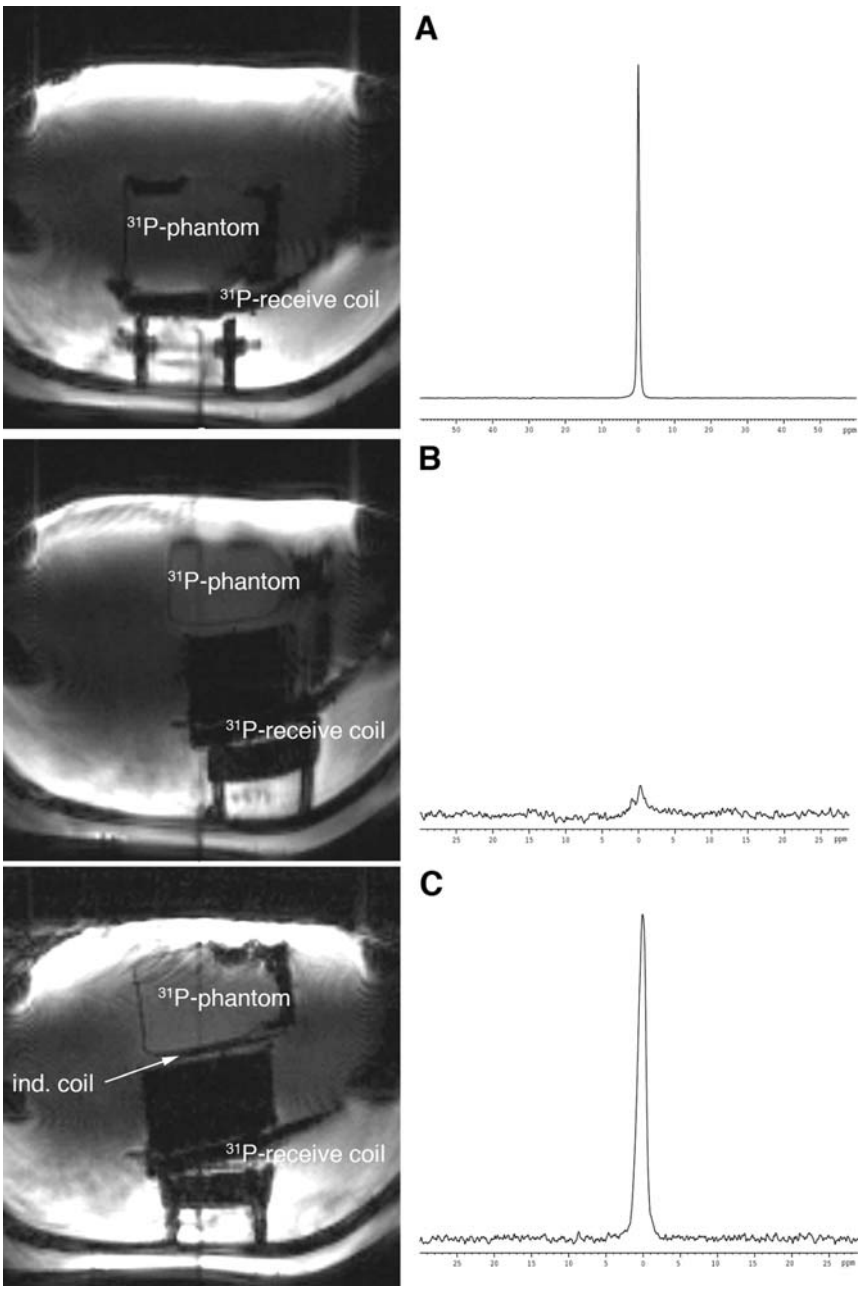

Figure 3 Comparison of ^{31}P NMR spectra from a ^{31}P-phantom solution placed into the center of the swim tunnel together with corresponding sagittal MR images of the set-up. (A) ^{31}P NMR spectrum of the phantom positioned directly onto the surface coil (for acquisition parameters see text, *S/N* ratio = 100). (B) ^{31}P NMR spectrum of the phantom placed at a distance of around 5 cm relative to the surface coil. Almost no signal could be detected from the phantom. (C) ^{31}P NMR spectrum of the phantom at the same position with attached inductive coil (the arrow indicates the location of the coil in the sagittal view, *S/N* = 35).

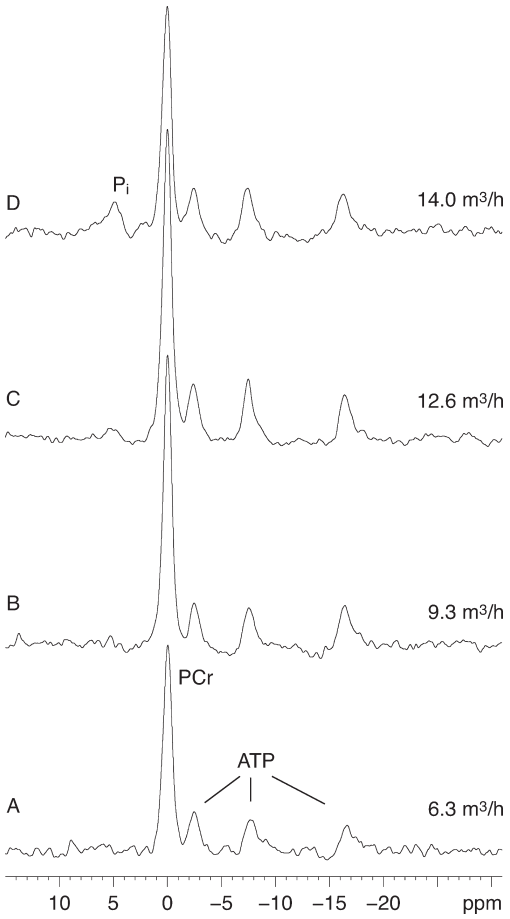

Figure 4 Accumulated in vivo ^{31}P NMR spectra from Atlantic cod at different swimming speeds. Figure 4(A) presents a ^{31}P NMR spectrum at a flow of 6.3 m^3/h corresponding to a swimming speed of 0.2 body length/s. Phosphocreatine (PCr) and the three ATP signals can be clearly resolved (for acquisition parameters see text, line broadening 30 Hz). (B,C) In vivo ^{31}P NMR spectra from the tail muscle of swimming cod at moderate swimming speeds (9.3 and 12.6 m^3/h, respectively), resulting in no obvious differences to spectra at slower swimming speeds, despite a higher *S*/*N* ratio. Figure 4(D) shows the energetic situation at critical swimming speed, when cod started to use kick and glide swimming (14 m^3/h, around 1.0 body length/s). Note the decrease in PCr accompanied by the onset of inorganic phosphate (Pi) accumulation.

were reached, the quality of the spectra decreased again. In Fig. 4(D), for instance, an in vivo ^{31}P NMR spectrum of the same animal swimming near the critical swimming speed is presented (14 m^3/h, 1.0 body length/s). The critical swimming speed has recently been defined as the point, when the fish started using ''kick and glide'' bursts and exploited high energy phosphates while maintaining position in the swim tunnel (*16*, *18*). ''Kick and glide'' bursts are typically used during escape swimming or hunting prey and are powered by anaerobic white muscle, whereas ''normal'' subcarangiform swimming employs red aerobic muscle. Using white musculature for faster swimming is fuelled by anaerobic metabolism and therefore time limited. The metabolic situation during swimming bursts and the use of anaerobic white muscle is clearly reflected in the spectrum displayed in Fig. 4(D). PCr decreased in comparison to moderate swimming and the accumulation of inorganic phosphate (Pi, see spectrum 4D) is detected. Furthermore, intracellular pH, derived from the position of the inorganic phosphate signal, decreased. A drop in intracellular muscle pH values during exercise despite the alkalizing effect of phosphocreatine depletion gives strong evidence for lactate production (*24*) and is therefore indicative of limited aerobic energy supply and the onset of anaerobic metabolism. A brief quantitative analysis of in vivo ^{31}P NMR data from four individuals is presented in Fig. 5. The graph displays concentration changes of the high-energy phosphates [PCr, ATP, and inorganic phosphate (Pi)] from muscle tissue at increasing swimming speeds. Swimming speeds are expressed as mean water flow in m^3/h. A significant increase of inorganic phosphate could be observed at swimming speeds around 13 m^3/h, confirming the use of white musculature, the onset of anaerobic metabolism, and the reaching of critical swimming speed. No significant changes in PCr and ATP levels could be

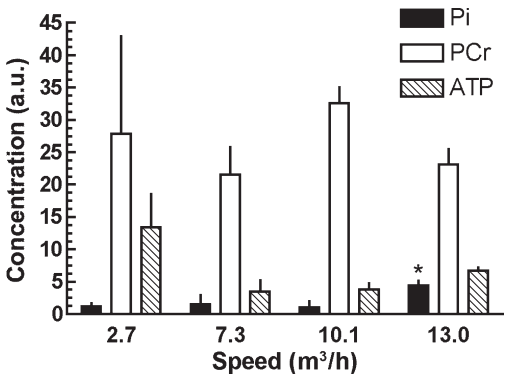

Figure 5 In vivo ^{31}P NMR metabolite concentration changes of fish tail muscle at different swimming speeds (n = 4). At a mean swimming speed of 13 m^3/h, a significant increase of inorganic phosphate could be observed indicating the onset of anaerobic metabolism.

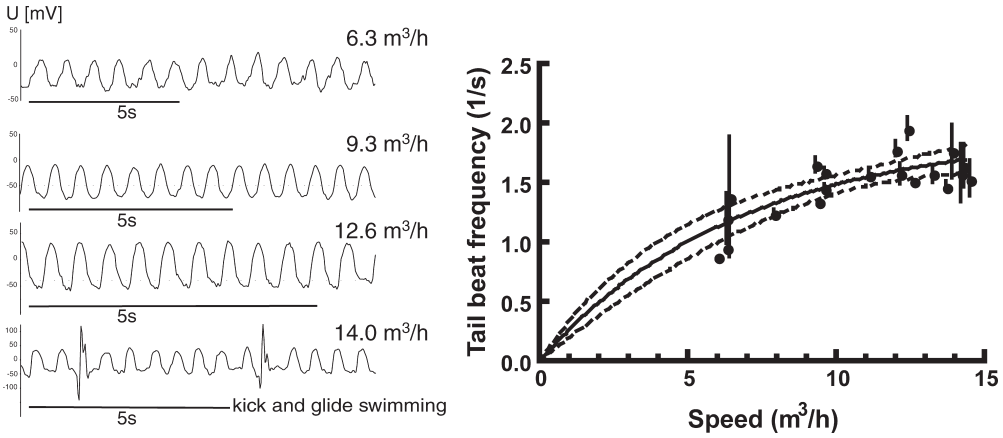

Figure 6 Left: Individual pressure traces from same individium as in Fig. 4 at different swimming speeds. Note the spikes in the trace at 14 m^3/h mark the onset of ''kick and glide'' swimming. Right: Mean tail beat frequency is presented in relation to swimming speed ($n = 4$). Tail beat frequency increased with swimming speed until the critical swimming speed was reached. Maximum values are indicating the onset of kick and glide swimming near the critical swimming speed. The point when critical swimming speed is reached fits perfectly with the onset of intracellular acidification and the onset of inorganic phosphate accumulation as shown in Figs. 4 and 5 (see also *16*, *18*).

observed, most likely due to high individual variability. Indeed, in our accompanying paper presenting a more complete and time-resolved analysis of the bioenergetics; the observed increase in Pi was coupled with a stoichiometric decrease in the relative proportion of PCr, whereas free ATP did not change significantly during the swimming trials. Intracellular pH decreased from around pH 7.48 to a minimal pH of 6.81 at critical swimming speeds indicating lactate formation (*16*).

Results from the initial set of experiments using NMR spectroscopy together with the differential pressure transducer are presented in Fig. 6. On the left part of the figure, typical pressure recordings from increasing swimming speeds are presented. The smooth sine curve of the traces is reflecting the tail movement and indicates controlled swimming. The spikes in the trace of the highest swimming speed resulted from the additional ''kick and glide'' swimming trials. On the right part of the figure, the results from the pressure traces are summarized. Mean tail beat frequency increased with swimming speed and leveled off when critical swimming speed was reached. Maximum critical swimming speed was around 70% of that compared to literature values from swimming cod (*17*, *20*, *25*, *26*), but are in line with observations from experiments on similar sized cod using pressure transducers (Gamperl, personal communication). Additionally, the recordings obtained with the pressure transducer allowed us to set trigger values for the acquisition of ^{31}P NMR spectra. In this way, spectra could be recorded at specific tail and therefore inductive coil positions relative to the receiver coil resulting in improved *S/N* ratios together with narrower line widths of the signals. Figure 7 shows an example of three gated in vivo ^{31}P NMR spectra at a flow rate of 9.3 m^3/h. The lower spectrum was triggered on the maxima of the pressure trace; the middle spectrum was triggered at the rising edge and the upper spectrum was gated on the minima of the pressure trace. The various trigger settings resulted in substantial differences of the *S/N* ratio with the best quality showing the spectrum gated on the minima. This trigger set point reflected the optimal position of inductive coil in relation to the receive coil resulting in the highest *S/N* ratio. Gating is especially advantageous at higher swimming speeds, i.e. near the critical swimming speed when animals started to use ''kick and glide'' bursts (see spikes in pressure trace at 14.0 m^3/h of Fig. 6). Furthermore, triggering allows acquisition of time-resolved ^{31}P NMR spectra for analyzing the metabolic machinery during exercise as for instance previously has been carried out in squid (*27*).

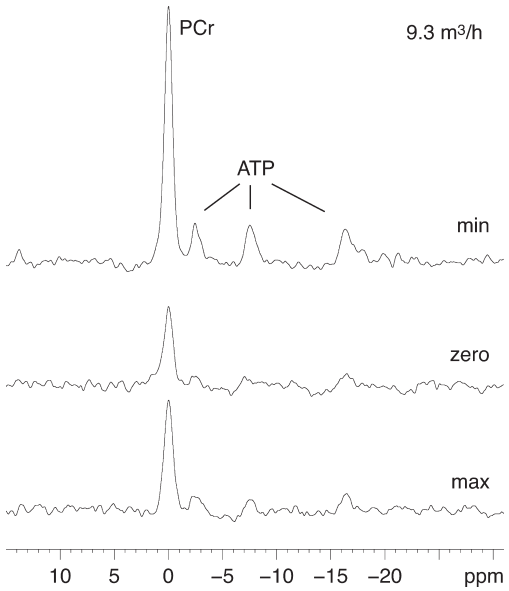

Figure 7 Example of three gated in vivo ^{31}P NMR spectra at a flow rate of 9.3 m^3/h and different trigger settings according to the pressure trace from Fig. 6. Note the different *S/N* ratios obtained with different trigger set points.

CONCLUSIONS AND PERSPECTIVES

This paper describes an integrated swim tunnel setup for online in vivo ^{31}P NMR spectroscopy studies of exercising marine fish. ^{31}P spectra were accumulated at sufficient *S/N* ratios within minutes allowing the characterization of energy metabolism and acid base regulation of working tail muscle (see *16*, *18*) in combination with oxygen consumption measurements (data not shown). Using differential pressure transducers improved spectra quality and allowed to trigger and monitor spectra during specific contraction phases and the resulting metabolic situation (*18*, *27*). As the use of differential pressure transducers is invasive, a next step will use the integrated camera system combined with online video analysis software for noninvasive triggering and online determination of swimming performance.

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4 Discussion

The ability of Atlantic cod to acclimate to different temperatures is considered within the hierarchical model of biological organisation proposed by Pörtner (2002). The mechanisms involved in acclimation at different levels are considered in relation to each other and data from previous experiments, to elucidate any trade-offs that may result.

4.1 Long-term thermal acclimation

Before we examine the processes underlying thermal acclimation in Atlantic cod, it is necessary to define and consider some terms and processes. The ability of animals to adapt to changes in temperature can be thought of in two ways. Adaptation is a process of permanent adaptation to a specific thermal niche that involves fundamental changes in the physiological, biochemical and/or molecular constitution of the animal, which are not readily reversible. This is a long-term process that typically spans several generations. Acclimatization, on the other hand, is a response seen in many ectotherms to seasonal fluctuations in the environmental. Mechanisms employed during thermal acclimatization to grant thermal independence are typically reversible upon reversal of the temperature change. Acclimation, as used in the present study, exploits these same processes under artificial conditions. Precht (1958) outlined 5 different types of thermal compensation that may be evident as a result of acclimatization/acclimation: type 1 = supra-optimal compensation; type 2 ideal (a.k.a. perfect) compensation; type 3 = partial compensation, type 4 = no compensation; and type 5 = inverse compensation.

4.1.1 Mitochondrial proliferation in the cold

Previous reports have found increased activity of the mitochondrial markers enzymes citrate synthase (CS) and cytochrome-*c*-oxidase (COX) in the white skeletal muscle from cold (4 °C) acclimated NSC and NEAC (Lucassen et al., 2006). Subsequent work has also found that CS activity is similarly increased in NSC cardiac muscle as a result of acclimation to 4 °C (Bremer, 2007). An increase in mitochondrial density is typical of cold acclimation in skeletal muscle (Egginton & Sidell, 1990). Indeed it is also a phenomenon exhibited as a result of cold adaptation (Johnston et al., 1998) with Antarctic icefishes having some of the highest mitochondrial volume

densities (~50%) reported for fish. Using stereological methods in the current study, it was possible to establish that the red skeletal muscle mitochondrial volume density was elevated 1.7 fold in NEAC and 1.3 fold in NSC as a result of acclimation to 4 °C (figure 4.1).

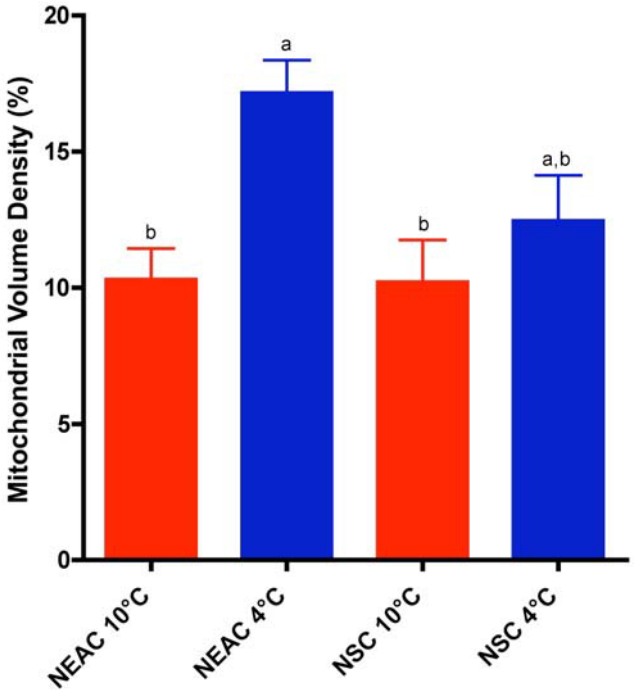

Figure 4.1. The mitochondrial volume density as a percentage of total red skeletal myocyte volume in 4 and 10 °C acclimated North East Arctic cod (NEAC) and North Sea cod (NSC). Dissimilar letters indicate a significant difference. Error bars represent SEM.

Two not necessarily exclusive hypotheses have been proposed to account for the increase in mitochondrial volume density typically seen during cold acclimation and adaptation. The first hypothesis states that with decreased temperature there is a higher proton leak rate (Hardewig et al., 1999), leading to a higher basal oxygen demand (Rolfe et al., 1999). The increased proton leak may be due to a) homeoviscous adaptations in the membranes which allow the membranes to retain flexiblity at lower temperatures, with the cost being that they are 'leakier' (Guderley, 2004), and/or b) an active method for reducing the membrane potential using uncoupling proteins, thus avoiding excessive production of reactive oxygen species (Brand, 2000; Guderley, 2004). An examination of respiration rates and membrane potential (proxies for the proton leak rate) in NEAC and NSC liver mitochondria found no differences either as a result of acclimation temperature, or between the two populations (Polleichtner, 2006).

The second hypothesis proposed by Sidell (1983), states that the increase in mitochondrial volume density serves three functions, all related to diffusion. Firstly, the reduced oxygen diffusion rate is reduced at low temperature and mitochondrial

proliferation may serve to mitigate this by simply increasing the absolute surface area exposed to oxygen. Secondly, the increased number of lipid membranes associated with mitochondrial proliferation aids intracellular oxygen diffusion by acting as an oxygen conduit. And thirdly, more mitochondria distributed throughout the cell may aid in the diffusion of ATP to sites of use (Jones, 1986; Sidell & Hazel, 1987; Kinsey et al., 2007). This limited diffusion hypothesis is also supported by the fact that despite the high mitochondrial volume densities exhibited by the haemoglobinless icefishes, the cristae volume density (i.e. the reactive core of the mitochondrion) is reduced compared to red-blooded Antarctic fishes (Johnston et al., 1998). Thus, icefishes increase the muscle cell volume devoted to mitochondria, and therefore membranes available for conduction of oxygen, while reducing the reactive surface area, minimizing cellular cost attributable to proton leak (Guderley, 2004).

Nevertheless, mitochondrial proliferation as a result of cold acclimation has down stream effects, two of which were hypothesized to impact on swimming performance. Firstly, mitochondrial proliferation may lead to a relative increase in the standard metabolic rate (Fischer, 2002), and this may in turn reduce the aerobic scope, thus reducing the critical swimming speed. Furthermore, a trade-off for mitochondrial proliferation typically seen during cold acclimation is a reduction in the number of myofibrils due to space and diffusion constraints, the so called 'optimal fibre number hypothesis' (Johnston et al., 2003, 2004, 2005), which leads to increased red muscle mass and compression of the recruitment order (see section 1.1). Ultimately, the compression of recruitment order means that a gait change to kick and glide swimming occurs earlier, i.e. at lower speeds. The effects of both SMR and recruitment are dealt with in publication III and below (section 4.1.3).

4.1.2 Thermal acclimation in the heart

We must first consider the potential impacts mitochondrial proliferation may have had on cardiac performance because the heart is of such importance with respect to swimming performance (Farrell, 2002). The heart appears to be the limiting factor during exercise (Kiceniuk & Jones, 1977; Farrell & Clutterham, 2003), and in setting acute thermal tolerance limits in fish (Pörtner, 2002; Gollock et al., 2006). At a cellular level, the mitochondrial proliferation seen in 4 °C acclimated cod would have increased the cellular oxygen demand (Rolfe et al, 1999). Within myocytes, myoglobin facilitates oxygen diffusion and is known to respond to increases in mitochondrial

oxygen demand, with myocytes increasing the expression of myoglobin (Wittenberg & Wittenberg, 2007). An increase in the myoglobin expression as a result of cold acclimation was seen in NEAC and NSC in the current study (publication I), indicative of the increased oxygen demand due to mitochondrial proliferation within cardiac myocytes.

According to the optimal fibre number hypothesis (see section 1.1), the increase in mitochondrial volume density after cold acclimation reduces the number of contractile fibres. To compensate for this, and reductions in enzyme activity, some fishes are able to express different myosin heavy chain ATPase (the enzyme that powers muscle fibre contraction) isoforms, which exhibit different thermodynamic properties. For example, the extremely eurythermal, polyploidal fish, the crucian carp (*Cyprinus carpio*), possesses 28 different MHC genes in skeletal muscle (Gerlach et al., 1990) that are differentially expressed depending on temperature and/or developmental stage (Goldspink et al., 1992; Cole & Johnston, 2001; Nihei et al., 2006). Myosin heavy chain isoform switching has also been seen as a result of acclimation temperature in the heart (Vornanen, 1994). Different isoforms are also known to be expressed in Atlantic cod skeletal muscle during development (Hall et al., 2003). In the current study a reduction in MHC activity was seen due to reduced assay temperature, however, no differences were seen in the cardiac myosin heavy chain activity as a result of acclimation temperature (figure 4.3), indicating there was no MHC isoform switching between 4 and 10 °C.

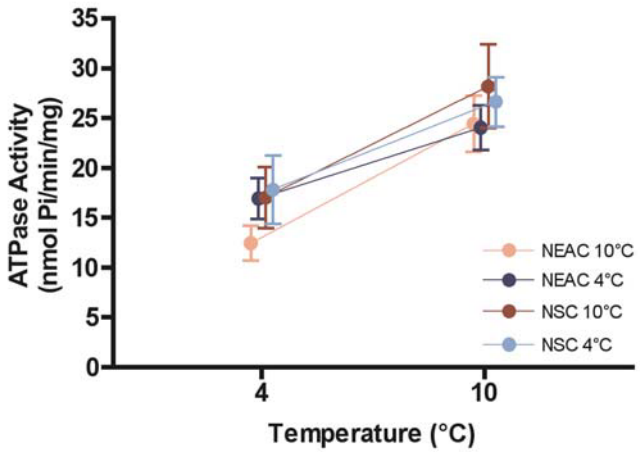

Figure 4.3. Myosin heavy chain ATPase enzymatic activity from 4 and 10 °C acclimated North East Arctic cod (NEAC) and North Sea cod (NSC) measured at 4 and 10 °C. Error bars represent SEM.

Another mechanism typical of cold acclimation that compensates for the reduced cardiac performance with reduced temperature is an increase in the heart size,

which in turn increases stroke volume (Graham & Farrell, 1989; Kolok & Farrell, 1994; Farrell, 1996). This is a phenomenon common to both cold acclimatized/acclimated fishes and cold adapted fishes (Farrell, 1996). No significant temperature effect was seen on relative ventricle size in NEAC and NSC, or from Atlantic cod from Newfoundland (NFC, see below, section 4.1.3). However, there was a tendency towards cardiac hypertrophy with reduced temperature that was more pronounced in NEAC and NSC (figure 4.2). This may be due to the longer acclimation period (>1) year for the NEAC and NSC versus 6 - 8 weeks for the NFC (see table 2.1).

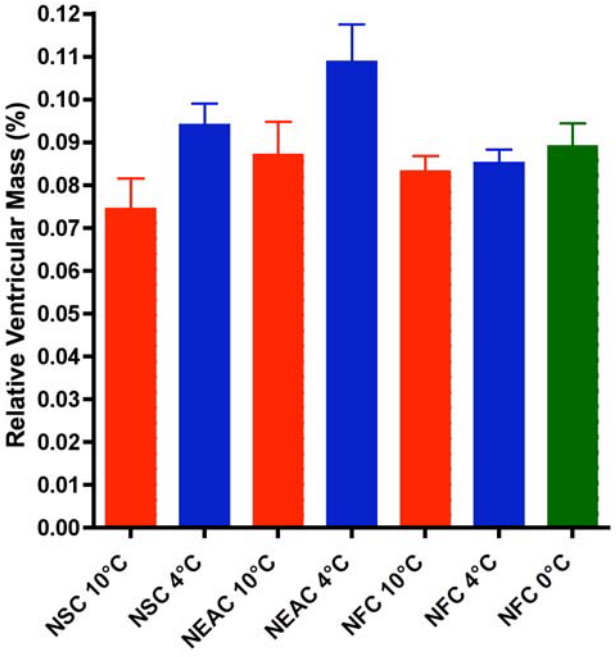

Figure 4.2. The relative ventricular mass in 10 and 4 °C acclimated North East Arctic cod (NEAC), North Sea cod (NSC), and 10, 4 and 0 °C acclimated Newfoundland cod (NFC). Both the NEAC and NSC were acclimated for >1yr and the NF for a minimum of 6 weeks. Error bars represent SEM.

4.1.3 *In situ* cardiac performance and temperature

At an organ level, a series of experiments examining *in situ* cardiac performance in 10, 4 and 0 °C acclimated NFC found that, in general, the maximum cardiac output was significantly reduced as acclimation temperature was lowered (figures 4, publication II), and that this reduction was the result of the significant effect of temperature primarily on heart rate. The heart rate and cardiac output data obtained with the *in situ* heart preparation compare well with *in vivo* data previously published for exercising Atlantic cod from Newfoundland and Nova Scotia. Webber et al. (1998) saw a tight relationship between the whole animal oxygen consumption rate and the cardiac output at 10 and 5 °C. This was also reflected by an increase in the heart rate. During swimming, cardiac output and heart rate increased from 12 to 35 mL kg^{-1} min^{-1}

and 28 to 40 bpm respectively at 10 °C and from 9 to 31 mL kg^{-1} min^{-1} and 16 to 28 bpm respectively at 5 °C (Webber et al., 1998). In contrast to the current study and that by Gollock et al (2006; see below), the stroke volume was also observed to increase concomitantly with heart rate and cardiac output (Webber et al., 1998). Claireaux et al. (1995) also saw an increase in the heart rate at 5 °C from 33 bpm to 40 bpm with increasing swimming speed, however this was not significant, perhaps owing to the sub-critical speeds at which the fish were swum, as was previously seen in brown trout (Butler et al., 1992).

The data in the current study (publication II) also compares well with recent *in vivo* data in Newfoundland cod where an acute temperature increase from 10 °C (acclimation temperature) up to the critical temperature caused a steady increase in heart rate and cardiac output, but no change in stroke volume (Gollock et al., 2006). It also differs substantially from the *in vivo* data from Lannig et al. (2004) who, during the same acute temperature increase from 2 up to 18 - 19 °C in 10 °C acclimated NSC, found an increase in blood flow from 2 up to only 6 - 7 °C when it plateaued. Consequently, the authors ascribed the ensuing oxygen limitation of thermal tolerance to the progressive mismatch between the increased oxygen demands of the various tissues and the stagnation of cardiac output and therefore oxygen supply by the heart above 7 °C.

The differences between these studies were subsequently ascribed to population differences (Gollock et al., 2006), but it is worth commenting on the two different techniques used to determine cardiac output in these experiments. Gollock et al. (2006) used a standard self-calibrating Transonic® flow probe around the ventral aorta. Lannig et al. (2004) used the NMR snapshot FLASH imaging technique, typically used in medical studies to measure increases in blood flow to various organs (Laub et al., 1998). This technique was not calibrated against a known flow rate and the authors also conceded that it may have underestimated peak blood flow (Lannig et al., 2004). Additionally, axial images were obtained from the aorta dorsalis and vena cordalis at approximately 2/3 of the body length. The different locations where blood flow was measured may contribute to different responses to temperature. It may be that as temperature is increased, blood flow to non-essential tissues, i.e. the anaerobic white musculature, may be reduced to a minimum while flow to more vital organs, i.e. brain, opecular and bucal pumps and the heart are increased, in a pattern opposite to the regional redistribution of blood flow seen during exercise (Taylor et al., 1996a;

Taylor et al., 1996b). Consequently, the blood flow to the white muscle may not have changed during rising temperatures, but this does not mean cardiac output was unaffected by increasing temperature.

The Q_{10}s for heart rate, cardiac output and power output in NFC acclimated to and tested at 10 and 4 °C indicated ideal, if not slight supra optimal-compensation (table 4.1). Between 4 and 0 °C however there was no compensation in power output and only partial compensation of CO and HR. This reflects a similar observation in Atlantic cod of a much greater thermal sensitivity of SMR and AMR at extremely low temperatures, (<2 °C) in modelled data from many different cod populations (Claireaux et al., 2000). The enhanced thermal sensitivity seen here was most likely because the lower thermal limit had been reached. The effects of temperature on cardiac performance during acute thermal challenge are dealt with in a separate section (4.1) below.

Table 4.1. Calculated Q_{10} values for the cardiac performance parameters, power output (PO), cardiac output (CO), heart rate (HR), and stroke volume (SV), as a function of long-term thermal acclimation (native) and acute thermal challenge (acute) and adrenaline (AD) dose in Newfoundland cod.

		AD (nM)	PO (mW g^{-1})	CO (mL min^{-1} kg^{-1})	HR (bpm)	SV (mL kg^{-1})
Native	Q_{10} 10-4 °C	5	0.77	0.56	0.60	1.12
		200	0.69	0.52	0.47	1.13
Acute	Q_{10} 10-4 °C	5	1.37	0.78	0.67	1.17
		200	1.28	0.77	0.67	1.24
Acute	Q_{10} 4-10 °C	5	0.18	0.20	0.40	0.57
		200	0.19	0.22	0.40	0.65
Native	Q_{10} 4-0 °C	5	1.98	1.30	1.52	0.82
		200	1.61	1.25	1.35	0.82
Acute	Q_{10} 4-0 °C	5	0.58	0.94	1.18	0.66
		200	0.62	0.77	1.17	0.64

As mentioned above, the ability for stroke volume to be compensated while heart rate and cardiac output were not reflects what was seen *in vivo* in these cod (Gollock et al., 2006). What was most surprising is that the preservation of stroke volume extended down to 0 °C, regardless of acclimation or acute exposure to 0 °C! This is surprising given the lethargy of these fish at 0 °C (Brown et al., 1989; pers. obs.). We can conclude that any preservation of the stroke volume is not achieved by cardiac hypertrophy. Nor was it attributable to adrenaline (publication II). We must therefore

conclude that the compensation of stroke volume in Atlantic cod with decreasing temperature is due to other mechanisms. These may include alterations in the ion channel conductivity, calcium flux/handling (Taylor et al., 1996b).

4.1.4 Swimming performance and temperature

An examination of swimming performance was undertaken in 10 and 4 °C acclimated NEAC and NSC (publication III) to examine potential effects of acclimation on swimming performance. In publication III a clear reuction in the critical swimming speed was seen at 4 °C, regardless of acclimation temperature or population. From table 4.2 it can clearly be seen that the kick and glide duration was significantly reduced from approximately 55 minutes at 10 °C to approximately 35 minutes at 4 °C. Furthermore, this pattern was consistent irrespective of acclimation temperature (for a full discussion of acute temperature change effects, see section 4.2 below). The compression of recruitment order was such that kicks are used at lower speeds and for a shorter duration. The crucial swimming speeds were also lower at 4 °C than at 10 °C (figure 6, publication III).

Table 4.2. Kick and glide duration (in minutes). Mean ± SEM. An asterisk indicates significantly different to 10 °C.

Test temperature	NEAC 10 °C	NSC 10 °C	NEAC 4 °C	NSC 4 °C
10°C	53.8 ± 18.0	58.5 ± 12.1	52.3 ± 11.7	54.2 ± 13.0
4°C	38.0 ± 5.9	36.6 ± 7.2 *	33.2 ± 4.9 *	33.8 ± 8.2 *

Because kick and glide swimming is anaerobically fuelled (see section 4.1.4 and publication IV), the shorter duration of kicking also reflects a shift in the preferred metabolic pathway to aerobic metabolism with decreasing temperature. This shift has previously been observed in Norwegian coastal Atlantic cod in the form of reduced LDH activity at lower temperatures and taken to imply a reduction in the anaerobic capacity (Zakartsev et al. 2004). Similarly, Martinez et al. (1999) found aerobic pathways to be enhanced in cold acclimatzed Newfoundland cod. Indeed different aerobic and anaerobic capacities were documented in different populations of cod from Nova Scotia, where the authors concluded that despite of the similar U_{crit}s, Bras d'Or cod were more reliant on anaerobic metabolism, as evidenced by a significantly

greater build up of anaerobic end products, and greater ion disturbances during critical swimming than oceanic Nova Scotian cod (Nelson et al., 1994).

In addition to the compression of the recruitment order, evident in the reduced kick and glide duration, the swimming efficiency was also affected by temperature and may therefore have reduced U_{crit}. The swimming efficiency was higher in fish at 10 °C, i.e. the oxygen consumption increment was lower as a function of speed (figure 4.4). From table 4.3 we can also see that two groups predominate, i.e. fish swum at 4 and at 10 °C and that the slopes from these groups differ significantly from each other and fall into two temperature groups. We can conclude that in addition to compressed recruitment order, the trade off for decreased swimming efficiency at lower temperatures was a decrease in the critical swimming speed.

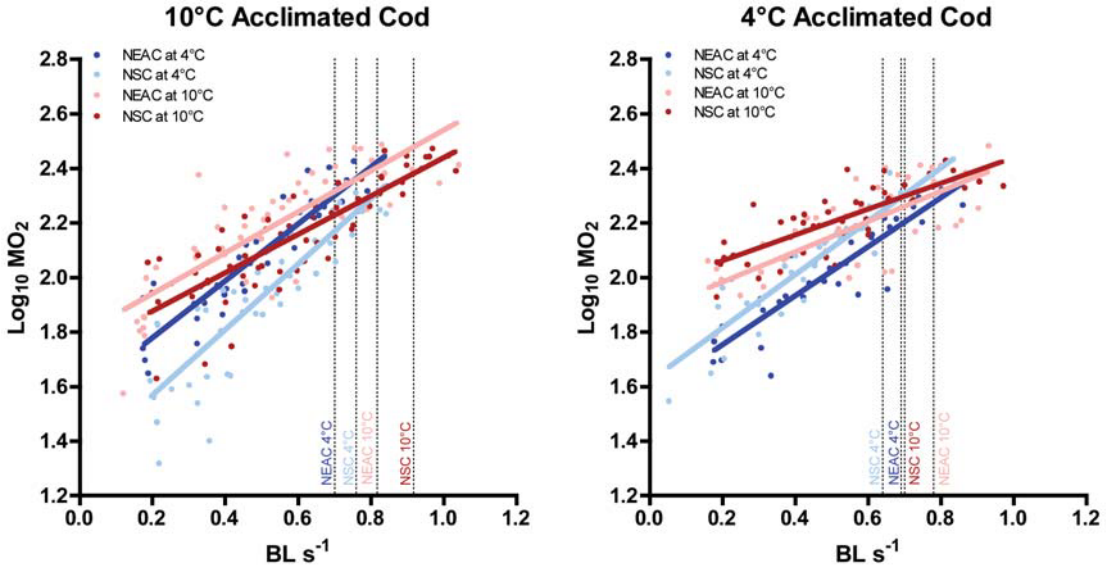

Figure 4.4. Log_{10} of the oxygen consumption rate (MO_2) as a function of swimming speed in 4 and 10 °C acclimated North East Arctic cod (NEAC) and North Sea cod (NSC). Temperatures given in the legend are those at which the fish were swum. Dotted vertical lines represent the mean U_{crit} for the respective group at the given swim temperature.

While the y-intercepts are comparable with other studies (Bushnell & Steffensen, 1994; Schurmann & Steffensen, 1997; Bremer, 2007), the slopes observed in the current study are considerably higher than those previously reported for smaller, juvenile cod, e.g. 0.12 for 4 °C Greenland cod (Bushnell & Steffensen, 1994), 0.36, 0.35 and 0.28 for 5, 10 and 15 °C acclimated NSC respectively (Schurmann & Steffensen, 1997), and 0.16 - 0.25 for 5 and 16 °C acclimated NSC respectively

(Bremer, 2007). This may be a) attributed to the increased drag and turbulence as a result of the NMR inductive coil and/or b) an artefact due to the size differences between the studies. Although the actual cost of transport may be significantly higher in smaller fish due to increased relative water viscosity and reduced inertia (Videler, 1981 & 1993), the higher basal metabolic rate seen in smaller fish combined with the smaller aerobic scope will reduce the apparent efficiency.

Table 4.3. Equations and regression coefficients for $\log_{10}$ of respiration. Values are mean ± SEM.

Population	NEAC	NSC	NEAC	NSC	NEAC	NSC	NEAC	NSC
Acclimation Temperature	4 °C	4 °C	10 °C	10 °C	10 °C	10 °C	4 °C	4 °C
Swim Temperature	4 °C	4 °C	4 °C	4 °C	10 °C	10 °C	10 °C	10 °C
Slope	0.90 ± 0.07	0.98 ± 0.07	1.04 ± 0.07	1.20 ± 0.10	0.78 ± 0.07	0.70 ± 0.06	0.57 ± 0.08	0.47 ± 0.05
Y-intercept	1.57 ± 0.04	1.62 ± 0.04	1.57 ± 04	1.33 ± 0.05	1.82 ± 0.04	1.74 ± 0.04	1.92 ± 0.05	1.97 ± 0.03
R^2	0.8088	0.8389	0.8504	0.7686	0.7075	0.7136	0.5374	0.6367

The higher relative oxygen demand at 4 °C may have reduced the kick and glide duration, with the consequence being that the U_{crit} was reduced after cold acclimated cod. We may therefore interpret this to mean that there was a mismatch between oxygen demand and oxygen supply, i.e. an oxygen limitation that set in earlier at 4 °C. This then begs the question, how did this oxygen limitation result in fatigue?

4.1.5 How does oxygen limitation result in fatigue?

The use of a specifically developed ^{31}P-NMR spectroscopic technique in combination with a swim tunnel, as detailed in publications IV and V, gave us new insight into the real time metabolic processes that are involved across the spectrum of different swimming gaits employed by Atlantic cod. A transition point was seen between the use of subcarangiform swimming, which proved to be fuelled aerobically and the use of kick and glide, which was fuelled anaerobically. This was evidenced by a significant increase in inorganic phosphate, a product of the hydrolysis of ATP, and decreases in phosphocreatine and intracellular pH. Furthermore, these changes were clearly visible after the exercise bout had stopped, i.e. during the initial stages of recovery, indicating that an 'oxygen debt' had been accumulated. Thus, unlike other

critical parameters, such as temperature, which are marked by a transition from aerobic to anaerobic metabolism (see definition in section 1.1), U_{crit} marks the point of complete exhaustion when both aerobic and aerobic resources have been fully expended.

With respect to the immediate cause of fatigue, it was found that the increase in inorganic phosphate concomitant with a significant decrease in the intracellular pH and phosphocreatine once kicking was initiated (all indicators of anaerobic metabolism), led to a drop in the Gibbs free energy. The result of this drop in the Gibbs free energy was that cellular processes, in particular muscular contraction via the myosin heavy chain ATPase, could not derive enough energy from ATP hydrolysis to continue functioning resulting in fatigue.

4.2 Acute temperature change

In their natural environment, Atlantic cod are exposed to a wide range of temperatures (see section 1.5). These variations are not only the result of seasonal changes. Atlantic cod also experience diel changes in temperature, (e.g. Gollock et al., 2006). Therefore it was also necessary to see what effect acute temperature change had on the same physiological functions. As acclimation generally shifts a thermal window and consequently the optimal temperature for a given physiological function (Pörtner, 2001), differences in cardiac performance and swimming performance were expected between cod acclimated to a given temperature and cod acutely exposed to the same temperature.

In NFC, an acute reduction in temperature from 10 to 4 °C resulted in only slight reductions in *in situ* cardiac performance. An acute decrease from 4 to 0 °C resulted in supra-compensation of all parameters except heart rate which was almost ideally compensated (table 4.2). Acute warming from 4 to 10 °C lead to significant supra-compensation in cardiac performance and power output (figure 3C and 4 in publication II, table 4.2 above). This may well be a product of changes in the electrophysiological state of the heart (as discussed in publication II).

There were slight differences seen in swimming efficiency, where 10 °C acclimated cod at 4 °C were slightly more efficient than the 4 °C acclimated cod at 4 °C, and the 4 °C acclimated cod were less efficient than 10 °C acclimated cod at 10 °C. This was also reflected in the net and factorial aerobic scopes which were consistently higher in both 10 °C acclimated cod populations. However, in general, this had no effect on their performance as all physiological parameters quantified

during swimming, i.e. SMR, AMR, kick and glide duration and U_{crit}, were similar for both NEAC and NSC swum at 4 or 10 °C, regardless of their acclimation temperature. Gulf of St. Lawrence cod acclimated to 7 °C and swum at 3, 7 and 13 °C also had essentially the same SMR, AMR and U_{crit} at 7 and 13 °C, however these parameters were significantly lower at 3 °C (Sylvestre et al., 2007), indicating again that cod are much more thermally sensitive at extremely low temperatures than they are at moderately warmer temperatures.

All of the cod populations used in the current study are likely to experience variations of 6 degrees or more during their daily activities, therefore it is not entirely surprising that the physiological functioning is maintained between 4 and 10 °C, particularly because this lies within their preferred thermal window (Tat'yankin, 1972; Bøhle, 1974; Jobling, 1988; Brown et al., 1989; Björnsson, 2001; Despatie et al., 2001; Petersen & Steffensen, 2003; Lafrance et al., 2005). A drop down to 0 °C is less likely. Woodhead and Woodhead (1959) state that NEAC generally avoided staying in water below 2 °C for extended periods, although they were known to venture into sub-zero waters for short periods. It would appear that 2 - 3 °C represents a threshold for Atlantic cod below which physiological function, and with it presumably the ability to reproduce deteriorate.

4.3 Metabolic cold adaptation: Fact or artefact?

Initial evidence for metabolic cold adaptation (as outlined in section 1.1) in NEAC was first presented by Fischer (2002), where he directly measured SMR in both NEAC (weight range 207-559g) and NSC (169-325g) at 4 and 12 °C. Fischer found that 4 °C acclimated NEAC had a significantly higher SMR than the 4 °C acclimated NSC. The method used in the current study to measure SMR was by extrapolation back from various swimming speeds to a swimming speed of zero, i.e. inactivity, a method reputedly more robust and reproducible (Reidy et al., 2000). No differences were found between any of the groups at either 4 °C or 10 °C (figure 4A, publication III). Subsequent work measuring SMR directly has also failed to find any difference between NEAC and NSC SMR at any given temperature (Zittier, 2006; Miest, pers. comm.), refuting any claim of MCA, at least in its' traditional sense. The only respiratory differences seen between NEAC and NSC in the current study were between the Q_{10}s for SMR, which clearly showed that SMR was uncompensated in NEAC, but partially

compensated in NSC. This may be due to differing abilities to regulate cellular mitochondrial volume density during long-term acclimation.

Contentious evidence indicates that there may be a form of MCA evident at a cellular/biochemical level. Both Lannig et al. (2003) and Lucassen et al. (2006) found CS and COX activity to be significantly higher in 4 °C acclimated NEAC compared to 4 °C acclimated NSC. A cautionary note must be sounded, that the former study suffered from large differences in the size ranges of the fish between populations and their diet, while the latter also had issues regarding significantly different hepatosomatic indicies. While the current study also found differences in the degree to which red skeletal muscle mitochondria proliferated, i.e. 1.7 fold in NEAC compared to 1.3 fold in NSC at 4 °C (figure 4.1), these differences were not significant, and the liver mitochondrial oxygen consumption and proton leak rates in the very same fish did not differ between populations (Polleichtner, 2006). We can conclude that the evidence for metabolic cold adaptation in NEAC is weak at best, and only manifest at a cellular level.

4.4 Conclusions

One of the primary conclusions that can be drawn from the current data set, when taken in the context of other recent studies with cod, is that there is a definite shift in a preference from anaerobic to aerobic metabolic pathways as temperature is reduced. This is evident on several functional levels. The mitochondrial density increased during cold acclimation (figure, 4.1) to maintain aerobic scope in the face a thermodynamically driven reduction in mitochondrial efficiency (Pörtner et al., 2000) and/or diffusion (Sidell, 1983). Myoglobin expression was also up-regulated to aid the increased oxygen demand and facilitate oxygen diffusion within myocytes (publication I). This was concomitant with other molecular and biochemical changes previously documented in Atlantic cod. For example, the activity of aerobic metabolic enzymes such as CS and COX (Martinez et al., 1999; Lannig et al., 2003, Lucassen et al., 2006; Bremer, 2007) were demonstrably up-regulated during cold acclimation, while anaerobic enzymes, e.g. LDH (Martinez et al., 1999; Zakhartsev et al., 2004) were down-regulated. At the functional level, the shift towards aerobic pathways means that there was a reduced reliance on anaerobic swimming methods such as kick and glide and an increase in the factorial aerobic scope (publication III). Combined with a certain degree of thermodynamic slowing of metabolic processes, the increased oxygen demand would also reduce oxygen available for growth and reproduction, contributing

to the reduced growth rates and fecundity seen at lower temperatures (Kjesbu et al., 1998; Björnsson et al., 2001; Pörtner et al., 2001; Fisher, 2002).

Despite a compensatory shift in the preference for aerobic metabolism with decreasing temperature, acclimation to 4 or 10 °C had no significant effect on SMR or AMR (publication III). The SMR was approximately equal in fish acclimated to or acutely exposed to a given temperature. However, other important physiological parameters were compensated. The Q_{10} for the critical swimming speed between 10 °C acclimated cod at 10 °C and 4 °C acclimated cod at 4°C was 1.3 for NEAC and 1.6 for NSC. The Q_{10}s for U_{crit} during the acute thermal challenge were if anything, slightly lower, i.e. between 1.1 and 1.4, more indicative of thermal independence, or ideal compensation. A similar temperature effect was also seen on cardiac performance in NFC. While there was evidence of thermal compensation of most parameters measured (table 4.3), the high Q_{10}s for power and cardiac output between 0 and 4 °C from NFC tested at their acclimation temperatures contrasted sharply with the ideal/supra-compensation of power and cardiac output during an acute cooling from 4 to 0 °C.

We can therefore conclude that acclimation to low temperatures typical of winter extremes, (less than ~2 °C), will lead to more severe reductions in Atlantic cod performance than during an acute decrease to low temperatures. It is difficult to gauge whether this is a trait typical of cold acclimation as there are currently no other data on acclimation or acute change to such an extreme low temperature in other fishes. Such a phenomenon is thought to trigger dormancy (Bailey & Lazaridou-Dimitriadou, 1991), a phenomenon exhibited by many aquatic fishes at extreme temperatures that is used to conserve dwindling energy reserves (Guderley, 2004). This goes someway in explaining how NEAC are capable of short excursions into polar waters (Woodhead & Woodhead, 1959), but do not truly acclimate to temperatures below approximately 2 °C (Brown et al., 1989). It is reasonable to expect that the more severe reductions in performance seen following acclimation could be the result of a higher proportion of the cellular energy budget devoted to cellular maintenance at lower temperatures following acclimation, and therefore less remains for maximal performance.

This is one clear example of how, as a trade-off, thermal acclimatory process on one level can affect physiological functioning on another. In the hierarchy outlined by Pörtner (2002), there are different levels of biological organisation, each of which displays differing degrees of susceptibility to the effects of temperatures. The lowest

levels of biological organisation have the broadest temperature range due to their simplicity. As the levels of biological complexity increase, i.e. as the hierarchy is climbed, the thermal tolerance windows narrow. This explains why MHC is active at 22 °C and higher, mitochondria from 4 °C acclimated cod function up to 20 °C (Polleichtner, 2006), while 4 °C acclimated cod *in situ* cardiac performance is compromised at approximately 15 - 16 °C (Garmperl, pers comm.) and 10 °C acclimated cod die at temperatures between 18 and 22 °C (Sartoris et al., 2003; Lannig et al., 2004, Gollock et al., 2006). The ultimate product of this hierarchy is the relatively narrow set of optimal temperatures for growth and reproduction seen for cod (Kjesbu et al., 1998; Björnsson et al., 2001; Pörtner et al., 2001; Fisher, 2002), while still retaining the ability to withstand both seasonal and acute temperature changes.

4.5 Future Prospects

There is an absolute dearth of data for any fish at 0 °C other than Antarctic fishes. Many temperate water and Arctic fishes must withstand temperatures below 0 °C for extended periods, e.g. during winter. Evidence presented here indicates that 2 °C is a critical threshold for Atlantic cod, below which compensation of physiological function, that is to say acclimation ability becomes severely limited. The most immediate experiment required to complement the current data set would involve swimming cod at 0 °C to see whether swimming performance is also more severely affected at this extreme. Moreover, a data set for Atlantic cod at temperatures higher than 10 °C, e.g. 16 - 18 °C would also provide valuable insight into how they are able to withstand such extreme temperature ranges. These experiments must be undertaken in the field with large numbers of acclimatized wild fish, rather than artificially acclimated in a laboratory, thus significantly enhancing the data's ecological relevance.

A very large data set exists for Atlantic cod with respect to their ecophysiology, however, by far the biggest problem is in its' interpretation. The way fish are held and sampled has a significant impact on the results. As mentioned previously in this thesis (section 4.3) the enzyme activity for Atlantic cod, i.e. CS and COX is affected by diet, among other parameters. Starvation decreases the activity, while *ad libitum* feeding increases it (Martinez et al., 2002; Martinez et al., 2003). The composition of the diet, i.e. lipid to protein ratio will similarly influence enzyme activity (Brito et al., 1992; Iossa et al., 2002). Furthermore, the anatomical position on the fish from which white muscle samples are taken and the size of the fish also influence activity (Martinez et al.,

2000; Martinez et al., 2003). All these issues cloud any conclusions about thermal acclimatory effects on enzyme activity.

A similar case can be made for the way in which physiological parameters are measured. A classic example is the thermal preferences for Atlantic cod. Data from various sources point to two fundamentally different thermal preferences of 4 - 7 °C and 13 - 14 °C for Canadian and European Stocks (Tat'yankin, 1972; Bøhle, 1974; Jobling, 1988; Brown et al., 1989; Björnsson, 2001; Despatie et al., 2001; Petersen & Steffensen, 2003). This vast difference is more likely a product of the many and varied techniques used to establish the preference, ranging from optimal growth, to thermal shuttle boxes. And that is to say nothing of the effect of acclimatization (Clark & Green, 1991) or ontogeny (Lafrance et al., 2005) on thermal preference. Standardisation of holding, sampling and measurement techniques is sorely needed and would vastly improve interpretation of data produced into a coherent picture.

While the data set for Atlantic cod is indeed extensive, it can be seen there is still much work needed to unravel and understand the intricate and incredibly complex interplay between the molecular biochemical, cellular, physiological and ultimately ecological processes that determine the biological success of this hugely important resource.

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