Aerobic exercise training for improving robustness of Atlantic salmon (*Salmo salar*)

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Aerobic exercise training for improving robustness of Atlantic salmon (*Salmo salar*)

Aerobisk trening for å bedre robusthet hos atlantisk laks (*Salmo salar*)

Philosophiae Doctor (PhD) Thesis

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Vicente
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Articles 1 to 3
1 List of articles

I. Aerobic training stimulates growth and promotes disease resistance in Atlantic salmon (Salmo salar).

II. Exercise training effects on disease resistance are dependent on training regimes and inherent swimming performance in Atlantic salmon.
Submitted manuscript

III. Cardiac molecular-acclimation mechanisms in response to swimming-induced exercise in Atlantic salmon.
Submitted manuscript
# Abbreviations

<table>
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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AMPK</td>
<td>adenosine monophosphate-activated protein kinase</td>
</tr>
<tr>
<td>bls$^1$</td>
<td>body lengths per second</td>
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<td>COT</td>
<td>cost of transport</td>
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<tr>
<td>COX</td>
<td>cyclooxygenase</td>
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<tr>
<td>CPT1</td>
<td>carnitine palmitoyltransferase-1</td>
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<tr>
<td>DHPR</td>
<td>dihydropyridine receptor</td>
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<tr>
<td>EPO</td>
<td>erythropoietin</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
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<tr>
<td>IPN</td>
<td>infectious pancreatic necrosis</td>
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<td>IPNV</td>
<td>infectious pancreatic necrosis virus</td>
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<tr>
<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
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<tr>
<td>PGC1α</td>
<td>PPAR gamma co-activator 1-alpha</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>qPCR</td>
<td>real-time quantitative reverse transcription PCR</td>
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<tr>
<td>RVM</td>
<td>relative ventricular mass</td>
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<tr>
<td>RyR</td>
<td>ryanodine receptor</td>
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<tr>
<td>SR</td>
<td>sarcoplasmic reticulum</td>
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<tr>
<td>TNFα</td>
<td>tumor necrosis factor-alpha</td>
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<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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3 Summary

Exercise training produces a wide range of benefits to fish kept in captivity. Research has shown that among several other parameters, growth, feed efficiency, feed intake and cardiac capacity are improved. In higher vertebrates, exercise training is known to improve performance in terms of resistance to infectious and life-style associated diseases. In fish, nevertheless, the latter has not been acknowledged so far. Despite this knowledge, and the high potential for increasing overall robustness of farmed fish species, regular aquaculture practices do not encourage swimming activity as a way to promote exercise training. Such a measure might become especially relevant in the actual fish farming industry scenario, where mortalities due to infectious diseases have maintained high for a long period of time and comprise a principal constrain to the sector’s sustainability. Production losses are particularly high the first months after sea-transfer, thus preventive health measures must be initiated in juvenile fish during the freshwater stage.

To assess the potential of exercise training for improving robustness of Atlantic salmon smolts, two experimental trials were performed, consisting of several training regimes differing in their duration, intensity and modality during the fresh water rearing stage. Robustness was measured in terms of growth, cardiac capacity and disease resistance.

The effects of exercise training on disease resistance were assessed by challenging the trained fish with Infectious Pancreatic Necrosis (IPN) after transfer to sea water (papers 1 and 2). It was found that exercise training at swimming velocities around one body length per second resulted in improved performance when compared to untrained fish. Furthermore, exercise training consisting on a daily change in swimming velocity (interval training) produced greater effects than continuous velocity training, though only when the velocity changes were mild. In the contrary, interval training regime with relatively large daily changes in water velocity, gave reduced disease resistance probably due to the formation of a stressful environment.

A step further in uncovering the effects of exercise training on resistance to infectious diseases was addressed in paper 2, as the inherent swimming capacity of fish within a population was taken into account. After splitting the population in two groups (poor and good) according to their swimming capacities, it was found that such inherent differences associated positively with disease resistance later in life. Furthermore, training conferred no disease resistance effects on the inherently good swimmers, while performance of poor
swimmers was either improved through an optimal regime (achieving similar resistance level as good swimmers), or worsened through a deleterious regime.

To get an insight into the cardiac acclimation response to exercise training, the molecular mechanisms underlying this were studied in papers 1 and 3 through the use of gene expression (microarrays and real-time quantitative RT-PCR) and protein expression (western blot and immunohistochemistry) analyses. The selected tissue was the heart given its central role as a main exercise-target organ as well as its immunological relevance. In paper 1, exercise-induced improved survival to IPN was associated with a reduction in the expression of genes related to inflammatory mechanisms, including cytokines and enzymes producing eicosanoids. Further, exercise-induced disease resistance was linked to a consistent up-regulation of complement components (immune effectors) as well as antioxidants and xenobiotics clearance molecules. This suggests that training modulates the cardiac molecular response, generating an immune competent tissue. In paper 3, the cardiac acclimation process was investigated for those cellular mechanisms known to strengthen the cardiac muscle in higher vertebrates. It was found that mRNA and protein levels of compounds participating in cardiac growth (both through cardiomyocyte hypertrophy and hyperplasia), contractility, blood supply and lipids metabolism were elevated by exercise training on an intensity-dependent manner, resembling the molecular signature behind the mammalian exercise-induced enlarged heart.

Finally, exercise training improved the growth rate of Atlantic salmon as seen in both experimental trials (papers 1 and 2). Such an effect was mainly ascribed to an increased feed intake (paper 1) and a mixture of feed intake and feed conversion efficiency (paper 2).

Overall, this thesis demonstrates that exercise training Atlantic salmon pre-smolts has a strong potential for producing more robust fish, with improved disease resistance associated with a strengthened cardiovascular system and improved somatic growth. Future research with an emphasis on further optimizing the training regimes presented here will undoubtedly generate the required knowledge to implement protocols with the potential of bringing large benefits to the fish, the industry and the consumers.
4 General introduction

4.1 Aquaculture towards sustainability

The aquaculture industry has a fundamental objective of achieving a sustainable growth and development. The World Commission on Environment and Development has defined sustainability as the kind of development that meets the needs of the present without compromising the ability of future generations to meet their own needs [1]. In this context intensive aquaculture systems possess great challenges, due to its relatively higher impact on the environment and on the welfare of the cultivated species when compared to less intensive systems. The most advanced and intensified aquaculture operation today is the farming of salmonids, with Atlantic salmon (Salmo salar L.) being the most important species in terms of industrial production and revenue. Production levels have increased rapidly in the last few decades, mainly supported by optimization of feeding efficiency, growth and environmental factors, selective breeding and the application of sanitary measures. Despite these improvements, sub-optimal rearing conditions caused by production strategies focused on fast growth and low costs may result in multiple and interacting stress factors with detrimental effects on fish health.

One of the greatest challenges for the Atlantic salmon industry is to reduce the high levels of mortalities observed during the seawater rearing stage. In Norway, world leader in Atlantic salmon production, diseases account for nearly 90% of all losses, which have bordered 15-20% of total production for the last 18 years [2]. Infectious viral diseases, such as infectious pancreatic necrosis (IPN), pancreas disease (PD), infectious salmon anemia (ISA), as well as the sea lice parasite (Lepeophtheirus salmonis) represent some of the most hazardous pathogens [3,4]. Furthermore, new emerging diseases have a great economical impact, such as heart and skeletal muscle inflammation (HSMI) and cardio-myopathy syndrome (CMS) are probably the most important ones. In addition, fish farmers experience fish losses associated with lifestyle diseases such as cardiac failures, including atherosclerosis, hypoplasia and malformations [5,6]. These are prompted by the sedentary way of living fish are forced to in the rearing facilities and potentially worsened by the use of high energy diets to boost growth.
4.2 Robust fish

Fish robustness can be defined as the capability to combine fast growth with normal organ development and improved resistance to both disease and physiological challenges. Improved robustness is intimately associated with higher welfare, hence, promoting fish robustness should have a strong impact on the industry’s economy and reputation, as well as in satisfying the consumer’s increasing request for sustainable and ethical foods.

Fish robustness can be improved by adequate management of most farming-related input factors including, breeding, nutrition and husbandry practices [7–11]. In terms of resistance to infectious diseases the most important strategy is vaccination, which reduces the need for antibiotics with the consequent reduction in environmental impact [12,13]. While vaccination of Atlantic salmon has proven effective against bacterial diseases such as vibriosis and furunculosis [14–16], efficiency of vaccines against viruses is more uncertain [4]. Furthermore, the development of new vaccines is slower than the appearance of new diseases, and vaccination itself imposes a challenge for the fish as it produces side-effects with strong welfare implications, including intra-abdominal lesions, vertebral deformities, behavioral changes, reduced feed intake and reduced weight at slaughter [17–21].

Intriguingly, Atlantic salmon seems to have a rather strong innate immunity against viruses, as controlled laboratory challenge trials have failed to produce high mortality levels against IPNV and PDV [4]. Despite of this and the use of antiviral vaccines and breeding programs aiming at improving resistance against certain viral diseases, the salmon industry still faces relatively high numbers of viral disease outbreaks every year [22].

It has been demonstrated that sub-optimal rearing conditions generate high stress levels, which drives the fish into an immunosuppressed state favoring viral pathogenicity [23,24]. Nevertheless, a stress free environment seems an impossible task in the heavily industrialized intensive Atlantic salmon aquaculture, where regular operational processes such as grading, crowding, bath treatments and transporting impose severe physiological challenges. This is further affected by a continuous exposure to potential pathogenic agents in the seawater grow-out stage. Therefore, improving the fish’s strength and robustness by stimulating its capability to maintain homeostasis under stressful situations, at the same time of possessing an alert immune system should be promoted.
4.3 Exercise training

Increasing the water velocity inside the rearing tanks is an efficient way of promoting the fish to perform physical activity by swimming against the current. If this is done for a prolonged time, farmed fish will be actually exercise training, which has the potential to confer a series of beneficial effects to the fish (welfare), the producer (fish performance), the consumer (product quality) and the industry (reputation and sustainability). Forcing fish to swim results in a series of morphometric, physiological, biochemical and behavioral changes, including growth, feed efficiency, flesh quality, muscle cellularity, fiber type switching, capillarization, metabolism, ultrastructural changes, sexual maturation, stress and welfare, neurogenesis, growth hormones, skin properties, swimming capacity and a series of cardiovascular and respiratory parameters [7,25,26]. While research has involved fish species with different lifestyles and swimming capacities, most of the efforts have been set on salmonids due to their well known athletic-like properties as well as their economical importance and ease of experimentation.

Under current aquaculture conditions, juvenile Atlantic salmon are usually reared in tanks where water velocities are not set according to any biological requirement of the fish, rather, low water exchange rates are used according to tank self-cleaning and oxygen distribution parameters [27]. This results in a sedate way of life were active swimming is not promoted, radically differing from the active behavior of salmonids in the wild. This is evidenced by wild salmonids possessing a significantly higher swimming capacity than reared ones [28–30]. When this is further coupled with the use of high energy diets to stimulate growth, the result is a farmed fish with poor cardiovascular conditions associated with health problems impacting the overall welfare condition. As a consequence, farmed fish are prone to lifestyle diseases similar to those occurring in humans in the industrialized world [31].

The following sub-sections will focus on the most commercially relevant of the exercise training effects found so far in fishes; increased somatic growth and enhanced cardiovascular capacity.

4.3.1 Exercise training and growth

Significant increases in body weight as a response to sustained-moderate exercise training have been found for most of the fish species examined to date. Benefits from exercise seem to
be maximized at speeds close to the optimal swimming speed ($U_{\text{opt}}$), where energy use is more efficient and the cost of transport (COT: energy spent on swimming a given distance) is minimized [32]. Among salmonids, weight gain occurs in response to exercise training in the three genera *Salmo*, *Oncorhynchus* and *Salvelinus* [33–45], while no effect, or even growth retardation has been found for Pacific salmon species [46–49]. Similar positive effects have been found in non-salmonid species such as Seabream (*Sparus aurata*), striped bass (*Morone saxatilis*), Danube bleak (*Chalcalburnus chalcoides mento*), Ayu (*Plecoglossus altivelis*), whiting (*Merlangius merlangus*), yellowtail (*Seriola quinqueradiata*) and zebrafish (*Danio rerio*) [50–58]. On the contrary, no growth effects have been found for goldfish (*Carassius auratus*), red sea bream (*Pagrus major*), Atlantic cod (*Gadus morhua*), and zebrafish [59–65]. Increasing water flow to promote swimming behavior in the rearing tanks also results in growth modulation in several flatfish species, both previous to and after metamorphosis [66].

Exercise-induced growth effects are not only species dependent, but also the intensity and duration of the training regime, the life stage of the exposed individuals as well as several environmental factors appear to be fundamental. For most salmonids, training at moderate speeds between 0.5 and 1.5 body lengths per second (bls$^{-1}$) appears to be optimal [7,25]. For example, Atlantic salmon pre-smolts trained at 1 bls$^{-1}$ grew 15% larger than fish kept at 0.3 bls$^{-1}$ [67], while training adult Atlantic salmon for a period of 8 months at 0.45 bls$^{-1}$ resulted in a 38% higher increase in body mass compared to control fish held in nearly stagnant water [40]. Brook trout (*Salvelinus fontinalis*) displayed higher growth when subjected to swimming intensities of 0.85 bls$^{-1}$, while higher velocities (1.72 and 2.5 bls$^{-1}$) resulted in reduced growth compared to individuals kept in still water [38]. Water velocities around 1 bls$^{-1}$ also resulted in higher growth for brown trout (*Salmo trutta*) [33] and rainbow trout [34] compared to untrained fish. Thus, it seems that exercise training salmonids by induced-swimming at moderate intensity (water speeds ~ 1 bls$^{-1}$) promotes growth, while higher or lower intensities result in poorer performance due to an increased energetic cost.

In salmonids, most of the studies reporting improved growth in response to exercise training have found that this effect is given, mainly, by higher feed conversion efficiency linked to increased appetite (feed intake), though some works have found increased feed intake to be the principal cause [7]. While forcing fish to swim at higher than optimal speeds results in lower growth and feed efficiency associated to an unsustainable high energy demand to maintain station, rearing salmonids at low speeds or static water results in high amounts of spontaneous activity, aggressive behavior and the formation of dominance hierarchies which would result in an overall higher use of energy than fish held at moderate
swimming speeds [7]. The latter reflects a behavioral benefit of rearing salmonids at moderate water flows. Actually, long-term training results in less stressed fish as suggested by lower levels of cortisol in exercise trained rainbow trout [68], cortisol returning to basal levels faster in trained than in untrained striped bass after handling stress [52] and by cortisol returning to lower than pre-training levels in 24 h exercised Atlantic salmon [69]. Furthermore, salmonids swimming at moderate speeds tend to form schools, reducing the amount of fin lesions due to a reduction in aggressive behavior [42,70]. Higher water velocities will favor a better distribution of water quality parameters and feed, further dampening social activities and promoting a desirable homogeneous growth of the population [7].

Another factor potentially affecting a better feed efficiency resulting in higher growth is the initiation of ram ventilation at moderate swimming speeds. Ram ventilation is the capacity of some fish species to passively ventilate by opening their mouths when swimming or facing high water speeds, allowing water to pass through the gills with enough pressure for gas exchange to occur without the need for branchial pumping [71,72]. This has been found to result in a significant net energy sparing effect of around 10% in rainbow trout [73].

Finally, improved growth in response to exercise training has been found to be related to increased levels of circulating growth hormone [74].

Mechanistically, exercise training induced-growth is explained mostly by an increase in the size of muscle fibers (hypertrophy), while the recruitment of new fibers (hyperplasia), though possible, has not been yet determined [75]. Such changes in the muscle fiber number and distribution (cellularity) have been further linked to improvements in fillet texture and quality [40,76].

4.3.2 Exercise training and the fish cardiovascular system

Given the importance of cardiac function for the aerobic swimming performance of fish [77,78], research on the effects of exercise on the fish heart and the cardiovascular system has been acknowledged for over 50 years [79]. Alongside skeletal muscle, the heart and the cardiovascular system are most probably the main systemic features resulting affected by exercise training, and while cardiac physiological acclimation has been extensively studied [80], a relevant gap exists in relation to the underlying molecular responses driving such acclimation.
4.3.2.1 The fish heart

In vertebrates, the heart is the principal driving force behind the most fundamental role of the cardiovascular system: to supply nutrients and \( \text{O}_2 \) to the tissues at the same time of collecting and removing \( \text{CO}_2 \) and cellular metabolic waste products [81]. The fish heart is a system of chambers and valves maintaining a unidirectional blood flow (Fig. 1). Venous blood is received by the first chamber; the *sinus venosus*. This is a thin-walled sac that, depending on the species, may or may not be surrounded by a muscular layer. For example, the European eel (*Anguilla anguilla*) has an almost complete muscular layer, while this is practically absent in brown trout and zebrafish [82] reflecting the high anatomic and functional diversity of the cardiac apparatus among teleosts. After the *sinus venosus*, blood is sequentially transported into the atrium and the ventricle. Both of these chambers are lined by cardiac muscle, though it is in the ventricle were the pumping pressure is generated to supply blood for the whole system. Previous leaving the heart via the ventral aorta and into the gills for oxygenation, blood passes through the last cardiac chamber; a pressure valve named *bulbus arteriosus*. Some primitive fish species, as well as the elasmobranchs have retained this last chamber with contractile capacity, being specifically named *conus arteriosus* [83].

The ventricle takes the largest percentage of the heart mass, and relative to body weight, it comprises around 0.12% in salmonids [49,81,84], though it may vary from 0.04% in flatfishes like the flounder (*Pleuronectes platessa*) to up to 0.38% as in the skipjack tuna (*Katsuwonus pelamis*) [85]. The fish ventricle may take one of two main architectures depending on the species lifestyle and physiological needs. The most common is a sac-like ventricle consisting solely of one type of myocardium called spongiosa, which occurs in about two thirds of all fish species. The spongiosa is a fine arrangement of trabeculae spanning the ventricle’s lumen forming a sponge-like network. In this heart morphology, all nutrients and oxygen are supplied to the myocardium by the returning systemic blood [86,87]. The second ventricular architecture is possessed by athletic-like fish species, including tunas and salmonids which have to perform extensive migratory journeys. In this case, the spongiosa is surrounded by an outer compact layer of circumferentially arranged cardiomyocytes termed compact myocardium [88] and the ventricle may adopt a pyramidal shape. Furthermore, the compact myocardium is always associated to the presence of coronary circulation irrigating it with freshly oxygenated blood [87,89]. These features (pyramidal shape and coronary irrigation) are thought to confer athletic-like properties to the heart supporting the increased cardiovascular demand of the fish by generating higher blood pressures and flow [90].
The proportion of compact myocardium relative to ventricular mass increases with age and can be up to 65% in highly active species such as skipjack, yellowfin (*Thunnus albacares*) and bigeye (*Thunnus obesus*) tunas, anchovy (*Engraulis encrasicolus*) and the Pacific tarpon (*Megalops pacificus*), while it rarely exceeds 50% in salmonids [91–93].

**Figure 1:** The Atlantic salmon heart. Venous blood flows into the heart through the *sinus venosus* (S) into the atrium (A). From there, it flows into the ventricle (V) and leaves the heart through the *bulbus arteriosus* (B) into the gills. The outer layer of the ventricle is the *epicardium* (E). The picture on the right shows a longitudinal section of the heart. Within the ventricle, the two different myocardial layers are perfectly distinguishable. V-c= compact myocardium; V-s= spongy myocardium. Photo: Trygve Poppe.

Several cardiac malformations and abnormalities have been seen in farmed salmonids, including atherosclerosis, hypoplasia of the compact myocardium, formation of cysts and malformed ventricles [5,6,94–96]. For example, the specialized pyramidal ventricular shape becomes rounded, resembling the morphology of a sedentary fish species ventricle, while fat depositions may appear around the ventricle and *bulbus arteriosus* (Fig. 2). Furthermore, both prevalence and severity of coronary artery lesions are correlated with growth rate [87,97]. While the coronary circulation does not seem to be important during routine cardiac function, as suggested by studies were the coronary artery was ligated [80], coronary oxygen supply does become important under situations requiring higher cardiac performance, such as during swimming or under hypoxic conditions [87]. This evidence suggests that aquaculture-induced high growth rates potentially resulting in a higher degree of coronary lesions would inflict a serious hazard on fish when under stressful situations, such as during crowding, transporting, etc.
Figure 2: A: Normal wild steelhead trout ventricle displaying a typical pyramidal shape. B: Abnormal heart from a farmed rainbow trout after sudden death in a net pen in Norway. Note the more rounded shape and excess fat deposition in ventricle and bulbus arteriosus. C: Farmed Atlantic salmon heart (sudden death) with fat depositions around the bulbus arteriosus and ventricle plus mild loss of pyramidal shape. Picture from Trygve Poppe (modified from [80]).

4.3.2.2 Exercise training effects on the cardiovascular system

The cardiovascular system, as well as the cardiac muscle itself, expresses an important degree of morphological and physiological plasticity to environmental and biological factors [80]. Aerobic exercise training has consistently been shown to positively affect cardiovascular performance as well as other parameters of the oxygen convection cascade. Maximum oxygen consumption increases in response to exercise training [49] and is closely related to the capacity of the cardiovascular system to transport oxygen [98], reflecting the capacity to perform sustained aerobic exercise. Examples of this are tunas, which have exceptionally high levels of maximum oxygen consumption compared to other fish species [99]. Furthermore, the relative ventricular mass (RVM) increases in response to sustained exercise training in most studied species [43,49,79], though some studies have failed to demonstrate this [36,39,100]. It seems that the duration, intensity and modality of the training protocol plays an important role in this response, with longer and more intense training regimes producing relatively larger hearts. In salmonids, relative cardiac mass increases in the range from 10% [79] to as much as 46% [34]. Highly related to the RVM is maximum cardiac output, i.e. the product of stroke volume and heart rate. While stroke volume is dependent on cardiac size and ventricular filling pressure, heart rate will depend upon the contractile efficiency of the cardiomyocytes [101]. In the perfused rainbow trout heart, exercise training produced an 18% higher cardiac
output compared to untrained hearts, an effect mostly given by an increment in the stroke volume. Higher maximum oxygen consumption and cardiac output as a result of aerobic exercise training probably results in an enhanced capacity for oxygen convection into the skeletal muscle [101]. Supporting this idea, exercise training in fish has been found to target and enhance other levels related to oxygen distribution cascade, including haematocrit [46,49,79] and capillarization of the skeletal muscle [36,102]. These effects result in an increment of the oxygen diffusional surface area further reducing the distances between the capillaries and the mitochondria, as well increasing transit time of red blood cells in the capillaries allowing more time for oxygen to be extracted by the tissues [49,103]. The final aim of efficiently driving and internalizing oxygen into the tissues is to use it for generating energy in the form of ATP. The oxidative capacity of the cardiac and skeletal muscles is increased in response to exercise training, as suggested by elevated levels of enzymes involved in aerobic metabolism [36,43,104–106]. This would confer the trained individual’s muscle with higher power production capacity and, hence, a better performance [106].

All of these effects are thought to provide the exercise trained fish with a fitter cardiovascular system and an increased aerobic scope (maximal range by which oxygen consumption can be increased above the basal metabolism demand) [107], resulting not only in a better swimming capacity, but also in performing better when faced to environmental and disease challenges, thus potentially improving the welfare of farmed fish.

### 4.3.2.3 Exercise-induced molecular acclimation mechanisms

Despite the relatively extensive current knowledge on cardiovascular effects of exercise training in fish, there is scarce knowledge on the underlying molecular changes leading to acclimation. This is contrary to the mammalian literature, where both the cardiac and skeletal molecular signature associated with the exercise-induced benefits is well on the way of being established [108,109]. For example, improved cardiac performance and growth in response to exercise in humans, referred to as “athlete’s heart”, is supported by a specific molecular signature composed of higher levels of proteins and their coding mRNA’s involved in processes such as excitation-contraction (E-C) coupling, angiogenesis and lipids metabolism. In this scenario, cardiac molecular acclimation underlies a beneficial “physiological” cardiac growth, resulting in a heart with increased cardiac output and a greater ability to deliver oxygen to the contracting skeletal muscle [110]. On the contrary, cardiac growth occurring in
disease settings, such as hypertension, ischemia or myocardial infarction, is associated with an increased risk of heart failure and is referred to as “pathological” (Fig. 3). In this case, the molecular signature differs from that seen on physiological growth [108] and further reflects the molecular changes seen in the ageing heart [111,112].

![Figure 3: Physiological and pathological modes of cardiac growth as known for mammals. Physiological cardiac growth is a beneficial acclimative reversible response to stimuli such as exercise training and pregnancy, resulting in a healthy heart with an improved pumping capacity. On the other hand, pathological cardiac growth occurs in settings of diseases such as hypertension and results in structural and functional modifications leading to cardiac failure. The molecular mechanisms involved in both types of cardiac growth differs radically (box).](image)

**Mechanisms of cardiac growth**

At the cellular level, cardiac growth can be given by one or a mix of two mechanisms: cardiomyocyte hypertrophy and cardiomyocyte hyperplasia. The first refers to an enlargement of the cell, while the second involves cell proliferation. For example, chronically induced anemia in rainbow trout [113] and zebrafish [114] stimulated cardiac growth via both mechanisms. Compared to untrained individuals, cardiac muscle of exercise trained zebrafish displayed higher transcript levels of proliferating cell nuclear antigen (PCNA), a cofactor of DNA polymerase delta and a reliable marker for cell division activity [115]. It seems then plausible to suggest that both hypertrophic and hyperplastic mechanisms take part in the exercise-induced cardiac growth, though this assumption has not been thoroughly examined so far in exercised fish. While the above mentioned cardiac growth is though to be a physiological acclimation to the higher oxygen demand by the tissues, pathological cardiac
growth has also been found to occur in fish. The heart of rainbow trout results enlarged in response to post-stress cortisol production and this has been associated to increases in several cardiac molecular markers at the mRNA level [116].

**Contractility in cardiomyocytes**

The mammalian cardiac and skeletal muscles are known to respond to aerobic exercise training with an improved contractile performance, which is reflected by elevated cardiomyocytes levels of gene transcripts and proteins involved in the E-C coupling and Ca\(^{2+}\) handling process [117–121]. In mammals, after depolarization (excitation) of the sarcolemmal membrane, the cardiomyocyte E-C coupling process (Fig. 4) begins with the influx of Ca\(^{2+}\) through the dihydropyridine receptors (DHPR), a voltage-dependent L-type Ca\(^{2+}\) channel. The entry of Ca\(^{2+}\) ions triggers the release of Ca\(^{2+}\) from the sarcoplasmic reticulum (SR) via the ryanodine receptors (RyR) in a calcium-induced calcium release mode (CICR), elevating the cytosolic Ca\(^{2+}\) concentration which activates troponin C for finally initiating myofilaments contraction (reviewed by Fill and Copello [122]). A deviation from this model occurs in most fish species, where the entry of extracellular Ca\(^{2+}\) appears to be sufficient to stimulate the contractile machinery [123], by-passing the need for internal (SR) Ca\(^{2+}\) to be released.

Ca\(^{2+}\) must then be removed from the cytosol to reduce its concentration and allow muscle relaxation. In mammals there are two main routes acting simultaneously to achieve this. Ca\(^{2+}\) is recycled back into the SR via the SR Ca\(^{2+}\) -ATPase (SERCA2) and is further removed to the extracellular space through the Na\(^+\)/Ca\(^{2+}\) exchanger (NCX). The preferred mechanism for reducing the cytosolic Ca\(^{2+}\) concentration varies considerably among species and during the ontogenic development of an individual, reflecting different cardiac E-C coupling strategies. For example, rat ventricle recycles 92% and 7% of Ca\(^{2+}\) through SERCA2 and NCX, respectively, while the values for rabbit ventricle are 70% and 28%, respectively [124]. In fish, few studies have acknowledged the importance of SERCA2 in Ca\(^{2+}\) recycling after contraction, though there seems to be a relation between higher swimming performance and higher dependence on SR stored Ca\(^{2+}\) to stimulate contraction [125].
Figure 4: Excitation-contraction coupling in cardiac muscle. The electrical impulse is sensed by the voltage dependent L-type Ca\(^{2+}\) channel (DHPR). Ca\(^{2+}\) entry generates a transient rise in intracellular free Ca\(^{2+}\) which in most fish species is sufficient to activate the contractile apparatus (sarcomere). In mammals and in highly athletic fish species, the influx of extracellular Ca\(^{2+}\) triggers Ca\(^{2+}\) release from the sarcoplasmic reticulum (SR) via the ryanodine receptors (RyR) which will further stimulate sarcomere contraction. Muscle relaxation is achieved after Ca\(^{2+}\) is recycled back into the SR via SR Ca\(^{2+}\) ATPase (SERCA2) and/or to the extracellular space through the Na\(^{+}/Ca\(^{2+}\) exchanger (NCX). Calsequestrin 1 (Calseq1) is a SR binding protein, while Fkbp1b is a RyR regulatory protein. Modified from Fares and Howlett [112].

Energy metabolism

A heart subjected to a higher work-load will undoubtedly require more energy to satisfy its increased pumping requirements. In oxidative muscles, such as heart and red muscle, the energy demands are met almost exclusively by mitochondrial aerobic metabolism. In cases of chronically increased energy demand, as during exercise training, the tissue undergoes physiological changes allowing it to enhance its efficiency. Such acclimative responses include mitochondrial ultrastructural changes as well as changes in the content and activity of metabolism-related enzymes [126].

Studying the shifts in the underlying molecular signature can give fundamental information to assess the cellular fuel preference in response to a given stimulus. 5’ AMP
activated protein kinase (AMPK) is probably the most important sensor of cellular energy status and its metabolic functions appear to be well conserved across living organisms (Fig. 5) [127–130]. An increase in the AMP:ATP ratio in response to cellular or metabolic stress due to ATP depletion (exercise training), or due to inhibited ATP synthesis (hypoxia, glucose deprivation, etc), will directly activate AMPK and further induce AMPK’s activation by upstream kinases [131]. Activation of AMPK results in both acute (phosphorylation-driven) and chronic (gene expression-driven) metabolic effects directed towards inhibition of non-fundamental energy-consuming anabolic pathways and activation of catabolic ATP-restoring pathways. In the mammalian skeletal muscle, AMPK is known to inhibit glycogen synthase (GS) and acetyl-CoA carboxylase (ACC), down-regulating glycogen and lipid synthesis, respectively. On the contrary, AMPK stimulates the intake of glucose and fatty acids in skeletal and cardiac muscles by inducing the translocation of the glucose transporter type 4 (GLUT4) and the fatty acid translocase (FAT/CD36) to the cell membrane [131–133]. Furthermore, AMPK will induce the transcription and/or activate proteins involved in mitochondrial biogenesis and fatty acid and glucose oxidation within many others, suggesting AMPK’s participation in the long term acclimative metabolic response to exercise training [134].

Figure 5: Metabolic effects of AMPK. By controlling the activity and/or expression (black arrows) of key proteins involved in either catabolic or anabolic pathways, AMPK regulates the cellular energy homeostasis. Fish cartoons represent those AMPK signaling mechanisms that have been found, so far, to be well conserved.
between fish and higher vertebrates. Modified from Hardie 2004. EF2: elongation factor-2; PGC1α: PPARγ co-activator 1α; FAT: fatty acid translocase; GLUT4: glucose transporter 4; PFK2: phosphofructokinase 2; ACC: acetyl-CoA carboxylase; GS: glycogen synthase; HMGR: 3-hydroxy-3-methyl-CoA reductase; FAS: fatty acid synthase; PEPCK: phosphoenolpyruvate carboxykinase; G6Pase: glucose-6-phosphatase.

There is a wide phylogenetic diversity among fishes in terms of cardiac preference for substrate utilization, and different studies have shown how the cardiac tissue is proficient in oxidizing lipids, carbohydrates, protein and lactate, though the first two substrates appear as the most important ones [43,101,126,135–138].

Fatty acid oxidation: In the mammalian tissues, and after hydrolysis of circulating lipoproteins by lipoprotein lipase (LPL), cellular uptake of the released fatty acids occurs either by passive diffusion or may be facilitated by membrane associated proteins, including fatty acid transport protein (FATP), FAT/CD36 and fatty acid binding protein (FABP). In fish, most of these molecules have been found, and appear to play similar roles [139–141]. Once in the cytosol, the enzyme fatty acyl-CoA synthetase (ACS) converts the fatty acids into acyl-CoA esters which may be transported into the mitochondria via the carnitine palmitoyltransferase (CPT) shuttle system for further entering the β-oxidation pathway, which has been well studied in several fish species [142,143]. During each β-oxidation cycle, a process involving four enzymes acting sequentially, each acyl-CoA molecule is shortened in two carbons and a single molecule of acetyl-CoA is produced which may then enter the TCA cycle. NADH produced during both β-oxidation and TCA cycle is then used to generate ATP in the electron transport chain. Importantly, each of the β-oxidation process enzymes is prone to feed-back inhibition by their products, especially by the acetyl-CoA/CoA ratio [144]. Another fundamental control point in the process of mitochondrial fatty acid oxidation is the allosteric inhibition of CPT1 by malonyl-CoA. The latter is produced from acetyl-CoA by the enzyme acetyl-CoA carboxylase (ACC). ACC activity is therefore inversely related to β-oxidation. The opposite role of ACC is played by malonyl-CoA decarboxylase (MCD), favoring the presence of acetyl-CoA for its use in the TCA cycle. As mentioned earlier, AMPK inhibits ACC activity by phosphorylation, and further activates MCD promoting a catabolic environment.

Several of the enzymes involved in the fatty acid oxidation process are controlled at the gene expression level. Among the most important regulators are the peroxisome proliferator-activated receptor (PPAR)α and the PPARγ co-activator (PGC)1α [145]. These mediate the
transcription of a set of genes controlling cellular fatty acid uptake (lpl, fatp and fat/cd36),
esterification (acs), import (cpt1) and oxidation in both mitochondria (mcad), and
peroxisomes (acyl-CoA oxidase - aco) [146]. Recently, pparα was found to be up-regulated in
cardiac muscle of Atlantic salmon treated with tetradecylthioacetic acid (TTA), a TTA agonist
and well-known enhancer of lipid metabolism and β-oxidation. Associated to it, levels of
cpt1, aco and lpl were also up-regulated, suggesting a conserved role for this transcription
factor among vertebrates [147].

Glucose oxidation: Biochemically, glucose metabolism appears to be a well conserved
mechanism among vertebrates (Fig. 6) [130,148–150]. Glucose is imported into the cells via
the glucose transporters (GLUTs). Once in the cytosol, it is phosphorylated by the enzyme
hexokinase (HK) into glucose 6-phosphate (G6P) which may then be used either to build up
glycogen stores (glycogenesis), to produce reducing agents (pentose pathway) or to generate
energy. The catabolic breakdown of glucose takes part in two stages. The first involves the
anaerobic-cytoplasmic glycolytic phase which results in the production of two pyruvate
molecules with a net yield of two ATP molecules. Pyruvate may then be transported into the
mitochondria where it is transformed into acetyl-CoA by the pyruvate dehydrogenase (PDH)
complex, which then may be further oxidized in the TCA cycle or be transformed into
malonyl-CoA by ACC. Inhibition of the PDH complex occurs after activation of the enzyme
pyruvate dehydrogenase kinase (PDK) in response to high ATP, NADH and acetyl-CoA
levels [151], similarly as it occurs for β-oxidation inhibition. If anorexigenic conditions are
present, pyruvate can be reduced into lactate in the cytosol for further supplying NAD+ to
glycolysis, a process performed by lactate dehydrogenase (LDH). Interestingly, such a
reaction is reversible, so circulating lactate may be transformed into pyruvate for further
feeding the TCA cycle.

The PDH complex plays a fundamental role in the interaction between fatty acid and
glucose/lactate metabolism. Higher rates of β-oxidation resulting in accumulation of acetyl-
CoA will activate PDK down-regulating the PDH activity. On the contrary, pyruvate
oxidation by PDH is increased when plasma fatty acid concentrations are low, or the β-
oxidation process is inhibited [152]. There is then, a tightly regulated system for energy
substrate usage or preference, which is modulated by the concentration of substrates and
products [153].
4.3.3 Exercise training and disease resistance

While there is a relatively large amount of knowledge on the beneficial effects of exercise training in the cardiovascular system of fish, basically, there are no studies regarding the effects of exercise training on the immune system and disease resistance. Resistance to infectious diseases may potentially be given by inherited or acquired characteristics at different levels affecting the host-pathogen interaction. These include behavioral, physico-chemical (integument) and physiological (immune system and tissue protective/cleaning factors) barriers [154]. Human sports medicine has addressed the latter issue as central and a series of acclimative molecular mechanisms have been found to explain how the external stimulus (exercise) couples with the internal response (transcriptome-proteome) in producing the well-known exercise training health benefits [155,156]. In humans, physical activity results in increased protection against all kinds of diseases, including both lifestyle-associated as well as infectious diseases. Within the first, physical activity provides protection against diabetes type 2, colon and breast cancer, dementia, and cardiovascular diseases [155].
In humans, there is a common consensus in that moderate-regular exercise training enhances the immune function and confers resistance to infections. On the contrary, endurance-high intensity exercising provokes a short-term immunosuppression referred to as “open-window” when the host has a higher susceptibility to infections [157]. Among the factors explaining the latter case, are suppressed functions of several components of both the innate (natural killer cell activity and neutrophil oxidative burst) and the adaptive (T and B cell function) immune systems, as well as increased plasma levels of pro and anti-inflammatory cytokines [158].

Searching for the muscle contraction-induced factors or mechanisms driving the positive effects of moderate exercise training has been a major challenge during the last decade. It was in this scenario that the term myokine was coined for those cytokines which are produced and released by the contracting muscle, acting as signaling molecules in an auto-, para- and/or endocrine fashion [155]. Among the several myokines identified so far in mammals, interleukin (IL)6 was the first to be discovered and is the most studied one so far. Usually though as a pro-inflammatory cytokine, muscle contraction-derived IL6 has strong systemic anti-inflammatory and metabolic effects and it is suggested as one of the main drivers of the exercise training health effects [159]. A reduction in the degree of systemic low-level inflammation appears as a main role for the exercise-derived myokines. Systemic low-level inflammation is defined as a two to fourfold increase in the levels of circulating pro- and anti-inflammatory cytokines, cytokine agonists and acute-phase proteins including tumor necrosis factor (TNF)α, IL1β, IL6, IL1 receptor antagonist (IL1ra) and soluble TNF receptor (sTNF-R) [160]. This type of inflammation is strongly associated with ageing, smoking and obesity, and with the occurrence of cardiovascular failures, diabetes type 2 and muscle wasting. Exercise training would reduce systemic low-level inflammation via production and release of IL6 from the contracting muscles [161]. IL6 produced in this way appears to orchestrate the formation of an anti-inflammatory environment by increasing the plasmatic levels of the anti-inflammatory IL1ra, IL10 and sTNF-R at the same time of inhibiting the production of TNFα [162–164]. IL6 also drives some of the metabolic effects of exercise training, as it will activate muscle AMPK through the gp130/IL6Rα receptor, stimulating fatty acid oxidation and glucose uptake. At the systemic level, IL6 stimulates fatty acid oxidation resulting in visceral fat reduction [165] with the consequent reduction in visceral’s fat production of TNFα [166].
Overall, engaging in physical activity seems to enhance the immune system and induce an anti-inflammatory state coupled to a fatty acid oxidation dominant environment. All of the above would be directly implicated in conferring the exercise-induced benefits in terms of resistance to infections as well as in lower prevalence of lifestyle diseases. Nevertheless, none of these effects have been so far examined in fish. Furthermore, and in addition to above mentioned immune and anti-inflammatory potential effects exercise training may have on fish, other mechanisms affected by exercise further argues for an improved robustness and overall health status. Specially, an improved cardiac performance with an overall higher oxygen distribution capacity (see section 3.3.2.2) is highly suggestive of an improved resistance to metabolic and life-style associated diseases as seen for humans.

4.3.4 Inherent individual variability in robustness

An important factor affecting the robustness level of a fish population is the high inherent or naturally occurring individual variation in physiological performance and responses to environmental challenges [167–169] For example, Claireaux et al. [168] found that the inherent differences in swimming capacity of juvenile rainbow trout (Oncorhynchus mykiss) were maintained nine months later as reflected by differences in several cardiovascular and performance parameters. In humans there is a significant individual variation in the responsiveness to exercise training which can range from no gain to up to 100% improvement in cardiorespiratory fitness. The level of pre-training and the heritability effect are the main factors explaining these response [170,171].

A difficulty with interventional experiments like exercise training is the degree of inherent individual diversity that exists in physiological performance traits, which can often be greater than the change elicited by the experimental intervention. Hence such inherent trait variations may be large enough to mask the actual effects of training. Further, it may be important to identify the set of individuals in which exercise training has the potential to cause significant changes. The latter would be of particular importance in an aquaculture facility where minimizing the costs of the procedure is always desirable.
5 Aims of the study

Principal aim
Evaluate the potential of exercise training as a measure to improve robustness of farmed Atlantic salmon smolts.

Specific aims
1- Evaluate the effects of exercise training on the resistance to infectious diseases.

2- Assess the impact of inherent swimming capacities on disease resistance, and the interaction of these with exercise training.

3- Characterize the exercise-induced cardiac molecular signature underlying a robust heart.

4- Evaluate the effects of exercise training on growth.
6 Results and discussion

The effects of exercise training on robustness were investigated through a series of two experiments in which Atlantic salmon pre-smolts were trained at different intensities (water velocities), durations and modalities (continuous vs. interval training).

The overall effects of exercise training on robustness were assessed by measuring disease resistance, the cardiac molecular acclimation response as well as growth performance. Growth and disease resistance are the two most relevant operational parameters in the fish farming industry. Linked to this, an improved cardiac capacity would further argue for higher robustness given not only the heart’s role in efficiently pumping oxygen and nutrients through the system, but also due to its potential association with reduced levels of life-style diseases as seen in mammals.

6.1 Disease resistance

Given the widely known health effects exercise training produces in mammals as well as the importance of improving health conditions in farmed fish, the potential effects of exercise training on disease resistance were assessed for Atlantic salmon, hypothesizing a similar beneficial response as in higher vertebrates. To evaluate disease resistance, trained fish were transferred to seawater and challenged by co-habitation with IPNV infected fish to simulate a natural-like infection transmission mechanism. IPN was the selected model given its position as a principal viral disease in salmon aquaculture, especially affecting smolts after sea water transfer [4].

6.1.1 Exercise effects on disease resistance

A striking result of this thesis was the finding of exercise training conferring higher disease resistance capabilities to Atlantic salmon smolts. This was reflected by trained fish displaying enhanced survival to IPN in comparison to untrained fish (paper 1) or fish trained at sub-optimal regimes (paper 2). Such an effect was found to be highly dependent on the intensity and modality of the regime, with higher survival tending to occur in response to moderate training intensities.
In humans, the benefits gain of going from no exercise to moderate exercise are potentially much larger than when just increasing the intensity of a previous exercise regime [172]. Due to this, in trial I (paper 1) the main objective was to assess the sole effect of exercise training the fish in comparison to individuals being held in, practically, standing waters (control). In that trial, fish were trained for six weeks and then allowed a further six weeks to detrain and smoltify at control water velocity. Two modes of training were performed and compared against the control group. Intriguingly, improved survival was found for fish being trained at an interval regime with an average water velocity of 0.85 bls$^{-1}$ and 0.25-fold daily changes in water velocity (0.8 + 1 bls$^{-1}$) compared to fish trained at a similar average but constant velocity and to untrained fish. In good agreement with this, another trial performed by our research group (unpublished results) showed that higher survival (~20%) to a natural outbreak of winter ulcer was displayed by Atlantic salmon previously trained for nine weeks at a similar interval regime (0.8 + 1.2 bls$^{-1}$) as in trial I. In that case, survival was higher compared to a continuous velocity regime (1 bls$^{-1}$) and to the control group (0.5 bls$^{-1}$).

With the knowledge gained in trial I, trial II (paper 2) was designed as to expand the duration and the range of the exercise intensities. Two continuous (0.65 and 1.31 bls$^{-1}$) and two interval (0.3 + 1.31 bls$^{-1}$; differing between them on the quantity of velocity changes during a day) training regimes were performed for ten weeks, and disease resistance (IPN) was compared against a control group trained at 0.3 bls$^{-1}$ continuously. This control was selected, instead of still water as in trial I, as to mimic a currently common fish farming condition, hence allowing for the assessment of the potential impact that exercise could have on the industry. Importantly, the best survival of this trial was achieved by fish being trained at constant water velocity averaging 0.65 bls$^{-1}$ throughout the trial, while the second best group was trained constantly at 1.31 bls$^{-1}$. The interval regimes with large magnitude fold changes (3-fold) resulted in the worst survival even though their average water speeds were also 0.65 bls$^{-1}$.

Beside the different control group velocities as well as the training regimes used, trials I and II further differed in the duration of the exercise stimulus (6 and 10 weeks, respectively) and of the detraining period, i.e. time between end of the training regimes and beginning of disease challenge test in sea water (6.5 and 2 week, respectively). Among other implications, this means that fish from trial II were exercise trained during most of the smoltification period, and it is currently unknown how this may affect the disease resistance performance of fish after sea transfer.
It can be concluded that optimal exercise training of Atlantic salmon pre-smolts in the fresh water rearing stage resulted in improved robustness reflected by a higher resistance to infectious diseases after sea water transfer. The best results were obtained when interval exercise was performed with mild daily changes in water velocity (<0.5-fold) with an average velocity around 1 bls\(^{-1}\). On the contrary, the relatively large changes in water velocity (trial II) appeared to worsen the disease resistance performance, probably due to higher stress levels resulting in a poor capacity of the fish to reach allostatic. Future research should then include trials focused on fine-tuning the training intensities, as well as in determining optimal durations for both training and detraining periods. Importantly, such an optimal regime must meet other requirements from the industry beside disease resistance, such as growth, overall fitness and its technical applicability.

6.1.2 Disease resistance is dependent on inherent swimming capacity

In humans, exercise training differently affects individuals with different fitness backgrounds given by pre-training fitness status and/or heritability [171]. Since fish have shown to possess a wide degree of inherent swimming capacity which is related to their cardiovascular performance [168], we evaluated if the effects of exercise training in disease resistance were population-wide, or only a part of the population (i.e. either the inherently poor or good swimmers) would result more benefited than the other.

Strikingly, fish that were categorized as good swimmers before commencement of exercise training (paper 2) displayed a significantly higher resistance to IPN than those initially categorized as poor swimmers. Another finding was that, though the inherent swimming capacity appeared to predict disease resistance after sea transfer, exercise training was sufficient to modify this (paper 2). While exercise training was not found to have a considerable effect on disease resistance in the inherently good swimmers, disease resistance of the inherently poor swimmers appeared to be highly affected by the exercise stimulus (Fig. 7). This was demonstrated by poor swimmers responding positively to an optimal training regime and negatively to a non-optimal regime. This further suggests that the overall disease resistance differences found between the different training regimes was mainly given by an exercise training effect upon the inherently poor swimmers.

To search for gene expression correlates between inherent swimming capacity and disease resistance at the end of the disease challenge test, a microarray platform containing 21
thousand unique sequences [173] was used. Analyses detected that a set of genes involved in the host response to viral infections, known as VRGs (virus responsive genes [174]), were the only significantly regulated genes between poor and good swimmers in cardiac tissue (paper 2), reflecting a higher infection status in poor swimmers. This further confirms a role for the heart in either being directly involved in responding to the infectious agent, or at least reflecting the infectious status and host response.

**Figure 7**: Interaction between inherent swimming performance, training regimes, and their association with survival on a disease challenge test. The trends shown here are valid for the training intensities and types used in this thesis and it should be expected to change if more intense training regimes are used.

Overall, promoting aerobic sustained swimming in aquaculture rearing facilities represents a non-invasive husbandry strategy towards increasing fish disease resistance, at the same time of being economical and in line with tougher ethical requirements for animal production. Furthermore, as an association seems to exist between inherent swimming performance and disease resistance, splitting the population according to their swimming performance in a simple screening test allows for separating those fish in which benefits are to be obtained by exercise training from those which may not. The relation between inherent swimming performance and disease resistance may also be exploited by fish breeders to reduce the proportion of unfavorable gene variants in the breeding nucleus.
6.2 Cardiac molecular acclimation response

Given that exercise training promoted higher disease resistance and that training strengthens cardiac performance [80], the cardiac molecular acclimation response was analyzed to gain a deeper insight into the driving mechanisms behind such beneficial exercise-induced effects. In the first trial (paper 1), emphasis was set on finding sets of genes whose regulation in response to training would correlate with the observed differences in disease resistance seen after sea transfer. This was done by using real-time quantitative RT-PCR (qPCR) as well as a microarray platform specially designed for studies involving the immune response (SFA 2.0 immunochip [175]). In the second trial (paper 3), several key cellular mechanisms known to participate in the physiological cardiac enlargement response to exercise, as seen in mammals, were studied. In this case, only those training regimes consisting on continuous water velocities were selected to further assess the effect of the intensity, or cardiac workload, on the given changes.

6.2.1 Effects on cardiac immune-related mechanisms

The emphasis on analyzing the cardiac molecular response was not only because of the heart’s central position as a main exercise target organ, but also because of the fish heart being an immune-relevant organ where many viral pathogens manifest themselves [175–178]. Hence, we looked for those underlying transcriptional changes which occurring in association with disease resistance could explain the acclimation response to the exercise stimulus.

In trial I (paper 1) we found a significant modulation in the cardiac transcription of genes directly involved in promoting protection against infectious agents as well as in conferring tissue protection against both endogenous and exogenous toxic compounds. Among these, down-regulation in the expression of genes involved in the inflammatory response was of special interest given the known anti-inflammatory effects of training in mammals [155]. Both continuous and interval training showed significantly lower transcript levels of *tnf*α and *il1β* when compared to the untrained control group as evidenced by qPCR analyzes at the end of a six week detraining period. Nevertheless, only those fish belonging to the interval training regime showed further down-regulation of genes participating in other inflammatory pathways, especially those related to the production of eicosanoids (microarray data). This provided valuable information, as interval training showed higher disease resistance than the
continuous training regime. Expression of several other immune relevant groups of genes demonstrated to correlate with disease resistance, including components of the complement system and other immune effector molecules, cell adhesion and recruitment, chemokines and cytokines (Fig. 8). The expression of genes not directly linked with the immune system was also associated with higher survival. These included genes involved in protection against different tissue damaging products such as oxidants and xenobiotics, as well as mRNA of genes coding for tissue reparation and differentiation.

In paper 3, a significant up-regulation of TNFα was found at both the protein and mRNA level when analyzed immediately at the end of the training period for the highest intensity regime (1.3 bls\(^{-1}\)) compared to the control. This was accompanied by higher mRNA levels of proteins involved in both pro- and anti-inflammatory mechanisms such as cyclooxygenase2 (COX2), IL1ra and sTNF-R, suggesting a balanced cardiac inflammatory environment. Thus, on the contrary to the moderate training regimes in trial I and the 0.65 bls\(^{-1}\) regime in trial II, the higher intensity training of 1.3 bls\(^{-1}\) was sufficient to promote a significant pro- and anti-inflammatory response in the heart at end of training. Still, due to the lowered expression of inflammatory genes after six weeks of detraining in trial I, the results suggest that training may have long term effects on the cardiac inflammatory status during detraining even without a strong inflammatory response during training. Unfortunately, the cardiac inflammatory status was not examined at the very beginning of the IPN challenge tests, which could have revealed potential detraining effects also for the high intensity fish in trial II. The significant effect of detraining on cardiac immune status may be important for future rearing strategies of fish before the severe stressful sea transfer of smolts. Another intervening factor when comparing trial I and II results was the difference in treatment of controls. Holding the control fish in standing waters as in trial I may result in higher stress levels due to the formation of social hierarchies and increased levels of aggression [7], potentially leading to higher inflammation. In the contrary, the control group in trial II was continuously trained at a low speed, but it may have been sufficient for dampening the above mentioned social effect. Thus, to clarify the individual importance of factors as intensity, duration, modality and recovery of training programs these must be isolated and tested independently.

Overall, exercise training modulates the cardiac expression of inflammation-related molecules. Lower levels of these appear to positively associate with higher disease resistance when further linked to transcription modulation of participants involved in other immune and tissue protective mechanisms. Although the mechanisms are far from being acknowledged in fish, it is possible to argue for the existence of a similar anti-inflammatory effect of exercise
training among vertebrates, which might be the driver of several of the long-term exercise-induced health benefits as has been found for humans [161].

Future studies should include the immune response in other tissues, such as head kidney and spleen, as these are the fish’s primary immune organs.

6.2.2 Effects on cardiac performance-related mechanisms

Given the well-known effects of exercise training in cardiac performance of salmonids [25,43,49,101], one of the aims of this thesis was to characterize the underlying molecular signature driving the exercise-induced cardiac acclimation. Further, the existence of an intensity (cardiac workload) factor in conferring such effects was addressed by comparing three regimes of similar style (continuous velocity), but of increasing intensity including the low (control), medium and high intensity groups (0.32, 0.65 and 1.31 bls$^{-1}$, respectively). For both RVM and gene expression, the effects displayed intensity dependence, with the higher intensity regime inducing larger changes than the medium regime, compared to the controls.

A typical acclimation response to exercise training in mammals and fish, is an increment in RVM. Larger hearts promote an improved cardiac output (stroke volume x heart rate), facilitating the delivery of oxygen and nutrients to the contracting skeletal muscles. In this thesis, we report that higher RVM was seen in response to exercise training under some circumstances (paper 3) but not others (paper 1). A plausible explanation for this difference is that in paper 1 fish were exercise trained for 6 weeks, while the duration was extended to 10 weeks in paper 3, allowing more time for acclimation changes to be seen. Furthermore, in paper 3 the intensity of the group presenting higher RVM (1.3 bls$^{-1}$) compared to the controls was higher than that used in paper 1 (~0.85 bls$^{-1}$). Thus, it could be suggested that increases in RVM in response to exercise training are dependent on both duration and intensity of the training regime. This is in line with previous studies of salmonids showing that longer and more intense regimes result in higher RVM than shorter and less intense ones [36,39,43,49,79,100]. Similarly, the 20% increase in RVM seen in paper 3 falls within the range previously found for exercised salmonids. In fish, increments in RVM are seen in response to different environmental and physiological factors involved in increasing the cardiac functional demand, including acclimation to low temperatures [78,92,179–181], induced chronic anemia [113,114,182] and sexual maturation [93,183,184].
The expression of 38 molecular markers involved in mechanisms known to be associated with physiological-beneficial cardiac growth in mammals was analyzed in paper 3. Exercise-induced modulation of these markers reflected the molecular signature underlying cardiac growth and, potentially, the exercise-induced cardiovascular effects in fish. Markers were related to growth, cardiomyocyte contractility, blood supply (capillarization and erythropoiesis) and energy metabolism.

**Cardiac growth:** Since exercise training results in cardiac enlargement, the cellular mechanisms potentially involved in such a response were examined. For this, protein and gene expression levels of both cardiomyocyte hyperplasia and hypertrophy were analyzed. The training regime of higher intensity (1.3 bls-1) showed to have elevated protein levels of PCNA compared to control fish, which is strongly suggestive of cell division and, thus, cardiomyocyte hyperplasia in response to training. Furthermore, increased expression of the transcription factors *mef2c* and *gata4* was suggestive of cardiomyocyte hypertrophy, as these are known to govern the cardiac growth response to external stimuli in mammals [185]. Ideally this suggestion should be confirmed by direct cardiomyocyte size measurements, but that is difficult in the intricate cardiac tissue. While for mammals cardiac growth is given almost uniquely by cardiomyocyte hypertrophy after birth, the heart of fish seems to maintain both types of cardiac growth mechanisms throughout life facilitating the remodelling process in response to physiological cardiac growth inducers.

**Contractility:** An improvement in the pumping performance of the cardiac muscle is a key feature for several of the beneficial cardiovascular effects elicited by training, including cardiac output. Higher levels of molecules participating in the E-C coupling process reflect an improved contractile capacity in the skeletal muscles of fish and in the skeletal and cardiac muscles of mammals. This appears to be valid for both between and within vertebrate species [105,106,117–121,125,186,187]. For example, the skeletal muscle of reared Atlantic salmon possesses significantly lower levels of DHPR and RyR than its wild counterpart, reflecting the higher swimming capacity of the latter [30]. Because of this, analyzing the expression levels of such markers in the heart of exercise trained Atlantic salmon allowed us to indirectly assess its contractile capacity. Results demonstrated that exercise training affected the mRNA amount of proteins involved in the E-C coupling process, as suggested by significant transcription up-regulation of *dhpr*, *fkbp1b* and *calsequestrin1*. DHPR is a voltage dependent L-type Ca\(^{2+}\) channel present in the sarcolemmal membrane. FKBP1B and Calsequestrin1 are present in the sarcoplasmic reticulum (SR) and modulate the release and recycling of Ca\(^{2+}\) in association with RyR.
The rate of contraction-relaxation is regulated by Ca\(^{2+}\) cycling into and out of the cardiomyocyte cytoplasm. In mammals and other higher vertebrates the SR functions as an intracellular Ca\(^{2+}\) store, reducing the diffusional distances for Ca\(^{2+}\) movement and increasing the efficiency of the system [188]. Not surprisingly, cardiomyocyte contraction in species displaying high cardiac and swimming performance, such as the yellowfin and skipjack tunas, do appear to rely on SR stored Ca\(^{2+}\) for contraction [189,190]. Other athletic-like fish species, such as rainbow trout and mackerel (Scomber japonicus) have been also found to depend (to a certain degree) on SR Ca\(^{2+}\) release for contraction, as suggested by a decreased force production on cardiac strips whose RyR channels have been blocked with ryanodine [78,191,192]. Altogether, it is plausible to argue that exercise training results in a better cardiac contractile capacity in Atlantic salmon. An improved myocardium performance is further suggested by an exercise-induced higher involvement of the SR Ca\(^{2+}\) stores in the contractile process.

**Blood supply:** As mentioned in section 3.3.2.1, a specialized coronary irrigation system delivering freshly oxygenated blood to the compact myocardium layer results fundamental for its proper function in times of high cardiovascular demand. In the current work, exercise training induced the cardiac transcription of genes with fundamental roles in oxygen convection, such as *vascular endothelial growth factor* (vegf), its receptor (vegf-r) and *erythropoietin* (epo). VEGF is an endothelial cell mitogenic factor and probably the most important angiogenic factor in vertebrates [193]. In mammals, exercise training increases coronary vascular supply in the healthy, the ageing and the recovering heart [194–198]. This effect has been directly linked to higher gene and protein expression levels of VEGF, its cardiac receptors, as well as the phosphorylation levels of VEGF’s downstream mediators [199].

EPO is the main regulator of red blood cell production; hence, it is involved in maintaining an adequate oxygen supply to the tissues. Interestingly, while kidney is the primary EPO producing organ in humans, this role is appears to be played by the heart in fish [200]. Since the spongy myocardium layer receives oxygen depleted blood returning from the systemic circulation, it is perfectly arguable for a role of this in sensing \(O_2\) levels and mounting a response in the form of EPO production and release. While EPO has been also found to be produced in the heart of juvenile zebrafish, its transcript levels were not modified by exercise training [64], contrary to what was found in this study.

Interestingly, EPO signaling through EPO receptor induces VEGF production and myocardial endothelial proliferation in the mammalian heart resulting in improved cardiac
performance [201]. Hence, higher levels of vegf and vegf-r on one side and epo on the other suggest both independently and in conjunction that heart of exercise trained fish is proficient in generating new blood vessels for increasing the efficiency of the cardiomyocyte O$_2$ uptake process.

**Figure 8:** Exercise induces a series of molecular acclimative responses in the cardiac muscle of Atlantic salmon. These are thought to result in a robust heart with an improved performance as well as with beneficial immune effects.

*Energy metabolism:* Understanding the cardiac energy substrate of preference in response to exercise in fish is important, as this is known to be a main difference between physiological and pathological cardiac growth in humans. While the first relates to an increased reliance on lipids with minor effects on glucose, the second mechanism is supported by a higher reliance on glucose and a reduced usage of lipids (Fig. 9).

The overall oxidative capacity of the cardiomyocytes was increased in response to exercise training as suggested by higher levels of phosphorylated-AMPK sub-unit α (pAMPKα) on a western blott analysis. Furthermore, immunofluorescence staining of cardiolipin, a mitochondrial marker, revealed that mitochondrial area was augmented. The
oxidative capacity is increased in response to training in fish for both cardiac and skeletal muscles, as seen by higher levels of metabolic enzymes participating in the process, including succinate dehydrogenase [105,106] and citrate synthase [43,101]. Furthermore, higher mitochondrial density was seen in response to training in skeletal red muscle of zebrafish larvae [202]. Given the results from the present and previous works, it seems clear that the higher demand for energy generation during exercise training results in an improved capacity for obtaining energy aerobically.

Cardiac muscle of exercise trained fish showed a preference towards using lipids, not carbohydrates, for energy generation. This was strongly suggested by exercise-induced transcription of a series of genes involved in both mitochondrial biogenesis and fatty acid oxidation. Within these, up-regulation of pparα and pgc1α, together with ampkα, reflect a higher capacity for lipid uptake, activation and oxidation by the cardiomyocytes. PPARα is a transcription factor controlling cardiac lipid metabolism in mammals by inducing the transcription of a series of genes involved in fatty acid metabolism [203], while PGC1α co-activates PPARα and further mediates several of the AMPK effects in response to exercise training [204]. In this work, exercise training also resulted in elevated mRNA levels of aco, cpt1 and mcd, all of which are known to be pro fatty acid oxidation molecules.

![Figure 9](image_url)

**Figure 9:** In mammals, physiological and pathological cardiac growth mechanisms are associated with distinct profiles of substrate preference (from Bernardo et al. [108]). Given the results reported in this thesis, physiological cardiac growth as an acclimation response to training results in a higher dependence on lipids metabolism, similar as it occurs in mammals.

In conclusion, exercise training of sufficient intensity and duration stimulates cardiac growth as an acclimative physiological response. As suggested by gene and protein expression results obtained in this work, a higher pumping efficiency of the enlarged heart is
supported by an improved capacity for contractility as well as for oxygen supply to the compact myocardium. The increased requirement of energy is satisfied by a higher reliance on lipids over carbohydrates. All of these changes resemble the molecular signature behind the larger and strengthened “athlete’s heart” of exercise trained humans and have, then, the potential to be employed as molecular markers of cardiac status in fish.

6.3 Growth

Achieving optimum growth is probably the single most important parameter involved in defining production strategies in the industry, at the same time of being a central feature of a robust fish.

Increased growth is a well known effect of exercise training in salmonids, especially when moderate swimming speeds between 0.5 and 1.5 bls\(^{-1}\) are promoted [7,25]. An increment in somatic growth was a consistent result in these studies (papers 1 & 2). The effect was much greater in paper 1, as gains in thermal growth coefficient (TGC) were in the range of 20% for both trained groups compared to the untrained control fish. In paper 2 though, improved growth was in the range of 6% for all groups, but only significant between low (control) and highest intensity. The differences seen between the trials are most probably explained by the velocity at which the control group was reared; the control group described in paper 1 was held in water at velocities below 0.1 bls\(^{-1}\), while control group in paper 2 was held at 0.32 bls\(^{-1}\). Thus, the magnitude of the growth effect would partly correspond to the intensity difference between the control and target group.

Growth seen in the present study is comparable to that reported by Jørgensen and Jobling [67] in Atlantic salmon, who found a 15% higher growth rate in pre-smolts trained at 1 bls\(^{-1}\) compared to control fish reared at 0.3 bls\(^{-1}\). Nevertheless, increments in growth has been found to be as high as 38% [40] in Atlantic salmon and 76% [33] in brown trout. Different explanations may be found for such large increments in growth in comparison to those reported in this thesis. The work by Totland et al. [40] used fish averaging 2 kg and training lasted for a long period (8 months). Further, trained and control fish were reared in different facilities (trained on raceways and control on a standard cages with water velocities below 0.1 bls\(^{-1}\)). In the work by Davison and Goldspink [33], though it lasted one month only, control (static water) and trained fish were also reared differently and control fish did hardly grow at all during the experiment (3% compared to 79% of fish trained at 1.5 bls\(^{-1}\)). It is valid to argue
that the fish strains used in those studies were probably not as adapted as the current ones to captivity due to increments in stress tolerance and the strong selective breeding for growth performance has likely exploited much of the total growth capacity. The strong domestication of Atlantic salmon may further be the main underlying reason explaining why we found increased feed intake to be the key explanation for training-induced growth, while older reports have documented also strong positive effects on the feed conversion efficiency [7]. Still, in *paper 2* we found improved feed efficiency for fish trained continuously at moderate intensity.

Altogether, this may suggest that COT is at its lowest between ~0.6-1.2 bls\(^{-1}\) and that the optimum swimming speed (\(U_{\text{opt}}\)) for Atlantic salmon pre-smolts would be found in this range, in good agreement with previous results seen for salmonids [7,25].
7 Conclusions

This thesis represents an important step forward into the acknowledgement of the robustness effects that exercise training may exert upon farmed fish. Previous studies on salmonids have found that intensities between 0.5 and 1.5 bls\(^{-1}\) appeared as optimal in terms of growth and feeding efficiency, among others. Results from this thesis as well as from other studies within our research group, have narrowed the intensity range which appears to be optimal for overall robustness to be improved. Training Atlantic salmon pre-smolts around 1 bls\(^{-1}\) would result in sounder benefits including not only growth, but higher disease resistance as well (Fig. 10). Further, training in intervals appeared as highly beneficial, but only if the difference in magnitude between the high and low velocities is small. Large changes in velocity probably cause stress with consequent deleterious effects on disease resistance.

Fish can be divided according to their inherent swimming (cardiovascular) capacity into poor or good swimmers, which further associates with disease resistance. While good swimmers performed better than poor irrespective of the exercise training regime used, poor swimmers appeared to be more affected by training in either a positive or negative way. The possibility to improve the disease resistance of unfit fish by optimal training programs is of great interest for the aquaculture industry. Likewise, sorting fish based on swimming performance can be utilized to improve the genetic material in breeding programs.

Several of the molecular mechanisms driving the cardiac acclimation response to exercise training were uncovered. These included protective-related mechanisms (immune system, inflammation, antioxidants and xenobiotics) as well as cardiac performance-related (growth, contractility, vascularization and metabolism). Improved cardiac capacity as suggested by molecular expression was seen in fish trained at high intensity (1.3 bls\(^{-1}\)) when compared to the low intensity group (0.3 bls\(^{-1}\)). As fish trained in medium intensity regime (0.65 bls\(^{-1}\)) already showed signs of higher cardiac capacity than the low intensity group, it remains unknown which is the intensity threshold for such effects to become evident.

Exercise training was proficient in promoting growth. The range at which this was found goes from 0.65 to 1.31 bls\(^{-1}\), though relative differences appeared to be mostly dependent on the control group training level.

Overall, optimal exercise training improves robustness of Atlantic salmon pre-smolts, including better disease resistance, a strengthening of the cardiovascular system and a better growth, all of which are though to be highly interesting in a fish farming industry context.
Figure 10: Exercise training exerts a series of effects on Atlantic salmon. In this thesis we have added further steps into uncovering the robustness effects of training including different fundamental parameters such as growth, cardiac performance and disease resistance. Nevertheless, much more research is required to find optimum training regimes giving optimum overall robustness for salmonids and other commercially relevant species.
8 Future perspectives

Even though the new knowledge generated with this thesis sets a promising future for employing exercise training as a measure to increase overall robustness of farmed Atlantic salmon, there is still a large scope for improvement as well as a wide range of areas that should be further investigated.

Analyzing the potential effects of training on the earlier life stages becomes relevant, as it could generate robust fast-growing individuals from early-on. For example, exercise training improved the efficiency of the swimming muscle of free-swimming larvae of zebrafish, suggesting that muscle fibers were already sufficiently plastic at such an early stage [202]. Similarly, the effects of exercise training on post-smolts and up to slaughter size should be further investigated. In the former case, the use of closed systems for raising salmon up to 1 kg size would result highly favorable if promoting optimal swimming activity is made possible.

From the results in this thesis, the existence of a detraining period was found to be important in the immune acclimative response of cardiac gene expression, which was further positively associated to improved disease resistance. The duration of such a period as well as the necessary detraining time required for the transcriptional immune changes to appear should be then acknowledged.

Other disease models must be performed as to expand on the protective effects of exercise. The viral agents of highest interest are those currently affecting farmed salmonids such as CMS, HSMI, ISA and PD. While we have also seen a positive response of trained fish against a bacterial infection (*Moritella viscosa*) causing a natural winter ulcer outbreak (unpublished), controlled trials for this and other relevant bacteria may become more informative. Exercise training effects upon the sea lice (*Lepeophtheirus salmonis*) is also an interesting area to be explored.

In terms of the driving molecular acclimations behind exercise-induced disease resistance, future research should include the response of other immune relevant tissues such as the spleen and head kidney as to investigate potential shifts in the cellular immune response. Similarly, studies should in general be broadened to include effects in peripheral organs as skin and gills to gain insight into the system effects of exercise training. The interaction of exercise training with other factors such as nutrition and breeding, as well as in the fish’s response to regular aquacultural handling procedures should be further evaluated.
Finally, each research group working with exercise in fish species has, of course, its own questions and ways to tackle these. Nevertheless, given the numerous ways in which fish can be exercise trained (e.g. rearing facility type, exercise intensity, exercise type, fish size, duration, etc.), it becomes hard to make reliable between-studies comparisons, hence making it difficult to achieve consensuses. Thus, standardization of methods would be highly helpful, especially when trying to answer similar questions as, for example, exercise effects on disease resistance.
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Paper 1
Aerobic training stimulates growth and promotes disease resistance in Atlantic salmon (Salmo salar)

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A B S T R A C T

Improving fish robustness is of utmost relevance to reducing fish losses in farming. Although not previously examined, we hypothesized that aerobic training, as shown for human studies, could strengthen disease resistance in Atlantic salmon (Salmo salar). Thus, we exercised salmon pre-smolts for 6 weeks at two different aerobic training regimes; a continuous intensity training (CT; 0.8 bl s⁻¹) and an interval training (IT; 0.8 bl s⁻¹ 16 h and 1.0 bl s⁻¹ 8 h) and compared them with untrained controls (C; 0.05 bl s⁻¹). The effects of endurance training on disease resistance were evaluated using an IPN virus challenge test, while the cardiac immune modulatory effects were characterized by qPCR and microarray gene expression analyses. In addition, swimming performance and growth parameters were investigated. Survival after the IPN challenge was higher for IT (74%) fish than for either CT (64%) or C (61%) fish. While both CT and IT groups showed lower cardiac transcription levels of TNF-α, IL-1β and IL-6 prior to the IPN challenge test, IT fish showed the strongest regulation of genes involved in immune responses and other processes known to affect disease resistance. Both CT and IT regimes resulted in better growth compared with control fish, with CT fish developing a better swimming efficiency during training. Overall, interval aerobic training improved growth and increased robustness of Atlantic salmon, manifested by better disease resistance, which we found was associated with a modulation of relevant gene classes on the cardiac transcriptome.

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1. Introduction

The Atlantic salmon (Salmo salar L.) farming industry has a long-term goal of improving the strength and robustness of the production fish. A robust fish can be defined by a general strong disease resistance, an ability to cope with environmental challenges and capability of combining fast growth with normal organ development. Importantly, improved robustness gives better growth, survival and fish welfare, which accommodates profitability in harmony with consumers increasing request for sustainable and ethical food. Thus, improving robustness has a strategic impact on the industry’s economy and reputation. One of the main challenges of the salmon industry is the unacceptable high mortality rates observed during the sea water period. In 2008, 34 out of 235 million fish (15%) died after sea transfer in Norway (Directorate of Fisheries, http://www.fiskeridir.no/english). The annual fish health report from the Norwegian Veterinary Institute noted that the situation was similar for 2009. Associated with this critical phase of the rearing cycle are particular viral diseases such as infectious pancreas necrosis (IPN), infectious salmon anemia (ISA), pancreas disease (PD) and cardiomyopathy syndrome (CMS), as well as the salmon louse (Lepeophtheirus salmonis K.). In addition, farmers experience significant unspecific losses that are not explained by the presence of any known pathogen. These mortalities have partly been associated with

Abbreviations: IPN, Infectious Pancreas Necrosis; TNFα, Tumor Necrosis Factor-alpha; IL-1β, Interleukin-1 beta; IL-6, Interleukin 6; RT-qPCR, Real Time quantitative PCR; ER, Expression Ratio; MO₂, Oxygen consumption; COT, Cost of Transport; TGC, Thermal Growth Coefficient.

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different cardiac failures such as atherosclerosis, hypoplasia of the outer compact layer of the ventricle, aberrant ventricle morphology and malformed hearts with cysts (Baeverfjord, 1998; Poppe and Taksdal, 2000; Brocklebank and Raverty, 2002; Poppe et al., 2002, 2003; Tørud and Hillestad, 2004).

An increasing body of evidence suggests that aerobic endurance training is an effective proactive preventive strategy to improve robustness of farmed fish. Exercise induced benefits, including improved growth performance and reduced stress, are generally restricted to active species like Atlantic salmon. These benefits may also be ascribed to the behavioral effects caused by increasing the water speed in the rearing tanks, were a better distribution of both, feed and water quality, will dampen the formation of dominance hierarchies with the consequent reduction in aggressive behavior and losses of energy due to spontaneous activity (Kalleberg, 1958; Totland et al., 1987; Christiansen et al., 1991; Boegaard et al., 1993; Jobling et al., 1993; Jørgensen and Jobling, 1993, 1994; Davison, 1997).

In agreement with mammalian studies, exercise may also improve cardiac capacity of fish. Sustained aerobic exercise has been found to alter various components of the salmonid cardiovascular system, e.g. inducing cardiac ventricular muscle growth (Hochachka, 1961; Farrell et al., 1990), and to increase mass-specific maximum cardiac output (Qmax), haematocrit, arterial O2 content, skeletal muscle capillarity and tissue O2 extraction (Hochachka, 1961; Davie et al., 1986; Farrell et al., 1990, 1991; Thorarensen et al., 1993. Cousins et al., 1997; Gallaugher et al., 2001; Camper and Farrell, 2004; Anttila et al., 2008). It is important to know how different aerobic training intensities influence adaptations in physiological parameters when selecting an optimum training regimen. Both in healthy humans and in cardiovascular disease patients superior cardiovascular effect of aerobic interval training compared to continuous moderate and low intensity training has been documented (Helgerud et al., 2007; Helgerud et al., 2010). Which exercise intensity yields maximal beneficial adaptations in Atlantic salmon is however still unknown.

In the absence of studies on the effects of exercise on immune system performance in fish, numerous studies with humans have shown that moderate regular exercise strengthens disease resistance. In contrast to a sedentary way of living, exercise training results in a reduction in the incidence of infections (Gleeson, 2007), and provides protection against lifestyle diseases like cardiac diseases, diabetes II, obesity, colon and breast cancer and dementia (Boule et al., 2001; Piepoli et al., 2004; Petersen and Pedersen, 2005; Mathur and Pedersen, 2008). In humans, many of the long-term benefits of exercise have been suggested to come from its effects on lowering systemic, low-grade inflammation levels by modulation of key cytokines like tumor necrosis factor (TNF)-α and interleukines IL-1β and IL-6 (Bruunsgaard, 2005; Mathur and Pedersen, 2008). Hence, exercise counteracts the increased systemic inflammation found in relation to lifestyle factors such as inactivity, diet, obesity, as well as aging (Bruunsgaard, 2005). In spite of these reported benefits of exercise, farmed juvenile Atlantic salmon are normally confined to a tank environment where water velocity is dictated by low water exchange rates (Kristensen et al., 2009) which are set according to the requirements for self-cleaning and oxygen distribution, and not for specific biological reasons (Rosten et al., 2007).

To test the hypothesis that aerobic endurance training of pre-smolts improves robustness and disease resistance after sea-transfer, Atlantic salmon were trained under two different swimming intensity regimes in freshwater. Their disease resistance was compared with untrained fish by conducting a controlled IPN virus challenge test on post-smolts. As heart ventricle has proven to be an immune-relevant organ in which many viral pathogens manifest themselves (Smail et al., 2006; Jørgensen et al., 2007, 2008; Ellis et al., 2010; Lovoll et al., 2010; Palacios et al., 2010) and at the same time is one of the main target organs for the exercise induced benefits, we further investigated how training modulates the cardiac expression of genes involved in inflammation with the use of microarray and quantitative RT-PCR. In addition, we searched for other exercise-responsive immune-related gene classes that could be involved in the generation of robust fish. Swimming efficiency and growth were also investigated.

## 2. Materials and methods

### 2.1 Fish and experimental design

The trial was conducted in agreement with the ‘European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes’ (EST 123). Juvenile Atlantic salmon (S. salar L.) of the Salmobreed strain were produced at Nofima Marin, Sunndalsora, Norway. All fish were tagged (Passive Integrated Transponder (PIT), Glass tag Unique 2.12 x 12 mm, Jojo Automasjon AS, Sola, Norway) before the start of the trial. After 2 days of fasting, fish were individually weighed (49.1 ± 0.6 g), fork-length was measured (16.0 ± 0.2 cm) and 120 fish were randomly distributed in each of 9 cylinder-conical tanks (500 l, 82 cm in diameter). The center of each tank was fitted with a plastic pipe (31.5 cm diameter) which reduced the area in the tank with lowest water speed. A wire mesh fence was attached between the pipe and the edge of the tanks to prevent the fish from drifting back over. Water velocity was controlled by increasing the inlet water pressure and the number and size of the holes in the inlet water pipe of each tank. Six-week endurance aerobic training programs were tested in triplicate tanks (Fig. 1). Control (C) untrained fish swam at a constant average water speed of 0.05 body lengths/second (bl s\(^{-1}\)). The continuous intensity training (CT) program exposed salmon to a constant average water speed of 0.8 bl s\(^{-1}\), while an interval training (IT) program exposed fish to an average speed of 0.8 bl s\(^{-1}\) for 16 h per day (including 4 h light−12 h dark photoregime) and an increased water speed of 1.0 bl s\(^{-1}\) for 8 h (light). The fish in the control tanks seemed to position themselves randomly around the tank. At the higher speeds, the fish swam behind the fence in the lower 2/3 of the tank, distributing themselves back over. They were fairly evenly distributed along the radius. Fish swimming speed (bl s\(^{-1}\)) was estimated from the average water speed (measured by a Höntzsch HFA propeller (Waiblingen, Germany) with HLOG software) in the tank divided by the average of the fish initial and final lengths during training. During the 6-week training period fish were fed an experimental, extruded diet based on fish meal, fish oil and wheat (produced at Nofima Ingrediens, Fyllingsdalen, Bergen, Norway). Tanks were supplied with freshwater (11.3 ± 0.1 °C) under a short-day light regime (12:12 LD). At the end of the training period, the PIT-tags were registered when fish were individually weighed and fork length measured. An equal number of fish from each tank were distributed into two (2 m\(^2\)) tanks supplied with freshwater (6.7 ± 0.5 °C) and maintained under continuous light (24 L) for a further 6 weeks at a water speed of 0.05 bl s\(^{-1}\) to smoltify and detrain. During this period, the fish were fed a commercial diet (S eksudd Nutra Parr) to satiation using automatic feeders, after which the smolts were reweighed and remeasured. Fish from one tank were randomly distributed to 3 (1 m\(^3\)) tanks supplied with seawater (7.4 ± 0.3 °C) and fed to satiation with a commercial diet (S eksudd Nutra Olympic) prior to swim tunnel respirometry tests after a total of 11 weeks of detraining. The fish in the other tank were subjected to a disease challenge test. A 24 L photoregime was maintained until the end of the experiment.

### 2.2. Disease challenge test

After 6 weeks of detraining, the second group of smolts were transferred to VESO research station, Viken, Norway and acclimated for three days in freshwater (12 ± 0.2 °C). At that time water was changed to seawater (12 ± 0.2 °C, 3.3 ± 0.5% salinity and 0.51 kg \(\text{m}^{-3}\) \(\text{min}^{-1}\) open flow). The next day 315 fish equally representing the three experimental
groups were challenged in a 1.5 m³ tank by cohabitation with IPN-infected challenger fish (n = 60), previously marked and i.p. injected with a dose of ~10⁷ TCID₅₀ of IPNV. Challenger fish were of same origin as test fish. The IPNV strain (Y-1244) was cultured at the Norwegian School of Veterinary Sciences and delivered as a fresh supernatant. During the challenge test, fish were observed daily and mortality per group was recorded until termination five weeks after challenge. Bacteriological examination was performed on a representative selection of dead fish using blood agar plates with 2% NaCl (VESO Vikan).

2.3. Growth and feed utilization

During training, the fish were fed in excess and the accumulation of waste feed was collected daily from the effluent water of each tank in a wire mesh box, weighed and stored at −20 °C. The waste feed expressed as dry matter (DM) content was used to recalculate daily feed intake in order to adjust ration level every second day (Helland et al., 1996). Water temperature was measured daily and oxygen saturation (>85%) was measured weekly. Any dead fish were removed with daily inspections and weighed.

Whole-body composition (DM, crude protein, crude lipid (without HCl hydrolysis), ash, minerals and energy, as described below) was measured on 10 fish from each tank taken at the start and completion of training. The fish were anesthetized (tricaine methanesulfonate, MS 222, Argent Chemical Laboratories Inc., Redmont, WA, USA), killed with a blow to the head and weighed. Feed and feces were removed from intestines and stomach before fish were stored at −20 °C until analysis.

The experimental diet was analyzed for DM (105 °C, until constant weight), crude lipid (Soxtec HT6 after hydrolysis with HCl, Tecator, Höganäs, Sweden), nitrogen (crude protein (CP) = nitrogen × 6.25; Kjeltec Auto System, Tectar, Höganäs, Sweden), ash (550 °C, overnight) and minerals (inductively coupled plasma mass spectroscopy at Bioforsk, Ås, Norway). Gross energy was measured using an adiabatic bomb calorimeter (Parr 1271, Parr Instrument Company, Moline, IL, USA). The 3 mm pellets contained 93.5% DM and on a DM basis, 52.6% crude protein, 23.3% crude lipid, 10.8% ash and 24.1 MJ kg⁻¹. The diet contained 14.43 g kg⁻¹ P, 21.39 g kg⁻¹ Ca, 21.20 g kg⁻¹ Mg, 86.25 g kg⁻¹ Na, 142 mg kg⁻¹ Fe, 28.3 mg kg⁻¹ Mn, 133.8 mg kg⁻¹ Zn and 7.8 mg kg⁻¹ Cu.

Measurement of gill ATPase was conducted by a commercial laboratory (Havbruksinstituttet AS, Bergen, Norway). After killing 14–18 fish from each group after 6 weeks of detraining and prior to seawater transfer (as above), the second gill branch was dissected from the right side of the fish and immediately frozen at −80 °C before shipment on dry-ice.

2.4. Cardiac muscle gene expression

Heart ventricles for gene expression analyses were sampled at the end of the training period and after 6 weeks of detraining. Hearts were dissected and blood excess was removed by gently squeezing the ventricle in paper towel. After cutting off the atrium and bulbus arteriosus, ventricles (n = 12 per treatment) were individually weighed on an analytical scale and frozen in liquid nitrogen prior to storage at −80 °C until analysis. Total RNA was extracted using TRIzol and purified with PureLink RNA Mini Kit (Invitrogen, Carlsbad, CA, USA) following manufacturer’s guidelines and stored at −80 °C until use. RNA concentration was quantified using NanoDrop 1000 spectrophotometer (Thermo Fischer Scientific, Waltham, MA, USA), and for microarrays, RNA integrity was tested by Bioanalyzer 2100 (Agilent Technologies, Waldbronn, Germany).

2.4.1. Quantitative real-time RT-PCR (qPCR)

Expression of the genes coding for the inflammation-related cytokines TNF-α, IL-1β and IL-6 was analyzed by qPCR for fish sampled immediately after 6 weeks of training and following 6 weeks of detraining. The cDNA synthesis was performed on 0.2 μg of DNase treated (DNA-free; Ambion, Austin, TX, USA) total RNA using TaqMan® reverse transcription reagents (Applied Biosystems, Foster City, CA, USA) and random hexamer priming. The qPCR primers were designed using either the Vector NTI software (Invitrogen) or the ePrimer software (Rice et al., 2000) and synthesized by Invitrogen (Table 1). PCR efficiencies were assessed by tenfold serial dilution of pooled templates for each primer pair, and specificity of the amplified product was confirmed by melting curve analysis (Tm calling; LightCycler® 480, Roche Diagnostics, Mannheim, Germany). All qPCR assays were performed on 96-well optical plates on LightCycler® 480 using 2× SYBR Green Master Mix (Roche), by using 5 μl of 1:10 diluted cDNA templates in 12 μl reactions, where concentration of both primers together was 0.83 μM. PCR conditions were as follows: 5 min pre-
cDNA was performed at 46 °C for 3 h in a 2× SSC, 0.3% SDS, 0.67
hybridized overnight at 60 °C in a cocktail containing 1.3× Denhardt’s,
(3 min) followed by 0.2× SSC (3 min) at room temperature and
5× SSC and 0.1% SDS for 30 min at 50 °C and then washed with 2× SSC
combined and puri

List of primers used for RT-qPCR.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primers 5′ to 3′</th>
<th>Accession number</th>
<th>PCR efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>F-CTGGGACACTGTTCTGGAAGA R-TGCTTCCCTCGTCGTTAG</td>
<td>NM001123582</td>
<td>1.95</td>
</tr>
<tr>
<td>IL-6</td>
<td>F-ATGGCTCTTCACAGCTTACC R-TAATCTGACGACCTTTCCTG</td>
<td>NM001124657</td>
<td>1.79</td>
</tr>
<tr>
<td>TNF-α</td>
<td>F-AGTTGGCTCATTAGAGCCTGT          R-TCTGCTCAATGATGTTGAGG</td>
<td>NM001123589</td>
<td>1.87</td>
</tr>
<tr>
<td>Cathepsin C-3</td>
<td>F-GGTCTGACTGATGAGCCTGT          R-GTATCCCTAACAGAGCTCAGG</td>
<td>BT057941</td>
<td>1.89</td>
</tr>
<tr>
<td>Complement factor D</td>
<td>F-AGGGCTCGCTGATGAGCCTGT          R-ATACCTGCGCTGATGAGCAGG</td>
<td>BT049738</td>
<td>1.89</td>
</tr>
<tr>
<td>Matrix metalloproteinase 17</td>
<td>F-AAACCTGAGAGTGTTGTCAGT          R-TAGGCGTCTGCAGAGATGTTG</td>
<td>CA377360</td>
<td>1.70</td>
</tr>
<tr>
<td>C type lectin receptor B</td>
<td>F-GACCATCCTCTGATGAGCTGT          R-GTCTGCTGATGATGTTGAGG</td>
<td>CA353501</td>
<td>1.77</td>
</tr>
<tr>
<td>MHC class I heavy chain-1</td>
<td>F-CTGTGATGAGTGTTGTCAGT          R-ATGATCCCTACGAAGTGACG</td>
<td>AF504022</td>
<td>1.59</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>F-GGCTCTACCTTCTCTGCTGATG          R-AGGATGCTTACCATCGTATG</td>
<td>AJ472679</td>
<td>1.62</td>
</tr>
<tr>
<td>RNA-polymerase II</td>
<td>F-TAAAGCTGCTTCACAGTCTG          R-ATGAGCCATCCTACGAAGTG</td>
<td>CA049789</td>
<td>1.85</td>
</tr>
<tr>
<td>Eukaryotic translation initiation factor 3a</td>
<td>F-ACCCACTGGGAGAGTGTTGGT          R-CAGGATGCTTCTGCTGGATG</td>
<td>DW542195</td>
<td>1.79</td>
</tr>
</tbody>
</table>

incubation at 95 °C, followed by 45 amplification cycles (95 °C for 10 s,
60 °C for 15 s and 72 °C for 15 s). Melting curve consisted of 95 °C for 5 s,
60 °C for 60 s and then 97 °C continuously. Fluorescence was measured at
the end of every extension step and continuously during the melting
curve step. Expression ratios (ER) were calculated using the relative
expression software tool (REST©; Pfaffl et al., 2002) with normalization
against the expression level of three averaged reference genes; RNA
polymerase 2, Eukaryotic translation initiator factor 3 and 18S rRNA
(templates of the latter were diluted 1:1000 before use). Stability of
reference genes was verified using NormFinder (Andersen et al., 2004).
Significance between the groups was tested by the pair-wise fixed
reallocation randomization test© (Pfaffl et al., 2002). Real-time qPCR
was further performed on five genes of interest from the microarray
results for confirmation purposes.

2.4.2. Microarray analyses

The salmonid fish microarray (SFA 2.0 immunochip), containing 1800 unique clones printed each on 6 spot replicates, was used. The
cloned were selected based on functional roles, including immune
responses, cell communication, response to oxidative stress and cell
cycle (Jørgensen et al., 2008). The complete sequences and compo-
sition of the array can be found at GEO depository GPL6154. Twelve
two-color microarray analyses were performed on RNA samples
(templates of the latter were diluted 1:1000 before use). Stability of
reference genes was verified using NormFinder (Andersen et al., 2004).
Significance between the groups was tested by the pair-wise fixed
reallocation randomization test© (Pfaffl et al., 2002). Real-time qPCR
was further performed on five genes of interest from the microarray
results for confirmation purposes.

2.5. Swimming respirometry

The effect of exercise on oxygen consumption (MO2) and the cost of
transport (COT) during swimming were examined using two Brett-type
intermittent flow-through swim-tunnel respirometers. Six groups of
five fish from each treatment were tested during the last week of the 6-
week training period, (freshwater; 11.2±0.1 °C). A further 6 groups of
two fish from each treatment were tested after 11 weeks of detraining
(seawater; 10.9±0.2 °C). The swimming respirometers had a total
volume of 30 l with a swimming section of 215 cm2 cross-sectional area
(length 47 cm). Each respirometer was submerged in an outer tank
filled with temperature-controlled water. Laminar-like flow was obtained
using a honeycomb grid placed upstream of the swimming
section, while a propeller attached to a variable speed motor maintained
the water flow. A roller pump (Ismatec) continuously moved a small
volume of water from the respirometer past an oxygen electrode
(OxyGuard, Birkerød, Denmark) and back to the respirometer. In a
closed circulation mode, the decline in oxygen saturation in the swim
section was recorded using Labtech Notebook and stored in an Excel
spreadsheet, was used to calculate oxygen consumption (MO2, mg O2·
kg−1·min−1). The oxygen electrode was zero-calibrated using a
saturated solution of sodium sulfite. At the start of each experiment,
the electrode was calibrated using 100% air-saturated water. Without
fasting, the fish were randomly taken from a tank and quickly placed in
the respirometer chamber. Measurements of MO2 were started immediately. The speed was initially set at 16 cm s⁻¹ and was increased by 4 cm s⁻¹ steps. In the experiments done during the last week of training, each trial was ended when any of the fish were unable to remove themselves from the back screen. For the experiments performed after 11 weeks of detraining, the maximum speed tested was 28 cm s⁻¹, or approximately 1.3 bl s⁻¹. Thus, maximum swimming speed was not evaluated. MO2 was measured during a 5 min closed recirculation period at each speed. This was followed by a 15 min period of open water flow, when the respirometer was flushed with aerated water and the fish rested at a water speed of 6 cm s⁻¹. At the end of the test fish were removed from the respirometer and killed with a blow to the head. The body mass, fork length, width and height of the fish were measured. The solid blocking effect of the fish (Bell and Terhune, 1970) was accounted for (which ranged from 3.5 to 4.7%) when converting absolute speed to relative speed to permit comparisons among fish of different length. MO2 was calculated based on the weight of the fish, the volume of the respirometer and oxygen solubility in water at the temperature and salinity used in the trial. Only slopes with r² > 0.95 were used in the calculations. Cost of transport (COT) was calculated for each swimming speed (U) as MO2/U.

2.6. Calculations and statistics for growth, feed, VSI, ATPase and respirometry analyses

Relative feed intake: 100 × (dry feed intake/mean body mass (BM)/days fed).

Thermal growth coefficient (TGC): 1000 × [(BM1.33 − BM0.33)/∑(day − degrees)], where BM1 and BM0 are final and initial body masses, respectively.

Feed efficiency ratio: (Wet fish gain + dead fish mass)/dry feed intake.

Condition factor: 100 × BM (g)/fork length (cm)³.

Ventricular-somatic index (VSI): 100 × heart mass (g)/BM (g).

Retention of nitrogen and energy in whole fish (%): 100 × (BM1 − final body nutrient content)/((BM0 × initial body nutrient content)/(total intake × nutrient content in diet)).

For growth, feed, VSI and ATPase analyses, the individual fish data were analyzed by analysis of variance in a hierarchical model including the fixed effect of training method and the random effect of tank within treatment. The mean data for each tank were tested by variance analysis. The mean weights of the fish were transformed (arcsine square root) before being subjected to analysis. Differences between treatments were considered significant at the p < 0.05 level, and are presented as mean ± SEM.

For respirometry the effect of one increment in swimming speed on MO2 and COT in each training protocol was tested using the GLM procedure in SAS (version 9, SAS Institute Inc., Cary, NC, USA). The overall effect of swimming speed on MO2 and COT in each training protocol was determined using a repeated-measures MIXED procedure and comparisons of MO2 and COT at each swimming speed between training protocols were made using the GLM procedure in SAS.

3. Results

3.1. Improved disease resistance in interval-trained fish

We used a challenge model for IPN since this is one of the most significant viral diseases in salmon aquaculture, mainly affecting post-smolts after transfer to seawater (Ariel and Olesen, 2002). Infection by cohabitation allowed us to study the effects of exercise training on survival to an infectious disease that was transmitted mimicking a natural disease outbreak. Mortalities due to IPN started 11 days after the experimental fish had been mixed with challenger fish and continued until termination at 31 days post challenge, when cumulative mortality reached a plateau phase. Only four challenger fish died during the experiment. For the exercised groups, IT fish showed better survival (74%; p = 0.07) than continuously trained (CT) (64%) and untrained controls (C) (61%) when 105 fish per group were challenged by cohabitation in one tank.

3.2. Aerobic endurance training induces beneficial changes in cardiac gene transcription

The improved performance of IT fish against IPN was further supported by examination of cardiac transcription of immune related genes prior to the disease challenge. Expression of TNF-α, IL-1β and IL-6 was examined immediately after 6 weeks of training and again after 6 weeks of detraining. While transcription of the three genes was unaffected immediately after training (results not shown), real-time qPCR analysis showed lowered transcription levels of the cytokines in both CI and IT groups compared to non-exercised controls after 6 weeks of detraining (Fig. 3). The reduction was most pronounced for IT fish, which showed significantly lower TNF-α, IL-1β and IL-6 expression (ER = 0.35, 0.42 and 0.56, respectively; p < 0.05); whereas CT fish only showed significantly lower expression for TNF-α and IL-1β (ER = 0.42 and 0.25, respectively; p < 0.05).

Fig. 3. Real-time qPCR analyses of TNF-α, IL-1β and IL-6 in continuously trained (CT) and interval trained (IT) fish after 6 weeks of detraining. Data are log transformed Expression Ratio (logER) ± SEM (n = 12). Significant difference from untrained control fish is indicated by * (p < 0.05; Pair-wise fixed reallocation randomization test©).
To further explore if other transcriptional changes could explain the enhanced disease performance of endurance trained groups, we applied gene expression profiling with the SFA 2.0 cDNA microarray using a hypothesis-free approach. Analysis of the cardiac ventricle following 6 weeks of detraining identified a suite of genes involved in immune responses (Table 2) and other processes that are known to affect disease resistance (Table 3). These effects were pronounced for immune responses (Table 2) and other processes that are known to affect disease resistance (Table 3). These effects were pronounced for

<table>
<thead>
<tr>
<th>Genbank</th>
<th>Probe</th>
<th>CT</th>
<th>IT</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA369900</td>
<td>Leukotriene A-4 hydrolase</td>
<td>1.35±0.17</td>
<td>1.91±0.17</td>
</tr>
<tr>
<td>CA359906</td>
<td>Thromboxane A synthase 1</td>
<td>0.88±0.06</td>
<td>1.89±0.12</td>
</tr>
<tr>
<td>CA359467</td>
<td>5-lipoxygenase activating protein</td>
<td>0.99±0.05</td>
<td>0.63±0.04</td>
</tr>
<tr>
<td>CA352474</td>
<td>Prostaglandin endoperoxide synthase-2</td>
<td>2.12±0.58</td>
<td>0.54±0.04</td>
</tr>
<tr>
<td>CA346166</td>
<td>Arachidonate 5-lipoxygenase-1</td>
<td>1.46±0.16</td>
<td>0.33±0.02</td>
</tr>
</tbody>
</table>

Table 2 Effect of 6 weeks of aerobic endurance training on the expression of genes with an immune function. CT: continuous training; IT: interval training. Data are presented as fold difference in gene expression relative to untrained fish (± SE). All genes presented in the table showed differential expression in IT relative to control (p<0.05).

<table>
<thead>
<tr>
<th>Genbank</th>
<th>Probe</th>
<th>CT</th>
<th>IT</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA370968</td>
<td>Chitinase 1 (Eosinophil chemotactic cytokine)</td>
<td>1.17±0.12</td>
<td>2.59±0.15</td>
</tr>
<tr>
<td>CA387884</td>
<td>Chemokine C-1</td>
<td>0.92±0.19</td>
<td>1.97±0.07</td>
</tr>
<tr>
<td>CA370601</td>
<td>Cytokine I</td>
<td>0.98±0.04</td>
<td>1.90±0.10</td>
</tr>
<tr>
<td>CA386149</td>
<td>Small inducible cytokine SCYA104</td>
<td>1.05±0.15</td>
<td>1.82±0.15</td>
</tr>
<tr>
<td>CU068239</td>
<td>Leukocyte cell-derived chemotaxin 2</td>
<td>0.67±0.07</td>
<td>0.47±0.02</td>
</tr>
<tr>
<td>CA347562</td>
<td>CC chemokine CCL</td>
<td>1.58±0.13</td>
<td>0.34±0.02</td>
</tr>
<tr>
<td>CA377397</td>
<td>C-C chemokine receptor type 3</td>
<td>0.96±0.07</td>
<td>2.05±0.27</td>
</tr>
<tr>
<td>CA345725</td>
<td>IL-1-receptor-like protein 1</td>
<td>0.66±0.10</td>
<td>1.84±0.17</td>
</tr>
<tr>
<td>CA362179</td>
<td>IL-1 receptor-like protein 2</td>
<td>0.63±0.08</td>
<td>1.74±0.11</td>
</tr>
<tr>
<td>CA372391</td>
<td>Chemokine receptor-2</td>
<td>1.20±0.15</td>
<td>1.68±0.17</td>
</tr>
<tr>
<td>CA348091</td>
<td>Chemokine receptor-1</td>
<td>1.70±0.16</td>
<td>0.47±0.02</td>
</tr>
</tbody>
</table>

Table 3 Effects of 6 weeks of aerobic endurance training on the expression of genes involved in the protection and reparation of tissues (results of microarray analyses). CT: continuous training; IT: interval training. Data are presented as fold difference in gene expression relative to untrained fish (± SE). All genes presented in the table showed differential expression in IT relative to control (p<0.05).

<table>
<thead>
<tr>
<th>Genbank</th>
<th>Probe</th>
<th>CT</th>
<th>IT</th>
</tr>
</thead>
<tbody>
<tr>
<td>CU071220</td>
<td>Peroxiredoxin 1</td>
<td>0.94±0.06</td>
<td>2.14±0.24</td>
</tr>
<tr>
<td>EG829317</td>
<td>Peroxiredoxin 2</td>
<td>0.87±0.04</td>
<td>1.69±0.12</td>
</tr>
<tr>
<td>CA346623</td>
<td>Selenium-binding protein 1</td>
<td>0.91±0.07</td>
<td>1.81±0.09</td>
</tr>
<tr>
<td>CA346256</td>
<td>Transaldolase</td>
<td>0.83±0.07</td>
<td>2.01±0.11</td>
</tr>
<tr>
<td>D737446</td>
<td>Apolipoprotein A-IV</td>
<td>0.76±0.07</td>
<td>1.98±0.08</td>
</tr>
<tr>
<td>CA366835</td>
<td>Serum albumin precursor</td>
<td>0.65±0.09</td>
<td>2.51±0.30</td>
</tr>
</tbody>
</table>

To further explore if other transcriptional changes could explain the enhanced disease performance of endurance trained groups, we applied gene expression profiling with the SFA 2.0 cDNA microarray using a hypothesis-free approach. Analysis of the cardiac ventricle following 6 weeks of detraining identified a suite of genes involved in immune responses (Table 2) and other processes that are known to affect disease resistance (Table 3). These effects were pronounced for immune responses (Table 2) and other processes that are known to affect disease resistance (Table 3). These effects were pronounced for...
was observed in tyrosine kinases SYK and FRK that control differentiation of immune cells (Turner et al., 2000). Down-regulated CCAAT/enhancer binding protein delta (C/EBPδ) is involved in inflammatory pathways by inducing the transcription of acute phase reactants (Ali et al., 2010). Co-ordinated down-regulation was shown by genes associated with anti-viral responses including toll-like receptor (TLR)-3, a receptor of double stranded RNA, components of the antigens presenting major histocompatibility complex (MHC)-I and three interferon (IFN) dependent proteins. With respect to the immune effectors, the greatest expression changes were seen in the complement system, which is an important part of the immune humoral response playing key roles in bridging innate and adaptive immunity (Boshra et al., 2006). Only one gene (properdin) was down-regulated, while genes associated with the three complement pathways were up-regulated: classical (C1rs-A), alternative (factor H) and lectin (C-type mannose-binding lectin). C3 protease is the effector, on which the three complement pathways converge, while the down-regulated C6 and C7 form a part of the membrane attack complex which the three complement pathways converge, while the downstream factors C6 and C7 form a part of the membrane attack complex (Murphy et al., 2008). The IT regime increased expression of several antibacterial effectors, including lectin receptors, liver expressed amidase, an enzyme destroying cell wall. Down-regulation was shown by pyrin-2, a component of the inflammasome (Bertino and DiStefano, 2000).

In addition to the immune genes, IT stimulated several functional groups that protect organisms from different hazards. Peroxiredoxins and selenium-binding protein regulate the redox status by removing free radicals and transaldolase has an essential role in responses to oxidative stress (Banki et al., 1998). Albumin and lipoproteins work as scavengers of oxidized lipids in blood plasma. Up-regulation was also shown by a group of genes involved in xenobiotics metabolism including a transporter (multidrug resistance protein) and several enzymes that transform toxic compounds by increasing their solubility (UDP-glucuronosyltransferase (UGT)-2B15) and oxidation (aldehyde oxidase (AOX), Cytochrome (CYP)-450 2F1 and epoxide hydrolase (EPHX)-1). The role of alanine-glyoxylate aminotransferase (AGXT)-2 is not known precisely, however this gene has shown response to various aquatic contaminants in salmonid fish (Krasnov et al., 2007). Aryl hydrocarbon receptor nuclear translocator (ARNT) regulates expression of genes involved in biotransformation. Interval training also stimulated genes taking part in the remodeling and reparation of heart. Beta-2-glycoprotein I (B2GP1) protects tissues by binding to phospholipids of damaged cells (Keeling et al., 1993). Notch 2 controls formation of various organs and structures including heart (Pedrazzini, 2007) and deltex protein-1 is a regulator of notch (Matsuno et al., 1998). Midkine-related growth factor (Mdk)-2 and peristin, are involved in reparation of arterial and myocardial injuries (Horiba et al., 2006; Stansfield et al., 2009). Cysteine-rich angiogenic inducer (CYR)-61, latent transforming growth factor-β binding protein (LTBP)-4 and signal peptide CUB-EGF-like domain containing protein (SCUBE)-1 regulate differentiation of diverse cell lines.

Microarray results were validated with qPCR analysis of five genes present on the array, using the same RNA samples from the IT treatment after 6 weeks detraining (Fig. 4). Results showed a significant correlation (p = 0.036) in expression levels produced with the two methods.

### 3.3. Aerobic endurance training improves growth performance

The effect of aerobic endurance training on growth and nutrient utilization was assessed immediately after training and following 11 weeks of detraining. After training, TGC was 20 and 22% higher in CT and IT fish, respectively, as compared with untrained controls (p < 0.05; Fig. 5), a difference that was maintained through the 11 weeks of detraining. Relative feed intake during the training period was 23 and 26% greater for the CT and IT groups, respectively, compared with C (p < 0.05), but feed efficiency was unchanged.

The condition factor of trained fish (CT and IT) increased significantly compared with controls (p < 0.05) at end of training (Table 4), a difference that was lost during 6 and 11 weeks of detraining. Training had no effect on macronutrient and energy composition of the fish, but the ash and sodium concentrations in the CT fish were significantly lower than in the other groups (Table 5). In
Table 4
Growth of the aerobic endurance trained and untrained Atlantic salmon (mean±SEM). C: untrained fish; CT: continuous training; IT: interval training. Week (W) 6 = end of 6 weeks of training; W 12 = end of 6 weeks of subsequent detraining (Weeks 1–12). C: untrained fish; CT: continuous training; IT: interval training. Different superscripts indicate significant differences based on one-way ANOVA (p<0.05).

<table>
<thead>
<tr>
<th>Weeks in trial</th>
<th>C</th>
<th>CT</th>
<th>IT</th>
</tr>
</thead>
<tbody>
<tr>
<td>W 6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W 12</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W 17</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fish mass (g)</td>
<td>49.6±0.9</td>
<td>49.5±0.5</td>
<td>48.1±0.4</td>
</tr>
<tr>
<td></td>
<td>82.3±3.5</td>
<td>88.8±1.6</td>
<td>89.1±1.1</td>
</tr>
<tr>
<td></td>
<td>108.2±1.5</td>
<td>115.7±1.5</td>
<td>116.0±1.6</td>
</tr>
<tr>
<td>Fish length (cm)</td>
<td>142.2±3.1</td>
<td>151.0±3.2</td>
<td>147.5±3.4</td>
</tr>
<tr>
<td>Fish length (cm)</td>
<td>161.0±1.0</td>
<td>161.0±1.0</td>
<td>158.4±0.4</td>
</tr>
<tr>
<td>Fish length (cm)</td>
<td>18.3±0.2</td>
<td>18.4±0.1</td>
<td>18.4±0.1</td>
</tr>
<tr>
<td>Fish length (cm)</td>
<td>20.3±0.1</td>
<td>20.7±0.1</td>
<td>20.8±0.1</td>
</tr>
<tr>
<td>Fish length (cm)</td>
<td>22.4±1.0</td>
<td>22.9±1.0</td>
<td>22.8±1.0</td>
</tr>
<tr>
<td>Relative feed intake</td>
<td>0.77±0.03</td>
<td>0.95±0.03</td>
<td>0.97±0.01</td>
</tr>
<tr>
<td>Condition factor</td>
<td>1.18±0.02</td>
<td>1.21±0.01</td>
<td>1.19±0.01</td>
</tr>
<tr>
<td>Condition factor</td>
<td>1.38±0.01</td>
<td>1.43±0.01</td>
<td>1.42±0.01</td>
</tr>
<tr>
<td>Condition factor</td>
<td>1.27±0.01</td>
<td>1.29±0.01</td>
<td>1.28±0.01</td>
</tr>
<tr>
<td>Condition factor</td>
<td>1.24±0.01</td>
<td>1.23±0.01</td>
<td>1.23±0.01</td>
</tr>
<tr>
<td>Feed efficiency</td>
<td>1.50±0.06</td>
<td>1.41±0.01</td>
<td>1.45±0.02</td>
</tr>
</tbody>
</table>

Table 5
Whole body composition and protein and energy retention of salmon at the end of 6 weeks of aerobic endurance training (means±SEM; n=3). C: untrained fish; CT: continuous training; IT: interval training.

<table>
<thead>
<tr>
<th>Component</th>
<th>Initial</th>
<th>CT</th>
<th>IT</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry matter (%)</td>
<td>29.9</td>
<td>31.1±0.4</td>
<td>31.2±0.1</td>
<td>31.2±0.3</td>
</tr>
<tr>
<td>Protein (%)</td>
<td>17.2</td>
<td>17.2±0.2</td>
<td>16.9±0.2</td>
<td>16.8±0.1</td>
</tr>
<tr>
<td>Lipid (%)</td>
<td>10.0</td>
<td>11.0±0.4</td>
<td>11.6±0.1</td>
<td>11.4±0.2</td>
</tr>
<tr>
<td>Ash (%)</td>
<td>1.73</td>
<td>1.96±0.07</td>
<td>1.91±0.02</td>
<td>2.01±0.01</td>
</tr>
<tr>
<td>Energy (MJ kg⁻¹)</td>
<td>8.18</td>
<td>8.53±0.09</td>
<td>8.73±0.02</td>
<td>8.62±0.09</td>
</tr>
<tr>
<td>Phosphorus (mg kg⁻¹)</td>
<td>3807</td>
<td>4145±10</td>
<td>3879±16</td>
<td>3955±58</td>
</tr>
<tr>
<td>Calcium (mg kg⁻¹)</td>
<td>3244</td>
<td>4173±44</td>
<td>3418±48</td>
<td>3707±80</td>
</tr>
<tr>
<td>Magnesium</td>
<td>293</td>
<td>290±6</td>
<td>286±6</td>
<td>294±2</td>
</tr>
<tr>
<td>Sodium (mg kg⁻¹)</td>
<td>987</td>
<td>1304±29</td>
<td>1051±55</td>
<td>1313±83</td>
</tr>
<tr>
<td>Iron (mg kg⁻¹)</td>
<td>11.5</td>
<td>8.67±0.09</td>
<td>8.03±0.43</td>
<td>9.03±0.38</td>
</tr>
<tr>
<td>Copper (mg kg⁻¹)</td>
<td>0.75</td>
<td>0.86±0.08</td>
<td>0.85±0.03</td>
<td>0.83±0.04</td>
</tr>
<tr>
<td>Zinc (mg kg⁻¹)</td>
<td>28.3</td>
<td>28.7±2.2</td>
<td>28.7±2.0</td>
<td>28.1±0.4</td>
</tr>
<tr>
<td>Protein retention (%)</td>
<td>52.4±1.2</td>
<td>47.7±1.1</td>
<td>48.2±0.5</td>
<td>0.03</td>
</tr>
<tr>
<td>Energy retention (%)</td>
<td>60.6±2.7</td>
<td>59.2±0.5</td>
<td>58.8±2.1</td>
<td>0.82</td>
</tr>
</tbody>
</table>

Significance probability associated with the F statistic. Means in the same row with different superscripts are significantly different based on one-way ANOVA (p<0.05).

Fig. 5. Thermal growth coefficient (TGC) for the 6-week period of aerobic endurance training (Weeks 1–6), including 6 weeks of subsequent detraining (Weeks 1–12). C: untrained fish; CT: continuous training; IT: interval training. Different superscripts indicate significant differences based on one-way ANOVA (p<0.05).

contrast, whole body phosphorus and calcium concentrations were significantly lower in CT and IT groups compared with C. Protein retention in the body (Table 5) was lower in both CT (47.7±1.1%) and IT (48.2±0.5%) treatments in comparison with C (52.4±1.2%, p<0.05). No effect of treatment was found on energy retention. Training had no effect on VSI (data not shown).

Endurance training was not found to affect the smoltification process, as it had no effect on gill ATPase levels measured just prior to seawater transfer (data not shown).

3.4. Effects of aerobic endurance training on swimming respirometry

3.4.1. Respirometry during exercise training

The fish used for the respirometry experiments during the 6th week of training had similar body masses and lengths (78.7±2.7, 85.7±3.1 and 84.9±3.2 g, and 17.8±0.2, 18.2±0.2 and 18.0±0.2 cm, for C, CT and IT, respectively) (mean±SEM). Swim efficiency was compared among test groups at speeds that were performed by all three groups up to 1.6 bl s⁻¹. MO₂ increased significantly with increasing speed in control fish but did not in any of the two trained groups (Fig. 6a).

Importantly, there was an overall effect of swimming speed on COT in control and CT groups, but not in IT fish. COT decreased for CT fish at speeds between 0.9 and 1.1 bl s⁻¹ (p<0.05) (Fig. 6b). Similarly, COT tended to decrease (p<0.1) in control fish over the same swimming speeds. As a result, and for all swimming speeds tested below 1.6 bl s⁻¹, there was no significant effect of training on either MO₂ or COT.

Fig. 6. Relationships between (a) oxygen consumption (MO₂) and (b) cost of transport (COT) and swimming speed (in body lengths per second; bl s⁻¹) for Atlantic salmon after 6 weeks of aerobic endurance training (N=4–6). C: untrained fish; CT: continuous training; IT: interval training. Different superscripts indicate significant differences in MO₂ or COT between training protocols at 1.6 bl s⁻¹ (p<0.05).
However, at 1.6 bl s\(^{-1}\) MO\(_2\) was higher in control fish than after the CT regime and COT was higher in both control and IT fish compared with CT fish (p<0.05). Therefore, continuous training, but not interval training, resulted in more efficient swimming at the highest test speed (1.6 bl s\(^{-1}\)) when compared with untrained fish.

3.4.2. Respiration rate after detraining for 11 weeks

The fish used for the respirometry experiments after 11 weeks of detraining had similar body masses and lengths (146.8 ± 5.2, 152.1 ± 5.4 and 149.2 ± 6.2 g, and 23.0 ± 0.3, 23.2 ± 0.2 and 22.7 ± 0.3 cm, respectively, for C, CT and IT fish). An overall effect of swimming speed on MO\(_2\) was evident in for CT fish (p<0.05; Fig. 7a), but not for C or IT fish. COT tended (p<0.1) to change overall with increases in swimming speed (Fig. 7b) and specifically decreased significantly for IT fish when the swimming speed was increased from 0.7 to 0.9 bl s\(^{-1}\). There was no effect of training protocol on MO\(_2\) or COT at any speed (p>0.05). Therefore, the benefit of continuous training on the efficiency of swimming at the fastest speed was lost during detraining.

4. Discussion

Reducing mortalities caused by infectious pathogens is an important challenge for the salmon aquaculture industry, and improving the general robustness of fish may be an efficient strategy to achieve this. To our knowledge, this is the first report to show positive effects of aerobic endurance training on survival after a disease challenge. Cardiac gene transcription analysis in exercised fish revealed that improved survival could be explained by modulation of the immune system. Further, endurance training for a period of just six weeks increased growth performance that lasted for at least eleven weeks after detraining.

Sustained aerobic exercise training in the range of 1–1.5 bl s\(^{-1}\) in juvenile Atlantic salmon has been found to elicit a number of positive effects. Care should be taken as for ascribing some of these effects to either the physical activity per se, to the increased water speed in the rearing tanks or to a mix of both. Most of the cardiovascular effects like improved cardiac capacity and growth, ability of oxygen extraction in the tissues, higher haematocrit and capillarization are logically attributable to exercise, while others like a decrease in agonistic interactions due to a better water quality and feed distribution, resulting in a more homogeneous population growth are more obviously assigned to the increased water current (reviewed by Jobling et al., 1993; Davison, 1997). Nevertheless, beneficial effects like higher growth and reduced stress can be thought as a result of both behavioral and exercise-induced molecular/physiological responses.

In this study we introduced aerobic interval training to salmons since this type of training regime is documented to have superior beneficial effects in humans (Helgerud et al., 2007; Helgerud et al., 2010). The interval-trained salmon had much better survival in the IPN challenge test compared to both the continuously trained and the untrained control fish. The IT fish trained at a 25% higher swimming speed than the CT group during 8 h of the 12 h of daylight. Thus, the beneficial effect of the interval regime could be equally well explained by either the higher intensity of swimming exercise that these fish performed during the six weeks of training or by the potential benefit of reduced swimming speed during nighttime, or by a combination of both. The IT regime may have also benefited by having a richer environment, as the changes in water speed would mimic a more natural situation.

In order to gain a better understanding of underlying differences that could explain the better survival of the interval trained fish, we investigated cardiac gene expression. The functional relation between increased cardiac workload through exercise and modulation of immune function has not been addressed previously in fish. The consensus from human medicine is that regular exercise reduces low-grade systemic inflammation, an effect driven by myokines. These are cytokines produced and released by the active skeletal muscle, with IL-6 playing a central metabolic and anti-inflammatory role at both local and systemic levels (Pedersen et al., 2007; Brandt and Pedersen, 2010). However, the 6-week training regimes used in the present study did not induce up-regulation of IL-6. Perhaps the training regimes were not long or strenuous enough to trigger such a response. Even so, both training regimes significantly reduced the mRNA expression of TNF-α and IL-1β after 6 weeks of detraining. Thus, aerobic endurance training showed a similar long-term anti-inflammation effect in the cardiac muscle of Atlantic salmon as it does at the systemic level in mammals. One could then argue that this effect on cardiac muscle would also extend to skeletal muscle. If so, this will likely provide a systemic anti-inflammatory effect as has been found in humans (Gleeson, 2007; Mathur and Pedersen, 2008).

The similar cardiac myokine response produced by both CT and IT regimes clearly argues for other exercise-induced changes accounting for the improved survival of interval trained fish against IPN. Indeed, transcriptional changes in IT fish were greater than in the CT group as revealed by microarray and we suggest that interval training improved protection of salmon against pathogens by suppression of potentially harmful processes, while stimulating favorable ones.

Beside the down-regulation of inflammatory cytokines, expression of IL-1 receptors increased suggesting that heart was better prepared to perceive noxious stimuli. In addition to TNF-α, IL-1β, and LECT-2, endurance IT decreased expression of key genes that control production of eicosanoids. These molecules are autocrine and paracrine mediators which have been reported to be involved in the inflammatory response (Funk, 2001). Leukotrienes, synthesized after oxidation of arachidonic acid by 5-LO, have chemotactic and activating properties in leukocytes (De Caterina and Basta, 2001). Prostaglandins and thromboxanes are produced after the oxidation of arachidonic acid by the enzyme COX-2, which was down-regulated in trained fish. The pivotal role of COX-2 is well illustrated with the fact that this enzyme is the molecular target of
non-steroidal anti-inflammatory pharmaceuticals including aspirin and ibuprofen (Cryer and Dubois, 1998). In endothelial cells, prostaglandins act as vasodilators and participate in endothelium activation favoring leukocyte extravasation, while thromboxanes are potent coagulating factors released by platelets (De Caterina and Basta, 2001). COX-2, as well as CEBP/α, is induced by cytokines such as TNF-α and IL-1β via Nuclear Factor-κB (NF-κB) activation; a transcription factor with fundamental roles in the immediate early pathogen response and in promoting inflammation (Steer and Corbett, 2003; Ali et al., 2010). Down-regulation of virus-responsive genes probably can be interpreted in the context of the anti-inflammatory status since the IFN axis responds to the same stimuli as TNF-α and IL-1β.

The up-regulation of genes implicated in the recruitment and trafficking of immune cells suggests an increased influx of leukocytes or an enhanced capability for their rapid mobilization. However, this was not associated with activation of tissue damaging processes, which is commonly recognized by a distinct transcription signature that includes up-regulation of TNF related genes, matrix metalloproteases and the components of respiratory burst complex (Iliev et al., 2010). Overall, interval training stimulated the effector mechanisms that might improve resistance, like antibacterial and complement proteins, while maintaining minimum risk for the host.

The components of the complement system are present in plasma as inactive proteins whose activation is triggered by pathogen recognition. It acts as one of the first lines of host defense and is immediately ready to target and eliminate bacteria and viruses (Lachmann and Davies, 1997) mainly by amplifying the immune response, labeling for destruction (opsonization) and directly killing pathogens by the formation of the membrane-attack complex (Murphy et al., 2008). There are several studies showing the importance of the complement system in providing resistance against infectious diseases in fish. Complement molecules were the first to appear in sea bass (Dicentrarchus labrax) in response to a parasite (Henry et al., 2009), while, Petram et al. (2008) found up-regulation of several complement factors in catfish (Ictalurus furcatus) challenged with a gram-negative bacteria. Zhang et al. (2011) demonstrated that elevated levels of complement activity prior to an infection challenge with Aeromonas salmonicida was decisive for having a higher survival rate in Atlantic salmon, similarly to what we have found in this study for up-regulated complement transcripts and IPNV resistance. Information regarding the effects of complement factors on IPN resistance in salmonids is limited and contradictory. While some authors (Ellis, 2001) state that complement is not required for antibodies to neutralize non-enveloped viruses like IPNV, others have reported that the presence of complement in serum of chum salmon (Oncorhynchus keta) made the fish less susceptible to IPNV and infectious hematopoietic necrosis virus (IHNV) (Boshr et al., 2006). Moreover, the components of the complement system were the only immune genes in the liver of Atlantic salmon that showed a strong relationship with survival when challenged with furunculosis (Skugor et al., 2009). That study used the same microarray platform (SFA2.0) for comparison of fish with high and low resistance to the pathogen and revealed a suite of genes associated with survival. Altogether, it is evident that elevated levels of complement mediators found in IT fish could strengthen the general pathogen resistance.

Outcome of infectious diseases is not determined exclusively with immunity and may depend greatly on the ability to protect against various impacts including free radicals and toxic compounds and to repair damaged tissues (Skugor et al., 2009). In this respect, stimulation of genes involved in xenobiotics metabolism and scavengers of reactive oxygen species might mean better protection of IT salmon. A significant regulation of genes related to oxidative stress was of particular interest, as it has been shown that aerobic training increases production of reactive oxygen species (ROS) due to higher levels of oxygen required to satisfy the increased ATP demands (Urso and Clarkson, 2003). Several reports have demonstrated a beneficial effect of exercise training in terms of decreased oxidative stress in species such as mice (Navarro et al., 2004; Silva et al., 2009), rats (Itsh et al., 1998; Hollander et al., 2001), horses (Avellini et al., 1999) and humans (Koja and Hambrecht, 2005; Dane et al., 2008). In the present study, IT salmon hearts showed stronger antioxidant capacity than salmon hearts exposed to CT or C treatments. Mild increases in ROS produced during moderate exercise generate an adaptation process where ROS themselves can act as intracellular signaling molecules (Sachdev and Davies, 2008). On the other hand, high levels of ROS produced during intense to heavy exercise overwhelms the endogenous cellular antioxidant system, causing tissue damage and inducing an inflammatory response via activation of transcription factors such as NF-κB (Conner and Grisham, 1996; Allen and Tresini, 2000). It has been demonstrated that several antioxidants inhibit the activation of NF-κB not only in response to oxidants such as H2O2, but also in response to different inducers, including TNF-α and IL-1 (Müller et al., 1997). Hence, different mechanisms might be involved in lowering the inflammatory gene expression in heart of endurance trained salmon. Further, the results indicate that interval trained fish had good tissue reparation capacities. Collectively, a combined improvement in antioxidant capacity, removal of toxic compounds and tissue repair will strengthen the capacity of the heart to cope with any physiological or disease challenge as seen in this study.

While a few reports show lower growth in some fish species after exercise, the general consensus is that moderate aerobic training stimulates growth in fish, especially in salmonid species at water speeds between 1 and 2 bl s⁻¹ (Jobling et al., 1993; Davison, 1997). The present work extends this general finding by showing a training effect that lasted at least until 11 weeks beyond the training regime. Enhanced growth in salmonids is often achieved primarily due to an improved efficiency of feed conversion (Davison, 1997). Yet here, and when Atlantic salmon were trained at 1 bl s⁻¹ for 122 days before seawater transfer (Jørgensen and Jobling, 1994), training had no significant effect on feed conversion efficiency. Hence increased growth is explained mainly with an increase in feed intake during the training period. Previous authors have emphasized the need for more standardized protocols in fish-training studies (Davison, 1989, 1997; Jobling et al., 1993) to account for dissimilar results with highly variable types of exercise training regimes.

Macronutrient composition in the fish was unaffected by training in the present study. Yet untrained fish retained significantly more protein than trained fish, while both trained fish had significantly higher condition factors after the training period. Similar trends in condition factors were obtained for trained Atlantic salmon (Jørgensen and Jobling, 1994) and brown trout (Bugeon et al., 2003). A training effect of increased development of muscle fibers in the cross-section, as proposed by Azuma et al. (2002) who found higher condition factor in 1-year-old Masou salmon continuously exercised for 11 months at an average water speed above 0.5 bl s⁻¹, would represent a paradoxical situation because we would expect to see higher protein levels as well, which was not the case. Higher condition factors could also be explained by a relative slower growth rate of the vertebral column compared to the skeletal muscle growth. It is well established that increased mechanical load imposed by sustained exercise training alters vertebral architecture by inducing bone mineralization in combination with lowering the total bone area in teleosts (Deschamps et al., 2009). The amount of mineralized extracellular matrix depends mainly on calcium and phosphorus availability, as they are needed to form hydroxyapatite crystals (Francillon-Vieillot et al., 1990). However, these minerals are also required for other physiological processes including osmoregulation and skeletal muscle growth. Thus, the significantly lower calcium and phosphorus level in the trained Atlantic salmon smolts suggests that they may have had a mineral deficiency due to an increased demand. This deficiency may also have contributed in reducing the vertebral bone area (for a review, see Sugiu et al., 2004), hence increasing the condition factors of the trained fish. Such deficiencies might be ameliorated in seawater, where there is calcium...
and phosphorous available in the surrounding water as well as in the food.

The results of the swimming respirometry are predicated by the limitation that fish were challenged to only 1.6 bl s⁻¹ and not their maximum capacity. McKenzie et al. (1998) reported that Atlantic salmon in seawater fed a diet containing fish oil had critical swimming speeds the same as the highest speed attained by the present fish. In contrast, salmon studied by Wagner et al. (2004) and Lijalad and Powell (2009) attained critical swimming speeds of 2.6–3.0 bl s⁻¹. Maximum MO₂ determined during the exercise tests was almost 8 mg kg⁻¹ m⁻¹ in the study of McKenzie et al. (1998); whereas it was only a third of that level in the salmon studied by Lijalad and Powell (2009). Thus, there seems to be a high level of variation in the swimming capacity of salmon and in their maximum MO₂. Nevertheless, continuous training resulted in more efficient swimming of juvenile Atlantic salmon at the highest, but not lower swimming speeds, a benefit that was quickly lost during detraining, unlike the changes in growth and gene expression. A similar detraining effect was previously reported for striped bass (Young and Cech, 1994). The limited increases in MO₂ with increasing speed is not surprising because of the relatively low test speeds and an expected reduction in COT at these speeds (e.g. Lee et al., 2003a), even though the cost of moving through water increases exponentially with swimming speed. Different confounding effects could have blunted potential effects of training. One factor is that the fish were not starved prior to the swim trials because we did not wish to interrupt the growth study. As discussed by Eliason et al. (2007), routine MO₂ may be doubled prior to the swim trials because we did not wish to interrupt the growth and potential effects of training. One factor is that the fish were not starved prior to the swim trials because we did not wish to interrupt the growth study. As discussed by Eliason et al. (2007), routine MO₂ may be doubled prior to the swim trials because we did not wish to interrupt the growth study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi: 10.1016/j.cjpa.2011.06.013.

References


5. Conclusions

Both aerobic endurance training regimes tested here significantly stimulated growth performance of juvenile Atlantic salmon by increasing the feed intake. Interval training had a higher impact on improving robustness in the form of higher disease resistance (a challenge against IPN after sea transfer) compared to continuous intensity training at a lower intensity. Improved robustness was associated by a stronger activation of protective mechanisms in the cardiac transcriptome, including up-regulation of immune effectors, antioxidant capacity, removal of xenobiotics, tissue reparation and lowered inflammatory status.

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Paper 2
Exercise training effects on disease resistance are dependent on training regimes and inherent swimming performance in Atlantic salmon

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Abstract

**Background:** Fish, just like humans, can be classified according to their athletic performance. Further, fish improve their growth and fitness by aerobic exercise training, and recent results have documented improved disease resistance in exercised Atlantic salmon. In this study we investigated to what extent disease resistance of fish is dependent on the interaction between inherent swimming capacity and the response to an exercise regime. Atlantic salmon were first classified as either poor or good according to their swimming performance in a screening test and then exercise trained for 10 weeks using one of two constant-velocity or two interval-velocity training regimes for comparison against untrained fish. Disease resistance was assessed by a viral challenge test and gene expression analyses identified among-group immune correlates of disease response.

**Results:** An inherently good swimming performance was strongly associated with disease resistance, as good swimmers out-performed poor swimmers in the viral challenge test. Furthermore, the constant-velocity training regimes conferred higher protection compared to interval-velocity training. However, training-induced effects on disease resistance were significant only for poor swimmers. While a medium-intensity, constant-velocity regime boosted resistance to infections in poor swimmers, an interval-training regime produced the opposite effect. Apparently, the poor swimmers had a reduced capacity to cope with the higher allostatic load imposed by the repeated changes in swimming speed. Differences in mortalities were correlated by mRNA expression of virus responsive genes reflecting the infection status. Finally, all training regimes had a positive effect on growth.

**Conclusions:** In conclusion, disease resistance in Atlantic salmon is enhanced by an inherent good swimming capacity, while low disease resistance associated with an inherent poor swimming capacity results improved by exercise training. Furthermore, balanced levels of feed intake and efficiency seem to be a requirement for an association between exercise-induced growth and disease resistance.

Background

Diseases represent the main constrain for the success of an expanding aquaculture industry. Atlantic salmon (Salmo salar) farmers can experience severe fish losses due to both infectious and non-infectious diseases, usually during the seawater growth stage (Directorate of fisheries, http://www.fiskeridir.no/english).

Infectious pancreatic necrosis (IPN), pancreas disease (PD), infectious salmon anemia (ISA), as well as the sea lice parasite (Lepeophtheirus salmonis K) represent some of the most hazardous diseases [1,2], but losses have also been associated with life-style
diseases such as cardiac failures [3,4]. Biosecurity countermeasures to control the disease situation include vaccines and pharmaceuticals, as well as improvements of the genetic material, feeds and husbandry practices. The aim of current and future countermeasures is to strengthen the fish robustness, which is the capability to combine fast growth and normal organ development with improved resistance to both disease and physiological challenges.

Aerobic exercise training has been documented to impose a range of robustness skills for cultured fish, including increased somatic and cardiac growth, cardiac performance, aerobic capacity of the muscle, oxygen carrying and extraction capacity and improved bone quality [5-10]. Khovanskiy et al. [11] found that exercised chum salmon (Oncorhynchus keta) displayed lower mortality associated to an improved osmoregulatory capacity after seawater transfer. Going further, we have recently demonstrated direct effects of aerobic exercise on disease resistance, showing that survival of Atlantic salmon challenged with infectious pancreatic necrosis virus (IPNV) was 13% higher for fish subjected to a moderate interval-training regime for six weeks prior to smoltification when compared with fish held at a low, constant swimming speed [12]. Thus, aerobic exercise in fish can induce a similar robustness effect as in humans, where a moderate aerobic training is also known to decrease the risk of infections and chronic life-style diseases [13,14].

It has been observed that exercised salmonids [12,15] and non-salmonids, (Plecoglossus altivelis [16]; Chalcarburnus chalcoides mento [17]; Morone saxatilis [18]; Sparus aurata [19]; Danio rerio [20]) exhibit improved growth due to improved feed efficiency, higher feed intake or a mix of both. Several studies have reported on the relationship between improved growth performance and disease resistance in fish (see reviews by Merrifield et al. [21,22]). For example, Gjedrem [23] suggested that a breeding program selecting for growth also induced a positive genetic response for disease resistance, although conflicting results exist [24]. Recently, an association between exercise-induced growth and improved disease resistance was shown in Atlantic salmon [12]. The possibility of a linkage between these two factors, growth and disease resistance, is of obvious importance for the fish farming sector.

Migratory fish such as salmonids have a great inherent capacity for sustained aerobic swimming. Benefits from exercise seem to be maximized at speeds close to the optimal swimming speed ($U_{opt}$), where energy use is more efficient and the cost of transport is minimized [25]. Exercising fish at speeds other than $U_{opt}$ results in additional energy usage for locomotion, even at low speeds due to behavioral changes (e.g. increased aggression and spontaneous activity). Further, the highest speeds may prove stressful and unsustainable compared with $U_{opt}$ [20]. Because of their natural swimming behavior and high aerobic capacity, salmonids are naturally amenable to long-term continuous exercise training, provided sustainable water velocities are used. This is in contrast to terrestrial animals that more typically require resting periods between bouts of exercise training. In humans, where most exercise training research has been performed, the intensity seems to be a fundamental factor affecting the individual’s systemic immunity. While engaging in regular moderate exercise activity seems to enhance immune functions [13], high intensity aerobic training results in acute and chronic states of impaired immunity [26].

On top of training effects, there seems to be an equally large inherent variation in fitness among fish and humans. For example, juvenile rainbow trout (Oncorhynchus mykiss) can be classified according to their inherent swimming performance as either poor or good swimmers. Interestingly, such classification was associated with several cardiac and metabolic capacities after 9 months of common rearing [27]. This study aimed to identify the effects of four exercise-training regimes on robustness of Atlantic salmon smolts. Juvenile fish were identified according to their inherent swimming performance by pre-screening them on a swim challenge test. Fish classified as either poor or good swimmers were then trained to investigate if training differentially affected them. After smoltification, a controlled disease challenge with IPNV allowed us to assess differences in disease resistance among and within the four training regimes and two performance groups. This was further confirmed by analyzing expression levels of sensitive virus responsive genes (VRGs) in head kidney and cardiac tissues. In addition, exercise-induced effects on robustness were evaluated by growth performance and feed efficiency.

**Results**

**Growth is stimulated by different training regimes**

After six weeks of exercise training in freshwater, no significant differences in thermal growth coefficient (TGC) were found among training regimes and control fish. At the end of the freshwater phase of the experiment (10 weeks of training plus 1 week detraining), TGC was significantly higher ($p<0.05$) in the high intensity (H) training regime (1.61) compared with the control (C) group (1.50) (Table 1). The tendency of the other training regimes to have higher TGC than the control did not reach statistical significance ($p<0.1$). At the end of exercise training, medium intensity (M) trained fish showed a significantly higher condition factor (CF) than C. Feed intake showed significant differences among training regimes, with the higher values belonging to the H and short interval (Sint) regimes at the end the first six
training weeks, while H and long interval (Lint) had the highest feed intake in the second part of the training experiment (Table 1). After six weeks of training, the only significant differences for feed efficiency ratio (FER) among training groups were H and Sint groups being lower than both C and M (Table 1). No effects of training were found for whole-body concentrations of protein, lipid and energy at the end of the training period. Phosphorus, calcium and sodium concentrations were highest in Lint, but not significantly greater than in the C group (Table 2). Protein retention was also affected, being significantly lower in the Sint and H training regimes when compared with control. No differences for any of the above parameters were found when grouping the fish according to swimming performance.

**Disease resistance is dependent on both the training regime and inherent swimming performance**

Mortality following the IPN challenge started 18 days after the introduction of virus-shedding fish and reached a plateau around day 38 post-challenge. When survival was examined independent of the inherent swimming performance, differences among training regimes did not reach statistical significance (p=0.21). The continuous-velocity training regimes tended to improved survival compared with untrained control group (87.1, 84.2 and 82.2% survival for respectively M, H and C), whereas interval-velocity training regimes tended to negatively impact survival (78.2 and 75.3% survival for Sint and Lint, respectively; Figure 1A).

In contrast, inherent swimming performance showed a strong association with survival after the IPN challenge test. Fish initially categorized as good swimmers had significantly better survival than poor swimmers (86.1 and 77.6%, respectively; p=0.02) when analyzed across all groups (Figure 1B).

Moreover, exercise training seemed to have a stronger impact on disease resistance of poor swimmers, with minimum effect on good swimmers (Figure 2). Within the poor swimmers (p=0.2), M training reduced the mortality by 6.4% compared to untrained fish (C), while Lint training increased fish mortality by 7.9%.

In comparison, exercised and untrained good swimmers showed similar levels of mortality (p=0.9) that was overall reduced compared with poor swimmers (Figure 2).

**Virus responsive gene expression reflects and supports mortality data**

To verify mortality data from IPNV, infection level and immune status were analyzed in challenged fish at termination of the disease trial. Quantification of IPNV (by real-time qPCR in head-kidney) in surviving fish showed low prevalence of virus-positive fish (33%) and low level of viral transcripts in positive fish. Heart tissue was also tested and found negative for all fish. Thus, sampled fish were in a late stage of infection with either low levels or no virus replication.

Gene expression analysis was performed to investigate among-groups differences in host immune correlates of disease response. For initial screening of correlates, transcriptome analysis by microarray of poor and good swimmers was assessed, since the strongest contrast in mortality was associated with swimming performance. This resulted in 21 genes with significantly higher expression ratios in poor versus good swimmers (t-test, p<0.05) (Figure 3). By function, all genes were previously identified as virus-responsive genes (VRGs) having a common activation to most of the known viruses infecting Atlantic salmon, being sensitive antiviral markers reflecting the infection status and the level of viral transcripts in cells [28]. Thus, poor swimmers seemed to have higher activation of antiviral immune genes and sustained infection status compared to good swimmers at the end of the infection trial.

To further substantiate these results and to evaluate effects of the different training regimes with sufficient biological replication, expression of six VRGs was analyzed in heart ventricle tissue of poor and good swimmers from the C, M and Lint exercise-trained groups using qPCR. Results showed that induced levels of VRGs in poor swimmers were mainly explained by a strong expression in Lint exercised fish (Figure 4A). Untrained controls and M trained poor swimmers had equal expression level of VRGs. Within the good swimmers, VRG expression levels were higher in M and Lint trained compared to untrained fish.

We further analyzed expression of eight VRGs in head kidney, where IPNV replication was observed. Expression levels within exercised good swimmers showed a similar trend as observed for heart tissue (Figure 4B). Within poor swimmers, VRG expression levels were significantly lower in M compared to both untrained and Lint trained fish (p<0.05). In contrast to heart tissue, VRG expression levels in head-kidney of untrained good swimmers were significantly lower compared to untrained poor swimmers (p=0.03). Thus, the increased mortality observed for poor swimmers was reflected by stronger expression levels of antiviral immune genes and sustained infection in Lint trained (heart) and untrained C (kidney) fish as compared to good swimmers. While no evidence for any positive effects of exercise training on mortality and infection status was observed for good swimmers, results implied beneficial effects in terms of reduced infection status (lower VRG expression) from M trained poor swimmers.

Results produced for 8 genes by microarray and qPCR were in close concordance (Figure 5, Pearson r = 0.92; p=0.001).
Discussion

In this study we found that constant-velocity and interval-velocity training had opposite effects on survival against IPN. Furthermore, we showed that inherent swimming performance of juvenile Atlantic salmon is associated with differences in survival to an infectious disease challenge after seawater transfer. Interestingly, exercise affected resistance to IPN in fish initially classified as poor swimmers but not in good swimmers. Mortalities were supported by mRNA expression levels of a subset of VRGs reflecting the infection status. All tested training regimes had a positive effect on growth, but feed efficiency was regime dependent. Swimming performance did not affect growth performance.

The impact of training regimes on disease resistance

Continuous and interval training had opposite effects on survival against IPNV in this study. Overall, the three continuous training regimes (including C) resulted in better survival than the two interval regimes. The continuous 0.65 bodylengths (BLs)⁻¹ M regime gave the best results, which is in agreement with our previous finding where Atlantic salmon pre-smolts trained at a similar intensity for six weeks (0.8 BLs⁻¹ for 16 h and 1 BLs⁻¹ for 8 h per day) showed 13% higher survival following an IPN challenge test when compared to untrained fish [12]. Such improvements are very important in an industry context. In contrast to the improvement in disease resistance from utilizing an interval training regime with mild speed changes as in the previously mentioned study, the ~3-fold daily changes in swimming speed applied for the Sint and Lint regimes reduced disease resistance against IPN compared to controls kept continuously at 0.32 BLs⁻¹. Since Sint and Lint trained fish had theoretically swum the same distance as the M trained fish, it could be argued that the relatively strong daily changes in water speed for both interval regimes may be the cause of the negative impact on disease resistance of these fish. A possible explanation would be a detrimental effect of the potential stress enforced on the fish by the repeatedly strong changes in water speed between 0.32 and 1.31 BLs⁻¹, which certainly increases the allostatic load. An increased cost of the allostatic load is possibly indicated by the lower FER of the two interval groups as well as for H trained fish. Furthermore, the inherent capacity to maintain allostasis in changing environments is also highly variable within a population of fish, as further discussed below.

Exercise-induced effects on disease resistance are dependent on inherent swimming performance

The inherent swimming performance of individual juvenile salmon was positively associated with disease resistance. Fish classified as good swimmers showed 8.5% better survival against IPN virus than poor swimmers when challenged 3 months after the swimming performance classification. Claireaux et al. [27] demonstrated that good swimmers of a cohort of rainbow trout, classified by a similar methodological approach as used in this work, retained this advantage nearly 9 months later, despite a common rearing environment and similar growth performance, and displayed a significantly better cardiac capacity and morphology. This and the present study collectively suggest that a simple screening test for swimming performance can efficiently distinguish between fish with low and high robustness, with the latter possessing better cardiac capacity and disease resistance. It must be noted that none of these studies could find differences in growth performance between poor and good swimmers.

In addition to the effects of the inherent swimming performance of fish on robustness, exercise training was able to modulate disease resistance, particularly for the poor swimmers. While the M training regime improved the survival rate of the poor swimmers, the Lint regime, which resulted in the overall lowest survival rate, clearly worsened the survival of the poor swimmers. The observed interaction effect between inherent poor swimming performance and training regime on survival was supported by expression analysis of VRGs in surviving fish from the different training regimes and performance groups at the end of the IPN challenge. Results showed that improved survival was associated with lower expression levels of virus responsive genes, probably reflecting an overall lower level of infection pressure, a more rapid or efficient viral clearance and/or a reduced antiviral status to recover and regain homeostasis. Thus, the ability to rapidly clear or reduce virus replication and antiviral immunity at the end of a viral infection might be important for survival. In a previous study, we demonstrated that the improved survival, induced by aerobic training of juvenile Atlantic salmon, was related to a specific cardiac transcriptome signature, suggesting lower levels of inflammation and higher levels of immune effector molecules, antioxidant enzymes and xenobiotics clearance capacity prior to an IPN challenge [12].

The reduced disease resistance of the inherently poor swimmers trained with the Lint regime is probably due to lower adaptation capacity of these fish to the relatively strong changes in swimming velocity compared to the good swimmers. There might be several different reasons explaining the weaker plasticity of the poor swimmers, including an inherently lower aerobic scope or stress tolerance,
unknown physical disadvantages related to locomotive power possibly related to skeletal muscle composition or vertebral flexibility, and an undisclosed behavioral context. Independent of the causality, it seems that the allostatic cost by following the Lint regime was too high for the poor swimmers, thus their immune defense system was impaired when challenged with IPN. Inherently good swimmers, however, had enough plasticity to avoid significant impairment in immune competence. Intriguingly, the poor swimmers obtained a better effect from the M training regime than the good performance group. An explanation for this is that the intensity of M training promoted an adaptive response in the poor swimmers, boosting their disease resistance to the level of the good performance group. Thus, the overall profit of conducting M regime training appears to be the achievement of a more homogenous population when it comes to disease resistance. This would imply an indisputable benefit for salmon producers.

The impact of training regimes on growth and feed utilization

By definition, a robust fish must possess a good combination of both high growth performance and disease resistance. Jobling et al. [29] and Davison [15] stated that higher growth may be achieved for fish when training intensity lies between 0.75 and 1.5 BLs$^{-1}$. Our results are in agreement with this; the H training regime (1.31 BLs$^{-1}$) had significantly higher TGC than the control group. Interestingly, the other three regimes (M, Sint and Lint), which had an average water speed of 0.65 BLs$^{-1}$, showed a strong tendency towards improved growth compared to control fish, suggesting the existence of a correlation between growth and total work load of the swimming-induced exercise. Here we suggest two scenarios to explain this effect. The first scenario applies for the three most intense training regimes (H, Sint and Lint) where higher growth was mainly due to increased feed intake associated with a lower feed efficiency and protein retention. This suggests that fish subjected to those exercise regimes required more energy to satisfy the increased demand. Despite a lowered feed efficiency, increases in feed intake were sufficient to overcompensate the needs of simultaneous swimming and growth. It could be argued, that training at these intensities stimulated the regulation of neuroendocrine factors involved in controlling feeding, resulting in an anabolic dominant state. It is logical to think though, that growth will be compromised at higher water speeds than those tested here, as has been found in salmonids [30,31] and other species, such as striped bass Morone saxatilis [32]. Indeed, routine gut blood flow, which is a basic requirement for effective digestion, is reduced in salmon as they swim progressively faster and can stop with abrupt stresses [33,34].

The M-trained fish represent the second scenario, where higher growth might be explained by modest improvements (though not statistically different from the control) in both appetite and feed efficiency during the last six weeks of training, which coincided with the energy-demanding smoltification period. The combination of high CF, feed intake and efficiency, coupled to the higher disease resistance, suggests that the M regime increased the proportion of robust individuals.

Another effect that may contribute to exercise-induced growth is the possibility of salmon juveniles changing from active to passive (ram) ventilation. Ram ventilation is the capacity of some fish species to ventilate passively by opening their mouths when swimming or facing high water currents, allowing water to pass through the gills with enough pressure for gas exchange to occur without the need for active branchial pumping [35,36]. The energy sparing effect of ram ventilation ranged from 8.4 to 13.3% in adult rainbow trout, which shifted ventilation mode when swimming above 0.5-1 BLs$^{-1}$ [37]. Nevertheless, we cannot know for certain if this is also the case in this study.

Gross composition of fish was unaffected by training in the present study, while training reduced the retention of protein, which is consistent with our previous findings [12]. Although not addressed in this experiment, studies specifically directed towards investigating energy substrate use during sustained exercise in salmonids, have found that the main fuel burned is lipid, followed by carbohydrates, and proteins playing a minor role [38-40]. Castro et al. [12] found that continuous or interval training had a negative effect on whole-body P and Ca concentrations compared with untrained salmon. In this study, lowered P and Ca levels in M and Sint suggest that fish subjected to those regimes could have had an increased mineral demand, resulting in mineral deficiency. Nevertheless, the mineral content of all groups was within normal range for salmon [41].

Conclusions

This study provides the first evidence demonstrating that inherent swimming performance in juvenile fish is predetermining disease resistance later in life. In contrast to fish classified as good swimmers, which show low mortality when challenged with IPN, the inherently poor swimmers are more affected by training regimes of different intensities and design. We show that continuous-velocity aerobic exercise training tends to improve disease resistance; while interval-training regimes with high daily variation in swimming speeds have an opposite effect, especially on the survival of poor swimmers. Furthermore, our results confirm that aerobic exercise independent of
training regime stimulates growth performance of Atlantic salmon. Exercise-induced growth produced by a balanced effect of feed intake and feed efficiency might show a positive association with improved disease resistance as found for the continuous M-trained fish.

The results of this study reveal that stabilizing the water current conditions in land-based salmon production sites may significantly reduce disease losses.

The great variability in swimming performance within populations of fish opens up novel possibilities for phenotype or marker-assisted selection in breeding programs and as a discrimination tool to sort out poor juvenile fish when it is still cost-effective. Furthermore, early screening of fish would identify subgroups that might benefit more from exercise training of the type used here (the poor swimmers) than others (the good swimmers) for which few gains are to be made.

**Materials and Methods**

**Swimming performance screening and training experimental setup**

Juvenile Atlantic salmon belonging to the Salmobreed strain were produced and reared at Nofima AS, Sunndalsora, Norway. The trial was conducted in agreement with the Norwegian Animal Research Authority (NARA) regulations.

A total of 1355 fish were individually tagged (Passive Integrated Transponder (PIT), Glass tag Unique 2.12 x 12 mm, Jojo Automasjon AS, Sola, Norway) and measured (mass = 40.7±0.2 g and fork lengths = 15.0±0.3 cm) before being graded according to their swimming performance. Groups of approximately 100 fish were placed in a preconditioned 1.5 m diameter circular tank with an inner ring to reduce the swimming area to a 40 cm radius (Figure 6). The water inlet to the swimming arena was tangentially situated on the side of the outer tank so that it generated the maximum water velocity. The inner ring was placed on four pieces of PVC (1 cm high) that allowed the water to drain back-mesh grid. They were removed with a dip-net, increasing water current typically laid against the back-mesh grid. The fish were removed with a dip-net, identified (PIT tag reading) and placed back in their rearing tank. During a trial, fish were regularly and gently repositioned in the arena to ensure that they would all be exposed to exercising conditions and would not evade from the high speed outer portion of the swimming ring. Based on their swimming performance, fish were then allocated to one of two groups. The first 50% that stopped swimming were categorized as “poor swimmers”, and the last 50% still swimming were the “good swimmers”. Both poor and good swimmers were randomly mixed among 16 cilindro-conical tanks (500 l, 82 cm in diameter, 77-86 fish tank⁻¹) and left undisturbed for one week before the start of the training regimes. The center of each experimental tank was fitted with a plastic pipe (31.5 cm diameter), which reduced the area in the tank with lowest water speed. A frequency-controlled pump (Hanning Elektro Werke, PS 18-300; Oerlinghausen, Germany) directed the water current and a wire mesh fence, attached between the pipe and the edge of the tank, prevented the fish from drifting backwards. The water speeds were calibrated by using the average speed measured at twelve points in the tank (four horizontal locations and three depths at each location (Höntzsch HFA propeller, Waiblingen, Germany with HLOG software). Five different aerobic exercise-training regimes were tested; the control regime in quadruplicate tanks and the other regimes in triplicate tanks. Three of the training regimes were continuous velocity: the control (C; 5.7 cm s⁻¹), medium intensity (M; 11.5 cm s⁻¹) and high intensity (H; 23 cm s⁻¹) regimes. At start of the 10-week training experiment, these speeds were equivalent to 0.38, 0.77 and 1.53 BLs⁻¹ for C, M and H, respectively. As fish grew during the trial, average relative water speeds were reduced to 0.32 (C), 0.65 (M) and 1.31 (H) BLs⁻¹. The two remaining training regimes used interval training, with daily increments in the relative water velocity from 0.32 to 1.31 BLs⁻¹ for either a single 8 h period (Long interval; Lint) or four 2-h periods for a total training period of 8 h (short interval; Sint). Theoretically, both interval groups swam the same distance as the M group. The 10 weeks of training were followed by a one-week recovery at control speed prior transferring the fish to seawater. To stimulate smoltification during the experiment, fish were exposed to a short daylight regime (12-12 Light-Dark) for the first six weeks, followed by continuous light for the remaining five weeks. Water temperature was measured daily (10.5±0.8°C) while oxygen saturation was measured weekly and was maintained over 85% with oxygen supplementation. Dead fish were removed with daily inspections and weighed.

**Growth, feed intake and body composition**
During the training experiment, fish were fed to excess an extruded diet based on fish meal, ground wheat and fish oil (produced at Nofima AS, Fyllingsdal, Bergen, Norway), using automatic feeders. The effluent water of each tank was led into a wire mesh box to enable sieving of waste feed. To minimize feed leaching, the effluent water was directed to two different areas of the wire box using pinch valves on the water pipes, dependent on whether feeding was occurring. The waste feed, expressed as dry matter (DM) content, was used to recalculate daily feed intake in order to adjust ration level every second day (Helland et al., 1996). After the change in the photoperiod at the sixth week of training, feeding regime was increased from 12 to 24 times per day.

Fish were weighed in bulk after six weeks of training and a two-day fast. At the end of the trial, fish were individually re-weighed and re-measured. Whole-body composition (DM, crude protein, crude lipid (without HCl hydrolysis), ash, minerals and energy, as described below) was determined on 15 fish at the start of the trial and 10 fish from each tank at the completion of 10 weeks of training. The fish were anesthetized (tricaine methanesulfonate, MS 222, Argent Chemical Laboratories Inc., Redmont, WA, USA), killed with a blow to the head and weighed. Feed and feces were removed from intestines and stomach before fish were stored at -20°C until analysis.

The experimental diet was analyzed for DM (105°C, until constant weight), crude lipid (Soxtect HT6 after hydrolysis with HCl, Tecator, Höganas, Sweden), nitrogen (crude protein (CP) = nitrogen x 6.25; Kjeltec Auto System, Tecator, Höganas, Sweden), ash (550°C, overnight) and minerals (inductively coupled plasma mass spectroscopy at Bioforsk, Ås, Norway). Gross energy was measured using an adiabatic bomb calorimeter (Parr 6300, Parr Instrument Company, Moline, IL, USA). The 2.5 mm pellets contained 92.7% DM and on a DM basis, 52.5% crude protein, 23.4% crude lipid, 9.2% ash and 24.1 MJ kg⁻¹ of IPN (strain V-1244 cultured at the Norwegian School of Veterinary Science). Throughout the challenge test, fish were observed and mortalities were recorded daily for 45 days, when the trial was terminated. A representative selection of dead fish was bacteriologically examined on 2% NaCl blood agar plates at VESO.

**Gene expression analyzes**

Fish for gene expression analyzes were sampled at the end of the IPN challenge test (day 45), when mortality of all groups had leveled off. Challenged fish belonging to both swimming performance categories (poor and good swimmers) were sampled for each of the treatments C, M and Lint. While nine unchallenged untrained (C) fish were used as hybridization controls for microarray analyzes. Cardiac ventricle and head kidney tissues were immediately dissected from fish killed by a blow to the head and stored in RNAlater (Ambion, Austin, TX, USA). Total RNA was extracted using TRIzol and purified with PureLink RNA Mini Kit (Invitrogen, Carlsbad, CA, USA) following manufacturers guidelines. RNA concentration was measured using NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and RNA integrity was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). All samples had RIN values above 9.

Microarray analysis were performed with the salmonid oligonucleotide microarray (SIQ2.0, NCBI GEO platform GPL10679), consisting of 21K features printed in duplicate on 4 x 44K arrays from Agilent Technologies [42]. Eighteen two-color microarray hybridizations were performed. Individual heart ventricle samples of challenged fish from poor and good swimming performers (n=9 each; 3 from each training regime) were competitively hybridized against a pooled sample consisting of equal amounts of RNA from the controls (n=9) per array. Unless specified otherwise, all reagents and equipment were from Agilent Technologies and used according to manufacturer’s protocol. Amplification and labeling of RNA (200 ng) were done using the LowInput-QuickAmp Labeling Kit. Cy5 (test) and Cy3 (control) labeled RNA was purified with RNeasy Mini Kit (Qiagen, MD, USA), and the Gene Expression Hybridization Kit was used for RNA fragmentation. Hybridizations were performed for 17 h at 65°C and rotation speed of 10 rpm in a hybridization oven. Arrays were washed twice (Gene Expression wash buffers 1 & 2) and immediately scanned with a GenePix 4100A (Molecular Devices, Sunnyvale, CA, USA) at 5 μm resolution and with manually adjusted laser power to ensure an overall intensity ratio close to unity between Cy3 and Cy5 channels and with the change in the photoperiod at the sixth week of training.
minimal saturation of features. The GenePix Pro 6.1 software was used for spot-grid alignment, feature extraction of fluorescence intensity values and spot quality assessment. Low quality spots were filtered with aid of GenePix flags as well as by the criterion \( (I-B)/(S_I-S_B) \geq 0.6 \) where \( I \) and \( B \) are the mean signal and background intensities, respectively, and \( S_I \) and \( S_B \) are the respective standard deviations. Data were exported into the STARS platform [42] for data transformation and Lowess normalization of log2-expression ratios (ER). Differentially expressed genes were selected based on spot signal threshold, \( \log_2-\text{ER average} > 0.6 \) in at least two individuals and significant difference between groups (swimming performers) \( p<0.05, \ n=9 \) (Student’s t-test). Expression of single genes (VRGs) was assessed by quantitative real-time RT-PCR (qPCR) on heart ventricle and head kidney samples from swim performers of the three groups C, M and Lint at day 45 post-challenge. Expression levels were compared against the same 9 control samples used for microarrays. A total of six and eight VRGs were analyzed for heart (n=16 per regime; 8 good and 8 poor swimmers) and head kidney (n=10 per regime; 5 good and 5 poor swimmers), respectively. The same samples from both tissues were further scanned for viral presence by using a set of specific IPNV primers (Table 3). Confirmation of array results by qPCR was based on eight genes (4 up- and 4 down-regulated) in the same individual samples as for microarray.

All qPCR primers were designed using the ePrimer3 from the EMBoss online package [43], except for the IPNV primers [44] and synthesized by Invitrogen (Table 3). Synthesis of cDNA was performed on 0.5 µg of DNase treated (DNA-free; Ambion) RNA samples using TaqMan® Reverse Transcription reagents (Applied Biosystems, Foster City, CA, USA) and primed with an equal mix of oligo dT and random hexamers. PCR reactions were prepared manually and run in duplicates in 96-well optical plates on a LightCycler® 480 (Roche Diagnostics, Mannheim, Germany) using 2X SYBR Green Master mix (Roche), 5 µl of cDNA samples and a primer concentration of 0.42 µM each in a final volume of 12 µl. For all genes, cDNA was previously diluted 1:10 (1:1000 for 18S). qPCR thermal cycling was as follows: 5 min pre-incubation at 95°C, followed by 45 amplification cycles consisting of 95°C for 10 s, 60°C for 15 s and 72°C for 15 s, followed by a melting curve protocol (95°C for 5 s, continuous increase from 65°C to 97°C) to assess specificity of the amplicon. Fluorescence was measured at the end of every extension step and throughout the melting curve step. Cycle threshold (C_T) values were calculated using the second derivative method. Duplicate reactions differing more than 0.5 C_T values were discarded, and values were averaged for relative quantification. PCR efficiency was assessed by six 10-fold serial dilutions of pooled sample templates for each primer pair. Relative expression ratios were calculated by the Pfaffl method [45] with the averaged C_T values of two genes as normalization factor (18S and Elongation factor 1α). An index value of VRG expression was calculated for each group (training regime or swimming performance) by averaging the relative expression ratio of the single genes.

**Calculation and statistics**

Relative feed intake: 100 x (dry feed intake/mean body mass (BM)/days fed).

TGC: 1000 x [(BM_i^0.33 – BM_0^0.33)/Σday-degrees], where BM_i and BM_0 are final and initial body masses, respectively.

FER: (Wet fish gain + dead fish mass)/dry feed intake.

CF: 100 x BM (g) x fork length (cm)^3.

Retention of nitrogen and energy in whole fish (%): 100 x [(BM_f x final body nutrient content) – (BM_i x initial body nutrient content)]/ (total intake x nutrient content in diet).

For growth and CF analyses, the individual fish data were analyzed by analysis of variance in a hierarchical model including the fixed effect of training regime and the random effect of tank within regime. The mean data for each tank were tested by variance analysis (means compared using the least-squares means procedure) (SAS software, version 9.1, SAS Institute, Inc., Cary, NC, USA). Percentage data were transformed (arc sine square root) before being subjected to analysis. The mean weights of the fish in each tank at the end of the trial were used as a covariate in the analysis of whole-body composition. Differences between training regimes were considered significant at the p<0.05 level, and are presented as means±SEM.

Differences in survival during the IPN challenge test were evaluated using the Mantel-Cox test in GraphPad Prism (version 5.01, GraphPad Software, Inc., San Diego, CA, USA). For the microarray analyses, expression differences between the groups where assessed by Student’s t-test; \( p<0.05 \), and data are presented as \( \log_2\text{ER±SEM} \). Difference in expression levels for the indexed values of pooled VRGs, was assessed by paired Student’s t-test, \( p<0.05 \); between target and control groups in qPCR. Correlation between microarray and qPCR results for selected genes was assessed by Pearson’s \( r \).

**Authors’ contributions**

VC carried out the molecular studies, participated in samplings, data interpretation and drafted the manuscript. BG performed the growth and nutrition
studies analyses. SMJ interpreted the microarray data and drafted the corresponding section. JH participated in the design of the study and revised the manuscript. GC designed the screening test facilities, performed the classification of fish according to swimming capacities, and revised the manuscript. APF participated in designing the study and critically revised the manuscript. AK analyzed the microarray data and revised the manuscript. SJH designed the fish training facilities and performed and participated in nutrition analyses. HT obtained the funding, conceived and designed the study, coordinated and participated in samplings, data interpretation and drafting of the manuscript. All authors read and approved the final manuscript.

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References


Figures

Figure 1 - Atlantic salmon survival in IPN challenge test
A: Fish exercised at constant speeds for 10 weeks showed a trend towards higher disease resistance than those subjected to interval training regimes (Medium intensity (M)=87.1%; High intensity (H)= 84.2%; Control (C)= 82.2%; Short interval (Sint)= 78.2% and Long interval (Lint)= 75.3%) (p=0.21). B: The inherent swimming performance of the fish had a significant effect on disease resistance, with the Good swimmers showing a higher survival (86.1%) than the Poor swimmers (77.6%). *indicates significant difference, Mantel-Cox test; p< 0.05.
**Figure 2 - Cumulative mortality reflects different effects of exercise and swimming performance to an IPN challenge test.**

Only those regimes analyzed by gene expression are displayed. These reflect the lower (Medium= 12.9%), middle (Control= 17.8%) and higher (Long interval= 24.7%). Data is shown independently of swimming performance (Poor & Good, p=0.09); within Poor swimmers (p=0.2) and within Good swimmers (p=0.87) for the different training regimes or independent of training regime (Poor vs. Good swimmers, red bars, p=0.08). Difference in mortality was larger between poor and good swimmers for the Long interval (12.9%, p=0.12), smaller for Control (6.6%, p=0.39) and minimum for the Medium intensity regime (2%, p=0.74; Mantel-Cox test). Initial number of fish on the challenge ranged from 100 to 112/regime.
Figure 3 – Microarray results.
Microarray analyses resulted in 21 genes being significantly up-regulated in poor swimmers compared to good swimmers. By function, all of these genes have been previously identified as virus responsive genes (VRGs). Data is log$_2$ER±SEM (n=9). ER= Expression ratio.

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<td>tetraspanin-3</td>
<td>0.43</td>
<td>0.11</td>
<td>0.05</td>
<td>0.08</td>
<td>0.58</td>
<td>0.01</td>
</tr>
<tr>
<td>Ssa#S47726724</td>
<td>XIAP-associated factor 1</td>
<td>0.84</td>
<td>0.15</td>
<td>0.46</td>
<td>0.10</td>
<td>0.37</td>
<td>0.05</td>
</tr>
<tr>
<td>Ssa#GRASP223647619</td>
<td>CD9 antigen</td>
<td>0.23</td>
<td>0.12</td>
<td>-0.13</td>
<td>0.08</td>
<td>0.36</td>
<td>0.03</td>
</tr>
</tbody>
</table>
Figure 4 – Expression of VRGs showed that effect of training on survival depended on swimming performance. Improved survival to an IPN challenge test associates with the level of virus responsive genes (VRGs). Higher expression levels of VRGs in cardiac tissue of poor swimmers from the Lint regime (A), and in head kidney from the control fish (B) reflects the overall higher mortality of poor swimmers in comparison to the good swimmers. Further, VRGs expression in both tissues was in concordance with differences in survival between interval (Lint) and constant speed (Control and Medium). abc: Denotes significant difference (p<0.05; paired t-test) between treatments (Poor & Good), only between the poor swimmers (Poor) and only between the good swimmers (Good) from each treatment. Similar symbol (*#§) denotes significant difference (p<0.05; paired t-test) between Poor and Good swimmers within -or independent of- training regime. For A and B, each bar is a composed index of 6 (n=8 fish/swim-performance/regime) and 8 (n=5 fish/swim-performance/regime) genes, respectively. ER: Expression ratio. Genes included: RSAD2 (radical S-adenosyl methionine domain containing protein2), IFIT5 (interferon-induced protein with tetratricopeptide repeats 5), STAT1 (signal transducer and activator of transcription 1), VHSV2 (viral haemorrhagic septicaemia virus-inducible protein), BAF (barrier-to-autointegration factor), GIG1 (interferon-inducible Gig1), RIG-I (DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide), MDA5 (interferon induced with helicase C domain 1).
Figure 5 - Microarray results validation by qPCR.
Eight genes were chosen for confirmation of microarray results with qPCR. Expression levels of 9 challenged fish (poor swimmers) were compared by both methods. BNP: B-type natriuretic peptide, LEI: leukocyte elastase inhibitor, put_collA1: putative collagen alpha1, IFIT5: interferon-induced protein with tetratricopeptide repeats 5, VHSV2: viral haemorrhagic septicaemia virus-inducible protein, GIG1: interferon-inducible Gig1, RSAD2: radical S-adenosyl methionine domain containing protein2_viperin, GIG2: interferon-inducible protein Gig2. Pearson $r$: 0.92; $p$: 0.001.

![Graph showing microarray vs qPCR results for selected genes.](image-url)
Figure 6 – Swimming performance screening test.
Groups of approx. 100 fish were placed in the screening tank each time and allowed to acclimate for 15 minutes at minimum water speed before start of the water speed increments. A plastic mesh (green, left side) prevented fish from drifting backwards. Inner ring was lifted one centimetre with the help of PVC pieces (not on diagram) to allow water drainage into the centre of the tank where the main outflow was located. Blue arrows indicate direction of water flow (clockwise).
### Tables

**Table 1 - Growth parameters and dry matter intake of exercise trained Atlantic salmon.**

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>M</th>
<th>H</th>
<th>Sint</th>
<th>Lint</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body weight (BW) (g)</strong></td>
<td>Start</td>
<td>40.9±0.2</td>
<td>40.7±0.2</td>
<td>40.6±0.2</td>
<td>40.2±0.4</td>
</tr>
<tr>
<td></td>
<td>W 6</td>
<td>70.5±1.3</td>
<td>70.9±0.7</td>
<td>72.4±2.4</td>
<td>72.3±0.6</td>
</tr>
<tr>
<td></td>
<td>W 11</td>
<td>95.4±0.3</td>
<td>99.9±1.1</td>
<td>99.4±2.4</td>
<td>98.2±2.2</td>
</tr>
<tr>
<td><strong>Length (cm)</strong></td>
<td>Start</td>
<td>15.1±0.03</td>
<td>15.0±0.04</td>
<td>15.0±0.01</td>
<td>15.0±0.05</td>
</tr>
<tr>
<td></td>
<td>W 11</td>
<td>20.0±0.04</td>
<td>20.2±0.07</td>
<td>20.2±0.16</td>
<td>20.2±0.14</td>
</tr>
<tr>
<td><strong>CF</strong></td>
<td>Start</td>
<td>1.18±0.003</td>
<td>1.18±0.002</td>
<td>1.18±0.005</td>
<td>1.18±0.002</td>
</tr>
<tr>
<td></td>
<td>W 11</td>
<td>1.18±0.004b</td>
<td>1.20±0.004a</td>
<td>1.19±0.004ab</td>
<td>1.18±0.002b</td>
</tr>
<tr>
<td><strong>TGC</strong></td>
<td>W 1-6</td>
<td>1.56±0.05</td>
<td>1.59±0.04</td>
<td>1.66±0.09</td>
<td>1.68±0.02</td>
</tr>
<tr>
<td></td>
<td>W 6-11</td>
<td>1.44±0.08</td>
<td>1.64±0.06</td>
<td>1.53±0.05</td>
<td>1.47±0.12</td>
</tr>
<tr>
<td></td>
<td>W 1-11</td>
<td>1.50±0.01b</td>
<td>1.58±0.03ab</td>
<td>1.61±0.02a</td>
<td>1.59±0.05ab</td>
</tr>
<tr>
<td><strong>Relative feed intake (%) BW d⁻¹)</strong></td>
<td>W 1-6</td>
<td>0.87±0.02c</td>
<td>0.88±0.02c</td>
<td>0.98±0.03ab</td>
<td>0.99±0.01a</td>
</tr>
<tr>
<td></td>
<td>W 6-11</td>
<td>0.65±0.02c</td>
<td>0.69±0.01bc</td>
<td>0.74±0.01a</td>
<td>0.70±0.02ab</td>
</tr>
<tr>
<td><strong>FER</strong></td>
<td>W 1-6</td>
<td>1.43±0.04a</td>
<td>1.42±0.02a</td>
<td>1.32±0.00b</td>
<td>1.34±0.00b</td>
</tr>
<tr>
<td></td>
<td>W 6-11</td>
<td>1.66±0.08</td>
<td>1.70±0.03</td>
<td>1.52±0.06</td>
<td>1.56±0.11</td>
</tr>
</tbody>
</table>

C: Control; M: medium intensity; H: high intensity; Sint: short interval; Lint: long interval. CF: condition factor; TGC: thermal growth coefficient; week (W) 6: End of first six weeks of training under a short day light photoperiod. W 11: End of 10 weeks of training and one week of detraining. a,b,c: Significance probability associated with the F statistic. Means in the same row with different superscripts are significantly different based on one-way ANOVA (p<0.05). Data are means±SEM.
Table 2 - Body composition and protein and energy retention of Atlantic salmon subjected to five different training regimes.

<table>
<thead>
<tr>
<th></th>
<th>Initial</th>
<th>C</th>
<th>M</th>
<th>H</th>
<th>Sint</th>
<th>Lint</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture, %</td>
<td>69.3</td>
<td>68.8±0.3</td>
<td>68.8±0.1</td>
<td>68.8±0.3</td>
<td>68.3±0.2</td>
<td>68.4±0.2</td>
</tr>
<tr>
<td>Crude protein, %</td>
<td>17.0</td>
<td>17.6±0.1</td>
<td>17.2±0.1</td>
<td>17.7±0.3</td>
<td>17.5±0.3</td>
<td>17.4±0.5</td>
</tr>
<tr>
<td>Crude lipid, %</td>
<td>11.3</td>
<td>11.1±0.2</td>
<td>11.5±0.0</td>
<td>11.5±0.5</td>
<td>11.7±0.2</td>
<td>11.8±0.2</td>
</tr>
<tr>
<td>Ash, %</td>
<td>2.4</td>
<td>2.2±0.0</td>
<td>2.2±0.0</td>
<td>2.2±0.1</td>
<td>2.2±0.1</td>
<td>2.2±0.0</td>
</tr>
<tr>
<td>Gross energy, MJ kg⁻¹</td>
<td>8.71</td>
<td>8.60±0.04</td>
<td>8.70±0.06</td>
<td>8.60±0.26</td>
<td>8.86±0.09</td>
<td>8.88±0.15</td>
</tr>
<tr>
<td>Phosphorus, mg kg⁻¹</td>
<td>4381</td>
<td>4255±110ᵇ</td>
<td>3934±143ᶜ</td>
<td>4056±169ᵇ</td>
<td>3894±198ᵇ</td>
<td>4549±65ᵃ</td>
</tr>
<tr>
<td>Calcium, mg kg⁻¹</td>
<td>4762</td>
<td>4392±113ᵇ</td>
<td>4008±190ᵇ</td>
<td>4053±270ᵃ</td>
<td>3817±425ᵇ</td>
<td>4800±48ᵃ</td>
</tr>
<tr>
<td>P:Ca ratio</td>
<td>0.92</td>
<td>0.97±0.02</td>
<td>0.98±0.03</td>
<td>1.00±0.03</td>
<td>1.03±0.06</td>
<td>0.95±0.01</td>
</tr>
<tr>
<td>Iron, mg kg⁻¹</td>
<td>11.5</td>
<td>10.8±0.3</td>
<td>11.1±0.3</td>
<td>11.1±0.3</td>
<td>11.4±0.2</td>
<td>11.2±0.2</td>
</tr>
<tr>
<td>Copper, mg kg⁻¹</td>
<td>1.4</td>
<td>1.7±0.6</td>
<td>2.2±0.6</td>
<td>1.4±0.3</td>
<td>1.6±0.5</td>
<td>1.7±0.4</td>
</tr>
<tr>
<td>Magnesium, mg kg⁻¹</td>
<td>266</td>
<td>265±4</td>
<td>262±5</td>
<td>250±5</td>
<td>253±4</td>
<td>268±8.5</td>
</tr>
<tr>
<td>Manganese, mg kg⁻¹</td>
<td>1.6</td>
<td>1.8±0.1</td>
<td>1.9±0.1</td>
<td>1.9±0.1</td>
<td>1.8±0.1</td>
<td>1.9±0.0</td>
</tr>
<tr>
<td>Sodium, mg kg⁻¹</td>
<td>1430</td>
<td>1331±153ᵃ</td>
<td>1008±58ᵇ</td>
<td>1244±124ᵃ</td>
<td>1222±234ᵃ</td>
<td>1500±129ᵃ</td>
</tr>
<tr>
<td>Zinc, mg kg⁻¹</td>
<td>45.7</td>
<td>41.1±1.1</td>
<td>40.0±1.5</td>
<td>40.0±2.5</td>
<td>39.0±9.9</td>
<td>41.7±1.5</td>
</tr>
<tr>
<td>Protein retention, %</td>
<td>56.6±2.6ᵃ</td>
<td>51.2±0.4ᵃ</td>
<td>49.0±1.8ᵇ</td>
<td>48.1±2.6ᵇ</td>
<td>51.1±1.0ᵇ</td>
<td></td>
</tr>
<tr>
<td>Energy retention, %</td>
<td>58.1±3.7</td>
<td>55.7±0.4</td>
<td>50.2±1.6</td>
<td>52.5±1.7</td>
<td>56.6±3.0</td>
<td></td>
</tr>
</tbody>
</table>

C: Control; M: medium intensity; H: high intensity; Sint: short interval; Lint: long interval. ab: Significance probability associated with the F statistic. Means in the same row with different superscripts are significantly different based on one-way ANOVA (p<0.05). Data are means±SEM.
Table 3 - List of primers used for the qPCR reactions.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Short name</th>
<th>Sequence 5' to 3'</th>
<th>Accession</th>
</tr>
</thead>
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<tr>
<td>radical S-adenosyl methionine domain-containing protein 2</td>
<td>Rsad2</td>
<td>F-GTACCGCAGATGCAACACAC R-TTGACACTGTGTTGGATGTA</td>
<td>AF076620</td>
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<tr>
<td>interferon inducible protein Gig1</td>
<td>Gig1</td>
<td>F-GGCAACCTGAATCCAGAAGA R-GCTCGAGGCAAGCATGTA</td>
<td>DW569595</td>
</tr>
<tr>
<td>VHSV-inducible protein</td>
<td>Vhsv2</td>
<td>F-GTGAAGACCTGGACCTGA R-TGACCCCTGGTCACTGGTC</td>
<td>BT072288</td>
</tr>
<tr>
<td>interferon-induced protein with tetratricopeptide repeats 5</td>
<td>Ifi5</td>
<td>F-CAGAGAGGGCCAGGCTAAC R-TGACCATCGTCGCAGT</td>
<td>BT046021</td>
</tr>
<tr>
<td>B-type natriuretic peptide</td>
<td>BNP</td>
<td>F-TGAGAACATGCAATGGTCGCAGT R-GTGGCCAGTTTCAGGATG</td>
<td>CK83650</td>
</tr>
<tr>
<td>putative collagen alpha 1</td>
<td>Coll-a1</td>
<td>F-TGACCTGAGACCCCTCAGT R-TGGTCCTACGTCGTTC</td>
<td>CA038317</td>
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<tr>
<td>leukocyte elastase inhibitor [Salmo salar]</td>
<td>LEI</td>
<td>F-TCACAGATGCAAGGACGTC TCTGAATG R-GTGGCCAGTTTC</td>
<td>BT045959</td>
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<tr>
<td>interferon induced with helicase C domain 1</td>
<td>Mda5</td>
<td>F-CAGAGGTTGCAATGCTGA TATGAT R-AGCTCAGTCCTGCA</td>
<td>NM00195179</td>
</tr>
<tr>
<td>DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide RIG-I</td>
<td>Rig-I</td>
<td>F-GAGGTCAGACGGGTGTTAC R-GCTCGGGCTACGTC</td>
<td>DY714827</td>
</tr>
<tr>
<td>barrier-to-autointegration factor</td>
<td>Baf</td>
<td>F-ACGAGGCTCAGGACAGTAGT R-CGTCATGCACTTACTG</td>
<td>BT049316</td>
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<tr>
<td>signal transducer and activator of transcription 1 isoform alpha</td>
<td>Stat1a</td>
<td>F-CGGTGTCGACGCCTACACTAAG R-GGATCGGTGGTGAAGGTA</td>
<td>CB513054</td>
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<tr>
<td>interferon-inducible protein Gig2-like</td>
<td>Gig2</td>
<td>F-GATGTTCATGCGCTGCAA R-CTTTTCAGGTGACGTCA</td>
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<td>elongation factor 1a</td>
<td>EF1a</td>
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<td>infectious pancreatic necrosis virus_polyprotein</td>
<td>IPNV</td>
<td>F-CGACCGGAGAAGA</td>
<td>AJ877117</td>
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</table>
Paper 3
Paper 3
Cardiac Molecular-Acclimation Mechanisms in Response to Swimming-Induced Exercise in Atlantic Salmon

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Abstract

Cardiac muscle is a principal target organ for exercise-induced acclimation mechanisms in vertebrates, given that sustained aerobic exercise training improves the cardiac output in both fish and mammals. Nevertheless, the molecular mechanisms underlying exercise-induced cardiac acclimation have been scarcely investigated in teleosts. Consequently, we studied mechanisms related to cardiac growth, contractility, vascularization, energy metabolism and myokine production in Atlantic salmon pre-smolts that had been exercise-trained for 10 weeks at three different swimming intensities: 0.32 (control), 0.65 (medium intensity) and 1.31 (high intensity) body lengths s⁻¹. Cardiac effects were examined by means of somatic index, immunofluorescence, western-blotting and qPCR analysis of a large number of target genes encoding proteins with significant and well-characterized function. Exercise had an overall stimulatory effect on the cardiac muscle that was dependent on training intensity. High intensity training produced changes of greater magnitude than either medium or control intensity. Cardiac growth involved a mixture of both cardiomyocyte hypertrophic and hyperplasic mechanisms as suggested by elevated mRNA levels of MEF2C, GATA4 and ACTA1, as well as protein levels of PCNA. The larger heart in high intensity-trained fish was supported by improvements to the contractile machinery (as suggested by an up-regulation of EC coupling transcripts) and the oxygen supply network (as suggested by elevated mRNA levels of VEGF and EPO). Also, increased cardiac lipid oxidation to service enhanced energetic requirements was suggested by higher levels of phosphorylated AMPKα as well as mRNA copies of PPARα, PGC1α and CPT1 among others. Training also elevated transcription of a set of myokines and other gene products related to the inflammatory process, such as TNFα, NFκB, COX2, IL1RA and TNF decoy receptor. This study provides the first characterization of the underlying molecular acclimation mechanisms in the heart of exercise-trained fish, which resemble those reported for mammalian cardiac growth.

Introduction

Aerobic exercise training imposes an intensity-dependent cardiac load in vertebrates as a result of the increased need for internal oxygen transport. In humans, aerobic training results in physiological cardiac growth which is typically associated with improved physical capacity [1–3]. On the other hand, pathological cardiac growth occurs in response to volume overload in disease settings (e.g. hypertension), to myocardial infarctions and ischemia, within others [4]. Similar to humans, the cardiovascular system in fish is an important target for the training-induced effects. This is a plastic system [5] where training intensity plays an important modulatory role influencing cardiac performance and other physiological systems, such as osmoregulation [6,7]. Cardiovascular variables known to improve with exercise in fish include maximum oxygen consumption [7], maximum cardiac output [8,9], capillarization [10,11], oxygen extraction by tissues [12], haematocrit [7,13,14], tissue oxidative capacity [8] and relative cardiac size [5,15]. Thus, many levels of the oxygen cascade are targeted with exercise training in fish. Cardiac stroke volume is highly dependent on cardiac growth, which has proven to be
highly plastic to environmental and physiological stressors in fish, e.g. sexual maturation, cold temperature acclimation and anemia [5,16–18]. During sexual maturation [19,20] or under anemic conditions [21], cardiac muscle growth involves both cardiomyocyte enlargement (hypertrophy) and proliferation (hyperplasia), while in response to exercise training, the heart of zebrafish (Danio rerio) grows by hyperplasia as evidenced by elevated transcription of proliferating cell nuclear antigen (PCNA) [22]. In mammals, cardiomyocytes practically lose their ability to proliferate after birth, hence cardiac growth thereafter involves mainly cellular hypertrophy [23], a response which is governed by the expression of transcription factors such as myocyte enhancer factor (MEF)2C, GATA4 and the homeobox protein NKX2.5 [24].

In mammals, exercise training improves cardiac muscle contractile capacity by elevating the transcript and protein levels of a suite of proteins involved in the excitation–contraction (EC) coupling process, as well as in the handling of Ca2+ fluxes required for contraction activation [25–27]. Within these, a special role is played by the voltage dependent L-type Ca2+ channel (dihydropyridine receptor; DHPR), the sarcoplasmic reticulum (SR) Ca2+ release channel (ryanodine receptor; RYR) and the SR Ca2+ ATPase (SERCA) which re-sequesters Ca2+ back into the SR allowing muscle relaxation. In fish, a direct relation has been found between DHPR or RYR and skeletal muscle contractile performance [28–30]. However, to our knowledge, no information is published on the exercise training regulation of these molecules in cardiac muscle of fish.

An adequate oxygen supply would support a robust myocardium. Exercise training improves capillarity in fish skeletal muscles increasing the oxygen diffusional surface area and reducing the distance between the capillary and mitochondria [7,10,31,32]. Such an effect is probably driven by vascular endothelial growth factor (VEGF), the most important angiogenic factor in vertebrates [33]. Furthermore, exercise training stimulates erythropoiesis in fish, as evidenced by increased haematocrit levels [14]. The principal regulator of erythropoiesis is the hormone erythropoietin (EPO), which was recently cloned in fish and was found to be mainly produced in the heart and not in the kidney, as for mammals [34].

In high performance fish, as well as in humans, cardiac muscle prefers lipids as a fuel under normal conditions [35,36]. When tunas and salmonids face a higher energetic demand, as during aerobic swimming-induced exercise, the reliance of cardiac muscle on lipids increases while the use of glucose remains limited [37]. AMP activated protein kinase (AMPK) is a master-switch energy sensor, which under activation by upstream kinases, promotes catabolic and inhibits some anabolic processes [38]. An increase in the ratio of AMP/ATP, as during exercise training, is the main signal promoting the activation of AMPK by phosphorylation. Recently, several AMPK effects have been found to be replicated in fish [39,40] suggesting a well conserved role of this protein in vertebrates. Some of the metabolic effects of AMPK, are decreases in glycogen synthesis by inactivation of glycogen synthase, a decrease in fatty acid synthesis by phosphorylation of acetyl CoA carboxylase (ACC), as well as higher glucose intake by increased expression and membrane translocation levels of glucose transporter type 4 (GLUT4) [40,41]. Peroxisome proliferator activated receptor (PPAR)α is a key cardiac transcription factor regulating lipid catabolism pathways by inducing the transcription of genes such as carnitine palmitoyltransferase I (CPT1) and others involved in fatty acid transport, esterification, mitochondrial import as well as mitochondrial and peroxisomal fatty acid oxidation [42]. PPARγ co-activator (PGC)1α is a cardiac-enriched PPAR co-activator that directly activates PPARα, boosting its effects at the same time of co-activating other transcription factors involved in mitochondrial biogenesis.

The contracting skeletal muscle produces and releases a set of cytokines, termed myokines, which are involved in both short and long term beneficial acclimation processes induced by regular exercise training in mammals [43]. Furthermore, a modulation in cardiac myokines production in response to training was recently shown for Atlantic salmon (Salmo salar) [44]. Thus, exercise-induced myokine regulation may be an important molecular acclimation mechanism affecting cardiac performance.

Since sustained exercise training strengthens the cardiovascular capacity of athletic fish such as Atlantic salmon, we hypothesized that the pathways involved are conserved among fishes and terrestrial vertebrates. Further, we hypothesized that exercise-induced activation of underlying gene transcription mechanisms must be dependent on the cardiac workload. To test our hypotheses, we trained Atlantic salmon pre-smolts at three different exercise intensities for 10 weeks and then analyzed key markers of pathways affecting the traits known to be involved in physiological cardiac growth in mammals. Hence, processes such as cardiomyocyte growth and proliferation, contractility, capillarization, oxygen transport, myokine production, energy metabolism and fuel preference were examined.

**Results**

**Cardiac growth and contractile capacity**

After 10 weeks of training, average mass and length of sampled fish were 99.6±5.7 g and 19.9±0.3 cm for the control (C); 91.7±1.7 g and 19.2±0.16 cm for the medium intensity (M), and 92.8±5.2 g and 19.6±0.13 cm for the high intensity (H) groups, respectively (p>0.05). Relative to body mass, cardiac ventricles of trained fish grew larger than control fish
by 11.1% for M and 19.4% for H, with relative ventricular mass (RVM) values of 0.087±0.004%; 0.097±0.007% and 0.104±0.007% for C, M and H, respectively. Hence, there was a clear tendency (p=0.06, Student’s t-test) in cardiac growth response to be intensity-dependent between C and H. To evaluate if cardiac growth was dependent on hyperplasia, cardiomyocyte proliferation was examined by PCNA immunofluorescence which was ~7 fold higher in cardiac ventricle of H relative to C (p=0.05; Figure 2).

Gene expression of growth and contractility markers were assessed to further define the molecular signature behind cardiac growth. Ten genes directly related with the processes of cardiac muscle growth, development, contraction machinery and EC coupling were studied by qPCR (Figure 3). Cardiac growth markers showed a trend towards higher transcription levels in the trained than in the control fish suggesting hypertrophic cardiomyocyte growth (Figure 3A). The H regime showed significant differences when compared to C for the genes encoding MEF2C and actin alpha 1 (ACTA1). Conversely, only M had up-regulated levels of GATA4 in comparison to C. Examination of key marker genes for evaluation of the contractile machinery suggested improved performance of exercised hearts. Significant higher transcription was found between H and C for the genes encoding DHPR, FK-506 binding protein (FKBP1B) and calsequestrin 1 (CALSEQ1) (Figure 3B).

Capillarization and oxygen carrying capacity
An improved oxygen carrying capacity together with a larger bed of coronary vessels would result in better cardiac oxygen and nutrient delivery. Five genes involved in oxygen transport and blood diffusion were studied by qPCR (Figure 4A; inducible nitric oxide synthase –iNOS- was undetectable). Transcription levels of EPO were significantly higher in ventricle of H-trained fish, while the EPO receptor (EPOR) showed no variation. Training induced a significant increase in the transcription levels of VEGF and its cell surface receptor VEGF-R2 in the H group compared to C. The spatial expression of VEGF was localized in the epi- and myocard of both H and C cardiac ventricles, while difference in protein levels was not found by IF (Figure 4B).

Energy metabolism
To address if cardiac ventricle fuel handling was affected in exercise-trained fish, we analyzed the levels of phosphorylated (active) AMPK subunit alpha (pAMPKα) by western blotting due to its fundamental role in energy sensing. Protein levels of pAMPKα were 57% higher in H than in C (p=0.005), although at the mRNA level, the elevated AMPKα in H compared with C did not quite reach statistical significance (p=0.058; Figure 5). A greater oxidative capacity was confirmed by average area of mitochondria being significantly larger (46%) in H compared with C (1.23±0.015 μm²; n=15620 and 0.84±0.09 μm²; n=17780, respectively). The mitochondrial to genomic DNA ratio (mtDNA/gDNA) was unaffected by exercise (Figure 6).

To gain insight into the fuel preference of the cardiac muscle performing aerobic exercise, we examined the expression of 18 genes involved in the metabolism of lipids and glucose by qPCR. Exercise training had a profound effect on the cardiac capacity for lipid metabolism in Atlantic salmon. A set of 7 out of 10 genes involved in the control of lipid metabolism showed consistent up-regulation in H-trained salmon compared with C (Figure 7A). These included genes encoding proteins promoting mitochondrial biogenesis and fatty acid oxidation such as PPARα, PGC1α, CPT1, and malonyl-CoA decarboxylase (MCD). Furthermore, mRNA of proteins participating in lipogenic pathways were also up-regulated by H, including malonyl CoA-acyl carrier protein transacylase (MCAT), ACC and fatty acid synthase (FAS). Despite showing a tendency towards up-regulation in M, no genes were differentially expressed between M and C.

In contrast, two out of eight genes involved in glucose metabolism were significantly induced by training (Figure 7B). Expression of hexokinase HK and pyruvate dehydrogenase kinase (PDK3) was significantly up-regulated in the H regime compared to C.

Cardiomyokine expression
Aerobic exercise increased cardiac concentrations of pro- and anti-inflammatory cytokines in an intensity-dependent manner. Protein and mRNA levels of tumor necrosis factor (TNF)α were significantly up-regulated in H compared with C, as shown by IF and qPCR, respectively (Figures 8A & 8B). Further, IF analysis allocated TNFα production to the cardiomyocytes and not to potential resident leukocytes. Other significantly up-regulated pro-inflammatory-related gene in H was the interleukin (IL)6 receptor subunit alpha (IL6Raα), while M promoted elevated transcription of cyclooxygenase (COX)2 only. Within the genes with anti-inflammatory properties, H significantly induced the transcription of TNF decoy receptor and IL1 receptor antagonist (IL1RA). Further, IL15, which has been linked to muscle growth [45], was up-regulated by H (Figure 8C).

Exercise Intensity
Overall, intensity of the training regime was directly related to the magnitude in the gene expression response (Pearson correlation; r=0.99; p=0.032). Furthermore, significant differences were found between the three groups (p<0.0001), with M and H displaying a 10.9 and 37.8% higher gene
expression than C, respectively. Correlation between exercise intensity and RVM, though high (r = 0.96), was not statistically significant (p=0.18).

Discussion

In this study, we demonstrate how known genes and proteins that are a key components of the molecular signature for the physiological cardiac growth stimulated by exercise training in mammals, also underlie the exercise-induced responses of the Atlantic salmon heart. Aerobic exercise training stimulated cardiac hypertrophy mechanisms, and the larger heart was apparently supported by a more efficient contractile machinery, as well as an improved capillarization and oxygen transport capacity. An increased metabolic capacity was orchestrated by AMPK activation and enhanced lipolysis. A similar molecular signature lies behind the enlarged “athlete’s heart” produced as an acclimation to chronic exercise training in humans [4,42].

Training intensity influenced the cardiac response at several levels, stimulating cardiac growth and gene expression, with lower values in C, a low-to-moderate effect in M and higher magnitude in H. Previous work on this subject has proven that intense training results in more clear cardiac improvements, while programs that are not intense enough or too short may not elicit cardiovascular changes in mammals and fish [7,46,47]. Previous works on salmonids have shown that optimal effects of exercise training, from a sound perspective including improved growth, reduced stress and higher disease resistance, are achieved at swimming speeds between 1 and 1.5 body lengths (BL) s$^{-1}$ [15,44,48]. The present study is in agreement with this, as fish subjected to the H training regime (1.31 BL s$^{-1}$) reflected the most significant effects.

There is growing consensus regarding increased RVM in response to exercise training in fish, although some studies have found opposite results [49]. The RVM increment of 19.4% found for the H group is generally higher than that reported previously for salmonids. Hochachka [13] found a 10% increase in trained rainbow trout (Oncorhynchus mykiss) ventricle, while Gallaugher et al. [7] observed a 12% increase in Chinook salmon (Oncorhynchus tshawytscha), though a gain as high as 46% was reported for rainbow trout trained at high speeds [30]. Overall fish robustness would be enhanced by a relatively larger cardiac mass, with the resulting improved cardiac capacity playing an important role in conferring the individuals with a well-equipped cardiovascular system to satisfy the energetic and nutritional demands for both swimming and growth simultaneously.

In the present work, we found that exercise-induced cardiac enlargement was given by a mixture of cardiomyocyte hyperplasic and hypertrophic mechanisms, as suggested by higher expression levels of PCNA, as well as of the morphogenetic and hypertrophic related genes MEF2C, GATA4 and ACTA1. This indicates a similar cardiac molecular acclimation mechanism in fish in response to a series of factors affecting the oxygen supply demand, such as under induced anemia or during sexual maturation in salmonids [19–21].

We also demonstrated that exercise induced an improved cardiac contractile capacity, as suggested by higher transcription activity of genes involved in calcium handling during EC coupling. Improved contractile capacity is a feature of physiological cardiac growth in mammals, and is reflected by higher expression of Ca$^{2+}$ channels and a better Ca$^{2+}$ homeostasis [4,51]. Conversely, ageing-induced repression of cardiac contractility is related to a disrupted Ca$^{2+}$ homeostasis [52]. In mammals, after sarcolemmal depolarization, the influx of a small extracellular Ca$^{2+}$ amount via the DHPR voltage sensitive channel is necessary to stimulate a larger Ca$^{2+}$ release from the SR. This takes place through RYR channels present in the SR membrane in a calcium-induced calcium release CICR mode [53–55]. In most fish, however, extracellular Ca$^{2+}$ entry via DHPR is sufficient to stimulate cardiomyocyte contraction, suggesting a minimum reliance on intracellular Ca$^{2+}$ stores in the process [56]. The exception to this would be the highly athletic tuna species such as yellowfin (Thunnus albacares) and skipjack (Katsuwonus pelamis), where strong evidence supports the dependence on SR Ca$^{2+}$ release and recycling for contraction [57–59]. In other athletic fish species, such as rainbow trout and the Pacific mackerel (Scomber japonicus), reliance on SR Ca$^{2+}$ has only been found under suboptimal conditions [59–61]. The present work showed induced transcription of FKBP1B and CALSEQ1, which suggests an increased reliance on SR-stored Ca$^{2+}$ to support the higher contractility requirements of salmon swimming at elevated speeds. FKBP1B is a RYR modulator in cardiac muscle, while CALSEQ1 is the most important Ca$^{2+}$ binding protein inside the SR and aggregates in regions where RYR is present, suggesting a modulatory effect [55]. We suggest that, as an acclimation process to the increased contractility requirements imposed by exercise, cardiac muscle of Atlantic salmon stimulates the transcription of relevant EC coupling genes. We further provide molecular evidence for an increased reliance on SR stored Ca$^{2+}$ to support the cardiomyocyte contractile machinery.

Our results suggest increased capillarization in the exercise-trained Atlantic salmon heart, as reflected by up-regulated mRNA levels of both VEGF and its receptor. In salmonids, as well as in other athletic fish species, the ventricle is powered by a compact external myocardium layer, which preferentially receives freshly oxygenated blood from a coronary circulatory system [62]. A proper function of the
compact myocardium under high workloads is highly
dependent on such a coronary circulation, as ligation
of the coronary artery resulted in reduced compact
myocardium size and respiratory capacity in trained
rainbow trout [8]. Furthermore, exercise training is
known to ameliorate the ageing-related decrease in
cardiac capillarization and blood supply in mammals
[63,64]; an effect that has been directly associated
with increased amounts of VEGF and its receptor at
the mRNA and protein levels [65]. Hence, exercise
training would alter cardiac vascular tissue, resulting
in improved capacity to deliver oxygen and nutrients
to contracting cardiomyocytes.

This conclusion is further supported by the
increased transcription levels of EPO in the H-trained
group, suggesting increased production of red blood
cells for oxygen delivery into the heart and swimming
muscles. Even though the mechanisms behind EPO
production have not been completely uncovered in
fish, there is evidence for a conserved biological role
of this within vertebrates [66,67]. Increased EPO
levels in response to training, as seen in this study,
partly explains the molecular mechanism behind the
consensus that aerobic exercise training increases
haematocrit levels, haemoglobin concentration and
the oxygen carrying capacity in teleosts [15].
Intriguingly, EPO is also produced and released by
the human skeletal muscle having potent metabolic
effects, including weight reduction related to
increased lipid and reduced glucose metabolism
[68,69]. Furthermore, EPO over-expression resulted
in muscle hypertrophy and higher capillarization,
confirming previous evidence in support of EPO as
an angiogenic factor with EPO receptors being
expressed in endothelial cells [68,70,71].

An enlarged heart, with better contractile
machinery and a more efficient system for delivering
oxygen to the cardiomyocytes, necessarily requires an
improved metabolic capacity. Here, the respiratory
capacity was increased in response to exercise
training, as suggested by the relatively larger
mitochondria in the H-trained group at the end of the
training experiment. Furthermore, the exercise-
induced increases in energetic demand were intensity
dependent and supported largely by a higher reliance
on lipid oxidation, as indicated by increased
transcription of PPARα and its co-factor PGC1α in
the H group, as well as the exercise-induced
transcription and activation of AMPKα. The first two
are key drivers of mitochondrial biogenesis as well as
the β-oxidation processes [72,73]. AMPK has a
central role in maintaining cellular energetic
homeostasis and activates and induces the
transcription of PGC1α, which has been suggested to
mediate some of the beneficial effects of exercise in
mammals [74]. It should be noted that pathological
cardiac growth is associated with repressed PPARα
and PGC1α transcription [42]. Training up-regulated
the transcription of CPT1, whose transcription is
stimulated by PPARα and is involved in the transport
of long-chain fatty acids into the mitochondria [72].
MCD, which is directly stimulated by AMPK [75],
converts malonyl-CoA into acetyl-CoA, favoring the
presence of acetyl-CoA which can be further oxidized
for energy in the citric acid cycle, at the same time as
reducing the inhibitory effects that malonyl-CoA
imposes on β-oxidation. Intriguingly, expression of a
set of genes involved in lipogenesis was also up-
regulated in response to training, including MCAT,
ACC and FAS. These are involved in the synthesis of
fatty acids from acetyl-CoA and malonyl-CoA and
their up-regulation may be interpreted as a
compensatory mechanism for the increased
accumulation of lipolytic enzymes and further argues
for cardiac muscle of trained fish potentially building-
up lipid stores, suggesting an optimized feeding
efficiency in response to training. In agreement with
our results, molecular signs of increased lipid β-
oxidation in response to exercise have been found in
heart of rainbow trout [8,9]. Similarly, a shift towards
a cardiac lipolytic dominant state also occurs in the
enlarged heart of rainbow trout during sexual
maturation [20] and cold acclimation [76].

Exercise training did not affect the transcription
of enzymes involved in glucose metabolism as heavily
as those for lipid metabolism. Only two genes were
significantly up-regulated by training (HK and PDK)
and these do not necessarily operate in the same
metabolic direction. HK transforms glucose into
glucose 6-phosphate (G6P), making it available to be
further metabolized for energy generation via
glycolysis. Farrell et al. [8] found that training induced
elevated levels of enzymes involved in both lipid and
glucose oxidation in heart of trained rainbow trout,
suggesting an increased dependence on both fuels to
cope with the higher demand. In this study, though,
we found that together with HK, exercise induced
higher levels of PDK. This enzyme inactivates the
pyruvate dehydrogenase complex by phosphorylation,
thereby reducing the transformation of the glycolysis
end product, pyruvate, into acetyl-CoA. This
information, added to the lack of exercise-induced
regulation in other proteins involved in glucose
metabolism, argues for maintenance, but not an
increase, in the myocardium use of glucose as energy
source during exercise training. It must be mentioned
that the fish heart is also proficient in the use of other
substrates, such as proteins and lactate [9,77,78],
though assessing their participation in energy
generation was not in the scope of this study.

The expression of myokines, as well as other
inflammation-related factors, was further modulated
by the intense H training regime, suggesting
participation of these molecules in providing a cardiac
acclimation response to exercise. The modulation in
expression of cardiac myokines in response to
exercise training was previously demonstrated in fish
[44]. In that study, cardiac down-regulation of TNFα,
IL1β and IL6 after six week of de-training was
associated with higher survival of Atlantic salmon in a
In mammals, the contracting skeletal muscle is heavily involved in the production and release of myokines, which seem to further drive the exercise-induced local and systemic acclimation process to exercise. In this scenario, one of the main actors is IL6, which, after being induced by exercise, plays a fundamental role in both metabolic and anti-inflammatory mechanisms [43,79]. IL6 receptor was significantly up-regulated by training in this study. After binding and signaling through its receptor complex gp130Rβ/IL6Rα, IL6 activates AMPK in the myocytes resulting, within others, in an increased lipid oxidation capacity [43,80]. Accordingly, IL15 expression in response to training has been linked to muscle growth as well as reduced adipose tissue mass [43]. While in the mammalian skeletal muscle model IL6 up-regulation is not associated with higher levels of pro-inflammatory mediators, in the present work we did find higher levels of cardiac TNFα and COX2. COX2 has a pivotal role in the production of prostaglandins, inflammatory mediators of lipid origin [81,82]. Possible inducers of inflammatory mediators are reactive oxygen species (ROS), as it is well known that aerobic exercise results in increased leakage of ROS from mitochondria [83]. ROS can induce the activation of NFκB [84], resulting in increased transcription of inflammation-related mediators including TNFα and COX2 [82,84]. Though it was not addressed in this study, regular aerobic exercise stimulates the production of endogenous antioxidant enzymes, which not only counteract the oxidative stress imbalance, but further create a beneficial antioxidant environment [83]. Interestingly, such an exercise-induced acclimation effect has already been seen in exercised Atlantic salmon [44]. Following the same concept, exercise-induced expression of anti-inflammatory genes such as IL1RA and TNF decoy receptor in this study, probably responds to the presence of pro-inflammatory molecules. Both molecules block the inflammatory effects of IL1β and TNFα, respectively. While IL1RA antagonistically binds the IL1 receptor in the cell surface inhibiting its activation by IL1 [85], TNF decoy receptor binds TNFα in solution with a similar effect. Thus, the overall net result of myokine activation in the exercised heart may be induced muscle growth and lipid oxidation.

In light of the present results, as well as others studies showing the production of myokines in the cardiac muscle in response to different stimuli [86–90], we suggest that the term “cardiomyokine” should be used when referring to cytokines/myokines which are directly expressed and produced by the cardiac muscle cells. Further research is needed though, to establish if these molecules are actually being released to the extracellular space.

In conclusion, we document that aerobic exercise training induces a wide range of cardiac molecular responses. These correspond to those underlying physiological cardiac growth in mammals, validating our hypothesis of a conserved exercise-induced cardiac response mechanism within lower and higher vertebrates. Further, this response most probably represents the molecular driving force behind the exercise-induced cardiovascular effects in fish. An increased cardiac mass, as an acclimation response to the higher cardiac output required by the skeletal muscle, was given by a mixture of cardiomyocytes enlargement and proliferation. This was further associated with a more efficient contractile machinery, as well as higher capacity for oxygen carrying and distribution. The exercise-associated increases in energetic requirements were satisfied by a higher reliance on lipid oxidation in enlarged mitochondria, while the use of glucose as energy substrate was not affected. Interestingly, the swimming-induced production of cardiomyokines may suggest an important role of these in the cardiac acclimation process to exercise. The reported molecular cardiac signature strongly suggests an exercise-induced strengthening of the heart, with improved capacity for supplying the necessary oxygen and nutrients to the swimming muscle. Furthermore, and in line with our second hypothesis, most of the exercise-induced responses were manifested in an intensity-dependent fashion.

Materials and methods

Experimental fish
Juvenile Atlantic salmon belonging to the Salmobreed strain were produced and reared at Nofima AS, Sunndalsøra, Norway. Freshwater stage experimental procedures took place on the same research station, which is an approved facility under the Norwegian Animal Research Authority (NARA). Stunning and sampling of fish was done in agreement with the Norwegian regulations. As fish were exposed to different sustainable water velocities that did not induce an un-physiological state, no specific NARA approval was required according to Dr. G. Baeverfjord, member of the national NARA board and local NARA officer at Nofima AS.

Exercise training regimes
Before the start of the trial, all fish were tagged (Passive Integrated Transponder (PIT), Glass tag Unique 2.12 x 12 mm, Jojo Automasjon AS, Sola, Norway). After individual measurements of body mass (40.7±0.2 g) and length (15.0±0.3 cm), 77-86 fish were set in each of 9 cylindro-conical tanks (500 l; 82 cm in diameter) and allowed to acclimate for one week under minimum disturbance prior beginning of the experimental trial. The center of each experimental tank was fitted with a plastic pipe (31.5 cm diameter), which reduced the area in the tank with lowest water speed. A frequency-controlled pump (Hanning Elektro Werke, PS 18-300; Oerlinghausen, Germany) directed the water current and a wire mesh
Relative ventricular mass (RVM)

At the end of the exercise-training period, a total of 30 fish per training regime were killed by a blow to the head; body mass and length were measured, and hearts were dissected out. Ventricles were weighed on an analytical scale after removal of the atrium, the bulbus arteriosus and any excess blood. RVM was calculated as ventricle weight (g) \cdot \frac{[\text{body mass (g)}]}{\text{cm}^3} \cdot \text{BL s}^3. Using average fish length during the trial, relative water speeds averaged 0.32 (C), 0.65 (M) and 1.31 (H) BL s\(^{-1}\) (Figure 1). To stimulate smoltification, fish were exposed to a short daylight regime (12-12 Light-Dark) the first 6 weeks, followed by continuous light (24 L) the remaining 4 weeks. Tanks were supplied with fresh water and temperature was measured daily (10.5±0.8 °C). Oxygen saturation was measured weekly and was maintained over 85% with oxygen supplementation. Measurement of ATPase in gills (n=10) sampled from each group was conducted in a commercial laboratory, Haukbruksinstituttet AS, Bergen, Norway, and confirmed that all fish were sampled within the smolt-window (data not shown).

Gene expression

A total of 45 genes were analyzed for expression levels by quantitative real-time RT-PCR (qPCR). For this, ventricle samples (n=12/regime) were sampled at this, ventricle samples (n=12/regime) were sampled at the end of the training experiment as previously described, and immediately frozen on liquid nitrogen. Each regime was run in triplicate tanks with constant water speeds of either 5.7 (C), 11.5 (M) and 23 (H) cm s\(^{-1}\). At start of the experiment, these speeds were equivalent to 0.38, 0.77 and 1.53 BL s\(^{-1}\) for C, M and H, respectively. Due to increases in fish length during the trial, relative water speeds were slightly reduced towards the end of training. Because fish had a similar length across the three groups, this reduction in relative water speed was similar among groups (75% of initial speed in BL s\(^{-1}\)). Using average fish length during the trial, relative water speeds averaged 0.32 (C), 0.65 (M) and 1.31 (H) BL s\(^{-1}\) (Figure 1). To stimulate smoltification, fish were exposed to a short daylight regime (12-12 Light-Dark) the first 6 weeks, followed by continuous light (24 L) the remaining 4 weeks. Tanks were supplied with fresh water and temperature was measured daily (10.5±0.8 °C). Oxygen saturation was measured weekly and was maintained over 85% with oxygen supplementation. Measurement of ATPase in gills (n=10) sampled from each group was conducted in a commercial laboratory, Haukbruksinstituttet AS, Bergen, Norway, and confirmed that all fish were sampled within the smolt-window (data not shown).

Mitochondrial quantification

The ratio of mitochondrial DNA to genomic DNA (mtDNA/gDNA) was calculated for the C and H regimes by qPCR, as an estimate of the number of mitochondria per cardiac cell. The same individual fish used for the gene expression study were analyzed (n=12/regime). Total DNA was isolated using Dneasy kit (Qiagen) according to manufacturer instructions and diluted 200-fold before use. Concentration was measured on NanoDrop 1000 spectrophotometer (Thermo Fischer Scientific) and quality was checked on a 1% agarose gel. The relative difference in the abundance of mitochondria per cell was calculated after qPCR amplification of specific mitochondrial (D-loop) and nuclear (myoD) DNA sequences. For the latter, primers spanned an intron-exon region (Table S1). Comparisons among groups, as well as the amplification reactions and primer efficiency calculations were performed similarly to the gene expression analyses described above, but using myoD as reference gene against which the levels of D-loop were normalized and quantified.

Immunofluorescence and mitochondrial staining

Hearts sampled for immunofluorescence (IF) were embedded in paraffin and 7 µm sections were prepared. After paraffin removal and dehydration, microwave facilitated antigen retrieval was carried out for 20 min in 10 mM Tris-HCl pH 10. After rinsing in PBST (phosphate buffered saline with 0.02% Tween 20), the tissue sections were permeabilized for 20 min in 1 x PBST with 1% Triton X100. Blocking was carried out for 2 h in 1x PBST with 5% skimmed dry milk. Polyclonal TNFα (100-fold dilution; obtained according to the method described in Bethke et al. [93]
and provided by L. Mercado at Pontificia Universidad Católica de Valparaíso, Chile) PCNA (70-fold dilution; PMID 17349083; Zymed Laboratories Inc., CA, USA) and VEGF (50-fold dilution; PMID 15177948; Santa Cruz Biotechnology, CA, USA) primary antibodies were diluted in 1 x PBST with 2% dry milk and 0.01% Triton X 100 and applied to the sections over night at 4°C. After extensive washing in 1 x PBST, the sections were incubated for 2 h with Alexa conjugated secondary antibodies diluted 200-fold (Invitrogen, Carlsbad, CA, USA). Finally, the sections were mounted after several washes in 1 x PBST and nuclear staining with DAPI. All images were captured using a Zeiss Axioplan Z1 microscope and post processed using the Zeiss Axiovision software. Identical exposure and image manipulation settings were applied to the images to enable comparison between swimming regimes. For visualization of TNFα, image stacks were deconvoluted using an iterative algorithm in the Axiovision software.

Staining of cardiolipin in the mitochondrial membranes was carried out using 10-nonyl acridine orange (NAO; PMID 16172211). Briefly, paraffin was removed from 3 µm sections before rehydration and permeabilization with 1% Triton X100 in 1 x PBST. Quenching of auto fluorescence was achieved by incubating the sections for 2 min in 0.1% Sudan black dissolved in 70% ethanol. After washing in 1 x PBST, the sections were incubated for 10 min in 10 µM NAO diluted in 1 x PBST, before washing and mounting. A total of 17700 and 15600 mitochondria from the control (n=5) and exercised fish (n=6), respectively, were analyzed for fluorescence intensity and size using a semiautomatic script which isolated the mitochondria from the background using fluorescence intensity and size segmentation.

SDS-PAGE and western blot
Following ventricle dissection and storage, approx. 50 mg of tissue spanning the whole vertical axis of the ventricle including both compact and spongy myocardium layers, (n=10/regime; only C and H) was homogenized in 500 µl of a 50 mM Tris-HCl, 10 mM EDTA buffer at pH 8.3, containing 5 µl of a protease inhibitor cocktail (Halt™, Thermo Scientific, Rockford, IL, USA). Protein concentration was assessed by the method developed by Bradford [94] using Bradford reagent (Sigma-Aldrich, St.Louis, MO, USA). Samples were denatured for 7 min at 75°C and 25 µg of protein per sample were used for SDS-PAGE [95] on 8% polyacrylamide gels (Novex® Tris-Glycine Gels, Invitrogen). Electrophoresis was performed for 80 min at 80 V. Precision Plus Protein™ Standards (Bio-Rad, Hercules, CA, USA) was used as molecular marker on each gel, and a random sample was loaded on each gel for normalization, allowing between gel comparisons. Dry-blotting of the separated proteins in the gel into a nitrocellulose membrane was done with the iBlot® system (Invitrogen). Membranes were blocked for 1 h in 5% skimmed milk powder in TBS and immediately set for overnight incubation at 4°C in a shaker with primary antibody (rabbit monoclonal anti-phospho-AMPKα, Cell Signaling Technology, Danvers, MA, USA) 1000 x in TBST (TBS plus 0.1% Tween 20) with 5% BSA. On the next morning, membranes were washed with TBST and incubated at room temperature for 2 h with an AP-conjugated secondary antibody (polyclonal goat anti-rabbit IgG, Cell Signaling Technology). Detection was done using a buffer containing BCIP/NBT (Bio-Rad) substrate for 30 min in the dark, and band intensities were measured with the ImageLab™ software (Bio-Rad).

Statistics
Statistical analyses among the different groups were assessed by analysis of variance (ANOVA) and comparison between groups with Tukey’s HSD post-hoc test. Student’s t-test was performed between C and H groups for those analyses on which only these two groups were of interest (a priori comparisons for mtDNA/gDNA ratio, mitochondrial area, immunofluorescence, western blot and RVM). We have further used Student’s t-test between C and H groups for those highly interesting genes p-value after ANOVA was close to significance (0.05<p<0.08). Correlation between training intensity, RVM and gene expression was assessed by Pearsons ‘r’ (SAS 9.1; SAS Institute Inc., NC, USA). Differences and correlations were considered significant at p<0.05.

References


Figure 1. Exercise training experimental design. Atlantic salmon pre-smolts were exercise trained at different relative water speeds (body lengths per second (BL s$^{-1}$)) for ten weeks (w). Throughout the trial, swimming speeds experienced a constant decrease due to fish length increase. Speeds shown in the middle of the figure (bold) are average, while start and end speeds are shown in the left and right, respectively (brackets). The first six weeks took place under a short day-light photoregime (12L–12D), while the last 4 weeks were on a continuous light photoregime (24L) to induce the smoltification process.
Figure 2. Exercise training induces cardiomyocyte proliferation. Immunofluorescence detection of PCNA (green) in spongy myocardium. Control fish showed modest cell proliferation (*) with an average of three positive cells per frame (left image). PCNA staining of exercised fish from the high intensity-training regime (right image) shows a considerable increase (20 positive cells per frame) in cell proliferation over specimen from the Control group. Nuclei are stained with DAPI (blue). n=12/group.
Figure 3. Cardiomyocyte growth and contractile capacity are affected by exercise. Gene expression related to the growth and contractile capacity of Atlantic salmon cardiomyocytes was analyzed by qPCR. A: Genes related to cardiomyocyte growth. B: Genes involved in the EC-coupling process as well as in Ca\(^{2+}\) handling. * denotes statistical difference (p<0.05; one-way ANOVA performed on log2 transformed expression ratio values followed by Tukey’s HSD; n=9-12/group) between either of the training regimes and the Control. # denotes significant difference between C and H (Students t-test; p<0.05). Bars represent SEM. MEF2C: Myocyte-specific enhancer factor 2C; GATA4: GATA binding protein 4; NKX2: NK2 homeobox 5; ACTA1: Actin; TNNT2: Troponin; DHPRa1D: Voltage dependent L-type Ca\(^{2+}\) channel alpha1D subunit (dihydropyridine receptor); RYR1: Sarcoplasmic reticulum Ca\(^{2+}\) release channel (ryanodine receptor) isoform1; FKBP1B: FK506 binding protein B; CALSEQ1: Calsequestrin 1; SERCA2: Sarcoplasmic reticulum Ca\(^{2+}\) ATPase 2.
**Figure 4. Oxygen carrying and distribution capacity is enhanced by exercise training.** A: High intensity exercise training stimulated up-regulation of genes coding for EPO, involved in erythropoiesis, and for VEGF and VEGF receptor (VEGF-R2), which mediates angiogenesis. B: Immunofluorescence detection image of VEGF (arrows, red staining) showing its production in both the epicardium (right image) and compact myocardium (left image) suggesting the formation of new blood vessels. No apparent differences were found between high intensity trained and control fish (nuclei: blue (DAPI)). * denotes statistical difference (p<0.05; one-way ANOVA performed on log2 transformed expression ratio values followed by Tukey’s HSD; n=9-12/group) between either of the training regimes and the Control. Bars represent SEM. ER: Expression ratio. EPO: erythropoietin; EPOR: EPO receptor; VEGF: Vascular endothelial growth factor; VEGF-R2: VEGF receptor 2.
Figure 5. Exercise promotes activation of cardiac catabolic pathways via AMPK. Protein levels of the phosphorylated form (pAMPKα), as well as the transcription levels of the catalytic sub-unit AMPKα were measured by western blotting and qPCR, respectively. Phosphorylation of AMPKα was significantly induced by the high-intensity regime compared with control, while stimulation of transcription of AMPKα did not quite reach statistical significance (p=0.058). * denotes statistical difference (Student’s t-test; p<0.05; n=10-12/regime). Only relative values are shown to allow visual comparisons between both techniques. A representative picture of the western blot gel is shown where C: Control and H: High-intensity regime.
Figure 6. Exercise effects on mitochondrial size and density. A: Fluorescence microscopy of Acridine orange 10-nonyl bromide (NAO) stained mitochondria (pseudo coloured red) in spongy myocardium. The segmentation masks are shown as green lines around the mitochondria. B: Quantification of fluorescence intensity inside the segmentation masks showed that mitochondrial area within the cardiomyocytes of Atlantic salmon was significantly increased by the high-intensity training regime (approx. 16000 mitochondrial from sections of 5 and 6 hearts from control and high-intensity regimes, respectively). The number of mitochondria per cell though, was not significantly different as shown by that the ratio between mitochondrial DNA and genomic DNA (mtDNA/gDNA). *: Student’s t-test p<0.05, performed on metric and log2ER values for area and mtDNA/gDNA respectively. n= 12/group.
Figure 7. Cardiac muscle relies on increased lipid metabolism as fuel during prolonged-intense exercise training. A: A strong trend towards up-regulation of genes participating in the metabolism of lipids was seen only for the high-intensity regime compared with control, while the metabolism of carbohydrates (B) was not necessarily affected. * denotes statistical difference (p<0.05; one-way ANOVA performed on log2 transformed expression ratio values followed by Tukey’s HSD; n=9-12/group) between either of the training regimes and the Control. # denotes significant difference between C and H (Students t-test; p<0.05). Bars represent SEM. ER: Expression ratio. PPARa: Peroxisome proliferator activated receptor (PPAR)α; PPARb: PPARβ; PGC1a: PPARγ coactivator 1α; ACO: Acyl-CoA oxidase; CPT1: Carnitine palmitoyltransferase 1; LPL: Lipoprotein lipase; MCD: Malonyl-CoA carboxylase; MCAT: Malonyl-CoA acyl carrier protein transacylase; ACC: Acetyl-CoA carboxylase; FAS: Fatty acid synthase; GLUT4: Glucose transporter type 4; HK: Hexokinase; PFK: Phosphofructokinase; PKM2: Pyruvate kinase isoform 2; PDHB: Pyruvate dehydrogenase E1 component subunit β; PDK3: Pyruvate dehydrogenase kinase 3; PEPCK: phosphoenolpyruvatecarboxykinase; PYGM: Glycogen phosphorylase.
Figure 8. Cardiac inflammatory response to exercise training. Both protein (A) and transcript (B) levels of TNFα were significantly upregulated by the high-intensity training regime. A: Immunofluorescence detection of TNFα (red) in cardiomyocytes of Control (left picture) and High intensity (right) shows how TNFα is strongly induced by exercise training in the compact myocardium of Atlantic salmon (n=12/regime). Nuclei were labelled with DAPI (blue). B: qPCR revealed mRNA levels of TNFα being up-regulated by the high-intensity regime compared with control. C: Expression of a set of genes associated with both pro- and anti-inflammatory mechanisms was consistently induced by the High intensity regime, while expression of only two genes was significantly stimulated by Medium intensity training in relation to Control. * denotes statistical difference (p<0.05; one-way ANOVA performed on log2 transformed expression ratio values followed by Tukey’s HSD; n=9-12/group). Bars represent SEM. ER: Expression ratio. IL1b: Interleukin 1β; IL6: Interleukin 6; COX2: Cyclooxygenase 2; FLAP: 5-lipoxygenase-activating protein; NFKBp105: Nuclear factor κBp105; NFκB inhibitor: Nuclear factor κB inhibitor α1; IL6Rα: IL6 receptor subunit α; IL1RA: IL1 receptor antagonist; TNF decoy-R (receptor); IL15: Interleukin 15.
### Supplementary file

**Table 1.** Genes and primer sequences used for the qPCR analyzes.

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