MASTER THESIS

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BI309F Master thesis in Marine Ecology

Thermal tolerance of the lumpsucker (*Cyclopterus lumpus* L.): association between cardiac physiology and upper thermal limits

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Preface

This master thesis is a part of the degree 'Master of Science in Marine Ecology' at Nord University, and is the result of one year of work (60 credits). The thesis is a part of the research project CycLus, in cooperation with Nord University, to assess basic physiological capacity of the lumpsucker. First of all, I would like to thank my supervisor Torstein Kristensen for putting together an exciting study, and excellent guidance throughout the whole period. To my co-supervisors Martin Haugmo Iversen and Harald Takle, thank you for your input and proofreading. I would also like to thank the staff at Mørkvedbukta Research Station, especially Bjørnar Eggen for technical help and Heidi Hovland Ludviksen for training in histology. Nofima and Gerrit Timmerhaus, thank you for experimental training and borrowing of equipment. My family, boyfriend, and fellow students deserves a huge thanks for motivation and inputs to my final draft.

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Contents

Abstract

Organisms need optimal physical conditions for performance, and temperature is the master abiotic driver affecting all levels of biological organization. In an era of accelerating climate change, an understanding of how aquatic animals will respond to environmental stressors is crucial. The aim of this study was to examine the thermal tolerance through the association between physiological capacity and upper thermal limits of the lumpsucker (*Cyclopterus lumpus* L.), a semi-pelagic teleost that is increasingly being utilized in the salmon aquaculture industry as a sea lice grazer. Maximum heart rate (fH_{max}) related measures of thermal tolerance were compared between 7 days cold acclimated (N = 20) and warm acclimated (N = 20) fish, to determine the effects of acclimation on the optimal temperature for performance (T_{opt}), the maximal temperature for performance (T_{max}), and the critical temperature for performance by cardiac arrhythmias (T_{arr}). In addition, morphological variables of the ventricle were examined in response to temperature acclimation. Results were finally compared with Atlantic salmon because of the relevance in aquaculture settings where the two species are to coexist. The cold and warm acclimated lumpsuckers differed notably in their systemic responses to warming, where warm acclimation significantly increased T_{opt} with 2.2-2.9 °C, T_{max} with 1.2 °C, and an increase in the average highest maximum heart rate (Max fH_{max}) of 8.2 beats per minute (bpm). A small increase in the compact myocardium thickness of the ventricle with warm acclimation was also observed, which indicates a somewhat strong effect of the short acclimation period, although not statistically significant. As expected, the comparison with Atlantic salmon revealed that the two species differed markedly in their cardiac responses to acute warming and thus their thermal limits. The species responses of fH_{max} to warming were dissimilar in Max fH_{max}, with 150 bpm (salmon) versus 105 bpm (lumpsuckers), and lumpsuckers experienced a rapid decline in fH_{max} after achieving Max $fH_{max,}$ before reaching T_{arr}. The thermal limits of lumpsuckers were generally lower compared to that of salmon, regarding all three temperature variables investigated. In conclusion, this study demonstrates cardiac plasticity of the lumpsucker in response to warming, indicating that thermal acclimation is a possibility for survival in a warmer future. In addition, the physiological capacity and thermal limits found for the lumpsucker should be considered in aquaculture settings, to ensure optimal conditions for performance and taking the physiological differences between lumpsuckers and salmon into account.

1. Introduction

1.1 Status aquaculture

The aquaculture industry is the fastest growing food production system in the world, and while traditional capture fisheries has remained more or less stationary for a couple of decades, the rapid development in the aquaculture sector continues to increase (Beveridge et al. 2013; Ellis et al. 2016; Godfray et al. 2010). This is especially true for the global production of Atlantic salmon (*Salmo salar* Linnaeus, 1758), a key industry in providing economic security and employment in several countries like Norway and Scotland, which are two good examples of how trends within production systems have developed (Ellis et al. 2016). The importance of providing enough food for a growing population (FAO 2014) together with technological innovation (Asche & Bjorndal 2011; Ellis et al. 2016), namely volume and production, are two main factors that have promoted the aquaculture, or blue "revolution", which has been commercially significant since the 1980s (Asche & Bjorndal 2011; Hovland 2014).

The salmonid aquaculture industry in Norway has experienced a tremendous development since the very beginning in the 1970s (Kolle 2014c). Atlantic salmon and rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792) production accounted for 94% and 5.1%, respectively, of the 1.35 million tons of fish and shellfish produced in Norway in 2014. With products of a value of 43.5 billion NOK and 6350 employees the same year (SSB 2015), there is no doubt that salmonid farming is of great economic importance for Norwegian coastal communities (Kolle 2014b).

Along with rapid expansion and many opportunities in the industry, challenges emerged already at an early stage in the aquaculture "adventure" (Hovland 2014; Kolle 2014a). Salmon biomass production in Norway today is currently limited, primarily due to environmental impacts on wild salmonid populations. The possibility for growth, which includes the number and size of fish farms, is controlled by governmental regulations and demands regarding sea lice combats (Asche et al. 2013; Winther et al. 2015). More specifically, the abundance of sea lice associated with wild fish populations is suggested as an index to evaluate the environmental effect of Norwegian aquaculture, because the number of dispersed lice is increasing along with the increase of hosts in fish farms (Karlsen et al. 2016). Other environmental challenges are also contributing to the restrictions, and The Institute of Marine Research (IMR) in Norway is conducting a yearly risk assessment of environmental impacts of open sea-cage farming.

Taranger et al. (2015) based on Anon. (2009) with the 'Strategy for an Environmentally Sustainable Norwegian Aquaculture Industry', focus upon problems associated with genetic interactions between farmed and wild salmon, the spread and impact of the salmon louse (*Lepeophtheirus salmonis* Krøyer, 1837), disease transfer from farmed to wild salmonid populations, and the impacts of nutrient and organic load on benthic ecosystems in the vicinity of farms. Other ecological aspects of concern are harvest of wild fish resources for use in aquaculture as fishmeal, (Asche & Bjorndal 2011; Bendiksen et al. 2011; Davenport et al. 2009; Deutsch et al. 2007) and the ecological footprints sea-cages and constructions might leave (Beveridge 2008; Ervik & Hansen 2007; Hersoug 2014). A more recent topic raising concern is the welfare of farmed fish (Ashley 2007). In other words, to be able to increase the aquaculture production further, a reduction in environmental pressure and increased fish welfare and biological knowledge must follow (Ellis et al. 2016; Garnett et al. 2013).

Sea lice (Copepoda, Caligidae), especially *Lepeophtheius salmonis* and *Caligus elongatus* Nordmann (1832), are the two most common pathogenic marine parasites in the North Atlantic, causing infestations in salmonid farms as well as in wild populations (Boxaspen 2006; Costello 2006; Costello 2009b). *L. salmonis* has the greatest economic impact, and are reported to cause damages on salmonids ranging from mild skin lesions to stress induced responses and subsequent mortality (Bjørn et al. 2001; Heuch et al. 2005; Tully & Nolan 2002).

The control of sea lice in salmon farms has mainly been in the form of bath treatments or through inputs in feed (Boxaspen et al. 2007; Burridge et al. 2010; Denholm et al. 2002). A disadvantage with previous and current chemical treatment is the ability of sea lice to acquire resistance. The salmon louse has shown increased resistance or reduced sensitivity to several delousing agents, including avermectins (Burridge et al. 2010; Lees et al. 2008), organophosphates (Fallang et al. 2004; Jones et al. 1992; Tully & McFadden 2000), pyrethroids (Burridge et al. 2010; Sevatdal & Horsberg 2003), and hydrogen peroxide (Treasurer et al. 2000). In addition, the drugs used can potentially disturb ecosystem resilience and have harmful side effects on non-target organisms where farms are situated (Burridge et al. 2010). The economic costs are also very high (Costello 2009a), and the stress of the fish when exposed to different chemicals is important to consider (Burka et al. 1997).

1.2 Biological delousing with cleaner fish

The need for a sustainable and environmental friendly alternative to the use of chemicals in lice control, has led to use of wrasse species (Labridae) in co-culture with salmon in sea-cages. Already in 1976, the Norwegian magazine *Fiskaren* published an article describing how a farmer used the ballan wrasse (*Labrus bergylta* Ascanius, 1767) for picking sea lice off farmed salmon (Kvenseth & Øien 2009; Nævdal & Hovland 2014). Since then, three other temperate wrasse species have been used as 'cleaner fish' in salmonid farms. These includes the goldsinny wrasse (*Ctenolabrus rupestris* Linnaeus, 1758), corkwing wrasse (*Symphodus melops* Linnaeus, 1758), and the rock cook (*Centrolabrus exoletus* Linnaeus, 1758). These wrasse have been found to control sea lice infestation in trials and commercially already from the late 1980s and early 1990s in Norway (Bjordal 1988; Bjordal 1991).

The use of wrasse in delousing to keep lice numbers at a minimum level is less costly for the industry, and regarded more safe for the consumer than chemical usage alone (Denholm et al. 2002; Treasurer 2002). Despite the advantages of wrasse as a delousing agent, there is a great deal of variability in their effectiveness. The ecology and life-history characteristics are poorly documented, but they have high site fidelity (Espeland et al. 2010; Skiftesvik et al. 2014) and populations seems to be genetically differentiated (Cowx et al. 1998). This makes wild populations vulnerable to exploitation and a transfer to new places (Sayer et al. 1996a; Skiftesvik et al. 2014; Varian et al. 1996). The distribution of wrasse in Northern arctic waters is scarce, and an inactive state during winter makes temperature-dependent activity a limiting factor to the use in delousing (Cowx et al. 1998; Lein et al. 2013; Sayer et al. 1996b).

A more recent species that has been observed grazing lice off salmon, is the lumpsucker (*Cycopterus lumpus* Linneaus, 1758) (Imsland et al. 2014a). The lumpsucker has a circumpolar distribution in the Northern hemisphere (Figure 3A), and seems to tolerate the year around temperature fluxes in aquaculture settings (Figure 1) better than wrasses, thereby having a greater appetite for lice during winter months (Nævdal & Hovland 2014).

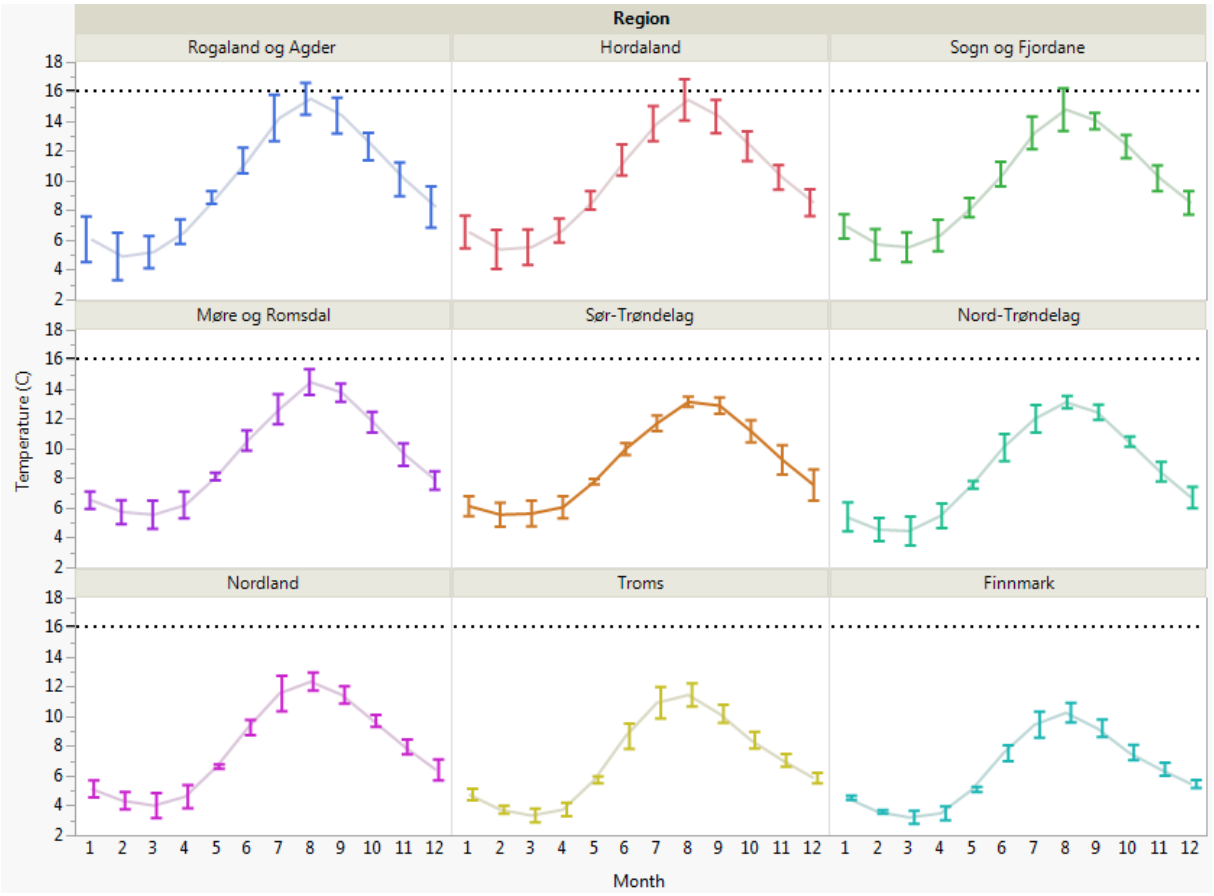

Figure 1. Monthly temperature (°C) averages (means ± standard deviation) reported from all active aquaculture facilities in 9 regions of Norway, from 2011 to 2015. Number of month (1-12) is plotted on the x-axis and temperature on the y-axis. The dotted lines indicate a reference temperature of 16 °C (Lusedata 2016).

1.3 The lumpsucker *Cyclopterus lumpus*

The lumpsucker has generally been considered an unpalatable fish for human consumption, but a fishery for its roe started in the 1950s and has since been commercially significant in Norway, Island, Canada, and Denmark. Indications of over-exploitation since the mid-1980s have led to reduced catch quotas in the Barents Sea (Durif 2014; Goulet et al. 1986; Sunnanå 2007).

The appearance of the lumpsucker is very characteristic and unique, it has no close relatives and is the only species in the genus *Cyclopterus* (Davenport 1985). The compressed body shape makes it look thick and short, and there are no scales or prominent skin layer. A high dorsal crest covers the dorsal fin entirely, modified pelvic fins constitutes a sucker disc, and there are three longitudinal rows of tubercles on each side of the body (Figure 2) (Davenport 1985).

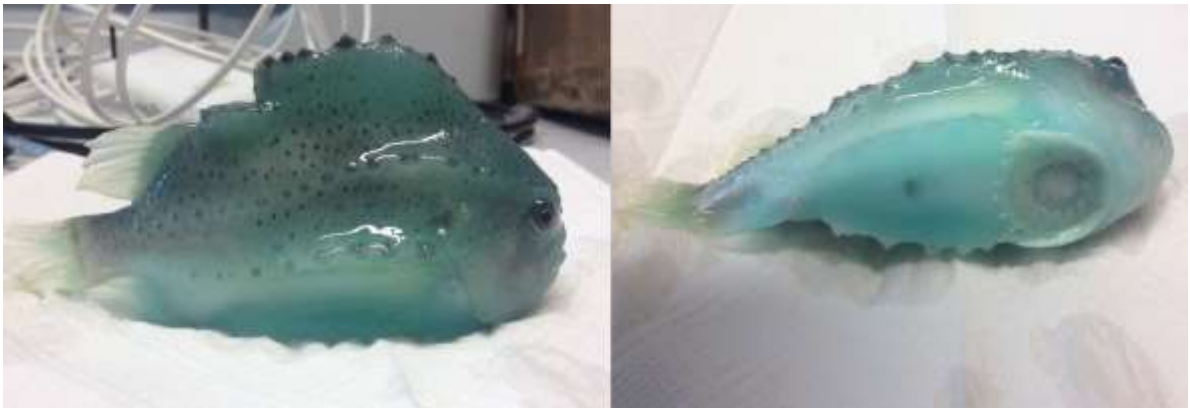

Figure 2. A characteristic lumpsucker, with prominent tubercles alongside the body, a distinct dorsal crest, and a ventral sucker disc (Photo: Nina S. Iversen).

A pronounced sexual dimorphism characterizes the maximum size, and also coloration of lumpsuckers during spawning. The female is usually larger than the male, and males turns red-pink during spawning while females are blue-green (Davenport 1985; Davenport & Thorsteinsson 1989). Because the lumpsucker lacks a swim bladder, buoyancy is achieved mainly through a cartilaginous skeleton and subcutaneous jelly tissue in the dorsal crest, which together makes the density of the body close to that of seawater (Davenport & Kjørsvik 1986). The largest individuals are usually around 60 cm and 5-6 kg, and can live to become 7-8 years (15 maximum) (Durif 2014).

The lumpsucker is widely distributed on both sides of the North Atlantic (Figure 3A). From Svalbard, the Barents Sea, and the White Sea to Portugal on the eastern side, and Greenland and Canadian waters to Cape Cod on the western side (approximately 80 °N – 32 °N, 95 °W – 49 °E) (Blacker 1983; Cox & Anderson 1922; Davenport 1985). The yearly mean sea surface temperatures of the North Atlantic Ocean are shown in Figure 3B, which indicates the thermal regime occupied by the lumpsucker.

Adult lumpsuckers lack Mauthner cells (Hale 2000), which are neurons triggering the fast escape response and are thus affecting the survival of fish (Domenici 2010; Eaton et al. 2001; Zottoli 1977). Despite having poorly developed morphological swimming capabilities and sharing many morphological features with benthic fish, the lumpsucker is regarded a semi-pelagic species that migrates long distances between offshore feeding areas and coastal spawning grounds (Davenport 1985; Thorsteinsson 1983). Spawning takes place in the sublittoral zone mainly during spring, where males must attract females to their nesting sites and are thereby left to guard the egg masses laid by several females (Daborn & Gregory 1983;

Davenport 1985). It is suggested that females spawn several batches over various locations, leaving the males with the eggs for about two months while migrating in and out of the coast and fjord systems (Goulet et al. 1986; Mitamura et al. 2007; Mitamura et al. 2012).

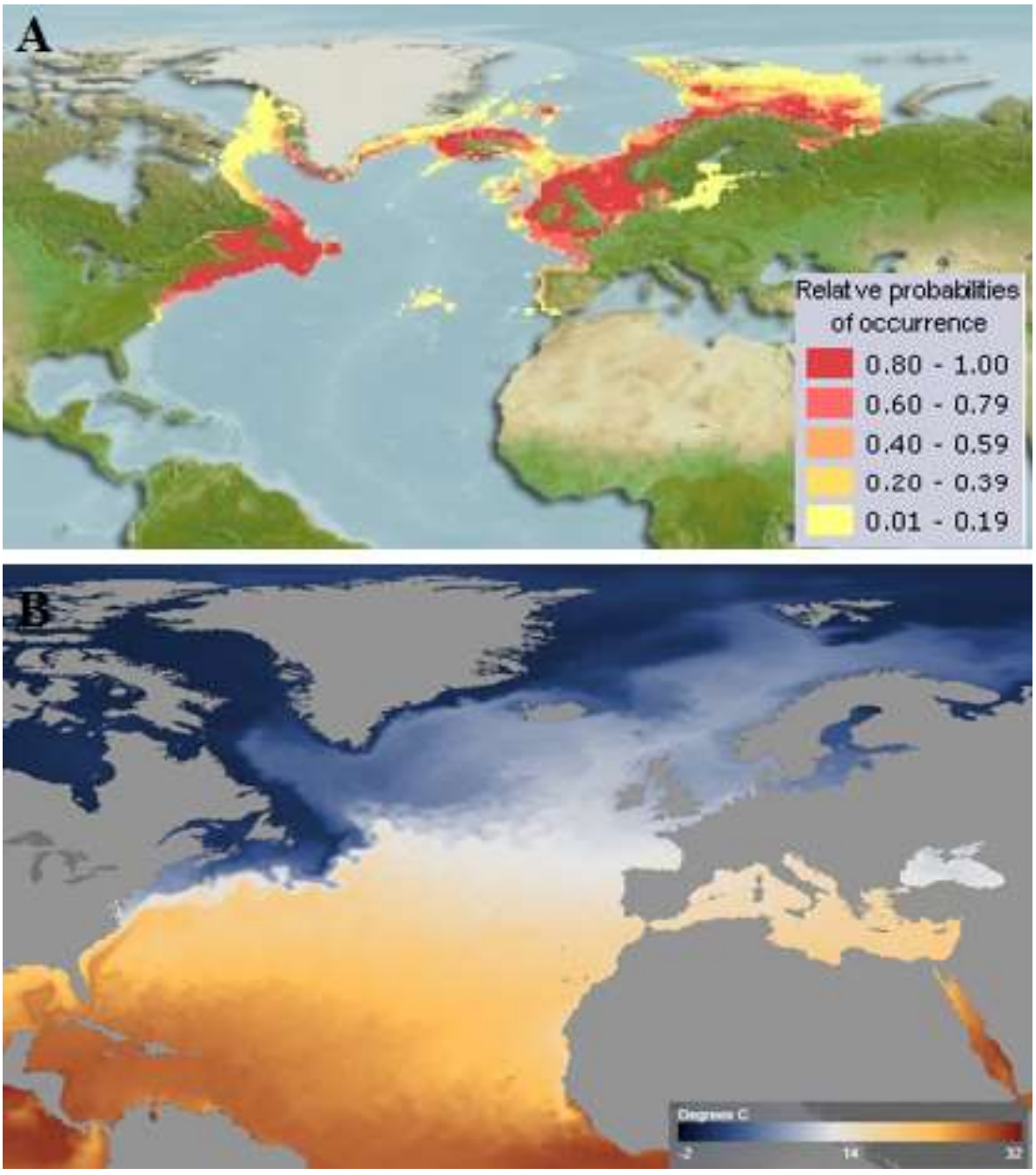

Figure 3. (A) Map of the distribution of the lumpsucker (*Cyclopterus lumpus*) with highest probability of occurrence in red areas (FishBase 2016) , and **(B)** the thermal regime of the North Atlantic Ocean, where the yearly mean sea surface temperatures of the North Atlantic are shown (NOAA 2016).

After hatching, juveniles spend the first 1-2 years feeding in the kelp forest of the intertidal zone and uses their sucker disc to attach to substrates for shelter and rest (Ingólfsson & Kristjánsson 2002; Moring 2001). Adults are often found in pelagic water masses 50-60 meters above abyssal depths (Blacker 1983), mainly feeding on planktonic organisms (Durif 2014; Sunnanå 2007). They migrate to coastal spawning grounds once mature, but the age of maturity is somewhat obscure. Individuals of 4-8 years are commonly found in spawning grounds, and Albert et al. (2002) suggest that males might be 2-3 years and females 3-4 years old at maturity in the wild. There have been observations of early maturation in the production cycle, and the growth rate of farmed lumpsuckers could probably be manipulated in order to affect the timing of maturity.

1.4 Implications to the use of fish in aquaculture

When a new species is introduced to aquaculture settings, there are a number of factors that determines the initial success. Cleaner fish are put into an environment where they normally do not exist, both in hatcheries and sea-cages, so their survival and welfare requires careful monitoring. Much of the lumpsucker production still depends upon wild brood stock, but the first attempts to produce farmed lumpsucker by the Norwegian NORDLUS-project seemed to be without major negative implications. Growth of larvae and fry was satisfactory and individuals put in sea-cages with sea lice infested salmon were observed to graze lice even at temperatures of 4-7 °C (Nævdal & Hovland 2014). However, control of diseases is an emerging problem in farmed lumpsucker and several parasitic and bacterial infections have been described (Alarcón et al. 2016; Poppe et al. 2013).

The biology of the lumpsucker is scarcely studied, and many approaches to the use in aquaculture are therefore based on learning along the way. Research is mainly constrained to wild populations and their migrations (Mitamura et al. 2007; Mitamura et al. 2012), foraging behavior (Ingólfsson & Kristjánsson 2002; Killen et al. 2007; Moring 2001), population genetics (Pampoulie et al. 2014; Skirnisdottir et al. 2013), and age reading (Albert et al. 2000; Albert et al. 2002; Hedeholm et al. 2014; Kasper et al. 2014). This is important for stock assessments and fisheries, but at the same time, much research necessary for optimization of production and use of farmed lumpsucker is lacking. Studies on the coexistence of lumpsuckers and salmon in sea-cages, with emphasis on feeding behavior, lice control, and suitable substrates for rest (Imsland et al. 2014a; Imsland et al. 2014b; Imsland et al. 2014c; Imsland et al. 2015a; Imsland et al. 2015b) have been performed. Other than that, little information on

optimal conditions for lumpsuckers in aquaculture facilities exists and few ecologically relevant measures have been investigated.

The success of cultured fish is mainly dependent on the physical responses to the environment, and fish needs optimal physiological conditions for growth and performance (Myrick 2011). Optimal temperatures varies regarding feed intake, growth, and general performance, with salmonids having an optimal growth at around 13-15 °C in early life stages (Handeland et al. 2008; Jonsson & Jonsson 2009; Ojanguren et al. 2001), while optimal temperatures for food intake and general performance is generally lower and higher, respectively. Optimal temperatures for growth in juvenile lumpsuckers have been suggested somewhere between 13-16 °C, with decreasing temperature optima with increasing fish size (Nytrø et al. 2014). Aquaculture facilities in Norway experiences a lot of variability during the seasons, with water temperatures averaging from 3 to 15 °C depending on the month and region, with summer peaks often higher than 16 °C in certain areas (Figure 1). Temperature-related performance of farmed fish in the sea phase of the production cycle is dependent upon the time of year and the temperatures previously experienced by the fish in the land phase of the production cycle, in order to cope with changing temperatures and surroundings.

1.5 Linking the ecology and physiology of fish

Natural variability and periodic changes have always characterized the world's climate, and most species developed adaptations to deal with the involved environmental effects. Research based on anthropogenic climate change increases, and the evidence of an accelerating rate of change is accumulating (Brander 2007; Brander 2010; Walther et al. 2002). The resilience of species and marine ecosystems is therefore of great concern. Ongoing climate change is expected to affect all life stages of individual organisms, through both direct impacts on physiology and behavior and indirect impacts on the composition and function of ecosystems (Brander 2010; Drinkwater et al. 2010; Pörtner & Peck 2011). Anthropogenic climate drivers adding extra pressure on ecosystems are emission of greenhouse gases (especially CO_2), which in turn promotes global warming and ocean acidification (Hofmann & Todgham 2010). The main responses of organisms facing these environmental drivers are dispersion to better suited habitats, phenotypic or physiological plasticity to tolerate environmental stressors, or adaptation to the new conditions through natural selection (Fields et al. 1993; Helmuth et al. 2005; Hofmann & Todgham 2010). If the environmental changes are intensifying over small

temporal and spatial scales, there is a risk of local extinction of wild species or even whole ecosystems (Helmuth et al. 2005; Perry et al. 2005; Pörtner & Knust 2007).

When explaining how and why species respond to variability and changes in the environment as they do, many authors argue that physiology is the mechanistic function that can link environmental conditions to behavior and fitness (Helmuth et al. 2005; Helmuth 2009; Horodysky et al. 2015; Seebacher & Franklin 2012). Through physiological processes on molecular-through-population-level, fisheries and aquaculture management can be placed in an appropriate ecosystem context. Physiological abilities or tolerances of individuals and populations can help forecast how increases in global temperatures will affect whole ecosystems (Horodysky et al. 2015).

1.6 Thermal performance of fish

Temperature is the climate variable that influences marine ecosystems and aquaculture systems the most (Drinkwater et al. 2010; Myrick 2011; Perry et al. 2005). Climate change does not only increase the global average temperature, but also involves changes in diurnal, seasonal, and geographic patterns of heat flux (Helmuth et al. 2005). All of these are important to consider in conservation of wild stocks and when assessing temperature related performance in aquaculture settings. The predicted temperature increase from global warming over the next century might seem small (1.4-5.8 °C), but the magnitude of the impact will have dramatic effects on individuals and communities (Roessig et al. 2004). An understanding of physiological limitations (Helmuth et al. 2005; Pörtner & Knust 2007; Wang & Overgaard 2007) and an integration of physiological approaches with behavioral studies and fisheries survey or aquaculture production data (Horodysky et al. 2015), may help predict how organisms will fare in the face of a warming ocean.

Environmental temperature is the master abiotic driver which affects the biochemistry, physiology and behavior of all life stages of ectothermic fish (Brett 1971), and it has been well established that fish have optimal and sub-optimal temperatures for performance (Fry 1971). Thermal strategies of most fish relies on heat exchange with the environment and some degree of behavioral thermoregulation, with a quite advanced thermoregulatory system both on a cellular and behavioral level (Crawshaw & Podrabsky 2011; Schulte 2011).

Organisms typically have tolerance limits or thresholds of physiological capacity, which means that below or above these thresholds, performance is not optimal and will eventually fail

(Hofmann & Todgham 2010). Three compensatory responses that contribute to setting thermal tolerance limits are acclimatization or acclimation, behavior, and genetic adaptation (Farrell 2016). Important rate functions such as oxygen consumption (MO_2) is typically maximal at an optimal temperature, which is species-specific and varies in time and space (Brett 1971; Casselman et al. 2012; Eliason & Farrell 2016).

Fry (1947) introduced the term 'scope for metabolic activity', now termed aerobic scope (Eliason & Farrell 2016) to describe the capacity of a fish to deliver additional oxygen to support activities beyond the basics of standard metabolic rate (SMR). Further, Fry and Hart (1948) found that SMR in fish increases exponentially with temperature, until the upper incipient lethal temperature (ILT). As maximum metabolic rate (MMR) peaks at a lower temperature than SMR and reaches a plateau before declining when temperature increases, the aerobic scope can be calculated from the difference between MMR and SMR (Farrell 2016). A concept of oxygen and capacity-limited thermal tolerance (OCLTT hypothesis) is suggested to explain why a fish's capacity for oxygen supply to tissues becomes limited at temperature extremes (Pörtner 2001; Pörtner & Farrell 2008; Pörtner 2010). This hypothesis is supported by several studies on salmonids (Anttila et al. 2013b; Eliason et al. 2011; Farrell 2009).

The thermal sensitivity of fish is often illustrated graphically using a tolerance or performance curve, a Fry curve for aerobic scope (Farrell 2009). These curves summarize the temperature and MO_2 relationship to assess performance at different acclimation temperatures. Information is also given on (1) the optimal temperature for performance (T_{opt}) where aerobic scope is maximal, (2) the thermal breadth or window within which performance meets specific thresholds, and (3) maximum and minimum temperatures the fish can tolerate for short-term survival (T_{max} and T_{min}) (Hofmann & Todgham 2010; Ojanguren et al. 2001; Pörtner & Peck 2010; Schulte 2011). The temperature where aerobic scope is below zero is termed the critical temperature for performance (T_{crit}) and is where fish becomes dependent upon anaerobic metabolism for short term survival. T_{crit} can be recognized by cardiac arrhythmias (Pörtner & Knust 2007).

Because generating a Fry curve normally takes several weeks with MO_2 measurements over a wide range of temperatures in resting and maximally swimming fish (Steffensen 1989), a high-throughput method to estimate T_{opt} has been suggested and confirmed using maximum heart rate (fH) as surrogate (Brett 1964; Farrell 2009; Fry 1947; Steinhausen et al. 2008). The heart is the key organ supplying oxygen to tissues, and is important in physiological plasticity and

acclimation to different thermal limits in fish (Jørgensen et al. 2014b). Heart rate is therefore the main factor that responds to a need for increased internal oxygen during acute warming, peaking at the optimal temperature, but plateaus and decreases above the T_{opt} (Farrell 2016). Thermal responses of the heart is therefore argued to be ecologically relevant, as the rate-transition temperatures from generating maximum heart rate are found similar to those generated from aerobic scope measurements (Casselman et al. 2012).

1.7 Objectives

The aim of this study was to examine the thermal tolerance of juvenile lumpsuckers and to determine the effects of temperature acclimation on associated physiological variables and thermal limits. After a 7days acclimation period, an acute warming protocol was performed on lumpsuckers to generate maximum heart rate in response to a temperature increase of 1 °C every 6 minutes, to derive several rate-transition temperatures for performance. More specifically, it was hypothesized that the temperature acclimation groups of nominally 8 °C and 14 °C differed in their (1) optimal temperature for performance, (2) maximal temperature for performance, (3) critical temperature, (4) maximum heart rate, (5) the relative ventricular mass, and the (6) compact myocardium thickness of the ventricle. The cardiac capacity and thermal limits of lumpsuckers were then compared to Atlantic salmon because of the use of lumpsuckers in salmonid aquaculture as cleaner fish.

2. Materials and methods

2.1 Study design

This study on lumpsuckers was performed in accordance with The Norwegian Regulation on Animal Experimentation (FOR-1996-01-15-23) and the Animal Welfare Act (LOV-2009-06-19-97), FDU application number 7835. Personnel executing the procedures were approved with respect to the FELASA C category.

All experiments were conducted in Mørkvedbukta Research Station (WGS84: 67°16'41.7"N 14°33'26.8"E), Bodø, Norway, during spring 2015.

To find the thermal tolerance of the lumpsucker, individuals were acclimated at two different holding temperatures for a week before an acute warming protocol of 2-3 hours was performed on each fish. This took place over two experimental periods (Replicate 1: 16-27 Mars 2015, replicate 2: 5-15 May 2015).

2.2 Experimental animals

Hatchery reared lumpsuckers were brought to Mørkvedbukta Research Station by the end of May 2014 from Arctic Cleanerfish in Stamsund, Lofoten (WGS84: 68°7'5.0"N 13°47'14.2"E). Lumpsuckers were kept at a water temperature of 10-12 °C the first 60 days after arrival, before the holding temperature was lowered to an ambient seawater temperature of approximately 7-8 °C. Oxygen content of the water was kept above 80% at all times, and LD 24:0 from 60 days after hatching and throughout the experimental period.

2.3 Acclimation conditions

The acclimation set-up (Figure 4) consisted of four circular acclimation tanks of grey fiber-glass units, each with a volume of 400 L with a flow of 150 L/hour. A total of 160 (256.7 g ± 12.6) lumpsuckers were randomly allocated from holding tanks to the four acclimation tanks. Of these available fish, N = 40 used in this study (N = 20 cold acclimated, N = 20 warm acclimated) and N = 48 used in another study conducted at the same time. During the first replicate 18 fish were held in each of the four acclimation tanks, and 22 fish were held in each tank during replicate 2. The acclimated fish not used in the acute warming experiment were euthanized with an overdose of 300 mg/L MS-222 (Finquel vet. 100%, ScanVacc) at the end of the experimental period. Two of the acclimation tanks were held at ambient seawater temperature of approximately 8 °C, while the other two received additional heated seawater

from a header tank to keep the temperature at 14 °C. The temperature in the warm acclimation tanks were gradually increased from the ambient temperature when lumpsuckers first were transferred and to a final temperature of 14 °C during 48 hours, to give the fish time to acclimate to the temperature increase.

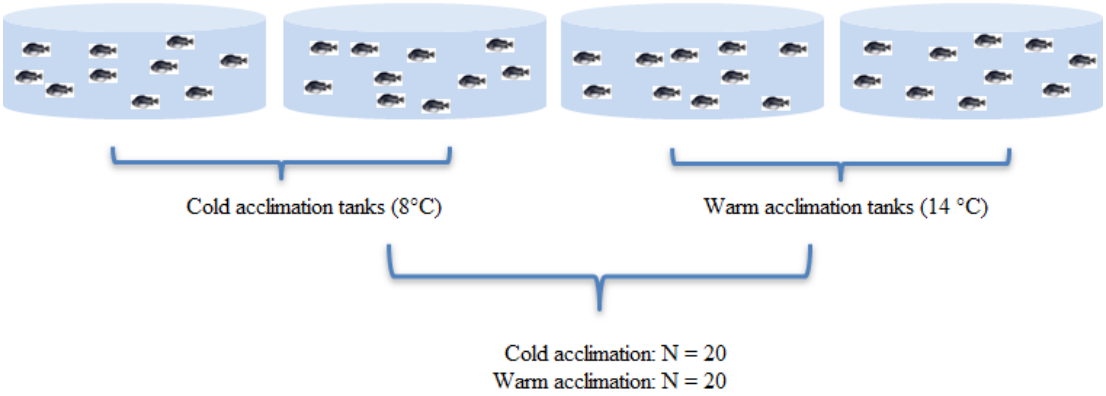

Figure 4. An overview of the acclimation set-up. Two cold acclimation tanks of 8 °C and two warm acclimation tanks of 14 °C were used during the acclimation period, and total N = 20 for the cold acclimation group and N = 20 for the warm acclimation group.

Each acclimation tank had an automatic feeder, which released feed once an hour for 3 seconds. The feed during the experimental period was the commercial feed Amber Neptun 3 mm (Skretting, Norway).

Daily routines during the acclimation period included monitoring of fish health, behavior and mortality, as well as cleaning and flushing of excess feed and faeces in all four tanks. Temperature and oxygen saturation were measured manually with a handheld Optical Dissolved Oxygen Meter (YSI ProODO) in each tank outlet every morning, and adjusted if necessary to keep the temperature within an SD of ± 0.5 °C (in the warm acclimation tanks) and the oxygen content above 80%. Table 1 summarizes the experimental conditions during the acclimation period.

Table 1. Experimental conditions during the acclimation period. Means ± standard deviation of temperatures (°C) and oxygen saturation (%), measured in the tank outlet. Fish (Total N) is the total number of fish per acclimation tank prior to the acute warming experiment.

Replicate	Tank	N	Temperature (°C)	Oxygen saturation (%)
1	1	18	7.3 ± 0.1	96.9 ± 1
1	2	18	7.3 ± 0.1	97.7 ± 1
1	3	18	13.7 ± 0.2	90. 6 ± 9
1	4	18	13.7 ± 0.3	99.1 ± 4
2	1	20	7.6 ± 0.1	105.7 ± 8
2	2	20	7.6 ± 0.1	103.1 ± 8
2	3	20	13.9 ± 0.1	99.2 ± 14
2	4	20	13.9 ± 0.1	95.1 ± 14

2.4 Experimental protocol

2.4.1 Acute warming experiment

Prior to the acute warming experiment, all fish were starved for 24 hours to ensure no effects of metabolism on heart rate. The methods in the acute warming experiment were used with permission and based on a protocol described by Anttila and Casselman (2013).

The response of maximum heart rate (fH_{max}) to acute warming was tested by pharmacological means (injection of Atropine) on N = 20 control fish from 8 °C acclimation tanks and N = 20 experimental fish from the 14 °C acclimation tanks, which were allocated and tested in a randomized order. The experimental set-up (Figure 5), which is also based on Jørgensen et al. (2014a), consisted of a water reservoir of 10 L with a heating unit (Heto, Birkerød, Denmark). Drain tubing from the back of the heater was connected to two rectangular chambers of 3 L each, as well as tubing back into the water reservoir for recirculation. The total water volume of the reservoir and chambers was 16 L, and the flow rate of the tubing (1.5 L min^{-1}) was sufficient to provide water over the gills and a water temperature regulation of ± 0.1 °C. The chambers were each put in a Faraday cage to eliminate field noise, and further placed in a Styrofoam box to isolate for heat gain from the surroundings. Water from the acclimation tanks was transferred to the heart rate apparatus, and mixed with 90-100 mg/L MS-222 prior to placement of the fish. The pump of the heater was then turned on to fill the chambers and start the circulation, and an air stone was added to reservoir to keep the O_2 content above 90% in the circulating water.

Figure 5. Overview of the experimental set-up, with all parts and their connections. The computer with the LabChart software was connected to the PowerLab unit, which again was connected to amplifiers. Electrodes were extended from the amplifiers to the fish for recording of ECG signals.

Two and two fish were anesthetized with 90-100 mg/L MS-222 (depending on size), weighted, and then transferred to the chambers when no movement was observed and the fish did not respond to any handling. The fish were secured to a polyethylene plate to ensure a vertical position below water level. The end piece of the draining tube was carefully put in the mouth of the fish so water flow was maintained over the gills. When no operculum movement was observed, electrodes were placed in the fish to detect the electrocardiogram (ECG). One electrode was surgically inserted with a 6xG needle syringe below the pelvic fin near the heart, while the reference electrode was inserted caudally in the dorsal crest. The electrode terminals were connected to amplifiers (Grass P55 AC, Astro-Med Inc.) to filter noise, and adjusted to receive a clear ECG signal. Good settings were amplification of x1000-10000, filtering of low-pass 10-30 Hz, and high-pass 0.1-0.3 kHz. The amplifiers were connected to a PowerLab unit (8/35, ADInstruments), which again was connected to a computer with a LabChart software (ADInstruments) that was reading real-time heart rate measurements.

When successful ECG signals were obtained (Figure 6A), the anaesthesized fish was kept in the chambers for 30 minutes to stabilize the heart rate. Atropine (Takeda Nycomed AS, Asker) was then injected intraperitoneally to ensure vagal nerve blockage and elicit maximum heart rate. Atropine sulphate from a stock solution of 1 mg/ml was diluted in 50 ml of a saline solution (0.9% NaCl) to make a working solution of 5 mg/ml. An injection dose of 1.2-2.4 mg/kg atropine is common among juvenile salmonids, and from testing with lumpsuckers a dosage 2.0

mg/kg was found appropriate as no further increase in heart rate was observed at higher dosages. After the increase in heart rate with atropine injection, the fish were allowed to stabilize for about 15 minutes.

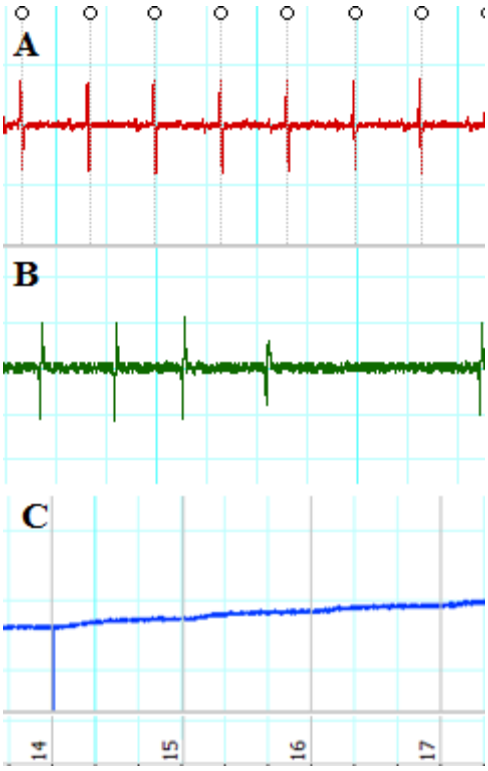

Figure 6. **(A)** Trace of a clear ECG signal with P wave and QRS complex at any temperature, each heartbeat is shown. **(B)** Cardiac arrhythmia at the critical temperature, where a heartbeat is missing at the end. **(C)** Heart rate after atropine injection, with a temperature increase each 6 min (temperature at x-axis).

The acute warming started after the atropine injection, and was performed by a temperature increase of 1 °C every 6 minutes (Figure 6C), which was found sufficient to allow heart rate to stabilize between each temperature change and has been used on salmonids (Anttila & Casselman 2013). Temperature and O_2 in the water reservoir and chambers were monitored during each temperature increment with a Handy Polaris (OxyGuard), to control correct temperature and O_2 levels above 90%. The temperature increase was noted in LabChart every time the heater was manually turned up 1 °C. The temperature increase was continued until cardiac arrhythmias (a lack of P wave or QRS complex) were observed in the ECG signals (Figure 6B), and then the experiment was terminated and fish removed from the heart rate apparatus. Arrhythmia temperature was noted, and the heart rate recordings were stopped and saved. Fish were then euthanized with a blow to the head, and length measurements were taken. The heart was removed and excised to measure wet ventricular mass, and was gently squeezed

on a piece of paper to get rid of all excess blood before fixation on 4% formalin for histological analyses (1 parts tissue:10 parts formalin). Sex was noted by checking the gonads.

2.4.2 Histological analyses

After fixation on formalin, lumpsucker ventricles were put in sample cassettes and dehydrated in alcohol and Xylen with the following procedure: 1 h 50% EtOH, 1 h 70% EtOH, 1 h 80% EtOH, 1.5 h 96% EtOH, 1.5 x 2 h 100% EtOH, and 1.5 x 2 h Xylen, before placed in paraffin for 2 + 3 h in a processing machine (Shandon Citadel 2000). The sample cassettes were afterwards embedded in 60 °C paraffin wax in a metal form (Leica EG 1150 H), before but on an ice block (Leica EG 1150 C) to harden. The ventricle samples were then sectioned into thin slices (5 µm) with a microtome (HM 355S Automatic microtome, Thermo Scientific). The slices were placed on microscopy slides and dried at the edge of a hot water bath. Paraffin had to be removed and the slides stained with Hematoxylin and Eosin (HE-staining) with a slide stainer (Robot Stainer HMS 760X, Microm). The bath lay out of the staining procedure was: 45 min heating, 5 min Xylen, 5 min Xylen, 1.5 min Xylen, 3 min 100% EtOH, 1.5 min 100% EtOH, 1.5 min 96% EtOH, 3 min 80% EtOH, 1.5 min 50% EtOH, 5 min rinsing, 1.5 min Hematoxylin, 5 min Rinsing, 1.5 min Eosin, 3 min rinsing, 1.5 min 50% EtOH, 1.5 min 70% EtOH, 2 min 96% EtOH, 2 min 100% EtOH, 2 min 100% EtOH, and 1 min Xylen. The slides were kept in the Xylen bath until mounting of cover slides with Pertex on top of the ventricle specimen. After mounting, the slides were ready for microscopy.

The histology sections were examined with an Olympus BX51 microscope. The thickness of the compact layer was measured from each ventricle from the distal edge to the spongy myocardium. The least variable area was found to be the middle part between the apex and the entrance of the Bulbus arteriosus, and at least 10 random measurements were taken from each ventricle in that area.

2.5 Data processing

Physiological variables and temperature-related heart rate measurements were determined from each individual fish, and then a mean group analysis was performed to statistically compare cold and warm acclimated fish. The response of maximum heart rate (fH_{max}), in beats per minute (bpm), to acute warming was used to derive several temperature limits. The 50 ECG values (in bpm) prior to every temperature increase were used to calculate the mean heart rate for each temperature, on each fish.

The optimal temperature for performance (T_{opt}) was determined using an Arrhenius plot analysis to find the Arrhenius breakpoint temperature (T_{AB}), where fH is transformed to its natural logarithm and plotted against temperature in inverse degrees Kelvin (1/K). The breakpoint analysis was based on (Yeager & Ultsch 1989) and performed with an R-package (CRAN, 'Package segmented') by fitting two-segment linear regression lines to the plot and calculating the intercept. Because fH_{max} often fails to increase in a logarithmic way at high temperatures, T_{opt} was also calculated with a Q_{10} analysis to find the T_{QB} breakpoint temperature. This was based on the incremental Q_{10} values for fH_{max} every 1 °C temperature step (Q_{10} temperature coefficient = rate of change in fH_{max} as temperature are increased by 10 °C), and is where Q_{10} decreases below 1.9 and temperature is no longer exponential.

The maximum temperature (T_{max}) was calculated as the temperature associated with the highest fH_{max} (Max fH_{max}).

The critical temperature for performance was determined from the temperature where cardiac arrhythmias appeared (T_{arr}).

Relative ventricular mass (RVM) was calculated as a percentage according to the equation:

$$\frac{Ventricular\ mass}{Body\ mass} \times 100\%$$

2.6 Statistical analyses

All statistical analyses in this study were done using R Software™ R 3.2.2 (R Core Team 2015, Vienna, Austria). The normality of distributions was assessed using a Shapiro-Wilk normality test, and the homogeneity of variances was tested using an F-test (variance ratio test). Correlations (parametric data: Pearson's product-moment and non-parametric data: Spearman's rank correlation) and linear regression analyses were performed to examine the relationships between continuous variables within each temperature acclimation group.

A parametric two-tailed Student's t-test (Welsh Two sample t-test) or a non-parametric Wilcoxon rank sum test was performed on morphological parameters, Max fH_{max} and rate-transition temperatures (T_{opt}, T_{max}, and T_{arr}) to statistically compare them between cold and warm acclimated fish. Analysis of covariance (ANCOVA), with body mass as the covariate, was used to rule out any interaction effect between body mass and treatment on the response of Max fH_{max}.

A significance level of $\alpha = 0.05$ was used unless stated otherwise.

3. Results

3.1 Mortality and behavior

No mortality occurred neither during the acclimation period or during the acute warming experiment. No health issues or abnormal behavior were observed in any of the acclimation tanks.

3.2 Physiological variables and temperature acclimation

3.2.1 Thermal tolerance and heart rate transition temperatures

Figure 7A shows the average maximum heart rate at each temperature for both acclimation groups, during acute warming. The warm acclimated group had significantly higher fH_{max} values at temperatures 18 °C, 21 °C, and 22 °C, but both groups followed a more or less similar slope. The Arrhenius plot in Figure 7B indicates where the Arrhenius breakpoint temperature falls for each of the two groups of lumpsuckers, plotted as ln of fH_{max} against inverse temperature (Kelvin) between the groups. The T_{AB} from this plot shows how the optimal temperature has increased with temperature acclimation, from 3.48 (~ 14 °C) in the cold acclimated group to 3.45 (~ 17 °C) in the warm acclimated group. The T_{QB} breakpoint temperature was impossible to calculate using Q_{10} = 1.9 as threshold value, as the rate transition temperature for fH did not cross the 1.9 line after an exponential phase of Q_{10} values with increasing temperature, but rather stayed below this threshold value the whole time (Figure 7C). When using Q_{10} = 1.6 as threshold value the T_{QB} could be calculated, and was approximately 14 °C in the cold acclimated group and 16 °C in the warm acclimated group (Figure 7C).

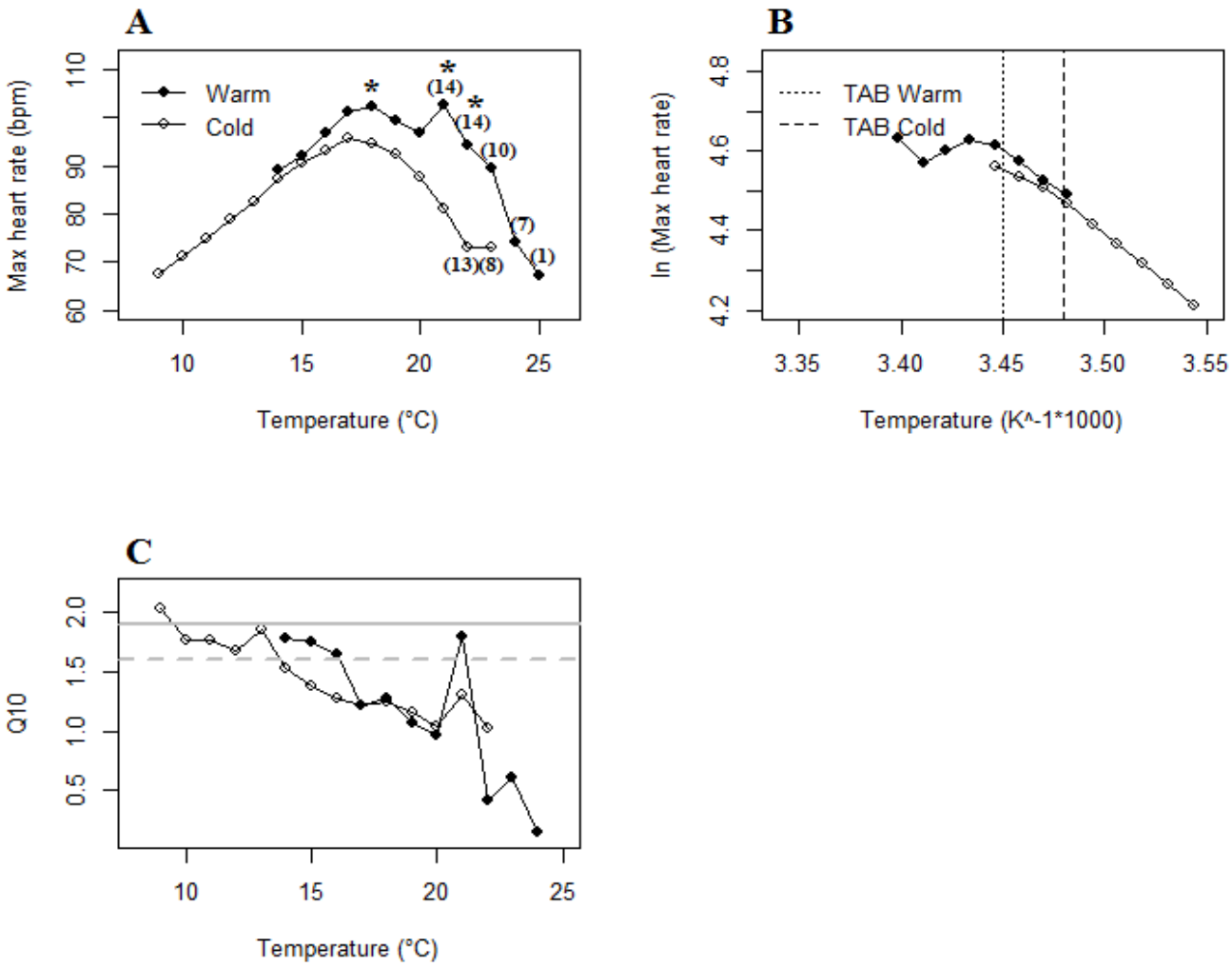

Figure 7. The cardiac responses to warming. **(A)** Average maximum heart rate of two groups of cold (8 °C) and warm (14 °C) acclimated lumpsuckers during temperature increase (N ≥ 15 for both groups, otherwise indicated in parenthesis). Significant difference between acclimation groups are indicated with * (Analysis of covariance, corrected for body mass, $p < 0.05$). Note: Standard error (SE) is not given due to clarity of the figure, but can be found in Appendix I: Table H. **(B)** Arrhenius plot of maximum heart rate. The large dotted vertical line indicates the Arrhenius breakpoint temperature (T_{AB}) for the cold acclimated group and the smaller dotted line represents the warm acclimated group. **(C)** Average Q_{10} of the maximum heart rate during acute warming of the two acclimation groups. The grey bar ($Q_{10} = 1.9$) indicates where the Q_{10} breakpoint temperature can be found, and the grey dotted bar ($Q_{10} = 1.6$) indicates an alternative threshold value of the Q_{10} breakpoint temperature.

There was a significant negative correlation between body mass and Max fH_{max}, T_{AB} and T_{max} within the cold acclimated group, and a linear model then revealed that the size of the fish accounted for 27%, 26%, and 46%, respectively, (all $p < 0.05$, Appendix I: Table E, F and G) of the variation observed in these response variables. Within the warm acclimated group on the other hand, no such correlations were observed. Relative ventricular mass (RVM) had a significant positive correlation with Max fH_{max} ($R^2 = 0.30$, $p < 0.05$), T_{AB} ($R^2 = 0.39$, $p < 0.05$),

and T_{max} (R^2 = 0.31, $p < 0.05$) within the cold acclimated group, but the only significant relationship within the warm acclimated group was between RVM and Max fH_{max} (R^2 = 0.21, $p < 0.05$). Compact myocardium thickness did not correlate with any of response variables within either of the two groups, with an exception of T_{max} for cold acclimated fish (R^2 = 0.28, $p < 0.05$).

No significant interaction effect between body mass and treatment on Max fH_{max} were found (Appendix I: Table I) in the analysis of covariance (where body mass includes the effect of ventricular mass). Based on the results from the correlation analyses and ANCOVA, with no correlation between response variables and body mass in the warm acclimated group and no interaction effect between body mass and acclimation treatment, tests for a difference were performed to decide effects of the acclimation treatments on response variables shown in Table 2.

Table 2. Heart rate and temperature tolerance differences between 7 days cold acclimated and warm acclimated lumpsuckers. Means ± standard deviation are shown for each parameter. N = 20 for cold acclimate fish, and N = 20 for warm acclimated fish. The * indicates a significant difference between acclimation temperatures at $p < 0.05$ (Welsh sample t-test or Wilcoxon rank sum test).

Acclimation temperature	8 °C	14 °C
T_{AB} (°C)	14.2 ± 1.2 *	17.1 ± 1.0 *
T_{QB} (°C)	13.7 ± 1.5 *	15.9 ± 1.0 *
T_{max} (°C)	17.3 ± 1.9 *	18.5 ± 1.4 *
T_{arr} (°C)	21.6 ± 1.9	22.2 ± 1.9
Max fH_{max} (bpm)	96.9 ± 12.3 *	105.1 ± 11.0 *
fH before atropine (bpm)	64.5 ± 4.2 *	84.3 ± 8.3 *
fH after atropine (bpm)	71.1 ± 4.6 *	88.2 ± 8.0 *

The lumpsuckers in the cold and warm acclimated groups differed markedly in their thermal tolerance. Acclimation to 14 °C for a week significantly increased the optimal temperature (T_{AB}) by 2.9 °C, from 14.2 ± 1.2 to 17.1 ± 1.0 (Table 2, Figure 7B). When using Q_{10} = 1.6, the T_{QB} of the cold acclimated group gave an optimal temperature of 13.7 ± 1.5, and 15.9 ± 1.0 for the warm acclimated group, which was a significant increase of 2.2 °C (Table 2). Warm acclimation did also significantly increase the maximum heart rate (Max fH_{max}) from 96.9 ± 12.2 bpm to 105.1 ± 11.0 bpm, along with a significant 1.2 °C increase in the associated maximal temperature (T_{max}) (Table 2). Cardiac collapse at T_{arr} occurred for both acclimation groups at an average of 21-22 °C, and did not differ significantly (Table 2).

There was a significant effect on the response of fH to atropine injection within both groups, which is necessary to generate maximum heart rate and thus determine the thermal limits investigated. The fH before and after atropine injection were both significantly different between the acclimation groups, where the warm acclimated group had an overall higher heart rate prior to and after the injection (Table 2), as this procedure is executed on different starting temperatures for the acclimation groups (8 °C vs. 14 °C).

Individual variation was observed for several variables, like the Max fH_{max} that varied from 75.5 to 119.6 bpm (cold acclimated group) and from 86.4 to 121.2 (warm acclimated group). T_{max} varied from 13 to 22 °C (cold acclimated group) and 17 to 21 °C (warm acclimated group), T_{opt} varied from 11.4 to 16.8 °C (cold acclimated group) and 15.6 to 19.6 °C (warm acclimated group), and T_{arr} varied from 16 to 23 °C (cold acclimated group) and 19 to 25 °C (warm acclimated group).

Max fH_{max} had a significant positive correlation with T_{AB} and T_{max} in both acclimation groups, and in addition T_{arr} in the warm acclimated group and T_{QB} in the cold acclimated group (Appendix I: Table E, F, and G). Their relationships are further shown with regression analyses in Figure 8. Max fH_{max} is responsible for 20% (cold acclimated group, $p < 0.05$) and 24% (warm acclimated group, $p < 0.05$) of the variation in the optimal temperature performance, 30% (cold acclimated group, $p < 0.05$) and 45% (warm acclimated group, $p < 0.05$) of the variation in the maximal temperature performance, and 16% (cold acclimated group, $p > 0.05$) and 30% (warm acclimated group, $p < 0.05$) of the variation in the arrhythmia temperature.

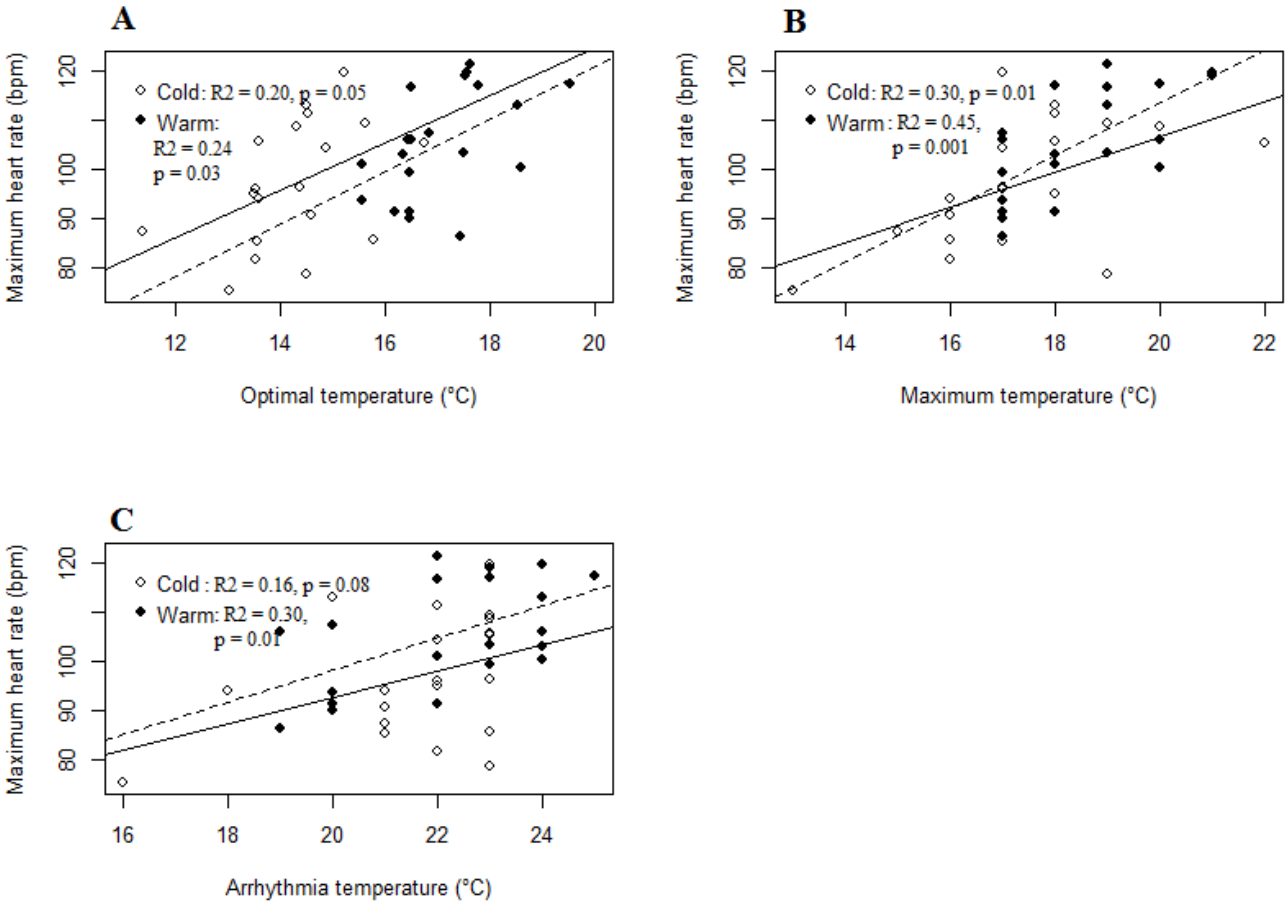

Figure 8. The relationships between temperature performance and maximum heart rate. **(A)** The optimal temperature for performance (T_{AB}) and average maximum heart rate (Max fHmax). **(B)** The maximal temperature for performance (T_{max}) and average maximum heart rate (Max fH_{max}). **(C)** The arrhythmia temperature (T_{arr}) and average maximum heart rate (Max fH_{max}). Note: Open circles and a continuous regression line represents the cold acclimated group, and the filled circles and dotted regression line represents the warm acclimated group. The multiple R-squared (R^2) and the significance (p) values for each group are shown in the legends.

3.2.2 Morphological variables and gender differences

Body mass and total length of the two acclimation groups of lumpsuckers were comparable, with no significant differences in the means (balanced experimental design). The mean ventricle mass and relative ventricle mass were almost exactly the same in both groups, but the mean compact myocardium thickness was ~ 10 µm thicker in the warm acclimated group, though this difference was not regarded significant according to statistical tests (Table 3). There was some variation in the morphometric variables, with a range in body mass from 89 to 439 g (cold acclimated group) and 175 to 389 g (warm acclimated group), a range in ventricular mass from

0.10 to 0.32 g (cold acclimated group) and 0.14 to 0.30 g (warm acclimated group), and a range in mean compact myocardium thickness from 71.2 to 238.6 µm (cold acclimated group) and from 91.3 to 253.5 µm (warm acclimated group).

Table 3. Morphometric variables for the two different acclimation groups used in this study. Means ± standard deviation are shown for each variable. N = 20 in the cold acclimated group and N = 20 in the warm acclimated group, except for the total length (N = 15 in each group), and the compact myocardium thickness (N = 18 in cold acclimated group, N = 20 in warm acclimated group). There were no significant differences between the acclimation groups at $p < 0.05$ (Student's t-test or Wilcoxon rank sum test).

Acclimation temperature	8 °C	14 °C
Body mass (g)	259.3 ± 97.8	254.1 ± 58.5
Ventricular mass (g)	0.21 ± 0.1	0.20 ± 0.1
Relative ventricular mass (%)	0.08 ± 0.01	0.08 ± 0.01
Compact myocardium thickness (µm)	171.5 ± 57.4	181.0 ± 45.9
Total length (cm)	19.9 ± 1.5	19.9 ± 1.4

Body mass was significantly correlated with all morphometric variables for cold acclimated lumpsuckers (all $p < 0.05$), while this was only true for body mass versus ventricular mass and total length for warm acclimated fish (Appendix I: Table E and F).

Gender did not significantly affect any of the response variables within either of the groups (all $p > 0.05$), and the composition was approximately the same between the groups (N = 8 females and N = 12 males in the cold acclimation group, N = 6 females and N = 14 males in the warm acclimation group).

Figure 9 illustrates histological sections of the ventricle and the compact myocardium thickness, and visualizes how the lumpsucker heart is pyramidal in shape. A ventricle from the cold acclimation group is shown in Figure 9A, a ventricle from the warm acclimated group in Figure 9B, a relatively thin compact myocardium from a cold acclimated fish in Figure 9C, and an example of a thicker compact myocardium of a warm acclimated fish Figure 9D.

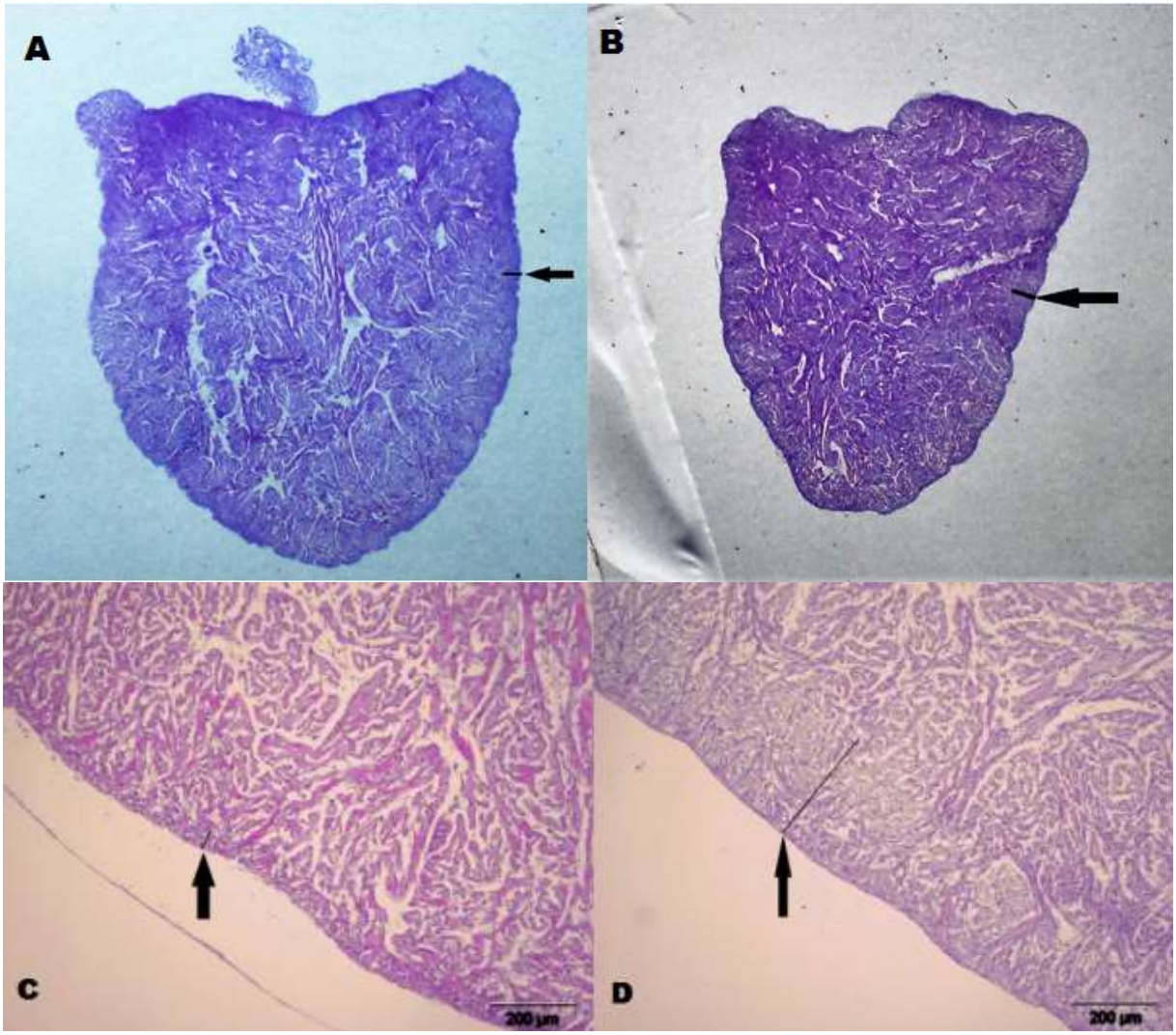

Figure 9. Histological sections of ventricles of lumpsuckers. The cross section of ventricles of **(A)** a cold acclimated fish and **(B)** a warm acclimated fish. Arrows pointing towards the lines indicates the middle area between apex and entrance of Bulbus arteriosus, where measurements were taken. **(C)** The cross section of the compact layer of a cold acclimated fish versus **(D)** the compact layer of a warm acclimated fish. The arrows points toward the lines which indicates how measuring lines were placed, and the scale bar is 200 µm.

3.3 Species differences in thermal tolerance

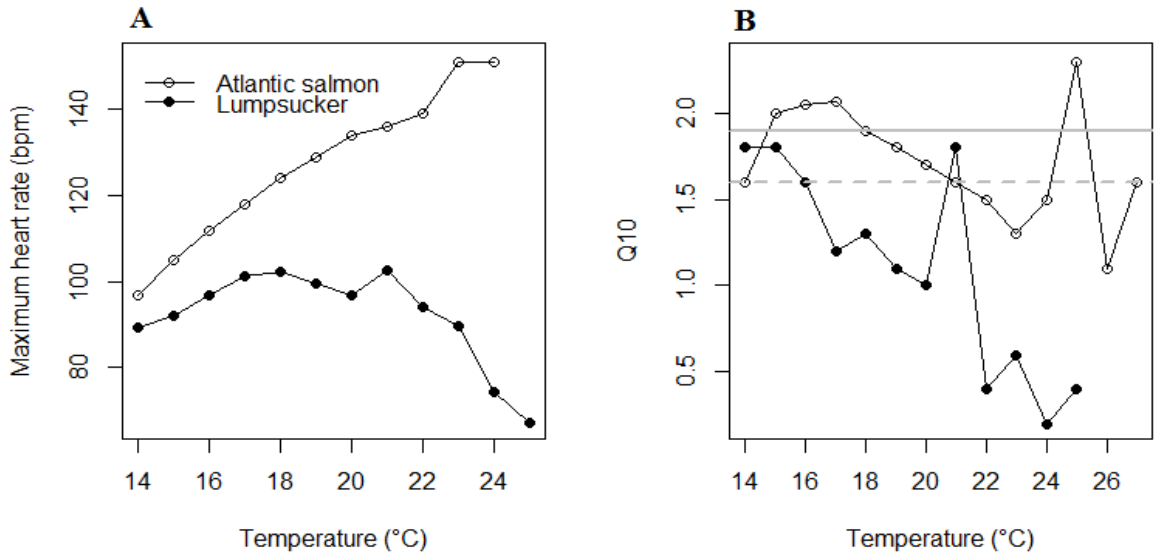

Figure 10. The differences between lumpsucker (warm acclimated, this study) and 'good swimmers' of Atlantic salmon (post-smolt, reproduced with permission from Jørgensen et al. (2014a)) in response to acute warming. **(A)** Differences in average maximum heart rate at each temperature. **(B)** Differences in Q_{10} changes during warming. The grey bar illustrates where Q_{10} = 1.9, and grey dotted bar illustrates where Q_{10} = 1.6. Note: Open circles represents the lumpsucker, and filled circles represents Atlantic salmon.

Figure 10 illustrates the differences between Atlantic salmon (reproduced with permission from Jørgensen et al. (2014a)) and warm acclimated lumpsuckers in response to acute warming. The slopes of average maximum heart rate per temperature increment in the two species (Figure 10A) differ both in shape and values of fH_{max}. The salmon gained a much higher Max fH_{max} (~150 bpm) and had an increase in heart rate all the way up to the arrhythmia temperature, making T_{max} and T_{arr} appear close to each other. Lumpsuckers followed a quite different slope, with a Max fH_{max} of ~ 105 bpm, and fH_{max} decreased gradually after reaching $T_{max,}$ and down to T_{arr} which appeared at a much lower fH_{max} (~70 bpm) than the T_{arr} of salmon. The Q_{10} analysis in Figure 10B shows that the T_{QB} of Atlantic salmon was ~18 °C (where Q_{10} crossed the grey bar of Q_{10} = 1.9), but the T_{QB} of lumpsuckers could not be assessed because the values were below the threshold value of 1.9 all the time. Average maximum heart rate increased only about 20 bpm from prior to the atropine injection and to when Max fH_{max} was achieved in lumpsuckers, while salmon had an increase of about 70 bpm (nearly a doubling of heart rate). The T_{QB} analysis of lumpsuckers when the threshold value of Q_{10} was 1.6 (Figure 10B), gave a T_{QB} of ~ 16 °C, which is comparable to the T_{QB} of ~ 18 °C in Atlantic salmon.

4. Discussion

Results from this study on temperature tolerance revealed that juvenile lumpsuckers (*Cyclopterus lumpus* L.) had a thermal window ranging from an optimal temperature for performance (T_{opt}) of around 14 °C, a maximal temperature for performance (T_{max}) of 17 °C and a critical temperature for performance (T_{crit}) of 22 °C. In addition, acclimation for 7 days at 14 °C significantly increased the T_{opt} with 2.2-2.9 °C and the T_{max} with 1.2 °C, an indication of phenotypic plasticity and cardiac remodeling in response to a short-term temperature change. The results are not surprising given the lumpsucker being a cold-water species, and that previous studies supports the possibility of physiological plasticity of the fish heart in face of changes in environmental temperatures (Gamperl & Farrell 2004). The average highest fH_{max} (Max fH_{max}) did also significantly increase with warm acclimation, where warm acclimated fish increased their Max fH_{max} with 8 beats per minute (bpm). The heart rate of the two acclimation groups of lumpsuckers increased with temperature increment at comparable rates as the fH_{max} values followed the same type of slope in response to warming (Figure 7A), and differed significantly only at certain temperatures above 18 °C, when the N started to decrease due to some fish reaching their individual thermal limits before others. The observed minimal increase in T_{crit} (indicated by the arrhythmia temperature, T_{arr}), and compact myocardium thickness (CMT) of the ventricle was not statistically significant, but somewhat strong given the short acclimation period. The lack of correlation between the relative ventricular mass (RVM) and heart rate (fH) or thermal limits within the warm acclimation group, might indicate that the morphological responses to warming are slow and a remodeling is taking place. The physiological responses of lumpsuckers to warming differed from that of Atlantic salmon, with lower thermal limits in general and different responses of fH_{max} to increasing temperatures.

Acclimation can be thought of as a reversible type of phenotypic plasticity that fish can utilize when experiencing changing environmental variables (Anttila et al. 2013b), and this has been observed modifying the thermal tolerance in some fish species (Eme & Bennett 2009; Ford & Beitinger 2005; Fu et al. 2011; Petersen & Gamperl 2011; Rees et al. 2001). The upper tolerance limits of the thermal window of a fish is considered plastic, and can change with life stage (Pörtner & Farrell 2008) and temperature acclimation (Ferreira et al. 2014). The lumpsuckers used in this study were yearlings and confirmed not sexually mature by visual inspection of gonads. Their life stage is comparable with Atlantic salmon post-smolt, and they are kept in

sea-cages together throughout the production cycle of the salmon, which would require the tolerance windows of both species to be compatible with the fluctuations in surrounding temperature. The relatively short acclimation period used in this study was simply because of an interest in the effect of a short acclimation time, but also because of practical reasons due to the experiment. The juvenile life stage and the relatively low N of the experiment also eliminated gender differences.

The two acclimation temperatures of 8 °C and 14 °C in this study may predict how the thermal tolerance differs between a distribution of lumpsuckers in northern arctic areas where water temperatures rarely rise above 8 °C and a more southern distribution of higher temperatures. The different acclimation temperatures can also illustrate how seasonal and diurnal variation in temperature may affect lumpsuckers both in sea-cages and at spawning grounds. When kept in sea-cages, possibilities of escaping sub-optimal temperatures are limited. At the same time, in the spawning season of lumpsuckers, males guarding the eggs face a difficult option if temperatures at the spawning ground becomes too high. The effect of thermal acclimation demonstrated here is in accordance with expectations from previous work on other species, where the cold acclimation group (illustration of northern distribution) has a lower thermal tolerance than the warm acclimated group (illustration of southern distribution), reflecting possible population differences. The possibility of acquiring a higher thermal tolerance with warm acclimation shown in this study, also tells that acclimation and plasticity might aid in survival of lumpsuckers if temperatures change abruptly. Information regarding the thermal acclimation capability of salmonids is contradicting (Anttila et al. 2014; Baroudy & Elliott 1994; Beitinger et al. 2000), but Anttila et al. (2013b) demonstrated that Atlantic salmon exhibit high phenotypic variation, and may have the potential of responding well to artificial selection in aquaculture and adapt to a warming ocean. The current study indicates that lumpsuckers are capable of acquiring a better thermal tolerance if presented the possibility of acclimation, and with further research on the genetic components together with molecular data, this could reveal if genetic and/or phenotypic traits underlie the differences.

Cardiac function is increasingly used as an important tool in determining the thermal window in a variety of aquatic organisms, and heart rate is found to be a reliable estimate and the key cardiovascular response to an increase in temperature (Anttila et al. 2013a; Casselman et al. 2012). The relationship between upper thermal tolerance limits of fish and fH_{max} have been studied and established in several species (Anttila et al. 2013a; Casselman et al. 2012; Chen et al. 2013; Clark et al. 2011) and are further supported by the correlation analyses in this study

(Figure 8) where fH_{max} is positively correlated with T_{opt}, T_{max} and T_{arr}. Various responses of the fH beyond T_{opt} has been observed, though; it can reach a maximum point, decrease, or become arrhythmic soon after (Eliason et al. 2011; Sandblom et al. 2009; Steinhausen et al. 2008). When achieving T_{opt}, both groups of lumpsuckers reached T_{max} within 1-3 °C, and fH decreased after the maximum temperature until reaching T_{arr} 4-6 °C later (Table 2). A small decrease in fH prior to T_{arr} have been observed in a few other species, e.g. in Arctic char (*Salvelinus alpinus*) (Penney et al. 2014) and rainbow trout (*Oncorhynchus mykiss*) (Chen et al. 2015). T_{arr} is usually reached within 0.5-1 °C of T_{max}, but in lumpsuckers fH_{max} decreased for quite a while after T_{max} (Figure 7A), and this may be due to other adaptations or unknown responses related to a temperature increase.

The use of atropine to generate maximum heart rate instead of maximal exercise is found a reliable method in studies of thermal tolerance (Anttila et al. 2013a; Casselman et al. 2012), and the fH of lumpsuckers significantly increased after the atropine injection along with a reduction in heart rate variability (Table 2, Figure 6C). The use of atropine can also avoid problems related to stress and behavior, as the fish are anaesthesized and handling is minimized. A limitation with the method is that it is impossible to make measurements of a resting heart, and thus calculate the scope of heart rate (Eliason et al. 2011).

The Arrhenius breakpoint analysis for estimation of T_{opt}, and T_{arr} as an estimate for the upper critical temperature have been established as reasonable indices in juvenile salmonids (Anttila et al. 2013a; Casselman et al. 2012). This is further supported by this study, although the curve of fH_{max} in response to acute warming in lumpsuckers posed some restrains for calculation of the breakpoint temperature. This was especially true for the T_{QB} analysis, which was practically impossible due to the low average increase in heart rate observed in both acclimation groups. The low Max fH_{max} compared to that of juvenile salmonids (~ 97 and 105 bmp versus ~ 150 bpm), was not enough to calculate the breakpoint of the Q_{10} values in response to warming, as Q_{10} was constantly below the threshold value. This might indicate a lower dependence of fH on temperature in lumpsucker than other species, as highly temperature dependent biological reactions usually doubles or more in response to an increase in temperature of 10 °C ($Q_{10} > 2$). The threshold value of 1.9 is the one most commonly used in studies of salmonids, but when using $Q_{10} = 1.6$ (Chen et al. 2015) on lumpsuckers their T_{QB} could be calculated and might be a better threshold value for the breakpoint than $Q_{10} = 1.9$. Another reason for the low overall fH of lumpsuckers might be that while salmonids regulate cardiac output mainly through heart rate (frequency), stroke volume is also an important aspect of cardiac output (Farrell 1991), and

this volumetric efficiency might be a greater regulator than fH in lumpsuckers. The potential problem with a T_{AB} analysis on optimal temperature is that fH_{max} does not always increase in a logarithmic way as assumed for the breakpoint analysis (Anttila et al. 2014). Therefore, the T_{AB} analysis gave a more precise estimate when calculating the breakpoint of values between starting fH and T_{max}, ignoring the values between T_{max} and T_{arr} as these made a curve on the slope and would interfere with the true location of the intercept of the regression lines of T_{opt} (Figure 7B). For comparison, Atlantic salmon post-smolt gained a T_{AB} of ~18 °C, a T_{QB} of ~17°C, a T_{max} of 21 °C, and a T_{arr} of 25 °C in a study by Jørgensen et al. (2014a), which all are 1-3 °C higher than that of the same variables in lumpsuckers.

Large individual differences regarding many aspects of lumpsucker production are observed, for instance in growth. The individuals used in this study were all from the same cohort, but their size ranged from 89 to 277.5 g during the first replicate of the experiment. Size variation is suggested to explain some of the phenotypic variation in upper thermal limits, even within the same size range as in this study (Jørgensen et al. 2014a). Thus, there was great variation in most of the response variables within the same cohort. Body mass was negatively correlated with Max fH_{max}, which is common among fish, and vertebrates in general (Lillywhite et al. 1999), together with a reduction in heart rate during development (Barrionuevo & Burggren 1999). The heart rate of large lumpsuckers is therefore expected to be lower than that of smaller fish and juveniles. This could further have been verified by additional experiments with different size classes.

The relative ventricular mass (RVM) is an important morphological aspect of cardiovascular capacity in fish, and usually constitutes around 0.1% of the body mass (Farrell & Pieperhoff 2011). This is quite similar to the RVM of 0.08% in this study (Table 3). Fish with larger ventricles have been observed having a higher thermal tolerance (Penney et al. 2014), as this is related to a higher stroke volume and heart rate. There is also a notion that cold acclimated fish could acquire larger relative ventricular mass than warm acclimated fish, where there is an increase in connective tissue and spongy myocardium that increases stroke volume in a response to a reduction in heart rate (Farrell et al. 1996; Klaiman et al. 2011). In warm acclimation, there is often observed an increase in the thickness of the compact myocardium, which in turn does not necessarily translate into a heavier ventricle, but is rather a compensatory response to elevated temperatures that can compromise oxygen supply to the heart itself (Farrell et al. 1996; Jørgensen et al. 2014a; Klaiman et al. 2011). There were no significant differences in RVM or CMT between the control and warm acclimated group of lumpsuckers, although a

small difference of ~ 10 µm in CMT was observed. This could again indicate that the acclimation period was not long enough to affect the cardiac morphology, and that a greater degree of plasticity in ventricular anatomy might have been apparent with a longer acclimation time. Also, systemic changes as changes in fH are known to respond more rapidly to environmental changes than do morphological changes as RVM or CMT.

Little information exists in general on the lumpsucker heart, and the results from this study demonstrated a pyramidal shaped ventricle (Figure 9A and B). This is common among actively swimming fish such as salmonids. Salmonids receive oxygen supply to the heart by a coronary circulation in addition to the cardiac circulation (Farrell 2002; Farrell 2011). An example at the other extreme is the Atlantic cod (*Gadus morhua* Linnaeus, 1758) which lacks a coronary circulation and only has a spongy myocardium. Lumpsuckers seems be somewhere in between, with no apparent coronary circulation, but still has a pyramidal ventricle and a compact myocardium layer. This cardiac anatomy makes sense because of their migrations and off-shore feeding behavior above abyssal depths (Blacker 1983) , which would require a certain cardiac performance. It should also be noted that the lumpsuckers in this study were hatchery reared for aquaculture, so studies on wild populations for comparison could help gain insight into the cardiac differences between these two. There have been observations of morphological changes of the heart in salmonids raised for aquaculture, as fish become less active and are often being feed well (Gamperl & Farrell 2004). In addition, migrating wild Atlantic salmon smolt were found to have more symmetrical hearts and without abnormalities, compared to that of aquaculture reared smolt (Kristensen et al. 2012). The cardiac health in salmon aquaculture is therefore of great importance, and should also be considered in the breeding programs and in other production conditions of lumpsuckers. The similarity in shape of the lumpsucker and salmonid heart might be beneficial for their coexistence in aquaculture facilities, as lumpsuckers might be better swimmers than their compressed body shape gives the impression of. The ventral sucker disc, the lack of a swimbladder and a cottoid shape together suggest that there is a selection for a strong and powerful heart rather than a hydrodynamic shape of the lumpsucker.

The lumpsucker occupies a relatively broad thermal niche (Figure 2B), with yearly mean temperatures ranging from ~ 0 to 20 °C (NOAA 2016). Because of the foraging and migration patterns, it is tempting to speculate that many populations use horizontal and vertical behavioral thermoregulation to avoid sub-optimal temperatures (Crawshaw & Podrabsky 2011). This way they can stay in the open ocean if coastal waters are too cold during winter and migrate back to

natal shores when temperatures increase, or swim at greater depths during the day when surface temperatures are highest, utilizing the thermocline. Seasonal and diurnal variation in heat flux are therefore likely variations that affects populations and their thermal tolerances differently, and with the extra pressure expected from global warming, the ability to adjust to changing conditions is crucial. Local adaptation has been considered important in thermal tolerance of fish (Eliason & Farrell 2016), as fish from warmer areas are expected to have a higher thermal tolerance than fish from colder areas. If thermal acclimation too is considered, physiological plasticity has shown promise of being an option than can replace the acute need for local adaptation if temperatures change quickly over ecologically relevant times (Anttila et al. 2014). If there is a strong cardiac plasticity of the lumpsucker heart, as indicated by the increase in certain thermal limits with warm acclimation, southern populations would be more vulnerable to climate change as they are already living at the limits of their thermal tolerance window. The capacity for cardiac warm acclimation would then still reside in the northern populations, who then would have larger temperature ranges to live within. Also, based on yearly averages in mean temperatures in Norwegian aquaculture facilities (Figure 1), it is very likely that lumpsuckers used in sea-cages for delousing are experiencing temperatures above 14 °C at some point during the year. Cardiac plasticity is then crucial for short-term survival, as an exposure to the upper thermal limits could lead to reduced survival, reduced delousing activity, and a reduction in general performance, among other things.

The optimal temperatures for performance of Atlantic salmon post-smolt and warm acclimated lumpsucker are comparable, 16-17 °C versus ~ 18 °C (Figure 10B) , which may support the use of lumpsucker and salmon together in sea-cages. Still, there are some basic physiological differences between salmon and lumpsucker that have to be considered and one cannot simply expect the same responses from both species if environmental conditions change. For example, the large gap in fH_{max} that appears after T_{opt} (Figure 10A) between the two species confirms a quite different regulatory system in cardiac output, where fH in salmon is a key cardiovascular responder to warming, but is not so apparent in the lumpsucker response as fH decreases.

This study was not only important for the investigation of basic physiology and thermal performance of lumpsuckers, but also for the applicability of the methodology used in acute warming, as well as the two different analyses of optimal temperature performance and the use of arrhythmia temperature as the critical thermal limit. Although this experiment only investigated certain aspects of thermal performance and might be regarded as a small scale

simplified study, the results are comparable to similar studies on other species, and thus supports the already established ecological relevance of the methods.

As no other published studies have yet looked into temperature related performance in lumpsuckers, this is the first information on thermal tolerance limits, investigated from a cardiac perspective. The current study demonstrates how warm acclimation increases the cardiac performance and thus the thermal limits, indicating physiological plasticity in this trait. There are also differences in physiological capacity between lumpsuckers and salmon, and the thermal limits in lumpsuckers are generally lower. This thermal dependence of the heart in lumpsuckers is an important finding and needs to be considered in farming situations, and conservation of a healthy heart in future breeding should be a goal. The genetic basis of thermal tolerance and cellular responses to acclimation and warming is still largely unknown, but is important as these mechanisms cause differences in both organ and whole animal performance (Anttila et al. 2013a; Anttila et al. 2013b; Munday et al. 2012; Penney et al. 2014; Perry et al. 2005). The cardiac plasticity and effect of acclimation seen, may aid northern distributions of lumpsuckers to compensate for a warmer future and cope with fluctuating conditions in aquaculture settings, given the thermal limits of ~16-17 °C (T_{opt}), ~ 18.5 °C (T_{max}), and ~ 22 °C (T_{arr}) found with warm acclimation in this study.

In conclusion, based on the current study it is likely that lumpsuckers from northern areas might have a lower thermal tolerance than more southern populations, but the cardiac plasticity observed with 7 days warm acclimation demonstrates how flexible lumpsuckers might be in acquiring higher thermal tolerances over short temporal scales. Species differences in thermal tolerance are well established, and the differences between juvenile Atlantic salmon and juvenile lumpsuckers observed are very interesting in an evolutionary perspective, and their thermal limits investigated differ only by 1-3 °C (with warm acclimatin). There are currently no studies published on the thermal tolerance of other species used as cleaner fish in salmon aquaculture industry, so it would have been interesting to compare the thermal limits and cardiac capacity of wrasse species with the ones found for lumpsuckers. For the use of farmed lumpsuckers in sea-cages, the timing of transfer to the sea should be in accordance with temperatures experienced in land-based facilities, to ensure optimal performance. Growth and lice grazing (food intake) of the lumpsucker is more likely to find place at temperatures below the T_{opt} for performance, as activity is maximal at the T_{opt}. Exposure to temperatures above T_{opt} might yield an inactive lumpsucker, but short term survival is possible thorough phenotypic

plasticity. As the lower thermal limits of the lumpsucker still is unknown, a complete thermal window would require research into this by cold acclimation at temperatures below 8 °C. For future research on ecologically relevant temperature limits, especially in response to climate change, the thermal preference of wild stocks of lumpsuckers with the application of telemetry tags might be a mechanistic link to couple the physiology and ecology of lumpsuckers.

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Appendix I

Table A. Results from the Shapiro-Wilk normality tests on all variables used in the experiment, within both treatments.

Treatment	Factor	W	p
Cold	Body mass	0.942	0.267
Cold	Ventricular mass	0.965	0.650
Cold	RVM	0.968	0.719
Cold	CMT	0.892	0.042
Cold	Total length	0.890	0.171
Cold	T_{AB}	0.946	0.308
Cold	Tmax	0.950	0.365
Cold	Tarr	0.753	0.000
Cold	Max fHmax	0.971	0.784
Cold	fH before atropine	0.963	0.615
Cold	fH after atropine	0.940	0.244
Cold	T_{QB}	0.921	0.153
Warm	Body mass	0.945	0.298
Warm	Ventricular mass	0.896	0.034
Warm	RVM	0.955	0.446
Warm	CMT	0.967	0.691
Warm	Total length	0.950	0.646
Warm	T_{AB}	0.931	0.158
Warm	Tmax	0.868	0.011
Warm	Tarr	0.901	0.043
Warm	Max fHmax	0.938	0.222
Warm	fH before atropine	0.966	0.670
Warm	fH after atropine	0.931	0.159
Warm	T_{QB}	0.886	0.049

Table B. Results from the F-test for equal variances of variables between treatments.

Variable by treatment	F	df	p
Body mass	2.793	19	0.030
Ventricular mass	1.724	19	0.244
RVM	2.311	19	0.076
CMT	1.556	19	0.352
Total length	1.096	10	0.881
T_{AB}	1.256	19	0.624
Tmax	1.888	19	0.175
Tarr	0.974	19	0.954
Max fHmax	1.248	19	0.634
fH before atropine	0.257	19	0.005
fH after atropine	0.330	19	0.020
T_{QB}	2.136	16	0.149

Table C. Student's t-test (Welsh Two Sample t-test) on parametric variables, to test for a difference between acclimation groups.

Variable by treatment	T	df	p
Body mass	0.204	31	0.840
RVM	0.933	33	0.358
Total length	0.068	19	0.946
T_{AB}	-8.252	38	5.883E-10
Max fHmax	-2.238	38	0.031
fH before atropine	-9.480	28	2.912E-10
fH after atropine	-8.266	30	2.928E-09

Table D. Wilcoxon rank sum test on non-parametric variables, to test for a difference between groups.

Variable by treatment	W	p
Ventricle mass	211	0.7763
Tmax	116	0.021
Tarr	164	0.326
CMT	166	0.693
T_{QB}	28.5	0.0001

Table E. Pearson's product-moment correlation between different parametric variables, within each acclimation group.

Treatment	Comparison factors		T	df	p	cor
Cold	Body mass	MaxfHmax	-2.570	18	0.019	-0.518
Cold	Body mass	T_{AB}	-2.152	18	0.045	-0.452
Cold	Body mass	RVM	-4.606	18	0.0002	-0.736
Cold	Body mass	Total length	6.969	8	0.0001	0.927
Cold	Body mass	fH before atropine	-1.128	18	0.274	-0.257
Cold	Body mass	fH after atropine	0.739	18	0.470	0.172
Warm	Body mass	MaxfHmax	-0.456	18	0.654	-0.107
Warm	Body mass	T_{AB}	-0.058	18	0.954	-0.014
Warm	Body mass	RVM	-1.350	18	0.194	-0.303
Warm	Body mass	Total length	3.357	9	0.008	0.746
Warm	Body mass	fH before atropine	0.358	18	0.724	0.084
Warm	Body mass	fH after atropine	0.620	18	0.543	0.145
Cold	MaxfHmax	T_{AB}	2.139	18	0.046	0.450
Cold	MaxfHmax	Tmax	2.785	18	0.012	0.549
Warm	MaxfHmax	T_{AB}	2.410	18	0.027	0.494
Cold	RVM	T_{AB}	2.732	18	0.014	0.541
Cold	RVM	MaxfHmax	2.804	18	0.012	0.551
Cold	RVM	fH after atropine	-0.357	18	0.725	-0.084
Cold	RVM	fH before atropine	1.814	18	0.086	0.393
Warm	RVM	T_{AB}	1.621	18	0.122	0.357
Warm	RVM	MaxfHmax	2.205	18	0.041	0.461
Warm	RVM	fH after atropine	1.415	18	0.174	0.316
Warm	RVM	fH before atropine	1.224	18	0.237	0.277
Cold	RVM	T_{QB}	2.693	15	0.017	0.571
Cold	Body mass	T_{QB}	-2.163	15	0.047	-0.488
Cold	MaxfHmax	T_{QB}	6.605	15	8.36E-06	0.863

Table F. Spearman's rank correlation rho between different non-parametric variables, within each acclimation group.

Treatment	Comparison factors		S	p	rho
Cold	Body mass	Ventricular mass	84.532	1.275E-09	0.9364423
Cold	Body mass	Tmax	2223.6	0.001176	-0.6719026
Cold	Body mass	Tarr	1978.1	0.0293	-0.4873175
Cold	Body mass	CMT	152.58	0.00001145	0.8425401
Warm	Body mass	Ventricular mass	272.81	0.00002821	0.7948815
Warm	Body mass	Tmax	1223.2	0.7364	0.08033349
Warm	Body mass	Tarr	1049	0.3711	0.2113118
Warm	Body mass	CMT	1029.8	0.3386	0.2257336
Cold	MaxfHmax	Tarr	859.38	0.1259	0.3538506
Warm	MaxfHmax	Tmax	420.88	0.0008915	0.6835478
Warm	MaxfHmax	Tarr	689.6	0.03159	0.4815025
Cold	RVM	Tarr	751.66	0.05536	0.434843
Cold	RVM	Tmax	539.7	0.005731	0.5942109
Warm	RVM	Tarr	1107.7	0.4812	0.1671458
Warm	RVM	Tmax	948.19	0.2197	0.2870745
Warm	MaxfHmax	TQB	524.83	0.3953	0.2281908
Warm	RVM	TQB	576.15	0.5723	0.1527197
Warm	Body mass	TQB	534.1	0.4249	0.2145652

Table G. Regression analyses between continuous variables, within each acclimation group.

Treatment	Comparison factors		R2	F	p
Cold	Body mass	MaxfHmax	0.27	6.6	0.02
Cold	Body mass	T_{AB}	0.20	4.6	0.05
Cold	Body mass	Tmax	0.46	15.6	0.00
Cold	MaxfHmax	T_{AB}	0.20	4.6	0.05
Cold	MaxfHmax	Tmax	0.30	7.8	0.01
Cold	MaxfHmax	Tarr	0.16	3.4	0.08
Warm	MaxfHmax	T_{AB}	0.24	5.8	0.03
Warm	MaxfHmax	Tmax	0.45	14.8	0.00
Warm	MaxfHmax	Tarr	0.30	7.8	0.01
Cold	RVM	T_{AB}	0.29	7.5	0.01
Cold	RVM	Tmax	0.31	8.2	0.01
Cold	RVM	Tarr	0.18	3.9	0.06
Cold	RVM	MaxfHmax	0.30	7.9	0.01
Warm	RVM	MaxfHmax	0.21	4.9	0.04
Warm	RVM	T_{AB}	0.13	2.6	0.12
Warm	RVM	Tmax	0.04	0.8	0.39
Warm	RVM	Tarr	0.03	0.6	0.47
Cold	MaxfHmax	T_{QB}	0.74	43.6	8.359E-06
Cold	Body mass	T_{QB}	0.24	4.7	0.05
Cold	RVM	T_{QB}	0.33	7.3	0.02
Warm	Body mass	T_{QB}	0.07	1.1	0.31
Warm	RVM	T_{QB}	0.05	0.8	0.39
Warm	MaxfHmax	T_{QB}	0.04	0.6	0.45

Table H. Results from the analysis of cardiac responses to warming, with average maximum heart rate per temperature increment for both acclimation group, along with standard deviation and standard error.

Treatment	Temperature (°C)	fHmax	SD	SE
Cold	9	67.4	2.9	0.7
Cold	10	71.1	3.3	1.0
Cold	11	74.9	3.8	1.0
Cold	12	78.9	4.7	1.1
Cold	13	82.6	6.0	1.4
Cold	14	87.2	7.1	1.7
Cold	15	90.7	8.8	2.1
Cold	16	93.2	10.5	2.5
Cold	17	95.9	10.8	2.6
Cold	18	94.7	13.0	3.1
Cold	19	92.4	13.8	3.3
Cold	20	87.5	14.2	3.3
Cold	21	81.2	14.7	3.4
Cold	22	72.9	20.6	5.4
Cold	23	73.0	23.3	8.1
Warm	14	89.2	8.6	2.7
Warm	15	92.2	8.9	2.2
Warm	16	96.9	8.6	1.9
Warm	17	101.2	8.2	1.8
Warm	18	102.2	10.3	2.3
Warm	19	99.4	17.8	4.0
Warm	20	96.8	23.9	5.8
Warm	21	102.6	12.6	3.4
Warm	22	94.2	16.3	4.6
Warm	23	89.7	15.9	5.6
Warm	24	74.3	20.9	8.6

Table I. One-way ANCOVA with body mass (BM) as co-variable, to assess true effects of treatments (cold and warm acclimation) on maximum heart rate (Max fHmax).

Response: MaxfHmax					
	df	SS	MS	F	p
BM	1	745.8	745.83	6.1353	0.01808
Treatment	1	636.29	636.29	5.2343	0.02812
BM:Treatment	1	97.2	97.16	0.7993	0.37725
Residuals	36	4376.3	121.56		