UNIVERSITAT DE BARCELONA

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Memòria presentada per
Mireia Rovira i Berger
per optar al grau de Doctora
Tesi realitzada sota la direcció de Dr. Josep Planas Vilarnau

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FACULTAT DE BIOLOGIA
DEPARTAMENT DE BIOLOGIA CEL·LULAR, FISIOLOGIA I IMMUNOLOGIA

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Programa de doctorat d'Aqüicultura

Dr Josep Planas Vilarnau Mireia Rovira i Berger

Barcelona, 21 de setembre de 2016

Photography by Pablo Bou

Pulvus eris et in pulverem reverteris

"Stardust you were and in stardust you will return"

A en Joan, en Jaume
i en Miquel

AKNOWLEDGEMENTS

En primer lloc haig de donar les gràcies al Dr. Josep Planas Vilarnau, per haver-me donat aquesta oportunitat. Gràcies per tenir la porta sempre oberta i sobretot per entendre la investigació com a un enriquiment que va més enllà del nostre laboratori, sempre disposat a impulsar-nos més amunt. Acabo la tesi però sé que me'n vaig encara amb moltes coses per aprendre. Gràcies i a reveure!

Als meus pares, als que estimo moltíssim tot i que de vegades no ho digui. Ho heu donat sempre tot per nosaltres i aquest treball, per tant, també és vostre. I a la meva Txarmander, la més forta i més dolça del món.

Al Dani, per ser el meu contrapunt i seguir aquí al peu del canó.

A mis compañeros del "lab". A Rubén, por no perder la sonrisa y el buen humor y enseñarme a parar y a reflexionar. Gracias por apoyarme en estos últimos años. A Diego, por contarme historietas y tener siempre una rato para dudas y estar dispuesto a salir improvisadamente. Y no me olvido de Xico, "maco!", por ser alegre y estar siempre dispuesto a charlar un rato de las penas y las glorias de la vida. Al Gerard, que ara sóc jo que hauré d'aprendre de tu! i a la Carol, futura empresària, que em vau ajudar en un any de molta feina i no vau defallir!

Als companys del Departament: Chilla, Cristina, Emilio, Esmail, Jonathan, Marta, Mónica, Sara, There, Pablo, Pepe, Sara i Vanesa, que tot i que ja en quedem ben pocs, hem fet una bona colla i hem compartit moments de festa, amics invisibles, videos de tesi, aniversaris, caps de setmana al laboratori, pràctiques al Parc, filipinos, sèries, trompetes, *room scapes*, tiramissú, sessions fotogràfiques, perruques, pràctiques de fisiologia, plaques d'LB, etc.

A la Paula, per ser companya de viatge, llenguados, *in situs*, bons vins i ostres. Cada cop que trenco un ou jo també penso en tu.

A la colla de la UAB, per no deixar de seguir fent plans junts, encara que amb alguns no coincidim tant. Ana, Aurora, Marina, Montse i Nere, gràcies pels sopars "terapèutics" i les bones estones, consells i suport. També al Joan, per tenir sempre un bon dia, una broma sota la màniga i estar disposat a ajudar (photoshop!), i al Roca, per enviar-me beques dels laboratoris de L'Oréal.

A les amigues de tota la vida que tot i el temps encara esteu aquí. A la Berta, per posar-ho tot tan fàcil i per ensenyar-me Barcelona de nit i de dia, a la Maria, per tenir aquest riure i animar-me, a la Mariona, que tot i estar lluny sempre torna per escoltar, i a la Clara, per compartir llargues tardes de treballs i les preocupacions d'un futur incert.

Finalment, a la gent que, sense saber-ho també m'ha ajudat en algun moment d'aquests anys, a la Marga per tenir sempre un favor a mà, als Guassos, secretàries del Departament, personal de l'estabulari, i a la resta de gent que he conegut durant aquests cinc anys que, poc o molt han influit en la meva feina.

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INTRODUCTION

1.-Exercise and health benefits

Exercise, defined as bouts of physical activity -muscular activity that induce a significant increase in the oxygen consumption of the skeletal muscles- adressed to improve or maintain personal health (Mooren, 2012), has a great impact on human health. There is evidence that regular activity is associated with a reduced risk of mortality and prevalence of chronic diseases such as diabetes, cancer, obesity and metabolic syndrome, diseases that, in turn, usually compromise the cardiovascular system (Booth *et al*, 2012; Carroll & Dudfield, 2004; Laaksonen *et al*, 2005; Lee *et al*, 2012a; Pate *et al*, 1995; Renehan & Howell, 2005; Strasser, 2013).

Therefore, chronic diseases are a concern for the public health and there is an urgent need for their prevention and treatment. In this regard, several risk factors are known to improve with the performance of regular exercise such as reducing body weight and fat deposition, improving HDL/LDL cholesterol balance, reducing blood pressure or hypertension, reducing high blood glucose by increasing insulin sensitivity, reducing systemic inflammation, enhancing endothelial function andimproving coronary blood flow and cardiac function (Braith & Stewart, 2006; Kokkinos & Myers, 2010; Davidson *et al*, 2009; Ross *et al*, 2004). Although to evaluate the beneficial effects of exercise in health and disease it is important to consider the type, duration and intensity of exercise, it is clear however that physical inactivity is an important factor in the increase in the incidence of chronic diseases (Booth *et al*, 2012). Indeed, physical inactivity is the fourth leading global mortality risk factor worldwide, after high blood pressure, tobacco and high blood glucose (Worlh Health Organization, 2009). Some of the relevant physiological changes of exercise performance occur in the skeletal muscle as the primary organ involved in locomotion. For instance, the increased use of carbohydrates as energetic fuel (caused by the increase in glycogenolysis or gluconeogenesis from the liver) as well as increased glucose uptake and glycogenolysis from the working skeletal muscle are changes of particular benefit in patients with metabolic diseases such as diabetes. In addition, as exercise continues and glycogen stores become depleted, lipid metabolism is increased resulting from fatty acid uptake in the muscle cells(Hawley *et al*, 2014). Some of the physiological effects during exercise performance are represented in **Figure I.1**.

In 2004, the World Health Organization rated cardiovascular diseases (CVD) as the first cause of death worldwide, accounting approximately for 29% of global deaths, and are projected to be the major cause of death in 2030 (World Health Organization, 2008). CVD generally refer to hypertension, myocardial infarction (ischemic or coronary heart disease), stroke (cerebrovascular disease), heart failure, peripheral vascular disease, congenital heart disease or cardiomyopathies. Myocardial infarction and stroke are the two top leading causes of death and are estimated to be so in the future (Mathers & Loncar, 2006). Together with other recommendations, exercise is a key element in supervised cardiac rehabilitation programs for patients with cardiovascular diseases(Smith *et al*, 2011), with evidence from meta-analyses studies that performance of exercise results in increased quality of life, reduced mortality and re-infarction in patients with myocardial infarct or heart failure under clinical guidelines and recommendations established in the past few years (van Tol *et al*, 2006; Crimi *et al*, 2009;

Lawler *et al*, 2011; Garza *et al*, 2015). Cardiac growth, myocardial contractility, increased coronary blood flow, reduced myocardial infarction in diseased hearts and possibly cardiomyocyte renewal, are some of the reported effects of exercise in the heart (Wisløff *et al*, 2001; Duncker & Bache, 2008; Boström *et al*, 2010; Platt *et al*, 2015).

Taken together, research has established a role for exercise in physical wellness either as a primary or secondary prevention. However, the mechanisms by which exercise promotes health benefits are still incompletely understood. Moreover, research on exercise physiology is also determinantin order to elucidate the molecular signature for disease prevention.

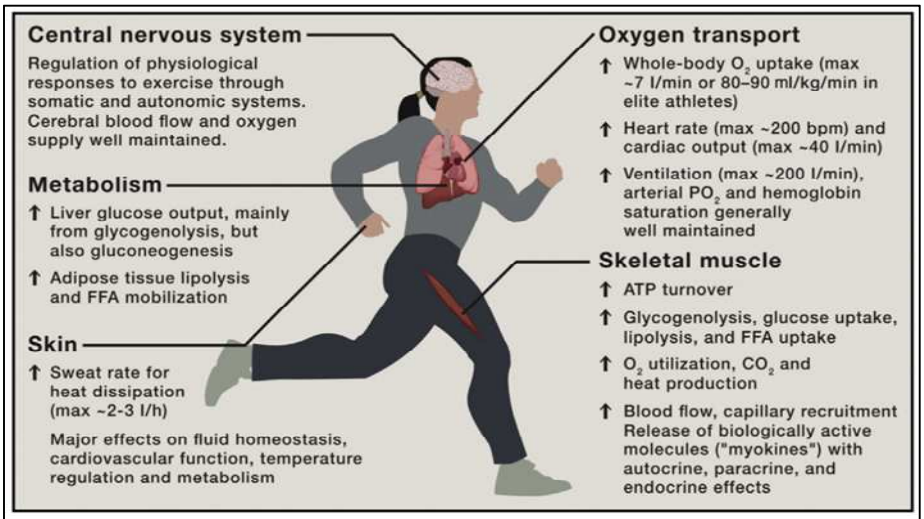

Figure I.1. Physiological effects of exercise in humans. Hawley *et al*. (2014)

2.- Skeletal muscle and exercise in mammals

2.1.-Skeletal muscle adaptation to exercise

Due to the known beneficial effects of exercise-induced skeletal muscle activity for preventing cardiovascular (e.g. coronary heart disease, hypertension), metabolic (e.g. type 2 diabetes mellitus, obesity) and age-related (e.g. sarcopenia) conditions (Booth *et al*, 2012; Fiuza-Luces *et al*, 2013) in humans, knowledge on the pathways that participate in the adaptation of skeletal muscle to exercise-induced activity is of crucial importance for understanding the basic mechanisms involved in this process.

Muscle contractions are initiated by nervous stimuli from the motor neurons that innervate the muscle cells through the neuromuscular junctions. Action potential generation by neural activation results in the depolarization of the muscle cell membrane (or sarcolemma) and the generation of mechanical tension, that triggers intracellular signaling pathways directed to regulate muscle contraction and transcriptional programs relevant for the physiological adaptation of skeletal muscle to exercise. The particular duration and intensity of exercise-induced contractile activity result in a determinate set of physiological adaptations to adjust the phenotype of the muscle cell to exercise training. Exercise is, in general lines, classified in two types: *endurance* (or aerobic) exercise and *resistance* (or strength) exercise, that result in different muscle phenotypes. Endurance exercise involves high frequency and low power output muscle contractions and is characterized by long-term adaptations that include

increases in mitochondrial content and vascularization as well as fiber type transformation. In contrast, resistance exercise involves low frequency and high muscle power output muscle contractions and is characterized by stimulated fiber hypertrophy to increase muscle strength. These two extreme modes of exercise have been thought to be regulated by different molecular mechanisms (**Figure I.2)**.

Besides generating muscle contraction, transient intracellular calcium concentrations within the muscle cell as a result of action potential also play a role in skeletal muscle plasticity. Indeed, increases in intracellular calcium activate downstream pathways including calcium/calmodulin-dependent proteins (CAMKs) and calcium/calmodulin-dependent phosphatase (calcineurin), responsible for a transcriptional response in skeletal muscle cells. Calcium-dependent transcriptional signaling pathways have been shown to modulate fiber type transformation and oxidative capacity in muscle cells. For instance,MEF2 activity (in lacZ reporter transgenic mice) was increased after exercise-induced activity in skeletal muscles (mainly slow type), through a calcineurin-dependent manner (Wu *et al*, 2001) and CAMKIV has been shown to induce mitochondrial biogenesis through activation of PGC1α (Wu *et al*, 2002).

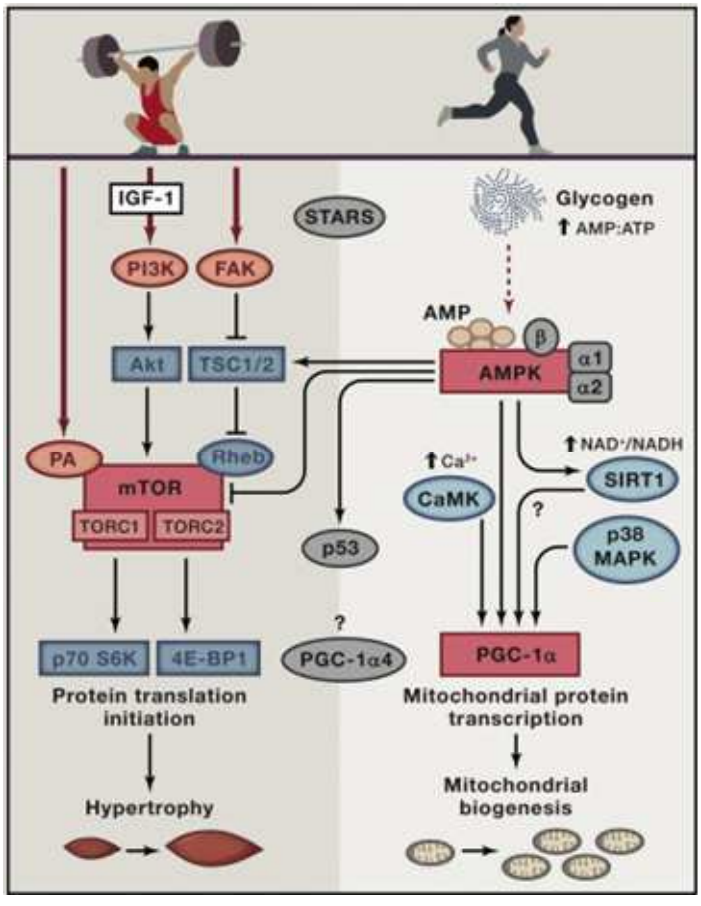

Figure I.2. Signalling pathways relevant for exercise-induced skeletal muscle adaptation. Hawley *et al* (2014)

Apart from calcium-dependent pathways, changes on the cellular energy status of the contracting muscle cells also promotes adaptative responses through AMP-activated protein kinase (AMPK), also named the "energy sensing enzyme" (**Figure I.2)**. AMPK is activated by

increases in the AMP/ATP and creatine/phosphocreatine ratios that reflect an energy depletion status in the cell and, in turn, stimulates metabolic adaptations that increase ATP generation processes such as glucose uptake or lipid oxidation (Kahn *et al*, 2005). As a kinase, AMPK modulates the activity of several factors such as PGC1α through direct phosphorylation, therefore controlling the transcription of oxidative genes (Jäger *et al*, 2007), but also AMPK indirectly activates PGC1α through deacetylation via sirtuin 1 (SIRT1) by increasing intracelular NAD^+ levels (Cantó *et al*, 2009). In addition, AMPK regulates the expression of genes mainly by modulating the activity of transcription factors such as PPARs, NRF1, MEF2 and HDACs. AMPK is activated in response to muscle contraction or endurance exercise (Winder & Hardie, 1996; Nedachi *et al*, 2008) and, although its role in resistance exercise has not been widely explored, AMPK has shown to be activated in an intensity and duration dependent manner (Bergeron *et al*, 2001; Egan *et al*, 2010). Moreover, exercise activates the p38 MAPK pathway which stimulates PGC1α expression through ATF2 and MEF2, two transcription factors that bind to the PGC1α promoter (Akimoto, 2005). Additionally, as the tumor suppressor protein p53 has also been proposed to modulate cellular oxidative capacity during exercise (Bartlett *et al*, 2014). Loss of p53 affects mitochondrial respiration in skeletal muscle, impairs exercise performance and it has been suggested to be activated by AMPK and/or p38 MAPK (Saleem *et al*, 2009; Bartlett *et al*, 2014).

A frequent adaptation following resistance exercise is increased fiber cross-sectional area (or muscle cell hypertrophy) in order to provide strength or force. The increase in cell size results from an increase in protein synthesis over a reduction in protein degradation in the muscle cell. Activation of the mammalian target of rapamycin complex (mTORC), which exists as two different complexes named mTORC1 and mTORC2, is known to be the main signaling enhancing protein synthesis pathway (Drummond *et al*, 2009; Laplante & Sabatini, 2012). Importantly, the mTOR pathway integrates many intracellular signals originating from growth factor action, stress, energy status and amino acids in order to control anabolic processes (protein and lipid synthesis), cell cycle regulation and degradation (autophagy) (Laplante & Sabatini, 2012). Particularly, contraction-induced activation of mTORC1 (composed of mTOR, Raptor, GβL and DEPTOR), promotes protein synthesis by targeting two relevant proteins involved in translational control: eukaryotic translation initiation factor 4E binding protein (4E-BP1) and ribosomal protein S6 kinase (p70S6K) (Drummond *et al*, 2009) (**Figure I.2**). Phosphorylation of 4E-BP1 de-represses eIF4E from 4E-BP1, thus allowing the formation of eIF4E complex to initiate cap-dependent translation, a rate-limiting step in translation initiation. In turn, p70S6K phosphorylates the 40S ribosomal protein S6 and eIF4B, increasing mRNA translation (Baar & Esser, 1999; Kubica *et al*, 2005). However, mTORC1 can also stimulate protein synthesis by promoting interaction of the regulatory element tripartite motif-containing protein-24 (TIF1A) with RNA ploymerase I and upregulating rRNA (Mayer *et al*, 2004) or inhibiting a polymerase III repressor, Maf1 (Kantidakis *et al*, 2010). However, the implication of these factors in exercise-induced hypertrophy have been less investigated (Figueiredo *et al*, 2015).

With regard to growth factors, insulin growth factor 1 (IGF1), an important anabolic factor, has been shown to induce muscle hypertrophy and to signal through mTOR by PI3K and protein kinase B (Akt) (Rommel *et al*, 2001; Bodine *et al*, 2001) (**Figure I.2)**. However, there known

growth factor independent pathways that lead to protein synthesis activation. Mechanosensing by focal adhesion kinase (FAK), a kinase involved in the transmission of contractile force between the cytoskeleton and the extracellular matrix, or by the membrane phospholipid phosphatidic acid (PA) upon disruption of the sarcolemma, are alternative signals reported to be induced during contraction or mechanical loading and to activate mTOR signaling (Fang *et al*, 2001; O'Neil *et al*, 2009; Philp *et al*, 2011) (**Figure I.2)**.

Regulation of mTORC1 is essential under energetic stress conditions. A negative regulation is mediated by the upstream heterodimer tuberous sclerosis complex (TSC1 and TSC2), which inactivates the small GTPase Rheb, an activator of mTORC1 (Laplante & Sabatini, 2012) (**Figure I.2**). Therefore, upstream stimuli that activate mTOR pathway such as growth factors, phosphorylate and inactivate TSC complex through Akt, an mTORC1 upstream kinase. Akt also signals directly to activate mTORC1 by inhibiting the activity of PRAS40 (Sancak *et al*, 2007). Another mechanism to regulate mTOR activity is through AMPK. Indeed, AMPK can regulate mTORC1 activity either by phosphorylating TSC2 an therefore inactivate Rheb or by directly phosphorylating the Raptor subunit of the mTORC1 complex to negatively regulate mTORC1 activity (Inoki *et al*, 2003; Gwinn *et al*, 2008) (**Figure I.2**). Conversely, under high nutritional status such as increased levels of aminoacids, AMPK activity is suppressed whereas mTOR activity is enhanced in muscle cells (Du *et al*, 2007), therefore suggesting a cross-regulation between these two pathways that is still incompletely understood.

Interestingly, Spiegelman and collegues identified an alternative promoter of PGC1α and found a PGC1α isoform (PGC1α4) that, rather than promoting the expression of oxidative genes, it promoted hypertrophy of cultured myotubes by inducing the IGF1 pathway while suppressing the myostatin pathway (Ruas *et al*, 2012). Moreover, this hypertrophic isoform was expressed during resistance exercise or combination of resistance and endurance exercise (Ruas *et al*, 2012) (**Figure I.2)**. Therefore, besides the well-known mTOR pathway in regulating muscle hypertrophy through protein synthesis, other mechanisms may exist to promote muscle hypertrophy.

Contribution of muscle stem cells (satellite cells) has also been reported in (adult) muscle hypertrophy, although their role has been disputed (Wagers & Conboy, 2005; Serrano *et al*, 2008; Blaauw *et al*, 2009; McCarthy *et al*, 2011). Satellite cells (SC) are resident adult skeletal muscle stem cells, located between the plasma membrane and the basal lamina that surrounds each muscle fiber (Mauro, 1961). All SC express the paired box transcription factor Pax7, which is used as a marker for SC identification, and remain quiescent in adult skeletal muscle unless when in response to injury or a growth stimulus they become activated (McCroskery *et al*, 2003; Lee *et al*, 2012b; Relaix & Zammit, 2012). Once activated, SC express myogenic regulatory factors (MRFs), specially MyoD, migrate to the muscle fiber surface, proliferate and differentiate (or self-renew) (Otto *et al*, 2011; Bentzinger *et al*, 2012). SC function during muscle hypertrophy is still unclear. For instance, after knockout of more than the 90% of the satellite cells in the adult skeletal muscle mouse, muscle hypertrophy was not impaired after synergist ablation (i.e. removal of a synergist muscle) but it was suggested that SC may be required for long term hypertrophy maintenance (McCarthy *et al*, 2011; Wackerhage, 2014). Therefore, besides its yet unresolved contribution to adult muscle

hypertrophy, satellite cells are considered to be essential during muscle regeneration and maintenance (Relaix & Zammit, 2012). However, exercise (both endurance and resistance) has been reported to increase satellite cell number, both in young and old animals (Shefer *et al*, 2010; Leiter *et al*, 2011; Lee *et al*, 2012b; Smith & Merry, 2012; Shefer *et al*, 2013) and, thus, the potential ability of exercise to induce satellite cell activation is still a matter of investigation.

Thus far, AMPK and mTOR are the two main pathways described in skeletal muscle exercise-induced contractile activity and appear to regulate a different set of adaptations. Moreover, although a great deal of research has been conducted, further knowledge on the intricate signaling networks that promote adaptation is still necessary as well as the role of other potential factors that may participate in this process, such as the reported muscle secreted factors or *myokines* (Pedersen & Febbraio, 2012). Understanding of the molecular mechanisms that induce exercise-induced adaptations in skeletal muscle may also be important for assessing possible modulatory effects of exercise on muscle regeneration and for identifying potential pharmaceutical targets useful for the treatment of muscle disorders.

2.2.- Myokines and cross-talk between skeletal muscle and other organs

The existence of a humoral factor released under exercise conditions was pointed out by M.Goldstein in 1961, from the observations that muscle contraction drives hypoglycemia in an insulin-independent manner (Goldstein, 1961). Years later, the term "myokine" appeared in the literature to define "cytokines and other peptides expressed, produced and/or released by muscle fibers that exert autocrine, paracrine or endocrine effects" (Pedersen *et al*, 2003) and skeletal muscle was subsequently referred to as an endocrine organ (Pedersen & Febbraio, 2012). Under exercise conditions, certain myokines expressed in the contracting skeletal muscle can act locally in the muscle tissue whereas others are released into the circulation to function at a systemic level, influencing the metabolism and function of several organs such as adipose tissue, liver, and blood vessels. In that sense, it is believed that myokines can mediate, at least in part, some of the beneficial effects of exercise. Nowadays, the myokines identified are numerous and they will probably grow in number over time. Some of the myokines are better characterized than others and there is still lack of experimental evidence for some of them. The relevant myokines for the present study are described below, with special attention in interleukin 6 (IL-6).

Interleukin 6 (IL-6)

Probably the most studied myokine is *IL-6* and it is the first cytokine released into the circulation during exercise. IL-6 is defined as a pleiotropic cytokine because apart from its pro- and anti-inflammatory properties it also plays a role in non immune tissues such as liver, adipose tissue and skeletal muscle. Skeletal muscle has been shown to produce IL-6 under basal conditions and in response to different stimuli *in vitro* including electrical-pulse induced contraction, Tiff and lipopolysaccharide (LPS) (Bartoccioni *et al*, 1994; De Rossi *et al*, 2000; Frost *et al*, 2002; Nedachi *et al*, 2008; Roher *et al*, 2008; Seyoum *et al*, 2011). During exercise, circulating IL-6 can increase in an exponential manner to (a maximum of) 100-fold, decreasing rapidly during the recovery period (Steensberg *et al*, 2002). IL-6 plasma levels are influenced by the duration, intensity and, to a lesser extent, to the mode of exercise (Fischer, 2006).

Circulating levels of IL-6 have been shown to decrease after a prolonged training and some authors suggested that this pattern was related to reduced carbohydrate utilization (Croft *et al*, 2009), whereas some other authors suggested that IL-6 may have a role in training adaptation by the observation that muscular IL-6 receptor (IL-6R) mRNA expression was up-regulated, improving sensitivity for IL-6 signaling (Fischer, 2006). Plasma IL-6 found at increased concentrations in during exercise was demonstrated to not arise from circulating monocytes (Ullum *et al*, 1994; Starkie *et al*, 2001) and there are several reports demonstrating IL-6 expression in the contracting skeletal muscle. For instance, studies exercising one leg and not the other reported an increase in plasma levels and IL-6 mRNA levels only in the exercised leg (Steensberg *et al*, 2000; Jonsdottir *et al*, 2000). Furthermore, a higher transcription rate of IL-6 in muscle fiber nuclei isolated from muscle biopsies under exercisewas also reported (Keller *et al*, 2001). Moreover, Hiscock *et al* (2004) demonstrated that IL-6 was expressed at the mRNA and protein levels in muscle fibers after exercised-induced muscle contraction and, interestingly, that IL-6 was expressed in all type of fibers pre-exercise but was predominantly localized in high glycogen containing cells post-exercise, coinciding with fast-type fibers (Hiscock *et al*, 2004) (**Figure I.3**). Although controversy still exists among the scientific community, it is recognized that skeletal muscle might be the principle source of this cytokine under exercise conditions.

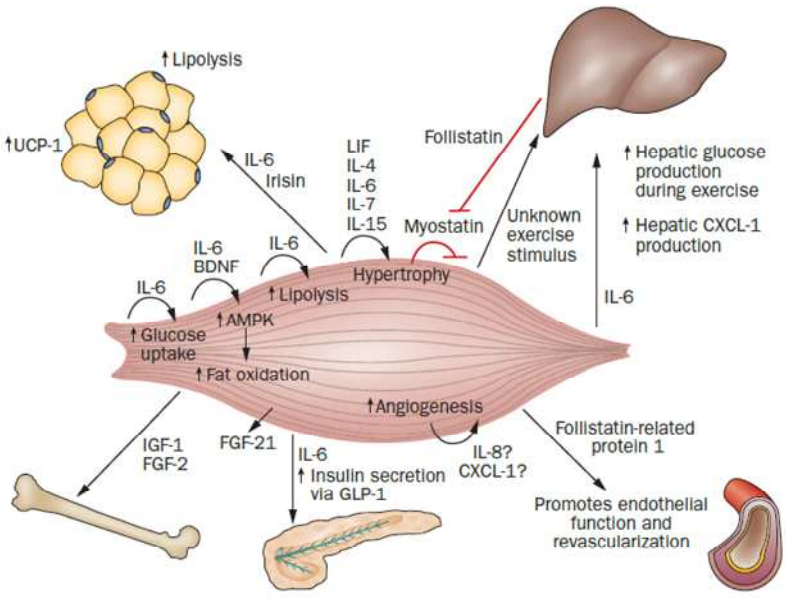

Figure I.3. Proposed myokines in skeletal muscle (Pedersen & Febbraio, 2012)

Several reports have evidenced that IL-6 could act as an energy sensor in response to exercise and that it is dependent on substrate availability. That was suggested from the observations that low intramuscular glycogen levels dramatically increased IL-6 mRNA and protein expression in humans, or that ingestion of glucose during exercise decreases the circulating levels of IL-6 from contracting muscles (Keller *et al*, 2001; Steensberg *et al*, 2001; Febbraio *et al*, 2003). In cell culture of human and murine myotubes, IL-6 increased glucose uptake, GLUT4 translocation, fatty acid uptake and fatty acid oxidation with an increase of AMPK activity (Carey *et al*, 2006; Al-Khalili *et al*, 2006). Therefore, it has been suggested that IL-6 activates

AMPK in murine and human muscle cells *in vitro* (Kelly *et al*, 2009). However, the exact mechanism of action ofIL-6 on muscle cells is still unknown since some authors pointed to the opposite trend, namely, that AMPK regulatesIL-6 secretion from muscle cells (Glund *et al*, 2009). Interestingly, IL-6function has been related to muscle growth. Under hypertrophy induced by mechanical load, mice deficient in IL-6 exhibited a weak increase in cell size due to impaired satellite cell proliferation (Serrano *et al*, 2008). Moreover, IL-6 has been shown to promote muscle differentiation in human myotubes (Al-Khalili *et al*, 2006) and to participate in skeletal myogenesis dependent on p38 MAPK activation (Baeza-Raja & Muñoz-Cánoves, 2004b).

Our knowledge on IL-6 function is expanding since emerging reports indicate that this cytokine has many physiological or systemic roles by acting on peripheral tissues. During exercise, it has been suggested that IL-6 released from working muscles may promote its own production in the liver to locally increase hepatic glucose production in response to a decrease in blood glucose induced by exercise (Pedersen & Febbraio, 2012), and that this effect is independent from circulating hormones such as insulin, glucagon or growth hormone (Febbraio *et al*, 2004). A hyperglycemic action of IL-6 has been shown from studies using primary cultures of rat hepatocytes that reported direct stimulatory effects of IL-6on gluconeogenesis and glucose release (Ritchie, 1990; Blumberg *et al*, 1995) and from studies showing increased glucose production in humans upon administration of recombinant IL-6(Stouthard *et al*, 1995). In summary, IL-6 could drive some of the beneficial effects of exercise locally in the contracting muscle by potentiating muscle growth or increasing glucose uptake and utilization but also could have a role at a whole systemic level through its cross-talk on liver and adipose tissue.

Interleukin 15 (IL-15)

IL-15 in skeletal muscle is known for its anabolic effects. In *in vitro* experiments using myotubes, overexpression of IL-15results in an increase of sarcomeric proteins, increased protein synthesis and decreased protein degradation, suggesting a potential role of this cytokine for muscle wasting protection *in vivo* (Quinn *et al*, 2002; Argilés *et al*, 2009). In cachectic rats bearing a tumor, IL-15 administration resulted in a decrease in protein degradation and a reduction in apoptosis in skeletal muscle (Figueras *et al*, 2004), confirming a role of this cytokine in muscle protein turnover. Furthermore, IL-15 has been related with the regulation of glucose metabolism in skeletal muscle. In rat muscle incubations and C2C12 cells, IL-15 increased glucose uptake and GLUT4 content (Busquets *et al*, 2006), that could explain the improved insulin sensitivity and glucose response (Barra *et al*, 2012). Muscle secreted IL-15 also couldplay a role on the regulation of white adipose tissue(Pedersen & Febbraio, 2012; Carbó *et al*, 2001; Quinn *et al*, 2005)as shown by the effects of administration of IL-15 in reducing lipogenesis and reducing lipid deposition without altering food intake (Carbó *et al*, 2001) and increasing lipid oxidation in muscle (Almendro *et al*, 2006). In genetic models, IL-15 transgenic mice show a lean phenotype whereas IL-15-deficient mice show increased body weight and visceral fat with an increase in adipocyte cell size (Barra *et al*, 2010). Using the obese mouse line *ob/ob* (leptin-deficient), IL-15 administration resulted in a reduction of white adipose tissue mass however, no changes in *fa/fa* mice (leptin receptor deficient) white adipose tissue were reported (Alvarez *et al*, 2002). Moreover, Nielsen *et al* negatively correlated circulating IL-15 levels with trunk fat mass in humans, an observationthat was

supported by the decreased concentration in plasma IL-15 in obese subjects and suggested that skeletal muscle was the major source of IL-15 (Nielsen *et al*, 2008; Barra *et al*, 2010), although this was not observed in another report (Pierce *et al*, 2015). Under exercise conditions, IL-15 levels are not as well correlated as in the case of IL-6. Probably due to the mode of exercise, several reports found either differences or no changes in plasma or skeletal muscle mRNA and protein IL-15 levels under aerobic or resistance conditions (Riechman *et al*, 2004; Nielsen *et al*, 2007; Tamura *et al*, 2011; Rinnov *et al*, 2014). Quin *et al*, observed that IL-15overexpression in mice presented a phenotype resembling an exercise adaptation, with an increase of oxidative metabolism in skeletal muscle (SIRT1, PPARδ or PGC-1α), preferential fuel utilization for lipids and, interestingly, exhibiting more endurance capacity by running a longer period of time (Quinn *et al*, 2013). Therefore, IL-15 could act locally in skeletal muscle by promoting muscle growth and regulating energy metabolism in adipose tissue.

Myostatin

Myostatin (MSTN) or growth differentiation factor 8 (GDF8), belongs to the TGF-β superfamily and in mammals is expressed primarily in the skeletal muscle. It functions by controlling muscle mass by negatively regulating muscle growth. Indeed, MSTN-deficient mice and cattle showed an enhanced muscle mass, known as the "double-muscling" phenotype, due to combination of an increase in hyperplasia (increase in fiber number) and hypertrophy (increase in fiber size) accompanied by a reduction of adipose tissue mass (McPherron *et al*, 1997; McPherron & Lee, 1997). MSTN down-regulates myogenic regulatory factors (MRFs) such as MyoD, Myf5 or myogenin, affecting myoblast differentiation and proliferation in a mechanism that may involve satellite cell inactivation (McCroskery *et al*, 2003; Manceau *et al*, 2008). Moreover, there is evidence that the negative regulation of muscle mass by MSTN affects protein synthesis *in vitro* and *in vivo* by acting on the Akt/mTOR pathway (Sartori *et al*, 2009; Lipina *et al*, 2010). Follistatin and decorin have been found to inhibit myostatin signaling in muscle, suggesting that other factors than myostatin also play a role in the control of muscle growth. These factors, when overexpressed, cause a similar phenotype of enhanced muscling (Lee & McPherron, 2001) and point to a more complex regulation of myostatin signaling. In contrast, overexpression of MSTN in mice produced muscle atrophy (Zimmers *et al*, 2002). Furthermore, several reports suggest that myostatin is probably more than a regulator of muscle mass and that is also involved in energy metabolism. Myostatin deficiency in MSTN null mice produced a decrease in force generation with a higher fatigability, an increase in fast type fibers, and a decrease in oxidative capacity with a shift from aerobic towards anaerobic metabolism (Amthor *et al*, 2007; Mouisel *et al*, 2014). Exercise training in MSTN null mice resulted in an improvement of muscle function with an increase in muscle force-generating capacity, decreased cross-sectional area of fast fibers (predominant in null MSTN), increased capillarity and angiogenic factors and oxidative capacity (Matsakas *et al*, 2012), suggesting that exercise is able to improve some of the detrimental effects of myostatin deficiency. Indeed, in human and murine muscle studies, aerobic and resistance exercise decreases mRNA expression of myostatin and it has been beneficial to prevent loss of muscle mass during aging (Louis *et al*, 2007; Allen *et al*, 2011; Ziaaldini *et al*, 2015). Moreover, myostatin has also reported to act directly on adipose tissue inhibiting adipogenesis in 3T3L1 preadipocytes, producing smaller adipocyte cell size and resistance to a diet-induced obesity (Feldman *et al*, 2006). Under cell culture conditions, myostatin action resulted in increased AMPK activivty and

increased glucose uptake and glycolysis in skeletal muscle cells which could explain the reported effects of myostatin on glucose homeostasis (Chen *et al*, 2010).

Decorin and SPARC

Secreted protein acidic cysteine-rich (SPARC) is a glycoprotein also known as ostenonectin because of its function in bone calcification, whereas decorin is a proteoglycan, but both factors are present in the extracellular matrix (ECM) and interact with components of the ECM such as collagen or growth factors. Decorin is secreted by human muscle cells *in vitro*(Henningsen *et al*, 2010)and also in response to muscle contraction *in vitro* under electrical stimulation and *in vivo* in humans and mice under exercise conditions(Kanzleiter *et al*, 2014). Overexpression of decorin by *in vivo* electroporation in mice muscle resulted in an increase of decorin and myostatin mRNA expression in muscle, an up-regulation of follistatin and MyoD and a reduction in atrophy marker genes (Kanzleiter *et al*, 2014). Decorin modulates growth factor activities and interacts with several members of the TGFβ family(Yamaguchi *et al*, 1990). In fact, administration of decorin reduces fibrosis and enhances regeneration in treatments for muscle injury primarily because itsinteraction with TGFβ1 neutralizing the binding with TGFβ1 receptor (Garg *et al*, 2015). Interestingly, it was reported that decorin binds to myostatin (Miura *et al*, 2006) and decorin peptides *in vitro* have been shown to block myostatin signaling upon binding to its receptor activin II, with no effect on other members of the TGFβ superfamily such as TGFβ2, GDF-11 and Activin A (El Shafey *et al*, 2016). However, muscle hypertrophy induced by decorin can be mediated by other interactions than myostatin inhibition since it has also been shown that decorin binds IGF-1R with high affinity in endothelial cells (Schönherr *et al*, 2005) and increases Akt phosphorylation in C2C12 myoblasts, enhancing their differentiation (Suzuki *et al*, 2013). Taken together, it is thought that decorin participates in muscle growth induced by exercise possibly by modulating the activity of myostatin; however, their regulation under exercise conditions has not been extensively investigated.

In vertebrates, SPARC is expressed during development and in adult tissues with constant regeneration (skin, gut, glands) (Sage *et al*, 1989; Rotllant *et al*, 2008). SPARC deficient mice develop early osteopenia, cataracts, accelerated cutaneous wound healing and more fat accumulation despite similar body weight(Gilmour *et al*, 1998; Bradshaw *et al*, 2003). In skeletal muscle, SPARC is expressed in C2C12 myoblasts undergoing differentiation (Cho *et al*, 2000; Chan *et al*, 2007). Moreover, SPARCmight beinvolved in response to muscle remodeling as shown in human muscular dystrophy biopsiesand muscle disease models where its expression is elevated(Chen *et al*, 2000; Jørgensen *et al*, 2009). Interestingly, Song et al suggested a role for SPARC in regulating glucose metabolism in muscle via AMPK (Song *et al*, 2010). SPARC was identified as a novel myokine in humans and mice in response to exercise by microarray analysis and is secreted from skeletal muscle with no expression in other organs observed and released to circulation (Aoi *et al*, 2013; Catoire *et al*, 2014). One reported role for this myokine under exercise conditions was an inhibitory effect on colon tumorigenesis (Aoi *et al*, 2013) but under physiological conditions its role has not been explored.

Irisin

Irisin is the secreted and membrane cleaved form of FNDC5 into the circulation. Interest in irisin emerged because of its potentialas a therapeutic factor for the treatment of obesity and

diabetes or related metabolic disorders, as it was proposed initially by Boström *et al* (2010). However, there are several gaps on the biology of irisin that need to be filled, such as the existence of a receptor, the elimination or clearance rates and, very importantly, its detection in humans, which is a topic of controversy in the scientific community.

Fibronectin type III domain containing 5 (FNDC5) was first cloned and identified in mice by two groups as a novel gene of the fibronectin type III superfamily. FNDC5was found to be expressed during development and in the adult heart and brain (Ferrer-Martínez *et al*, 2002; Teufel *et al*, 2002). Years later, Bruce Spielgman's group identified FNDC5 as a candidate protein responsible for the browning of adipose tissue (thus, resembling the effects of exercise on this tissue) in transgenic mice overexpressing PGC-1α in muscle. These mice were generated to mimic the muscle activation of PGC-1α in response to exercise (Boström *et al*, 2012).Boström et al observed that recombinant FNDC5 adminitration resulted in a dramatic effect on inducing the thermogenic gene expression program in adipose tissue at a low dose in comparison with other muscle candidate proteins such as BMP7 (a known potent inducer of browning). Moreover, they found an increase in plasma irisin in wild-type mice and humans under exercise conditions. Therefore, their hypothesis was that contracting skeletal muscles express and secrete irisin, in a PGC-1α-dependent manner, into the circulation to act on white adipose tissue to promote browning. Beside the metabolic effects of irisin on adipocyte cells, FNDC5 has been shown to act locally in muscle cells by inducing AMPK activity and glucose uptake (Huh *et al*, 2014; Lee *et al*, 2015a). With regards to metabolic disorders such as obesity, the role of irisin is again controversial, since increased circulating levels have been used as a marker in obese patients.

Brain-derived neurotrophic factor (BDNF)

In the adult brain, exercisemodulates brain function by increasing synapse plasticity and neurogenesis and it has been shown to be neuroprotective in neurodegenerative disorders. One of the putative mechanisms is the induction of growth factors such as BDNF (Vaynman *et al*, 2004; Cotman *et al*, 2007). Despite its role and expression in the nervous system, BDNF and other neurotrophins have been found to be expressed in several tissues such as skeletal muscle (Gómez-Pinilla *et al*, 2002). BDNF is secreted in an activity-dependent manner at the neuromuscular junction and its release is regulated by muscle activity (Garcia *et al*, 2010; Je *et al*, 2012). In particular, BDNF has been shown to be expressed in skeletal muscle satellite cells and its expression inhibits myogenic differentiation *in vitro* (Mousavi & Jasmin, 2006). Deficient muscle-BDNF transgenic mice showed abnormal muscle cell development and delayed muscle regeneration, suggesting that BDNF regulates satellite cell differentiation (Clow & Jasmin, 2010). Moreover, Matthews et al, showed thatduring exercise, muscle BDNF expression was expressed and producedin skeletal muscle and not released into the circulation. Cultured cells in response to contraction-induced activity increased BDNF expression and induced fatty acid oxidation upon AMPK activation (Matthews *et al*, 2009). Therefore, BDNF as a myokine acts locally in muscle cells. Some authors state thatthe brain is the major contributor of the circulating BDNF levels in exercise (Rasmussen *et al*, 2009). In fact, Spiegelman's group proposed a mechanism by which exercise increases the expression of BDNF in the brain. They showed that, PGC1α expression in the brain induced by exercise

stimulates FNDC5 that, in turn, induces BDNF expression in hippocampal neurons which will drive some of the beneficial effects of exercise in the brain (Wrann *et al*, 2013).

Apelin

In mammals, apelin is predominantly expressed in the brain but is widely distributed in heart, lung, liver, gastrointestinal tract, adipose tissue, vascular endothelium, skeletal muscle andgonads and. This peptide signals through its receptor APJ or AGTRL1. Many biological functions have been attributed to the apelin/APJ system, including regulation of fluid homeostasis, angiogenesis, cardiovascular function and, more recently, metabolic functions. Indeed, apelin deficient mice show increased insulin levels, impaired glucose tolerance and whole body insulin resistance(Yue *et al*, 2010). It has been shown that apelin plays a role in glucose and lipid metabolism mainly because of its role in adipose tissue. Apelin administration reduces hyperglycemia in both normal and insulin resistant mice, and stimulates glucose uptake in muscle involving AMPK and Akt signaling (Dray *et al*, 2008). Moreover, lipid oxidation and mitochondrial biogenesis in muscle increased after apelin administration in obese and insulin resistance. These and other studies suggest that apelin could play a role in improving insulin sensitivity (Xu *et al*, 2011; Attané *et al*, 2012; Habchi *et al*, 2014). The identification of apelin as a myokine was reported by Besse-Patin *et al* in a study with obese men subjected to exercise training. They found changes in apelin mRNA expression but did not find significant changes of other myokines such as IL-6, FGF21, MSTN in human muscle *in vivo* (Besse-Patin *et al*, 2014). Recently, it has been suggested that exercise could regulate apelin signaling in a tissue specific manner in adipose and muscle tissue (Yang *et al*, 2015).

To summarize this section, the different myokines described here have been reported to have a role in muscle function. Some of these myokines had not been initially related with muscle development or energy metabolism, such as in the case of cytokines (e.g. IL-6 and IL-15).Moreover, myokines possibly act in other tissues in an autocrine, paracrine or endocrine manner. However, their role in muscle or in the crosstalk with other tissues and organs is still not clear and, in many cases, no clear picture is present due to conflicting results in the literature. However, some of these myokines offer promising expectations for improving our knowledge and treatment of several metabolic disorders.

2.3.- Exercise, skeletal muscle and the immune system

As early as 1893, an increase in the number of circulating white cells (leucocytosis) was reported after an acute bout of exercise and it was only years later that the field of “exercise immunology” was conceived during the 1990s (Shephard, 2010). Probably the most notable or best studied effects of exercise are observed in a number of chronic diseases that are accompanied by a systemic low-grade inflammation. Such associated diseases include type 2 diabetes mellitus, metabolic syndrome, obesity, cardiovascular diseases and even age-neurodegenerative disorders such as Alzheimer’s disease (Hotamisligil, 2006; Nichol *et al*, 2008). These diseases have been characterized by the presence of circulating pro-inflammatory cytokines, including interleukin 6 (IL-6), tumor necrosis factor alpha (TNFα) and C-reactive protein (CRP) among other markers of inflammation, as well as with an elevated number of circulating inflammatory monocytes. Moreover, patients with metabolic disorders

such as type 1 or type 2 diabetes have an increased susceptibility to acute infections than healthy subjects (Shah & Hux, 2003; Muller *et al*, 2005).
From studies in humans and in animal models, it is thought that the protective effects of exercise on the immune function lie on the anti-inflammatory environment produced in each bout of exercise and many possible mechanisms have been postulated. First, regular exercise can reduce whole body fat mass, particularly visceral and abdominal, and, consequently, reduces the levels of pro-inflammatory cytokines released from the adipose tissue (adipokines),thus reducing systemic levels of inflammation (You & Nicklas, 2008). Second, the production of myokines such as IL-6 by the working muscles can contribute to reducing circulating pro-inflammatory cytokines. Indeed, IL-6 stimulates the production of the circulating anti-inflammatory cytokines IL-1RA, an inhibitor of IL-1 signaling, and IL-10. The latter is an anti-inflammatory cytokine that inhibits the production of several cytokines such as IL-1β and TNF-α and regulates the activation of immune cells (Starkie*et al*, 2003; Steensberg*et al*, 2003). In view of this, Petersen et al (2005) proposed that exercise-induced increases of circulating IL-6, IL-1ra and IL-10 produce anti-inflammatory actions that may be beneficial for obesity and insulin resistance (Petersen & Pedersen, 2005). Third, exercise reduces the infiltration of macrophages in the adipose tissue, possibly by inhibiting the release of chemokines and decreasing adhesion molecules (Kawanishi *et al*, 2010). Moreover, exercise has been shown to alter the phenotype of the infiltrated macrophages in the adipose tissue of obese mice by inducing a phenotypic switch from the predominant pro-inflammatory macrophages (known as M1 type) to an anti-inflammatory (known as M2 type), therefore reducing the inflammatory state in the adipose tissue (Lumeng *et al*, 2007; Kawanishi *et al*, 2010).

On the other hand, the immunomodulatory effects of exercise have also been investigated during sepsis. Sepsis is a severe complication of an infection that, among other consequences, causes the development of muscle weakness and is related to an increase in muscle catabolism that leads to muscle wasting when the systemic inflammatory response persists. In particular, during the initial phase of infection, aminoacids released from muscle tissue constitute a fuel source for the immune cells to support gluconeogenesis in other tissues or acute phase protein synthesis in the liver (Rosenblatt *et al*, 1983; Vary & Kimball, 1992; Karinch *et al*, 2001). Different models of sepsis are used experimentally by injection of pathogen/bacterial toxins (i.e. lipopolysaccharide or LPS), administration of viable pathogens or alteration of the organism's defense barrier (Remick *et al*, 2000; Buras *et al*, 2005). Incidentally, the cytokine profile after LPS administration is not as strong as the other approaches (Remick *et al*, 2000). However, the first clinical trial in humans using a bolus of *Escherichia coli* LPS to induce low-grade inflammation evidenced that in previously exercised subjects the increase of TNFα was attenuated in comparison with the control group. The increased circulating levels of IL-6 in exercised subjects produced similar results as the administration of recombinant human IL-6. Therefore, this study suggested a possible role in humans of exercise-induced IL-6 to mediate the anti-inflammatory effects of exercise (Starkie *et al*, 2003).

However, the exact mechanisms by which exercise exerts the proposed beneficial effects on immune function in health and disease, as well as the determination of how much exercise is needed, remain unknown. In this regard, skeletal muscle has received the attention of the

scientific community because muscle cells have been shown to express immune factors that may contribute to the immune function and, therefore, to participate in the immunomodulatory effects of exercise as well. In fact, muscle cells have been found to express molecules typically present in dendritic cells, monocytes or B cells that function as antigen-presenting cells (APCs). APCs are responsible for the presentation of antigens to T cells in order to target or stimulate the immune response of other immune cells. Particularly, major histocompatibility complex molecules (MHC), cytokines, cell adhesion molecules or co-stimulatory molecules have been found to be expressed in mammalian skeletal muscle cells (Nagaraju, 2001; Wiendl *et al*, 2005). For instance, human myoblast cells are known to express MHC after in vitro stimulation with pro-inflammatory cytokines (such as IFN-γ, TNFα, IL-1a or IL-1b),as well as *in vivo* under inflammatory myopathies (Wiendl *et al*, 2005). Moreover, as mentioned above, cultured muscle cells have the necessary antigen-processing machinery to present antigens either from intracellular or extracellular sources. Different toll-like receptors, that are the main receptors of the innate immune response to recognize pathogen associated molecules (PAMPs) and that are typically expressed in macrophages and dendritic cells, have also been found to be expressed in muscle cells (Marino *et al*, 2011). These observations have been particularly interesting in relation to intramuscular DNA vaccinations based on the notion that muscle cells can process antigens and present them to APCs, once recruited under an inflammatory response. Therefore, muscle cells under inflammatory conditions can trigger cytokine, chemokine and adhesion molecule production in order to recruit or stimulate leukocytes. Thus, in addition to the ability of antigen presentation, muscle cells could function as non-professional APCs to modulate the innate and adaptive immune response. However, many of these observations have been achieved *in vitro* and more research is warranted to confirm their role *in vivo*.

Therefore, given the role of skeletal muscle in modulating the adaptive response to exercise, the molecular properties of muscle cells under an inflammatory environment and the pathologies associated with inflammation, its role on the reported beneficial effects of exercise in immune-related pathologies warrants future and promising investigations.

3.- Cardiac muscle and exercise in mammals

Regular exercise initiates cardiovascular and respiratory changes in order to supply and deliver oxygen and nutrients to working muscles and other tissues. During exercise conditions, the blood flow to the working muscles can increase by 60%. Thus, cardiac output (a product of stroke volume and heart rate) is increased and blood flow is redistributed from inactive organs or tissues (such as the digestive system) to the skeletal muscle. However, apart from the acute cardiac responses (e.g. increased cardiac output) that are put in place to meet the increased energetic demands, exercise training elicits cardiac adaptations and protective effects on the heart or the cardiovascular system. Moreover, several evidences point to a beneficial effect of exercise performance in patients that underwent a cardiovascular disease such as myocardial infarction or heart failure (van Tol *et al*, 2006; Crimi *et al*, 2009; Lawler *et al*, 2011; Garza *et al*, 2015) as well as in animal models of myocardial infarction or ischemia/reperfusion (I/R). Intriguingly, exercise has been related to a preconditioning effect in the heart with similar effects than ischemic preconditioning (Murry *et al*, 1986), thus protecting the heart from

cardiac insults (Maybaum *et al*, 1996; Domenech *et al*, 2002; Michaelides *et al*, 2003; French *et al*, 2008; Parra *et al*, 2015). However, the mechanisms driving exercise-induced cardioprotection in health and disease remain elusive. Nitric oxide, heat shock proteins (HSPs), ATP-dependent potassium channels (KATP), antioxidants, reactive oxygen species (ROS) or AMPK are some of the proposed mechanisms for exercise-induced cardioprotection in mammalian studies that will be introduced in this section.

Exercise training produces morphological changes in the heart that may result in cardiac growth. Hypertrophy (or increased cardiac mass) is an adaptive response of the heart in order to satisfy a hemodynamic stress such as that elicited by an increase in cardiac output during aerobic exercise or during a disease setting such as hypertension. However, in such cases when the mechanical stress is persistent or chronic, cardiac hypertrophy can no longer be favorable or cardioprotective and evolves to be maladaptive. This results in an increase in fetal gene expression genes (e.g. increased atrial or brain natriuretic peptide, β-myosin heavy chain and reduced α-myosin heavy chain and calcium-handling proteins such as SERCA2a), an increase in cardiomyocyte cell death followed by an increase in fibrosis, impaired cardiac function, increased glucose utilization over fatty acid oxidation and an increase in cardiomyocyte cell width that ultimately leads to cardiac dysfunction, elevating the risk of heart failure (Heineke & Molkentin, 2006). In contrast, none of these detrimental effects are present in a hypertrophic process elicited by developmental hypertrophy, exercise or pregnancy, processes in which cardiac mass is achieved by a reversible and more proportionate increase of myocyte length and width and improved or normal cardiac function (Heineke & Molkentin, 2006). Based on the growth of the heart chambers, cardiac hypertrophy can be distinguished between eccentric hypertrophy (increased chamber diameter with a proportional growth of the wall thickness) and concentric hypertrophy (normal or reduced left ventricular diameter over an increase in wall thickness), two forms of hypertrophy that under pathological signals can be even further expanded. Therefore, two states of hypertrophy have been defined: physiological and pathological hypertrophy, with distinct molecular and morphological signatures (Boström *et al*, 2010; Song *et al*, 2012a).

Physiological hypertrophy, such as in the case of exercise or postnatal growth, can involve the IGF-1/PI3K/Akt signaling pathway. Although IGF-1 is typically produced by the liver and is essential during development in response to growth hormone, it is also produced by the heart during exercise (Neri Serneri *et al*, 2001) to promote physiological hypertrophy and is dependent on signaling through the kinases PI3K and Akt, as it has been shown from studies in murine models (McMullen *et al*, 2004; Matsui *et al*, 2003; Kim *et al*, 2008). In addition, thyroid hormone, insulin, growth hormone or mechanical sensors have also been shown to initiate physiological hypertrophy, whereas pathological hypertrophy is characterized by signals induced by hormones such as endothelin 1 and angiotensin II (Heineke & Molkentin, 2006). Cardiomyocyte cell growth has been correlated with an increase in protein accumulation which involves the mTOR pathway as regulator of protein synthesis (Hannan *et al*, 2003).However, although the knowledge of mTOR signaling regulation in the heart is still not completely understood from loss and gain of function studies, many studies point to an adaptive role of mTOR in cardiac hypertrophy (Heineke & Molkentin, 2006). In addition to participating in IGF-1 or mTOR signaling, Akt down-regulates the transcription factor CCAAT/enhancer binding protein β (C/EBPβ) in response to exercise, a factor involved in the

regulation of cardiomyocyte growth and proliferation (Boström *et al*, 2010). Inactivation of C/EBPβ, which interacts with the serum response factor (SRF), leads to the activation of an "exercise gene set" (i.e. comprising of Gata4, α-MHC, Mef2c, Tbx5, Nkx2.5, TnT and TnI) to promote cardiomyocyte hypertrophy and differentiation. In turn, inactivation of C/ EBPβ activates the cbp/p300-interacting transactivator 4 (CITED4) to enhance cardiomyocyte proliferation (Boström *et al*, 2010; Mann & Rosenzweig, 2012). Moreover, neuroregulin 1 (NRG1) and its receptor ErbB4 promoted cardiomyocyte proliferation under cardiac injury (Bersell *et al*, 2009) and, during exercise-induced cardiac hypertrophy, NRG1 and other growth factors such as IGF-1, TGFβ1 and BMP-10 appeared up-regulated and to stimulate cardiac stem cell activation and differentiation (Waring *et al*, 2014). Recently, NRG1 produced during exercise was suggested to promote cardiac regeneration in rats by inducing cardiomyocyte proliferation and reduced cell death, a finding that shows NRG1 to be a putative factor involved in exercise-induced cardioprotection (Cai *et al*, 2016). Therefore, besides the limited capacity of the mammalian heart to induce cardiomyogenesis (Bergmann *et al*, 2009), exercise may be a mechanism to induce proliferation of cardiomyocytes. The source of these newly formed cardiomyocytes remains unknown, but cardiac stem cells may contribute to the cardiomyogenic role of exercise (Wahl *et al*, 2009; Waring *et al*, 2014).

Vascular remodeling is another adaptation of exercise in the heart in order to compensate for elevated blood flow and increased myocardial oxygen demand. For instance, coronary arteries increase in size in human athletes and trained animals (Wyatt & Mitchell, 1978; Kozàkovà *et al*, 2000) as well as cardiac capillary growth (White *et al*, 1998; Marini *et al*, 2008; Waring *et al*, 2015). Moreover, endurance exercise increases vascular shear stress that, in turn, increases the activity and phosphorylation of endothelial NO synthase (eNOS) increasing the production of NO. Null eNOS mice exhibited reduced exercise performance and, remarkably, exercise in these animals did not improve myocardial infarction outcomes such as fibrosis, apoptosis or (pathological) hypertrophy (Calvert & Lefer, 2013). eNOS activation in exercise cardioprotection has been proposed to take place upon activation of adrenergic receptor β3 by catecholamines and to signal through Akt (Calvert *et al*, 2011; Calvert & Lefer, 2013).

Exercise has also reported to increase the expression of stress proteins, heat shock proteins particularly from the HSP70 family (Hamilton *et al*, 2001; Lennon *et al*, 2004; Quindry *et al*, 2007; Esposito *et al*, 2011). For instance, HSP72 overexpression in hearts with I/R injury increased protection from myocardial cell death and increased antioxidant MnSOD activity (Jayakumar *et al*, 2001; Suzuki *et al*, 2002). However, the question if elevated levels of HSP72 are cardioprotective during exercise is controversial, with studies suggesting a beneficial role for stress proteins (Esposito *et al*, 2011) whereas other studies showing lack of protection against I/R (Hamilton *et al*, 2001; Lennon *et al*, 2004; Quindry *et al*, 2007).

During exercise, transient bursts of ROS are produced (in an intensity and duration dependent manner) and believed to be favorable for exercise adaptation, similarly to the increased ROS during the initial phase of late ischemic preconditioning (the first 24h after the stimulus)(Bolli, 2000). Conversely, ROS attenuation by antioxidants has been shown to suppress the preconditioning effect, thus suggesting a complex role for oxidative stress in ischemic preconditioning (Sun *et al*, 1996; Gomes *et al*, 2012). Yamashita *et al* (1999) showed that the

reduced infarct size after a single bout of exercise preconditioning was related to the increased activity the antioxidant manganese superoxide dismutase (MnSOD) after 30min and 48h, thus suggesting a biphasic cardioprotective effect of MnSOD (Yamashita *et al*, 1999). However, the cardioprotective effects of exercise-increased MnSOD as well as other antioxidants show discrepancies among different studies (Esposito *et al*, 2011; Lennon *et al*, 2004; Hamilton *et al*, 2001; French *et al*, 2008), possibly because of the lack of information on the ROS and antioxidant activity profile during exercise preconditioning.

ATP-dependent potassium channels (KATP) are plasma membrane channels that exist in many cell types and in cardiomyocytes KATPs have been localized in the sarcolemma and the mitochondria. KATPs act as cellular sensors to metabolic changes such as ATP levels. For instance, under a metabolic stress condition caused by decreased ATP levels, KATPs are activated and opened causing an outflow of K^+ that repolarizes the cell that can, in turn, inhibit Ca^{+2} entry into the cell. Activation of KATP is of particular interest to prevent intracellular Ca^{+2} overload in I/R injury that is known to cause contractile dysfunction, mitochondrial damage and cell death. In fact, through the use of pharmacological approaches involving administration of KATP blockers or agonists, a role for KATP in improving contractile recovery and reducing infarct size was demonstrated (Foster & Coetzee, 2016). In a few reports, the expression of KATP in cardiomyocytes has shown to increase in exercise training (Brown *et al*, 2005a; Zingman *et al*, 2011) and pharmacological inhibition of KATP resulted in increased infarct size, therefore reducing the exercise-induced cardioprotective effects in I/R injury without altering ventricular arrhythmias possibly due to the extended I/R challenge duration performed in the study (Quindry *et al*, 2012).

AMPK has been examined as a potential factor in cardioprotection due to its function in metabolic reprogramming. Several reports showed AMPK as a protective factor against oxidative stress, cardiac fibrosis and pathological hypertrophy (Kukidome *et al*, 2006; Li *et al*, 2009; Xiao *et al*, 2010), therefore suggesting a role for AMPK in exercise-induced cardioprotection. Indeed, similarly than in skeletal muscle, AMPK is activated in heart during exercise (Coven *et al*, 2003) and a recent report using AMPKα2 transgenic mice showed that four weeks of exercise training reduced pharmacologically-induced cardiac fibrosis and increased myocardial antioxidant expression (Ma *et al*, 2015).

In summary, exercise training produces physiological adaptations in the heart such as cardiac hypertrophy or vascular remodeling and exerts beneficial effects in a context of cardiac damage. However, the mechanisms responsible for the cardioprotective effects of exercise are incompletely understood due to the multifactorial events (e.g. neural, endocrine, paracrine, autocrine) that take place as part of the physiological responses to exercise.

4.- Zebrafish as an important vertebrate physiological model

4.1.- Introduction to the zebrafish model

The teleost fish zebrafish (*Danio rerio*) was discovered as a promising experimental model by George Streisinger (University of Oregon) in the 1970s, and nowadays it is used by more than a thousand laboratories around the world. Examples of research fields that include zebrafish as a

model include developmental biology, cardiovascular, immunity, cancer, ageing, metabolism or regeneration (Poss *et al*, 2002; Paik & Zon, 2010; van der Velden *et al*, 2011; Marín-Juez *et al*, 2015; Ruyra *et al*, 2014; Gilbert *et al*, 2014; Tulotta *et al*, 2016). Major advantages of this lower vertebrate include its external fertilization, the rapid development and translucid embryos that allow easy manipulation of the embryos during all the developmental stages. Because a great number of fertilized eggs can be collected easily, embryos can be genetically manipulated at the one-cell stage and visualize or analyze the resulting phenotype. Translucid embryos have allowed developing transgenic zebrafish, expressing constitutively fluorescent proteins (e.g. GFP) to trace developmental processes *in vivo* at the single cell level, organs or sub-cellular structures and as early as 5 days post-fertilization. Zebrafish offers an advantage in forward genetics approaches such as induced mutagenesis by chemicals or insertional elements in adults or embryos.

In the 1990s with the pioneering work of Nüsslein-Volhard, zebrafish was used in large-scale studies in order to screen for candidate genes responsible for particular phenotypes of human diseases (Haffter *et al*, 1996; Driever *et al*, 1996). The amenability of the zebrafish model in forward genetics allowed the discovery of novel genes relevant in vertebrate embryogenesis, infectious disease or regeneration (Stainier *et al*, 1996; Ransom *et al*, 1996). However, forward genetics are often time consuming and require of high-throughput screening strategies. Probably a fact that brought zebrafish as a world-wide experimental tool is the initiation of its genome sequencing in 2001 (Welcome Trust Sanger Institute). The latest genome assembly (GRCz10) has identified a total of 30,741 genes, including 14,019 known and 12,459 predicted protein-coding genes. Thus, in order to interrogate for a specific gene function, several gene-editing techniques previously used in mammalian models have been implemented in zebrafish over the past years. These include zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and, more recently, the bacterial nuclease Cas9 (Doyon *et al*, 2008; Cade *et al*, 2012; Hruscha *et al*, 2013). In early developmental stages, knocking down the expression of a gene at the mRNA level by antisense oligonucleotides (morpholinos) is a commonly used strategy to characterize gene function; however, its use in comparison with the mentioned DNA editing strategies remains controversial (Rossi *et al*, 2015). In biomedicine, zebrafish emerged as a model organism for human diseases and drug screening because of the similarity between the two genomes; approximately 70% of human protein-coding genes (including disease-causing genes) have at least one zebrafish ortholog (Howe *et al*, 2013). This teleost species is a lower vertebrate genetically and evolutionary more related to humans than the invertebrate models, the fly *Drosophila melanogaster* or the worm *Caenorhabditis elegans*, and more economical to maintain than mammalian models. Considerable numbers of zebrafish models are available to investigate human diseases such as cancer, cardiomyopathy, muscular dystrophy, Parkinson's disease, Alzheimer's disease (Gibbs *et al*, 2013).

As a model, the zebrafish has some disadvantages that have to be taken into consideration when using this species. For instance, the use of a teleost in translational studies has to consider the physiological differences between fish and mammals such as temperature regulation (poikilotherms), anatomical differences (lack of heart septation, lungs, etc) as well as the extra genomic duplication (Lieschke & Currie, 2007; Seth *et al*, 2013; Howe *et al*, 2013). Above all, the zebrafish model offers many advances for organismal science and from the

opinion of the scientific community its perspective is growing for biomedicine as well as for basic vertebrate biology research. Therefore, the selection of an experimental model (e.g. the zebrafish) for any specific research aim has to be made based on its ability to provide the best possible approximation to accomplish the research goals, taking into full account its advantages and disadvantages.

In the present work, fast skeletal muscle and cardiac muscle have been studied in response to exercise; therefore, a brief introduction on the cellular characteristics of these tissues is given.

4.1.1.- Skeletal muscle in fish

Skeletal muscle in fish, including zebrafish, comprises approximately 60-80% of the total body mass and, besides its function in locomotion, it contributes to maintain metabolic homeostasis as in the rest of higher vertebrates. In teleost fish, like mammals, skeletal muscle is composed of two distinguishable types of muscle fibers: slow-twitch (oxidative or red) and fast-twitch (glycolytic or white) with distinct functional properties and expression of different myosin isoforms. In contrast to mammals, fish muscle fiber types are anatomically separated as illustrated in **Figure I.4**. A fiber type with intermediate characteristics has been found in the intersection between slow and fast fibers in some fish species such as zebrafish. Slow and fast fiber types are specialized for distinct locomotor demands: slow fibers are used at low swimming speed and sustained swimming whereas fast fibers are used at short bursts of maximum speed such as escaping from a predator (Tudorache *et al*, 2013).

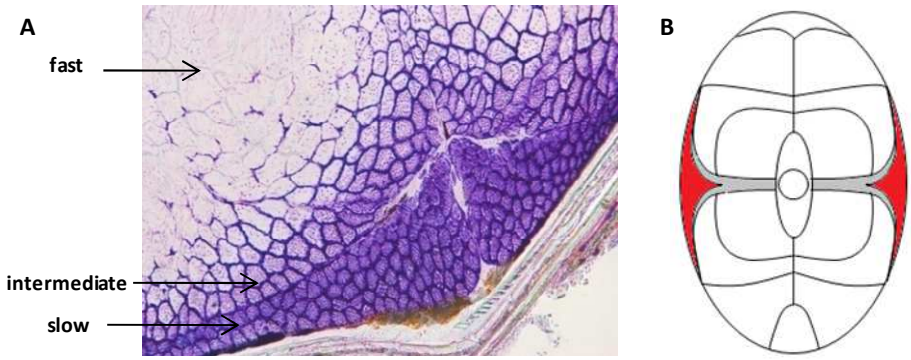

Figure I.4. Fiber type distribution in teleost fish. A Succinate dehydrogenase activity assay showing different fiber types from a tail section of an adult muscle zebrafish. Arrows indicate the three fiber types in fish: fast, intermediate, and slow. Staining method according to Nachlas *et al* (1957) performed in the laboratory. **B** Schematic representation of a tail section. Coloured areas represent fast (white), intermediate (grey) and slow (red) muscle fibers.

Slow-twitch fibers are located along the lateral line (**Figure I.4**) and constitute less than the 10% of the musculature. They are highly vascularized, have elevated glycogen content and abundant mitochondria. Concentration of myoblobin is elevated and they are more efficient at using oxygen to generate ATP by using lipid and carbohydrates as fuel (aerobic metabolism). They have a small diameter and the term “slow” refers to their lower contraction velocity in comparison with the fast-twitch fibers(Sänger & Stoiber, 2001; Tudorache *et al*, 2013).

In contrast, fast-twitch fibers are the major muscle fiber type and constitute more than the 70% of the fish musculature. They are poorly vascularized (therefore also referred as *white*),

have low glycogen content and few mitochondria. Levels of myoglobin and lipid droplets are low and energy is produced by anaerobic metabolism mainly from glycogen breakdown. They have a larger diameter and due to their metabolic properties (glycolytic) they supply energy (ATP) rapidly but are less resistant to fatigue (Sänger & Stoiber, 2001; Tudorache *et al*, 2013).

Another characteristic from fish is the continued growth throughout life that involves the process of recruitment of newly formed muscle fibers or *hyperplasia*, and the enlargement of the existing muscle cells or *hypertrophy*. Whereas in most vertebrates post-juvenile growth is achieved through hypertrophy only, in most fish species both hyperplasia and hypertrophy processes continue throughout adulthood. The relative contribution of hyperplasia in muscle growth is pronounced at early stages and decreases in post-larval stages with the increase in size of the fish (Zimmerman & Lowery, 1999; Johnston *et al*, 2011). From studies in more than ten different species of fish, Weatherley *etal*. (1988) determined that hyperplasia diminishes at a size corresponding approximately to 44% of the final total length; however, in some teleost fish species such as white seabass, hypertrophic growth persists far beyond this approximation (Weatherley *et al*, 1988). In the particular case of zebrafish, there is controversy about the contribution of hyperplasia in adult stages of muscle growth (Biga & Goetz, 2006; Johnston *et al*, 2011).

4.1.2.- The fish heart

The teleost fish heart is located in the pericardial sac, anterior to the body cavity and ventral to the esophagus, between the operculum and the pectoral bone of the pectoral fin (**Figure I.5**) (Menke *et al*, 2011). The teleost fish heart is composed by a single chambered ventricle and single chambered atrium. The ventricle shows anatomical variability among the teleost fish species, with either a larger compact layer, absence of coronary vessels in the myocardium or completely trabeculated ventricles (no compact layer) (Icardo, 2012). The heart is comprised of three specialized tissue layers (as in other vertebrates): the epicardium, the myocardium and the endocardium. The epicardium, the outer layer of the heart (or inner layer of the pericardium), is a thin and elastic layer that protects the heart from friction and composed of epithelial cells. The myocardium is composed of cardiomyocytes, specialized striated muscle cells responsible for the cardiac contractility. The endocardium, the inner layer of the heart, is composed of endothelial cells and provides a smooth surface in contact with the blood flow. Besides cardiomyocytes, other cells exist in the atrium and ventricle such as fibroblasts (maintenance of the extracellular matrix).

The circulatory system of fish differs from mammals in that the blood flows in a unique direction. The blood from the tissues (low in oxygen) flows to the atrium through the *sinus venosus* (mostly connective tissue) from which is separated by the *sinus valve*. Importantly, the sinus venosus contains the pacemaker cells responsible to initiate contraction. The atrium pumps the blood to the ventricle through the atrioventricular valves and then, the blood is pumped to the *bulbus arteriosus*. The bulbus arteriosus is an elastic chamber, mainly composed of elastin, and an external collagen layer that expands during ventricular ejection to reduce the systolic pressure generated by the ventricle in order to prevent damage of the thin-walled gill vasculature. Finally, the oxygen-rich blood flows to the tissues (Icardo, 2012; Evans *et al*, 2013). Interestingly, in fish the blood pressure between the dorsal aorta and the ventral

aorta is reduced (by 40% in adult zebrafish) due to the resistance exerted by the gills (Hu *et al*, 2001). In mammals, oxygen-rich blood irrigates the heart through coronary vessels that originate from the aorta (left ventricle) whereas in fish, the coronary circulation irrigates the compact layer and originates from the gills (at reduced pressure). Interestingly, zebrafish cardiomyocytes in the trabeculated myocardium contain abundant mitochondria due to the lack of coronary supply (Hu *et al*, 2001).

In zebrafish, as well as in other teleost species, a complete cardiac conduction system has not been identified and it has been suggested that trabeculae play a role in the conduction of the electrical signal such as the His-Purkinje system in mammals (Sedmera *et al*, 2003; Icardo & Colvee, 2011). Nevertheless, zebrafish electrocardiograms show electrical activity with the characteristic P, QRS and T waves (Liu *et al*, 2016).

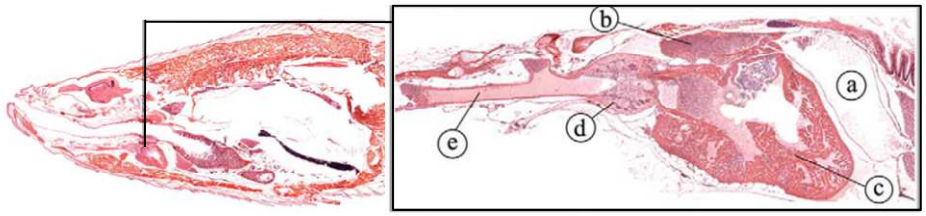

Figure I.5. Zebrafish heart and structures. a, sinus venosus; b, atrium; c, ventricle; d, bulbus arteriosus; e, ventral aorta. Adapted from Menke *et al*. (2011).

4.2. Zebrafish as an established model in muscle and cardiovascular research

Due to the above-mentioned features of zebrafish, this teleost fish is also widely used as a model for skeletal muscle and cardiovascular diseases (Curado *et al*, 2008; Stoletov *et al*, 2009; Berger *et al*, 2010). Zebrafish is established as a useful model for the study of human congenital diseases or other genetic disorders. Patophysiological diseases such as atherosclerosis. Due to its amenable disposition for transgenesis, many genetically modified zebrafish have been generated in order to serve as models for the study of human diseases such as Alzheimer's, Parkinson's, muscular dystrophy (Best & Alderton, 2008; Lieschke & Currie, 2007; Gibbs *et al*, 2013)

4.2.1. Skeletal muscle and the zebrafish model

After approximately two decades as a research model, the zebrafish has made important contributions to our current knowledge on skeletal muscle because the zebrafish skeletal muscle has many molecular features such as conserved transcriptional network regulating myogenesis (i.e. myogenic regulatory factors), as well as histological and ultrastructural features that are very similar to those in mammals. Specially, zebrafish has received special attention in the study of vertebrate myogenesis since, like in other teleost fish, muscle development occurs in distinct myogenic waves, involves different populations of progenitor cells, formation of a myotome (segment of a somite giving rise to skeletal muscle), conserved molecular signatures of cell commitment and differentiation and distinct muscle fiber types with distinct contractile properties. However, some differences exist in comparison to mammals, such as continued muscle growth or the presence of adaxial cells (that give rise to a slow myogenic program) whereas in mammals the determination to a slow fiber type depends on molecular signals and a specific slow myogenic population has not been identified (Devoto *et al*, 1996; Jackson & Ingham, 2013).

In addition, several phenotypes resembling human muscle diseases have been identified in zebrafish, allowing for the study of the pathological basis of neuromuscular disorders, such as muscular dystrophy and myopathies (Gibbs *et al*, 2013; Berger *et al*, 2010). For instance, taking advantage of the zebrafish model in imaging techniques, a recent study revealed by high-resolution imaging of the myofibers the sequential repair of the sarcolemma, which is a frequent lesion of myofibers in several myopathies with mutations in the membrane protein *dysferlin* (Roostalu & Strähle, 2012). A large-scale drug screen performed in the dystrophin-null zebrafish embryos *sapje*, considered models of human Duchenne muscular dystrophy, allowed the identification of seven compounds (e. g. phosphodiesterase inhibitors) over more than a thousand that restored zebrafish muscle integrity(Kawahara *et al*, 2011).

Nevertheless, most of the current knowledge on the regulation of skeletal muscle mass and development in zebrafish is derived from studies on the effects of muscle inactivity or injury and on genetic models of human muscle disorders (Gibbs *et al*, 2013; Yogev *et al*, 2013) and not based on models of increased skeletal muscle activity, such as induced by exercise.

4.2.2.- Cardiac regeneration and the zebrafish model

Recently, zebrafish has gained attention in the field of cardiac regenerative medicine. In particular, the finding that zebrafish can regenerate about 20% of the ventricle has raised the interest of the scientific community in this small teleost (Jopling *et al*, 2010b; González-Rosa *et al*, 2011; Schnabel *et al*, 2011; Chablais *et al*, 2011; Wang *et al*, 2011).

Several cardiac injury models have been developed in adult zebrafish: resection of the ventricular apex, cryoinjury and cardiomyocyte genetic ablation (**Table I.1**) (Poss *et al*, 2002; Schnabel *et al*, 2011; Chablais *et al*, 2011; González-Rosa & Mercader, 2012; Wang *et al*, 2011). Amputation or resection was the first implemented technique (Poss *et al*, 2002) and the most used since then. Similar than in urodele tail amputation, ventricular apex resection produces a blood clot that is sealed by the growth of new tissue. Instead, cryoinjury is also used in mammals as an alternative to coronary artery ligation, a model of myocardial ischemia. Cryoinjury causes the destruction of the cardiac tissue leading to a more severe apoptotic response than resection, extending to coronary vessels and the vasculature around the injured area. Moreover, a characteristic feature of cryoinjury is the development of a prominent fibrotic scar, scarcely present in models of amputation and resembling the pathological outcome after a myocardial infarction. Fibrotic tissue formed by fibrin and collagen is completely replaced by intact cardiac tissue after approximately 130 days post injury (dpi)although functional recovery has been shown to be impaired (González-Rosa *et al*, 2014; Hein *et al*, 2015). Ventricular resection, that produces minor deposition of fibrin particularly in collagen, is only localized at the amputated border and is rapidly eliminated in comparison with the cryoinjury model (Poss *et al*, 2002; González-Rosa *et al*, 2011). On the other hand, genetic cardiomyocyte ablation does not develop scarring (or occasionally according to authors) and is an non-invasive methodology (Wang *et al*, 2011). Conversely to the previous models, genetic ablation produces large cardiomyocyte or muscle loss of about 60% of the total ventricle area and cell death as well as proliferation is dispersed throughout the ventricle and atrium. Because of the specific depletion of the muscle tissue and the observed reduced swimming performance in comparison with the resection model, cardiomyocyte genetic

ablation has been associated to the pathological features of cardiac failure. In addition, cardiomyocyte proliferation index is massively elevated to approximately 42% in comparison with the resection and cryoinjury model(between 6-15%) at 7 dpi (Chablais *et al*, 2011; Kikuchi *et al*, 2011b; Sallin *et al*, 2014). Despite the differences among the three injury models, all of them evidence the remarkable ability of the zebrafish to completely regenerate the heart, a process still incompletely understood.

In contrast to mammals, zebrafish cardiomyocytes (and from some amphibian species) have the capacity to proliferate during adulthood, or at least to respond to injury. Although one report implicated un-differentiated progenitor cells, it is well accepted that complete regeneration of the heart occurs by a process of de-differentiation of pre-existing cardiomyocytes, thus, returning to a more immature form (Lepilina *et al*, 2006; Jopling *et al*, 2010b). This de-arrangement of the contractile apparatus, evidenced by the disassembly of sarcomeric structures, that is also shown in proliferating cardiomyocytes in the mouse embryo (Ahuja *et al*, 2004), is accompanied by the expression of developmental marker genes including *gata4*, *nkx2*.5, *hand2*, *tbx5* and *tbx20* (Jopling *et al*, 2010b; Kikuchi *et al*, 2010). Therefore, there is a need to understand the genetic reprogramming of cardiomyocytes that induces their proliferation.

For instance, NF-kB signaling is activated in cardiomyocytes upon injury and its disruption leads to reduced cardiomyocyte proliferation as well as fewer epicardial cells (Karra 2015). By applying ribosome affinity purification of translating mRNAs in cardiomyocytes, Fang *et al* (2013) identified activation of the Jak1/Stat3 downstream signaling factors that, upon inhibition, affected cardiomyocyte proliferation as well as fibrotic healing (Fang *et al*, 2013). Signaling from the epicardium has gathered the attention of many studies since the observations that epicardial cells actively participate in the regenerating response and promote proliferation of cardiomyocytes by the secretion of mitogens. For instance, the retinoic acid synthesizing enzyme *raldh2* is expressed in the epicardium upon injury and is required for cardiomyocyte proliferation. Also, it has been shown that the epicardium secretes *cxcl12a* to promote the migration of the cardiomyocytes into the injured area (Itou *et al*, 2012). Moreover, by lineage-tracing and transplantation studies, a population of epicardial cells infiltrating into the injury area has been identified and, although they are not a source for the growing myocardium(conversely to what has been shown in mammals(Smart *et al*, 2011)),they transdifferentiate into myofibroblasts and perivascular cells possibly to modulate cardiomyocyte proliferation and neoangiogenesis (Kikuchi *et al*, 2011a; González-Rosa *et al*, 2012). On the other hand, members of the Notch signaling pathway are expressed in the epicardium and endocardium during zebrafish heart regeneration. However, both inhibition and hyperactivation of Notch signaling impaired cardiomyocyte proliferation and regeneration (Zhao *et al*, 2014).Other factors described so far shown to be expressed during injury and to promote cardiomyocyte proliferation include *tgfb1* (Chablais & Jazwinska, 2012), *pdgf* (Lien *et al*, 2006), sonic hedgehog (Choi *et al*, 2013) or *igf2b* (Huang *et al*, 2013). Negative regulators of cardiomyocyte proliferation have also been found although to a lesser extent. For instance, p38α MAPK activation has been shown to arrest cardiac growth during development and to negatively regulate mammalian adult cardiomyocyte proliferation (Engel *et al*, 2005). In the zebrafish heart, similarly to mammals, constitutive expression of Mkk6, a p38 upstream kinase

in the myocardium, arrested cardiomyocyte proliferation (Jopling *et al*, 2012b).Besides identification of the cell lineage, it is also important to analyze the different zones of the heart that are affected when using ventricular resection or cryoinjury. In this regard, a spatial-temporal transcriptomic analysis revealed three different areas in the heart: the uninjured, the lesioned and the border zone, with distinct expression profiles (Wu *et al*, 2016).The authors observed that the border zone was enriched for BMP signaling molecules expressed in the cardiomyocytes (also in the epicardium and endocardium) that promoted their proliferation.

Table I.1. Summary of the principle characteristics reported for each cardiac injury model in adult zebrafish.

Time after injury	Resection	Cryoinjury	Genetic ablation
4h	Cell death at the border of the amputated area	Massive cell death (including vascular cells) Epicardium activation and thickness	
1 day	Blood accumulation	Blood accumulation Infiltration of immune cells	
3-5 days		Decreased cell death Sprouting of coronary vasculature Epicardial cells into injured area Increased cell proliferation (epicardial, endothelial, endocardial, cardiomyocytes) Thickening of epicardium	Cell death distributed in the ventricle Cell death of atrial myocytes Presence of immune cells
7 days	Cardiomyocyte proliferation Fibrotic scar at the border of the amputated area Minor collagen deposition	Massive collagen deposition Cardiomyocyte proliferation	Decreased cell death Massive and distributed cardiomyocyte proliferation in ventricle and atrial myocytes Epicardial cell proliferation
14 days	Increased cardiomyocyte proliferation Residual scar	Decreased injured area	Massive and distributed cardiomyocyte proliferation Occasional collagen deposition
21-30 days	Residual scar Decreased cardiomyocyte proliferation	Complete regeneration of coronary vasculature Decreased cardiomyocyte proliferation Compact layer closed	Fully myocardial recovery Decreased cardiomyocyte proliferation
90 days	Complete scar clearance	Residual scar at the interior of the injured area	
130 days		Complete regression of fibrotic scar Functional recovery incomplete	

Characteristics of each injury model at the approximate time post-manipulation (Time).

In zebrafish, hypoxia has been shown to stimulate cardiomyocyte proliferation (Marques *et al*, 2008; Jopling *et al*, 2012a) and particularly after injury (in a model of ventricular resection), a condition during which cardiac oxygen supply may be reduced creating an hypoxic environment. Moreover, disruption of HIF1α signaling leads to decreased cardiomyocyte DNA

synthesis and incomplete heart regeneration (Jopling *et al*, 2012a). In neonatal mice, reactive oxygen scavenging (ROS) production or hypoxia delayed cardiomyocyte cell-cycle arrest in a process likely involving HIF1α (Guimarães-Camboa *et al*, 2015), similarly than zebrafish. The authors suggested that the transition to an oxygen-rich environment at birth, together with the switching to an aerobic metabolism, impairs the proliferating capacity of mammalian cardiomyocytes and that the hypoxic environment of zebrafish could be a reason for the regenerative capacity of this teleost (Puente *et al*, 2014). Intriguingly, contrasting with the described findings, Han *et al* (2014) found that between 3 and 14 dpi, epicardial and myocardial cells at the amputated border produced H_2O_2 that was required for heart regeneration (Han *et al*, 2014), in accordance with other studies in lower vertebrates where oxidative stress favors regeneration (Love *et al*, 2013; Santabárbara-Ruiz *et al*, 2015)

It is important to keep in mind that post-transcriptional regulation may also modulate the expression of genes relevant for zebrafish heart regeneration. In an attempt to identify differentially expressed microRNAs during the regenerating process, Yin *et al* (2012) identified cell cycle and cell junction genes as targets for miR-133. miR-133 was shown as a negative regulator of cardiomyocyte proliferation by modulating the expression of connexin 43(Yin *et al*, 2012). Interestingly, another microRNA has been implicated in the scar clearance in zebrafish heart regeneration. Beauchemin *et al* (2015) observed that initial down-regulation of miR-101a is required for cardiomyocyte proliferation and subsequent up-regulation at 7-14 dpi was shown to be essential during the removal of the fibrotic scar (Beauchemin *et al*, 2015). The dynamic expression of miR-101a suggests that scar formation is an indispensable mechanism for the regenerating process and highlights the relevance of temporal approaches for the study of the mechanisms underlying heart regeneration. In this regard, Rodius *et al* (2016) performed a complete transcriptomic profiling study at specified time-points of the regenerating process, from 4 hours to 90 days, that identified a dynamic expression pattern of different genes. Further studies on the functional role of the genes identified will provide knowledge on the conserved genetic regulation of heart regeneration between zebrafish and mammals. Given the relevant role of the epicardium, a recent transcriptomic analysis from distinct epicardial lineage cells also contributed to further understand the epicardial-drived mechanisms for heart regeneration (Cao *et al*, 2016).

To date, relevant signaling pathways and intrinsic and extrinsic factors that contribute to cardiomyocyte proliferation and heart regeneration have been identified by applying different approaches (e. g. gain and loss of function studies, genetic lineage tracing, etc). More importantly, these studies revealed that the process of regeneration not only involves a cardiomyocyte cell response to injury but also different type of cells of the heart such as epicardial and endocardial cells as well as neural innervation (Mahmoud *et al*, 2015), suggesting paracrine regulation and, possibly, a complex systemic response to injury that is still poorly understood.

4.3. Zebrafish as a novel vertebrate exercise model

Research on exercise or swimming-induced exercise in fish has been particularly focused in migratory species to understand the migratory behavior, reproductive maturation as well as skeletal muscle growth, with especial attention to commercially relevant species for the

aquaculture industry (Davison, 1997). In fish, it is known that swimming-induced exercise has beneficial effects on muscle growth and survival rate, in reducing aggressive behavior and improving resistance to fatigue, heart performance and oxygen carrying capacity, although the effects may vary depending on the training protocol and the species used (Davison, 1997). As a method of standarization, swimming speed in fish is measured in body lengths per second (BL/s) since the length of the fish indicates a measure of distance that is relative to the developmental stage of the fish for a particular species. The optimal swimming speed (U_{opt}) is defined as the speed at which the energetic efficiency is highest and the cost of transport (i.e. oxygen uptake per unit of distance swum) is lowest (Weihs, 1973; Tudorache *et al*, 2013). Our group has established the swimming economy of adult zebrafish and its U_{opt} was determined at 13 BL/s (Palstra *et al*, 2010), a swimming speed that is surprisingly high in comparison with than that reported in salmonid fish (e.g. 1-2 BL/s), indicating that zebrafish is capable of sustaining high speeds of swimming without signs of fatigue (Plaut & Gordon, 1994).

Although our previous study (Palstra *et al*, 2010) was the first to investigate the physiological effects of swimming at U_{opt}in adult zebrafish, other studies have used zebrafish (mostly at larval stages) under swimming exercise conditions, establishing this small teleost fish as an emerging model in exercise physiology, comprising the areas of muscle growth and development, metabolism and aging research (Palstra *et al*, 2010, 2014; Pelster, 2002; Pelster *et al*, 2003; LeMoine *et al*, 2010a; Hasumura & Meguro, 2016). Remarkably, although several studies on exercise physiology using zebrafish have been performed, there has been very little research studying the effects of exercise on the cardiac muscle and none on heart regeneration.

Previous studies in adult zebrafish subjected to long-term training periods (4 and 8 weeks) did not report an exercise-induced growth potentiation in terms of weight, length or muscle fiber growth(McClelland *et al*, 2006; LeMoine *et al*, 2010a). Both studies trained the fish for 3 h twice a day during 6 days per week and used considerably lower swimming speeds than that described by Palstra *et al* (2010), with an initial swimming speed of 2 BL/s and increasing 1 BL/s weekly (reaching to 5-10 BL/s at the end of the training). In contrast, the study by Palstra et al (2010) observed an increase in body weight and length by exercising adult zebrafish at the determined U_{opt} for 6 h a day, 5 days per week during 4 weeks (20 days) but did not evaluate possible changes in muscle fiber cellularity. At the molecular level, previous studies suggested an adaptation of adult zebrafish skeletal muscle to a more aerobic phenotype as observed by the increased mRNA expression levels of the nuclear respiratory factor 1 (*nrf1*) and citrate synthase (*cs*) after 1 week of training. Although *pgc1a* expression was found to be up-regulated after 4 weeks, the authors suggested that this factor is not involved in mitochondrial biogenesis in fish but could play a role in the regulation of metabolism during the recovery period after exercise (McClelland *et al*, 2006; LeMoine *et al*, 2010a, 2010b).Therefore, a detailed comprehension of the transcriptomic changes that occur in skeletal muscle in response to swimming-induced exercise in adult zebrafish would provide insights into the regulatory mechanisms underlying the adaptive response to exercise. For instance, in salmonid fish exercised at their U_{opt} during 40 days, expression profiling by deep RNA-sequencing (in slow and fast muscle) revealed that exercise increased the expression of genes in both types of muscle (e. g. myogenic factors, cytoskeletal and contractile elements, metabolic and immune

factors) and interestingly, showed an important contribution of fast muscle in sustained aerobic swimming. Considering the available genomic resources in zebrafish, the use of high-throughput sequencing techniques would provide further knowledge of the exercise response in vertebrates by using the zebrafish as an exercise model.

On the other hand, giving the advantages of zebrafish for developmental research (i.e. conserved developmental processes, larvae manipulation, *in vivo* imaging, etc) and the energy consuming processes taking place during developmental stages, more studies have investigated the effects of exercise in zebrafish larvae. In terms of growth, contrasting observations have been reported. For instance, larvae at distinct ages (4-5, 9-15 or 21-32 days post fertilization, dpf) subjected to swimming training at 2 BL/s or 5 BL/s did not show significant differences in length, although exercised larvae showed a rapid reduction of the yolk sac volume (Bagatto *et al*, 2001). However, in one study performed under similar conditions, 14 dpf exercised larvae increased in length (Fiaz *et al*, 2012) and in another study performed with larvae up to 84 dpf (approximately 3 months of age) a transient significant increase in length and weight that was blunted after 35 dpf was reported (van der Meulen *et al*, 2006). Moreover, swimming-induced activity in zebrafish larvae increased volume density of mitochondria in slow (but not fast) fibers from the axial muscle together with an increase in vascularization in the tail at late developmental stages (32 dpf) (Pelster *et al*, 2003). Furthermore, cardiac function parameters such as heart rate, stroke volume or end-diastolic volume were not affected by exercise after 6 or 11 days of training (Pelster *et al*, 2003).

Considering the reported beneficial physiological function of myokines in mammals, as previously stated, zebrafish exercise physiology offers a promising area of study that has not yet been investigated in any other fish species. Indeed, several myokines are also found in zebrafish, although in comparison with its mammalian counterparts some of the zebrafish genes have two isoforms resulting from the extra genomic duplication in teleosts (and a third in salmonids). A majority of the myokines discovered in mammals and mentioned above, have their coding genes present in the zebrafish genome. **Table I.2** shows the mammalian myokine genes and the corresponding zebrafish homologs identified to date.

Besides the availability and potential use of transgenic zebrafish lines for functional studies on genes involved in the adaptive response to exercise (i.e. transgenic lines that due to their genetic background might have differences in swimming performance), another advantage of zebrafish as an exercise model is that exercise in fish is achieved only by swimming as they are easily stimulated to swim against a water current (i.e. rheotactic response) since it is part of their natural behavior. Thus, unlike mammals, that require a training or adaptation period to a be able to be exercised by treadmill or swimming (which is not a natural behavior of rodents), zebrafish do need to be previously trained to swim and only require acclimatization to the swim tunnel "environment" to minimize stress. Although this consideration may apparently seems obvious, the fact that swimming is a natural stimulus to induce exercise in zebrafish can be advantageous for exercise studies particularly when considering that different exercise training strategies are commonly used in mammals (e. g. voluntary exercise, treadmill running or swimming) that may complicate comparison of the results. Furthermore, it is important to stress that among the studies published to date on swimming-induction in zebrafish,, distinct

swimming protocols have been used that vary in the duration and intensity of exercise, similarly to exercise studies using mammalian species. Since zebrafish is still emerging as an exercise model, it would be important to work towards a standarization of distinct types of swimming protocols through consensus of the scientific community. Moreover, depending on the swim tunnel used, fish can be held in the swim tunnel until the end of the experiment (ensuring a recirculation system of the water), avoiding daily handling of the animals at every training session. Although different research groups have used custom swim tunnels in zebrafish and other teleost species, due to the growing interest in the use of zebrafish as an exercise model there has been an increase in the production of specialized swim tunnels for zebrafish or similar sized fish by professional companies that ensure a constant laminar flow and that provide the means to monitor oxygen consumption. However, as an exercise model, the zebrafish also presents some limitations such as the difficulty of obtaining blood samples to measure, for instance, metabolites or circulating myokines.

Table I.2. Zebrafish gene sequences from described mammalian myokines. The majority of them have been identified in zebrafish. The sequence status from GeneBank Database and the transcript accession number are shown.

Mammals	Available?	Zebrafish	Status	Accession number
interleukin 6 (IL-6)	Yes	*il6*	PROVISIONAL	NM_001261449.1
interleukin 15 (IL-15)	Yes	*il15*	PROVISIONAL	NM_001039565.1
interleukin 8 (IL-8)	Yes	*il8*	MODEL	XM_001342570.5
Interleukin 7 (IL-7)	Not identified	-	-	-
myostatin (MSTN)	Yes	*mstnb*	PROVISIONAL	NM_131019.4
secreted protein acidic cysteine-rich (SPARC)	Yes	*sparc*	PROVISIONAL	NM_001001942.1
meteorin-like	Yes	*metrnl*	PROVISIONAL	NM_212985.1
decorin (DCN)	Yes	*dcn*	PROVISIONAL	NM_131697.1
follistatin-like 1 (FSTL-1)	Yes	*fstl1a*	PROVISIONAL	NM_001017860.1
fibroblast growth factor 21 (FGF-21)	Yes	*fgf21*	PROVISIONAL	NM_001045324.1
irisin (FNDC5)	Yes	*fndc5b*	PROVISIONAL	NM_001044337.1
apelin (APLN)	Yes	*apln*	VALIDATED	NM_001166124.3
brain-derived neurotrophic factor (BDNF)	Yes	*bdnf*	VALIDATED	NM_001308648.1
leukemia inhibitory factor (LIF)	Yes	*lif*	PROVISIONAL (released dec2015)	NM_001079833.1
insulin growth factor 1 (IGF-1)	Yes	*igf1*	PROVISIONAL	NM_131825.2

The use of zebrafish as an exercise model could improve our knowledge on the beneficial effects (still not well characterized) of exercise in vertebrates in health and disease. Skeletal muscle is the main tissue responsible of locomotion and, although extensive research has been performed in this tissue, there is considerable interest on elucidating the role and the molecular mechanisms of muscle contractile activity in human pathologies as well as during development. Moreover, exercise has shown to provide benefits in cardiovascular diseases and thus, there is a need to study the role of exercise-driven cardioprotection in mammals, making the zebrafish a suitable model for complementing mammalian and human studies.

Taking advantage of the zebrafish model and continuing on our previous studies on swimming-induced exercise in adult zebrafish (Palstra *et al*, 2010), the purpose of the present study is to improve our knowledge on the cellular and molecular effects of exercise skeletal and cardiac muscle in this promising exercise experimental model.

OBJECTIVES

“The best scientist is open to experience and begins with romance - the idea that anything is possible”

Ray Bradbury (1920-2012)
Science-fiction writer

OBJECTIVES

In this thesis we pursued the following objectives:

1. To examine theadaptation of fast skeletal muscle fibers to exercise conditions by identifying the molecular and cellular changes related to fiber hypertrophy and proliferation.

2. To study the temporal gene expression of known muscle factors named "myokines"in response to exercise.

3. To study the exercised fast skeletal muscle expression profile after an immune challenge.

4. To study the effects of exercise in cardiac muscle by specifically evaluating the transcriptomic response, cardiomyocyte proliferation and changes on cardiac function.

5. To evaluate the cardioprotectiveeffects of exercise within the context of cardiac regeneration.

MATERIALS AND METHODS

“An experiment is a question which science poses to Nature, and a measurement is the recording of Nature’s answer”

Max Planck (1858 to 1947)

Nobel Prize in Physics in 1918

Ethical approval

All animal procedures described herein have been approved by the Ethical Committee of the University of Barcelona under protocols CEEA 400.14 and 719.15 to J.V.P.

Exercise training and experimental conditions

Adult wild-type zebrafish used in this study were purchased from a commercial supplier and grown to approximately 2.5 to 3 cm. Control and exercised group of fish were always similar in size in the same experiment in order to avoid deviations when calculating the swimming speed in Body Lengths per second (BL/s).

Adult zebrafish were housed in two large 127 L swimming tunnels in a recirculation system at the University of Leiden, The Netherlands (**Figure M1.A**). The non-exercised group of fish ("**non-exercised**") and the exercised group of fish ("**exercised**") were housed at the same population density. In a previous study, our group determined adult zebrafish *optimal swimming speed* (U_{opt}) at 0.4 m/s (13 BL/s), which is the speed with the highest energetic efficiency and the lowest oxygen uptake per distance swum(Palstra *et al*, 2010).As previously described, exercised fish were swam at their U_{opt} for 6h/day (10:00-16:00h) for 5 days/week (Monday to Friday) over 20 experimental days (over a four-week period). Control fish were kept in a resting condition in a separate tunnel at a flow rate of 0.1 m/s to ensure sufficient mixing of the water. Fish were fed *ad libitum* twice a day, before and after the swimming trials. This set-up was used only for the microarray experiments described in Chapter 1, 3 and 4. A video recording of the non-exercised and exercised zebrafish is available (**Movie1**).

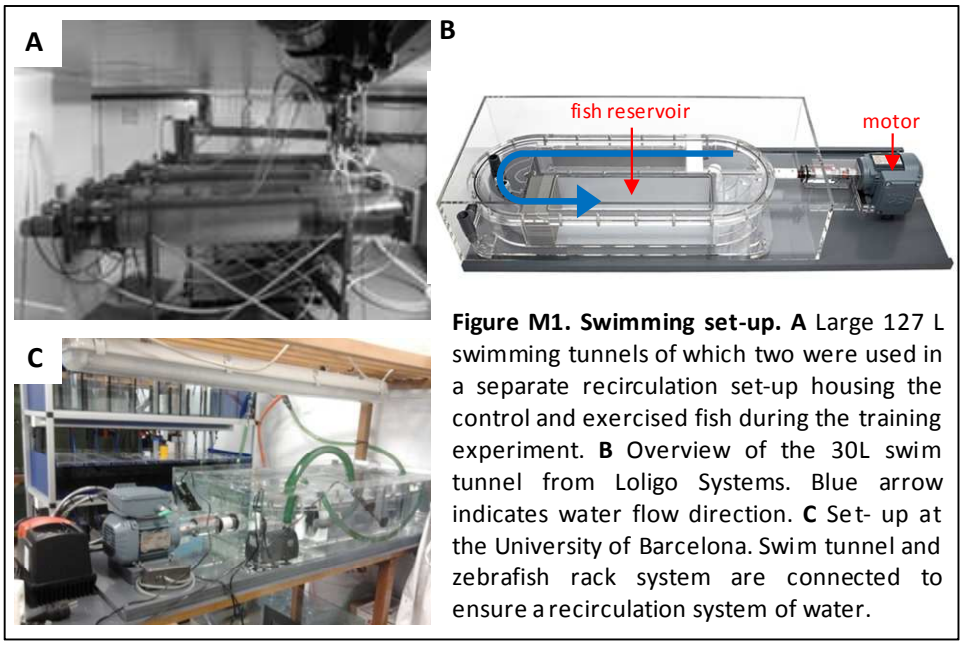

Figure M1. Swimming set-up. A Large 127 L swimming tunnels of which two were used in a separate recirculation set-up housing the control and exercised fish during the training experiment. **B** Overview of the 30L swim tunnel from Loligo Systems. Blue arrow indicates water flow direction. **C** Set- up at the University of Barcelona. Swim tunnel and zebrafish rack system are connected to ensure a recirculation system of water.

Subsequent swimming experiments were performed at the University of Barcelona under similar swimming conditions as detailed above by using a 30 L swimming tunnel (SW10150, Loligo Systems) (**Figure M1.B**). Control fish were maintained in an aquarium rack connected with the swimming tunnel through a water recirculation system (**Figure M1.C**). The control

exercised groups of fish were housed at the same population density. For the time-course experiments, fish were removed from the system at week 1 (5 days of swimming), week 2 (10 days of swimming) and week 4 (20 days of swimming) after the daily training. A video recording of the non-exercised and exercised zebrafish is available (**Movie2**).

Lipopolysaccharide (LPS) challenge

After the exercise training experiment, exercised and non-exercised fish were injected intraperitoneally with either 6 mg/kg body weight (Kaitetzidou *et al*, 2012) of purified LPS from *Escherichia coli* (E. coli clone 026:B6, L-8274 Sigma) or with the same volume of PBS. Fast muscle samples were obtained 72 hours post-injection and were flash-frozen in liquid nitrogen for RNA isolation.

In vivo bacterial challenge with Pseudomonas aeruginosa

After the four-week training period, exercised and control zebrafish were transferred to the *Universitat Autònoma de Barcelona* (UAB, Bellaterra) at the *Insitut de Biotecnologia i de Biomedicina*. Subsequently, exercised and non-exercised fish were housed in two different tanks and acclimated for two days before each experiment. The infection protocol previously developed by Ruyra *et aL* (2014) was performed (Ruyra *et al*, 2014). Briefly, fish were anaesthetized by immersion in tricainemethanesulfonate (0.168 mg/ml, MS-222) and 20 µl of a *Pseudomonas aeruginosa* (PAO1, sub-line MPAO1) suspension at a concentration of approximately 5.3×10^7cfu was injected intraperitoneally using a Hamilton syringe. Once fish were recovered, they were returned to the experimental tanks. The fish were observed daily and their survival was assessed for 5 days. The whole procedure (training + infection) was performed in three independent experiments in order to further corroborate the results. Survival curves were analyzed using the Kaplan–Meier method and the statistic differences were evaluated using the log-rank test (GraphPad, USA).

Induction of heart lesion by cryoinjury

Procedures described in Gonzalez-Rosa and Mercader (2012) were followed. Briefly, fish were anesthetized by immersion in0.168mg/ml of buffered MS-222 and immobilized under a stereomicroscope ventral side up. Scales around the incision area were removed to facilitate surgery. A small incision was made with microdissection scissors using the pericardium pigmentation as a landmark. Promptly, the ventricle was exposed by pressing the abdomen of the fish and the excess water was dried with a paper towel. The cryoprobe, previously frozen in liquid nitrogen, was applied to the ventricular wall and, once the cryoprobe had thawed, it was removed and the fish was placed in a tank with clean water to recover. Fish survival was equivalent to that reported by Gonzalez-Rosa and Mercader (2012).

Exercise training and subsequent recovery from heart injury

After the four-week training period, exercised (n=15) and non-exercised (n=15) fish were transferred to the zebrafish facility of the Centro Nacional de Investigaciones Cardiovasculares (CNIC, Madrid) and acclimated for two days. Subsequently, fish were housed individually in 1.5 L tanks to perform a longitudinal study on heart lesion recovery after exercise. For that purpose, control and exercised fish were lesioned as detailed above and kept in 1.5 L tanks for 28 days post injury (dpi). At approximately twenty-four hours before being sacrificed (27 dpi),

fish received an intraperitoneal injection of BrdU (5-bromo-2'-deoxyuridine). BrdU diluted in water at 2.5 mg/ml was administered intraperitoneally (30 µl) at approximately twenty four hours (27 dpi) before sampling (28 dpi). Cardiac function in control and exercised fish was assessed by echocardiography, as described previously(González-Rosa *et al*, 2014), before the myocardial lesion (0 dpi), at 7 days post-injury (7dpi) and at 28 days post-injury (28dpi). A schematic representation of the experimental design is shown in **Figure M2**. Briefly, fish were anaesthetized in 60 µM MS-222 and 3mM isoflurane dissolved in fish water and placed in a foam holder for immobilization. Two dimensional images were obtained using the Vevo 2100 Imaging System (Visual Sonics) equipped with a 50 MHz ultrasound probe that was immersed into the solution. Measurements at end-diastole and end-systole were performed in B-mode images by tracing the ventricle from the bulbo-ventricular valve annulus to the apex. Parameters measured were heart rate (bmp), systolic and diastolic area (mm^2), systolic and diastolic volume (µl), stroke volume (µl) and ejection fraction (%).

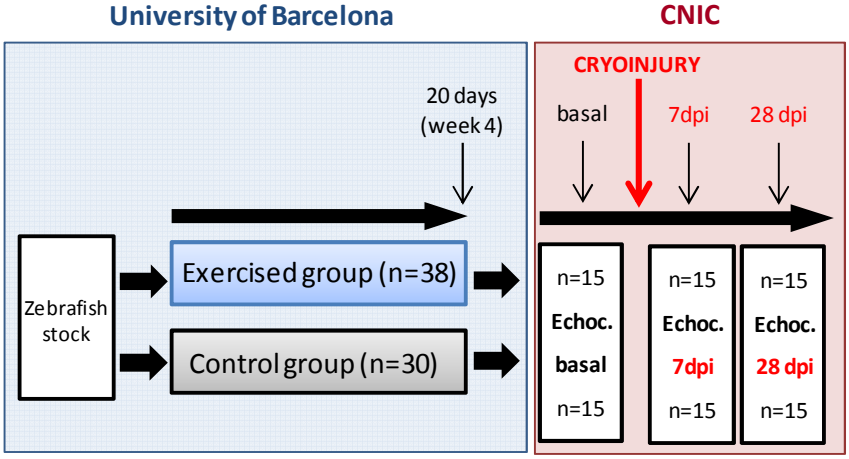

Figure M2. Schematic representation of the experimental design to evaluate adult zebrafish cardiac function. After the cryoinjury procedure, the mortality was very low, only two fish from the exercised group did not survive.

Exercise training during recovery from heart injury

In order to evaluate the effects of exercise training during recovery from a heart lesion, non-exercised fish were subjected to heart cryoinjury as described above and allowed to recover for 48 h. At 3 dpi, one group of cryoinjured fish (n =36) were introduced in the swimming tunnel and exercised daily following the training conditions described above (injured exercised), whereas a control group (n = 35) was maintained in resting conditions (injured control). An additional control group (sham) was included as a control for the myocardial injury (n = 31) by performing a small incision with the microdissection scissors without lesioning the ventricle. Fish were sacrificed at 7 dpi and 14 dpi. At 14 dpi, a group of exercised fish (n = 5) and control fish (n = 5) received an injection of EdU (1.25 mg/ml) and were sampled the next day (see below). Ventricle samples were obtained from the three experimental groups, flash frozen in liquid nitrogen and stored at -80ºC. Whole heart samples from exercised (n=5) and control (n=5) group at 7 and 15 dpi were obtained and processed for histological procedures as described below. **Figure M3** shows a schematic representation of the experimental design.

Sampling

Fish were sampled after the experiments and euthanized by immersion in 0.25mg/ml of buffered MS-222.

From fish that swam at the facility of the University of Leiden, dorsal epaxial fast muscle filets were dissected and either immediately frozen in isopentane cooled to -160°C and stored in liquid nitrogen until sectioned for histochemical analyses or stored at -20ºC in RNAlater (Life Technologies, Barcelona, Spain) for microarray analyses. Hearts from these fish were also dissected and stored at -20ºC in RNAlater for microarray analyses.

From fish that swam at the facility of the University of Barcelona, two fast muscle filets were dissected from the dorsal (epaxial) musculature taking care to avoid slow muscle contamination, flash frozen in liquid nitrogen and stored at -80ºC until processing for RNA and protein isolation. The heart was also dissected, placed in sterile PBS to remove the atrium or bulbusarteriosus and the remaining ventricle was subsequently flash frozen in liquid nitrogen and stored at -80ºC.Hearts were also collected for histology and kept in a solution containing KCl 0.1 M, 2 U/ml heparin in PBS buffer to arrest the heartbeat and avoid blood coagulation. Subsequently, hearts were fixed in 4% paraformaldehyde (PFA)/PBS at 4ºC overnight under agitation, washed in PBS, dehydrated through graded alcohols to 70% ethanol and stored at -20ºC.

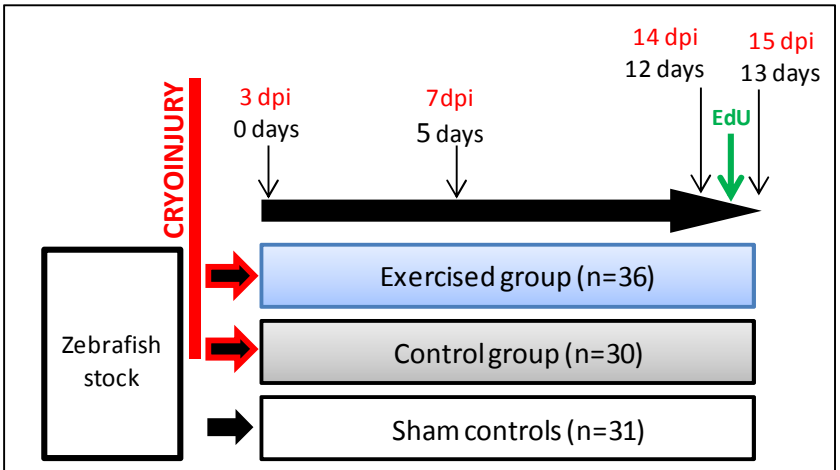

Figure M3. Experimental design to inverstigate the effects of exercise during the regeneration process. Black arrows on the time line represent the sampling time points. At 3 dpi lesioned heart samples were taken to evaluate the myocardial lesion progression before the training period. The exercise experiment started at 3dpci (0 days) and samples were taken at 7dpi (5 days exercising) and 14-15 dpi (12-13 days exercising). Green arrow represents the EdU injection administered the day before sampling.

RNA extraction

Total RNA from a single fast muscle filet was isolated by homogenizing the tissue with a manual homogenizer (ProScientific) with 500 µl TriReagent (Ambion) and following the manufacturer's specifications. The obtained RNA was subsequently precipitated with NaOAc (Fermentas) and resuspended in RNAse free water. Total RNA from two, three or four ventricles (except for microarray analysis where whole hearts were used) was isolated by quickly disrupting the tissue with a scalpel and homogenizing with a Pellet pestle (Sigma) in

150 µl of QIAzolLysis Reagent. Pooled samples were processed with the miRNeasy MicroKit (Qiagen). The miRNeasy Micro Kit DNAse treatment was included for RNA-seq analysis to remove genomic DNA, following the manufacturer's specifications. RNA concentrations were determined using a Nanodrop 2000 spectrophotometer (Thermo Scientific). For quantitative real-time PCR, DNAse treatment was performed as detailed below.

Quantitative real-time PCR

One microgram of RNA was treated with DNAse I Amplification Grade (Life Technologies) to remove any contaminating genomic DNA and reverse transcribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche) as specified by the manufacturer. Reactions were run in a CFX384™ Real-Time System (Bio-Rad) under the following thermal cycling conditions: 3 min at 95 °C and 40 cycles of 10 s at 95 °C, 30 s at the corresponding annealing temperature and a final melting curve of 81 cycles from 55 to 95 °C (0.5 °C increase every 30 s) to analyze the specificity of the reaction and absence of primer dimers. The reactions (5 ul) contained 2.5 µL of iQ SYBR Green Supermix (Bio-Rad), 500 nM of forward and reverse primers and 1 µL of cDNA for each sample (diluted 1:5) and followed the requirements of the MIQE guidelines. All PCR reactions were run in triplicate. Primer efficiency was calculated by analyzing serial dilutions of cDNA samples and was higher than 90%. The expression level of each gene was normalized to the two or three most stable reference genes tested, rps15, rps18 and rpl11 (M-value < 0.5) and quantified using CFX384 software (modified (Pfaffl, 2001)). Primer sequences were designed using PerlPrimer Software or Roche Universal Probe Library Assay Design Center. Primer sequences, amplicon sizes and accession numbers are listed in **Table M1**.

Microarray analysis

Single color microarray-based gene expression analysis was performed using an Agilent custom oligo microarray 4x44K with eArray design ID 021626 and containing a total of 43.863 probes of 60 oligonucleotides in length. RNA was amplified and labeled with Cy3 dye using single color Low Input Quick Amp Labeling kit (Agilent Technologies) following the manufacturer's indications using 200 ng of RNA in each reaction. Next, 1.65 µg of labeled cRNA were hybridized to the arrays. Overnight hybridization (17 h 65°C and 10 rpm rotation) was performed in a Microarray Hybridization Oven (Agilent Technologies). After hybridization, microarrays were washed with Gene Expression Wash Buffers 1 and 2 (Agilent Technologies) and scanned using the High-Resolution C Scanner (Agilent Technologies). Feature Extraction Software 10.7.3 (Agilent Technologies) was used for spot to grid alignment, feature extraction and quantification. Processed data were subsequently imported into GeneSpring GX 11.5 (Agilent Technologies). For the DEGs, gene IDs were converted to human ENSEMBL gene IDs using g:orth function from G: profiler (http://biit.cs.ut.ee/gprofiler), taking advantage of the more complete gene ontology (GO) annotations of the human genes and improving, in this way, the subsequent analysis of the functional categories.

For fast muscle analysis, total RNA from fast skeletal muscle samples of individual adult zebrafish from Experiment 1 (control, n = 8; exercised, n = 8) was isolated with TRIzol (Life Technologies). Significance cut-offs for the ratios of exercised vs control were set at $P < 0.01$ (sample t-test) and >1-fold change for differentially expressed genes (DEGs). The complete microarray data have been deposited in NCBI´s Gene Expression Omnibus and are accessible

through GEO Series accession number GSE58929 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE58929). GO enrichment analysis was performed using Database for Annotation, Visualization and Integrated Discovery (DAVID) software tools (Huang *et al*, 2009) (http://david.abcc.ncifcrf.gov) and the resulting categories were considered significant at $P < 0.05$. Pathway and network analyses were conducted using Ingenuity Systems Pathway Analysis (IPA) software. To analyze by IPA, annotated spots were mapped to zebrafish and human orthologs using BLASTN against the Ensembl *Danio rerio* gene database (v.Zv9.66) and the Homo sapiens transcript database (v.GRCh37.66) with an e-value ≤1.00E − 05. Human and zebrafish orthologs were then compared to the Ingenuity Knowledge Base (www.ingenuity.com) and significantly altered pathways and biological functions were determined using the Fisher exact test ($P < 0.05$).

For heart analysis, three to four adult zebrafish heart pools collected from the experiment detailed above, were used to extract total RNA using the RNeasy Micro Kit (Qiagen) and quantified using a Nanodrop2000 Spectophotometer. Data was normalized and filtered and significant cut-offs for the differentially expressed genes were set at a P-value <0.05 and 1.2 fold change.

RNA-sequencing

A total of 24 samples were sent to GenomeScan (Leiden, The Netherlands) for library preparation and sequencing. To assess the concentration and quality of the RNA, the Fragment Analyzer (Advanced Analytical) was used. All samples passed the quality checks, being the lowest RQN=8.9 (RNA Quality Number). The NEBNext Ultra Directional RNA Library Prep Kit was used to process the samples and resulting products measured between 300-700bp. Samples were sequenced using the next generation sequencing (NGS)platform IlluminaNextSeq 500. Finally, a minimum sequencing depth of 15M reads (>95% of base quality>30) per sample was obtained and the alignment was performed using the RNA aligner TopHat v.2.1.0 against the reference zebrafish genome GRCz10.

Muscle histochemical analyses

After placing the fast muscle frozen samples in OCT embedding medium at 22°C, serial transverse sections of 16 µm in thickness were obtained in a cryostat (Leica CM3050S) and mounted on 2% gelatinized slides. Two histochemical assays were performed on fast muscle serial sections: (1) succinate dehydrogenase (SDH) according to (Nachlas *et al*, 1957) in order to demonstrate the aerobic or anaerobic characteristics of muscle fibers; and (2) endothelial ATPase according to (Fouces *et al*, 1993) to reveal muscle capillaries. All morphofunctional measurements of fast muscle cellularity and vascularization were performed on the sections processed for endothelial ATPase activity. All the parameters listed below were empirically determined from windows of tissue of approximately 5.5×10^5 μm^2 from two different zones or muscle fields in each sample. After testing for the absence of differences between the two muscle fields from each sample, the data obtained from both fields were considered together. The mean results presented throughout tables and figures were obtained from a sample of n = 8 fish for each condition (control and exercised). In order to determine if swimming-induced exercise caused changes in the morphometric and vascularization characteristics of fast muscle fibers, the following parameters were counted or calculated: capillary density (CD; capillary

counts per unit cross-sectional area of muscle), fiber density (FD), capillary-to-fiber ratio (C/F = CD/FD; a parameter relatively independent of FCSA and a good indicator of muscle capillarization (Mathieu-Costello *et al*, 1996)), the number of capillaries in contact with each fiber (NCF) and the circularity shape factor ($SF = 4 \cdot \pi \cdot FCSA/FPER^2$), which is an estimation of the circular morphology of the fiber (with a value of 1 for a perfect circle). Capillary and fiber counts were calculated and expressed as capillaries and fibers per mm^2. The following fiber morphometric parameters were measured: fiber cross-sectional area (FCSA) and perimeter (FPER) and the maximal diffusion distance (MDD) between the capillary and the centre of the fiber. The total number of fibers analyzed in each muscle sample ranged from 200 to 250. The indices expressing the relationship between the number of capillaries per fiber and the fiber cross-sectional area ($CCA=NCF \cdot 10^3/FCSA$) or fiber perimeter ($CCP =NCF \cdot 10^2/FPER$) were also calculated. These indices are considered a measure of the number of capillaries per 1000 μm^2 of muscle FCSA and the number of capillaries per 100 µm of muscle FPER.

Data for all the parameters are expressed as sample means ± standard error of the mean (SEM). The histograms of FCSA (**Figure 1.1**) express the percentage frequencies of fibers grouped in intervals of 200 μm^2 and error bars represent the SEM. To obtain the superposed curves in the histograms, a dynamic fitting by nonlinear regression was performed for each group of fish (control and exercised). The approximation was done by a log-normal (four parameters) equation with a dynamic fit option of 200 for both total number of fits and maximum number of iterations.

Western blotting

Zebrafish fast muscle filet previously frozen were lysed in 300 µl using a manual homogenizer (ProScientific) whereas individual ventricles were lysed in 75 µl of RIPA buffer (Sigma) using a Pellet pestle (Sigma).The lysis buffer contained 1mM phenylmethylsulfonyl fluoride (PMSF), 1X protease inhibitor cocktail (Sigma) and 1X of phosphatase inhibitor cocktail 2 and 3 (Sigma). Lysates were incubated for 1h on ice and centrifuged at 10000 rpm for 5 min at 4ºC, the supernatant was recovered and stored at -80ºC. The BCA kit (Thermo Scientific) was used for total protein quantification. A minimum of 20 µg of protein lysates were loaded in a SDS-PAGE gel and transferred to a PVDF membrane (Millipore). Membranes were blocked in 5% milk diluted in PBS/0.5% Tween for 1h at room temperature and probed overnight at 4ºC with the primary antibody. Afterwards, membranes were washed in PBS/0.01% Triton (3 x 10min) and incubated for 2h at room temperature with an anti-rabbit or anti-mouse HRP-conjugated secondary antibody (1:10000, Jackson ImmunoResearch). Membranes were stripped with Restore Western Blot Stripping Buffer (ThermoScientific) following the manufacturer's indications to re-probe the membranes with the corresponding loading control. Effectiveness of the stripping procedure was checked everytime by incubating the membrane with the secondary antibody. Primary antibodies used were: PCNA (1:500, Anaspec), phospho-Histone 3 Ser10 (1:400, Millipore), phospho-mTOR Ser2448 (1:100, Cell Signaling), mTOR (1:1000, Cell Signaling), phospho-p70 S6 Kinase Thr389 (1:200, Cell Signaling), p70 S6 Kinase (1:200, Cell Signaling), phospho-4E-BP1 Thr37/46 (1:500, Cell Signaling), 4E-BP1 (1:500, Cell Signaling), phospho-p38 Thr180/Tyr182 (1:200, Cell Signaling), p38 (1:100, Santa Cruz), pax7 (1:20, DHSB), phospho-ACC (1:250, Cell Signaling), ACC (1:1000, Cell Signaling), PGC1a (1:100, Santa Cruz) and y-tubulin as a loading control (1:2000, Bethyl). The membranes were developed with the

enhanced chemiluminescence method using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare). Immunoreactive bands were visualized (LAS-3000; Fujifilm) and quantified with ImageJ software (http://rsb.info.nih.gov/ij/).

AMPK activity determination

AMPK activity from fast muscle lysates was determined using the CycLex AMPK Kinase Assay Kit (CycLex Co., Japan) following the manufacturer's specifications. Samples were incubated in a pre-coated plate containing mouse IRS-1 S789 substrate peptide with kinase reaction buffer (in the presence of 50 µM of ATP) containing DMSO or Compound C as inhibitor control (250 µM)for each sample during 30 min at 30ºC. Wells were washed 5 times with wash buffer and incubated with anti-mouse phospho-Ser-789 IRS-1 monoclonal antibody for 30min at room temperature to measure the amount of phosphorylated substrate. Wells were washed 5 times and incubated with an anti-mouse HRP antibody for 30min at room temperature and washed again. Tetra- methylbenzidine substrate (TMB) was added and incubated for 15min protected from light. Stop Solution was added to stop the peroxidase chromogenic reaction following the same pipetting order from the previous steps. The relative amount of AMPK activity was calculated as the difference between the absence (DMSO) or presence of Compound C at 450 nm and 550 nm. Activity was measured in duplicates, normalized to total protein in the muscle lysates, and expressed as fold induction with respect to the control group.

Staining techniques and quantification of myocardial area

Hearts stored in 70% ethanol were dehydrated to 100% ethanol, cleared in xylene and embedded in paraffin. Paraffin sections of 6 µm were cut using a Rotary 3003 microtome (Pfm Medical), mounted on Superfrost/Polylysine (VWR) and dried overnight at 37ºC. Conventional Hematoxylin and Eosin staining was performed in heart paraffin-embedded samples (C, n = 7; E, n =6). Briefly, slides were cleared in xylene (2x10min) and rehydrated by passing through serial dilutions of ethanol and distilled water. Slides were immersed in Hematoxylin for 7min, rinsed in tap water for 20 min to allow the stain to develop and followed by immersion in Eosin for 5 min. Subsequently, slides were dehydrated, cleared with xylene, and mounted in DPX (Scharlau). Hematoxylin and Eosin stained slides with 12 to 20 sections/slide were scanned with NanoZoomer 2.ORS (Hamamatsu) and total and compact myocardial areas were quantified using the Tissuemorph software (Visiopharm).

To reveal damaged tissue from cryoinjured heart samples, Acid Fuchsin Orange G (AFOG) staining was used. Slides were cleared in xylene (2x5 min) and rehydrated by passing through serial dilutions of ethanol and distilled water. Slides were fixed overnight in Bouin solution (Sigma) at room temperature and washed with distilled water. Nuclei were stained with Weigert's iron hematoxylin (Sigma) for 5 min and differentiated in 1% acid alcohol. Slides were rinsed in tap water and treated with aqueous 1% phosphomolybdic acid (Sigma) for 5min, rinsed with distilled water and stained with AFOG solution for 10 min (pH 1.09). Slides were rinsed and dehydrated and mounted as described before. Quantification of the injured area was performed using the ImageJ software.

In vivo labeling of cardiomyocytes

In an exercise trial performed at the University of Barcelona, exercised (n=38) and control fish (n=30) were anaesthetized by immersion in 0.168mg/ml of MS-222. 20 µl of EdU (5-ethynyl-2'-deoxyuridine, Click-iT kit) at 1.25 mg/ml (Life Technologies) diluted in sterile PBS were injected intraperitoneally using a Hamilton syringe once a week and24h before being sacrificed. After the 20 experimental days, fish were euthanized with an overdose of MS-222 (0.25 mg/ml) and hearts were collected and processed for histological analyses as described above. Paraffin slides were dewaxed by submerging in xylene (2x5min), absolute ethanol (2x3 min), and followed by a series of graded alcohols finishing with a destilledwater wash for 5min. Antigen retrieval was performed before the EdU reaction protocol by immersing the slides in 10 mM citrate buffer ph 6.0 and heating in a microwavefor 20 min and slides were subsequently allowed to cool in PBS. Slides were washed 2x3 min in 3%BSA/PBS and permeabilized in PBS-0.5% Triton for 20 min at room temperature. Slides were washed 2x3 min 3%BSA/PBS, the Click-iT reaction cocktail was prepared following manufacturer's indications and slides were incubated in a wet chamber for 30 min at room temperature. The following steps were carefully performed protected from light. Slides were washed 2x3 min in 3%BSA/PBS and blocking of non-specific binding for further antibody labeling was performed in 5%BSA, 5% sheep serum, 20mM $MgCl_2$ diluted in PBS for at least 1h in a wet chamber. Immunofluorescence was performed afterwards by incubating the slides with MEF2c antibody (1:100, Abcam) or MEF2 antibody (1:50, Santa Cruz) in a wet chamber at 4ºC overnight to specifically label cardiomyocyte cells. Slides were washed in PBS-0.1% Tween and incubated with secondary antibody AlexaFluor 555 (1:400, Life Technologies) for 45min at room temperature. Subsequently, slides were washed in 3x5 min PBS-0.1% Tween and incubated with Hoechst 33342 (1:2000, Thermo Scientific) for 30min at room temperature. Finally, slides were washed with PBS-0.1% Tween for 3x5 min and mounted in ProLong Gold Antifade mounting media (Life Technologies).

BrdU immunofluorescence

Imunofluorescence was performed as described above by incubating the slides with mef2 antibody (1:50, Santa Cruz) and BrdU antibody (1:100, BD Biosciences) in a wet chamber at 4ºC overnight to specifically label proliferative cardiomyocyte cells. Slides were washed in PBS-0.1% Tween and incubated with anti-rabbit secondary antibody AlexaFluor 555 and anti-mouse secondary antibody Alexa Fluor 488 (1:400, Life Technologies) for 45 min at room temperature. Washed in 3x5 min PBS-0.1% Tween and incubated with Hoechst 33342 (1:2000, Thermo Scientific) for 30min at room temperature. Slides were washed with PBS-0.1% Tween for 3x5min and mounted in ProLong Gold Antifade mounting media (Life Technologies).

In situ hybridization

Antisense probes for periostin b (*postnb*) and atrial natriuretic peptide (*nppa*) were kindly obtained from Dr. Nadia Mercader (University of Berne) and retinaldehyde dehydrogenase 2 (*raldh2*) probes from Dr. Carolin E. Burns (Massachusetts General Hospital). They were generated by linearizing 5 µg of target plasmid with EcoRI, SacI and XhoI (New England Biolabs) respectively. Digoxigenin-labeled antisense RNA probes were synthesized with T7 (*nppa*) or SP6 (*postnb* and *raldh2*) RNA polymerases (20u/µl, Ambion) and labeled with DIG RNA mix (Roche) for 2 h at 37ºC. Template DNA was digested by adding 2 µl of DNAse (10u/µl, Roche)

and incubated at 37ºC for 15 min and the enzymatic reaction was stopped by adding EDTA pH 8.0 to a final concentration of 8mM.ThecRNA products were precipitated with 4M LiCl and resuspended in DEPC water. RNA probes were quantified using Nanodrop2000 (Thermo Scientific) and confirmed integrity by agarose gel electrophoresis. Probes were stored at -20ºC with formamide. Slides were dewaxed, cleared in xylene and rehydrated through a graded series of ethanol dilutions until PBS. Slides were fixed in 4%PFA/PBS for 20 min at room temperature, washed for 2x5 min in PBS, permeabilized with proteinase K (10ug/ml) at 37ºC for 10 min, fixed in 4%PFA/PBS for 5min at room temperature, permeabilized with HCl 0.07N for 15 min at room temperature and blocked in 0.1M triethanolamine (pH 8.0) and 0.25% acetic anhydride for 10 min at room temperature. Slides were incubated in hybridization buffer for 2h at 65ºC in a wet chamber and hybridized overnight at 65ºC with hybridization buffer containing the probe. Slides were washed at 65ºC with hybridization buffer and MABT1X at room temperature. Slides were blocked for 2h at room temperature with blocking solution (10% blocking reagent, Roche; 20% sheep serum) in a wet chamber. Detection was done using an anti-DIG AP antibody (Roche) incubated at 4ºC overnight and probe hybridization was visualized using BM-purple substrate (Roche). Slides were fixed in 4%PFA/PBS for 15 min at room temperature, dehydrated and mounted in DPX (Sharlau).

Imaging

Fluorescent images were visualized by fluorescence microscopy using a Leitz DMIRB microscope and captured with a DFC360FX camera (Leica). White field images were obtained using a light microscope (Olympus) connected to a digital camera (DP70, Olympus). Muscle histochemical measurements, western blot densitometry analyses, myocardial area quantification and cardiomyocyte cell proliferation were performed using image J software.

In silico sequence analysis of zebrafish il6 promoter

Zebrafish *il6* gene cloning and characterization was first described by Varela et al. (Varela *et al*, 2012) and an upstream genomic region of approximately 1.5 kb upstream from the first start codon was retrieved in Ensembl database. Analysis of the promoter sequence for transcription factor binding sites was performed with the MatInspector (www.genomatrix.com) software, matrix family library version 8.4. Putative transcription binding sites were selected based on previous publications.

Statistical analysis

Results are expressed as mean±standard error of the mean (SEM). Statistical differences were analyzed by Student's t-test or one-way ANOVA for data following Gaussian distribution and considered to be significant at $P<0.05$. For non-normal distribution data and normalized data, Mann-Whitney or Klustall Wallis non-parametric tests were used and considered to be significant at $P<0.05$. All statistical analyses were performed using SigmaStat 4.0 (in SigmaPlot 11.0 Software) or GraphPad Prism6.

RESULTS

“Facts are the air of scientists. Without them you can never fly”
Linus Carl Pauling (1901-1994)
Nobel Prize in Chemistry in 1954

Chapter 1

Exercise training promotes changes in fiber morphometry and capillarization in fast muscle of adult zebrafish

In order to examine the changes in fast muscle fiber cellular characteristics between exercised and control zebrafish from a morphological perspective, different parameters were measured from histochemical images (Figure 1.1 A-B).

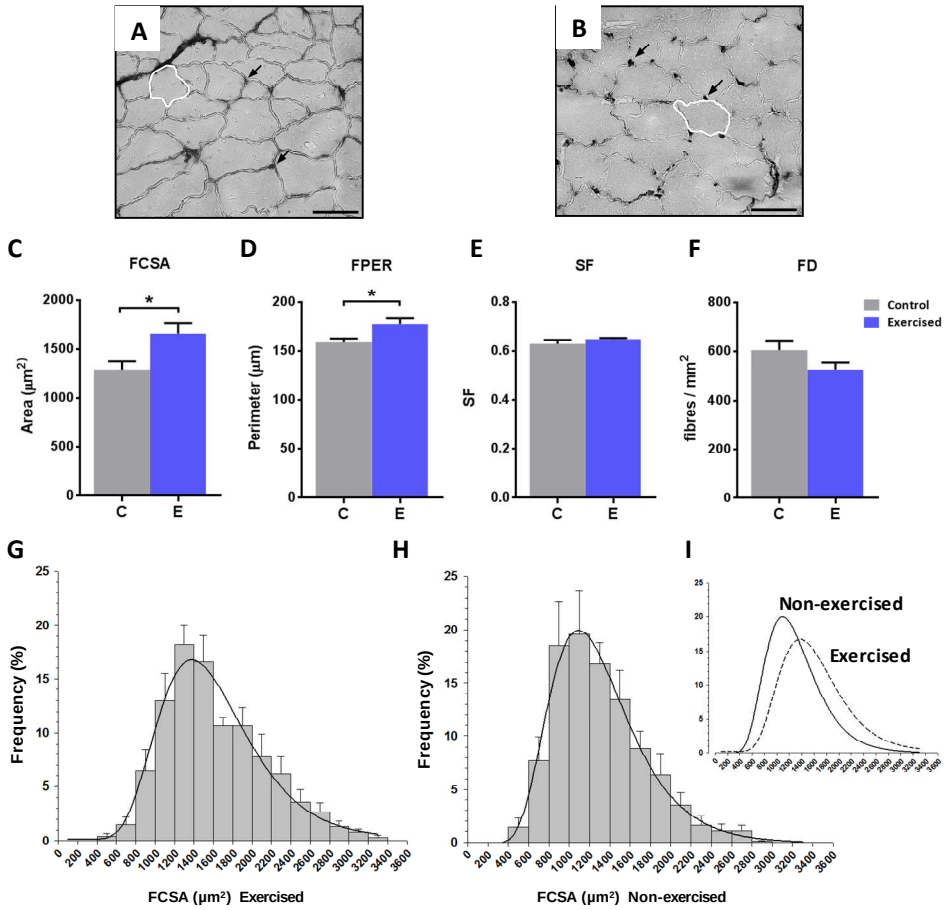

Figure 1.1. Morphometrical fiber parameters in fast muscle of exercised (E) and non-exercised (C) adult zebrafish. A-B Images of zebrafish cross-sectional white muscle for stained for ATPase for capillary demonstration (black arrows) and FCSA and FPER measures (white line) from a non-exercised **(A)** and an exercised **(B)** fish. Bar represents 50 µm. **C-D** Morphometric fiber parameters used in non-exercised and exercised zebrafish. FCSA, fiber cross-sectional area (µm²); FPER, fiber perimter (µm); FD, fiber density (fibres/mm²); SF, shape factor. P<0.05. **G-I** Fiber cross-sectional area histograms from fast muscle of exercised **(G)** and non exercised **(H)** fish. In **I**, the two overlapped log-normal regression curves are shown. Muscle fibers areas were grouped in intervals of 200 µm². Bars represent ± SEM of the mean frequency (E, n=6; C, n=6).

As shown in **Figure 1.1 C-F**, the exercise protocol significantly increased the fiber cross-sectional area (FCSA) by 29% and perimeter (FPER) by 12%. However, there were no differences in fiber density and shape factor suggesting that the number of the fibers and fiber circularity was not altered. The distribution of the frequencies of FCSA for both groups (**Figure 1.1 G-I**) showed that the exercised group had a significantly higher mean frequency value

(1400 µm2) over the control group (1100 µm2), indicating that the majority of the fast muscle fibers of exercised fish were larger (note the shift to the right of the regression curve in the exercised group, **Figure 1.1 I**). To standarize this observation, FCSA were grouped in small, medium and large and the mean percentages of FCSA were compared, confirming that after exercise there was a higher proportion of medium and large fibers (**Appendix Table A1**).

Interestingly, the exercised group of fish presented an increase in capillarisation as illustrated in **Figure 1.2**.

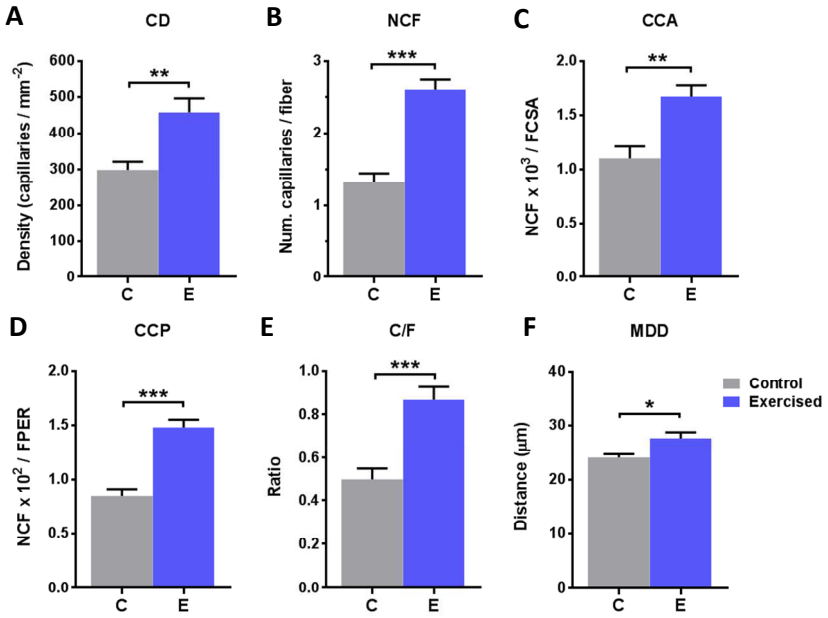

Figure 1.2. Morphometrical capillarity parameters in fast muscle of exercised and non-exercised adult zebrafish. **A** CD, capillary density (capillaries/mm^2); **B** NCF, number of capillaries in contact with each fiber; **C** CCA, relationship between NCF and the FCSA (NCF · 10^3/FCSA); **D** CCP, relationship between NCF and the FPER (NCF · 10^2/FPER); **E** C/F, capillary-to-fiber ratio (CD/FD); **F** MDD, maximal diffusion distance between the capillary and the centre of the fiber. Statistical significance values between non-exercised and exercised zebrafish: *P < 0.05, **P < 0.01, ***P < 0.001. Values are mean ± SEM from a sample size of n = 8 for each condition.

After exercise, the capillary density (CD) and the number of capillaries in contact with a fiber (NCF) significantly increased by 54% and 98%, respectively. The indices of capillarity were also significantly greaterin exercised fish as well as the capillary-to-fiber ratio (C/F). These results suggest that the applied exercise protocol resulted in an increase in capillarization in the fast muscle. However, there was a significant increase in maximal diffusion distance (the distance of a capillary to the center of a fiber), possiblysuggesting a decrease in oxidative capacity but that could also be a possibly a result of the FCSA increment.

Exercise training induces profound transcriptomic changes in fast muscle of adult zebrafish

In order to study the molecular mechanisms that might be responsible forthe increase in fast muscle mass and capillarization in response to four weeks of exercise training regime in adult zebrafish, we investigated the transcriptomic changes in fast muscle of exercised and non-exercised fish by microarray analysis. A strong transcriptomic response to swimming-induced exercise was identified, with 1625 genes down-regulated and 2851 genes up-regulated by

exercise when using a cut-off of 1.5 fold-change and P-value < 0.05.DAVID analysis of GO Biological processes revealed significant enrichment in functional groups involved in “striated muscle tissue development” and “cell differentiation”, “growth”, “apoptosis”, “regulation of proteolysis”, “response to oxidative stress”, “oxidative phosphorylation”, “cellular carbohidrate metabolic process”, “fatty acid metabolic process”, “blood vessel development (and) morphogenesis” and “vasculogenesis” (**Table 1.1**).

Specifically, gene expression profiling identified genes related with neuromuscular junction formation, where the motor neurons synapse with muscle cells (*ache, chrm2, scn4b*) and propagate action potentialsthrough the muscle cell triggering calcium release from the sarcoplasmic reticulum (*atp2a1, s100a4, fkbp1a, ryr1*). Genes involved in sarcomere contraction and relaxation (*acta2, actc1, capzb, casq1, myl2, mylpf, pvalb, tpm1, tnni2, tnnt3, ttn*) as well as calcium signaling-related transcripts were also represented (*calm1, camk2a, camk2b, capn8, capn2, ppp3ca*). In addition, genes coding for cytoskeletal elements involved in force transmission (*ank2, dag1, des, dmd, dtnbp1, flnc, itga2b, itgb4, lmna, myoz1, myoz2, sntb1, sptbn, vim*)ending at the extracelular matrix to ensure cell structure and maintenance (*bgn, col1a2, col4a1, col6a2, lamc3, loxl2, mmp14, sdcbp, tnc*) were also differentially expressed (**Table 1.2**).

Table 1.1. Functional annotation analysis based on GO terms in zebrafish fast muscle in response to swimming-exercise (DAVID)

GO Term		Count	P-value
GO:0014706	**Striated muscle tissue development**	41	0.001
GO:0051146	**Striated muscle cell differentiation**	31	0.004
GO:0030239	**Myofibril assembly**	10	0.022
GO:0031032	**Actomyosin structure organization**	14	0.002
GO:0032956	**Regulation of actin cytoskeleton organization**	28	0.031
GO:0040007	**Growth**	65	1.72E-05
GO:0045926	**Negative regulation of growth**	36	0.007
GO:0000278	**Mitotic cell cycle**	124	8.01E-08
GO:0051726	**Regulation of cell cycle**	92	0.005
GO:0006915	**Apoptosis**	161	0.001
GO:0043065	**Positive regulation of apoptosis**	110	0.029
GO:0006457	**Protein folding**	60	1.67E-04
GO:0030162	**Regulation of proteolysis**	19	0.024
GO:0006468	**Protein amino acid phosphorylation**	173	0.0038954
GO:0006511	**Ubiquitin-dependent protein catabolic process**	84	2.55E-06
GO:0006979	**Response to oxidative stress**	46	0.041
GO:0080135	**Regulation of cellular response to stress**	31	0.041
GO:0045454	**Cell redox homeostasis**	21	0.037
GO:0015980	**Energy derivation by oxidation of organic compounds**	51	1.68E-04
GO:0022900	**Electron transport chain**	41	5.67E-04
GO:0006754	**ATP biosynthetic process**	32	0.002
GO:0006119	**Oxidative phosphorylation**	44	5.45E-07
GO:0044262	**Cellular carbohydrate metabolic process**	111	6.80E-04
GO:0006096	**Glycolysis**	21	9.35E-04
GO:0044255	**Cellular lipid metabolic process**	157	4.84E-06
GO:0006635	**Fatty acid beta-oxidation**	15	7.99E-04
GO:0006631	**Fatty acid metabolic process**	75	2.13E-07
GO:0006633	**Fatty acid biosynthetic process**	30	0.001
GO:0006520	**Cellular amino acid metabolic process**	71	5.16E-04
GO:0042180	**Cellular ketone metabolic process**	190	1.95E-11
GO:0001568	**Blood vessel development**	78	1.68E-04
GO:0048514	**Blood vessel morphogenesis**	68	3.13E-04
GO:0001570	**Vasculogenesis**	16	0.019
GO:0045449	**Regulation of transcription**	593	0.042
GO:0043408	**Regulation of MAPKKK cascade**	35	0.011
GO:0051101	**Regulation of DNA binding**	42	0.001

For each enriched Gene Ontology term, count and p-value (P<0.05) are shown.

Furthermore, exercise training also altered the expression of genes involved in the control of muscle growth and development such as growth factors (*egfr, fgf13, fgf18, fgf20, fgfr1, fgfr2, fst, igfr1, igfbp1, igfbp3, igfbp7, igf2, mstn, ngf, tgfb1, tgfb2*), extracellular ligands (*bmp1, bmp4, bmpr1a, bmpr1b, ihh, nog, shh, wnt7a, wnt10a*), signaling molecules (*irs1, irs2, mapk1, mapk8, mapk13, mapk14, pik3c2b, smad6*) and transcription factors (*esrra, esrrb, esrrg, foxa1, foxo3, hdac4, hadc6, mef2a, mef2c, mef2d, pax3*) (**Table 1.3**).

Table 1.2. Selected differentially expressed genes in fast muscle of exercised zebrafish that participate in the contractile activation of skeletal muscle fibers

Name	Gene description	FC	Name	Gene description	FC
	Neuromuscular junction			**Muscle contraction** (continued)	
ache	**acetylcholinesterase**	**8.86**	*tpm1*	**tropomyosin 1 (alpha)**	**1.71**
vamp1	**vesicle-associated membrane prot. 1**	**3.78**	*atp2a1*	**ATPase, Ca transporting, cardiac muscle,**	**1.70**
chrm2	**cholinergic receptor, muscarinic 2**	**3.65**	*actc1*	**actin, alpha, cardiac muscle 1**	**1.68**
snap25	**synaptosomal-associated protein, 25kDa**	**3.08**	*cacng1*	**calcium channel, voltage-dependent,**	**1.61**
scn4b	**sodium channel, voltage-gated, type IV, beta**	**3.01**	*fkbp1a*	**FK506 binding protein 1A**	**-1.51**
syn2	**synapsin II**	**2.70**	*s100a4*	**S100 calcium binding protein A4**	**-1.63**
syt1	**synaptotagmin I**	**2.22**	*calm1*	**calmodulin 1 (phosphorylase kinase,**	**-2.04**
rims2	**regulating synaptic membrane exocytosis 2**	**1.93**	*actg2*	**actin, gamma 2, smooth muscle, enteric**	**-3.87**
scnm1	**sodium channel modifier 1**	**1.65**	*tnnt3*	**troponin T type 3 (skeletal, fast)**	**-7.01**
	Muscle contraction			**Cytoskeleton**	
capn8	**calpain 8**	**4.11**	*ank2*	**ankyrin 2, neuronal**	**11.01**
camk2n2	**Ca/calmodulin-dependent protein kinase II**	**3.38**	*plec*	**plectin**	**3.31**
pvalb	**parvalbumin**	**3.24**	*myoz1*	**myozenin 1**	**2.41**
tnni2	**troponin I type 2 (skeletal, fast)**	**3.12**	*myoz2*	**myozenin 2**	**2.28**
capn3	**calpain 3, (p94)**	**3.08**	*dag1*	**dystroglycan 1**	**2.26**
nfatc4	**nuclear factor of activated T-cells, calcineurin-**	**3.08**	*itgb4*	**integrin, beta 4**	**1.99**
capn2	**calpain 2, (m/II) large subunit**	**2.92**	*itga2b*	**integrin, alpha 2b**	**1.95**
tmod4	**tropomodulin 4 (muscle)**	**2.79**	*dmd*	**dystrophin**	**1.94**
nfatc1	**nuclear factor of activated T-cells, ,**	**2.76**	*filip1*	**filamin A interacting protein 1**	**1.88**
capzb	**capping protein (actin filament) muscle Z-line,**	**2.75**	*sntb1*	**syntrophin, beta 1**	**1.78**
casq1	**calsequestrin 1 (fast-twitch, skeletal muscle)**	**2.68**	*vim*	**vimentin**	**1.61**
myl2	**myosin, light chain 2, regulatory, cardiac, slow**	**2.64**	*lmna*	**lamin A/C**	**-1.51**
ppp3cc	**protein phosphatase 3, catalytic subunit,**	**2.61**	*dtnbp1*	**dystrobrevin binding protein 1**	**-1.76**
capn5	**calpain 5**	**2.60**	*flnc*	**filamin C, gamma**	**-1.88**
ttn	**titin**	**2.58**		**Extracelular matrix**	
ppp3ca	**protein phosphatase 3, catalytic subunit,**	**2.52**	*lamc3*	**laminin, gamma 3**	**10.06**
mylpf	**myosin light chain, phosphorylatable, fast**	**2.26**	*col1a2*	**collagen, type I, alpha 2**	**3.12**
mybph	**myosin binding protein H**	**2.17**	*bgn*	**biglycan**	**2.98**
capn10	**calpain 10**	**2.15**	*loxl2*	**lysyl oxidase-like 2**	**2.92**
cacna1s	**calcium channel, voltage-dependent, L type,**	**2.11**	*col6a2*	**collagen, type VI, alpha 2**	**2.73**
camk2a	**calcium/calmodulin-dependent protein kinase**	**1.98**	*mmp14*	**matrix metallopeptidase 14**	**2.69**
camk2d	**calcium/calmodulin-dependent protein kinase**	**1.97**	*sparc*	**secreted protein acidic and rich in**	**2.59**
nfatc3	**nuclear factor of activated T-cells, calcineurin-**	**1.92**	*tnc*	**tenascin C**	**2.53**
acta2	**actin, alpha 2, smooth muscle, aorta**	**1.92**	*p4ha1*	**prolyl 4-hydroxylase, alpha polypeptide I**	**2.31**
mylk	**myosin light chain kinase**	**1.87**	*col4a1*	**collagen, type IV, alpha 1**	**1.97**
tpm4	**tropomyosin 4**	**1.78**	*sdcbp*	**syndecan binding protein (syntenin)**	**-2.26**
ryr1	**ryanodine receptor 1 (skeletal)**	**1.77**			

Data are shown as fold change (FC)

Differentially expressed genes involved in metabolism were also represented in fast skeletal muscle, particularly inprocesses required for energy production such as glycolisis (*aldoa*, *eno1*, *gapdh*, *hk2*, *pfkm*, *pgk1*), fatty acid oxidation (*acacb*, *acadl*, *cpt1*, *cpt2*, *ppard*), citric acid cycle (fh, ogdh, mdh1), components of the oxidative phosphorylation (*atp5h*, *atp5o*, *mt-atp6*, *ndufaf4*, *ndufb1*, *ndufv1*, *cox4i1*, *cox5a*, *cox7c*) and energy dissipation (*ucp2*, *ucp3*). Genes responsible for the metabolic provision of ATPin muscle cells such as the ATP-phosphagen

system (ckm, *ckmt2*), genes that participate in energy metabolism (*adipor2*, *mb*, *ppara*, ppard) including genes coding for the energy-sensing AMPK (*prkaaq*, *prkag1*, *prkab1*) were also differentially expressed (**Table 1.4**). Exercise training also altered the expression of genes involved in protein synthesis and degradation (*eif4e*, *eif4ebp1*, *fboxo32*, *foxo3*, *trim63*) (**Table 1.4**).

Microarray also evidenced altered expression of genes related to cell cycle regulation such as cyclin kinases (*cdk9*, *cdk11b*, *cdkn1c*, *cdkn2aip*), regulators specific for muscle progenitor cell proliferation (*paxbp1*) and apoptosis such as tp53, members of the BCL family (e.g. *bag1* and *bax*) and caspases (*casp8*, *casp9*) (**Table 1.5**).

Table 1.3. Selected differentially expressed genes that participate in growth and devlopment of skeletal muscle fibers

Name	Gene description	FC	Name	Gene description	FC
		Muscle growth and development			
fgf20	**fibroblast growth factor 20**	**8.94**	*igfbp7*	**insulin-like growth factor binding protein 7**	**2.36**
hdac6	**histone deacetylase 6**	**6.42**	*esrrg*	**estrogen-related receptor gamma**	**2.36**
fgf18	**fibroblast growth factor 18**	**6.30**	*bmpr1b*	**bone morphogenetic protein receptor, IB**	**2.27**
wnt10a	**wingless-type MMTV integration site, 10A**	**6.25**	*erbb2*	**v-erbb2 erythroblastic leukemia 2**	**2.27**
pax3	**paired box 3**	**6.21**	*mapk13*	**mitogen-activated protein kinase 13**	**2.23**
tgfb2	**transforming growth factor, beta 2**	**5.35**	*fst*	**follistatin**	**2.17**
nog	**noggin**	**4.90**	*mapk8*	**mitogen-activated protein kinase 8**	**2.12**
esrra	**estrogen-related receptor alpha**	**4.73**	*smad6*	**SMAD family member 6**	**2.06**
wnt7a	**wingless-type MMTV integration site, 7A**	**4.60**	*fgfr1*	**fibroblast growth factor receptor 1**	**1.96**
mstn	**myostatin**	**4.41**	*irs2*	**insulin receptor substrate 2**	**1.91**
shh	**sonic hedgehog**	**3.78**	*runx2*	**runt-related transcription factor 2**	**1.90**
fzd2	**frizzled family receptor 2**	**3.08**	*igfbp1*	**insulin-like growth factor binding protein 1**	**1.89**
pik3c2b	**phosphatidylinositol-4-p- 3-kinase c2b**	**3.06**	*irs1*	**insulin receptor substrate 1**	**1.78**
fgf13	**fibroblast growth factor 13**	**3.01**	*acvr2b*	**activin A receptor, type IIB**	**1.74**
mapk1	**mitogen-activated protein kinase 1**	**3.00**	*tgfb1*	**transforming growth factor, beta 1**	**1.71**
fzd10	**frizzled family receptor 10**	**2.94**	*mef2d*	**myocyte enhancer factor 2D**	**1.71**
ihh	**indian hedgehog**	**2.91**	*hdac4*	**histone deacetylase 4**	**1.71**
fzd8	**frizzled family receptor 8**	**2.87**	*igfbp3*	**insulin-like growth factor binding protein 3**	**1.66**
esrrb	**estrogen-related receptor beta**	**2.61**	*mef2a*	**myocyte enhancer factor 2A**	**1.66**
bmpr1a	**bone morphogenetic protein receptor, IA**	**2.58**	*igf2*	**insulin-like growth factor 2**	**1.61**
ngf	**nerve growth factor (beta polypeptide)**	**2.55**	*pten*	**phosphatase and tensin homolog**	**-1.54**
igf1r	**insulin-like growth factor 1 receptor**	**2.53**	*mef2c*	**myocyte enhancer factor 2C**	**-1.59**
bmp1	**bone morphogenetic protein 1**	**2.46**	*egfr*	**epidermal growth factor receptor**	**-2.13**
dvl1	**dishevelled, dsh homolog 1 (Drosophila)**	**2.43**	*id3*	**inhibitor of DNA binding 3**	**-2.39**
smad2	**SMAD family member 2**	**2.40**	*srf*	**serum response factor**	**-2.68**
bmp4	**bone morphogenetic protein 4**	**2.38**	*mapk14*	**mitogen-activated protein kinase 14**	**-2.78**

Data are shown as fold change (FC)

Table 1.4. Selected differentially expressed genes that participate in metabolism, portein synthesis and degradation of skeletal muscle fibers

Name	Gene description	FC	Name	Gene description	FC
		Energy metabolism			
cpt1a	**carnitine palmitoyltransferase 1A (liver)**	**5.23**	*fabp3*	**fatty acid binding protein 3, muscle and heart**	**1.94**
pfkm	**phosphofructokinase, muscle**	**3.84**	*atp5h*	**ATP synthase, H+ transporting, mitochondrial Fo**	**1.91**
prkaa1	**protein kinase, AMP-activated, alpha 1 catalytic**	**3.66**	*ucp3*	**uncoupling protein 3 (mitochondrial, proton carrier)**	**1.88**

prkag1	**protein kinase, AMP-activated, gamma 1**	**3.50**	*cpt2*	**carnitine palmitoyltransferase 2**	**1.86**
acadl	**acyl-CoA dehydrogenase, long chain**	**3.20**	*ndufb1*	**NADH dehydrogenase (ubiquinone) 1 beta**	**1.82**
ppard	**peroxisome proliferator-activated receptor**	**3.19**	*mdh1*	**malate dehydrogenase 1, NAD (soluble)**	**1.77**
aldoa	**aldolase A, fructose-bisphosphate**	**3.18**	*sdha*	**succinate dehydrogenase complex, subunit A**	**1.74**
prkab1	**protein kinase, AMP-activated, beta 1**	**3.06**	*mt-atp6*	**ATP synthase F0 subunit 6**	**1.70**
mb	**myoglobin**	**2.95**	*acacb*	**acetyl-CoA carboxylase beta**	**1.70**
cox7c	**cytochrome c oxidase subunit VIIc**	**2.85**	*ucp2*	**uncoupling protein 2 (mitochondrial, proton carrier)**	**1.68**
ppara	**peroxisome proliferator-activated receptor**	**2.77**	*atp5o*	**ATP synthase, H+ transporting, mitochondrial F1**	**1.68**
tfb2m	**transcription factor B2, mitochondrial**	**2.42**	*eno1*	**enolase 1, (alpha)**	**1,68**
g6pc	**glucose-6-phosphatase, catalytic subunit**	**2.38**	*cox4i1*	**cytochrome c oxidase subunit IV isoform 1**	**1.68**
pdha1	**pyruvate dehydrogenase (lipoamide) alpha 1**	**2.24**	*atp5f1*	**ATP synthase, H+ transporting, mitochondrial Fo**	**1.65**
ckm	**creatine kinase, muscle**	**2.24**	*idh3b*	**isocitrate dehydrogenase 3 (NAD+) beta**	**1.64**
fh	**fumarate hydratase**	**2.20**	*nrf1*	**nuclear respiratory factor 1**	**1.62**
ogdh	**oxoglutarate (alpha-ketoglutarate)**	**2.19**	*adipor2*	**adiponectin receptor 2**	**1.56**
gapdh	**glyceraldehyde-3-phosphate dehydrogenase**	**2.19**	*lpl*	**lipoprotein lipase**	**-1.51**
pdk1	**pyruvate dehydrogenase kinase, isozyme 1**	**2.19**	*ndufaf4*	**NADH dehydrogenase (ubiquinone) complex I 4**	**-1.88**
cox5a	**cytochrome c oxidase subunit Va**	**2.12**	*aldoc*	**aldolase C, fructose-bisphosphate**	**-2.03**
pgk1	**phosphoglycerate kinase 1**	**2.02**	*pkm*	**pyruvate kinase, muscle**	**-2.24**
ndufv1	**NADH dehydrogenase (ubiquinone) flavoprotein**	**1.94**	*hk2*	**hexokinase 2**	**-2.36**
	Protein synthesis and degradation				
fbxo32	**F-box protein 32**	**6.01**	*rps6ka1*	**Ribosomal protein S6 kinase, 90kDa, polypt 1**	**1.96**
eef2k	**Eukaryotic Elongation Factor 2 Kinase**	**4.12**	*eif2b4*	**eukaryotic translation initiation factor 2B, sub 4**	**-1.58**
foxo3	**forkhead box O3**	**2.08**	*eif4e*	**Eukaryotic translation initiation factor 4E**	**-1.89**
trim63	**Tripartite motif containing 63, E3 ubiquitin protein ligase**	**2.02**	*eif4ebp1*	**Eukaryotic translation initiation factor 4E binding protein**	**-2.01**

Data are shown as fold change (FC)

Consistent with the increased vascularization of fast muscle by exercise training, the expression of a number of genes involved in angiogenesis was altered in fast muscle, including angiopoietins (e.g. *angpt2, angptl2, angptl3*), members of the ephrin family and receptors (e.g. *efna2, efna3, efnb2, efnb3, epha4, epha7, ephb4*), members of the notch family (e.g. *dll1, jag1, jag2, notch1, notch2*), hypoxia-inducible factors (e.g. *hif1an, hif3a*), *gata1*and *nrp1* (**Table 1.6**).

Table 1.5. Selected differentially expressed genes in fast muscle of exercised adult zebrafish that participate in cell cycle regulation and immune system

Name	Gene description	FC	Name	Gene description	FC
	Cell cycle			**Immune-related factors**	
rerg	**RAS-like, estrogen-regulated, growth inhibitor**	**7.50**	*traf6*	**TNF receptor-associated factor 6, E3 ubiquitin**	**10.78**
tp53	**tumor protein p53**	**2.53**	*il17D*	**interleukin 17D**	**6.51**
paxbp1	**PAX3 and PAX7 binding protein 1**	**2.31**	*ptgs1*	**prostaglandin-endoperoxide synthase 1**	**5.81**
cdkn1c	**cyclin-dependent kinase inhibitor 1C (p57, Kip2)**	**2.09**	*irf3*	**interferon regulatory factor 3**	**4.60**
cdkn2aip	**CDKN2A interacting protein**	**2.07**	*il29ra*	**interleukin 20 receptor, alpha**	**3.91**
atg9a	**autophagy related 9A**	**1.96**	*il12b*	**interleukin 12B**	**3.68**
tp53bp1	**tumor protein p53 binding protein 1**	**1.92**	*il11ra*	**interleukin 11 receptor, alpha**	**3.24**
cdk9	**cyclin-dependent kinase 9**	**1.84**	*ptgr1*	**prostaglandin reductase 1**	**3.21**
bcor	**BCL6 corepressor**	**1.84**	*ptgds*	**prostaglandin D2 synthase 21kDa (brain)**	**3.18**
mcl1	**myeloid cell leukemia sequence 1 (BCL2-related)**	**1.69**	*ptgis*	**prostaglandin I2 (prostacyclin) synthase**	**2.87**
bag1	**BCL2-associated athanogene**	**-1.53**	*il13ra2*	**interleukin 13 receptor, alpha 2**	**2.54**
cdk11b	**cyclin-dependent kinase 11B**	**-1.57**	*il20*	**interleukin 20**	**2.44**
atg3	**autophagy related 3**	**-1.62**	*tnfrsf19*	**tumor necrosis factor receptor superfamily 19**	**2.43**
casp8	**caspase 8, apoptosis-related cysteine peptidase**	**-1.91**	*nkrf*	**NFKB repressing factor**	**1.77**
cdk8	**cyclin-dependent kinase 8**	**-2.01**	*il17rd*	**interleukin 17 receptor D**	**1.74**
myc	**v-myc myelocytomatosis viral oncogene homolog**	**-2.27**	*mst1*	**macrophage stimulating 1**	**1.66**
casp9	**caspase 9, apoptosis-related cysteine peptidase**	**-2.33**	*mif*	**macrophage migration inhibitory factor**	**-1.50**
apaf1	**apoptotic peptidase activating factor 1**	**-2.54**			
mki67ip	**MKI67 (FHA domain) interacting nucleolar**	**-2.70**			
bax	**BCL2-associated X protein**	**-3.25**			

Data are shown as fold change (FC)

Table 1.6. Selected differentially expressed genes in fast muscle of exercised adult zebrafish that participate in angiogenesis

Name	Gene description	FC	Name	Gene description	FC
	Angiogenesis				
klf2	**Kruppel-like factor 2 (lung)**	**8.52**	*hif3a*	**hypoxia inducible factor 3, alpha subunit**	**1.92**
robo2	**roundabout, axon guidance receptor, homolog 2**	**4.30**	*epha7*	**EPH receptor A7**	**1.91**
angpt2	**angiopoietin 2**	**3.90**	*angptl2*	**angiopoietin-like 2**	**1.90**
angptl3	**angiopoietin-like 3**	**3.47**	*nos2*	**nitric oxide synthase 2, inducible**	**1.85**
efna3	**ephrin-A3**	**3.45**	*cdc42ep2*	**CDC42 effector protein (Rho GTPase binding) 2**	**1.83**
gata1	**GATA binding protein 1 (globin transcription factor 1)**	**3.00**	*efna2*	**ephrin-A2**	**1.83**
epha4	**EPH receptor A4**	**2.96**	*nr2f1*	**nuclear receptor subfamily 2, group F, member 1**	**1.83**
nrp1	**neuropilin 1**	**2.89**	*jag1*	**jagged 1**	**1.80**
mmp14	**matrix metallopeptidase 14 (membrane-inserted)**	**2.69**	*slit2*	**slit homolog 2 (Drosophila)**	**1.79**
nos1	**nitric oxide synthase 1 (neuronal)**	**2.65**	*hey1*	**hairy/enhancer-of-split related with YRPW motif 1**	**1.78**
notch1	**notch 1**	**2.60**	*hif1an*	**hypoxia inducible factor 1, alpha subunit inhibitor**	**1.73**
sema3f	**sema domain, immunoglobulin domain (Ig), 3F**	**2.57**	*foxc1*	**forkhead box C1**	**1.68**
slit3	**slit homolog 3 (Drosophila)**	**2.53**	*efnb2*	**ephrin-B2**	**1.63**
amot	**angiomotin**	**2.34**	*jag2*	**jagged 2**	**1.54**
hey2	**hairy/enhancer-of-split related with YRPW motif 2**	**2.20**	*vegfc*	**vascular endothelial growth factor C**	**1.36**
tp63	**tumor protein p63**	**2.16**	*dll1*	**delta-like 1 (Drosophila)**	**-1.25**
mmp10	**matrix metallopeptidase 10 (stromelysin 2)**	**2.06**	*rac1*	**ras-related C3 botulinum toxin substrate 1**	**-1.57**
s1pr1	**sphingosine-1-phosphate receptor 1**	**2.03**	*rock2*	**Rho-associated, coiled-coil containing protein**	**-1.61**
ephb4	**EPH receptor B4**	**1.97**	*notch2*	**notch 2**	**-1.89**
nr2f2	**nuclear receptor subfamily 2, group F, member 2**	**1.95**	*cdc42*	**cell division cycle 42**	**-1.97**
efnb3	**ephrin-B3**	**1.94**	*aggf1*	**angiogenic factor with G patch and FHA domains 1**	**-2.10**

Data are shown as fold change (FC)

Canonical pathway analysis identified 22 pathways that were significantly (P<0.05) over-represented in fast muscle of adult exercised zebrafish (**Table 1.7**).

Table 1.7 Significantly over-represented putative canonical pathways in fast muscle of exercised zebrafish

Ingenuity Canonical Pathways (IPA)					
	P-value	**Ratio**		**P-**	**Ratio**
Integrin Signaling	3.28E-16	94/208	**Calcium Signaling**	6.03E-	58/213
Protein Ubiquitination	3.62E-12	103/268	**Insulin Receptor Signaling**	1.22E-	44/142
Wnt/β-catenin Signaling	5.57E-11	75/175	**FGF Signaling**	1.53E-	31/92
mTOR Signaling	6.06E-06	68/211	**Chemokine Signaling**	1.72E-	26/73
TGF-β Signaling	7.22E-06	36/89	**PI3K/AKT Signaling**	1.75E-	41/144
Ephrin B Signaling	2.46E-05	32/82	**Fatty Acid β-oxidation**	1.99E-	14/45
Actin Cytoskeleton Signaling	3.04E-05	72/239	**Hypoxia Signal. Cardiovascular System**	4.35E-	24/67
IGF-1 Signaling	6.53E-05	38/105	**PDGF Signaling**	4.63E-	27/85
Glycolysis	1.01E-04	14/41	**HIF1α Signaling**	9.07E-	33/108
VEGF Signaling	1.03E-04	36/104	**Notch Signaling**	9.60E-	15/43
AMPK Signaling	4.67E-04	46/169	**Angiopoietin Signaling**	3.00E-	21/74

The associated p-value (Fisher's exact test P<0.05) and the ratio of the number of differentially expressed genes in fast muscle of exercised zebrafish over the total number of genes in each particular pathway in the Ingenuity Knowledge Base. Canonical pathway names are from Ingenuity Systems.

Regulated canonical signaling pathways associated with skeletal muscle contractile activity includedthe calcium, integrin, actin cytoskeleton, FGF, wnt/β-catenin and AMPK signaling pathways. Moreover, the IGF-1, insulin receptor, PI3K/AKT and mTOR signaling pathways were also significantly regulated in fast muscle, in accordance with the observed hypertrophy in fast

muscle of exercised zebrafish. Interestingly, the canonical TGFβ signaling pathway was also significantly altered by exercise in fast muscle. The metabolic effects of exercise training in the zebrafish fast muscle were exemplified by the significant regulation of the protein ubiquitination pathway, glycolysis and fatty acid β-oxidation. Furthermore, exercise training also caused a significant over-representation of signaling pathways involved in angiogenesis (e.g. ephrin B, VEGF, hypoxia, PDGF, HIF1α, Notch and angiopoietin signaling pathways) in the zebrafish fast muscle. Moreover, **Appendix Figures A1.1 to A1.3** show the observed gene networks corresponding to muscledevelopment, angiogenesis and proliferation by IPA, that allowed us to establish connectivity maps for these processes.

The results of microarray analysis were validated by qPCR for 7 differentially expressed genes in fast muscle: 4 down-regulated (*fabp7, tuba1b, psme3, psma5*) and 3 up-regulated (*capns1, fgfrl1, foxa1*) genes. All 7 genes showed a similar pattern of change with the two techniques used (**Appendix Table A1.2**).

Exercise training signals through mTOR and AMPK pathways

In view of the increase in fiber cross-sectional area after four weeks of swim training, we investigated the potential involvement of known molecular pathways involved in exercise-induced muscle growth. Therefore, we were interested to examine the activity of the mTOR protein synthesis pathway, comprising mTOR, 4EBP-1, that is inhibited by mTOR by hyperphosphorylation, and the transcription factor Mef2, a final downstream target of the pathway (**Figure 1.3 A**). The activity of mTOR (P=0.044) and 4EBP-1 (P=0.066) was increased, as well as the protein levels of Mef2 (P=0.045) (**Figure 1.3 B**), suggesting an activation of the mTOR pathway after four weeks of exercise.

In addition, we aimed at examining the expression of the energy sensing kinase AMPK, a key enzyme responsible for the inhibition of anabolic processes including inhibition of the mTOR pathway. We analyzed AMPK activity after 1, 2 and 4 weeks of swimming-induced exercise training. AMPK activity was increased, although not significantly, in response to exercise after 1 and 2 weeks of swimming and significantly after 4 weeks of swimming (P=0.038) (**Figure 1.4A**). We subsequently analyzed two known down-stream targets of AMPK, PGC1α and ACC after 4 weeks of exercise, the time pointat which AMPK activity was significantly elevated. We found a significant increase in mRNA expression of *pgc1α* (**Figure 1.4 B-C**), a main target of AMPK during the response to exercise, and a trend towards higher levels of Pgc1α at the protein level when analyzed by Western Blot (P=0.165). ACC is phosphorylated by AMPK inhibiting the synthesis of fatty acids; however, ACC phosphorylation (its inactive form) was unaltered in response to exercise (**Figure 1.4 B-C**).

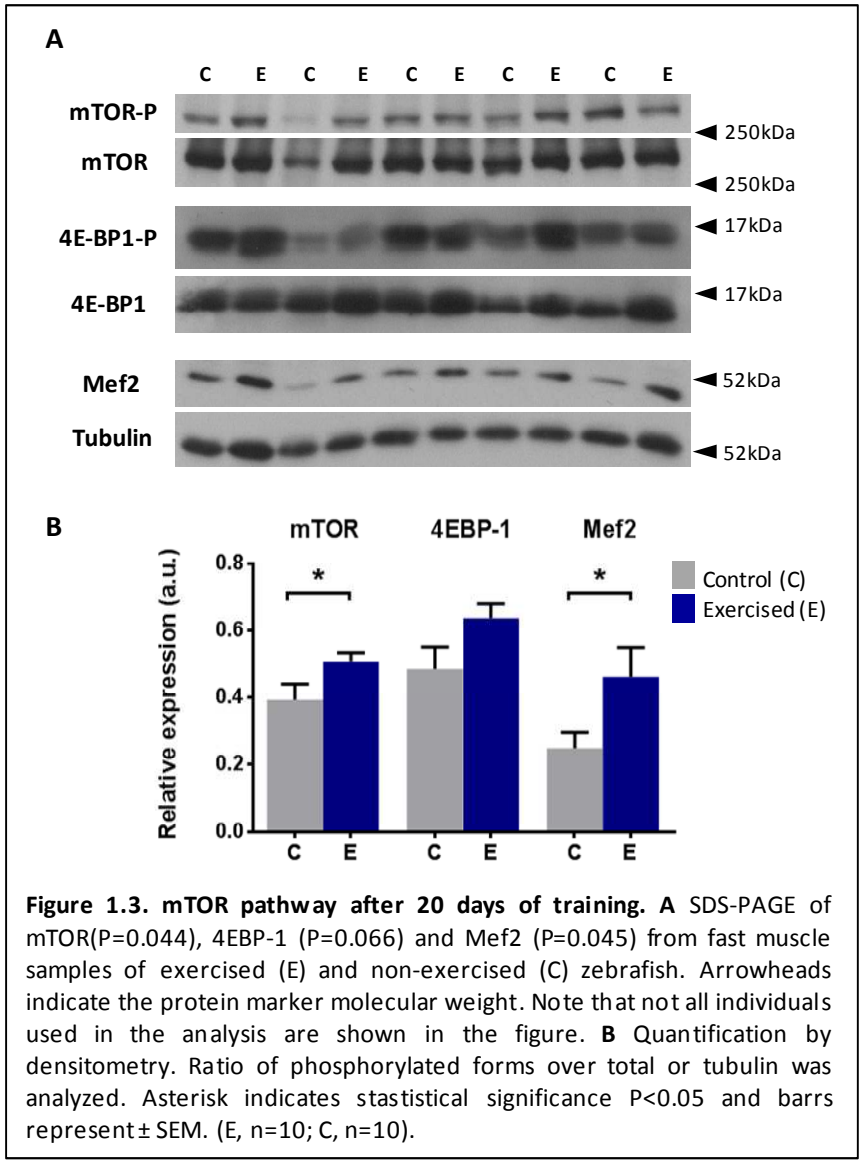

Figure 1.3. mTOR pathway after 20 days of training. A SDS-PAGE of mTOR(P=0.044), 4EBP-1 (P=0.066) and Mef2 (P=0.045) from fast muscle samples of exercised (E) and non-exercised (C) zebrafish. Arrowheads indicate the protein marker molecular weight. Note that not all individuals used in the analysis are shown in the figure. **B** Quantification by densitometry. Ratio of phosphorylated forms over total or tubulin was analyzed. Asterisk indicates stastistical significance P<0.05 and barrs represent ± SEM. (E, n=10; C, n=10).

Oxidative metabolism

To further investigate if AMPK activation and PGC1α increase could contribute to a more aerobic phenotype of the zebrafish fast muscle, we analyzed the expression of genes involved in oxidative metabolism, fiber type and mitochondrial biogenesis. Exercise produced temporal changes in myosin fiber type mRNA expression. In fast muscle fibers, smyhc1, a myosin heavy chain marker for slow fibers, showed dynamic expression changes over the exercise period, increasing after week 1 and 4 of swimming and significantly decreasing after week 2 (P=0.035)(Figure 1.5 A). In contrast, mRNA expression of myhz2, a myosin heavy chain marker for fast fibers remained unchanged until week 4 when it decreased significantly (P=0.029) (Figure 1.5 A). Two zebrafish isoforms of PPARδ were analyzed as markers for lipid oxidation. Whereas ppardb was unaltered, pparda mRNA expression increased significantly after 4 weeks of exercise (P=0.009), consistent with the up-regulation of Pgc1α shown before (Figure 1.5 B). We also analyzed the expression of mitochondrial genes in fast muscle: mitochondrial transcription A (tfam), the major activator of mitochondrial transcription, T7-like mitochondrial DNA helicase (twinkle or pao1), involved in mitochondrial DNA maintenance, and the

mitochondrial RNA polymerase (polmrt), responsible for mitochondrial gene expression. mRNA expression of the mitochondrial genes did not show any differences in expression during the exercise training period between the two groups (Figure 1.5 C).

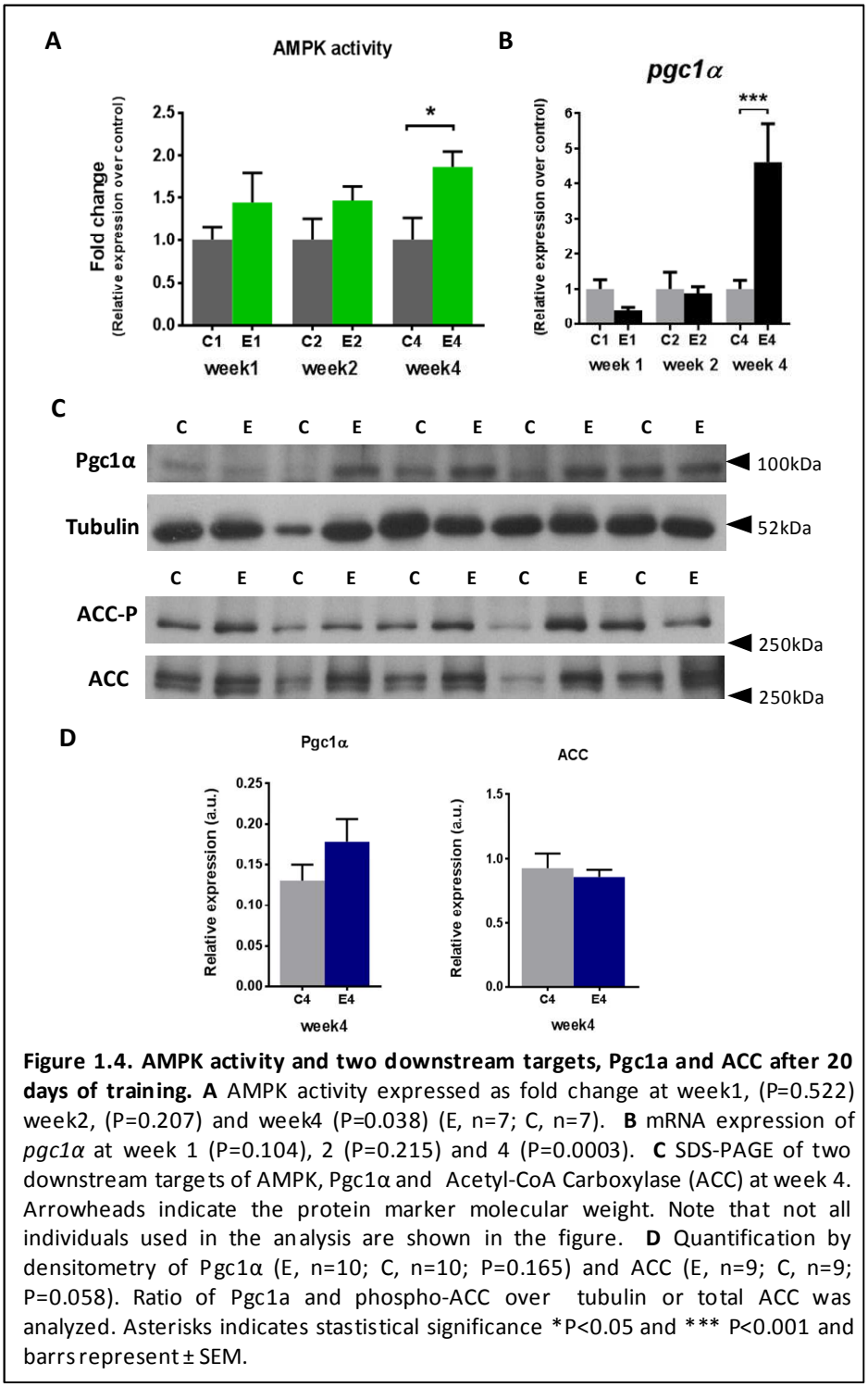

Figure 1.4. AMPK activity and two downstream targets, Pgc1a and ACC after 20 days of training. A AMPK activity expressed as fold change at week1, (P=0.522) week2, (P=0.207) and week4 (P=0.038) (E, n=7; C, n=7). **B** mRNA expression of *pgc1α* at week 1 (P=0.104), 2 (P=0.215) and 4 (P=0.0003). **C** SDS-PAGE of two downstream targets of AMPK, Pgc1α and Acetyl-CoA Carboxylase (ACC) at week 4. Arrowheads indicate the protein marker molecular weight. Note that not all individuals used in the analysis are shown in the figure. **D** Quantification by densitometry of Pgc1α (E, n=10; C, n=10; P=0.165) and ACC (E, n=9; C, n=9; P=0.058). Ratio of Pgc1a and phospho-ACC over tubulin or total ACC was analyzed. Asterisks indicates stastistical significance *P<0.05 and *** P<0.001 and barrs represent ± SEM.

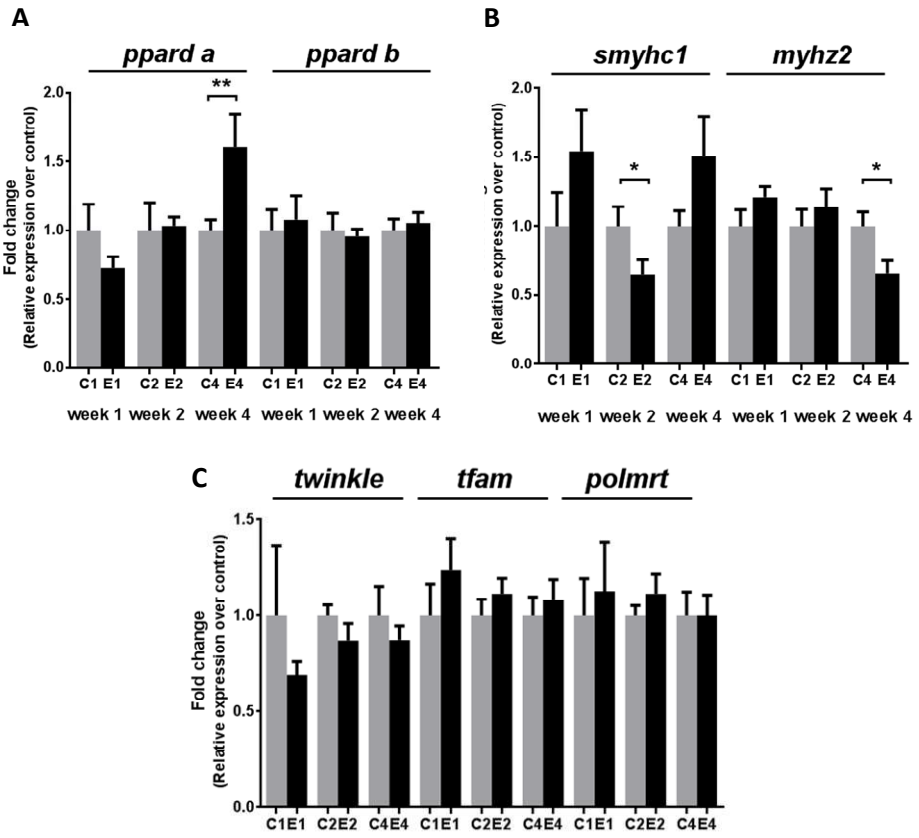

Figure 1.5. Expression of genes related with muscle oxidative capacity. A mRNA expression of peroxisome proliferator-activated receptor delta: *pparda* (week 4, P=0.009) and *ppardb*; **B** slow and fast fiber type myosins: slow myosin heavy chain 1, *smyhc1* (week 2, P=0.035) and myosin fast muscle specific polypeptide 2, *myhz2* (week4, P=0.029); **C** mitochondrial genes, mitochondrial twinkle protein, *twinkle* (or *peo1*), transcription factor A mitochondrial, *tfam* and polymerase RNA mitochondrial, *polmrt*. Data is represented as fold change in exercised (E, n=10) over non-exercised (C, n=10) adult zebrafish at 1,2 and 4 weeks (20 days) of exercise training. Significant differences are indicated by *P<0.05 and bars represent ± SEM.

Exercise increase skeletal muscle fiber proliferation and satellite cell marker expression

The observed increase in muscle mass or hypertrophy of the fast muscle fibers in exercised zebrafish prompted us to examine if skeletal muscle proliferation was affected. Fast muscle samples from non-exercised and exercised zebrafish after 1, 2 and 4 weeks of training were analyzed for phospholylated histone 3 (H3P), a marker of cell proliferation, p38 MAPK, a marker for muscle cell differentiation, and Pax7, a marker of muscle satellite cells. The protein levels of H3P were significantly up-regulated in the exercised fish after week 2 of training and remained significantly elevated until the end of training (Figure 1.6). No differences were found for p38 MAPK activity, although a trend in decreasing the expression was shown in the exercise group after the first week of training (Figure 1.7). The mRNA expression levels of the two isoforms of pax7 in zebrafish, pax7a and pax7b, were not altered by exercise training (Figure 1.8 A). However, the protein expression levels of Pax7 were significantly increased during the whole period of exercise training, after 1, 2 and 4 weeks (Figure 1.8 B). The mRNA expression levels of myod, a myogenic regulatory factor responsible of myogenic commitment and differentiation, were not altered by exercise at any of the measured time points (Figure

1.8A). Since no histological immunolocalization of markers of satellite cells on zebrafish muscle samples was performed, we cannot define the state of muscle satellite cell proliferation, over non satellite cells, in response to exercise.

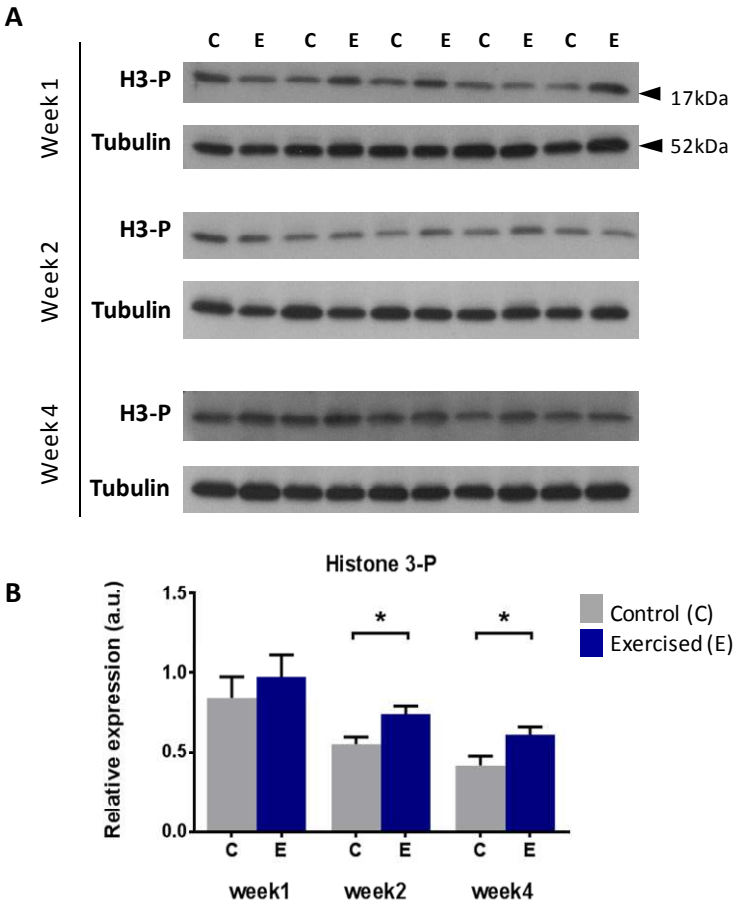

Figure 1.6. Temporal expression of cell proliferation by Histone 3-phosphorylated. A SDS-PAGE of the cell cycle marker Histone 3-phosphorylated at week 1, 2 and 4. Arrowheads indicate the protein marker molecular weight. Note that not all individuals used in the analysis are shown in the figure. **B** Quantification by densitometry at week 1 (P=0.491), week 2 (P=0.010) and week 4 (P=0.017). Tubulin was used as loading control. Asterisk indicates stastistical significance P<0.05 and barrs represent ± SEM. (E, n=10; C, n=10).

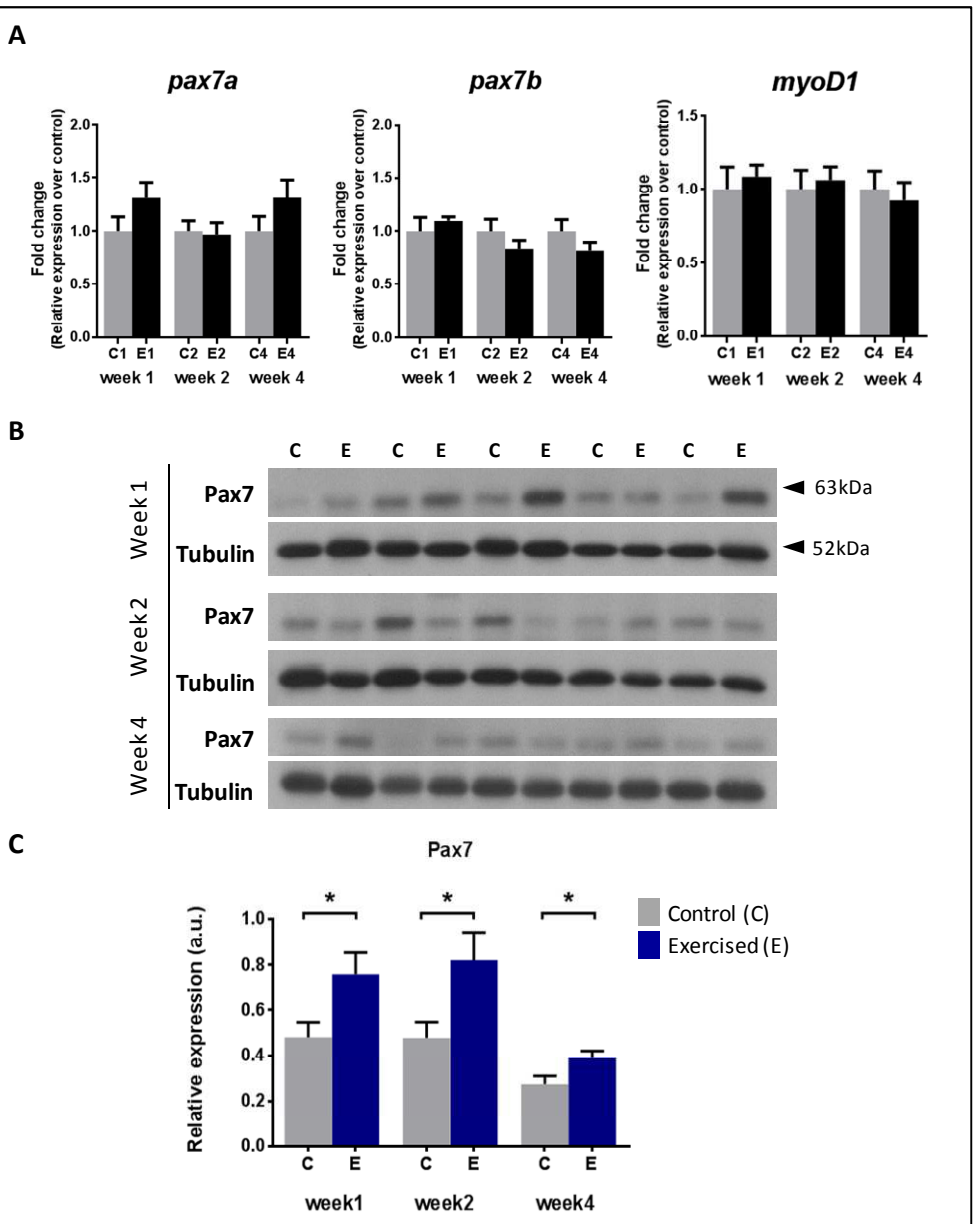

Figure 1.8. Expression of the stem cell marker Pax7. A mRNA expression of the two zebrafish isoforms *pax7*a and *pax7b* and myogenic factor m*yod1* represented as fold change in exercised (E, n=10) over non-exercised (C, n=10) adult zebrafish at 1, 2 and 4 weeks (20 days) of exercise training. Bars represent ± SEM. **B** SDS-PAGE of Pax7 at week 1, 2 and 4. Arrowheads indicate the protein marker molecular weight. Note that not all individuals used in the analysis are shown in the figure. **C** Quantification by densitometry at week 1 (P=0.026), week 2 (P=0.025) and week 4 (P=0.025). Asterisk indicates stastistical significance P<0.05 and barrs represent ± SEM. (E, n=10; C, n=10).

Chapter 2

Exercise training increases the expression of myokines in fast skeletal muscle

In fast muscle, the mRNA expression of several myokines was analyzed throughout the training period: after one week (5 days exercise), two weeks (12 days exercise) and 4 weeks (20days exercise) of swimming-induced exercise.

The mRNA expression of interleukin 6 (*il6*), one of the most interesting myokines evaluated because of its implications in muscle metabolism and satellite cell regulation, appeared significantly up-regulated after one week of training (P=0.004) as was its receptor, *il6ra* (P=0.009) (**Figure 2.1**).

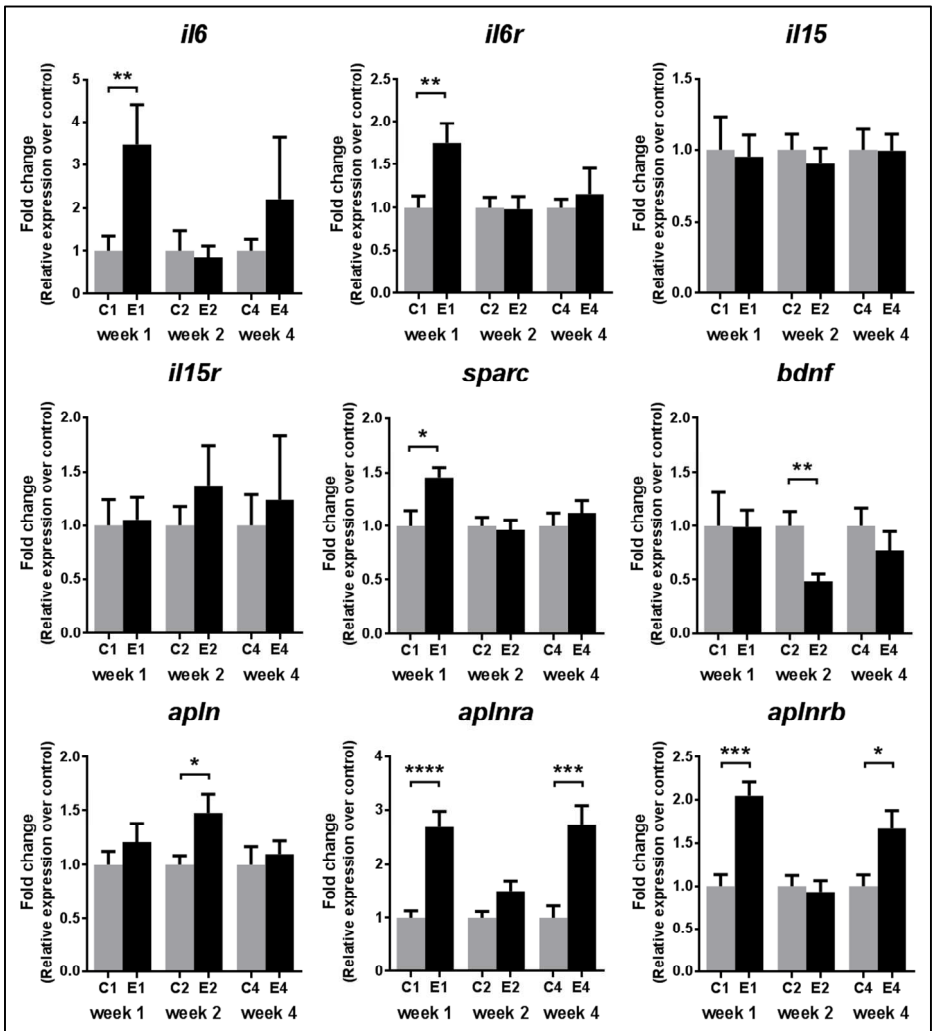

Figure 2.1. Expression of myokines principally involved in muscle metabolism and development. mRNA expression is represented as fold change in exercised (E, n=10) over non-exercised (C, n=10) adult zebrafish at 1,2 and 4 weeks (20 days) of exercise training. Significant differences are indicated by *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 and barrs represent ± SEM.

The second subunit of the receptor heterodimer, *gp130* or signal the transducer, was not analyzed because it is not specific for *il6* as it is shared with other receptor complexes from other genes. To demonstrate Il6 signaling we attempted to analyze the phosphorylation status of STAT3, a downstream target of Il6. However, the phosphorylated STAT3 polyclonal antibody used in Western blotting did not immunoreact with proteins in our zebrafish muscle samples. The mRNA expression levels of interleukin 15 (*il15*) and its receptor remained unchanged over the four week exercise period (**Figure 2.1**). The secreted protein acidic and rich in cysteine (*sparc*) mRNA expression levels were significantly higher only after one week of exercise (P=0.043) and remained unchanged after two and four weeks (**Figure 2.1**). In contrast, the brain derived neurotrophic factor (*bdnf*) decreased its mRNA expression levels significantly after two weeks of training in fast muscle of exercised zebrafish (P=0.006) but no effect of exercise was observed after one or four weeks of training (**Figure 2.1**). The mRNA expression levels of apelin (*apln*) were significantly increased after two weeks of exercise (P=0.029). Similarly, the mRNA expression levels of the receptor subunits *aplnra* and *aplnrb* were also significantly increased by training, showing higher level of change than their ligand but significantly increasing after one (*aplnra,* P<0.0001; *aplnrb,* P=0.0003) and four (*aplnra,* P=0.0004;*aplnrb* P=0.011) weeks of training (**Figure 2.1**).

Figure 2.2 shows the regulation by exercise training of the mRNA expression levels of myokines and other growth factors mainly involved in muscle mass regulation.

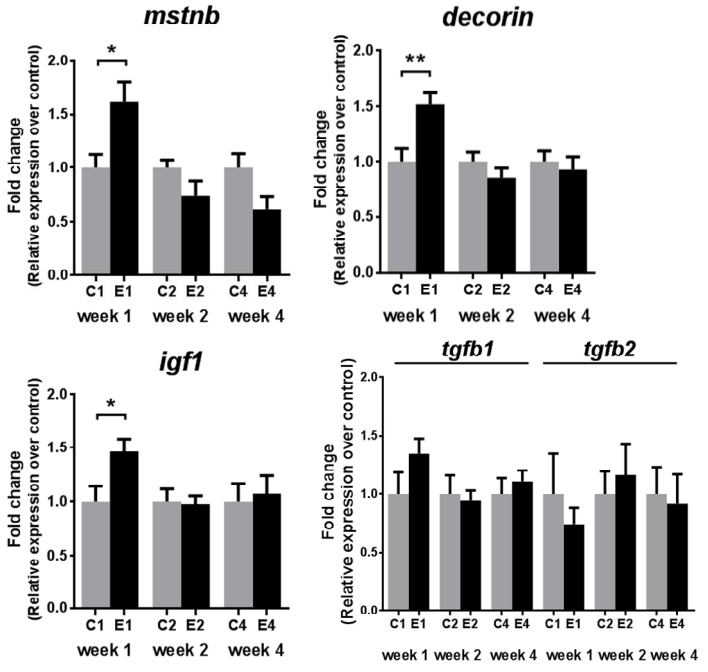

Figure 2.2. Expression of myokines and other growth factors involved in muscle growth. . mRNA expression is represented as fold change in exercised (E, n=10) over non-exercised (C, n=10) adult zebrafish at 1,2 and 4 weeks (20 days) of exercise training. Significant differences are indicated by *P<0.05, **P<0.01 and barrs represent ± SEM.

Decorin was only significantly up-regulated after one week of exercise (P=0.007), similar to myostatin b (*mstnb*) (P=0.023) (**Figure 2.2**). We also analyzed the expression of other *tgfb* family members such as *tgfb1* and *tgfb2* and their mRNA levels remained unaltered during the exercise training period, with a slight increase in *tgfb1* mRNA expression after the first week of training (P=0.089). Interestingly, *igf1* expression was also significantly up-regulated only after the first week of exercise (P=0.019) (**Figure 2.2**).

We were particularly interested in investigating the regulation of the expression of the myokine fibronectin type III domain-containing protein 5b (*fndc5b*), that codes for the soluble factor named irisin, by exercise. In this study, we measured for the first time in zebrafish the presence and the regulation of *Fndc5b* expression in zebrafish skeletal muscle. In zebrafish fast muscle, *fndc5b* was more highly expressed in muscle in comparison with *fndc5a* (**Figure 2.3**). Tissue distribution analyses of *fndc5a and fndc5b* transcripts show that both isoforms were widely distributed with a high level of expression in the brain (**Figure 2.3**).

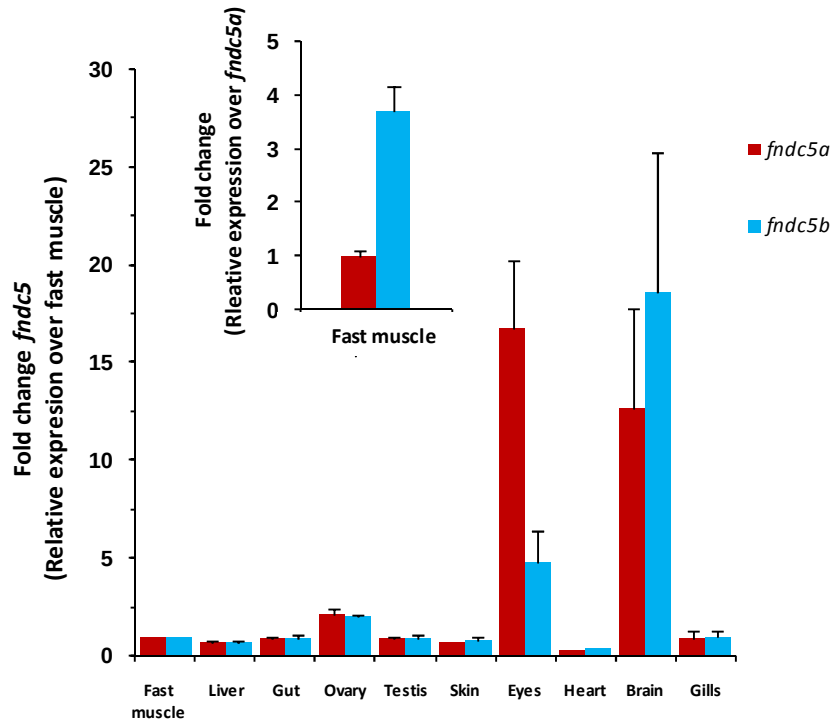

Figure 2.3. *fndc5a* and *fndc5b* mRNA expression tissue distribution. A mRNA expression differences between *fndc5a* and *fndc5b* in fast muscle, represented as fold change over fndc5a. **B** mRNA expression on *fndc5a* and *fndc5b* represented as fold change over fast muscle**.** Bars represent ± SEM.of >1 biological replicates per tissue.

During exercise conditions, *fndc5b* showed a trend towards an increase after four weeks of exercise (P=0.094) (**Figure 2.4**). Interestingly, as shown above, the expression of *pgc1α*, a target transcription factor for *fndc5b*, was significantly up-regulated after four weeks of training (**Figure 1.4**), suggesting a link between the induction of *fndc5b* and *pgc1α* at four weeks at least at the mRNA level. There is controversy in the literature regarding irisin

expression during exercise and therefore, we attempted to test a mammalian antibody in zebrafish samples. The polyclonal antibody used (USCS) was raised against the non-cytoplasmatic domain (FNIII domain) of human FNDC5 that is highly conserved in zebrafish fndc5b (see Appendix). However, we failed to detect antibody reactivity with muscle samples from exercised zebrafish as well as in rat gastroceminus muscle (**Figure 2.4 B**).

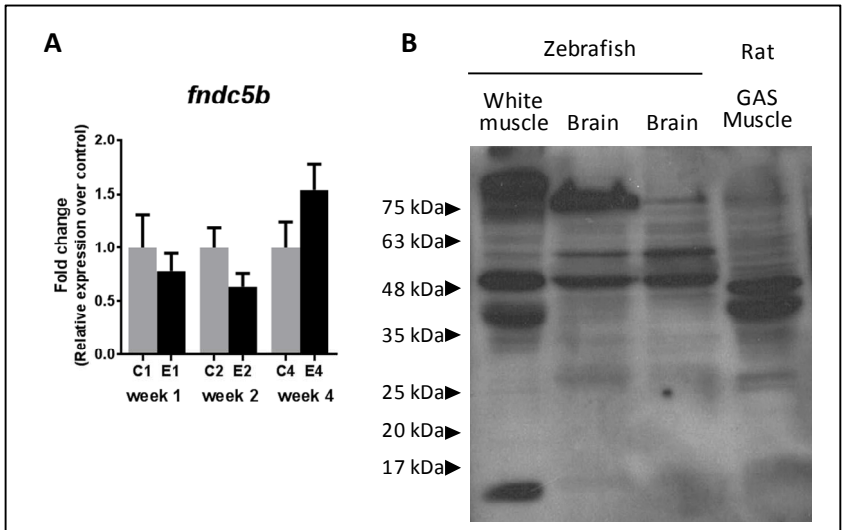

Figure 2.4. Expression of *fndc5b* in zebrafish.
A mRNA expression is represented as fold change in exercised (E, n=10) over non-exercised (C, n=10) adult zebrafish at 1 (P>0.999), 2 (P=0.104) and 4 weeks (P=0.093) (20 days) of exercise training. Bars represent ± SEM. **B** SDS-PAGE blot of polyclonal human FNDC5 antibody (USCN, Leu77-Met144, dilution 1:50) in zebrafish fast muscle and brain and rat gastrocnemius muscle (GAS) as positive control. Expected molecular weight should be arround 13 kDa, however, no clear bands could be identified for any tissue sample.

In silico characterization of the zebrafish *il6* promoter sequence

After the cloning and characterization of the *il6* gene in zebrafish (Varela *et al*, 2012) and the work on *il6* promoter characterization in our laboratory on *Sparus aurata*, another teleost fish species (Castellana *et al*, 2013), we interrogated the sequence of the zebrafish *il6* promoter for conserved elements. A genomic DNA sequence of approximately 1.5 kb upstream from the first codon (ATG) of the zebrafish *il6* gene (ENSDARG00000102318) was searched for putative transcription factor binding sites. As shown in **Figure 2.5**, a TATA box was found,similar to the human, chicken and seabream IL-6 promoters. Consensus binding sites among vertebrates were found in the zebrafish *il6* promoter, including NF-KB(-138 to -152), NFAT (-348 to -366, -806 to -824), AP-1 (-409 to -421), NF-IL6 or C/EBPb(-524 to -538), ETS-1 (-594 to -614), CRE (-729 to -749), GATA (-746 to -758, -826 to -838) and glucocorticoid response elements GRE (-840 to -854, -969 to 987). Interestingly, a MEF2 binding site was found in the core sequence of the promoter and was included in the analysis. Although this is based on an in silico analysis, muscle-response elements are likely present within the *il6* promoter sequence, particularly if we consider the reported roles of IL-6 in skeletal muscle (Pedersen, 2012; Muñoz-Cánoves *et al*, 2013). Similar positions of the transcription factor binding sites were found among the vertebrate promoters. Detailed information about transcription binding sequences are shown in Appendix Table A.3.

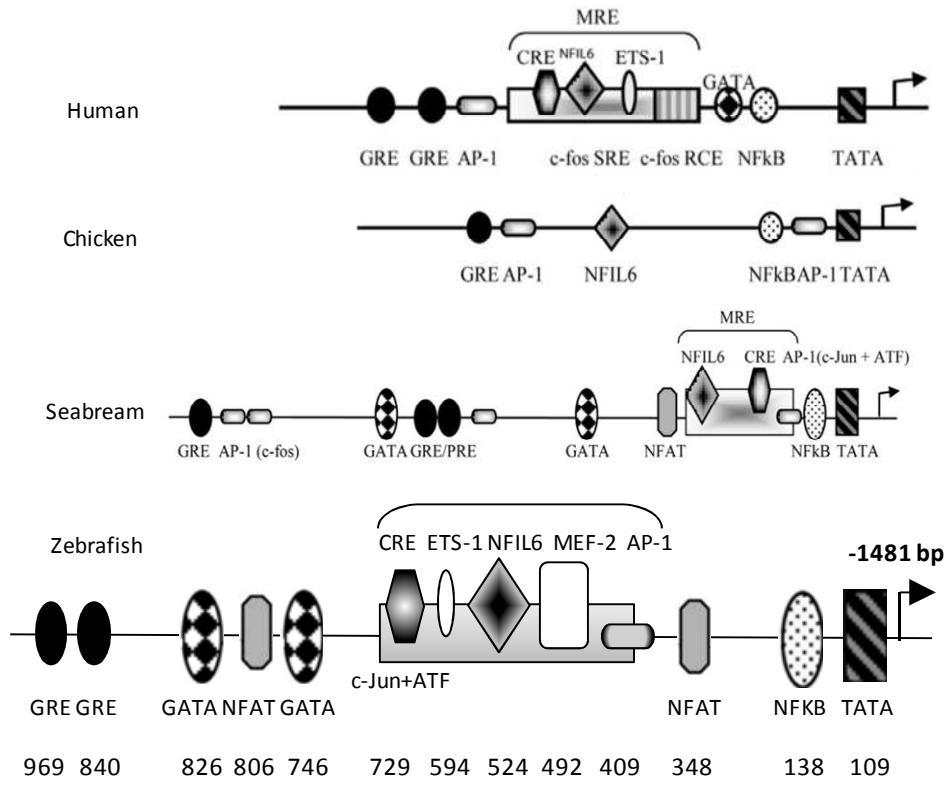

Figure 2.5 Characterization *in silico* of zebrafish *il6* proximal promoter (-1481 bp). Comparison with human, chicken and seabream IL6 promoters shows a high conservation of the transcription response elements. Allen et al. suggested a MEF-2 binding sequence in the human IL6 promoter (Allen et al. 2009) that was found in zebrafish sequence. Numbers indicate the position of the predicted binding sequences. Human (Dendorfer *et al*. 1994), chicken (Kasier et al. 2004) and seabream (Castellana *et al*. 2013).

Taking all these results together, we have shown for the first time that adult zebrafish fast muscle expresses several myokines at the mRNA level in response to exercise and that their regulation occurs in a time-dependent manner. Moreover, to the best of our knowledge, the promoter of *il6*, an important inflammatory cytokine and considered as the "prototype myokine", has been characterized for the first time in zebrafish.

Chapter 3

Transcriptomic response to LPS administration and exercise

In order to investigate if exercise training had any modulatory effects on the ability of fish to respond to an immune challenge, exercised and non-exercised fish were subjected to an intraperitoneals administration of lipopolysaccharide (LPS), a typical component of the external cell layer of Gram-negative bacteria. First, we aimed at investigating the transcriptomic response of zebrafish fast muscle to LPS in adult zebrafish subjected to exercise and resting conditions. Microarray analysis was performed using fast muscle samples from exercised and non-exercised fish and differential gene expression patterns were evaluated comparing the results from LPS-treated samples with the corresponding control PBS-injected samples (Non-exercised-LPS vs Non-exercised-PBS and Exercised-LPS vs Exercised-PBS). The selection of the sampling time at 72 hours post-LPS injection was chosen in view of results from previous studies in our laboratory that showed a robust transcriptomic response in skeletal muscle of fish treated with LPS at 72 hours post injection (Kaitetzidou *et al*, 2012; Magnoni *et al*, 2015). In exercised LPS-stimulated fast muscle, a total of 1141 differentially expressed genes were detected, with 679 up-regulated and 462 down-regulated genes by using a cut-off of 1.2 fold-change and a P-value of 0.05. Non-exercised LPS-stimulated fast muscle showed a total of 360 differentially expressed genes, with 151 up-regulated and 209 down-regulated genes using the same cut-off values as described above. Therefore, the number of differentially expressed genes in fast muscle from exercised LPS-treated fish was higher than that of non-exercised LPS-treated fish, suggesting that exercised zebrafish show a stronger transcriptomic response to LPS than non-exercised zebrafish.

Initial classification of differentially expressed genes was performed by following Gene Ontology categories using the bioinformatics software DAVID ($P<0.05$). Significant functional categories between the two datasets were very similar in nature but with the observation that exercised LPS-stimulated fast muscle contained a higher number of significant enriched functional categories. Both datasets had significant enrichment in functional categories related to muscle development, regulation of growth, intracellular signaling cascade, regulation of apoptosis, regulation of chemotaxis, catabolic process, carbohydrate and fatty acid metabolism. Interestingly, as shown in **Table 3.1**, exercised LPS-stimulated fast muscle was enriched in categories related to muscle contractile function that resembled the molecular profile identified in the transcriptomic response of adult zebrafish fast muscle to exercise described in Chapter 1. These categories included muscle contraction, cytoskeleton and ECM organization, angiogenesis and oxidative phosphorylation (**Table 3.1**). However, none of these functional categories were found in non-exercised LPS-stimulated zebrafish fast muscle and, contrary to the exercised LPS-stimulated group, functional categories related with the immune system and the immune response were not statistically enriched in this group.

By mining the Ingenuity Knowledge Base for canonical pathways, we identified pathways that were significantly ($P<0.05$) over-represented in LPS-stimulated fast muscle of adult zebrafish (**Table 3.2**). Notably, a higher number of significantly regulated canonical pathways were found

in the exercised than in the non-exercised LPS-stimulated fast muscle, such as mitochondrial dysfunction, actin cytoskeleton signaling, protein ubiquitination pathway, mTOR signaling, IGF-1 signaling or p53 signaling. Common regulated pathways were found in the two datasets, such as Wnt/β-catenin signaling, IL-8 signaling, fatty acid β-oxidation, calcium signaling or AMPK signaling; whereas other pathways such as glycolysis or LXR/RXR activation were not found in fast muscle of exercised LPS-stimulated zebrafish. In contrast to the fast muscle from non-exercised zebrafish stimulated with LPS, exercised LPS-stimulated fast muscle showed a number of significant canonical pathways involved in the immune response, such as T Cell Receptor signaling, complement system, B cell receptor signaling, leukocyte extravasation signaling or LPS-stimulated MAPK signaling. Using this initial approach we can conclude that the differentially expressed genes in exercised LPS-stimulated fast muscle showed a higher representation of canonical pathways, including immune-related pathways.

The Venn diagram displayed in **Figure 3.1** shows that 1070 differentially expressed genes were uniquely expressed in fast muscle of exercised LPS-stimulated zebrafish, whereas289 differentially expressed genes were uniquely expressed in fast muscle of non-exercised LPS-stimulated zebrafish. In addition, only 71 differentially expressed genes were common for the two exercise conditions tested. By examining the genes that appeared unique under each condition (**Figure 3.1**); we observed a more intense regulation of immune-related genes in fast muscle from exercised LPS-stimulated than in fast muscle from non-exercised LPS-stimulated zebrafish. The exercised LPS-stimulated group showed a more intense regulation of factors of the complement system (*c1qr*, *c3*, *c7*, *c8a*, *cfb*, *cfi*) in comparison with the non-exercised LPS-stimulated group (*c3ar1*) (**Table 3.3**). Cytokines (*hmbg1*), cytokine receptors (*il11ra*, *il12r*), TNF related factors (*litaf*, *tnfaip6, zfp36*), toll-like receptors (*tlr2*) and phagocytic or scavenger receptors (*cd302*, *scara5*, *scarf2*) were uniquely expressed in the exercised LPS-stimulated fast muscle (**Table 3.3**). Among the unique genes expressed in the non-exercised LPS-stimulated fast muscle there were a small number of genes involved in the immune response, although canonical pathways and functional categories did not statistically represent them. These genes included lyzozyme bacteriolytic factor *lyzl2* (a gene related to male reproductive system), the important chemokine *cxcl14*, toll-like receptor *tlr3*, interferon transcription factor *irf3*, oxidative defense *nos2* and *cd36,* that acts as a co-receptor for TLR to promote inflammation but also plays a role in lipid metabolism regulating fatty acid transport in the cell (**Table 3.3**). It is important to highlight that both datasets expressed genes from the innate (complement, toll-like, phagocytic receptors) and adaptive (*lck*, *mll5*, *hla-dqa1*, *igbp1*, *blk*, *hm13*, *spi1*) immune systems; however, the exercised fast muscle showed a representative number of genes implicated in the immune response as shown by the enriched categories and canonical pathways specific for the immune system.

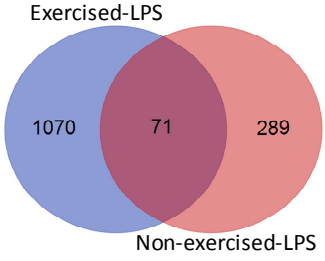

Figure 3.1. Venn diagram of the two gene sets from LPS administration. Significant differentially expressed genes with 1.2 fold change are represented. Exercised-LPS (blue) and non-exercised LPS (red) have common differentially expressed genes (intersection).

Among the unique genes differentially expressed in fast muscle from exercised LPS-stimulated zebrafish, many of them have been found previously under exercise conditions, such as genes related to muscle contraction (*camk2a*, *pvalb*, *ryr1*, *tmp4*, *tnni1*, *ttn*), cytoskeletal components involved in the transmission of the sarcomere force (*myoz2*, *sgcg*, *sgce*, *itga5*, *itgb5*) as well as ECM components (*dcn*, *fn1*,*mmp14*, *col1a1*) (**Table 3.4**). Interestingly, two myokines were found down-regulated, *sparc* and *dcn*. Furthermore, unique genes in exercised LPS-stimulated zebrafish fast muscle contained a number of muscle growth and developmental factors (*acvr2b*, *bmp1*, *fgf13*, *fgf18*, *fst*, *fstl1*,*fzd3*, *fzd7, igf1r*, *igf2bp1*,*mtor*,*tgfb2*,*wnt4*), including myogenic factors (*myod1*, *myf6*). In contrast, on-exercised LPS-stimulated fast muscle had less representation of genes in these categories (*fgf4*, *notch3*, *nrg1*,*wnt11*), as shown in the GO analysis. Moreover, there were genes related to angiogenesis that were also found in exercised LPS-stimulated fast muscle and that were reported in our previous transcriptomic analysis (*angpl2*, *angpl4*, *edn1*, *efna1*, *epha4*) (**Table 3.4**). Interestingly, up-regulation of genes related with autophagosome formation (*atg9a*, *atg13*, *hmgb1*, *gabarapl1*) and a down-regulation of the lysosomal enzymes (e.g. cathepsins)were observed in fast muscle of exercised LPS-stimulated zebrafish (**Table 3.4**). Moreover, in the exercised LPS dataset, genes related to protein degradation such as cathepsins (*ctsd*, *ctsk*), as indicated above, calpains (*capn5*) and ubiquitin enzymes (*cbl*, *znrf1*, *nedd4l*, *dtl*, *trim71*, *hspa1a, pmse4*) were found together with differentially regulated genes encoding translation factors (*eef1d*, *eif4a1*, *eif4e3*, *rps6ka6*) (**Table 3.4**). In the non-exercised LPS-stimulated fast muscle, there was a lower number of genes related to protein degradation but a strong down-regulation of genes encoding serine proteases (*prss1*, *prss2*) was found. Genes involved in carbohydrate and lipid metabolism were found to be expressed in fast muscle of LPS-stimulated zebrafish irrespective of their swimming condition; however, as observed previously, in the exercised LPS-stimulated fast muscle there was a higher number of differentially expressed genes related to energy metabolism (*ckmt1b*, *ckb*, *mb*, *ucp2*) and mitochondrial oxidation (*atp5s*, *sdhc*, *ndufa*, *cox5a*, *cox7c*) (**Table 3.4**). It is worth mentioning the altered expression of pro-apoptotic (*casp3*, *p53*, *tp53rk*, *bcl2l13*, *bnip3*) and anti-apoptotic (*clu*, *xiap*) genes, mostly in the exercised LPS-stimulated group.

From the 71 common differentially expressed genes (Figure 3.1), 21had an opposite pattern of regulation as highlighted in **Table 3.5**. For instance, an up-regulation of the expression of members of the Wnt pathway (*cby1*, *dvl2*, *fzd3, ptpn13*) was found in the exercised group, whereas in the non-exercised group these genes are found to be down-regulated, suggesting an activation of this pathway in exercised fish upon LPS infection. Moreover, genes involved in muscle contraction (e.g.*tnni2)* and metabolism (e.g. *ugp2*involved in glycogen biosynthesis and the aminoacid transporter *slc43a2),* were up-regulated in the exercised group (**Table 3.5**). From the common differentially expressed genes, genes involved in microtubule dynamics were also found, such as *mid1ip*1 (microtubule stabilization), *snx14* (expressed in motoneurons and involved in membrane receptor recycling) or *dynlrb1* (microtubule transport), the latter being strongly up-regulated in the non-exercised group (**Table 3.5**). Interestingly, the chaperone *hsp90aa1* (also known as Lipopolysaccharide-Associated Protein 2), that functions also as a component of the pattern recognition receptors (such as TLRs) that binds to LPS, was down-regulated in the exercised LPS-stimulated fast muscle but slightly up-regulated in non-exercised LPS fast muscle. The remaining 51 common genes that were differentially expressed

in a similar direction might give information about relevant genes that respond to LPS in fast muscle regardless of the training effect. These include immune-related genes (*lck*, *tcf7l1*, *traf4*), structural and contractile genes (*col11a1*, *gsn*, *loxl2*, *loxl3*, *lum*, *myh4*, *tnnt3*, *tpm4*, *tubb4a*) or metabolic genes (*etfdh, hif3a*, *pgam2*, *tgfb3*) (**Table 3.5**).

The results of microarray analysis were validated by qPCR for13 differentially expressed genes in fast muscle, 7 in exercised LPS-stimulated fast muscle and 6 in non-exercised LPS-stimulated fast muscle. The genes examined showed a similar pattern of change with the two techniques used, except for *ucp2* (Additional TableA.5)

Table 3.1. Functional annotation analysis based on GO terms in zebrafish fast muscle in response to LPS stimulation (DAVID).

		C-LPS		E-LPS	
GO Term		**Count**	**P-Value**	**Count**	**P-Value**
GO:0048634	Regulation of muscle development	8	1.53E-04	16	7.87E-05
GO:0006936	Muscle contraction			27	0.004
GO:0007010	Cytoskeleton organization			76	1.06E-06
GO:0030036	Actin cytoskeleton organization			43	4.10E-05
GO:0030198	Extracellular matrix organization			29	5.66E-07
GO:0001666	Response to hypoxia	10	0.004	50	5.37E-17
GO:0001525	Angiogenesis			47	5.80E-13
GO:0001568	Blood vessel development			73	3.68E-18
GO:0040008	Regulation of growth	15	0.026	75	4.28E-11
GO:0006366	Transcription from RNA polymerase II promoter	25	0.047	45	2.01E-05
GO:0007242	Intracellular signaling cascade	43	0.008	185	1.05E-08
GO:0042325	Regulation of phosphorylation	18	0.041	91	2.54E-10
GO:0043408	Regulation of MAPKKK cascade			25	1.29E-04
GO:0046425	Regulation of JAK-STAT cascade			10	0.007
GO:0016567	Protein ubiquitination			19	0.044
GO:0042981	Regulation of apoptosis	28	0.031	168	6.58E-22
GO:0002682	Regulation of immune system process			77	2.37E-09
GO:0006954	Inflammatory response			44	0.028
GO:0002253	Activation of immune response			20	0.002
GO:0050920	Regulation of chemotaxis	4	0.034	11	5.40E-04
GO:0007159	Leukocyte adhesion			9	0.004522
GO:0045321	Leukocyte activation			45	4.69E-05
GO:0046649	Lymphocyte activation			38	1.14E-04
GO:0042110	T cell activation			25	0.00
GO:0042113	B cell activation			16	0.007
GO:0030099	Myeloid cell differentiation			22	2.29E-04
GO:0002224	Toll-like receptor signaling pathway			5	0.042
GO:0034097	Response to cytokine stimulus			17	0.004
GO:0043122	Regulation of I-kappaB kinase/NF-kappaB cascade			19	0.017
GO:0016236	Macroautophagy			6	0.009
GO:0005975	Carbohydrate metabolic process	25	0.001	75	0.001
GO:0006631	Fatty acid metabolic process	14	6.67E-04	42	3.36E-06
GO:0009056	Catabolic process	50	1.50E-04	157	0.001
GO:0006090	Pyruvate metabolic process			11	0.007
GO:0006091	Generation of precursor metabolites and energy			79	5.75E-15
GO:0006094	Gluconeogenesis			7	0.031
GO:0006099	Tricarboxylic acid cycle			8	0.005
GO:0006119	Oxidative phosphorylation			30	3.75E-08
GO:0006754	ATP biosynthetic process			16	0.027

GO:0000302	Response to reactive oxygen species		18	8.38E-04
GO:0001836	Release of cytochrome c from mitochondria		9	5.53E-04

The two comparisons are indicated as C-LPS for the non-exercised and E-LPS for the exercised fish. For each enriched Gene Ontology term, count and P-value (P<0.05) are shown.

Table 3.2. Significantly over-represented putative canonical pathways in LPS-stimulated fast muscle of zebrafish.

	C-LPS		E-LPS	
Ingenuity Canonical Pathways	**P-value**	**Ratio**	**P-value**	**Ratio**
Wnt/β-catenin Signaling	2.51E-05	13/17	4.64E-04	23/175
IL-8 Signaling	9.49E-04	11/20	2.69E-02	19/208
Glycolysis I	1.03E-02	3/41		
eNOS Signaling	1.29E-02	7/152		
Fatty Acid β-oxidation I	1.91E-02	3/45	2.18E-03	7/45
Calcium Signaling	2.24E-02	8/213	8.14E-06	28/213
LXR/RXR Activation	2.85E-02	6/136		
Estrogen Receptor Signaling	3.49E-02	6/136		
AMPK Signaling	4.35E-02	6/169	7.33E-04	19/169
Mitochondrial Dysfunction			1.17E-15	41/192
Hypoxia Signaling in the Cardiovascular System			8.06E-08	18/67
Integrin Signaling			2.31E-07	33/208
Actin Cytoskeleton Signaling			5.49E-07	34/239
NRF2-mediated Oxidative Stress Response			8.14E-06	28/192
PI3K/AKT Signaling			2.61E-05	21/144
HIF1α Signaling			7.05E-05	18/108
GM-CSF Signaling			1.25E-04	13/68
Protein Ubiquitination Pathway			3.37E-04	31/268
Role of p14/p19ARF in Tumor Suppression			4.06E-04	8/32
mTOR Signaling			4.09E-04	25/211
JAK/Stat Signaling			8.77E-04	12/70
T Cell Receptor Signaling			1.05E-03	15/109
CNTF Signaling			1.32E-03	10/55
IL-15 Signaling			1.34E-03	11/67
IL-2 Signaling			1.54E-03	10/58
TGF-β Signaling			1.97E-03	13/89
PI3K Signaling in B Lymphocytes			2.78E-03	17/140
CXCR4 Signaling			3.51E-03	19/170
Complement System			3.88E-03	7/35
IL-3 Signaling			4.63E-03	11/74
PPAR Signaling			5.93E-03	13/105
Lymphotoxin β Receptor Signaling			6.17E-03	9/61
fMLP Signaling in Neutrophils			8.45E-03	14/130
Role of Macrophages, Fibroblasts and Endothelial Cells in R.Arthritis			1.04E-02	30/336
B Cell Receptor Signaling			1.13E-02	18/171
Glutamate Degradation II			1.14E-02	2/10
Aspartate Biosynthesis			1.14E-02	2/7
TCA Cycle II (Eukaryotic)			1.28E-02	5/41
Leukocyte Extravasation Signaling			1.34E-02	21/207
PPARα/RXRα Activation			1.52E-02	19/193
NF-κB Activation by Viruses			1.72E-02	10/83
IGF-1 Signaling			1.86E-02	12/105
Insulin Receptor Signaling			1.91E-02	15/142
Mitochondrial L-carnitine Shuttle Pathway			1.92E-02	4/22

Role of IL-17F in Allergic Inflammatory Airway Diseases		2.14E-02	7/48
Role of NFAT in Regulation of the Immune Response		2.24E-02	18/199
p53 Signaling		3.17E-02	11/96
Creatine-phosphate Biosynthesis		3.49E-02	2/9
LPS-stimulated MAPK Signaling		3.92E-02	9/82
Natural Killer Cell Signaling		4.40E-02	12/117

The associated p-value (Fisher's exact test P<0.05) and the ratio of the number of differentially expressed genes in fast muscle in response to LPS over the total number of genes in each particular pathway in the Ingenuity Knowledge Base. Canonical pathway names are from Ingenuity Systems.

Table 3.3. Selected genes with immune-related function from non-exercised (C-LPS) and exercised (E-LPS) fast muscle stimulated with LPS.

		C-LPS	E-LPS
Name	Gene description	FC	FC
	Immune-related factors		
lyzl2	lysozyme-like 2	**8.98**	
cfb	complement factor B		**4.04**
cfi	complement factor I		**3.01**
c3	complement component 3		**2.74**
c7	complement component 7		**2.69**
nfatc3	nuclear factor of activated T-cells, calcineurin-dependent 3		**2.63**
tlr2	toll-like receptor 2		**2.62**
litaf	lipopolysaccharide-induced TNF factor		**2.46**
lck	lymphocyte-specific protein tyrosine kinase	**2.38**	**2.34**
c8a	complement component 8, alpha polypeptide		**2.15**
igbp1	immunoglobulin (CD79A) binding protein 1		**2.04**
hmgb1	high mobility group box 1		**2.01**
cxcl14	chemokine (C-X-C motif) ligand 14	**1.96**	
cd302	CD302 molecule Type I Transmembrane C-Type Lectin Receptor DCL-1		**1.8**
blk	B lymphoid tyrosine kinase		**1.71**
il11ra	interleukin 11 receptor, alpha		**1.65**
nos2	nitric oxide synthase 2, inducible	**-1.46**	
tlr3	toll-like receptor 3	**-1.49**	
irf3	interferon regulatory factor 3	**-1.53**	
tcf7l1	transcription factor 7-like 1 (T-cell specific, HMG-box)	**-1.54**	**-1.62**
mll5	myeloid/lymphoid or mixed-lineage leukemia 5 (trithorax homolog, Drosophila)	**-1.55**	
c3ar1	complement component 3a receptor 1	**-1.57**	
hla-dqa1	major histocompatibility complex, class II, DQ alpha 1	**-2.04**	
traf4	TNF receptor-associated factor 4	**-2.16**	**-1.77**
cd36	CD36 molecule (thrombospondin receptor)	**-2.17**	
pglyrp1	peptidoglycan recognition protein 1	**-2.36**	
il21r	interleukin 21 receptor		**-2.45**
scarf2	scavenger receptor class F, member 2		**-2.68**
c1qc	complement component 1, q subcomponent, C chain		**-2.83**
hm13	histocompatibility (minor) 13		**-3.13**
spi1	spleen focus forming virus (SFFV) proviral integration oncogene spi1		**-3.73**
scara5	scavenger receptor class A, member 5 (putative)		**-5.71**
tnfaip6	tumor necrosis factor, alpha-induced protein 6		**-15.53**

Cell colour and intensity indicate up-regulated (red) and down-regulated (green) genes for a better comparison between the two groups.

able 3.4. Selected genes related with muscle function from non-exercised (C-LPS) and exercised (E-LPS) fast muscle stimulated with LPS.

Name	Gene description	C-LPS FC	E-LPS FC
	Muscle contraction		
tnni1	troponin I type 1 (skeletal, slow)		9.07
ryr1	ryanodine receptor 1 (skeletal)		6.35
myh7	myosin, heavy chain 7, cardiac muscle, beta		3.68
pvalb	parvalbumin		3.28
ttn	titin		3.16
calm1	calmodulin 1 (phosphorylase kinase, delta)		1.56
cacna1a	calcium channel, voltage-dependent, P/Q type, alpha 1A subunit		-1.54
cacna1c	calcium channel, voltage-dependent, L type, alpha 1C subunit	-2.00	
camk2a	calcium/calmodulin-dependent protein kinase II alpha		-1.98
	Cytoskeleton and ECM		
sgcg	sarcoglycan, gamma		2.43
myoz2	myozenin 2		2.02
postn	periostin, osteoblast specific factor		-1.88
itgb5	integrin, beta 5		-1.91
sparc	secreted protein, acidic, cysteine-rich (osteonectin)		-1.96
sgce	sarcoglycan, epsilon		-2.12
itga5	integrin, alpha 5 (fibronectin receptor, alpha polypeptide)		-2.31
mmp9	matrixmetallopeptidase 9 (92kDa type IV collagenase)	-1.33	
mmp14	matrixmetallopeptidase 14 (membrane-inserted)		-2.06
dcn	decorin		-3.32
col1a1	collagen, type I, alpha 1		-3.76
fn1	fibronectin 1		-4.54
tuba1a	tubulin, alpha 1a		-10.48
	Muscle growth and development		
fgf13	fibroblast growth factor 13		4.88
fgf17	fibroblast growth factor 17		3.5
esrrb	estrogen-related receptor beta		3.09
fgf18	fibroblast growth factor 18		3.06
wnt4	wingless-type MMTV integration site family, member 4		3.03
fzd8	frizzled family receptor 8		2.76
fst	follistatin		2.57
igf1r	insulin-like growth factor 1 receptor		2.38
myod1	myogenic differentiation 1		2.08
fgf4	fibroblast growth factor 4	2.03	
myf6	myogenic factor 6 (herculin)		1.9
wnt11	wingless-type MMTV integration site family, member 11	1.94	
acvr2b	activin A receptor, type IIB		1.89
pten	phosphatase and tensin homolog		1.52
tgfb2	transforming growth factor, beta 2		1.33
bmp1	bone morphogenetic protein 1		-1.19
fzd7	frizzled family receptor 7		-1.46
mtor	mechanistic target of rapamycin (serine/threonine kinase)		-1.64
bdnf	brain-derived neurotrophic factor	-1.69	
tgfb1	transforming growth factor, beta 1	-1.74	
pdgfa	platelet-derived growth factor alpha polypeptide		-1.85
ctnnb1	catenin (cadherin-associated protein), beta 1, 88kDa		-2.18
nrg1	neuregulin 1	-2.32	
fstl1	follistatin-like 1		-3.12
	Angiogenesis		
angptl4	angiopoietin-like 4		3.67
epha4	EPH receptor A4		3.45
edn1	endothelin 1		1.92
efna1	ephrin-A1		1.87
aggf1	angiogenic factor with G patch and FHA domains 1		1.74
efnb1	ephrin-B1	-1.42	
angptl3	angiopoietin-like 3	-1.71	

angptl2	angiopoietin-like 2		-1.8
notch3	notch 3	-2.16	
	Protein synthesis and degradation		
gabarapl1	GABA(A) receptor-associated protein like 1		5.96
znrf1	zinc and ring finger 1, E3 ubiquitin protein ligase		2.94
usp25	ubiquitin specific peptidase 25		2.54
capn5	calpain 5		2.24
vhl	vonHippel-Lindau tumor suppressor, E3 ubiquitin protein ligase		2.18
eif4e3	eukaryotic translation initiation factor 4E family member 3		2.15
atg9a	autophagy related 9A		2.1
hmgb1	high mobility group box 1		2.01
eef1d	eukaryotic translation elongation factor 1 delta		2.01
trim71	tripartite motif containing 71, E3 ubiquitin protein ligase		1.52
atg13	autophagy related 13		1.77
eif4a1	eukaryotic translation initiation factor 4A1		1.68
psme4	proteasome (prosome, macropain) activator subunit 4		1.63
ubc	ubiquitin C	1.49	
psmb2	proteasome (prosome, macropain) subunit, beta type, 2	1.32	
cbl	Cbl proto-oncogene, E3 ubiquitin protein ligase		-1.54
ctsd	cathepsin D		-2.62
ctsk	cathepsin K		-3.22
eif4b	eukaryotic translation initiation factor 4B		-3.64
hspa1a	heat shock 70kDa protein 1A		-4.75
prss1	protease, serine, 1 (trypsin 1)	-13.7	
ctrb2	chymotrypsinogen B2	-23.17	
prss2	protease, serine, 2	-29.79	
	Metabolism		
cpt1a	carnitinepalmitoyltransferase 1A (liver)		6.89
ppara	peroxisome proliferator-activated receptor alpha		5.75
mb	myoglobin		5.62
ucp2	uncoupling protein 2 (mitochondrial, proton carrier)		4.18
aldoa	aldolase A, fructose-bisphosphate		3.62
gyg1	glycogenin 1		3.49
pfkm	phosphofructokinase, muscle		3.44
atp5o	ATP synthase, H+ transporting, mitochondrial F1 complex, O		2.86
pdk4	pyruvate dehydrogenase kinase, isozyme 4	2.62	
cox7c	cytochrome c oxidase subunit VIIc		2.51
hk1	hexokinase 1	2.46	
fabp3	fatty acid binding protein 3, muscle and heart		2.34
eno3	enolase 3 (beta, muscle)	2.33	
slc2a1	solute carrier family 2 (facilitated glucose transporter), 1		2.26
hadhb	hydroxyacyl-CoA dehydrogenase, beta subunit		2.26
mt-coi	cytochrome c oxidase subunit I		2.24
ndufa4	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4		2.19
cox5a	cytochrome c oxidase subunit Va		2.15
cs	citrate synthase		2.15
sdhc	succinate dehydrogenase complex, subunit C, integral membrane		2.12
ndufa1	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 1		2.02
ndufc2	NADH dehydrogenase (ubiquinone) 1, subcomplex unknown, 2		1.97
etfdh	electron-transferring-flavoprotein dehydrogenase	1.72	
hadha	hydroxyacyl-CoA dehydrogenase, alpha subunit	1.54	
pfkl	phosphofructokinase, liver	-1.39	
prkab1	protein kinase, AMP-activated, beta 1 non-catalytic subunit	-1.48	
ckmt1b	creatine kinase, mitochondrial 1B	-2.08	
slc27a2	solute carrier family 27 (fatty acid transporter), member 2		-2.37
slc2a11	solute carrier family 2 (facilitated glucose transporter), 11	-5.88	
ckb	creatine kinase, brain		-7.91

Cell colour and intensity indicate up-regulated (red) and down-regulated (green) genes for a better comparison between the two groups

Table 3.5. Full list of overlapping genes between the two LPS-microarray analyses.

Name	Description	FC	FC
	Overlapping genes	**C-LPS**	**E-LPS**
dll1	delta-like 1 (Drosophila)	**-3.24**	**2.27**
col11a1	**collagen, type XI, alpha 1**	**-3.05**	**-14.63**
tnnt3	**troponin T type 3 (skeletal, fast)**	**-2.68**	**-4.80**
mid1ip1	**MID1 interacting protein 1**	**-2.59**	**3.78**
tpm4	**tropomyosin 4**	**-2.35**	**-2.28**
dact2	dapper, antagonist of beta-catenin, homolog	**-2.32**	**-2.87**
loxl2	**lysyl oxidase-like 2**	**-2.29**	**-3.33**
traf4	**TNF receptor-associated factor 4**	**-2.16**	**-1.77**
myh4	**myosin, heavy chain 4, skeletal muscle**	**-2.13**	**-10.95**
slc43a2	**solute carrier family 43, member 2**	**-2.12**	**2.49**
lum	lumican	**-1.82**	**-3.67**
tgfb3	**transforming growth factor, beta 3**	**-1.80**	**-2.78**
crmp1	collapsin response mediator protein 1	**-1.75**	**1.31**
pdia6	protein disulfide isomerase family A, member 6	**-1.75**	**-2.73**
ptpn13	**protein tyrosine phosphatase, non-receptor type 13 (APO-1/CD95 (Fas)-associated phosphatase)**	**-1.74**	**3.13**
cby1	**chibby homolog 1 (Drosophila)**	**-1.74**	**2.21**
stk25	serine/threonine kinase 25	**-1.71**	**-2.09**
gsn	gelsolin	**-1.70**	**-2.21**
dvl2	**dishevelled, dsh homolog 2 (Drosophila)**	**-1.67**	**1.49**
loxl3	lysyl oxidase-like 3	-1.65	-2.50
tnni2	**troponin I type 2 (skeletal, fast)**	**-1.62**	**2.99**
ugp2	**UDP-glucose pyrophosphorylase 2**	**-1.59**	**6.22**
mast2	microtubule associated serine/threonine kinase 2	**-1.54**	**2.38**
adh1b	alcohol dehydrogenase 1B (class I), beta polypeptide	**-1.54**	**1.91**
tcf7l1	**transcription factor 7-like 1 (T-cell specific, HMG-box)**	**-1.54**	**-1.62**
h2afz	H2A histone family, member Z	**-1.51**	**-2.00**
fzd3	**frizzled family receptor 3**	**-1.50**	**3.30**
snx14	**sortingnexin 14**	**-1.44**	**1.99**
ppia	peptidylprolylisomerase A (cyclophilin A)	**-1.43**	**2.36**
kdelr2	KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 2	**-1.37**	**-2.82**
rasa1	RAS p21 protein activator (GTPase activating protein) 1	**-1.26**	**-1.81**
hp1bp3	heterochromatin protein 1, binding protein 3	**-1.22**	**-1.57**
hsp90aa1	**heat shock protein 90kDa alpha (cytosolic), class A member 1**	**1.21**	**-3.13**
elf2	E74-like factor 2 (ets domain transcription factor)	**1.26**	**1.70**
axin1	axin 1	**1.27**	**1.54**
zc3h11a	zinc finger CCCH-type containing 11A	**1.28**	**1.72**
yy1	YY1 transcription factor	**1.28**	**1.41**
sh3bp5	SH3-domain binding protein 5 (BTK-associated)	**1.29**	**-1.84**
zfand2b	zinc finger, AN1-type domain 2B	**1.29**	**1.53**
eif4g1	eukaryotic translation initiation factor 4 gamma, 1	**1.30**	**-1.82**
ado	2-aminoethanethiol (cysteamine) dioxygenase	**1.31**	**1.45**
znf91	zinc finger protein 91	**1.32**	**2.77**
msl2	male-specific lethal 2 homolog (Drosophila)	**1.35**	**1.56**
esr2	**estrogen receptor 2 (ER beta)**	**1.37**	**-1.34**
ogt	O-linked N-acetylglucosamine (GlcNAc) transferase	**1.37**	**1.51**
rnf14	ring finger protein 14	**1.38**	**1.50**
med17	mediator complex subunit 17	**1.42**	**1.69**
rps6ka6	**ribosomal protein S6 kinase, 90kDa, polypeptide 6**	**1.53**	**1.50**
c20orf111	chromosome 20 open reading frame 111	**1.55**	**1.49**
gfod2	glucose-fructose oxidoreductase domain containing 2	**1.56**	**1.51**
tubb4a	**tubulin, beta 4A class IVa**	**1.60**	**2.59**
acadvl	acyl-CoA dehydrogenase, very long chain	**1.61**	**2.42**
ccnt2	cyclin T2	**1.63**	**1.49**
usp19	ubiquitin specific peptidase 19	**1.67**	**3.08**
pacsin2	protein kinase C and casein kinase substrate in neurons 2	**1.70**	**3.64**
etfdh	**electron-transferring-flavoprotein dehydrogenase**	**1.72**	**2.39**
hif3a	**hypoxia inducible factor 3, alpha subunit**	**1.74**	**3.62**
scd	stearoyl-CoA desaturase (delta-9-desaturase)	**1.79**	**2.28**

fam43a	family with sequence similarity 43, member A	**1.81**	**1.63**
ppp1r3c	protein phosphatase 1, regulatory subunit 3C	**1.84**	**2.79**
got2	glutamic-oxaloacetic transaminase 2, mitochondrial (aspartate aminotransferase 2)	**1.95**	**2.78**
dnajc18	DnaJ (Hsp40) homolog, subfamily C, member 18	**2.00**	**1.88**
actb	actin, beta	**2.02**	**-3.46**
ampd3	adenosine monophosphate deaminase 3	**2.06**	**3.15**
pgam2	**phosphoglyceratemutase 2 (muscle)**	**2.07**	**2.88**
chmp4b	chargedmultivesicular body protein 4B	**2.09**	**2.88**
slc26a5	solute carrier family 26, member 5 (prestin)	**2.10**	**3.00**
vegfa	**vascular endothelial growth factor A**	**2.37**	**2.66**
lck	**lymphocyte-specific protein tyrosine kinase**	**2.38**	**2.34**
nt5c2	5'-nucleotidase, cytosolic II	**2.65**	**3.19**
dynlrb1	**dynein, light chain, roadblock-type 1**	**19.97**	**-1.40**

Cell colour and intensity indicate up-regulated (red) and down-regulated (green) genes. Genes in bold have been described in the Results section.

Interleukin 6 expression in fast muscle from LPS-treated zebrafish

IL6 is an important cytokine with reported both pro- and anti-inflammatory effects and it has been described as a myokine in mammals as reported elsewhere(Pedersen & Febbraio, 2008). We have previously shown an up-regulation of *il6* mRNA expression in fast muscle in response to exercise in adult zebrafish (Chapter 2) and the microarray analysis described here showed differential expression of immune-related molecules in response to LPS. The microarray platform used for these studies was designed before zebrafish *il6* was cloned and characterized and, therefore, did not contain probes for *il6*. Thus, we wanted to analyze the differential expression of *il6* and its receptor *il6ra* in exercised and non-exercised fast muscle in response to LPS using the same samples used for the microarray analysis. Moreover, we also analyzed the expression of TNFα, a pro-inflammatory cytokine released in response to bacterial infection and upon administration of LPS.

As shown in **Figure 3.2** no statistically significant differences in *il6*, *il6ra* and *tnfα* expression were found in response to LPS treatment in non-exercised or exercised fast muscle. Strong variability was observed for *il6* and *tnfα* mRNA expression in non-exercised LPS-treated and for *il6* in exercised-LPS-treated fish but nevertheless the results showed a trend towards increased expression of these two genes in the LPS treated groups (C-LPS). In the exercised group of fish, *il6* expression was slightly elevated in response to LPS (E-LPS) in comparison with the exercised non LPS-treated muscles (E-PBS) and its receptor, *il6ra*, showed a trend towards an increase in its expression in the exercised non LPS-treated muscles and a similar level of expression in the exercised LPS-treated than in the non-exercised LPS-treated fish (C-LPS) . No change on *tnfα* expression was found in the exercised fast muscle fish. Therefore, in non-exercised fast muscles, a trend towards an elevated expression of inflammatory cytokines was found whereas this response appeared to be somewhat blunted in the exercised group.

Survival of exercised and non-exercised zebra fish to an in vivo immune challenge with PAO

LPS is an endotoxin that mimics a gram-negative bacterial infection. Therefore, in order to examine the effects of exercise on the survival after an immune challenge we performed an *in vivo* infection experiment with live bacteria. We took advantage of the established infection model for adult zebrafish developed by Roher's group at the *Institut de Biotecnologia i de Biomedicina* (IBB) in the Autonomous University of Barcelona using the opportunistic pathogen

Pseudomonas aeruginosa, a Gram-negative bacteria. From three independent experiments, exercised and non-exercised fish showed different survival responses to *P. aeruginosa* infection. In each experiment, an external control group with adult zebrafish from the IBB facility was included (C PAO).

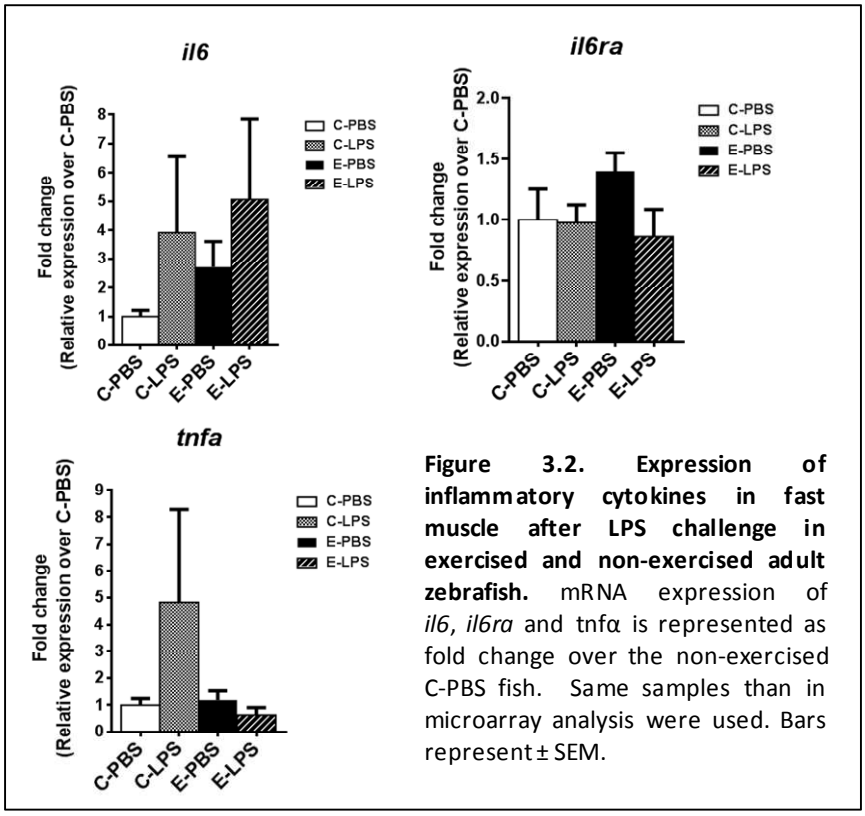

Figure 3.2. Expression of inflammatory cytokines in fast muscle after LPS challenge in exercised and non-exercised adult zebrafish. mRNA expression of *il6*, *il6ra* and tnfα is represented as fold change over the non-exercised C-PBS fish. Same samples than in microarray analysis were used. Bars represent ± SEM.

In experiment 1, fish that exercised during 20 days showed a significantly higher percent survival in comparison with the non-exercised at the end of the infection challenge period (P=0.046). At approximately 24h post-infection, survival of exercised and non-exercised fish decreased to 50.0% and 41.6%, respectively, which was followed by a decrease in survival of 25% in the non-exercised fish, comparable to the C PAO group (21.4%). Surprisingly, all fish from the non-exercised group died after 96 h of the *P. aeruginosa* infection and exercised fish maintained the percent survival at 41.6% during the last 24h.

In contrast to Experiment 1, an opposite pattern was shown in Experiment 2, with a lower survival of the exercised and non-exercised fish but without reaching statistical significance. At approximately 24h post-infection, survival of the exercised group declined to 27.2% whereas survival of the non-exercised group was maintained higher, at 53.8%, similar to the C PAO group, 57.1%. After 48h post-infection and until the end of the challenge, the survival of non-exercised fish remained higher than the exercised fish, 30.7% and 18.1%, respectively.

In Experiment 3, a distinct pattern was observed. After 24h post-infection, the percent survival of the non-exercised group was similar to C PAO, at 42.8% and 45.4%, respectively. The exercised group had a percent survival of 50.0% that dropped to 32.1% after 48h post-infection, similar to the non-exercised fish, at 32.1%. At the end of the challenge, both

exercised and non-exercised fish experienced similar percent survival, 32.1% and 28.5%, respectively.

In the three experiments, a sharp decrease in survival in all the experimental groups was observed before 24h post-infection, coinciding with the expected infection protocol designed by the collaborator group (Ruyra *et al*, 2014). The results with this opportunistic pathogen in zebrafish showed a non-consistent pattern in response to exercise due to the variability of the three independent experiments. Therefore, from these experiments we cannot firmly suggest an effect of previous exercise to an acute infection.

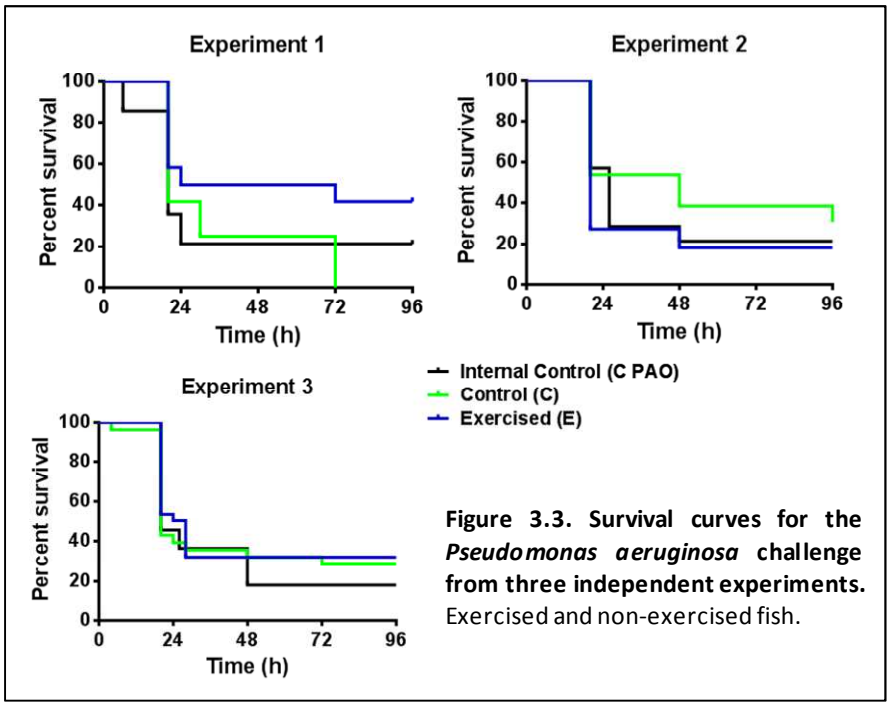

Figure 3.3. Survival curves for the *Pseudomonas aeruginosa* challenge from three independent experiments. Exercised and non-exercised fish.

Chapter 4

Exercise induces transcriptomic changes in the zebrafish heart

Exercise affected zebrafish fast skeletal muscle at the molecular and cellular level (Chapter 1) and we next interrogated how the zebrafish heart would respond to the exercise protocol. In order to study the molecular changes in the zebrafish heart after four weeks of swimming-induced exercise, we analyzed the heart transcriptomic response by performing a microarray analysis comparing exercised versus non-exercised zebrafish whole hearts (including the atrium and bulbus). A total of 765 genes were differentially expressed, with 421 genes up-regulated and 344 genes down-regulated by exercise when using a cut-off of 1.2 fold-change and P-value < 0.05. Initial classification of differentially expressed genes was performed by following Gene Onthology categories using the bioinformatics software DAVID (P<0.05). Functional categories showing significant enrichment were related to muscle contraction, actin cytoskeleton organization, axonogenesis, regulation of growth, catabolic process, glucose and fatty acid metabolic process, oxidative phosphorylation and ATP metabolic process (**Table 4.1**). In a KEGG pathway analysis for canonical pathways, we identified pathways that were significantly (P<0.05) over-represented such as oxidative phosphorylation, spliceosome, fatty acid metabolism, MAPK signaling, glycolysis/gluconeogenesis, axon guidance, TGF-β signaling, regulation of actin cytoskeleton, cardiac muscle contraction, Wntsignalingand mTOR (**Table 4.2**). It is interesting to notice that in comparison with the transcriptomic data obtained in skeletal muscle, zebrafish heart mRNA response in this microarray analysis is not as strong in terms of number of differentially expressed genes.

Table 4.1. Functional annotation analysis based on GO terms in zebrafish whole heart pools in response to swimming-exercise (DAVID).

GO Term		Count	P-Value
GO:0006936	Muscle contraction	15	0.002376
GO:0051592	Response to calcium ion	7	0.018677
GO:0051493	Regulation of cytoskeleton organization	15	7.65E-04
GO:0030036	Actin cytoskeleton organization	19	0.002901
GO:0007409	Axonogenesis	17	0.003294
GO:0040008	Regulation of growth	24	0.006666
GO:0009056	Catabolic process	61	0.045672
GO:0006006	Glucose metabolic process	12	0.034602
GO:0006631	Fatty acid metabolic process	15	0.021247
GO:0006119	Oxidative phosphorylation	14	9.57E-05
GO:0046034	ATP metabolic process	9	0.049731

For each enriched Gene Ontology term, count and p-value (P<0.05) are shown.

As observed in Chapter 1, exercise enhanced the up-regulation of muscle contractile elements (*tnnc1b*, *tnnt3b*, *ttnb*) as well as cytoskeletal and extracellular matrix components (*actb2*, *col1a1a*, *loxl1*, *postnb*, *tmsb*, *sgcd*, *sparc*) in the heart (**Table 4.3**). Moreover, skeletal and cardiac muscle developmental genes (*cited4a*, *cxcl12b*, *klf11a*,*jag1b*, *mef2ca*, *tgfb1a*, *tgfb2*, *wnt11r*) were also up-regulated, together with cell cycle and apoptotic-related genes (*bcl2l13*, *boka*, *gadd45ab*, *tp73*). Genes related with muscle oxygen delivery (*mb*), mitochondrial oxidation (*atp5a1*, *atp5g1*, *cox6a2*, *cox7a3*, *etfb*, *ndufa4*, *por*) and generation of ATP (*ckma*,

ckmat2) were up-regulated, similarly as in exercised fast muscle. Carbohydrate (*aldoab*, *gys1*, *pfkfb4l*) and lipid (*acadl*, *hadhaa*, *lpl*, *pparab*, *ucp3*) metabolism related genes were also differentially expressed. Factors involved in transcription (*polr1e*, *polr3h*, *ptrfa*), translation (*eif4a1a*, *eif4e3*, *eif4ebp1*, *eif4ebp3l*) and protein degradation (*atg12*, *calpain5a*, *ctrb1*, *psme4b*, *ubb*) were also found differentially expressed. Finally, a possible down-regulation of the mTOR pathway, involved in muscle growth and protein synthesis (*tsc1*, *rps6kb1b*, *eif4ebp1*) was suggested by the different regulation of the translation repressor proteins *eif4ebp1* and *eif4ebp3l* (**Table 4.3**). The results of microarray analysis were validated by qPCR for 6 differentially expressed genes using the same samples. The genes examined showed a similar pattern of change with the two techniques used (Additional Table A.4).

Table 4.2. Significantly over-represented KEGG pathways in adult zebrafish whole (pooled) hearts.

	ID KEGG pathway	P-value	Ratio
190	Oxidative phosphorylation	3.01e-12	18/132
3040	Spliceosome	1.31e-08	14/127
71	Fatty acid metabolism	1.78e-08	9/43
4010	MAPK signaling pathway	1.29e-06	17/268
10	Glycolysis / Gluconeogenesis	7.62e-06	8/65
4360	Axon guidance	3.64e-05	10/129
4350	TGF-beta signaling pathway	5.08e-05	8/84
4810	Regulation of actin cytoskeleton	0.0001	12/213
4260	Cardiac muscle contraction	0.0002	7/77
4310	Wnt signaling pathway	0.0006	9/150
4150	mTOR signaling pathway	0.0013	5/52

P-value (P<0.05) and the ratio of the number of differentially expressed genes in adult zebrafish hearts in response to swimming-exercise over the total number of genes in each particular KEGG pathway

Table 4.3. Selected significant (P<0.05) differentially expressed genes from adul zebrafish hearts obtained in the microarray analysis.

Gene name	Gene description	FC
	Muscle contraction	
atp1b1b	ATPase, Na+/K+ transporting, beta 1b polypeptide	**2.64**
casq2	calsequestrin 2	**2.31**
smtnb	smoothelin b	**2.11**
tnnt3b	troponin T3b, skeletal, fast	**1.74**
calm1b	calmodulin 1b	**1.72**
ttnb	titin b	**1.68**
camk2d2	calcium/calmodulin-dependent protein kinase (CaM kinase) II delta 2	**1.62**
tnnc1b	troponin C type 1b (slow)	**1.59**
myl6	myosin, light chain 6, alkali, smooth muscle and non-muscle	**1.28**
	Extracelular matrix and cytoskeleton	
col1a1a	collagen, type I, alpha 1a	**4.14**
postnb	periostin, osteoblast specific factor b	**2.83**
loxl1	lysyl oxidase-like 1	**2.10**
tmsb	thymosin, beta	**2.07**
sgcd	sarcoglycan, delta (dystrophin-associated glycoprotein)	**1.67**
sepp1a	selenoprotein P, plasma, 1a	**1.59**
sparc	secreted acidic cysteine rich glycoprotein	**1.52**
actb2	actin, beta 2	**1.42**
ankrd28b	ankyrin repeat domain 28b	**-2.11**
	Muscle growth and development	

klf11a	Kruppel-like factor 11a	**3.50**
insig1	insulin induced gene 1	**2.62**
map2k6	mitogen-activated protein kinase kinase 6	**2.32**
igfbp2a	insulin-like growth factor binding protein 2a	**2.17**
tgfb2	transforming growth factor, beta 2	**2.13**
stat1a	signal transduction and activation of transcription 1a	**1.98**
cxcl12b	chemokine (C-X-C motif) ligand 12b (stromal cell-derived factor 1)	**1.89**
efnb3a	ephrin B3a	**1.83**
jag1b	jagged 1b	**1.81**
tgfb1a	transforming growth factor, beta 1a	**1.81**
sema3aa	semaphorin 3aa	**1.75**
wnt11r	wingless-type MMTV integration site family, member 11, related	**1.70**
igfbp3	insulin-like growth factor binding protein 3	**1.65**
fstl1b	follistatin-like 1b	**1.61**
smad6b	SMAD family member 6b	**1.55**
mef2ca	myocyte enhancer factor 2ca	**1.55**
tsc1a	tuberous sclerosis 1a	**1.37**
cited4a	Cbp/p300-interacting transactivator, Glu/Asp-rich carboxy-terminal domain, 4a	**-1.60**
socs3b	suppressor of cytokine signaling 3b	**-1.65**
nle1	notchless homolog 1 (Drosophila)	**-1.74**
mdkb	midkine-related growth factor b	**-1.94**
	Cell cycle and apoptosis	
tp73	tumor protein p73	**2.05**
bcl2l13	BCL2-like 13 (apoptosis facilitator)	**1.60**
boka	BCL2-related ovarian killer a	**-1.86**
gadd45ab	growth arrest and DNA-damage-inducible, alpha, b	**-2.00**
mych	myelocytomatosis oncogene homolog	**-2.91**
	Metabolism	
ckmt2	creatine kinase, mitochondrial 2 (sarcomeric)	**3.25**
ckma	creatine kinase, muscle a	**3.03**
ndufa4	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4	**2.54**
etfb	electron-transfer-flavoprotein, beta polypeptide	**2.22**
mdh1b	malate dehydrogenase 1b, NAD (soluble)	**2.01**
cox6a2	cytochrome c oxidase subunit VIa polypeptide 2	**1.98**
atp5g1	ATP synthase, H+ transporting, mitochondrial Fo complex, subunit C1 9	**1.80**
mb	myoglobin	**1.79**
idh1	isocitrate dehydrogenase 1 (NADP+), soluble	**1.65**
atp5a1	ATP synthase, H+ transporting, mitochondrial F1 complex alpha subunit 1	**1.64**
cox7a3	cytochrome c oxidase subunit VIIa polypeptide 3	**1.60**
por	P450 (cytochrome) oxidoreductase	**-2.89**
	Carbohydrate metabolism	
aldoab	aldolase a, fructose-bisphosphate, b	**2.24**
gpib	glucose phosphate isomerase b	**2.61**
gys1	glycogen synthase 1 (muscle)	**1.94**
gapdhs	glyceraldehyde-3-phosphate dehydrogenase, spermatogenic	**1.84**
pkma	pyruvate kinase, muscle, a	**1.59**
pfkfb4l	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4, like	**-2.61**
	Lipid metabolism	
hadhaa	hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme	**2.88**
acadl	acyl-Coenzyme A dehydrogenase, long chain	**2.35**
pparab	peroxisome proliferator activated receptor alpha b	**2.28**
crata	carnitine O-acetyltransferase a	**1.66**
adipor2	adiponectin receptor 2	**-1.72**
ucp3	uncoupling protein 3	**-2.66**
ctps1a	CTP synthase 1a	**-2.87**
lpl	lipoprotein lipase	**-3.04**

	Protein synthesis and degradation	
ctrb1	chymotrypsinogen B1	**6.09**
ptrfa	polymerase I and transcript release factor a	**3.24**
ube2ql1	ubiquitin-conjugating enzyme E2Q family-like 1	**3.10**
capn5a	calpain 5a	**2.76**
atg12	ATG12 autophagy related 12 homolog (S. cerevisiae)	**1.76**
eif4e3	eukaryotic translation initiation factor 4E family member 3	**1.71**
eif4ebp1	eukaryotic translation initiation factor 4E binding protein 1	**1.63**
ubb	ubiquitin B	**1.52**
psme4b	proteasome (prosome, macropain) activator subunit 4b	**1.49**
tsc1a	tuberous sclerosis 1a	**1.37**
rps6kb1b	ribosomal protein S6 kinase b, polypeptide 1b	**-1.26**
psmd11b	proteasome (prosome, macropain) 216S subunit, non-ATPase, 11b	**-1.51**
eif4ebp3l	eukaryotic translation initiation factor 4E binding protein 3, like	**-1.60**
polr3h	polymerase (RNA) III (DNA directed) polypeptide H	**-1.65**
polr1e	polymerase (RNA) I polypeptide E	**-1.83**
eif4a1a	eukaryotic translation initiation factor 4A, isoform 1A	**-1.89**
hsp70l	heat shock cognate 70-kd protein, like	**-5.24**

Importantly, since the generation of the molecular data was performed in whole heats, we cannot exclude the possibility of contamination resulting from the presence of the bulbus and/or atrium in our samples. Thus, the results presented from now will be based only from zebrafish ventricles. In order to focus on transcriptomic responses in the heart ventricle, as opposed to the whole heart, we next evaluated in isolated zebrafish heart ventricles the expression of specific genes known to be induced during cardiac growth, stress or damage as well as other genes found to be differentially expressed in the microarray analysis using whole zebrafishhearts after four weeks of exercise. Atrial natriuretic peptide (*nppa)*, a marker for cardiac stress in adult hearts that is expressed after cardiac damage(Sergeeva & Christoffels, 2013), showed no differences in mRNA expression levels by qPCR between exercised and non-exercised ventricles (**Figure 4.1A**). Furthermore, the expression pattern of *nppa* by *in situ* hybridization evidenced higher expression levels in the atrium, and to a lower extent in the ventricle, but very similar *nppa* expression levels in exercised and non-exercised hearts (**Figure 4.1B**). Neuroregulin 1 (*nrg1)*, a gene with mitogenic functions that is induced during heart regeneration(Gemberling *et al*, 2015),showed a trend towards increased expression in the exercised ventricles but it was not significant (P=0.944). Moreover, we analyzed the expression of HIF1α, a gene induced under hypoxic conditions (Kopp *et al*, 2011) and that has two isoforms in zebrafish. Only *hif1aa*, which is more abundant than *hif1ab* in the developing zebrafish heart (Rytkönen *et al*, 2014), showed a trend towards decreased expression in the exercised group although it was close to statisticalsignificance (P=0.056).Thus,the observed decrease in *hif1aa* expression would suggest that our exercise training did not induce hypoxia in the zebrafish ventricles. Moreover, the mRNA expression levels of periostin (*postnb*), a fibroblast marker, and transforming growth factor beta 1 (*tgfb1*), a member of the TGF-β family that is involved in muscle development and cardiac fibrosis after a cardiac injury and that has been shown to play a role during zebrafish heart regeneration, showed no significant changes in response to exercise (P=0.310). In contrast, thymosin beta (*tmsb*), a gene found to be induced upon cardiac injury and involved in actin cytoskeleton organization,was significantly down-regulated in zebrafish ventricles after four weeks of swimming-induced exercise (P=0.032) (**Figure 4.1A**). It is worth mentioning that some of these genes (*postnb*,

tgfb1 and *tmsb*) were differentially expressed in exercised whole hearts as shown by microarray analysis but, when examined in isolated ventricles,show an opposite regulation or no change in expression. The possible reasons for this discrepancy are explained in the Discussion section.

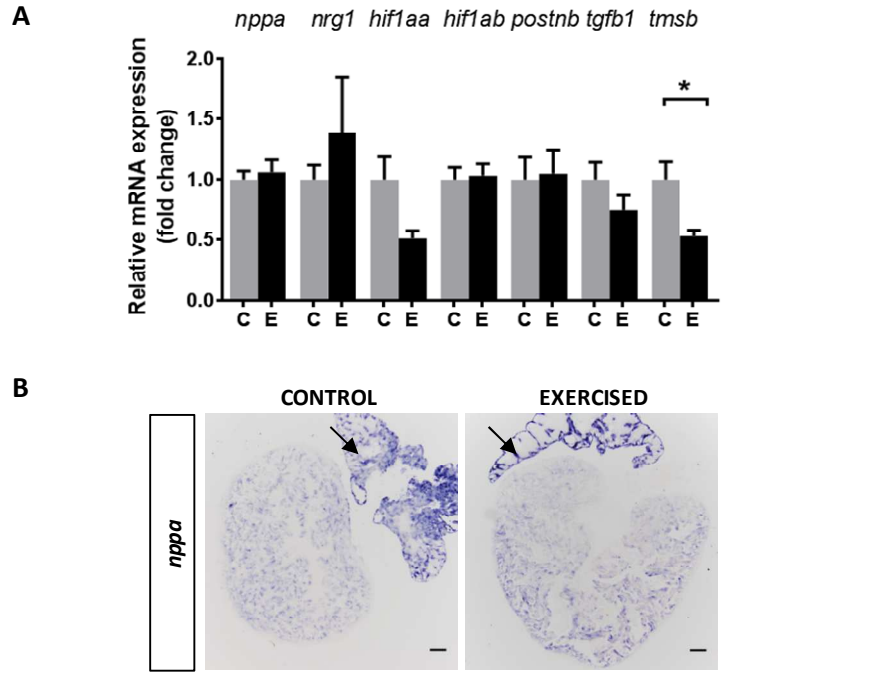

Figure 4.1. mRNA expression of relevant genes. A Relative mRNA expression represented as fold change in exercised fish over control. Bars represent ± SEM. Significant differences are P<0.05. *hif1aa*, hypoxia inducible factor 1 alpha subunit a (P=0.056); *hif1ab*, hypoxia inducible factor 1 beta subunit b; *nppa*, natriuretic peptide A; *nrg1*, neuroregulin 1; *postnb*, periostin b; *tgfb1*, transforming growth factor beta 1; *tmsb*, thymosin beta (P=0.032). **B** In situ hybridization of nppa to confirm no changes in expression between exercised and control ventricles. Nppa is highly expressed in the auricula (arrow). *nppa* is equally distributed in exercised and control ventricles with no apparent differences. Scale bar represents 100 µm.

Exercise-training influences cardiac hypertrophy and promotes cardiomyocyte proliferation but does not affect cardiac function

We were interested to examine whether swimming-induced exercise induced ahypertrophic cardiac response in adult zebrafishas it has been shown under exercise training conditions or during pregnancy in mammals.Here, we have investigated the adaptive cardiac responses to four weeks ofa swimming-induced exercise training protocol in adult zebrafish hearts, particularly in the ventricle. First, morphometric analyses of the ventricular myocardium revealed that exercised fish had a significantly larger (39.24%) total ventricular myocardium (TM) area than non-exercised fish (P=0.018) (**Figure 4.2 A**). Similarly, the compact myocardial (CM) or ventricular wall area was also increased (45.21%) in exercised over non-exercised fish, although not significantly (P=0.111) (**Figure 4.2 C**), and increased proportionally to the total ventricular myocardium area (**Figure 4.2E**). When normalized by the number of sections

counted per individual, both the total and the compact ventricular myocardial area were larger in exercised over non-exercised fish (20.21% and 18.21%, respectively), although without reaching statistical significance (**Figure 4.2 B,D**). These results suggest that swimming-induced exercise caused a mild increase in the ventricular myocardium area, possibly reflecting a hypertrophic and/or hyperplasic response of the zebrafish ventricle to exercise.

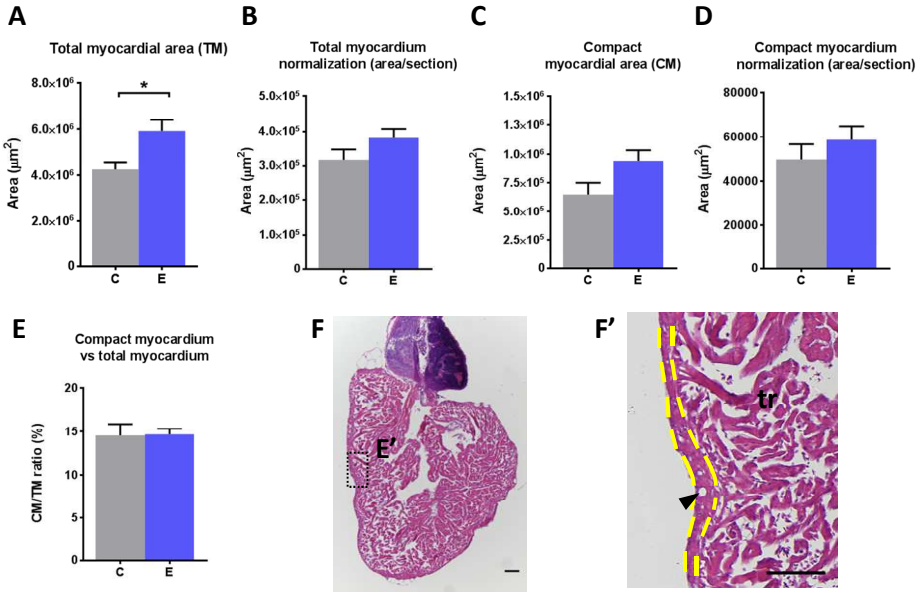

Figure 4.2. Evaluation of ventricle hypertrophy after four weeks of swimming-induced exercise. A. Quantification of the myocardial area in 12-20 sections per individual (exercised n=6 and control n=7) P=0.018. **B.** Normalization of the myocardial area per section quantified (P=0.145). **C.** Quantification of compact myocardium (CM) area (P=0.111) **D.** Normalization of the compact myocardium area per section quantified (P=0.412) **E.** Ratio of compact myocardium by the total myocardium (TM) in each individual (P=0.873). Barrs represent ± SEM. P<0.05. Asterisk represents P<0.05. **F.** An hematoxylin and eosin stained ventricle section. **F'** zoomed area boxed in F. Yellow lines delineate the compact myocardium. Arrowhead points to a coronary vessel in the ventricular wall. tr, traveculated myocardium. Scale bars represent 100µm.

Since an increase in muscle mass is associated, at least in part, with increased protein synthesis (Goodman *et al*, 2011), we next analyzed the activity levels of mTOR and its two downstream targets, p70S6K and 4E-BP1, in ventricles from exercised and non-exercised fish by Western blotting. Ventricular mTOR activity showed a significant increase in the exercised fish (P=0.017), but no changes were observed in p70S6K (P=0.669) and 4E-BP1 activity (P=0.791) (**Figure 4.3**). Moreover, the expression of myocyte enhancer factor 2 (Mef2), an important transcriptional factor in regulating the expression of genes in cardiac muscle differentiation, showed no differences in response to exercise (**Figure 4.3**).

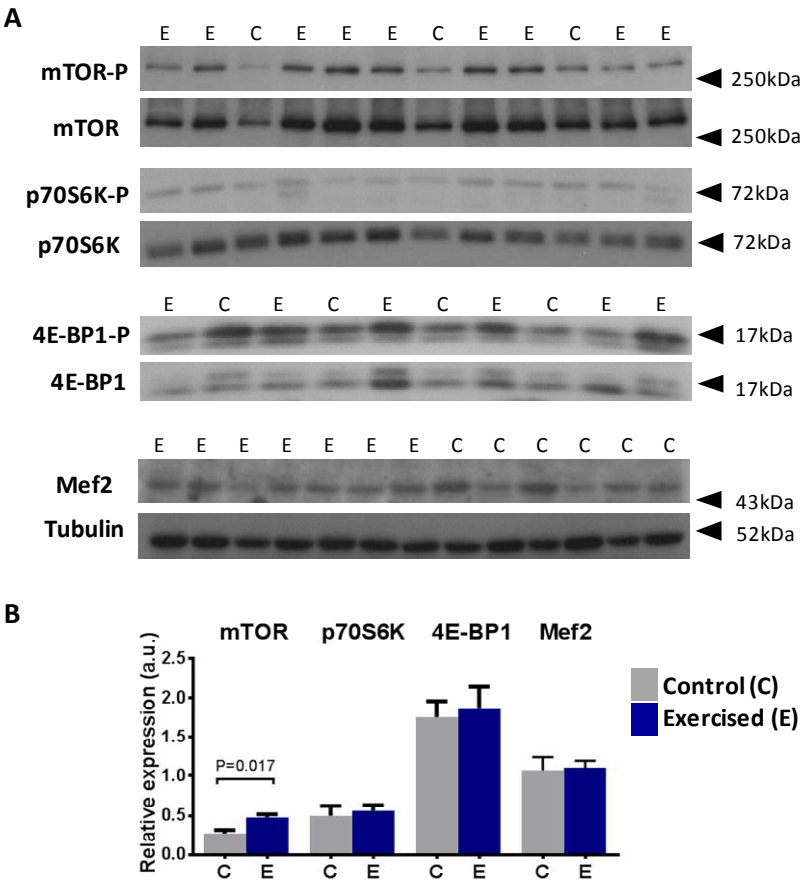

Figure 4.3. Protein expression of components of the mTOR pathway and MEF2 in individual ventricles after four weeks of swimming-induced exercise. **A.** SDS-PAGE of mTOR pathway components represented by mTOR, its downstream kinase p70S6K (E, n=9; C, n=3), the translation repressor protein 4E-BP1 (E, n=12; C, n=6) and the MEF2 transcription factor (E, n=7; C, n=6). Arrowheads indicate the protein marker molecular weight. Note that not all individuals used in the analysis are shown in the figure. **B.** Quantification by densitometry of Western blots shown in A. Activity levels of mTOR, p70S6K and 4E-BP1 represent the levels of the phosphorylated form (P) relative to the total form. MEF2 protein expression levels are shown relative to tubulin. Bars represent ± SEM. P=0.017.

In order to investigate whether exercise affected hyperplasia in the zebrafish ventricle, we next assessed cardiomyocyte proliferation. In order to quantify the proliferative response to exercise specifically in the cardiomyocyte cells, we identified proliferating cardiomyocytes by *invivo* labelling with EdU followed by immunofluorescent detection of Mef2 and measured the cardiomyocyte index (**Fig. 4.4 A-D**). Our results clearly evidence a significant increase in the number of proliferating cardiomyocytes (EdU+/Mef2+ nuclei) in exercised over non-exercised fish (P=0.02) (**Figure 4.4 F**). In contrast, swimming did not affect the proliferation of non-cardiomyocytes (EdU+/Mef2- nuclei) (**Figure 4.4 G**). Analysis of protein expression levels of two distinct proliferative cell cycle markers, the proliferating cell nuclear antigen (Pcna) and phospho-histone 3 (Ph3), in ventricles from exercised and non-exercised zebrafish revealed no differences between the groups (**Figure 4.5**). Interestingly, the activity of p38 MAP kinase, an

inducer of cell cycle arrest and promoter of differentiation that negatively regulates cardiomyocyte proliferation in cardiomyocytes (Engel *et al*, 2005), was decreased in zebrafish ventricles in response to swimming-induced exercise (P=0.056) (**Figure 4.5**).

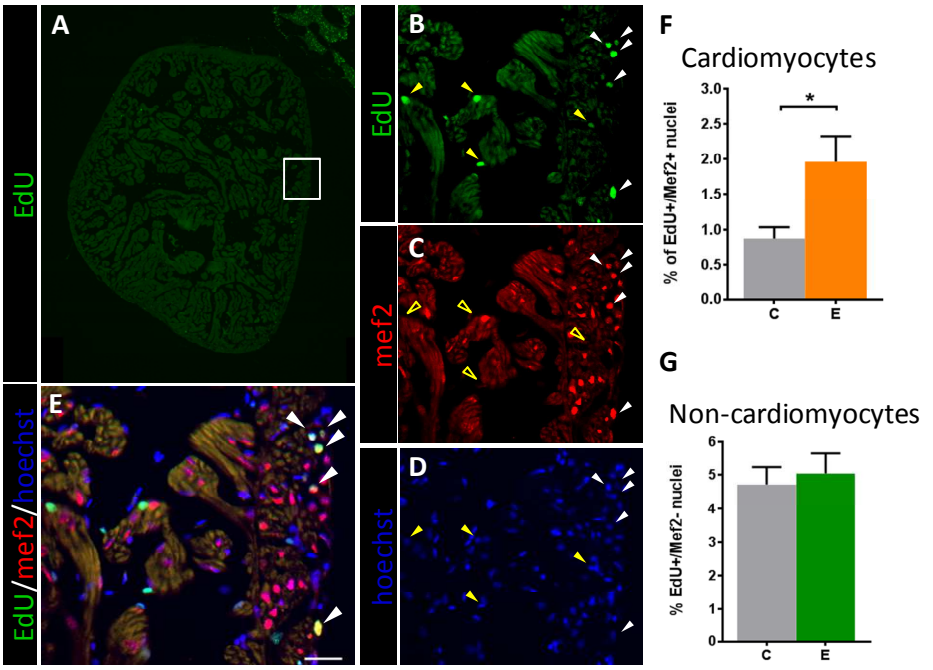

Figure 4.4. Cardiomyocyte and non-cardiomyocyte proliferation after four weeks of swimming-induced exercise. A-E. Labeling of proliferative cardiomyocyte and non-cardiomyocyte nuclei with EdU (5-ethynyl-2-deoxyuridine) combined with immunofluorescence. (A) Stitched image of a whole ventricle section labeled with EdU. Higher magnification views of the framed area in A: (**B**) EdU labeling, (**C**) mef2c antibody labeling, (**D**) nuclei staining with Hoetch, (**E**) merged image from A, B and C. White arrowheads indicate EdU-mef2c-positive nuclei of cardiomyocytes. Yellow arrowheads indicate EdU mef2-negative nuclei of non-cardiomyocytes, as shown by the absence of mef2 staining in (C). Scale bar represents 10µm. **F.** Percentage of proliferative cardiomyocyte nuclei from exercised (E, n=6) and control (C, n=6) fish (P=0.021). **G.** Percentage of non-cardiomyocyte proliferative nuclei (P=0.688).

To evaluate the functional effects of the applied swimming-induced exercise protocol, we measured the ventricle ejection fraction (percentage of blood ejected by the ventricle with each heartbeat calculated as stroke volume/end diastolic volume) in the zebrafish heart by 2D-echocardiography by taking advantage of the recent establishment of this technique in adult zebrafish (**Figure 4.6 A**). After four weeks of exercise, ejection fraction was similar between exercised (36.74 %) and non-exercised fish (37.35 %) (**Figure 4.6 B**). Similarly than ejection fraction, the heart rate was not different between the two groups (**Figure 4.6C**).

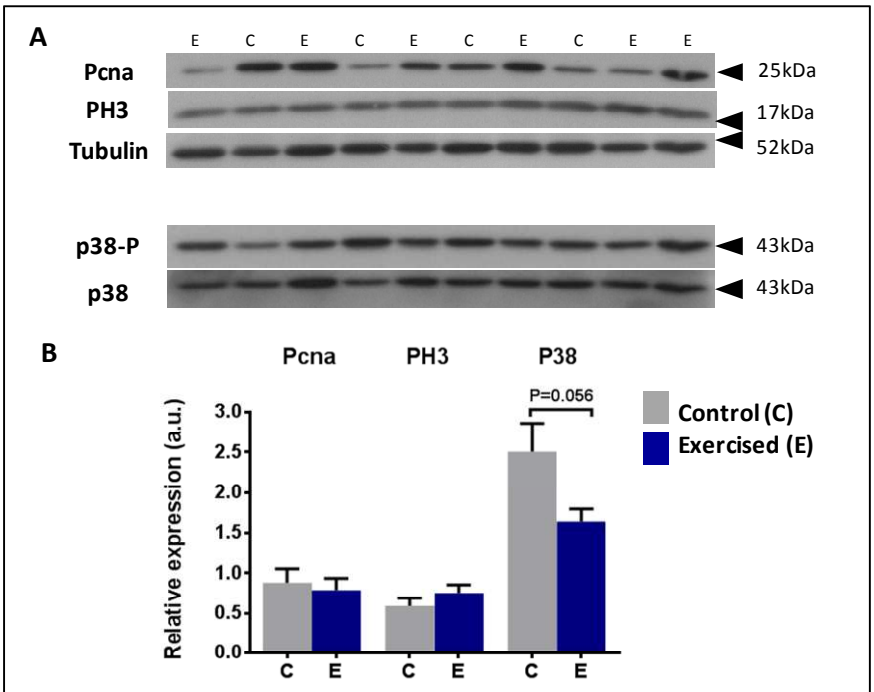

Figure 4.5. Protein expression of cell proliferation markers in individual ventricles after four weeks of swimming-induced exercise. A. SDS-PAGE of cell proliferation markers, proliferating cell nuclear antigen (Pcna) and phosphorylated histone 3 (Ph3) (E, n=12; C, n=6) and p38 MAPK as a cell differentiation and growth marker (E, n=12; C, n=6). Arrowheads indicate the protein marker molecular weight. Note that not all individuals used in the analysis are shown in the figure. **B.** Quantification by densitometry of Western blots shown in A. Expression of Pcna and Ph3 was analyzed relative to tubulin and the activity of p38 as the ratio between phosphorylated p38 relative to total p38. Bars represent ± SEM.

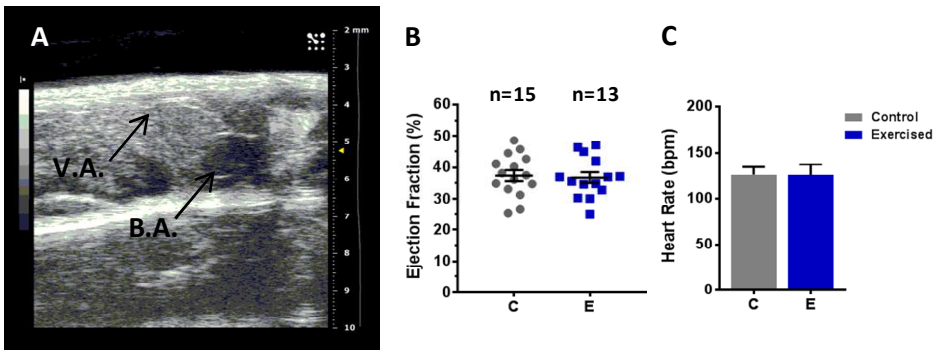

Figure 4.6. Evaluation of cardiac function by 2D-Echochardiography after four weeks of swimming-induced exercise. A. 2D echochardiography image of an adult zebrafish positioned ventrally (left side – caudal, right side – rostral) showing the ventricle apex (V.A) and the bulbus arteriosus (B.A.). Atrium is not shown yet is not easyly visible. **B.** Ejection fraction (%) parameter was measured to assess pumping ability of the ventricles. No significant diferences were found (P=0.806). **C** Heart Rate was not altered between groups (P=0.981). Barrs represent ± SEM. P<0.05. C, control; E, exercised; bpm, beats per minute.

Chapter 5

Given the observed effects of exercise on adult zebrafish hearts in the previous Chapter and the reported beneficial effects of exercise in the mammalian injured heart, we analyzed the effects of swimming-induced exercise in adult zebrafish hearts prior to or after a myocardial injury.

Swimming-induced exercise training influences heart regeneration by stimulating cardiomyocyte proliferation and improves cardiac function

Here, we sought to investigate the influence of exercise training on cardiac regeneration after cryoinjury. In fish that were exercised or not for four weeks (20 days) and subsequently subjected to a myocardial injury by cryolesioning the ventricle (**Figure 5.1 A, B**), we analyzed longitudinally cardiac function by echocardiography at 0 (basal), 7 and 28 dpi. Ejection fraction measurements during the regeneration process showed a significant decrease in exercised and non-exercised fish between 0 and 7 dpi, as expected after cryolesioning the heart.No significant differences in ejection fraction were observed between exercised and non-exercised zebrafish at basal, 7 or 28 dpi, indicating that both groups of fish had similarly compromised cardiac function after the myocardial injury. However, exercised fish showed a significant (P=0.029) and more pronounced increase in ejection fraction than non-exercised fish (P=0.053) between 7 and 28 dpi (**Figure 5.1 C**). Moreover, post-lesion recovery of the

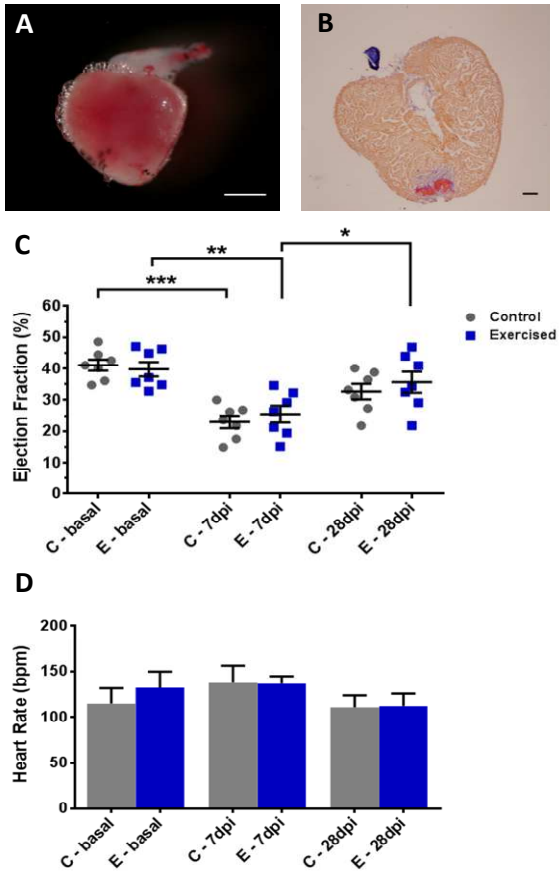

Figure 5.1. Evaluation of cardiac function over 28 days in control and exercised fish with lesioned ventricles.
A. Image from a dissected ventricle at 28 dpi. Scale bar represents 0.5 mm. The doted line shows approximately the injured area. **B.** Corresponding AFOG staining from the same ventricle shown in B. Scale bar represents 100 µm. **C.** Plot of the ejection fraction values taken from 2D echochardiography images for each individual. Grey dots represent the Control group (C, n=7) and blue squares the Exercised group (E, n=7) before and after the cryoinjury procedure at 7 and 28 dpis. Bars represent ± SEM. * P<0.05, ** P<0.01, *** P<0.001.

ejection fraction at 28 dpi was more similar to basal values in exercised fish than in the control group. However, heart rate was not different between the two groups at any time point comparison (**Figure 5.1 D**).

At 28 dpi, after echocardiography, hearts were collected and processed for AFOG staining to localize the injured area in order to confirm the echocardiographic measurements for each fish. The lesionedarea in AFOG images at 28 dpi was quantified and although the lesioned area appeared smaller in exercised than in non-exercised ventricles (4.5% and 5.7% P=0.460,

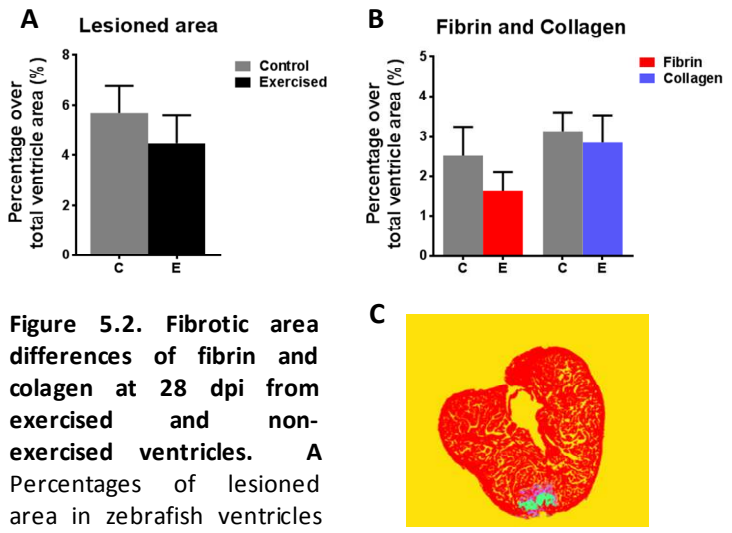

Figure 5.2. Fibrotic area differences of fibrin and colagen at 28 dpi from exercised and non-exercised ventricles. A Percentages of lesioned area in zebrafish ventricles (E, n=7; C, n=7). **B** Percentages of the lesioned area corresponding to fibrin (P=0.314) and collagen (P=0.730) revealed with AFOG staining. **C** Example of fibrin and colagen area identification with ImageJ. Same AFOG image from Figure 6B is shown. Red=myocardium, Green=fibrin, Purple=colagen. Bars represent ± SEM.

respectively), no significant differences were found betweenthe two groups (**Figure 5.2 A**). In order to analyze the extent of fibrosis in the regenerating ventricle, fibrin- and collagen-containing areas were quantified. Fibrin (1.6% exercised and 2.5% non-exercised) and collagen (2.8% exercised and 3.14% non-exercised) areas appeared again smaller in exercised than in non-exercised fish but no significant differences between the two groups were found (P=0.314 and P=0.729, respectively), suggesting that there were no differences in fibrotic tissue deposition at 28 dpi as a result of exercise training (**Figure5.2 B**).

We next investigated whether previous exercise training influenced cardiomyocyte proliferation subsequent to a myocardial injury. We performed *in vivo* BrdU labeling of exercised and non-exercised zebrafish subjected to cryoinjury to examine cardiomyocyteproliferation at 28 dpi from the same individuals subjected to echocardiography. As previously indicated, nuclei were co-immunolocalizedwith BrdU and Mef2 in order to differentiate cardiomyocyte from non-cardiomyocyte nuclei (**Figure 5.3 A-D**). At 28 dpi, there was a 3-fold higher percentage of proliferating cardiomyocytes (BrdU+/Mef2+ nuclei) in ventricles from exercised over non-exercised zebrafish (P=0.066) (**Figure 5.3 E**). In

contrast, no changes in the presence of non-cardiomyocytes were observed between ventricles from exercised and non-exercised zebrafish (P=0.188) (**Figure 5.3 F**).

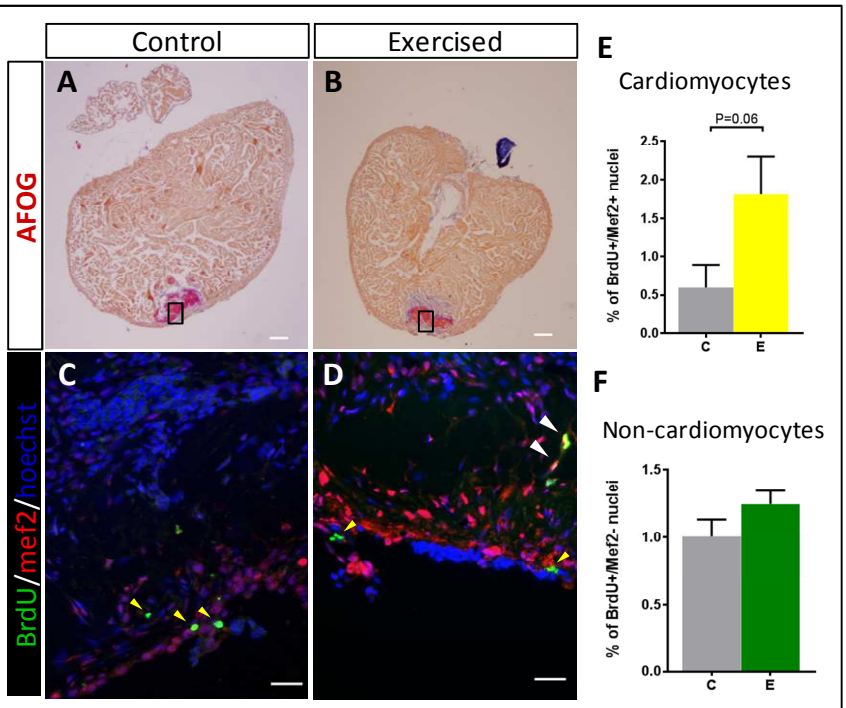

Figure 5.3. Cardiomyocyte proliferation at 28 dpi in pre-exercised adult zebrafish. A and **B** AFOG staining of a control and an exercised ventricle. Scale bar represents 100µm. **C and D** Higher magnification views of the framed area in A and B. Immunofluorescence to label proliferative cardiomyocytes with BrdU (5-bromo-2'-deoxyuridine) combined with mef2. White arrowheads indicate BrdU-mef2c-positive nuclei or cardiomyocytes. Yellow arrowheads indicate BrdU mef2-negative nuclei or non-cardiomyocytes. Scale bar represents 10µm. **E** Percentage of proliferative cardiomyocyte nuclei from exercised (E, n=7) and control (C, n=7) fish (P=0.066). **F** Percentage of proliferative non-cardiomyocyte nuclei from exercised (E, n=7) and control (C, n=7) fish (P=0.188)

Exercise training during heart regeneration post-injury increases cardiomyocyte proliferation

In view of the effects of exercise on cardiomyocyte proliferation, we set out to investigate the effects of concurrent exercise training *during* the regenerative process after ventricular cryoinjury. In order to do that, we first established that injured zebrafish were able to tolerate our swimming-induced exercise conditions at 3 dpi (data not shown). Next, we subjected (untrained) lesioned zebrafish to resting conditions or to the same exercise conditions used in previous experiments for 12 days (from 3 to 15 dpi) and assessed cardiomyocyte proliferation by EdU *in vivo* labeling (**Figure 5.4 A-E**). Our results clearly indicate that cardiomyocyte (EdU+/Mef2+) proliferation, both in the lesioned area as outside of it, increased significantly (4-fold; P=0.029) in exercised over non-exercised, lesioned zebrafish at 15dpi (**Figure 5.4 F**). Proliferation of non-cardiomyocyte cells showed no differences inside or outside the lesion area, as in the previous analyses (**Figure 5.4 G**).

As in previous experiments, we quantifed the extent of the lesioned area at 7 and 15 dpi. Although in exercised ventricles the lesioned area was reducedby 25.3% at 7 dpi and 53.3% at

15 dpi, no statistically significant differences were found between exercised and non-exercised zebrafish (7 dpi P=0.411 and 15 dpi P=0.200) (**Figure 5.5 A**). In order to compare the fibrotic scar progression in exercised and non-exercised zebrafish, we quantified fibrin- and collagen-containing areas (**Figure 5.5 B**). At 7 dpi, although lesioned exercised ventricles contained less fibrin (8.0% in exercised and 8.9% in non-exercised ventricles; P=0.713) or collagen (0.4% in exercised and 2.5% in non-exercised ventricles; P=0.825) there were no statistically significant differences in percentage area in lesioned ventricles between the two groups. Similar results were obtained when comparing lesioned exercised and non-exercised ventriclesat 15 dpi, with 2.4% and 3.9% fibrin areasin exercised and non-exercised ventricles, respectively (P=0.343) and with 1.5% and 4.7% collagen areas in exercised and non-exercised ventricles, respectively (P=0.314). Both groups had higher content of fibrin than collagen at 7 and 15 dpi as expected in the first days of the cardiac regeneration progresses.

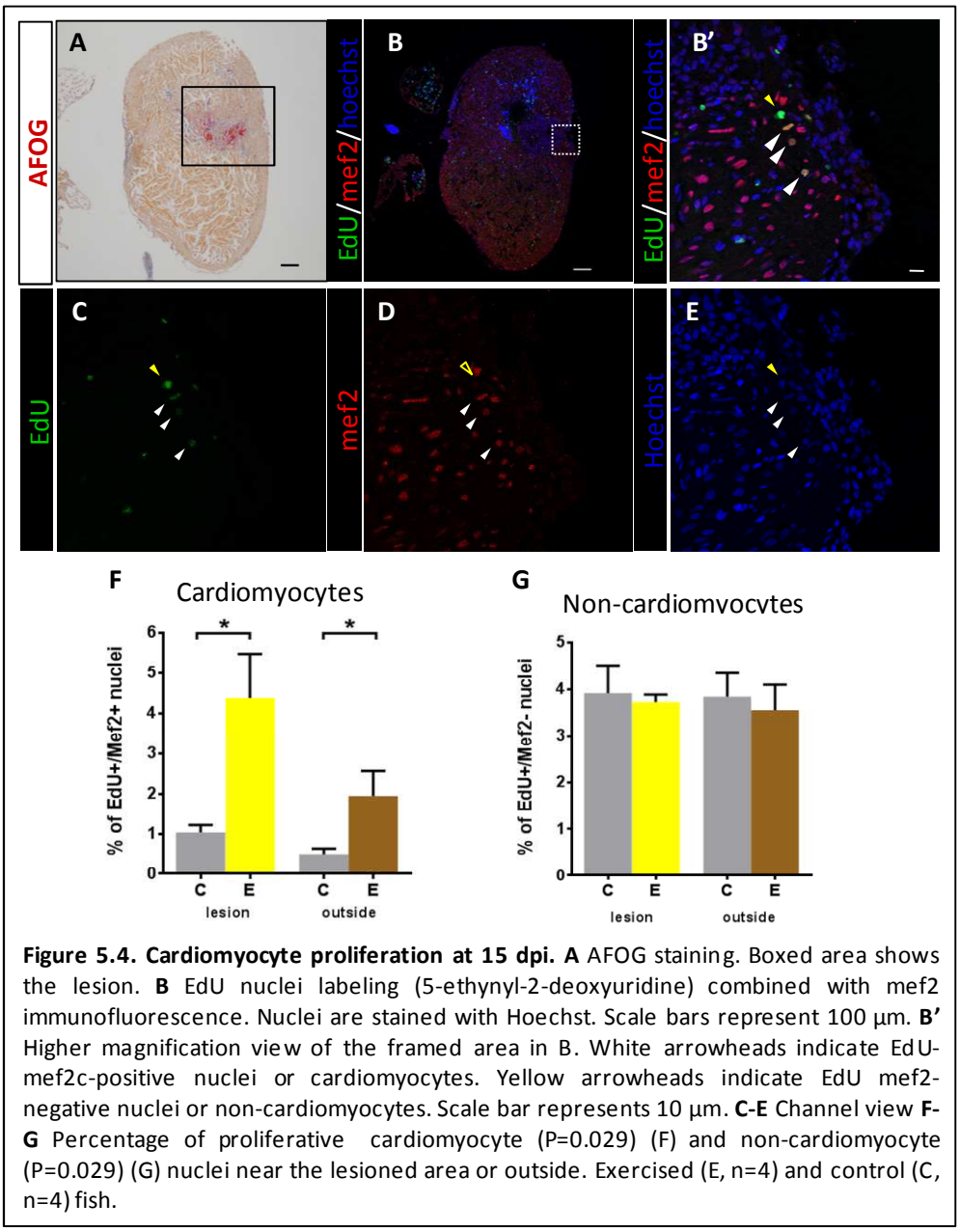

Figure 5.4. Cardiomyocyte proliferation at 15 dpi. A AFOG staining. Boxed area shows the lesion. **B** EdU nuclei labeling (5-ethynyl-2-deoxyuridine) combined with mef2 immunofluorescence. Nuclei are stained with Hoechst. Scale bars represent 100 µm. **B'** Higher magnification view of the framed area in B. White arrowheads indicate EdU-mef2c-positive nuclei or cardiomyocytes. Yellow arrowheads indicate EdU mef2-negative nuclei or non-cardiomyocytes. Scale bar represents 10 µm. **C-E** Channel view **F-G** Percentage of proliferative cardiomyocyte (P=0.029) (F) and non-cardiomyocyte (P=0.029) (G) nuclei near the lesioned area or outside. Exercised (E, n=4) and control (C, n=4) fish.

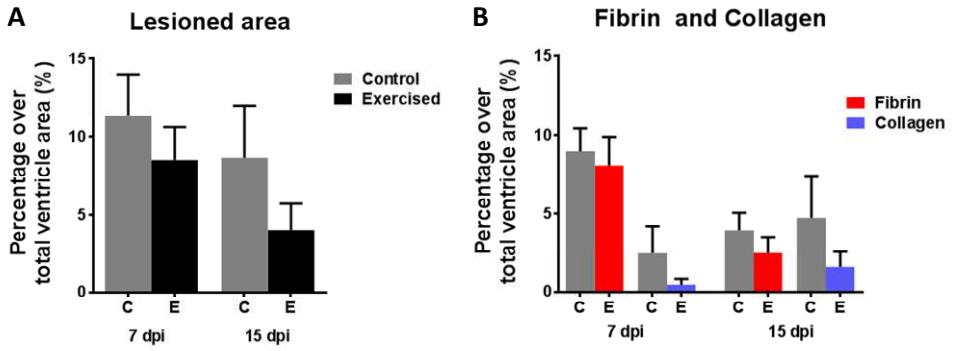

Figure 5.5. Fibrotic area differences of fibrin and colagen at 7 and 15 dpi from exercised and non-exercised ventricles. **A** Percentage of the lesioned area at 7 (E, n=5; C, n=5; P=0.411) and 15 dpi (E, n=4; C, n=4; P=0.200) in exercised and non-exercised ventricles **B** Percentages of fibrotic tissue fibrin and collagen revealed with AFOG staining. Bars represent ± SEM.

Identification of genes differentially regulated in exercised ventricles during the regenerating process

We analyzed the transcriptomic changes induced by exercise during the regenerating process from 7 to 14dpi. By RNA sequencing we obtained the transcriptomic profile from exercised and non-exercised regenerating ventricles at 7 and 14 dpi, using four pooled samples at each time point except in the non-exercised group at 14 dpi where two pooled samples were used. Sample clustering shows that samples are clustered by time (7 and 14 dpi) and condition (exercised or non-exercised) (**Figure 5.6 A**). Principal component analysis (PCA) shows that the distribution of the samples clearly represents the regenerative process over time from left to right over the x axis (**Figure 5.6 B**). To specifically detect the genes regulated by exercise during zebrafish heart regeneration, the expression profile was built to account for the biological

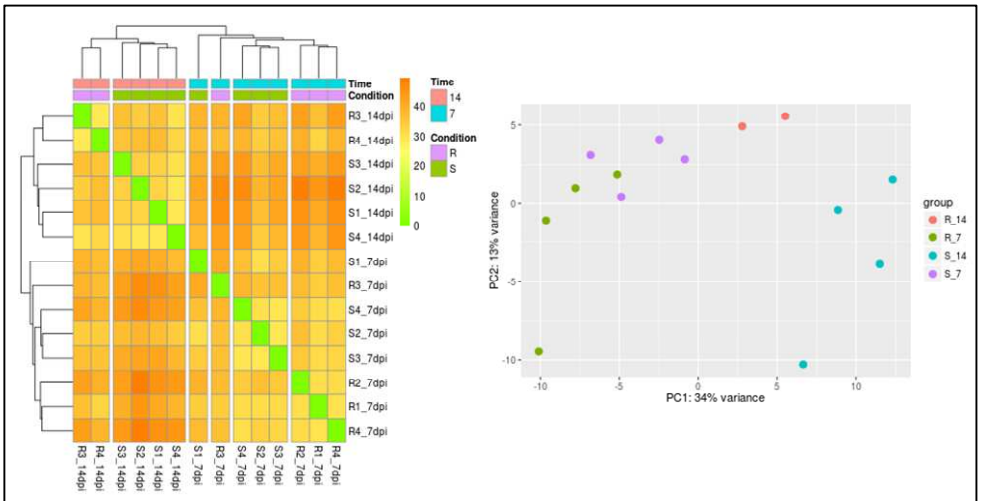

Figure 5.6. Sample distances and distribution. **A** Sample distance matrix shows that samples are correctly clustered by time, and as a second factor clustered by condition (exercised or non-exercised). Colors represent lower (green) to higher (orange) distance between samples. Sample condition and time are shown as annotation columns over the sample distance matrix. **B** Sample dependent principal component 1 of log2 transformed expression values is plotted against principal component 2, x and y axis, respectively. Sample groups are represented by colors. PCA component one reflects the effect of time grouping 14 dpi to the right and 7 dpi to the left.

differences found regarding the effect of exercise (exercise vs non-exercise) as well as regarding the effect of time after the cryolesion (7 and 14 dpi).A total of 512 differentially expressed genes (Padj<0.1) were significantly found, with 174 up-regulated genes and 338 down-regulated genes (fold-change cut-off 1.2). From the significant differentially expressed genes, 67 unknown or undescribed genes were found (**Table 5.1**) that could have a relevant role in the response of exercise in adult zebrafishheart regeneration. Next, KEGG pathway analysis identified pathways significantly (Padj<0.1) over-represented in injured ventricles from exercised zebrafish during the regenerating process. These included insulin signaling, Foxo signaling, vascular smooth muscle contraction, MAPK signaling, ubiquitin mediated proteolysis, regulation of actin cytoskeleton, mTOR signaling, Notch signaling, Wnt signaling, JAK-Stat signaling, TGFβ signaling (**Table 5.2**).

Based on the gene expression data, hierarchical clustering grouped the samples by time (7 and 14 dpi) and condition (exercised or non-exercised) in four different clusters with distinct dynamic expression patterns of the genes (**Figure 5.7 A**). Cluster 1 included 278 genes showing up-regulation in 7 dpi in both conditions and a different regulation at 14 dpi, where the genes in exercised ventricles were down-regulated. Cluster 2 included 60 genes showing a higher expression in non-exercised than in exercised ventricles at 7dpi that was accentuated even further at 14 dpi. Opposite to cluster 1, cluster 3 showed a similar regulation for both groups at 7 dpi, when genes were mainly down-regulated, that was reverted at 14 dpi when the clustered genes in exercised ventricles were strongly up-regulated whereas the expression levels of genes in non-exercised ventricles remained at similar levels as in 7 dpi. Lastly, cluster 4 included 74 genes and showed a similar regulation at 7 dpi for both groups, with genes being up- and down-regulated, but that varied at 14 dpi, with a general down-regulation of genes expressed in non-exercised ventricles and a slight up-regulation of genes expressed in exercised ventricles. The aforementioned regulatory changes for each cluster can be also visualized in **Figure 5.7 B**. Therefore, we can conclude from the clustering analysis that 14 dpi is the time-point with relevant gene expression changes in response to exercise.In **Figure 5.8** the top 20 significant differentiallyexpressed genes from the analysis were plotted to show the expression trend from exercised and non-exercised zebrafish between 7 and 14 dpi. This figure clearly shows the different expression trend of the genes between 7 and 14 dpi as it was previously shown in the hierarchical cluster analysis (**Figure 5.7 B**). Interestingly, all of them were down-regulated in response to exercise. To our knowledge, any of them has been described to date to have a relevant role in zebrafish heart regeneration and five are unknown (*Metazoa_SRP*, *si:dkey-153m14.1*, *si:dkey-18a10.3*, *zgc:158463*, *si:dkey-23n7.10*).
Among the 512 differentially expressed genes (Padj<0.1), relevant genes were selected into different categories according to scientific literature. In order to appreciate the differences in gene regulation between exercised and non-exercised ventricles, the difference in the slope between the two time points analyzed is given (at 7 and 14 dpi; as a percentage of change in exercised over non-exercised ventricles) as well as the cluster group assignment for each differentially expressed gene. Therefore, we identified genes related with the cell cycle such as cyclins (*cdkn1d*, *cdkn1bb*, *ccnd2a*) or apoptotic factors (*bcl6l*, *bag3*, *xiap*, *bcl3*) (**Table 5.3**). We selected genes found to be expressed in previous studies in animal models of regeneration, such as*rarga* and *klf17* (or *klf4b*) in zebrafish fin regeneration (White et al, 1994; Christen et al, 2010; Blum & Begemann, 2012), *fgf1a* and *klf17* (or *klf4b*) in limb regeneration in urodeles

(Dungan et al, 2002; Maki et al, 2009) or midn during hair cell regeneration in zebrafish (Jiang et al, 2014) (**Table 5.3**). Remarkably, we found factors that have been related to adult zebrafish heart regeneration and all of them grouped in cluster 3: a) members of the HIF-1α signaling pathway that positively regulates zebrafish heart regeneration and cardiomyocyte proliferation, such as *hif1aa* (up-regulated) and *hif1al* (down-regulated) (Jopling *et al*, 2012a), b) *hand2* (up-regulated) that is expressed during vertebrate cardiogenesis and also in the regenerating heart and that plays a role inducing cardiomyocyte proliferation (Lepilina *et al*, 2006; Schindler *et al*, 2014), c) *nfkb2* (up-regulated) that is a subunit of the NF-kB transcription factor which its activity modulates cardiomyocyte proliferation and de-differentiation (Karra *et al*, 2015) and d) *fgf17b* (up-regulated) that is suggested to be synthetized from the regenerating myocardium (including cardiomyocytes) in order to recruit epicardial cells and vascularize the new tissue (Lepilina *et al*, 2006) (**Table 5.3**).

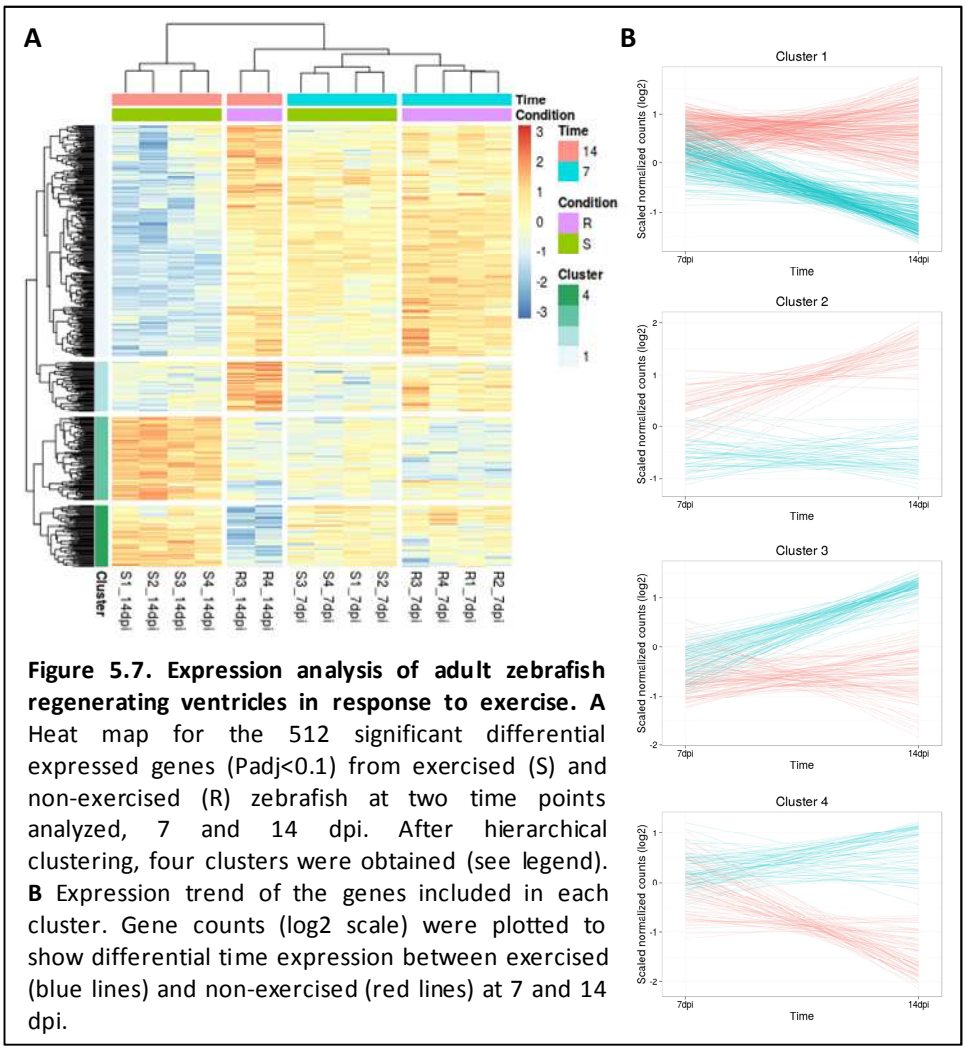

Figure 5.7. Expression analysis of adult zebrafish regenerating ventricles in response to exercise. A Heat map for the 512 significant differential expressed genes (Padj<0.1) from exercised (S) and non-exercised (R) zebrafish at two time points analyzed, 7 and 14 dpi. After hierarchical clustering, four clusters were obtained (see legend). **B** Expression trend of the genes included in each cluster. Gene counts (log2 scale) were plotted to show differential time expression between exercised (blue lines) and non-exercised (red lines) at 7 and 14 dpi.

Because cardiac injury is related to a hypoxic response both in zebrafish and mammals (Jopling *et al*, 2012a; Hashmi & Al-Salam, 2012), in addition to the HIF-1α family member indicated above, we identified genes that have been previously expressed or reported to have a relevant effect in models of ischemia or in response to hypoxia. Albeit these genes are based primarily from mammalian models (*in vitro* or *in vivo*), their expression in response to exercise in

zebrafish are worth mentioning. These include *agtr2*, *prkceb*, *ddit4*, *egln3*, *hsp90aa1*.1, *egr1*, *gpx1a*, immune factors such as *tnfaip2b*, *c4*, the carbohydrate metabolic factors *hk2*, *ppp1r3cb* and the adenosine receptors *adora2aa* and *adora2ab* (**Table 5.3**).

Moreover, we identified an important set of genes related with angiogenesis: *edn2* and its receptor *ednraa*, *mtus1a*, *adipor2*, *cd248a*, *notch3*, *nrarpa*, *cited2*, *btc*, *vezf1b*, *ptgds*, *hdac9b*, *runx1*, *antxr1a*, *hdac9b*and also endothelial cell adhesion molecules such as *antxr1*, *pcdh12*, *cldn11a* and even regulators of zebrafish cardiomyocyte cell junctions during development such as *rhoub* (**Table 5.4**). Genes coding for gap junctions, particularly important in cardiac electrical coupling, such as *cx41*.8 and *gja3,* were up-regulated. We also identified differentially expressed genes related with cardiac electrophysiology such as *kcnh6a*, *irx1a*, *nfat5a*, *hrc* (or *trpm4*), *atp2a2b*, *gria1a* as well as genes coding for muscle contracting elements such as myosins *myh7ba*, *mybphb*, *mylk4b* or sarcomeric elements such as *tcap* (**Table 5.4**). Interestingly, we found genes known to be relevant for cardiovascular development that have not been related with zebrafish heart regeneration (**Table 5.5**). For instance, frizzled receptors *fzd5*, *fzd9b*, *fzd10*, members of the Wnt pathway, weresignificantly up-regulated at 14 dpi and other cardiac developmental genes such as *klf13*, *heg1*, *klf2a*, *irx1a*, *bmi1a* and *nedd9*, the latter two expressed in progenitor cells (Aquino *et al*, 2008; Valiente-Alandi *et al*, 2015), were found to be differentially expressed. An up-regulation of genes related with cardiomyocyte growth or hypertrophy, such as *dyrk2*, *klf15*, *igflr1* and *cited4b,* was observed as well as strong up- and down- regulation of *gyg2* and *ppp1r3,* respectively, two genes involved in glycogen synthesis (**Table 5.5**). Finally, genes related with extracellular matrix remodeling were also differentially expressed, including *adamts15a*, *mmp13a* and *loxl2a*.

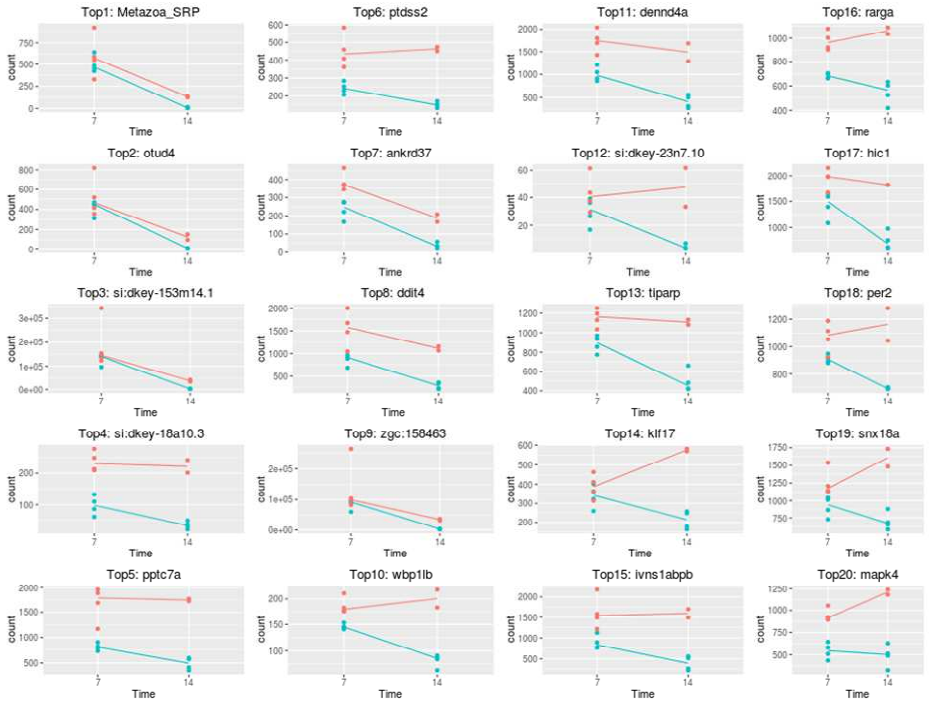

Figure 5.8. Expression profile of the top 20 significant differentially expressed genes. Gene counts were plotted to show differential time expression at 7 and 14 dpi between the exercised (blue dots) and non-exercised (red dots) regenerating ventricles.

Table 5.1. List of unkown differentially expressed genes in response to exercise during adult zebrafish regeneration. Gene name, ID number, fold-change (FC), slope rate (SR) percentage (%) between 7 dpi and 14 dpi and fold-change (FC) values for each time point are shown (Padj<0.1).

Gene name	ID number	FC	SR (%)	T7_FC	T14_FC
Metazoa_SRP	ENSDARG00000087732	**-18.90**	-2.68	-1.22	-24.75
si:dkey-153m14.1	ENSDARG00000096403	**-14.44**	-8.85	-1.03	-14.36
si:dkey-18a10.3	ENSDARG00000090814	**-6.81**	-20.43	-2.37	-7.02
zgc:158463	ENSDARG00000089382	**-12.97**	-9.00	-1.08	-13.26
si:dkey-23n7.10	ENSDARG00000104129	**-12.11**	-56.62	-1.29	-14.29
zgc:122979	ENSDARG00000004187	**-3.23**	-40.46	-1.44	-3.20
CR388047.1	ENSDARG00000101705	**10.27**	34.44	1.11	7.89
zgc:171704	ENSDARG00000018048	**-3.49**	4.70	-1.85	-3.53
si:dkey-4i23.7	ENSDARG00000094295	**-2.40**	-36.33	-1.24	-2.42
wu:fb55g09	ENSDARG00000088449	**-1.84**	17.94	-1.65	-1.88
si:ch211-261d7.6	ENSDARG00000104641	**2.74**	51.48	-1.00	2.75
CU019646.1	ENSDARG00000036414	**-2.35**	-8.88	-1.40	-2.30
si:ch73-112l6.1	ENSDARG00000093126	**2.72**	52.75	-1.02	2.66
si:dkey-162b3.5	ENSDARG00000094362	**1.85**	21.64	1.39	1.94
zgc:64189	ENSDARG00000033501	**-1.81**	-28.81	-1.12	-1.75
CABZ01033309.1	ENSDARG00000099178	**-3.04**	-25.57	-1.29	-3.39
si:dkey-250d21.1	ENSDARG00000042492	**2.89**	53.03	1.08	2.92
si:dkeyp-115e12.6	ENSDARG00000090986	**-1.73**	-9.00	-1.58	-1.81
si:ch73-334d15.2	ENSDARG00000087020	**1.74**	33.44	1.04	1.73
zgc:66448	ENSDARG00000022952	**2.11**	37.71	1.21	2.11
si:dkey-220o10.1	ENSDARG00000092448	**3.97**	48.20	-1.40	4.01
zgc:162509	ENSDARG00000070604	**1.98**	38.05	1.05	2.06
si:ch211-265g21.1	ENSDARG00000096508	**-2.03**	-38.50	-1.01	-2.12
si:rp71-1c10.7	ENSDARG00000096717	**-2.04**	-13.43	-1.39	-2.16
si:ch73-347e22.4	ENSDARG00000101029	**1.85**	34.83	1.15	1.89
si:dkey-19d21.2	ENSDARG00000097247	**241.51**	32.00	1.21	214.18
wu:fb52c12	ENSDARG00000088195	**-1.69**	-15.91	-1.38	-1.68
si:dkey-242k1.4	ENSDARG00000104899	**-4.06**	-50.43	1.15	-4.03
CABZ01070258.1	ENSDARG00000040503	**1.72**	40.68	1.01	1.77
C12H10orf71	ENSDARG00000086945	**1.62**	18.95	1.12	1.48
si:zfos-2131b9.2	ENSDARG00000100669	**9.30**	42.86	3.09	10.68
si:ch211-158d24.4	ENSDARG00000093048	**2.68**	66.08	-1.29	2.68
si:ch211-285e16.9	ENSDARG00000097466	**2.39**	11.76	1.92	2.29
si:ch211-71k24.7	ENSDARG00000102712	**2.07**	36.96	1.09	2.00
zgc:100829	ENSDARG00000015557	**-1.45**	-16.77	-1.11	-1.46
CT574587.12	ENSDARG00000090346	**2.14**	54.11	-1.08	2.13
si:ch73-386h18.1	ENSDARG00000073944	**1.98**	43.86	1.04	1.99
zgc:194312	ENSDARG00000077414	**2.12**	32.81	1.22	2.05
BX321875.1	ENSDARG00000090015	**12.15**	28.74	1.12	8.71
si:ch211-139k8.2	ENSDARG00000091946	**-2.62**	-10.03	-1.33	-2.37
zgc:172139	ENSDARG00000068830	**-1.84**	-34.41	1.01	-1.79
BX004816.2	ENSDARG00000069019	**7.31**	29.84	-1.05	5.31
si:ch211-203c7.2	ENSDARG00000089871	**-1.39**	-3.99	-1.33	-1.43
im:7147486	ENSDARG00000086626	**2.36**	41.49	1.18	2.51
si:ch73-52f24.4	ENSDARG00000100697	**-4.78**	-49.24	-1.19	-6.14
CABZ01102039.1	ENSDARG00000076357	**-3.32**	-70.13	1.49	-3.58
si:dkey-169i5.4	ENSDARG00000077862	**-4.35**	-58.36	1.26	-4.35
sb:cb1058	ENSDARG00000099819	**13.32**	56.78	-1.36	13.46
si:ch211-152p11.8	ENSDARG00000095427	**-1.60**	-20.38	-1.18	-1.57
zgc:113209	ENSDARG00000100294	**2.78**	15.09	1.49	2.61

C24H7orf50	ENSDARG00000045254	**2.16**	62.39	-1.40	2.17
si:rp71-1g18.1	ENSDARG00000053792	**2.74**	48.97	1.09	2.83
si:dkey-203a12.8	ENSDARG00000101290	**8.06**	78.01	-5.19	6.78
zgc:92066	ENSDARG00000031776	**-1.42**	-1.41	-1.29	-1.43
CABZ01079490.1	ENSDARG00000091595	**1.84**	56.26	-1.25	1.91
si:ch211-159i8.4	ENSDARG00000076332	**1.76**	56.08	-1.35	1.77
zgc:55512	ENSDARG00000003017	**-1.44**	-19.26	-1.11	-1.41
zmp:0000001085	ENSDARG00000052074	**2.32**	28.85	1.16	2.25
zgc:109744	ENSDARG00000070800	**1.99**	57.74	-1.12	2.03
CABZ01069595.1	ENSDARG00000024431	**-1.51**	0.24	-1.35	-1.54
CABZ01075268.1	ENSDARG00000104674	**-1.31**	-18.39	-1.04	-1.31
si:dkey-31n5.4	ENSDARG00000097738	**1.86**	32.90	-1.07	1.95
si:dkey-16p21.6	ENSDARG00000097646	**-1.89**	-20.18	-1.42	-1.89
si:ch211-210h11.7	ENSDARG00000095692	**-3.05**	-65.12	1.10	-3.21
si:dkeyp-2e4.6	ENSDARG00000093656	**6.81**	58.78	-1.08	7.04
si:dkey-15n5.6	ENSDARG00000099680	**1.93**	21.52	1.12	1.87
si:dkey-3n22.9	ENSDARG00000096273	**6.16**	14.81	1.60	5.94

The prefix im: indicates this gene is represented by an EST generated by the I.M.A.G.E. Consortium. The prefix 'si:' indicates that this gene is represented by annotated genomic sequence from the Sanger Institute. The prefix wu: indicates this gene is represented by an EST generated at Washington University School of Medicine. The prefix `zgc:' indicates that this gene is represented by cDNAs generated by the ZGC project.

Table 5.2. Significantly over-represented KEGG pathways in adult zebrafish regenerating hearts in response to exercise.

ID	KEGG pathway	P-value
4910	**Insulin signaling pathway**	9.8E-006
4068	**FoxO signaling pathway**	4.6E-005
4270	**Vascular smooth muscle contraction**	4.0E-004
4010	**MAPK signaling pathway**	5.2E-004
4120	**Ubiquitin mediated proteolysis**	1.6E-003
4810	**Regulation of actin cytoskeleton**	1.9E-003
4150	**mTOR signaling pathway**	2.3E-003
4510	**Focal adhesion**	3.2E-003
4330	**Notch signaling pathway**	5.3E-003
71	**Fatty acid degradation**	1.5E-002
4310	**Wnt signaling pathway**	1.9E-002
4630	**Jak STAT signaling pathway**	2.1E-002
4350	**TGF beta signaling pathway**	3.4E-002

P-value (P<0.05).

Table 5.3. List of differentially expressed genes related with cell cycle regulation and regeneration (Padj<0.1).

Name	Description	FC	SR (%)	7 FC	14 FC	Cluster
	Cell cycle regulation					
bcl6a	B-cell CLL/lymphoma 6	**-2.80**	-8.62	-1.62	-2.94	1
bag3	BCL2 associated athanogene 3	**-1.93**	-38.80	-1.11	-1.91	1
xiap	baculoviral IAP repeat containing 8 2]	**-1.54**	-14.01	-1.23	-1.53	1
cdkn1d	Cyclin-dependent kinase inhibitor 1D	**-1.86**	-34.51	-1.13	-1.89	1
bcl2l10	BCL2-like 10 (apoptosis facilitator)	**-1.51**	-21.89	-1.13	-1.47	1
ccng2	cyclin G2	**-1.73**	-17.23	-1.44	-1.76	1
cables2b	Cdk5 and Abl enzyme substrate 2	**-1.64**	-14.68	-1.25	-1.65	1
ccnt2a	cyclin T2	**-1.52**	-21.89	-1.05	-1.53	1
ccni2	cyclin I family member 2	**-1.60**	-23.36	-1.06	-1.60	1
cdk13	cyclin-dependent kinase 13	**-1.51**	-43.60	1.23	-1.43	1
pmaip1	phorbol-12-myristate-13-acetate-induced protein 1	**-2.14**	-59.73	1.17	-2.36	1
cdkn1bb	cyclin-dependent kinase inhibitor 1B (p27, Kip1)	**-1.82**	-26.37	-1.34	-1.84	2

ccnd2a	cyclin D2	**1.70**	19.72	1.38	1.71	3
bcl3	B-cell CLL/lymphoma 3	**4.07**	29.83	-1.05	3.90	4
casp8ap2	caspase 8 associated protein 2	**1.82**	25.50	1.29	1.85	4
	Regeneration					
klf17 (klf4b)	Kruppel-like factor 1 (erythroid)	**-2.70**	-54.59	-1.13	-2.69	1
rarga	retinoic acid receptor, gamma	**-1.95**	-19.96	-1.41	-1.88	1
hif1al/hif3a	hypoxia-inducible factor 1, alpha subunit, like	**-2.19**	-30.65	-1.24	-2.24	1
fgf1a	fibroblast growth factor 1	**-1.82**	-15.50	-1.45	-1.83	1
midn	midnolin	**-2.72**	-60.15	1.00	-2.63	1
nfkb2	nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)	**2.01**	36.12	1.19	1.99	3
fgf17b	fibroblast growth factor 17	**2.17**	28.44	1.78	2.16	3
hif1aa	hypoxia inducible factor 1, alpha subunit	**2.08**	25.50	1.32	1.95	3
hand2	heart and neural crest derivatives expressed 2	**1.69**	34.91	-1.02	1.64	3
	Response to hypoxia or ischemia					
ddit4	DNA damage inducible transcript 4	**-3.96**	-8.46	-1.72	-3.95	1
adora2ab	adenosine A2a receptor	**-2.14**	-7.72	-1.66	-2.52	1
egln3	egl-9 family hypoxia-inducible factor 3	**-3.98**	18.40	-1.96	-4.14	1
prkceb	protein kinase C, epsilon	**-2.24**	-42.31	1.10	-2.34	1
agtr2	angiotensin II receptor, type 2	**-1.79**	-41.80	-1.04	-1.83	1
cpt1b	carnitine palmitoyltransferase 1B	**-1.43**	-25.03	-1.03	-1.44	1
gpx1a	glutathione peroxidase 2	**-1.68**	-51.43	1.27	-1.57	1
adora2aa	adenosine A2a receptor	**-1.63**	-33.55	-1.03	-1.61	2
ppp1r3cb	protein phosphatase 1 regulatory subunit 3C	**1.68**	26.23	1.16	1.65	3
egr1	early growth response 1	**2.75**	54.54	-1.02	2.82	3
tnfaip2b	TNF alpha induced protein 2	**6.55**	33.37	-1.26	5.81	4
c4	complement component 4	**1.95**	32.02	-1.17	1.87	4
hk2	hexokinase 2	**2.56**	41.68	-1.09	2.71	4
hsp90aa1	heat shock protein 90, alpha (cytosolic), class A member 1	**1.65**	-9.85	1.71	1.70	4

Gene name, gene description, fold-change (FC), slope rate (SR) percentage (%) between 7 dpi and 14 dpi and fold-change (7 FC, 14 FC) values for each time point and cluster group are shown (Padj<0.1).

Table 5.4. List of differentially expressed genes related with angiogenesis and cardiac function (Padj<0.1).

Name	Description	FC	SR (%)	7 FC	14 FC	Cluster
	Angiogenesis and endothelial function					
adipor2	adiponectin receptor 2	**-1.67**	-26.01	-1.15	-1.67	1
runx1	runt-related transcription factor 1	**-4.62**	-30.89	-1.76	-4.16	1
nrarpa	NOTCH-regulated ankyrin repeat protein	**-1.61**	-22.65	-1.18	-1.69	1
mtus1a	microtubule associated tumor suppressor 1	**2.91**	46.94	1.54	2.97	3
cd248a	CD248 molecule, endosialin	**1.61**	42.67	-1.31	1.54	3
btc	betacellulin	**2.18**	45.87	-1.30	2.11	3
edn2	endothelin 2	**2.40**	33.29	-1.06	2.20	3
ednraa	endothelin receptor type A	**1.36**	16.32	1.10	1.35	3
hdac9b	histone deacetylase 9	**1.91**	26.17	1.31	1.87	3
notch3	notch 3	**1.99**	36.36	1.08	1.98	3
vezf1b	vascular endothelial zinc finger 1	**5.86**	70.19	1.12	5.95	4
ptgds	prostaglandin D2 synthase	**3.46**	51.12	-1.18	3.25	4
rhoub	ras homolog family member Ub	**-3.71**	-19.39	-1.95	-3.54	1
cldn11a	claudin 11	**-2.50**	-21.35	-1.56	-2.42	1
pcdh12	protocadherin 12	**1.64**	23.92	1.08	1.56	4
cntn6	contactin 6	**2.99**	68.21	-1.03	3.13	4
antxr1a	anthrax toxin receptor 1	**3.28**	82.60	-1.86	3.15	4
	Cardiac function					

kcnh6a	potassium voltage-gated channel, subfamily H, member 6a	**-2.05**	-25.75	-1.36	-2.22	1
nfat5a	nuclear factor of activated T-cells 5, tonicity-responsive	**-1.97**	-22.67	-1.06	-2.03	1
atp2a2b	ATPase, Ca++ transporting, cardiac muscle, slow twitch 2	**-1.42**	-4.78	-1.27	-1.41	1
klf2a	Kruppel-like factor 2a	**-2.29**	-45.89	-1.08	-2.21	1
mybphb	myosin binding protein H-like	**-1.90**	-57.68	1.66	-1.91	2
mylk4b	myosin light chain kinase family member 4	**-2.09**	-36.39	-1.36	-2.14	2
tcap	titin-cap	**-2.03**	-24.38	-1.41	-2.18	2
irx1a	iroquois homeobox 1	**2.01**	48.58	-1.28	2.02	3
hrc/trpm4	histidine rich calcium binding protein	**1.67**	43.63	-1.16	1.75	3
myh7ba	myosin, heavy chain 7B, cardiac muscle, beta	**1.63**	16.40	1.26	1.53	3
gja3	gap junction protein alpha 3	**2.15**	37.68	1.21	2.17	3
cx41.8	connexin 41.8	**1.71**	19.57	1.34	1.66	3
gria1a	glutamate receptor, ionotropic, AMPA 1	**4.83**	51.96	-1.32	3.90	4
boc	BOC cell adhesion associated, oncogene regulated	**1.55**	38.62	-1.12	1.59	4

Gene name, gene description, fold-change (FC), slope rate (SR) percentage (%) between 7 dpi and 14 dpi and fold-change (7 FC, 14 FC) values for each time point and cluster group are shown (Padj<0.1).

Table 5.5. List of differentially expressed genes related with cardiovascular system (Padj<0.1).

Name	Description	FC	SR (%)	7 FC	14 FC	Cluster
	Cardiovascular development and others					
cited2	Cbp/p300-interacting transactivator, Glu/Asp rich carboxy-terminal domain, 2	**-3.29**	12.29	-1.65	-3.90	1
klf13	Kruppel-like factor 13	**-2.87**	-23.13	-1.29	-2.56	1
ucp2	uncoupling protein 2 (mitochondrial, proton carrier)	**-1.74**	-15.65	-1.22	-1.71	1
nedd9	neural precursor cell expressed, developmentally down-regulated 9	**-2.63**	-36.74	-1.19	-2.63	1
sema3ab	semaphorin 3ab	**-1.58**	4.42	-1.40	-1.58	1
heg	heart development protein with EGF-like domains 1	**-1.50**	-4.45	-1.32	-1.45	1
loxl2a	lysyl oxidase-like 2a	**-2.00**	25.76	-1.97	-1.88	1
rorca	RAR-related orphan receptor C a	**2.06**	26.27	1.48	2.04	1
cxcl14	chemokine (C-X-C motif) ligand 14	**-1.81**	-21.35	-1.31	-1.78	2
gstk1	glutathione S-transferase kappa 1	**-3.73**	-44.73	-1.72	-3.58	2
ppp1r3g	protein phosphatase 1 regulatory subunit 3G	**-4.50**	-50.23	-2.45	-4.64	2
fgf3	fibroblast growth factor 3	**3.13**	40.59	1.29	2.98	3
fzd5	frizzled class receptor 5	**1.93**	54.74	-1.81	1.79	3
fzd9b	frizzled class receptor 9	**2.31**	43.89	-1.09	2.17	3
fzd10	frizzled class receptor 10	**3.46**	59.01	-1.76	3.34	3
dyrk2	dual specificity tyrosine-(Y)-phosphorylation regulated kinase 2	**1.99**	35.19	1.21	2.03	3
irx1a	iroquois homeobox 1	**2.01**	48.58	-1.28	2.02	3
bmi1a	BMI1 proto-oncogene, polycomb ring finger	**1.84**	32.92	-1.06	1.72	3
cited4b	Cbp/p300-interacting transactivator, with Glu/Asp rich carboxy-terminal domain, 4	**1.83**	49.82	-1.26	1.80	3
klf15	Kruppel-like factor 15	**2.20**	31.49	1.21	2.10	3
gyg2	glycogenin 2	**4.42**	69.95	-1.57	4.27	3
igflr1	IGF like family receptor 1	**1.69**	35.08	-1.07	1.65	4
adamts15a	ADAM metallopeptidase with thrombospondin type 1 motif 15	**1.95**	45.14	1.06	1.97	4
mmp13a	matrix metallopeptidase 13a	**7.16**	12.46	1.61	4.44	4

Gene name, gene description, fold-change (FC), slope rate (SR) percentage (%) between 7 dpi and 14 dpi and fold-change (7 FC, 14 FC) values for each time point and cluster group are shown (Padj<0.1).

DISCUSSION

“Science is wonderfully equipped to answer the question 'How?' but it gets terribly confused when you ask the question 'Why?'”

Erwin Chargaff (1905-2002)
Biochemist

Chapter 1

Exercise training induces growth of fast muscle fibers in adult zebrafish

The present study describes the cellular and molecular adaptive mechanisms that are responsible for the plasticity of fast skeletal muscle to exercise-induced contractile activity. Here, we have adopted swimming adult zebrafish as a muscle activity model and have shown, for the first time in adult zebrafish, that exercise training under sustained, aerobic conditions causes hypertrophy of fast muscle fibers. We hypothesize that this may explain, at least in part, the stimulation of muscle growth by swimming in adult zebrafish that we previously reported using the same experimental conditions (Palstra *et al*, 2010). Therefore, as in mammals (Braun & Gautel, 2011; Schiaffino *et al*, 2013) and in other fish species (Davison & Herbert, 2013), exercise promotes growth in adult zebrafish by increasing muscle mass as a result of increased fiber hypertrophy.

Our gene expression analysis of fast muscle of exercised adult zebrafish shows that the increase in fiber hypertrophy is associated with an important regulation of the fast muscle transcriptome. Here, we show for the first time in zebrafish that exercise-stimulated contractile activity in adult fast muscle induced significant and parallel changes in the expression of canonical pathways important for the regulation of protein turnover, namely the anabolic IGF-1/PI3K/Akt/mTOR signaling pathways that promote protein synthesis and the catabolic ubiquitination and atrophy pathways that are responsible for protein degradation (Schiaffino *et al*, 2013). The increase in the expression of genes involved in protein synthesis and in its regulation (e.g. *igfr1*, *irs1*, *pi3k*, *pdk1*, *pdk2*, *rps6ka1*) and the decrease in the expression of the translation inhibitor *eif4ebp1*, recently shown to be up-regulated in a zebrafish inactivity model (Yogev *et al*, 2013), is consistent with the up-regulation of the mRNA expression levels of a large number of genes that code for structural and regulatory contractile elements as well as components of the extracellular matrix in fast muscle of exercised zebrafish. Further support for the activation of this pathway in fast muscle of exercised zebrafish can be found in the down-regulation of the expression of *pten*, a known inhibitor of PI3K/Akt signaling (Song *et al*, 2012b). From research in mammalian models, exercise-induced hypertrophy has been shown to occur concomitantly with an activation of mTOR signaling (Bodine, 2006; Egan & Zierath, 2013). Thus, the increase in mTOR and Mef2 protein levels observed in fast muscle from exercised zebrafish could provide a mechanistic explanation for the hypertrophy of zebrafish fast muscle fibers through increasing protein synthesis, at least in part, by activation of mTOR signaling. These observations reinforce the notion that accretion of myofibrillar proteins is an important contributor to muscle growth in fish (Johnston *et al*, 2011) and strongly suggest that myofibrillogenesis can be stimulated by exercise-induced contractile activity in adult zebrafish. In support of this hypothesis, the increase in protein deposition in the fast muscle of rainbow trout by swimming-induced activity (Felip *et al*, 2012) has been associated with the transcriptional activation of a large set of genes involved in protein biosynthesis and in muscle contraction and development, including components of the sarcomeric structure of skeletal muscle (Magnoni *et al*, 2013a). Interestingly, in the present study exercise also increased the mRNA expression levels of known regulators of atrophy in

skeletal muscle, namely the E3 ubiquitin ligases *trim63* and *fbxo32* (Sandri, 2008) and their transcriptional activators *foxo3* (Sandri *et al*, 2004) and *traf6* (Paul *et al*, 2010), consistent with previous reports indicating that TRIM63 and FBXO32 mRNA expression levels increase in hypertrophied muscles in humans subjected to resistance training (Léger *et al*, 2006). These observations suggest that genes involved in the regulation of the degradation of skeletal muscle protein (i.e. atrogenes), in addition to a large set of genes belonging to the ubiquitin proteasome pathway or other proteolytic systems (e.g. calpains), may also participate in the hypertrophic response of the zebrafish fast muscle to exercise-induced contractile activity, possibly to facilitate the maintenance of normal skeletal muscle protein turnover during long-term training (Léger *et al*, 2006). Therefore, our results strongly indicate that exercise-induced hypertrophy of fast muscle fibers in adult zebrafish involves increased protein turnover, shown for the first time in this species by the parallel activation of the IGF-1/PI3K/mTOR signaling and atrophy pathways that, in turn, induce the expression of a number of downstream genes coding for myofibrillar elements, as illustrated by the molecular interactome of the muscle development process (Appendix Figure A.1).

One of the important and novel findings of our transcriptomic analysis of the hypertrophic fast muscle of exercised adult zebrafish is the activation of nearly all TGFβ superfamily signaling pathways known to regulate skeletal muscle mass in mammals. On one hand, we observed an increase in the mRNA levels of follistatin (*fst*), known to promote muscle hypertrophy in mammals by binding myostatin (MSTN) and preventing its interaction with activin receptors resulting in activation of the Akt/mTOR signaling pathway to stimulate protein synthesis (Lee *et al*, 2010). The MSTN signaling pathway, known in mammals and fish to exert a repressive action on muscle hypertrophy (McPherron *et al*, 1997; Xu *et al*, 2003) through its inhibition of IGF-1/Akt signaling (Trendelenburg *et al*, 2009), was also up-regulated in fast muscle of exercised zebrafish as evidenced by the increased expression of the extracellular ligand (*mstn*), corroborating the results of a previous study from our laboratory (Palstra *et al*, 2010), receptors (*acvr1b* and *acvr2b*) and signaling molecules (*smad2*). On the other hand, a number of components of the BMP signaling pathway, including extracellular ligands (*bmp1, bmp3, bmp4, bmp8b*), receptors (*mbpr1a, bmpr1b*), gene targets (*id1*) and antagonists such as *noggin* and *smad6*, were all up-regulated in fast muscle of exercised zebrafish. In mammals, BMPs promote skeletal muscle hypertrophy by stimulating mTOR-dependent anabolism (Sartori *et al*, 2013; Winbanks *et al*, 2013). The results from the present study are significant because they suggest, for the first time, that the BMP signaling pathway may be involved in exercise-induced hypertrophy of skeletal muscle. In mammals, it has been proposed that the regulation of muscle mass depends on the balance between the competing MSTN and BMP signaling pathways (Sartori *et al*, 2013). We hypothesize that the exercise-induced increase in muscle mass associated with hypertrophy of fast muscle in adult zebrafish may have resulted, at least in part, from alterations in the normal balance between negative (i.e. MSTN) and positive (i.e. FST, BMPs) regulators of skeletal muscle mass.

Exercise-induced activity in this study also altered the expression of other important myogenic differentiation factors in the zebrafish fast muscle, most notably Myocyte enhancer factor 2 (Mef2) proteins, found to be induced at the mRNA and protein level, and Serum response factor (Srf). MEF2 family members are transcription factors that do not have intrinsic myogenic

activity but control the differentiation of skeletal muscle during development through transcriptional cooperation with co-activators such as CREBBP(CBP)/p300, resulting in the potentiation of the function of myogenic regulatory factors (MRFs) and in the regulation of fiber type-specific gene expression programs in mammals (Potthoff & Olson, 2007). In the adult mammalian muscle, MEF2, in addition to NFAT proteins, is induced by contractile activity in a calcineurin- and CAMKIV-dependent fashion (Wu *et al*, 2001) to regulate the metabolic and structural (contractile) phenotype of skeletal muscle cells. Several *mef2* genes are expressed in the zebrafish skeletal muscle(Ticho *et al*, 1996), with *mef2a* being expressed in fast muscle after differentiation, *mef2c* after myoblast terminal differentiation and *mef2d* in muscle precursor cells (Hinits & Hughes, 2007). Although Mef2cand Mef2d proteins are not required for muscle fiber terminal differentiation, they are indispensable for myofilament expression and myofibril assembly in zebrafish fast muscle fibers(Hinits & Hughes, 2007).Recently, *mef2ca* was shown to be induced post-transcriptionally by the TOR pathway to regulate hypertrophic muscle growth in zebrafish (Yogev *et al*, 2013). Importantly, the observed increase in mTOR and Mef2 protein levels in fast muscle from exercised zebrafish could suggest an involvement of the proposed model by Yogev *et al* (2013) to regulate muscle activity in adult zebrafish under the present exercise conditions. Here, we observed an up-regulation of the mRNA expression levels of *ep300* and *crebbp*, two nuclear genes that occupy a central position in the transcriptional network in fast muscle of exercised zebrafish, and of *mef2a* and *mef2d*; however, the expression of *mef2ca* was decreased by exercise. In addition, genes involved in calcium signaling initiated by nerve-elicited electrical activity and that regulate MEF2 activity such as *ppp3ca* (calcineurin), its targets *nfatc1*, *nfatc3* and *nfatc4*, *camk4* and *hdac4* were all up-regulated by exercise in the zebrafish fast muscle. Another central molecule in the transcriptional network of regulated nuclear genes in the fast muscle of exercised zebrafish is SRF, a transcription factor that regulates myogenic fusion and differentiation and that is also required for overload-induced hypertrophy in the adult mammalian muscle by controlling satellite cell proliferation (Guerci *et al*, 2012). The altered expression of *srf* in fast muscle of exercised zebrafish, as well as that of the transcriptional repressor *hdac1*, is consistent with their role as regulators of skeletal myogenesis (Guerci *et al*, 2012; Puri *et al*, 2001).

Importantly, our study also provides molecular evidence to suggest that exercise in adult zebrafish may have activated a myogenic program resulting from the activation of satellite cells. Satellite cells, muscle precursor cells with stem cell characteristics (Wagers & Conboy, 2005), are known to contribute importantly to postnatal skeletal muscle growth and muscle regeneration after injury. However, their involvement in hypertrophic muscle growth in adult mammals is currently a subject of debate, particularly in the light of studies showing that hypertrophy does not require the presence of satellite cells (Wagers & Conboy, 2005) or their activation (McCarthy *et al*, 2011; Blaauw *et al*, 2009). In contrast, postembryonic muscle growth in zebrafish is accomplished by mosaic hyperplasia (i.e. new myotubes forming on the surface of existing muscle fibers) until fish achieve half of their final body length after which growth is only accomplished by hypertrophy (Johnston *et al*, 2011). To date, the exact role of satellite cells (referred to as myogenic precursor cells in fish) in exercise-induced activity in skeletal muscle or whether contractile activity of skeletal muscle fibers can modify the quiescent status of satellite cells and promote their activation in adult muscle is not

completely understood. However, there are reports showing that hypertrophy due to resistance training in humans is associated with an increase in the satellite cell pool probably as a result of increased proliferation (Lee *et al*, 2012b). Here, we show for the first time in fish that exercise-induced activity in adult zebrafish altered the expression of genes known to participate in the myogenic program, most notably the satellite cell marker *pax3* and its target gene *lbx1*. PAX3 is a key factor in skeletal muscle development thought to be responsible for the enlargement of the satellite cell population in muscle at least in part through its activation of the FGF signaling pathway (Braun & Gautel, 2011). PAX3 is important for the activation of the muscle regulatory factors MYOD and, together with the mesenchyme homeobox gene 2 (MEOX2) and SIX proteins (SIX1 and SIX4), of MYF5 (Braun & Gautel, 2011). PAX3 was recently shown to be up-regulated specifically in hyperplasic growth zones in the late embryonic myotome in rainbow trout (Rescan *et al*, 2013), another fish species with hyperplasic growth continuing into adulthood. In the present study, we show that the mRNA expression levels of a number of components of the FGF signaling pathway, including ligands (*fgf13*, *fgf18*, *fgf20*), receptors (*fgfr1*, *fgfr2*, *fgfrl1*) and signaling molecules (*mapk1*, *raf1*, *mapk13*, *crebbp*), as well as *meox2*, *six1* and *six4*, were increased in fast muscle in response to exercise training in adult zebrafish. All these factors interact with *pax3*, *sox9* and *rela* in a complex molecular network similar to that described in the exercise-trained human skeletal muscle (Thalacker-Mercer *et al*, 2013; Keller *et al*, 2011). Interestingly, the canonical Notch and Wnt signaling pathways, known to sequentially control the transition of satellite cells from a proliferative to differentiative phase (Brack *et al*, 2008), were significantly altered in fast muscle of exercised zebrafish. In accordance with the increased expression of *pax3*, the altered expression of ligands (*dll1*, *jag1*, *jag2*) and receptors (*notch1*, *notch2*) of the Notch signaling pathway, coupled with the significant alteration of the expression of genes involved in mitosis and cell cycle progression, suggests the possibility that satellite cells may have been activated by exercise in adult zebrafish fast muscle. Indeed, we have found an increase in a satellite cell marker during the four weeks of exercise and an increase in cell proliferation from the second week to the end of the training period as suggested by the induced expression of the protein markers Pax7 and phospho-histone 3, respectively. However, we have not evaluated the activation and localization of satellite cells and, thus, their possible contribution to swimming-induced muscle growth is unknown at the present time. Moreover, it has recently been shown that *pax7a* and *pax7b* satellite cells contribute differently to muscle repair in zebrafish larvae (Pipalia *et al*, 2016). Although we have not found differences between *pax7a* and *pax7b* at the mRNA level it would be interesting to further determine if these distinct satellite cell populations have a role in the response to exercise in adult zebrafish. The recent demonstration that satellite cells in adult zebrafish muscle fibers can be activated by mechanical stretch (Zhang & Anderson, 2014) and that both *pax3* and *pax7* are expressed in satellite cells isolated from adult zebrafish muscle (Alexander *et al*, 2011) provide support for the hypothesis that satellite cells may have proliferated in fast muscle of adult zebrafish in response to exercise-induced activity. In addition, exercise caused a significant increase in the expression of components of the Wnt (e.g. *wnt1, wnt2, wnt4, wnt6, wnt7a, wnt7b, wnt8a, wnt10a, wnt10b, wnt11, wnt16; fzd2 to 5, fzd8 to 10; dvl1, dvl2, ccnd1*) and the hedgehog (e.g. *shh, ihh*) signaling pathways, known to play a key role in the induction of myogenesis in vertebrates by promoting differentiation of satellite cells (Ochi & Westerfield, 2007; Montarras *et al*, 2013). Interestingly, hyperplasic growth in embryonic trout was also associated with an

important up-regulation of growth factors and soluble signaling molecules (including members of the Wnt pathway) (Rescan *et al*, 2013) but, to our knowledge, this is the first report of exercise regulating the expression of the hedgehog signaling pathway. However, the expression of various paralogs of fast skeletal myosin heavy chain (e.g. myhz1.1, myhz1.2, myhz1.3 and myhz2) that were reported to be markers for hyperplasic growth in zebrafish (Johnston *et al*, 2009) did not change in fast muscle of exercised adult zebrafish. Therefore, it will be important to investigate in future studies whether exercise can promote proliferation and/or activation of satellite cells in fast muscle of adult zebrafish.

Taken together, we report that swimming exercise in adult zebrafish resulted in the activation of myogenic and growth transcriptional programs as well as increased protein synthesis and possibly also cell proliferation that, in turn, may have been responsible for the observed hypertrophic phenotype of the exercised fast muscle.

Exercise training induces the activity of AMPK, an important energy sensing enzyme, and increases oxidative metabolism in fast muscle of adult zebrafish

Another important finding in this study is the swimming-induced activation of AMP-activated protein kinase (AMPK) in fast muscle of adult zebrafish. Our transcriptomic profiling of the fast muscle of adult zebrafish showed an up-regulation of genes coding for the three AMPK subunits (e.g. *prkaa1*, *prkab1* and *prkag1)* that correlated well with the observed increased activity of AMPK after four weeks of exercise training. AMPK activity has been measured in skeletal muscle in other fish species after nitrite and hypoxia exposure (Jibb & Richards, 2008; Xu *et al*, 2016) but under swimming-exercise conditions it has only been shown in trout (Magnoni *et al*, 2014) and in zebrafish (present study). It is well documented from muscle cell cultures and *in vivo* studies that AMPK is activated in response to muscle contraction or exercise in mammals (Winder & Hardie, 1996; Nedachi *et al*, 2008). AMPK regulates the expression of metabolic genes involved in ATP generation processes such as glycolysis or fatty acid oxidation in order to replenish the energy levels of the cells (Hardie *et al*, 2012). Indeed, our transcriptomic profiling of the fast muscle of exercised adult zebrafish showed an enrichment of genes involved in those processes and specifically evidenced the regulation of AMPK target genes (direct or indirect) such as *hk2*, *pdk4*, *cpt1a*, *hadhb*, *cox4l1* and *cat* (McGee & Hargreaves, 2010). From previous studies in trout, our group suggested a conserved mechanism of AMPK in fish in mediating glucose uptake and utilization through the glucose transporter GLUT4 in trout muscle cells (Magnoni *et al*, 2012, 2014) as it has been shown in mammals (Jessen & Goodyear, 2005; Hardie *et al*, 2012). These studies could suggest a similar mechanism in fast muscle from exercised zebrafish although the possible involvement of GLUT4 in the metabolic actions of AMPK is not known because the GLUT4 ortholog in zebrafish has not been identified in the genome of this species.

Remarkably, the induction of AMPK activity after four weeks of training coincided with an up-regulation of *pgc1α* at the mRNA and, to a lesser extent, at the protein level. PGC1α is an important co-transcriptional regulator that controls the expression of genes involved in oxidative metabolism and mitochondrial biogenesis and is phosphorylated by AMPK in mammals (Lin *et al*, 2002; Jäger *et al*, 2007). Previous studies have shown that *pgc1α* mRNA levels increase after swimming-induced exercise in fish (LeMoine *et al*, 2010a; Magnoni *et al*,

2013b, 2014) although it has been suggested that PGC1α may not be a regulator of mitochondrial biogenesis in fish (LeMoine *et al*, 2010a, 2010b) conversely to mammals (Olesen *et al*, 2010). Moreover, a recent study has reported the inability of human AMPK to phosphorylate a zebrafish Pgc1α peptide *in vitro* (Bremer *et al*, 2015), which brings into question the regulation of PGC1α by AMPK in fish. Another proposed mechanism by which AMPK activates PGC1α (and other nuclear proteins) is through the deacetylase enzyme SIRT1 (Cantó *et al*, 2009, 2010). SIRT1 activity was found to be elevated after exercise training in human and rodent muscle and it was related with increased PGC1α activity (Suwa *et al*, 2008; Gurd *et al*, 2011). Interestingly, In contrast to what we have shown in trout (Magnoni *et al*, 2014), our observation of increased mRNA expression levels of *sirt1* in exercised zebrafish fast muscle by transcriptomic profiling could suggest an AMPK-driven mitochondrial biogenesis program but these interactions have not yet been examined in fish. Besides its role in increasing mitochondrial biogenesis through PGC1α, AMPK has been involved in mitochondrial degradation (or mitophagy) by activating ULK1 (Egan *et al*, 2011; Sanchez *et al*, 2012), a gene found to be up-regulated in our gene expression analysis which could suggest an exercise-induced maintenance of functional mitochondria in zebrafish fast muscle as it has been proposed in higher vertebrates (Yan *et al*, 2012). Although the observed increase in *pgc1α* expression by exercise in the zebrafish fast muscle is consistent with results from studies in mammals, further research is needed to clarify the role of Pgc1α in zebrafish fast muscle. Interestingly, Lin et al (2002) reported a role for PGC1α in regulating fiber type determination by interacting with the Mef2c and Mef2dproteins, that are targets of calcineurin (Lin *et al*, 2002). However, PGC1α does not appear to influence fiber type transformation under exercise conditions (Geng *et al*, 2010), in contrast to PPARδ. PPARδ, a transcriptional regulator of lipid metabolism that is highly expressed in skeletal muscle and known to be up-regulated during aerobic exercise, regulates muscle fiber type under aerobic exercise conditions (Luquet, 2003; Wang *et al*, 2004) and the PPARδ-induced myogenic response could be mediated by a calcineurin-dependent pathway (Gaudel *et al*, 2008). As discussed above, our transcriptional analysis of exercised zebrafish fast muscle identified differentially regulated genes of the calcineurin signaling pathway (*ppp3ca*, *nfatc1*, *nfatc3*, *nfatc4*, *camk4*, *hdac4*), including *mef2* (also increased at the protein level) as well as *pparda*, that was up-regulated after four weeks of training. Therefore, it is tempting to speculate that the observed exercise-induced expression of *pgc1α* and *ppard* in this study, coupled with the down-regulation of *mhyz2*, a fast myosin heavy chain isoform, could have favored myofiber remodelling possibly towards a fast-to-slow fiber type switch after four weeks of training in order to promote the metabolic adaptation of zebrafish fast muscle to swimming-induced exercise. As a matter of fact, AMPK and PPARδ in rodents have been defined as “exercise mimetic” factors, since their pharmacological activation was demonstrated to enhance running endurance and to induce the expression of genes involved in oxidative metabolism, effects that are potentiated when their agonists are combined under exercise conditions (Narkar *et al*, 2008; Manio *et al*, 2016). Based on the results presented here, we hypothesize that exercise-induced fast muscle adaptation in zebrafish included metabolic remodelling by priming oxidative metabolic routes and that these changes may be mediated, at least in part, by AMPK, as it has been previously suggested in trout (Magnoni *et al*, 2014).

Exercise training promotes vascularization in fast muscle of adult zebrafish

In addition to the increased hypertrophy of fast muscle fibers, exercise increased vascularization of this tissue in adult zebrafish. This is consistent with the well-known increase in capillary number that accompanies fiber hypertrophy in humans and mammalian models (Egginton, 2009; Plyley *et al*, 1998) and also with previous reports that indicate that swim training increases muscle capillarity in several fish species, including larval zebrafish (Ibarz *et al*, 2011; Pelster *et al*, 2003; Sanger, 1992; Davie *et al*, 1986). In mammals, exercise-induced angiogenesis is believed to be induced by the contractile activity of skeletal muscle fibers that, through the combination of growth factor production, hypoxia and shear and mechanical stresses, results in the activation of pro-angiogenic signaling pathways (Prior *et al*, 2004). Importantly, our transcriptomic profiling of the fast muscle of exercised adult zebrafish clearly evidenced the activation of the majority of signaling pathways known in mammals and zebrafish to regulate angiogenesis (Adams & Alitalo, 2007; Potente *et al*, 2011; Gore *et al*, 2012), and identified for the first time the molecular programs responsible for the observed increase in vascularization of this tissue by exercise. Specifically, fast skeletal muscle of exercised zebrafish increased the mRNA levels of genes involved in vascular sprouting, including *sema3d*, *sema3f*, *netrin1* and *efnb2*, molecules known to be important for intersegmental vessel formation in zebrafish (Gore *et al*, 2012), as well as of *robo2* and *slit2*, an endothelial cell guidance receptor and its ligand, respectively. In addition, exercise also activated at the transcriptional level several canonical signaling pathways known to control the specification of arteries and veins (e.g. Vegf, Notch, Ephrin B2) (Hong *et al*, 2006; Herbert *et al*, 2009), as supported by the increased mRNA levels of *ssh*, of members of the Vegf signaling pathway including ligands (e.g. *vegfc*), co-receptors (*nrp1*) and downstream signaling molecules (*pik3c2a*, *pikc3b*, *pik3cg*, *plcg1*, *mapk1*), of *notch1* and of *efnb2* and its receptor *ephb4*. Furthermore, exercise altered the mRNA levels of genes involved in vascular lumen formation in zebrafish such as *integrins*, *cdc42*, *rac1* and *pax2* (Gore *et al*, 2012). Interestingly, to the best of our knowledge, we provide the first demonstration that exercise increases the mRNA levels in fast muscle of *klf2*, a shear stress-responsive transcription factor that is activated by the onset of blood flow in newly formed vessels and that induces vessel remodelling through alteration of PI3K and MAPK signaling in zebrafish (Nicoli *et al*, 2010). *klf2* occupies a central position in the angiogenic transcriptional network in fast muscle of exercised adult zebrafish with connections with soluble pro-angiogenic factors (e.g. endothelins, angiopoietins, IGF2, semaphorins), signaling molecules (e.g. *traf6*, *erbb2*) and transcriptional regulators (e.g. *id1*, *ctnnb1*, *crebbp*, *sirt1*). Remarkably, *klf2*, as well as other components of the angiogenic transcriptional network such as the IGF-1,TGFβ and Notch signaling pathways and the nuclear transcriptional regulator *crebbp*, also participate in the muscle development network. Thus, the molecular response to exercise in skeletal muscle may involve the coordinated activation of angiogenic and muscle development transcriptional programs.

The mechanisms by which angiogenesis is initiated under the normal conditions of adaptive remodelling imposed by exercise are complex and not entirely understood, even in humans. It has been proposed that mechanical and metabolic stimuli responsible for exercise-induced angiogenesis exert their effects by stimulating the production of VEGF, considered to be a central pro-angiogenic factor in the regulation of physiological angiogenesis (Egginton, 2009;

Hoier & Hellsten, 2014). In the present study, we report that exercise-induced contractile activity in adult zebrafish caused changes in the expression of the VEGF canonical pathway and of factors that participate in its regulation including members of the hypoxia-inducible factor family (*hif1an*, *hif3a*), nitric oxide synthases (*nos1* and *nos2*), *ppard*, known to increase VEGF production and skeletal muscle angiogenesis (Gaudel *et al*, 2008), and *esrra*, an important mediator of hypoxia-induced PGC-1α transcriptional regulation of VEGF (Arany *et al*, 2008). Therefore, these results suggest that exercise in adult zebrafish may have induced a transcriptional angiogenic program, at least in part, by activating VEGF and its signaling in fast muscle. In support of this hypothesis, swim training in larval zebrafish was recently reported to increase the expression of the HIF and VEGF pathways (Kopp *et al*, 2011). To the best of our knowledge, we provide the first evidence that exercise training in zebrafish activates a complex transcriptional program in fast muscle involving multiple signaling pathways (e.g. VEGF, HIF, TGFβ, Ephrin-B, PDGF, angiopoietin) known to participate in the induction and regulation of angiogenesis, resulting in an important increase in vascularization of this tissue.

We hypothesize that, as in mammals (Prior *et al*, 2004), the increase in capillarity as a result of exercise training may enhance the exchange of respiratory gasses, substrates and metabolites between the blood and fast muscle. Consequently, by increasing the oxygen exchange capacity and the ensuing oxidative capacity, exercise may induce a more aerobic phenotype in fast muscle in zebrafish, in agreement with previous studies that showed that swim training increased the aerobic capacity of the fast muscle by increasing the expression of respiratory genes in adult zebrafish (McClelland *et al*, 2006; LeMoine *et al*, 2010a) and in developing zebrafish, as shown by the increased expression of erythropoietin and myoglobin (van der Meulen *et al*, 2006). Support for an increased aerobic phenotype of fast muscle in exercised zebrafish is derived from the observed increased expression of a large set of genes that participate in oxidative metabolism in mitochondria (i.e. TCA cycle and oxidative phosphorylation) and of the oxygen transport protein myoglobin. It is interesting to point out that the relationship between capillary and fiber density (C/F ratio), shown here to increase in adult zebrafish in response to exercise as in mammals (Prior *et al*, 2004), is related to mitochondrial volume (Mathieu-Costello *et al*, 1996) suggesting that swimming-induced exercise could have improved mitochondrial function and number. Surprisingly, the theoretical maximum diffusion distance from the capillaries to the center of the fiber increased in fast muscle of exercised zebrafish. Although this finding could initially suggest a reduction in muscle oxidative capacity, it should be only seen as a consequence of fiber hypertrophy. Despite the fact that the expression of mitochondrial genes was unaltered by the four-week long training period, the exercise-induced increase in capillarization of fast fibers relative to their area and perimeter (i.e. CCA and CCP indexes) provides further support for the hypothesis of increased mitochondrial oxidative capacity of fast muscle fibers in adult zebrafish subjected to aerobic exercise training.

To summarize, in the present study we have shown that exercise-induced contractile activity in adult zebrafish promotes a coordinated adaptive response in fast muscle that leads to increased muscle mass by hypertrophy (and possibly also cell proliferation) and increased vascularization by angiogenesis. We hypothesize that these adaptations in fast muscle are the result of extensive transcriptional changes induced by exercise. Analysis of the transcriptional

networks that are activated in response to exercise in the adult zebrafish fast muscle allowed us to identify signaling pathways and transcriptional regulators that play an important role in the regulation of skeletal muscle mass, myogenesis and angiogenesis by exercise. The present study is the first to describe coordinated molecular programs regulating muscle mass and vascularization induced by exercise in any species other than humans and supports the notion that these programs may regulate “generic” features of exercise adaptation in the vertebrate skeletal muscle. The development of these adaptive responses to exercise in the zebrafish fast muscle, together with an important metabolic remodelling of this tissue, strongly suggest that exercise training may have caused the acquisition of a more aerobic phenotype in fast muscle in zebrafish. It will be interesting to determine in future studies if these changes result in improved aerobic work capacity. In summary, exercise-induced activity resulted in the transcriptional activation of a series of complex networks of extracellular and intracellular signaling molecules and pathways involved in the regulation of muscle mass, myogenesis and angiogenesis in adult zebrafish, some of which had not previously been associated with exercise-induced contractile activity. The results from this study demonstrate the utility of the adult zebrafish as an excellent exercise model for advancing our knowledge on the basic mechanisms underlining the regulation of skeletal muscle mass.

Chapter 2

In the present study we have investigated the regulation of the expression of known mammalian myokines in fast skeletal muscle of adult zebrafish subjected to four weeks of swimming-induced exercise training. One of the most relevant findings is the increased expression of *il6*, known as the prototype myokine in mammals, by swimming. These results clearly show that exercise-induced contractile activity in zebrafish enhances the mRNA expression of *il6* in skeletal muscle as it occurs in mammals (Jonsdottir *et al*, 2000; Keller *et al*, 2001; Febbraio *et al*, 2003; Hiscock *et al*, 2004). Functionally, IL-6 is known to be involved in myogenesis and muscle growth in mammals through the regulation of satellite cell proliferation and myonuclear accretion (Serrano *et al*, 2008). Furthermore, it has been suggested that changes in circulating or expression levels of IL-6 in muscle are related with training adaptation in humans (Fischer, 2006). In the present study we show that the muscle hypertrophic phenotype observed after four weeks of swimming-induced exercise training was accompanied by an increase in the expression of the satellite cell and proliferation markers Pax7 and phospho-H3 (Chapter 1) suggesting the possibility that Il-6 produced by the contracting fast muscle may have been involved in this response. Although we do not have any evidence for the production and secretion of the Il-6 protein nor for the biological activity of Il-6 in zebrafish fast muscle, further support for a possible role for Il-6 in the adaptive response of zebrafish fast muscle to swimming may be derived from several other observations. First, the presence of MEF2 binding sites in the zebrafish *il6* promoter, together with the increased expression of MEF2 mRNA and protein levels in fast muscle from exercised zebrafish (Chapter 1), support the notion that the increase in *il6* mRNA levels was likely associated with a swimming-induced myogenic program in fast muscle. Second, the increase in *il6* mRNA expression levels was associated with an increase in expression and activity levels of AMPK in fast muscle of exercised zebrafish (Chapter 1) that is consistent with the known mediation by AMPK of several actions of IL-6 in the mammalian skeletal muscle, particularly its potentiation of fat oxidation (Al-Khalili *et al*, 2006; Kelly *et al*, 2009). In support of the possibility that exercise-induced Il-6 production may have resulted in the activation of AMPK and of its metabolic targets, our transcriptomic analysis of the fast muscle of exercised zebrafish evidenced the activation of catabolic pathways such as fatty acid β-oxidation and glycolysis (Chapter 1). Third, although IL-6 has been shown to increase phosphorylation of p38 MAPK in the mammalian muscle (Baeza-Raja & Muñoz-Cánoves, 2004a), our study shows that the increase in *il6* mRNA levels after 1 week of swimming training is accompanied by a decrease in p38 MAPK activity. These results could lead to the suggestion that swimming may not potentiate skeletal muscle differentiation (i.e. a process dependent on p38MAPK) over muscle cell proliferation, supported in our study by molecular data and the increase in phosphorylated-H3 levels (Chapter 1). Interestingly, IL-6 mRNA and protein expression levels are increased in glycogen-depleted muscle cells after exercise in mammals (Keller *et al*, 2001; Steensberg *et al*, 2001; Febbraio *et al*, 2003). If such, the increased expression of zebrafish *il6* during the initial exercise period (i.e. after one week of training), could be indicative of an adaptative mechanism to exercise in zebrafish as reported in untrained (sedentary) or trained

subjects after an acute intensity bout of exercise (Fischer, 2006; Gokhale *et al*, 2007; Robson-Ansley *et al*, 2007).

It is interesting to highlight the early (after one week of training) increase in the mRNA expression levels of *mstnb* and *igf1*, both known negative and positive regulators of muscle mass, respectively, together with *decorin*, found to modulate the activity of members of TGFβ family including *myostatin* (Yamaguchi *et al*, 1990; Miura *et al*, 2006), in fast muscle of exercised zebrafish. Decorin can induce muscle hypertrophy by mechanisms other than myostatin inhibition since it has also been shown to bind IGF-1R with high affinity in endothelial cells (Schönherr *et al*, 2005) and to increase Akt phosphorylation and cell differentiation in C2C12 myoblasts (Suzuki *et al*, 2013). Although the up-regulation of the expression of *mstnb* and *igf1* observed in this study confirms the results of our transcriptomic analysis that showed a significant induction of *myostatin* signaling, *follistatin*, a myokine known to promote muscle hypertrophy in mammals by binding myostatin, and IGF signaling after four weeks of swimming-induced exercise (Chapter 1), temporal differences in *mstnb* expression between the two experiments may be attributed to differences in swimming-induction apparatus, source of fish, etc. Similar temporal differences in the stimulatory effects of swimming-induced exercise were observed with other muscle-derived growth factors such as *tgfb1,* increasing it mRNA levels (although not significantly) in the present study only after 1 week of swimming and significantly after four weeks of swimming (Chapter 1). Another myokine found to be differentially expressed after one week of swimming-induced exercise is *sparc*, an extracellular matrix component. The function of SPARC in the working muscle has been poorly investigated but it has been found to be expressed in human satellite cells during proliferation and differentiation *in vitro* and in the regenerating muscle (Jørgensen *et al*, 2009). Its significant increase in mRNA expression levels in the present study could suggest a role in muscle remodeling during the first week of swimming induction and its possible contribution to the regulation of muscle growth together with the other myokines and growth factors identified here (e.g. *myostatin*, *decorin* and *igf1*). Furthermore, the observed down-regulation of the expression of *bdnf* in this study is intriguing since *bdnf* has been shown to be expressed in muscle satellite cells and in exercising muscles (Mousavi & Jasmin, 2006; Matthews *et al*, 2009). From studies using muscle BDNF-deficient transgenic mice, it has been suggested that BDNF positively regulates satellite cell differentiation possibly through its regulation of Pax7 (Clow & Jasmin, 2010). It is interesting that the decrease in *bdnf* mRNA expression levels only after week 2 of swimming-induced exercise takes place in the context of increased Pax7 protein levels which suggests that Bdnf could indeed be involved in the regulation of muscle mass during the latter part of the exercise training period. Finally, *apelin*, a relatively novel myokine (Besse-Patin *et al*, 2014), and its two receptors (*aplnra* and *aplnrb*) appeared strongly up-regulated in response to exercise in this study, with distinct expression patterns between ligand and receptors. Under non-exercise conditions, apelin has been shown to impact glucose homeostasis by increasing glucose utilization in skeletal muscle and adipose tissue, reducing glycemia in normal and insulin resistant mice (Dray *et al*, 2008) and, consequently, is viewed as a promising factor in the treatment of insulin resistant type 2 diabetes and obesity. On the other hand, it is known that acute and long-term exercise increases insulin sensitivity and glucose uptake in muscle (Goodyear & Kahn, 1998). Therefore, from the recent evidence in mammals showing that exercise-induced expression of apelin in skeletal muscle resulted in

improved insulin sensitivity in insulin resistant or obese conditions (Besse-Patin *et al*, 2014; Yang *et al*, 2015), the highly significant induction of apelin and receptors in our study could suggest that apelinergic signaling in zebrafish could play a role in regulating glucose homeostasis under exercise conditions.

The molecular mechanism by which each the above-mentioned myokines exert their effects in mammals is still under investigation. However, several myokines such as IL-6, FNDC5, myostatin, decorin, apelin, BDNF or SPARC have been found to modulate AMPK activity and influence metabolism in muscle cells (Carey *et al*, 2006; Dray *et al*, 2008; Kelly *et al*, 2009; Glund *et al*, 2009; Matthews *et al*, 2009; Song *et al*, 2010; Chen *et al*, 2010; Das *et al*, 2012; Attané *et al*, 2012; Goyal *et al*, 2014; Huh *et al*, 2014; Lee *et al*, 2015a, 2015b). In Chapter 1 we have described transcriptomic changes involving metabolic genes relevant in lipid oxidation and glycolisis (Palstra *et al*, 2014) as well as a marked increase of AMPK activity at four weeks of training. Moreover, these findings suggest that muscle growth regulation by myostatin, decorin and IGF-1 in response to exercise may involve complex signaling among different growth factors and we believe that future studies on the temporal regulation at the protein level as well as under cultured conditions would clarify the interaction of these factors in adult zebrafish. Therefore, it would be of interest in future studies to explore the molecular mechanisms of the identified myokines in zebrafish and their impact in muscle cell metabolism in our exercise model.

Does FNDC5/irisin exist in fish?

In the present work, we have analyzed for the first time in a teleost species the expression of *fndc5b*, the mammalian ortholog of FNDC5 (coding for irisin), and, more importantly, the regulation of its expression under exercise conditions. Plasma increases of the irisin protein or of its expression in muscle tissue in response to exercise has been reported by several authors (Boström *et al*, 2012; Huh *et al*, 2012; Norheim *et al*, 2014; Daskalopoulou *et al*, 2014; Lee *et al*, 2014; Nygaard *et al*, 2015; Tsuchiya *et al*, 2015) but these effects have not been corroborated by other studies (Pekkala *et al*, 2013; Hecksteden *et al*, 2013; Aydin *et al*, 2014). The discrepancy in the results in detecting irisin/FNDC5 brings into question if is a matter of the different exercise protocols used and/or the specificity of the detection systems used (Albrecht *et al*, 2015). Additionally, it has been demonstrated that human FNDC5 (but not the other mammalian forms) has a mutation in the start codon from ATG to AUA which results in a low translation rate of the protein that, therefore, places into question the published results on the role or even the existence of irisin in humans (Raschke *et al*, 2013). In the present study, we have tested the immunoreactivity of a mammalian antibody in non-exercised zebrafish fast muscle but, despite the high homology between the antibody recognition sequence and the corresponding zebrafish sequence, nonspecific product was obtained. Specific antibodies against zebrafish *fndc5* should be generated to be able to conduct studies on the presence and regulation of irisin in this species.

The role of FNDC5 in mammals was first described by Bostrom *et al* (2012), suggesting that muscle exercise-induced contraction stimulated the production of irisin to promote browning of the white adipose tissue and thermogenesis through a mechanism involving PGC1α. However, teleost fish (including zebrafish) are ectotherms and brown adipose tissue,

responsible for thermogenesis and energy expenditure in mammals, has not been identified in this group of vertebrates (Seth *et al*, 2013). Therefore, instead of a role in browning, Fndc5 would be more likely to share the metabolic effects of FNDC5 by inducing AMPK activity and glucose uptake locally in muscle cells described in mammals (Huh *et al*, 2014; Lee *et al*, 2015a). Given the induction of *fndc5b*, *pgc1α* and AMPK activity in the zebrafish fast muscle by swimming-induced exercise in adult zebrafish, the possible metabolic role of Fndc5 in the zebrafish fast muscle warrants further investigation.

Under resting conditions, the strong expression of *fndc5b* over *fndc5a* in fast muscle could suggest a specific role for *fndc5b* in the glycolitic (fast) fibers. In this regard, functional characterization of both paralogs, *fndc5b* and *fndc5a*, should be addressed in order to elucidate the subfunctionalization or neofunctionalization resulting from the process of gene duplication as could be expected from the teleost genome (Howe *et al*, 2013). In comparison with mammalian studies where FNDC5 was highly expressed in heart and brain (and to a lower extent in skeletal muscle and testis) at the mRNA level (Teufel *et al*, 2002), both zebrafish *fndc5a* and *fndc5b* have a wide expression pattern amongst the tissues examined, being strongly expressed in eyes and brain. These expression patterns suggest a possible specific function for zebrafish *fndc5* paralogs in the respective tissues during adulthood. Our study provides evidence for the existence of FNDC5 in zebrafish but the role of *fndc5b* as well as *fndc5a* in zebrafish either in resting or exercise conditions is still unknown.

Taken together, the identification of an array of myokines in fast muscle of zebrafish and the regulation of their mRNA expression levels during exercise further demonstrates the potential of adult zebrafish as a useful exercise model to better characterize the function of these myokines in response to exercise. However, certain limitations such as the functional divergence of some of the myokine genes, the difficulty to obtain sufficient plasma and the lack of zebrafish immunoassays in order to evaluate the circulating or tissue levels of myokines, hamper the full establishment of adult zebrafish as an operational experimental model in exercise physiology.

Chapter 3

Exercise enhances the immune response in fast muscles at the transcriptional level

The work described in the present chapter addresses the question of the potential immunomodulatory effects of exercise in adult zebrafish. To our knowledge, the molecular regulation of skeletal muscle function in response to LPS under exercise conditions has not yet been studied in fish. Since skeletal muscle is the principle tissue involved in the adaptive response to exercise training (Egan & Zierath, 2013; Palstra *et al*, 2013, 2014) and muscle cells are able to express immune-related molecules in fish (Kaitzedou et al., 2012; Palstra et al., 2013; Magnoni et al., 2015), we investigated the transcriptomic response of exercised and non-exercised fast muscle to LPS, a component of the external layer of Gram-negative bacteria commonly used to mimic the effects of a bacterial infection.

A strong transcriptomic response has been observed in zebrafish fast muscle under exercise conditions (Chapter 1) and also after LPS administration in exercised fish (this section). Indeed, as shown by the Gene Ontology and canonical pathway analyses, fast muscle of LPS-stimulated exercised fish showed a higher representation of genes related with the immune function than non-exercised fish. For instance, the increased expression of factors of the complement system as well as genes related with leukocyte or lymphocyte activation suggest an enhanced activation of the immune response in exercised muscles. In fish, endotoxins such as LPS stimulate innate and adaptive immunity by stimulating macrophage inflammatory responses (i.e. cytokine production, phagocytosis, respiratory burst), by activating the complement system and by stimulating B-lymphocyte and T-lymphocyte proliferation and B-cell antibody production (Swain *et al*, 2008). However, we are aware that many of the immune-related genes identified in the zebrafish skeletal muscle in the present study are often expressed in immune cells or in the liver (i.e. in the case of complement system factors). This raises the question if the immune-related genes identified in this study were actually expressed by skeletal muscle cells or by infiltrated immune cells in muscle tissue. As it has been described in mammals, immune resident cells in skeletal muscle, especially macrophages, increase in number and are recruited to the site of injury during skeletal muscle regeneration (Arnold *et al*, 2007). Therefore, a possible contribution of immune cells in muscle function cannot be discarded in zebrafish, although this mechanism has been reported only in damaged muscle (e.g. muscular dystrophies) (Pillon *et al*, 2013) and in intramuscular vaccination (Castro *et al*, 2014). Moreover, several reports have found that exercise affects the function of the immune system in mammals by increasing circulating T regulatory cells or by reducing pro-inflammatory monocytes in the blood (Gleeson *et al*, 2011) that could provide support, at least in part, for the increased expression of immune-related genes in fast muscle from exercised in comparison to non-exercised zebrafish. To resolve this issue, future studies should perform immunohistochemical or cell sorting analyses of the zebrafish fast muscle tissue and/or muscle cells with immune cell markers to investigate the presence of immune cells in muscle after an infection.

More importantly, it is known that mammalian muscle cells as well as muscle tissue have the capability to express several immune molecules, including antigen-presenting-molecules, toll-like receptors, cytokines and chemokines when stimulated with inflammatory agents (including LPS) (Frost *et al*, 2002; Wiendl *et al*, 2005; Olesen *et al*, 2015), that are suggested to contribute to a systemic immune response (Nielsen & Pedersen, 2008; Chatterjee *et al*, 2016). Exercise has been postulated to exert anti-inflammatory effects for instance by reducing the pro-inflammatory cytokine levels after an acute immune challenge and ameliorating the status of disorders associated with elevated inflammatory molecules (Petersen & Pedersen, 2005). However, the immune response of skeletal muscle in exercised fish has been poorly investigated in the past. Our study represents, to the best of our knowledge, the first attempt at investigating the relationship between exercise and immune response in skeletal muscle in fish. In the present study, the lower mRNA expression levels of the pro-inflammatory cytokines *il6* and *tnfα* in exercised zebrafish fast muscle, when compared to non-exercised zebrafish, would suggest an effect of previous exercise training in reducing the levels of pro-inflammatory cytokine expression, reinforcing the role of exercise in promoting anti-inflammatory effects in zebrafish fast muscle. Nevertheless, the function of the immune-related genes that were differentially expressed in either exercised or non-exercised zebrafish fast muscle has to be further investigated in order to decipher the role of muscle tissue during infection.

A common phenomenon under systemic infection, such as induced by LPS administration, is an enhanced catabolic response in muscle that has been described as a combination of decreased protein synthesis, increased protein degradation and possibly apoptosis (Biolo *et al*, 1997; Callahan & Supinski, 2009; Gordon *et al*, 2013). In this study, altered expression of genes related with protein synthesis (e.g. eukaryotic translation factors) as well as different factors involved in protein degradation has been observed, consistent with previous studies on fish muscle after *in vivo* LPS administration (Johansen *et al*, 2006; Kaitetzidou *et al*, 2012; Magnoni *et al*, 2015). In non-exercised LPS-stimulated muscle, serine proteases, known to participate in muscle atrophy (Morris *et al*, 2005), were down-regulated; whereas in exercised-LPS muscle, factors from several degradation pathways were differentially expressed. These include genes related to the ubiquitin-proteasome system and lysosomal-mediated degradation, two molecular mechanisms involved in muscle atrophy (Bonaldo & Sandri, 2013). In mammals, activation of both the ubiquitin-proteasome system and lysosomal autophagy has been reported after LPS-induced muscle catabolism and were found to signal through the LPS receptor, TLR4 (Dehoux *et al*, 2003; Doyle *et al*, 2011). Although the receptor for LPS in fish is unknown (Pietretti & Wiegertjes, 2014), LPS increased the expression of several TLRs and induced muscle atrophy in trout cultured myotubes, which supports the notion that LPS may act on muscle tissue to induce catabolism in fish (Aedo *et al*, 2015). For instance, an important factor known to promote muscle atrophy in mammals in response to catabolic stimuli (such as fasting) is the deubiquitinase USP19, an enzyme that decreases protein synthesis, especially of muscle cell structural proteins, and increases protein degradation (Combaret *et al*, 2005). In the present study, the mRNA expression of *usp19* was up-regulated in response to LPS in fast skeletal muscle of exercised as well as non-exercised zebrafish, suggesting a role for this gene in regulating protein turnover in the zebrafish fast skeletal muscle. Taken together, although fast muscle from exercised and non-exercised zebrafish showed differential expression of

genes involved in muscle catabolism in response to LPS, fast muscle from exercised zebrafish showed a higher representation of distinct degradation processes, possibly leading to an enhanced catabolism or decreased protein synthesis, than non-exercised fast muscles. Indeed, in the exercised zebrafish fast muscle, the up-regulation of the expression levels of *tsc1,* that inactivates the mTOR complex (Laplante & Sabatini, 2012), coupled with the down-regulation of *mtor* expression and the slight increase in *rps6ka6* expression*,* suggests that the mTOR pathway and, thus, the regulation of the protein synthesis may have been attenuated in response to LPS. This observation is in agreement with a recent study showing that sepsis suppresses the rate of protein synthesis both in control and electrically-induced contracting muscles by inhibiting the mTOR anabolic pathway (Steiner & Lang, 2015). In the present study, the observed regulation in the expression of genes involved in protein metabolism, especially in muscle from exercised fish, suggests that this may be a likely necessary mechanism for zebrafish fast muscle to respond to an acute LPS challenge and we cannot discard the additional implication of muscle atrophy in this process as it has been shown in other fish species (Johansen *et al*, 2006; Kaitetzidou *et al*, 2012; Magnoni *et al*, 2015). However, since many of the degradation-related genes were down-regulated in both groups it is possible to suggest that muscle catabolism may have decreased after 72 hours of the LPS administration and that an analysis earlier than 72 hours could show greater differences between exercised and non-exercised fast muscles. Previous studies from our laboratory in other fish species reported time-related differential effects of LPS on skeletal muscle between 24 and 72h post-injection (Kaitetzidou *et al*, 2012; Magnoni *et al*, 2015). Therefore, the analysis of earlier immune stimulation times in fast zebrafish muscle should be considered in future studies in order to better clarify the temporal regulation of cellular processes during infection. We cannot discard the possibility that muscle proteins may be degraded in order to be distributed to other organs for fuel as it has been shown in mammalian models of sepsis (Rosenblatt *et al*, 1983; Vary & Kimball, 1992; Karinch *et al*, 2001).

On the other hand, the down-regulation by LPS of several cytoskeletal and ECM elements in fast muscle of exercised fish contrasts with the up-regulation of several contractile elements (*pvalb*, *ryr1*, *ttn*, *tnni2*), muscle growth and developmental factors (*acvr2b*,*fst*, *igf1r*, *myod1*), the majority of them previously found in response to exercise alone (Chapter 1). This finding is in contrast with the reported skeletal muscle response to sepsis in mammalian models, where muscle catabolism, partially induced by pro-inflammatory cytokines, is associated with suppression of muscle anabolic factors such as IGF-1 (Saini *et al*, 2006; Nystrom *et al*, 2009), thus, emphasizing the coordination of catabolic and anabolic processes. In support of the notion that exercise may regulate LPS-induced muscle remodeling, the reduced mRNA levels of *il6* and *tnfα* in exercised muscles in response to LPS might contribute to an improved regulation of muscle function after an LPS challenge, as described in mammals (Zoico & Roubenoff, 2002). In a previous study on trout, our group showed that LPS administration also increased the expression of genes related with muscle development in fast muscle after 72h, time at which it was suggested that LPS administration induced muscle remodeling (Magnoni *et al*, 2015). In contrast, in a fish model of chronic stimulation with LPS, muscle growth was decreased without significant changes in the mRNA expression levels of muscle developmental genes (e.g. *myoD*) (Johansen *et al*, 2006). When considering the response to LPS in fast muscle from exercised fish, it is tempting to speculate that, due to an elevated proportion of

contractile and structural elements resulting from the known exercise-induced fiber hypertrophy (Palstra et al. 2014), zebrafish may have to tightly balance their cellular demands at expenses of exercise-induced muscle growth in front of an immune challenge which results in the regulation of degradation processes. Moreover, decreased cell death in fast muscle from exercised zebrafish, as suggested by the down-regulation of *tp53*, could reinforce a process of muscle remodeling in previously exercised muscles, supported by the growth- and developmental-related genes expressed. Conversely, in non-exercised muscles, the transcriptional response to LPS was not as strong as in exercised fast muscle, suggesting that it may have not been necessary to make so many structural adjustments in the cell to face LPS administration when compared to exercised muscles.

Finally, it is worth highlighting the highly significant representation of the canonical pathway corresponding to mitochondrial dysfunction and the increased mRNA expression levels of mitochondrial genes (*cs*, *cpt1b*, *cox5a*, *sdhc* and NADH dehydrogenases) in exercised fast muscle, suggesting that mitochondrial function could be especially affected by the LPS challenge. It is well known in mammals that mitochondrial function is impaired by LPS or live bacteria by the increased production of reactive oxygen species that lead to energy depletion and cell death (Crouser *et al*, 2002; Cherry & Piantadosi, 2015). Although little is known about the effects of exercise on mitochondrial function during sepsis, it is possible that the oxidative metabolism acquired during the training period is maintained during sepsis, preventing mitochondrial dysfunction as it has been proposed in rats (Werlang-Coelho *et al*).

This study only evaluated the transcriptomic response of fast muscle and, therefore, further studies would be necessary in order to evaluate the cellular changes of fast muscle under the effects of LPS (with or without exercise). For instance, this could be accomplished by analyzing protein synthesis (e.g. mTOR) pathways at the protein level or by performing morphometric measurements to evaluate changes in fiber growth or fiber atrophy. Furthermore, we have to stress the importance in comparing zebrafish slow and fast muscle in future studies as it has been performed in other fish species (Kaitetzidou *et al*, 2012; Magnoni *et al*, 2015). These two muscle types have distinct properties and transcriptomic responses during swimming (Palstra *et al*, 2013) and could respond differently in front of an immune challenge under exercise conditions. Finally, although the use of purified endotoxins might not be comparable to a real infection process, LPS injection serves as a valid first approximation that has been used extensively in experimental studies.

Exercise does not affect survival against an acute *in vivo* immune challenge

In contrast to the observed differences on the transcriptomic response of fast muscle to LPS administration between exercised and non-exercised zebrafish, swimming-induced exercise did not improve survival upon *in vivo* infection with *Pseudomonas aeruginosa*, an opportunistic pathogen. Therefore, we question whether the potential immunomodulatory effects of exercise on skeletal muscle may be strong enough to be able to resolve an acute and –ultimately- lethal infection as it has been performed in this study. In view of the lack of effects of swimming on survival in front of a lethal pathogen load, we hypothesize that it is possible that the immunomodulatory effects of exercise may be better detected under low chronic models of inflammation, such as performing sublethal or chronic pathogen infections

(Johansen *et al*, 2006; van der Sar *et al*, 2009) or by using zebrafish models of obesity that have increased levels of inflammatory molecules(Morales Fénero *et al*, 2016). Therefore, zebrafish could be used as a model to study the impact of exercise in low chronic states of inflammation, characteristic of diseases such as T2D or rheumatoid arthritis in mammals (Gleeson *et al*, 2011; Booth *et al*, 2012). In support of the contribution of contracting muscles during infection, a recent study performed using *Drosophila* mutants for muscle structural genes has shown that the muscle tissue is essential for survival in front of bacterial agents and that this response may be conserved in zebrafish (Chatterjee *et al*, 2016). Thus, a new experimental approach has to be used to be able to answer this question in adult zebrafish, for instance by administering repeated injections of an attenuated pathogen (the choice of an appropriate pathogen would be another factor to consider) during exercise training. In our study, it is also possible that the lack of differences in survival to the *in vivo* pathogenic challenge between exercised and non-exercised fish are due to the high virulence of the pathogen used since LPS from *P. aeruginosa* is more lethal than *E. Coli* LPS in zebrafish larvae (Novoa *et al*, 2009). Another potentially interesting field of research that derives from the research outlined in this Chapter is regarding the potential effects of exercise on the absorption of vaccines or other currently developed delivery techniques such as nanoliposomes (Ruyra *et al*, 2014; Castro *et al*, 2014).

To summarize this section, we have shown that in fast muscle from exercised zebrafish, the transcriptomic response is stronger than in non-exercised fish in terms of the number and function of the differentially expressed genes after an immune challenge with LPS. The observed transcriptomic response suggests that in exercised muscles the regulation of the immune response is enhanced together with maintenance of the muscle function. Moreover, we tested the effects of exercise on the survival of adult zebrafish to an *in vivo* acute challenge with a bacterial opportunistic pathogen. However, in view of the negative results of exercise training on survival, we need to expand our studies in order to further our comprehension on the active role of skeletal muscle in regulating the immune response and on the coordination between muscle and immune cells.

Chapter 4

In mammals, it has been documented that regular exercise provides a set of physiological adaptations suggested to be cardioprotectiveand beneficial forthe prevention and treatment of cardiovascular diseases. Cardiac hypertrophy, cardiomyocyte renewal, vascular remodelling, improved calcium sensitivity and alterations in metabolism have been described as the main physiological adaptations of the cardiac tissue in response to exercise. The mechanisms underlying the stimulatory effects of exercise on cardiac hypertrophy are relatively well understood in mammals (Boström *et al*, 2010), however, although the zebrafish is regarded as an important experimental model in cardiovascular research, the response of the adult zebrafish heart to exercise has not been evaluated to date. Here, we have shown for the first time in adult zebrafish that exercise training causes cardiomyocyte cell proliferation accompanied by a modest induction of cardiac hypertrophy. In view of our previous results on exercise-induced skeletal muscle hypertrophy (Chapter 1), we hypothesize that under our exercise training condition during four weeks, exercise stimulated adult zebrafish cardiomyocyte hyperplasia and cardiac hypertrophy. From the best of our knowledge, this is the first report showing that swimming-induced exercise increased cardiomyocyte proliferation in the adult zebrafish heart. Similarly, exercise has been reported to enhance cardiomyocyte proliferation from existing (mitotic) cardiomyocytes in mammals (Boström *et al*, 2010) and also to increase the number of activated cardiac progenitor cells suggested to be the source of the newly formed cardiomyocytes (Waring *et al*, 2014).

In zebrafish, the cardiomyogenic potential of the heart to regenerate from a cardiac injury is well known, having been shown to occur through a process of de-differentiation and proliferation of pre-existing cardiomyocytes, with only one study reporting contribution from undifferentiated cells (Kikuchi *et al*, 2011a; González-Rosa *et al*, 2012; Foglia & Poss, 2016). Since exercise and injury are distinct stimuli, we cannot discard any possibility about the lineage or cellular source of the newly raised zebrafish cardiomyocytes by swimming-induced exercise in our study. There are very few published reports regarding cardiac adaptations to exercise in fish and most of them are centered in fish species of commercial interest. Studies on the effects of exercise on the heart in fish have reported a swimming-induced stimulation of cell proliferation by estimating *pcna* mRNA expression or the percentage of PCNA+/DAPI+ cells by immunohistochemistry, although identification of the specific cardiac cell type under proliferation was not attempted (van der Meulen *et al*, 2006; Jean *et al*, 2012; Castro *et al*, 2013a). In a similar manner in mammals, exercise induces proliferation of cardiomyocytes in mice (Boström *et al*, 2010), an observation that is related to the ability of the adult mammalian heart to exhibit (low) mitotic activity throughout its lifespan (Bergmann *et al*, 2009; Bersell *et al*, 2009; Kajstura *et al*, 2010). Therefore, exercise could be a conserved mechanism across vertebrates to stimulate proliferation of cardiomyocytes.

Besides exercise, several strategies have been designed to induce adult mammalian cardiomyocyte proliferation by targeting regulators of the cell cycle in order to find a therapeutic mechanism for heart regeneration (Foglia & Poss, 2016). In this regard, p38

mitogen-activated protein kinase (p38-MAPK) induces cell cycle exit and differentiation in many cell types and its activity is inversely correlated with cardiac growth during development. Interestingly, it has been demonstrated that p38α is a negative regulator of mammalian adult cardiomyocyte proliferation (Engel *et al*, 2005) and a similar mechanism in zebrafish cardiomyocytes has been found, in which active p38α can block cardiomyocyte proliferation and heart regeneration (Jopling *et al*, 2012b). Interestingly, in our study, the increase in cardiomyocyte proliferation induced by exercise is associated with a decrease in the activity of p38 in zebrafish ventricles. Elucidating the cardiac role of specific p38 isoforms could be a matter of study in exercise physiology since in mammals it has been recently shown that besides p38α, the δ and γ isoforms of p38 play an important role in the postnatal control of cardiac growth (González-Terán *et al*, 2016). In the adult zebrafish heart, chronic hypoxia has shown to be mitogenic (Marques *et al*, 2008) specially after a cardiac injury, during which the hypoxic response that occurs in the zebrafish ventricle positively regulates heart regeneration through a mechanism involving HIF1α (Jopling *et al*, 2012a). In the present study, the decreased mRNA expression levels of *hif1aa* and the lack of change in *hif1ab* expression in response to swimming-induced exercise do not support the notion of a HIF1α-driven mechanism in the induction of cardiomyocyte proliferation in the adult exercised heart, at least after four weeks of exercise training. Interestingly, studies in human skeletal muscle have shown that the transcriptional expression of HIF1α was increased after an acute exercise bout whereas its expression was unaltered in trained individuals (after a three month training period), suggesting a role for HIF1α in the early exercise adaptation (Ookawara *et al*, 2002; Lundby *et al*, 2006). This response differed in zebrafish, where larvae trained for six days had an increased *hif1* mRNA expression over the training period but HIF1α protein levels decreased (Kopp *et al*, 2011). Therefore, according to these studies and given the lack of effects of the four-week swim training period on *hif1a* mRNA expression levels, it will be interesting in future studies to evaluate HIF1α signaling in the exercised zebrafish heart at an earlier time, especially at the protein level since the activity of HIF1α is post-transcriptionally and post-translationally regulated (Kuschel *et al*, 2012). On the other hand, Nrg1 has also been related to cardiomyocyte proliferation in zebrafish and mammals and it would be relevant to further determine if the slight induction of ventricular *nrg1* mRNA expression levels observed in response to the applied swimming-exercise training protocol could be a relevant mechanism involved in exercise adaptation in the zebrafish ventricle. Remarkably, Cai *et al* (2016) have recently demonstrated that endogenous NRG1 produced during exercise promoted cardiac repair and regeneration in rats (Cai *et al*, 2016), a finding that highlights exercise as a potential tool to stimulate cardiac regenerative mechanisms in a physiological manner by the production of endogenous factors. In addition, Nrg1 partially rescues cardiac denervation inherent after a cardiac insult in both zebrafish and mammals (Mahmoud *et al*, 2015) and this observation, supported by our data on exercise-induced up-regulation of genes related to axonal growth (*cspg5b*, *ephn3b*, *sema3aa*) in the zebrafish heart, warrants future research to evaluate the possible effects of exercise on the plasticity of the neurons innervating the heart.

Next, we have shown a slight increase of the compact layer and the total myocardium after four weeks of swimming-induced exercise that would resemble a process of eccentric hypertrophy as occurs during long-term training in mammals, during which a uniform growth of the heart is achieved by a proportional increase of the heart chambers and myocardial walls

(Maillet *et al*, 2013). In teleost fish there are equivocal findings on cardiac growth responses to swimming-induced exercise (Davison, 1997) and a majority of the studies on cardiac exercise physiology have been performed on species that are distantly related from zebrafish. Cardiac hypertrophy induced by exercise in mammals has been proven to be dependent on the type of training regime (i.e. aerobic or anaerobic exercise) and its duration or intensity are factors that will affect heart growth (Kemi *et al*, 2005; Wang *et al*, 2010). In many reports in humans and mammals, low and moderate intensity training have reported lower induction of ventricular hypertrophy than high intensity training, as measured by magnetic resonance or left ventricle cardiomyocyte cell size measurements (Wisløff *et al*, 2009; Sipola *et al*, 2009). Therefore, it is plausible that the swimming-induced exercise protocol used in our study may not have been of sufficient intensity to induce a significant increase in cardiac hypertrophy, as fish swam at their U_{opt} (Palstra *et al*, 2010). Nevertheless, although the transcriptomic response in the heart was not as strong as in fast muscle, the observed regulation of the expression of genes coding for contractile elements and muscle developmental factors in the heart resembled the transcriptomic profile associated with fast muscle hypertrophy previously described in Chapter 1. Therefore, although further measures of cardiac hypertrophy at the cellular level would be required, our data suggests that a hypertrophic response had taken place in the exercised adult zebrafish ventricle.

Cardiomyocyte cell growth or hypertrophy has been correlated with an increase in protein accumulation resulting from a net increase of protein synthesis over protein breakdown (Hannan *et al*, 2003). The mTOR pathway, which is principally involved in regulating protein synthesis and cell growth (Miyazaki & Esser, 2009; Sciarretta *et al*, 2014), appeared to be differentially regulated in this study as suggested from the transcriptomic data in adult exercised zebrafish hearts and it has been further analyzed at the protein level. From our findings, mTOR protein activity significantly increased in the exercised zebrafish ventricles but no activation of the downstream targets (p70S6K or 4EBP-1) was observed, suggesting that protein synthesis may have not been affected in response to exercise. Several studies have linked mTOR pathway with physiological hypertrophy, which includes exercise-induced hypertrophy. Unlike in pathological animal models of cardiac hypertrophy, the mTOR pathway has been shown to be activated in exercise-hypertrophied hearts (Kemi *et al*, 2008; Maillet *et al*, 2013). Nevertheless, Liao *et al* (2015) found an activation of the mTOR pathway under long-term moderate intensity but not under high intensity exercise in the rat myocardium although both training regimes induced cardiac hypertrophy (Liao *et al*, 2015). The authors suggested that mTOR activation is restricted to physiological hypertrophy since the high-intensity training regime produced cardiac damage. Nevertheless, the unaltered mTOR pathway in zebrafish hearts observed in the present study is not related to cardiac damage as suggested by the non significant expression levels of fibrotic and stress genes in zebrafish ventricles. However, whole heart transcriptomics revealed a significant expression of fibrotic markers such as ECM molecules (periostin and collagens), possibly resulting from contamination from the bulbus arteriosus. It is interesting to highlight the regulation of the expression levels of eukaryotic initiation factor *eif4ebp3l* by swimming-induced exercise in our study, a factor previously characterized under muscle inactivity conditions as a key check point in muscle growth by reducing the activity of TORC1 - an mTOR complex - and ultimately regulating the transcription of *mef2ca* (Yogev *et al*, 2013). Therefore, we suggest an involvement of *eif4ebp3l* under

muscle activity or exercise in adult zebrafish; and thus, the role of this factor in the control of growth of cardiac (muscle) cells should be explored. Other signaling pathways have been described to be relevant in cardiac physiological growth (Maillet *et al*, 2013) and many of them converge onto a nodal mediator: AKT (protein kinase B). Such signaling pathways include IGF-1/PI3K/AKT, NRG1/ErbB4/AKT, downstream targets of AKT such as mTOR or important transcription factors such as C/EBPβ, that have been found to be regulated during exercise (Wilson *et al*, 2015). Specially, a valuable contribution on the molecular mechanisms underlying the effects of exercise in mammals was made by Boström *et al* (2010) by their identification of C/EBPβ, a target of AKT, as a negative regulator of cardiac hypertrophy and CITED4 as an inducer of cardiomyocyte proliferation. In our transcriptomic profiling of the exercised zebrafish heart, *cited4a* was down-regulated and, thus, we suggest that this gene could also play a role in the cardiac response to exercise in zebrafish. However, since many of the pathways involved in cardiac hypertrophy mentioned so far involve AKT, the analysis of its activity would be of relevance in the present study. From the best of our knowledge, no information on adult zebrafish cardiac growth in response to exercise is currently available in the literature since more emphasis has been made at investigating the hypertrophic response in zebrafish models of human disease and development (Sun *et al*, 2009; Bakkers, 2011) rather than in exercise physiology.

Together with cardiomyocyte proliferation and hypertrophy, we were interested in studying the effects of exercise on cardiac function in order to complement the cellular and molecular findings. Echocardiography and other non-invasive methodologies allow the assessment of cardiac function in the developing and adult zebrafish (Sun *et al*, 2008; Hoage *et al*, 2012; González-Rosa *et al*, 2014; Hein *et al*, 2015; Lee *et al*, 2016; Ernens *et al*, 2016). Importantly, this study is the first in analyzing cardiac function in adult zebrafish after swimming-exercise training using echocardiography. Our findings showed no significant differences in ejection fraction (contractile effectiveness) or heart rate, suggesting that an adaptation of cardiac function is not present after four weeks of training in adult zebrafish hearts. In other teleost fish species, exercise training improved cardiac performance by increasing stroke volume or cardiac output (Farrell *et al*, 1991) as it usually occurs in the mammalian heart (Poliner *et al*, 1980; Bar-Shlomo *et al*, 1982; Waring *et al*, 2015; Lavie *et al*, 2015). Therefore, it is plausible that the modest increases of the compact myocardium (or ventricle wall) and heart size in this study were not sufficient to increase contractility and stroke volume and, thus, ejection fraction. It has to be pointed out that these measurements were taken at rest because we could not measure the changes of ejection fraction or heart rate during the training period (i.e. meanwhile adult zebrafish were induced to swim in the swim tunnel) due to technical reasons. Therefore, acute changes of ejection fraction would be appreciated at the time point during the exercise performance. Moreover, the unaltered cardiac function in this study suggests that our training protocol at U_{opt} maintained the levels of oxygen delivery to the working skeletal muscles, possibly because of the compensation elicited by the increased capillarity in fast skeletal muscle shown previously in Chapter 1 (Palstra *et al*, 2014).

The mammalian heart has a high metabolic flexibility and is able to switch from different energy substrates to produce ATP in response to distinct physiological situations (i.e. hypoxia,

stress, etc) or developmental stages (Taegtmeyer, 2002). For instance, the developing mammalian heart, that operates in a hypoxic environment, favors carbohydrates over fatty acids, whereas after birth fatty acids are the predominant source of fuel in the adult heart (Makinde *et al*, 1998). Conversely to mammals, where a great effort has been made in the study of cardiac metabolism in health and especially in heart diseases, little is known about the metabolic needs of the zebrafish heart to date. In our study, exercise regulated metabolic processes intended to supply ATP to the adult zebrafish working heart, such as mitochondrial oxidation, and also regulated the expression of genes involved in lipid and carbohydrate metabolism. It is worth to highlight the up-regulation of *pparab* (PPARα) in our study, a transcriptional activator of fatty acid oxidation and a master gene in the control of heart metabolism (Barger & Kelly, 2000). Its elevated expression is usually related to a preference for fatty acid metabolism under exercise conditions as it has been shown in the exercised mammalian heart (Goodwin & Taegtmeyer, 2000), in contrast to under hypoxic conditions where its expression is decreased and metabolism switches to glucose utilization (Goodwin & Taegtmeyer, 2000; Razeghi *et al*, 2001; Iemitsu *et al*, 2002). Genes participating in the glycolytic pathway were also elevated (i.e. *aldoab*, *gapdhs* or *pkma*). In line with our results, gene expression and activity levels of key glycolytic and lipid oxidative enzymes were increased in trout and salmon exercised hearts (Farrell *et al*, 1990; Castro *et al*, 2013a), although in the latter study the authors suggested a stronger reliance in lipid than in glucose metabolism. Although we cannot determine if there is a preferential metabolic pathway in exercised zebrafish hearts only from transcriptomic data, our findings are similar to what has been reported in exercised fast muscle previously in Chapter 1, suggesting an important role of metabolism (either from carbohydrates or lipids) for the adaptation to exercise in the heart likely by increasing the aerobic or oxidative capacity. It was described by Murry *et al* (1986) that short and controlled periods of ischemia before a severe ischemic insult result in improved resistance and protection to subsequent episodes of ischemic damage, which is known as preconditioning (Murry *et al*, 1986). This is connected to exercise by the fact that from animal and human studies exercise has been shown to induce preconditioning of the myocardium, thus protecting the heart from cardiac insults (Maybaum *et al*, 1996; Domenech *et al*, 2002; Michaelides *et al*, 2003; French *et al*, 2008; Parra *et al*, 2015). However, the exercise preconditioning mechanism of this adaptive phenomena (including increased antioxidant capacity) is still a matter of research in which metabolic regulation could play an important role. Exercise influences metabolism in the heart and at the whole organism level and many cardiovascular diseases are accompanied by changes in cardiac metabolism. Taken together, it is easy to reason that the understanding of cardiac metabolic flexibility either in the diseased (such as in ischemia) or exercised heart would help to design therapeutic strategies for the treatment of heart diseases and zebrafish would appear as a valuable model for the study of heart energy homeostasis during exercise.

To summarize this section, we have described the response to exercise in adult zebrafish hearts and showed for the first time, using our exercise training protocol, that four weeks of training stimulated the proliferation of cardiomyocytes. Moreover, our transcriptomic data unveils a transcriptional program involving factors that are likely crucial in the response to exercise. We have proposed possible mechanisms by which exercise could have enhanced

proliferation and cardiac growth in adult zebrafish hearts. Importantly, our results are in accordance with the emerging concept that exercise can be an alternative strategy for cardiac therapy by stimulating endogenous proliferation in a physiological manner. Therefore, as we previously proposed, we reinforce the use of adult zebrafish as an exercise model to study vertebrate cardiovascular adaptations to exercise. However, despite many advances in the field, further research is required to elucidate the mechanisms responsible for the cardioprotective or preconditioning effects of exercise. Indeed, an important consideration is the determination of how much exercise is needed to achieve the beneficial effects of exercise and in the case of our study, how these effects would be improved. We believe that the current analysisafterfour weeks of training could be extended to earlier time points in order to identify the temporal relevance of specific regulatory mechanisms in the adaptation to exercise, as we have performed in fast skeletal muscle (Chapters 1 and 2). In addition, the application of different training regimes (in terms of duration and intensity) as it has been performed in mammals could have distinct effects, either beneficial or detrimental, that arealso worth investigating. Finally, due to the known differences in heart anatomy and physiology that exist between fish and mammals and, although zebrafish is an excellent model in biomedical research, caution should be taken when establishing comparisons between teleost fish and mammals.

Chapter 5

Performance of regular exercise (endurance or resistance) has been proven to have beneficial cardiovascular effects in humans and rodents (Wisløff *et al*, 2001; Duncker & Bache, 2008; Platt *et al*, 2015). However, our understanding of the exercise response under cardiac regenerating conditions remains very limited basically due to the predominantly mammalian animal models being used in biomedical research that are unable to regenerate the heart during adulthood. Besides fetal and neonatal stages (Porrello *et al*, 2011), mammals appear to have lost the regenerative potential that other lower vertebrates such as teleost fish or amphibians retain throughout their lifetime. In this context, the zebrafish, a teleost fish, brings light into the research field of cardiac regeneration and repair due to its innate ability to completely regenerate the ventricle after injury (Jopling *et al*, 2010a; González-Rosa *et al*, 2011; Schnabel *et al*, 2011; Chablais *et al*, 2011; Poss *et al*, 2002). In view of the effects shown in the previous Chapter and of the relevant role of zebrafish as a translational model for heart regeneration, the present study is the first in exploring the potential to investigate adult zebrafish heart regeneration by swimming-induced exercise. By applying the swimming-induced activity protocol established in our laboratory (Palstra *et al*, 2010) we examined the cardioprotective and preconditioning effects of exercise (during and after, respectively) in adult zebrafish subjected to a heart cryoinjury.

Exercise training in adult zebrafish induces a cardiac preconditioning-like effect

The beneficial health effects of regular exercise have been extensively reported in the literature (Braith & Stewart, 2006; Kokkinos & Myers, 2010; Davidson *et al*, 2009; Ross *et al*, 2004) and the performance of regular exercise is recommended as a prevention for the outcome of cardiovascular diseases in humans. However, the question of whether the cardio-modulatory effects of exercise are sustained after a period of detraining (e.g. this study) is under debate as shown by the available literature from mammals. Some groups argue that exercise training-induced adaptations (in the absence of cardiac injury) persist for some time, with time being a variable among the different reports. For instance, Marini *et al.* (2008) showed that cardiac capillarity after eight weeks of training is retained after four weeks of detraining (Marini *et al*, 2008). Using the same training and detraining duration, Carneiro-Junior *et al.* (2010) showed that exercise-induced eccentric hypertrophy was maintained after four weeks of detraining (Carneiro-Júnior *et al*, 2010). Furthermore, Lehnen *et al.* (2010) found that the reduction in blood pressure and improved insulin sensitivity developed after ten weeks of training was preserved following two weeks of detraining (Lehnen *et al*, 2010). In contrast, other studies showed loss of the stimulatory effects of exercise on cardiomyocyte size and contractility and endothelial function (Kemi *et al*, 2004; Carneiro-Júnior *et al*, 2013), left ventricular wall thickness, heart rate (Bocalini *et al*, 2010) or left ventricular hypertrophy (Weiner *et al*, 2012). Despite their seemingly contradictory findings, these reports indicate that cardiovascular adaptations to exercise are highly dynamic and that could be dependent on the training regime as well as the detraining time. It is noteworthy to mention that studies in fish examining the long-term effects of exercise training and detraining (with or without a cardiac insult) are limited (Young & Cech Jr., 1994; Liu *et al*, 2009; Castro *et al*, 2011).

Here, we investigated the effects of exercise, as a preconditioning of the adult zebrafish heart, on recovery from a cardiac injury after the training period. We have found that exercised hearts have an improved outcome, in terms of cardiomyocyte proliferation and cardiac function, at approximately one month after the last training session. In mammals, studies inducing myocardial injury, ischemia or ischemia/reperfusion injury have provided evidence for a role of exercise in protecting the heart from myocardial damage. Decreased infarct size, decreased cell death, increased vascularity and improved cardiac function are some of the improvements attributed to a previous bout of exercise, lasting days or weeks (McElroy *et al*, 1978; Powers *et al*, 1998; Domenech *et al*, 2002; Brown *et al*, 2003, 2005b; Frasier *et al*, 2013). However, few reports have investigated the effects of exercise after cardiac injury during a long period of recovery such as that examined in the present study (Lennon *et al*, 2004; Dayan *et al*, 2005; Freimann *et al*, 2005; Rahimi *et al*, 2015). To the best of our knowledge, the study by Freimann *et al.* (2005) is the only one performed in mammals reporting that a seven week exercise period elicited exercise-induced adaptations (e.g. decreased injured area, increased arteriole density, increased cardiac function) that were retained four weeks after induced myocardial infarction (Freimann *et al*, 2005). Other studies reported on the loss of the acquired exercise effects after nine or fourteen days post injury (Lennon *et al*, 2004; Rahimi *et al*, 2015). Therefore, given the proposed role of exercise in mammals, including humans, in mimicking an "ischemic preconditioning" phenomena (Maybaum *et al*, 1996; Domenech *et al*, 2002; Michaelides *et al*, 2003) and given the demonstration by Gamperl et al (2001) that fish hearts can be preconditioned (under anoxia) similarly to mammals (Gamperl *et al*, 2001), our results suggest that exercised adult zebrafish hearts may have acquired exercise-induced adaptive characteristics that conferred a preconditioning-like effect resulting in improved heart regeneration after cryoinjury.

The observed improvement o heart regeneration by exercise preconditioning may have been achieved through cardiomyocyte proliferation and reduced fibrosis, as shown by the increase in cardiomyocyte proliferation and the trend towards decreased fibrin content after 28 dpi. These observations suggest that the cardiomyocyte cells have retained the proliferative capacity that is triggered by exercise alone, as shown in the previous Chapter. Importantly, the results suggest that it is under a regenerating milieu when exercise plays a role in the recovery of cardiac contractility, since exercise alone did not induce changes on cardiac function (Chapter 4). A similar finding was observed in rats, in which cardiac functional parameters were equal between exercised and sedentary animals after three weeks of training but differences between the two groups appeared subsequent to a surgical myocardial infarction and were maintained even after four weeks (Dayan *et al*, 2005).

Although in the present study we only examined the preconditioning effects of exercise at 28 dpi, it would be interesting to evaluate the post-lesion regeneration process in hearts of exercise-preconditioned hearts in more detail by sampling time-points earlier than 28 dpi and by investigating the molecular mechanisms that could be responsible for the preconditioning-like effect of exercise in the adult zebrafish heart.

Exercise training during the cardiac regeneration process elicits changes at the cellular and molecular levels

By using adult zebrafish as a model for heart regeneration, we have applied swimming-induced exercise as a physiological stimulus to enhance cardiomyocyte proliferation. Importantly, exercise increased proliferation of cardiomyocytes in regenerating adult zebrafish ventricles at 15 dpi and this cellular response to exercise concurred with marked differences in gene expression in the ventricle, as shown in our transcriptomic analysis. We specifically performed the transcriptomic analysis of exercised versus non-exercised regenerating zebrafish ventricles in order to identify differentially expressed genes that are relevant for the response to exercise over genes relevant for the response to the cardiac lesion. Notably, exercise up-regulated the expression of the transcription factors *hand2*, *nfkb2* and *hif1aa*, related to the cardiomyocyte proliferative response in the regenerating adult zebrafish heart (Jopling *et al*, 2012a; Schindler *et al*, 2014; Karra *et al*, 2015), and also differentially regulated the expression of members of the FGF family such as *fgf17b* (Lepilina *et al*, 2006), *fgf3* and *fgf1a*. Giving the proposed role of FGF signaling in mediating neovascularization in the regenerating zebrafish heart by expressing *fgf17b* to recruit epicardial-derived cells to vascularize newly formed cardiomyocytes (Lepilina *et al*, 2006), and given the control of FGF signaling during zebrafish cardiogenesis (Simoes *et al*, 2011), it seems plausible that exercise may modulate the regenerative response by expressing different members of the FGF family. In support of a role for FGF signaling in the cardiac response to exercise, intramuscular FGF administration in rats induced cardiac capillarization to a similar extent than exercise alone (Efthimiadou *et al*, 2006). FGFs (particularly FGF-1), have been suggested to promote cardioprotection in the mammalian ischemic heart (Cuevas *et al*, 2001) by inducing angiogenesis and cardiomyocyte proliferation (effects that were potentiated with p38 inhibition) (Engel *et al*, 2006) and by decreasing apoptosis (Cuevas *et al*, 2001). Interestingly, we identified other differentially expressed genes in our transcriptomic profiling study that can be associated with the observed exercise-induced cardiomyocyte proliferation in regenerating ventricles and that had not been previously characterized during adult zebrafish heart regeneration. As discussed in the previous Chapter, the transcription factor CITED4 was found to induce cardiomyocyte proliferation in the mammalian exercised heart (Boström *et al*, 2010). Here, *cited4b* was found to be significantly up-regulated in regenerating exercised ventricles at 15 dpi and we suggest that this may represent a potentially interesting factor to study further in terms of its role during cardiac regeneration.

Several reports support the notion that the mammalian heart does exhibit limited endogenous cardiomyocyte turnover throughout life from pre-existing cardiomyocytes (Bergmann *et al*, 2009; Senyo *et al*, 2012) as well as activation of cardiac stem cells following injury (Hsieh *et al*, 2007). In contrast, the adult zebrafish heart responds to injury by inducing regeneration through cardiomyocyte de-differentiation and subsequent proliferation (Jopling *et al*, 2010b) and, to date, contribution from cardiac progenitor stem cells has been shown only in one report (Lepilina *et al*, 2006). The Polycomb protein Bmi1, was recently identified to label a resident population of non-cardiomyocyte stem cells in mice that contribute to cardiomyocyte turnover in the adult mouse heart (Valiente-Alandi *et al*, 2015), is essential for self-renewal of hematopoietic stem cells (HSCs) in mammals and suggested to be expressed also in the HSCs of the developing zebrafish (Park *et al*, 2003; Zhou *et al*, 2011). Therefore, it is tempting to speculate that the up-regulated expression of *bmi1* in the present study could indicate the

involvement of an unidentified population of stem cells in the adult zebrafish heart in response to exercise. The above described factors related with cardiomyocyte proliferation, together with the differential expression of cell cycle-related genes, provide support for the observed increase of cardiomyocyte proliferation in exercised regenerating zebrafish ventricles.

We not only observed an increase in cardiomyocyte number in the lesioned area but also outside of it that may indicate a contribution from the whole ventricle to the resolution of the injury. In support of this, a recent report identified three distinct areas in the adult zebrafish regenerating ventricle with distinct expression profiles (Wu *et al*, 2016). This result suggests that cardiomyocytes or other cell types from the uninjured myocardium could participate in the signaling mechanisms regulating heart regeneration and the response to exercise. In the present study, we have focused on the cardiomyocytes, the main cell type that confers the particular contractile properties to the heart and the spotlight for mammalian regenerative therapies. However, it is worth mentioning that other cell types participate in the regenerating process in the adult zebrafish heart (Kim *et al*, 2010; González-Rosa *et al*, 2011; Kikuchi *et al*, 2011b; Mahmoud *et al*, 2015) that may possibly be also relevant under exercise conditions. Interestingly, a recently identified interstitial cell type, the telocytes, has been identified in the heart and shown to increase in number in the exercised heart in mice (Xiao *et al*, 2016). Thus, although not examined here, it is possible that other cell types shaping the heart such as endothelial, fibroblast and epicardial cells, telocytes or even neurons could play a role in the adaptation of exercise to the regenerating process.

Apart from our evaluation of the induced cardiomyocyte proliferation and the robust transcriptomic signature found in exercised regenerating zebrafish ventricles, we have not examined the functional differences between exercised and non-exercised hearts and, thus, the question remains if exercise induction after cryoinjury, as reported in this study, is beneficial or detrimental towards zebrafish heart regeneration. However, based on the observations that (1) exercised hearts presented a trend towards a reduced injured area at 7 and 15 dpi that is indicative of a better regression of the scar, and that (2) genes related with angiogenesis or endothelial cell function (e. g. *vezf1b* (Bruderer *et al*, 2013), *btc* (Mifune *et al*, 2004), *ptgds* (Taba *et al*, 2000), *antxr1a* (Besschetnova *et al*, 2015), *pcdh12* (Philibert *et al*, 2012) and cardiac function (e. g. *rhoub* (Dickover *et al*, 2014), *trpm4* (Guinamard *et al*, 2015), *tcap* (Ibrahim *et al*, 2013), *cx41*.8 (Vozzi *et al*, 1999), *gja3* (Chi *et al*, 2010)) were differentially expressed in exercised regenerating zebrafish ventricles, we interpret these data to be indicative of an improved functional recovery of the exercised hearts. Remarkably, the response of the regenerating zebrafish ventricle to exercise has not been evaluated to date and, therefore, many genes found in our transcriptomic profiling study have not been previously associated with heart regeneration.

To summarize, in the present study we have shown that exercise induces cardiomyocyte proliferation in adult zebrafish and enhances, at least in part, the recovery upon a cardiac lesion by improving cardiac functionality. Performance of regular exercise in humans is increasingly recognized as a therapeutic complement to face recovery after ischemia or myocardial infarction as well as a recommendation to prevent the risk of cardiovascular

diseases even during elderly (Abete *et al*, 2000, 2001; Wang *et al*, 2014). Thus, considering the advances in understanding the effects of exercise against cardiac stress in mammalian research (Boström *et al*, 2010), the present work could suggest a cardioprotective function of exercise in zebrafish, as in mammals. We propose that exercise, as a physiological stimulus, triggered an endogenous transcriptional program to induce cardiomyocyte proliferation as suggested by the differential gene expression profile between the exercised and non-exercised regenerating ventricles. Thus, considering the observed role of exercise in improving cardiac regeneration in zebrafish as could be suggested from our results, it would be interesting to investigate in future studies the function of the products of the genes that were up-regulated by exercise in the regenerating ventricle as factors that could potentially modulate the regenerative capacity of the zebrafish heart and, importantly, that could have implications in human cardiovascular research. Therefore, further investigations are required to fully characterize the exercise cardioprotective or preconditioning response of exercise in the regenerating zebrafish heart.

GENERAL DISCUSSION

“Nothing in the universe can travel at the speed of light, they say, forgetful of the shadow's speed”

Howard Nemerov (1920-1991)
Poet

GENERAL DISCUSSION

The aim of this thesis was to study the effects of swimming-induced exercise at the molecular and cellular level in skeletal muscle and heart of adult zebrafish. The implementation of the exercise protocol at U_{opt} for this species, as previously reported (Palstra *et al*, 2010), allowed us to investigate some of the physiological adaptationsto exercise that, even in mammals,arestill not entirely understood. By comparing ourfindings on the responses of fast muscle and heart to swimming, we have reasons to believe that common responses to the two tissues may have been achieved under sustained swimming, the main one beingan activation of the proliferative response. In response to exercise, we have found that proliferation of the cardiomyocyte cells is increased in the normal and regenerating heart and, although in skeletal muscle this observation was deduced from the elevated expression of a mitotic marker (through increases in phosphorylated histone 3) and from increased mRNA and protein expression of satellite cell markers (*pax3* and Pax7, respectively), despite no changes in fiber density, our results may be suggestive of the possibility that swimming-induced exercise in adult zebrafishmay have stimulatedmuscle satellite cell proliferation. Therefore, we propose that exercise may have induced proliferation in fast skeletal muscle and cardiomyocytes in adult zebrafish. It is then crucial to determine in future studies if thisproliferative response involves (1) cardiac stem cells in the zebrafish heart, as it has been suggested in the mammalian heart through theactivation of cardiac stem cells or the recruitiment ofcirculating progenitor stem cells (Waring *et al*, 2014; Xiao *et al*, 2014; Leite *et al*, 2015; Vukusic *et al*, 2015); (2) skeletal muscle satellite cells,although they have been mostly related with injury and regeneration and theircontribution to the exercise response is still a matter of investigation (Shefer *et al*, 2010; Leiter *et al*, 2011; Lee *et al*, 2012b; Smith & Merry, 2012; Shefer *et al*, 2013) or (3) activation of other somatic stem cells that may contribute to the exercise response (Macaluso & Myburgh, 2012).

Moreover, despite that we have investigated in some detail the celullar and molecular events that occur in skeletal muscle and heart in response to exercise, this study warrants functional experiments to examine the differences in swimming performance between previously trained and untrained adultzebrafish, as it has been reported in other fish species (Davison, 1997; Zhao *et al*, 2012; He *et al*, 2013). In addition to the need for functional studies, the present work also warrants further studies that provide information about the metabolic adaptations to exercise, such as the likely transition towards a more aerobic phenotype in the skeletal muscle. Similarly, although we provided some data on the effects of swimming on cardiac function, it would have been interesting to evaluate swimming performance in fish that underwent exercise training during cardiac regeneration. In this regard, Wang et al (2011) reported that adult zebrafishafter 7 days of post-genetic ablation of cardiomyocytes exhibited decreased swimming performance (Farrell *et al*, 1990; Wang *et al*, 2011). In contrast, coronary artery ligation in swimming trained trout was not shown to affect maximum speed or critical swimming speed (i.e. speed at which the highest sustainable oxygen consumption occurs, nearly to fatigue) (Farrell *et al*, 1990; Wang *et al*, 2011).

Furthermore, an important fact to take into account in exercise research is the individual variability in physiological performance that could influence the adaptation to exercise. In fish, swimming performance has been used as a measure to differentiate between “poor” and “good” fish swimmers (Claireaux, 2005). Indeed, Castro *et al*. found a 13% increase in survival in “good” swimmers over “poor” swimmers when Atlantic salmon were subjected to a viral challenge after aswimming training period (Castro *et al*, 2013b). These differences in intrinsic swimming performance in fish have been postulated to be retained throughout the development of the fish, as suggested by a study showing that upon classification of juvenile trout in “poor” and “good” swimmers, critical swimming speed, maximum metabolic rate and cardiac output were significantly different between both groups after nine months,when fish reached the adult stage (Claireaux, 2005). Interestingly, anatomical differences in the heart ventricles between“poor” and “good” swimmers were also found, with rounded and longer ventricles, respectively, which may reflect differences between wild and hatchery-raised fish since wild-type trout have longer ventricles (Gamperl & Farrell, 2004). However, whetherthese individual differences that appear to be retained throughout development in fish were based on distinct genetic traits or influenced by environmental or behavioral factors was not investigated. This opens the question if experimental designs with fish subjected to swimming protocols should include a pre-screening of the experimental animals to be used in order to investigate and describe possibly differential responses such as in terms of survival upon infection. Exercise training can improve swimming performance in fish although thishas not been always achieved possibly due to the distinct swimming training protocols used (Davison, 1997) and, therefore, when studying exercise physiology, variability of the results obtained could be explained, at least in part, by differences in individual swimming performance. Indeed, the first genome-wide association study in humans identified several genetic variants associated with improved cardiorespiratory fitness (maximal oxygen uptake)(Bouchard, 2012). In order to eliminate the individual variability that has also been reported in response to exercise training in humans (Bouchard & Rankinen, 2001), a robust approach has been developedin mammals, where a rat model of exercise has been developed after 15 generations of artificial selection, resulting in low or high resistance training rats (Koch *et al*, 2013). Although there is no report to date, this mammalian model would be useful, for instance, to begin tocharacterize the immunomodulatory effects of exercise during sepsis or pathogen infection (as well as in other conditions) and, thus, screening approaches to select different performance phenotypes should be considered in fish as well. However, it should be taken into consideration that the discrimination between two performance phenotypes could underestimate the real variability inherent in a population for translational studies relevant to human health. Therefore, future exercise physiological studies should aim at integrating innate (i.e. sex, epigenetic, genetic) and acquired adaptations (i.e. health status, fitness level, environment, age) throughout life of an individual together with knowledge on the regulatory mechanisms that are involved in the response to exercise.

How much exercise is beneficial?

To achieve better knowledge on the adaptive mechanisms involved in exercise training, research has to focus in improving our understanding on exercise training parameters (e.g. optimal duration, intensity, mode, etc.) that are required for exercise to confer prolonged health benefits. The knowledge of the exercise “dose” would also be beneficial for those

patients who can benefit from an improved physical recovery with exercise. In this study, we have investigated the effects of exercise using the same swimming-induced exercise protocol in all the experiments with which we found remarkable molecular and cellular responses in skeletal and cardiac muscle, but we are aware that this protocol could be optimized. For instance, it would be interesting to experiment with an exercise training protocol at which cardiac function and cardiac hypertrophy weresignificantly inducedto further investigate the limitsof cardiac plasticity in zebrafish that this species appears to retain in view of its cardiac regeneration capabilities. Moreover, with regards to the adaptations of skeletal muscle involvin ghypertrophy and probably proliferation observed in this study, it would be interesting to examine, using the same or and optimized swimming protocol, the response to exercise of available zebrafish muscle disease models, such as those for muscle dystrophy or muscle wasting. However, although in such models of disease the performance of exercise would not improve the (genetic) pathological condition their study could be adressed to modulate the response of gene therapies in order to recover muscle function.

CONCLUSIONS

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1. Four weeks of swimming-induced exercise in adult zebrafish increases muscle fiber size (hypertrophy) and vascularization. The hypertrophic response to exercise in fast muscle may be regulated, at least in part, by the mTOR pathway and possibly also by increased cell proliferation.

2. Swimming-induced exercise increased AMPK activity and *pgc1α* expressionin fast muscle after four weeks of training. These findings suggest an important role for AMPK in modulating the proposed anabolic (i.e. hypertrophy) and oxidative adaptive responses to exercise.

3. The swimming-induced increase in fast muscle vascularization together with the differential expression of angiogenic and oxidative genes (i.e. oxidative metabolism and *pgc1α*) suggest a shift towards a more aerobic phenotype in adult zebrafish fast muscle after four weeks of training.

4. The mRNA expression levels of *il6*, *il6ra*, *decorin*, *myostatin*, *bdnf*, *sparc*, *apelin* and apelin receptors are dynamically regulated over four weeks of exercise training in zebrafish fast muscle. In mammals, these factors are described as muscle secreted factors or *myokines* that influence muscle metabolism and growth during exercise and that may modulate health beneficial effects of exercise. We have characterized for the first time the regulation of the expression of several myokines in adult zebrafish fast muscle that could have potentially important roles in mediating the physiological effects of swimming in zebrafish, as in mammals.

5. Swimming-induced exercise confers specific transcriptional responses to fast muscle of adult zebrafish exposed to an immune challenge. An immune challenge with bacterial lipopolysaccharide elicits a transcriptomic response in fast muscle that is enriched in immune factors, muscle developmental and growth factors in fast muscle from exercised zebrafish when compared with fast muscle from non-exercised zebrafish. Similarly than mammalian muscle cells, the fast muscle of adult zebrafish may modulate this tissue's immune response under exercise-induced contraction.

6. The established swimming-induced exercise protocol in adult zebrafish did not improve survival after an *in vivo* challenge with the opportunistic pathogen *P. aeruginosa* after four weeks of training.

7. Four weeks of exercise training stimulates cardiomyocyte proliferation and a transcriptomic response in the zebrafish heart accompanied by a trend towards increased cardiac growth. However, no changes in cardiac function, as measured by ejection fraction or heart rate, were observed in trained zebrafish.

8. Previous exercise training during four weeks improves cardiac function and increases cardiomyocyte proliferation after 28 dpi in the adult zebrafish ventricle. These findings suggest that exercise facilitates an improved recovery after a cardiac insult eliciting a preconditioning-like effect as it has been reported in mammals.

9. Performance of exercise during the regeneration process after a cardiac lesion stimulated cardiomyocyte proliferation after 11 days of swimming-induced exercise (14 dpi) concomitant with a differential transcriptomic profile. Swimming-induced exercise during regeneration regulated the expression of genes previously reported to be differentially expressed during adult zebrafish heart regeneration as well as new genes, suggesting that exercise induced a specific molecular response during cardiac regeneration and that some of the identified genes could represent potentially interesting targets for improving cardiac regeneration in zebrafish, with potential clinical application.

BIBLIOGRAPHY

Abete P, Calabrese C, Ferrara N, Cioppa A, Pisanelli P, Cacciatore F, Longobardi G, Napoli C & Rengo F (2000) Exercise training restores ischemic preconditioning in the aging heart. *J. Am. Coll. Cardiol.* **36:** 643–50

Abete P, Ferrara N, Cacciatore F, Sagnelli E, Manzi M, Carnovale V, Calabrese C, de Santis D, Testa G, Longobardi G, Napoli C & Rengo F (2001) High level of physical activity preserves the cardioprotective effect of preinfarction angina in elderly patients. *J. Am. Coll. Cardiol.* **38:** 1357–65

Adams RH & Alitalo K (2007) Molecular regulation of angiogenesis and lymphangiogenesis. *Nat. Rev. Mol. Cell Biol.* **8:** 464–78

Aedo JE, Reyes AE, Avendaño-Herrera R, Molina A & Valdés JA (2015) Bacterial lipopolysaccharide induces rainbow trout myotube atrophy via Akt/FoxO1/Atrogin-1 signaling pathway. *Acta Biochim. Biophys. Sin. (Shanghai).* **47:** 932–7

Ahuja P, Perriard E, Perriard J-C & Ehler E (2004) Sequential myofibrillar breakdown accompanies mitotic division of mammalian cardiomyocytes. *J. Cell Sci.* **117:** 3295–306

Akimoto T (2005) Exercise Stimulates Pgc-1 Transcription in Skeletal Muscle through Activation of the p38 MAPK Pathway. *J. Biol. Chem.* **280:** 19587–19593

Albrecht E, Norheim F, Thiede B, Holen T, Ohashi T, Schering L, Lee S, Brenmoehl J, Thomas S, Drevon CA, Erickson HP & Maak S (2015) Irisin - a myth rather than an exercise-inducible myokine. *Sci. Rep.* **5:** 8889

Alexander MS, Kawahara G, Kho AT, Howell MH, Pusack TJ, Myers JA, Montanaro F, Zon LI, Guyon JR & Kunkel LM (2011) Isolation and transcriptome analysis of adult zebrafish cells enriched for skeletal muscle progenitors. *Muscle Nerve* **43:** 741–50

Al-Khalili L, Bouzakri K, Glund S, Lönnqvist F, Koistinen HA & Krook A (2006) Signaling specificity of interleukin-6 action on glucose and lipid metabolism in skeletal muscle. *Mol. Endocrinol.* **20:** 3364–75

Allen DL, Hittel DS & McPherron AC (2011) Expression and function of myostatin in obesity, diabetes, and exercise adaptation. *Med. Sci. Sports Exerc.* **43:** 1828–35

Almendro V, Busquets S, Ametller E, Carbó N, Figueras M, Fuster G, Argilés JM & López-Soriano FJ (2006) Effects of interleukin-15 on lipid oxidation: disposal of an oral [(14)C]-triolein load. *Biochim. Biophys. Acta* **1761:** 37–42

Alvarez B, Carbó N, López-Soriano J, Drivdahl RH, Busquets S, López-Soriano FJ, Argilés JM & Quinn LS (2002) Effects of interleukin-15 (IL-15) on adipose tissue mass in rodent obesity models: evidence for direct IL-15 action on adipose tissue. *Biochim. Biophys. Acta* **1570:** 33–7

Amthor H, Macharia R, Navarrete R, Schuelke M, Brown SC, Otto A, Voit T, Muntoni F, Vrbóva G, Partridge T, Zammit P, Bunger L & Patel K (2007) Lack of myostatin results in excessive muscle growth but impaired force generation. *Proc. Natl. Acad. Sci. U. S. A.* **104:** 1835–40

Aoi W, Naito Y, Takagi T, Tanimura Y, Takanami Y, Kawai Y, Sakuma K, Hang LP, Mizushima K, Hirai Y, Koyama R, Wada S, Higashi A, Kokura S, Ichikawa H & Yoshikawa T (2013) A novel myokine, secreted protein acidic and rich in cysteine (SPARC), suppresses colon tumorigenesis via regular exercise. *Gut* **62:** 882–9

Aquino JB, Marmigère F, Lallemend F, Lundgren TK, Villar MJ, Wegner M & Ernfors P (2008) Differential expression and dynamic changes of murine NEDD9 in progenitor cells of diverse tissues

Arany Z, Foo S-Y, Ma Y, Ruas JL, Bommi-Reddy A, Girnun G, Cooper M, Laznik D, Chinsomboon J, Rangwala SM, Baek KH, Rosenzweig A & Spiegelman BM (2008) HIF-independent regulation of VEGF and angiogenesis by the transcriptional coactivator PGC-1alpha. *Nature* **451:** 1008–12

Argilés JM, López-Soriano FJ & Busquets S (2009) Therapeutic potential of interleukin-15: a myokine involved in muscle wasting and adiposity. *Drug Discov. Today* **14:** 208–13

Arnold L, Henry A, Poron F, Baba-Amer Y, van Rooijen N, Plonquet A, Gherardi RK & Chazaud B (2007) Inflammatory monocytes recruited after skeletal muscle injury switch into antiinflammatory macrophages to support myogenesis. *J. Exp. Med.* **204:** 1057–69

Attané C, Foussal C, Le Gonidec S, Benani A, Daviaud D, Wanecq E, Guzmán-Ruiz R, Dray C, Bezaire V, Rancoule C, Kuba K, Ruiz-Gayo M, Levade T, Penninger J, Burcelin R, Pénicaud L, Valet P & Castan-Laurell I (2012) Apelin treatment increases complete Fatty Acid oxidation, mitochondrial oxidative capacity, and biogenesis in muscle of insulin-resistant mice. *Diabetes* **61:** 310–20

Aydin S, Kuloglu T, Aydin S, Eren MN, Celik A, Yilmaz M, Kalayci M, Sahin İ, Gungor O, Gurel A, Ogeturk M & Dabak O (2014) Cardiac, skeletal muscle and serum irisin responses to with or without water exercise in young and old male rats: cardiac muscle produces more irisin than skeletal muscle. *Peptides* **52:** 68–73

Baar K & Esser K (1999) Phosphorylation of p70(S6k) correlates with increased skeletal muscle mass following resistance exercise.

Am. J. Physiol. **276:** C120-7

Baeza-Raja B & Muñoz-Cánoves P (2004a) p38 MAPK-induced Nuclear Factor- B Activity Is Required for Skeletal Muscle Differentiation: Role of Interleukin-6. *Mol. Biol. Cell* **15:** 2013–2026

Baeza-Raja B & Muñoz-Cánoves P (2004b) p38 MAPK-induced nuclear factor-kappaB activity is required for skeletal muscle differentiation: role of interleukin-6. *Mol. Biol. Cell* **15:** 2013–26

Bagatto B, Pelster B & Burggren WW (2001) Growth and metabolism of larval zebrafish: effects of swim training. *J. Exp. Biol.* **204:** 4335–43

Bakkers J (2011) Zebrafish as a model to study cardiac development and human cardiac disease. *Cardiovasc. Res.* **91:** 279–88

Barger PM & Kelly DP (2000) PPAR Signaling in the Control of Cardiac Energy Metabolism. *Trends Cardiovasc. Med.* **10:** 238–245

Barra NG, Chew M V, Holloway AC & Ashkar AA (2012) Interleukin-15 treatment improves glucose homeostasis and insulin sensitivity in obese mice. *Diabetes. Obes. Metab.* **14:** 190–3

Barra NG, Reid S, MacKenzie R, Werstuck G, Trigatti BL, Richards C, Holloway AC & Ashkar AA (2010) Interleukin-15 Contributes to the Regulation of Murine Adipose Tissue and Human Adipocytes. *Obesity* **18:** 1601–1607

Bar-Shlomo BZ, Druck MN, Morch JE, Jablonsky G, Hilton JD, Feiglin DH & McLaughlin PR (1982) Left ventricular function in trained and untrained healthy subjects. *Circulation* **65:** 484–8

Bartlett JD, Close GL, Drust B & Morton JP (2014) The Emerging Role of p53 in Exercise Metabolism. *Sport. Med.* **44:** 303–309

Bartoccioni E, Michaelis D & Hohlfeld R (1994) Constitutive and cytokine-induced production of interleukin-6 by human myoblasts. *Immunol. Lett.* **42:** 135–8

Beauchemin M, Smith A & Yin VP (2015) Dynamic microRNA-101a and Fosab expression controls zebrafish heart regeneration. *Development* **142:** 4026–37

Bentzinger CF, Wang YX & Rudnicki MA (2012) Building muscle: molecular regulation of myogenesis. *Cold Spring Harb. Perspect. Biol.* **4:**

Berger J, Berger S, Hall TE, Lieschke GJ & Currie PD (2010) Dystrophin-deficient zebrafish feature aspects of the Duchenne muscular dystrophy pathology. *Neuromuscul. Disord.* **20:** 826–32

Bergeron R, Ren JM, Cadman KS, Moore IK, Perret P, Pypaert M, Young LH, Semenkovich CF & Shulman GI (2001) Chronic activation of AMP kinase results in NRF-1 activation and mitochondrial biogenesis. *Am. J. Physiol. Endocrinol. Metab.* **281:** E1340-6

Bergmann O, Bhardwaj RD, Bernard S, Zdunek S, Barnabé-Heider F, Walsh S, Zupicich J, Alkass K, Buchholz BA, Druid H, Jovinge S & Frisén J (2009) Evidence for cardiomyocyte renewal in humans. *Science* **324:** 98–102

Bersell K, Arab S, Haring B & Kühn B (2009) Neuregulin1/ErbB4 signaling induces cardiomyocyte proliferation and repair of heart injury. *Cell* **138:** 257–70

Besschetnova TY, Ichimura T, Katebi N, St Croix B, Bonventre J V & Olsen BR (2015) Regulatory mechanisms of anthrax toxin receptor 1-dependent vascular and connective tissue homeostasis. *Matrix Biol.* **42:** 56–73

Besse-Patin A, Montastier E, Vinel C, Castan-Laurell I, Louche K, Dray C, Daviaud D, Mir L, Marques M-A, Thalamas C, Valet P, Langin D, Moro C & Viguerie N (2014) Effect of endurance training on skeletal muscle myokine expression in obese men: identification of apelin as a novel myokine. *Int. J. Obes. (Lond).* **38:** 707–13

Best JD & Alderton WK (2008) Zebrafish: An in vivo model for the study of neurological diseases. *Neuropsychiatr. Dis. Treat.* **4:** 567–76

Biga PR & Goetz FW (2006) Zebrafish and giant danio as models for muscle growth: determinate vs. indeterminate growth as determined by morphometric analysis. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **291:** R1327-37

Biolo G, Toigo G, Ciocchi B, Situlin R, Iscra F, Gullo A & Guarnieri G (1997) Metabolic response to injury and sepsis: changes in protein metabolism. *Nutrition* **13:** 52S–57S

Blaauw B, Canato M, Agatea L, Toniolo L, Mammucari C, Masiero E, Abraham R, Sandri M, Schiaffino S & Reggiani C (2009) Inducible activation of Akt increases skeletal muscle mass and force without satellite cell activation. *FASEB J.* **23:** 3896–905

Blum N & Begemann G (2012) Retinoic acid signaling controls the formation, proliferation and survival of the blastema during adult zebrafish fin regeneration. *Development* **139:** 107–116

Blumberg D, Hochwald S, Brennan MF & Burt M (1995) Interleukin-6 stimulates gluconeogenesis in primary cultures of rat

hepatocytes. *Metabolism.* **44:** 145–6

Bocalini DS, Carvalho EVA, de Sousa AFM, Levy RF & Tucci PJF (2010) Exercise training-induced enhancement in myocardial mechanics is lost after 2 weeks of detraining in rats. *Eur. J. Appl. Physiol.* **109:** 909–14

Bodine SC (2006) mTOR signaling and the molecular adaptation to resistance exercise. *Med. Sci. Sports Exerc.* **38:** 1950–7

Bodine SC, Stitt TN, Gonzalez M, Kline WO, Stover GL, Bauerlein R, Zlotchenko E, Scrimgeour A, Lawrence JC, Glass DJ & Yancopoulos GD (2001) Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo. *Nat. Cell Biol.* **3:** 1014–1019

Bolli R (2000) The late phase of preconditioning. *Circ. Res.* **87:** 972–83

Bonaldo P & Sandri M (2013) Cellular and molecular mechanisms of muscle atrophy. *Dis. Model. Mech.* **6:** 25–39

Booth FW, Roberts CK & Laye MJ (2012) Lack of exercise is a major cause of chronic diseases. *Compr. Physiol.* **2:** 1143–211

Boström P, Mann N, Wu J, Quintero P a, Plovie ER, Panáková D, Gupta RK, Xiao C, MacRae C a, Rosenzweig A & Spiegelman BM (2010) C/EBPβ controls exercise-induced cardiac growth and protects against pathological cardiac remodeling. *Cell* **143:** 1072–83

Boström P, Wu J, Jedrychowski MP, Korde A, Ye L, Lo JC, Rasbach KA, Boström EA, Choi JH, Long JZ, Kajimura S, Zingaretti MC, Vind BF, Tu H, Cinti S, Højlund K, Gygi SP & Spiegelman BM (2012) A PGC1-α-dependent myokine that drives brown-fat-like development of white fat and thermogenesis. *Nature* **481:** 463–8

Bouchard C (2012) Genomic predictors of trainability. *Exp. Physiol.* **97:** 347–352

Bouchard C & Rankinen T (2001) Individual differences in response to regular physical activity. *Med. Sci. Sports Exerc.* **33:** S446-51–3

Brack AS, Conboy IM, Conboy MJ, Shen J & Rando TA (2008) A temporal switch from notch to Wnt signaling in muscle stem cells is necessary for normal adult myogenesis. *Cell Stem Cell* **2:** 50–9

Bradshaw AD, Graves DC, Motamed K & Sage EH (2003) SPARC-null mice exhibit increased adiposity without significant differences in overall body weight. *Proc. Natl. Acad. Sci. U. S. A.* **100:** 6045–50

Braith RW & Stewart KJ (2006) Resistance exercise training: its role in the prevention of cardiovascular disease. *Circulation* **113:** 2642–50

Braun T & Gautel M (2011) Transcriptional mechanisms regulating skeletal muscle differentiation, growth and homeostasis. *Nat. Rev. Mol. Cell Biol.* **12:** 349–61

Bremer K, Kocha KM, Snider T & Moyes CD (2015) Sensing and responding to energetic stress: The role of the AMPK-PGC1α-NRF1 axis in control of mitochondrial biogenesis in fish. *Comp. Biochem. Physiol. Part B Biochem. Mol. Biol.* **in press:**

Brown DA, Chicco AJ, Jew KN, Johnson MS, Lynch JM, Watson PA & Moore RL (2005a) Cardioprotection afforded by chronic exercise is mediated by the sarcolemmal, and not the mitochondrial, isoform of the K ATP channel in the rat. *J. Physiol.* **569:** 913–924

Brown DA, Jew KN, Sparagna GC, Musch TI & Moore RL (2003) Exercise training preserves coronary flow and reduces infarct size after ischemia-reperfusion in rat heart. *J. Appl. Physiol.* **95:** 2510–8

Brown DA, Lynch JM, Armstrong CJ, Caruso NM, Ehlers LB, Johnson MS & Moore RL (2005b) Susceptibility of the heart to ischaemia-reperfusion injury and exercise-induced cardioprotection are sex-dependent in the rat. *J. Physiol.* **564:** 619–30

Bruderer M, Alini M & Stoddart MJ (2013) Role of HOXA9 and VEZF1 in endothelial biology. *J. Vasc. Res.* **50:** 265–78

Buras JA, Holzmann B & Sitkovsky M (2005) Model organisms: Animal Models of sepsis: setting the stage. *Nat. Rev. Drug Discov.* **4:** 854–865

Busquets S, Figueras M, Almendro V, López-Soriano FJ & Argilés JM (2006) Interleukin-15 increases glucose uptake in skeletal muscle. An antidiabetogenic effect of the cytokine. *Biochim. Biophys. Acta* **1760:** 1613–7

Cade L, Reyon D, Hwang WY, Tsai SQ, Patel S, Khayter C, Joung JK, Sander JD, Peterson RT & Yeh J-RJ (2012) Highly efficient generation of heritable zebrafish gene mutations using homo- and heterodimeric TALENs. *Nucleic Acids Res.* **40:** 8001–10

Cai M-X, Shi X-C, Chen T, Tan Z-N, Lin Q-Q, Du S-J & Tian Z-J (2016) Exercise training activates neuregulin 1/ErbB signaling and promotes cardiac repair in a rat myocardial infarction model. *Life Sci.* **149:** 1–9

Callahan LA & Supinski GS (2009) Sepsis-induced myopathy. *Crit. Care Med.* **37:** S354-67

Calvert JW, Condit ME, Aragón JP, Nicholson CK, Moody BF, Hood RL, Sindler AL, Gundewar S, Seals DR, Barouch LA, Lefer DJ, Brown D, Jew K, Sparagna G, Musch T, Moore R, Lennon S, Quindry J, Hamilton K, French J, et al (2011) Exercise Protects Against Myocardial Ischemia–Reperfusion Injury via Stimulation of β3-Adrenergic Receptors and Increased Nitric Oxide Signaling: Role of Nitrite and NitrosothiolsNovelty and Significance. *Circ. Res.* **108:** 548–552

Calvert JW & Lefer DJ (2013) Role of -Adrenergic Receptors and Nitric Oxide Signaling in Exercise-Mediated Cardioprotection. *Physiology* **28:** 216–224

Cantó C, Gerhart-Hines Z, Feige JN, Lagouge M, Noriega L, Milne JC, Elliott PJ, Puigserver P & Auwerx J (2009) AMPK regulates energy expenditure by modulating NAD+ metabolism and SIRT1 activity. *Nature* **458:** 1056–1060

Cantó C, Jiang LQ, Deshmukh AS, Mataki C, Coste A, Lagouge M, Zierath JR & Auwerx J (2010) Interdependence of AMPK and SIRT1 for Metabolic Adaptation to Fasting and Exercise in Skeletal Muscle

Cao J, Navis A, Cox BD, Dickson AL, Gemberling M, Karra R, Bagnat M, Poss KD, Bollini S, Vieira JMN, Howard S, Dubè KN, Balmer GM, Smart N, Riley PR, Cavallero S, Shen H, Yi C, Lien C-L, Kumar SR, et al (2016) Single epicardial cell transcriptome sequencing identifies Caveolin 1 as an essential factor in zebrafish heart regeneration. *Development* **143:** 232–43

Carbó N, López-Soriano J, Costelli P, Alvarez B, Busquets S, Baccino FM, Quinn LS, López-Soriano FJ & Argilés JM (2001) Interleukin-15 mediates reciprocal regulation of adipose and muscle mass: a potential role in body weight control. *Biochim. Biophys. Acta* **1526:** 17–24

Carey AL, Steinberg GR, Macaulay SL, Thomas WG, Holmes AG, Ramm G, Prelovsek O, Hohnen-Behrens C, Watt MJ, James DE, Kemp BE, Pedersen BK & Febbraio MA (2006) Interleukin-6 increases insulin-stimulated glucose disposal in humans and glucose uptake and fatty acid oxidation in vitro via AMP-activated protein kinase. *Diabetes* **55:** 2688–97

Carneiro-Júnior MA, Pelúzio MCG, Silva CHO, Amorim PRS, Silva KA, Souza MO, Castro CA, Roman-Campos D, Prímola-Gomes TN & Natali AJ (2010) Exercise training and detraining modify the morphological and mechanical properties of single cardiac myocytes obtained from spontaneously hypertensive rats. *Brazilian J. Med. Biol. Res. = Rev. Bras. Pesqui. médicas e biológicas / Soc. Bras. Biofísica ... [et al.]* **43:** 1042–6

Carneiro-Júnior MA, Quintão-Júnior JF, Drummond LR, Lavorato VN, Drummond FR, da Cunha DNQ, Amadeu MA, Felix LB, de Oliveira EM, Cruz JS, Prímola-Gomes TN, Mill JG & Natali AJ (2013) The benefits of endurance training in cardiomyocyte function in hypertensive rats are reversed within four weeks of detraining. *J. Mol. Cell. Cardiol.* **57:** 119–28

Carroll S & Dudfield M (2004) What is the relationship between exercise and metabolic abnormalities? A review of the metabolic syndrome. *Sports Med.* **34:** 371–418

Castellana B, Marín-Juez R & Planas J V (2013) Transcriptional regulation of the gilthead seabream (Sparus aurata) interleukin-6 gene promoter. *Fish Shellfish Immunol.* **35:** 71–8

Castro R, Martínez-Alonso S, Fischer U, Haro NÁ de, Soto-Lampe V, Wang T, Secombes CJ, Lorenzen N, Lorenzen E & Tafalla C (2014) DNA vaccination against a fish rhabdovirus promotes an early chemokine-related recruitment of B cells to the muscle. *Vaccine* **32:** 1160–8

Castro V, Grisdale-Helland B, Helland SJ, Kristensen T, Jørgensen SM, Helgerud J, Claireaux G, Farrell AP, Krasnov A & Takle H (2011) Aerobic training stimulates growth and promotes disease resistance in Atlantic salmon (Salmo salar). *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* **160:** 278–290

Castro V, Grisdale-Helland B, Helland SJ, Torgersen J, Kristensen T, Claireaux G, Farrell AP & Takle H (2013a) Cardiac molecular-acclimation mechanisms in response to swimming-induced exercise in Atlantic salmon. *PLoS One* **8:** e55056

Castro V, Grisdale-Helland B, Jørgensen SM, Helgerud J, Claireaux G, Farrell AP, Krasnov A, Helland SJ & Takle H (2013b) Disease resistance is related to inherent swimming performance in Atlantic salmon. *BMC Physiol.* **13:** 1

Catoire M, Mensink M, Kalkhoven E, Schrauwen P & Kersten S (2014) Identification of human exercise-induced myokines using secretome analysis. *Physiol. Genomics* **46:** 256–67

Chablais F & Jazwinska A (2012) The regenerative capacity of the zebrafish heart is dependent on TGFβ signaling. *Development* **139:** 1921–30

Chablais F, Veit J, Rainer G & Jaźwińska A (2011) The zebrafish heart regenerates after cryoinjury-induced myocardial infarction. *BMC Dev. Biol.* **11:** 21

Chan XCY, McDermott JC & Siu KWM (2007) Identification of secreted proteins during skeletal muscle development. *J. Proteome Res.* **6:** 698–710

Chatterjee A, Roy D, Patnaik E & Nongthomba U (2016) Muscles provide protection during microbial infection by activating innate immune response pathways in Drosophila and zebrafish. *Dis. Model. Mech.* **9:** 697–705

Chen Y, Ye J, Cao L, Zhang Y, Xia W & Zhu D (2010) Myostatin regulates glucose metabolism via the AMP-activated protein kinase

pathway in skeletal muscle cells. *Int. J. Biochem. Cell Biol.* **42:** 2072–81

Chen Y-W, Zhao P, Borup R & Hoffman EP (2000) Expression Profiling in the Muscular Dystrophies. *J. Cell Biol.* **151:** 1321–1336

Cherry AD & Piantadosi CA (2015) Regulation of mitochondrial biogenesis and its intersection with inflammatory responses. *Antioxid. Redox Signal.* **22:** 965–76

Chi NC, Bussen M, Brand-Arzamendi K, Ding C, Olgin JE, Shaw RM, Martin GR & Stainier DYR (2010) Cardiac conduction is required to preserve cardiac chamber morphology. *Proc. Natl. Acad. Sci. U. S. A.* **107:** 14662–7

Cho WJ, Kim EJ, Lee SJ, Kim HD, Shin HJ & Lim WK (2000) Involvement of SPARC in in vitro differentiation of skeletal myoblasts. *Biochem. Biophys. Res. Commun.* **271:** 630–4

Choi W-Y, Gemberling M, Wang J, Holdway JE, Shen M-C, Karlstrom RO & Poss KD (2013) In vivo monitoring of cardiomyocyte proliferation to identify chemical modifiers of heart regeneration. *Development* **140:** 660–6

Christen B, Robles V, Raya M, Paramonov I & Belmonte J (2010) Regeneration and reprogramming compared. *BMC Biol.* **8:** 5

Claireaux G (2005) Linking swimming performance, cardiac pumping ability and cardiac anatomy in rainbow trout. *J. Exp. Biol.* **208:** 1775–1784

Clow C & Jasmin BJ (2010) Brain-derived neurotrophic factor regulates satellite cell differentiation and skeltal muscle regeneration. *Mol. Biol. Cell* **21:** 2182–90

Combaret L, Adegoke OAJ, Bedard N, Baracos V, Attaix D & Wing SS (2005) USP19 is a ubiquitin-specific protease regulated in rat skeletal muscle during catabolic states. *Am. J. Physiol. Endocrinol. Metab.* **288:** E693-700

Cotman CW, Berchtold NC & Christie L-A (2007) Exercise builds brain health: key roles of growth factor cascades and inflammation. *Trends Neurosci.* **30:** 464–72

Coven DL, Hu X, Cong L, Bergeron R, Shulman GI, Hardie DG & Young LH (2003) Physiological role of AMP-activated protein kinase in the heart: graded activation during exercise. *Am. J. Physiol. Endocrinol. Metab.* **285:** E629-36

Crimi E, Ignarro LJ, Cacciatore F & Napoli C (2009) Mechanisms by which exercise training benefits patients with heart failure. *Nat. Rev. Cardiol.* **6:** 292–300

Croft L, Bartlett JD, MacLaren DPM, Reilly T, Evans L, Mattey DL, Nixon NB, Drust B & Morton JP (2009) High-intensity interval training attenuates the exercise-induced increase in plasma IL-6 in response to acute exercise. *Appl. Physiol. Nutr. Metab. = Physiol. Appl. Nutr. métabolisme* **34:** 1098–107

Crouser ED, Julian MW, Blaho D V & Pfeiffer DR (2002) Endotoxin-induced mitochondrial damage correlates with impaired respiratory activity. *Crit. Care Med.* **30:** 276–84

Cuevas P, Carceller F & Giménez-Gallego G (2001) Fibroblast growth factors in myocardial ischemia / reperfusion injury and ischemic preconditioning. *J. Cell. Mol. Med.* **5:** 132–42

Curado S, Stainier DYR & Anderson RM (2008) Nitroreductase-mediated cell/tissue ablation in zebrafish: a spatially and temporally controlled ablation method with applications in developmental and regeneration studies. *Nat. Protoc.* **3:** 948–954

Das AK, Yang Q-Y, Fu X, Liang J-F, Duarte MS, Zhu M-J, Trobridge GD & Du M (2012) AMP-activated protein kinase stimulates myostatin expression in C2C12 cells. *Biochem. Biophys. Res. Commun.* **427:** 36–40

Daskalopoulou SS, Cooke AB, Gomez Y-H, Mutter AF, Filippaios A, Mesfum ET & Mantzoros CS (2014) Plasma irisin levels progressively increase in response to increasing exercise workloads in young, healthy, active subjects. *Eur. J. Endocrinol.* **171:** 343–52

Davidson LE, Hudson R, Kilpatrick K, Kuk JL, McMillan K, Janiszewski PM, Lee S, Lam M & Ross R (2009) Effects of exercise modality on insulin resistance and functional limitation in older adults: a randomized controlled trial. *Arch. Intern. Med.* **169:** 122–31

Davie PS, Wells RM & Tetens V (1986) Effects of sustained swimming on rainbow trout muscle structure, blood oxygen transport, and lactate dehydrogenase isozymes: evidence for increased aerobic capacity of white muscle. *J. Exp. Zool.* **237:** 159–71

Davison W (1997) The effects of exercise training on teleost fish, a review of recent literature. *Comp. Biochem. Physiol. Part A Physiol.* **117:** 67–75

Davison W & Herbert NA (2013) Swimming-Enhanced Growth. In *Swimming Physiology of Fish* pp 177–202. Berlin, Heidelberg: Springer Berlin Heidelberg

Dayan A, Feinberg MS, Holbova R, Deshet N & Scheinowitz M (2005) Swimming exercise training prior to acute myocardial infarction attenuates left ventricular remodeling and improves left ventricular function in rats. *Ann. Clin. Lab. Sci.* **35:** 73–8

Dehoux MJ., van Beneden RP, Fernández-Celemín L, Lause PL & Thissen J-PM (2003) Induction of MafBx and Murf ubiquitin ligase mRNAs in rat skeletal muscle after LPS injection. *FEBS Lett.* **544:** 214–217

Devoto SH, Melançon E, Eisen JS & Westerfield M (1996) Identification of separate slow and fast muscle precursor cells in vivo, prior to somite formation. *Development* **122:** 3371–80

Dickover M, Hegarty JM, Ly K, Lopez D, Yang H, Zhang R, Tedeschi N, Hsiai TK & Chi NC (2014) The atypical Rho GTPase, RhoU, regulates cell-adhesion molecules during cardiac morphogenesis. *Dev. Biol.* **389:** 182–91

Domenech R, Macho P, Schwarze H & Sánchez G (2002) Exercise induces early and late myocardial preconditioning in dogs. *Cardiovasc. Res.* **55:** 561–566

Doyle A, Zhang G, Abdel Fattah EA, Eissa NT & Li Y-P (2011) Toll-like receptor 4 mediates lipopolysaccharide-induced muscle catabolism via coordinate activation of ubiquitin-proteasome and autophagy-lysosome pathways. *FASEB J.* **25:** 99–110

Doyon Y, McCammon JM, Miller JC, Faraji F, Ngo C, Katibah GE, Amora R, Hocking TD, Zhang L, Rebar EJ, Gregory PD, Urnov FD & Amacher SL (2008) Heritable targeted gene disruption in zebrafish using designed zinc-finger nucleases. *Nat. Biotechnol.* **26:** 702–8

Dray C, Knauf C, Daviaud D, Waget A, Boucher J, Buléon M, Cani PD, Attané C, Guigné C, Carpéné C, Burcelin R, Castan-Laurell I & Valet P (2008) Apelin stimulates glucose utilization in normal and obese insulin-resistant mice. *Cell Metab.* **8:** 437–45

Driever W, Solnica-Krezel L, Schier AF, Neuhauss SC, Malicki J, Stemple DL, Stainier DY, Zwartkruis F, Abdelilah S, Rangini Z, Belak J & Boggs C (1996) A genetic screen for mutations affecting embryogenesis in zebrafish. *Development* **123:** 37–46

Drummond MJ, Fry CS, Glynn EL, Dreyer HC, Dhanani S, Timmerman KL, Volpi E & Rasmussen BB (2009) Rapamycin administration in humans blocks the contraction-induced increase in skeletal muscle protein synthesis. *J. Physiol.* **587:** 1535–46

Du M, Shen QW, Zhu MJ & Ford SP (2007) Leucine stimulates mammalian target of rapamycin signaling in C2C12 myoblasts in part through inhibition of adenosine monophosphate-activated protein kinase. *J. Anim. Sci.* **85:** 919–27

Duncker DJ & Bache RJ (2008) Regulation of Coronary Blood Flow During Exercise. *Physiol. Rev.* **88:** 1009–1086

Dungan KM, Wei TY, Nace JD, Poulin ML, Chiu I-M, Lang JC & Tassava RA (2002) Expression and biological effect of urodele fibroblast growth factor 1: relationship to limb regeneration. *J. Exp. Zool.* **292:** 540–54

Efthimiadou A, Asimakopoulos B, Nikolettos N, Giatromanolaki A, Sivridis E, Lialiaris TS, Papachristou DN & Kontoleon E (2006) The angiogenetic effect of intramuscular administration of b-FGF and a-FGF on cardiac muscle: The influence of exercise on muscle angiogenesis. *J. Sports Sci.* **24:** 849–854

Egan B, Carson BP, Garcia-Roves PM, Chibalin A V., Sarsfield FM, Barron N, McCaffrey N, Moyna NM, Zierath JR & O'Gorman DJ (2010) Exercise intensity-dependent regulation of peroxisome proliferator-activated receptor γ coactivator-1α mRNA abundance is associated with differential activation of upstream signalling kinases in human skeletal muscle. *J. Physiol.* **588:** 1779–1790

Egan B & Zierath JR (2013) Exercise metabolism and the molecular regulation of skeletal muscle adaptation. *Cell Metab.* **17:** 162–84

Egan DF, Shackelford DB, Mihaylova MM, Gelino S, Kohnz RA, Mair W, Vasquez DS, Joshi A, Gwinn DM, Taylor R, Asara JM, Fitzpatrick J, Dillin A, Viollet B, Kundu M, Hansen M & Shaw RJ (2011) Phosphorylation of ULK1 (hATG1) by AMP-activated protein kinase connects energy sensing to mitophagy. *Science* **331:** 456–61

Egginton S (2009) Invited review: activity-induced angiogenesis. *Pflügers Arch. Eur. J. Physiol.* **457:** 963–77

Engel FB, Hsieh PCH, Lee RT & Keating MT (2006) FGF1/p38 MAP kinase inhibitor therapy induces cardiomyocyte mitosis, reduces scarring, and rescues function after myocardial infarction. *Proc. Natl. Acad. Sci. U. S. A.* **103:** 15546–51

Engel FB, Schebesta M, Duong MT, Lu G, Ren S, Madwed JB, Jiang H, Wang Y & Keating MT (2005) p38 MAP kinase inhibition enables proliferation of adult mammalian cardiomyocytes. *Genes Dev.* **19:** 1175–87

Ernens I, Lumley AI, Devaux Y & Wagner DR (2016) Use of Coronary Ultrasound Imaging to Evaluate Ventricular Function in Adult Zebrafish.

Esposito F, Ronchi R, Milano G, Margonato V, Di Tullio S, Marini M, Veicsteinas A & Samaja M (2011) Myocardial tolerance to ischemia-reperfusion injury, training intensity and cessation. *Eur. J. Appl. Physiol.* **111:** 859–68

Evans DH, Claiborne JB & Currie S (2013) The physiology of fishes 4th ed. Evans DH Claiborne JB & Currie S (eds) Boca Raton, USA: CRC Press

Fang Y, Gupta V, Karra R, Holdway JE, Kikuchi K & Poss KD (2013) Translational profiling of cardiomyocytes identifies an early Jak1/Stat3 injury response required for zebrafish heart regeneration. *Proc. Natl. Acad. Sci. U. S. A.* **110:** 13416–21

Fang Y, Vilella-Bach M, Bachmann R, Flanigan A & Chen J (2001) Phosphatidic acid-mediated mitogenic activation of mTOR signaling. *Science* **294:** 1942–5

Farrell AP, Johansen JA, Steffensen JF, Moyes CD, West TG & Suarez RK (1990) Effects of exercise training and coronary ablation on swimming performance, heart size, and cardiac enzymes in rainbow trout, Oncorhynchus mykiss. *Can. J. Zool.* **68:** 1174–1179

Farrell AP, Johansen JA & Suarez RK (1991) Effects of exercise-training on cardiac performance and muscle enzymes in rainbow trout,Oncorhynchus mykiss. *Fish Physiol. Biochem.* **9:** 303–12

Febbraio MA, Hiscock N, Sacchetti M, Fischer CP & Pedersen BK (2004) Interleukin-6 is a novel factor mediating glucose homeostasis during skeletal muscle contraction. *Diabetes* **53:** 1643–8

Febbraio MA, Steensberg A, Keller C, Starkie RL, Nielsen HB, Krustrup P, Ott P, Secher NH & Pedersen BK (2003) Glucose ingestion attenuates interleukin-6 release from contracting skeletal muscle in humans. *J. Physiol.* **549:** 607–12

Feldman BJ, Streeper RS, Farese R V & Yamamoto KR (2006) Myostatin modulates adipogenesis to generate adipocytes with favorable metabolic effects. *Proc. Natl. Acad. Sci. U. S. A.* **103:** 15675–80

Felip O, Ibarz A, Fernández-Borràs J, Beltrán M, Martín-Pérez M, Planas J V & Blasco J (2012) Tracing metabolic routes of dietary carbohydrate and protein in rainbow trout (Oncorhynchus mykiss) using stable isotopes ([^{13}C]starch and [^{15}N]protein): effects of gelatinisation of starches and sustained swimming. *Br. J. Nutr.* **107:** 834–44

Ferrer-Martínez A, Ruiz-Lozano P & Chien KR (2002) Mouse PeP: a novel peroxisomal protein linked to myoblast differentiation and development. *Dev. Dyn.* **224:** 154–67

Fiaz AW, Léon-Kloosterziel KM, Gort G, Schulte-Merker S, van Leeuwen JL & Kranenbarg S (2012) Swim-training changes the spatio-temporal dynamics of skeletogenesis in zebrafish larvae (Danio rerio). *PLoS One* **7:** e34072

Figueiredo VC, Caldow MK, Massie V, Markworth JF, Cameron-Smith D & Blazevich AJ (2015) Ribosome biogenesis adaptation in resistance training-induced human skeletal muscle hypertrophy. *Am. J. Physiol. Endocrinol. Metab.* **309:** E72-83

Figueras M, Busquets S, Carbó N, Barreiro E, Almendro V, Argilés JM & López-Soriano FJ (2004) Interleukin-15 is able to suppress the increased DNA fragmentation associated with muscle wasting in tumour-bearing rats. *FEBS Lett.* **569:** 201–6

Fischer CP (2006) Interleukin-6 in acute exercise and training: what is the biological relevance? *Exerc. Immunol. Rev.* **12:** 6–33

Fiuza-Luces C, Garatachea N, Berger NA & Lucia A (2013) Exercise is the real polypill. *Physiology (Bethesda).* **28:** 330–58

Foglia MJ & Poss KD (2016) Building and re-building the heart by cardiomyocyte proliferation. *Development* **143:** 729–40

Foster MN & Coetzee WA (2016) K ATP Channels in the Cardiovascular System. *Physiol. Rev.* **96:** 177–252

Fouces V, Torrella JR, Palomeque J & Viscor G (1993) A histochemical ATPase method for the demonstration of the muscle capillary network. *J. Histochem. Cytochem.* **41:** 283–9

Frasier CR, Moukdar F, Patel HD, Sloan RC, Stewart LM, Alleman RJ, La Favor JD & Brown DA (2013) Redox-dependent increases in glutathione reductase and exercise preconditioning: role of NADPH oxidase and mitochondria. *Cardiovasc. Res.* **98:** 47–55

Freimann S, Scheinowitz M, Yekutieli D, Feinberg MS, Eldar M & Kessler-Icekson G (2005) Prior exercise training improves the outcome of acute myocardial infarction in the rat: Heart structure, function, and gene expression. *J. Am. Coll. Cardiol.* **45:** 931–938

French JP, Hamilton KL, Quindry JC, Lee Y, Upchurch PA & Powers SK (2008) Exercise-induced protection against myocardial apoptosis and necrosis: MnSOD, calcium-handling proteins, and calpain. *FASEB J.* **22:** 2862–2871

Frost RA, Nystrom GJ & Lang CH (2002) Lipopolysaccharide regulates proinflammatory cytokine expression in mouse myoblasts and skeletal muscle. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **283:** R698-709

Gamperl AK & Farrell AP (2004) Cardiac plasticity in fishes: environmental influences and intraspecific differences. *J. Exp. Biol.* **207:** 2539–50

Gamperl AK, Todgham AE, Parkhouse WS, Dill R & Farrell AP (2001) Recovery of trout myocardial function following anoxia: preconditioning in a non-mammalian model. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **281:** R1755-63

Garcia N, Tomàs M, Santafe MM, Lanuza MA, Besalduch N & Tomàs J (2010) Localization of brain-derived neurotrophic factor, neurotrophin-4, tropomyosin-related kinase b receptor, and p75 NTR receptor by high-resolution immunohistochemistry on the adult mouse neuromuscular junction. *J. Peripher. Nerv. Syst.* **15:** 40–9

Garg K, Corona BT & Walters TJ (2015) Therapeutic strategies for preventing skeletal muscle fibrosis after injury. *Front. Pharmacol.* **6:** 87

Garza MA, Wason EA & Zhang JQ (2015) Cardiac remodeling and physical training post myocardial infarction. *World J. Cardiol.* **7:** 52–64

Gaudel C, Schwartz C, Giordano C, Abumrad NA & Grimaldi PA (2008) Pharmacological activation of PPARbeta promotes rapid and calcineurin-dependent fiber remodeling and angiogenesis in mouse skeletal muscle. *Am. J. Physiol. Endocrinol. Metab.* **295:** E297-304

Gemberling M, Karra R, Dickson AL & Poss KD (2015) Nrg1 is an injury-induced cardiomyocyte mitogen for the endogenous heart regeneration program in zebrafish. *Elife* **4:**

Geng T, Li P, Okutsu M, Yin X, Kwek J, Zhang M & Yan Z (2010) PGC-1alpha plays a functional role in exercise-induced mitochondrial biogenesis and angiogenesis but not fiber-type transformation in mouse skeletal muscle. *Am. J. Physiol. Cell Physiol.* **298:** C572-9

Gibbs EM, Horstick EJ & Dowling JJ (2013) Swimming into prominence: the zebrafish as a valuable tool for studying human myopathies and muscular dystrophies. *FEBS J.* **280:** 4187–97

Gilbert MJH, Zerulla TC & Tierney KB (2014) Zebrafish (Danio rerio) as a model for the study of aging and exercise: Physical ability and trainability decrease with age. *Exp. Gerontol.* **50:** 106–113

Gilmour DT, Lyon GJ, Carlton MB, Sanes JR, Cunningham JM, Anderson JR, Hogan BL, Evans MJ & Colledge WH (1998) Mice deficient for the secreted glycoprotein SPARC/osteonectin/BM40 develop normally but show severe age-onset cataract formation and disruption of the lens. *EMBO J.* **17:** 1860–70

Gleeson M, Bishop NC, Stensel DJ, Lindley MR, Mastana SS & Nimmo MA (2011) The anti-inflammatory effects of exercise: mechanisms and implications for the prevention and treatment of disease. *Nat. Rev. Immunol.* **11:** 607–15

Glund S, Treebak JT, Long YC, Barres R, Viollet B, Wojtaszewski JF & Zierath JR (2009) Role of adenosine 5'-monophosphate-activated protein kinase in interleukin-6 release from isolated mouse skeletal muscle. *Endocrinology* **150:** 600–6

Gokhale R, Chandrashekara S & Vasanthakumar KC (2007) Cytokine response to strenuous exercise in athletes and non-athletes—an adaptive response. *Cytokine* **40:** 123–127

Goldstein MS (1961) Humoral nature of the hypoglycemic factor of muscular work. *Diabetes* **10:** 232–4

Gomes EC, Silva AN & de Oliveira MR (2012) Oxidants, antioxidants, and the beneficial roles of exercise-induced production of reactive species. *Oxid. Med. Cell. Longev.* **2012:** 756132

Gómez-Pinilla F, Ying Z, Roy RR, Molteni R & Edgerton VR (2002) Voluntary exercise induces a BDNF-mediated mechanism that promotes neuroplasticity. *J. Neurophysiol.* **88:** 2187–95

González-Rosa JM, Guzmán-Martínez G, Marques IJ, Sánchez-Iranzo H, Jiménez-Borreguero LJ & Mercader N (2014) Use of Echocardiography Reveals Reestablishment of Ventricular Pumping Efficiency and Partial Ventricular Wall Motion Recovery upon Ventricular Cryoinjury in the Zebrafish. *PLoS One* **9:** e115604

González-Rosa JM, Martín V, Peralta M, Torres M & Mercader N (2011) Extensive scar formation and regression during heart regeneration after cryoinjury in zebrafish. *Development* **138:** 1663–74

González-Rosa JM & Mercader N (2012) Cryoinjury as a myocardial infarction model for the study of cardiac regeneration in the zebrafish. *Nat. Protoc.* **7:** 782–8

González-Rosa JM, Peralta M & Mercader N (2012) Pan-epicardial lineage tracing reveals that epicardium derived cells give rise to myofibroblasts and perivascular cells during zebrafish heart regeneration. *Dev. Biol.* **370:** 173–186

González-Terán B, López JA, Rodríguez E, Leiva L, Martínez-Martínez S, Bernal JA, Jiménez-Borreguero LJ, Redondo JM, Vazquez J & Sabio G (2016) p38γ and δ promote heart hypertrophy by targeting the mTOR-inhibitory protein DEPTOR for degradation. *Nat. Commun.* **7:** 10477

Goodman CA, Mayhew DL & Hornberger TA (2011) Recent progress toward understanding the molecular mechanisms that regulate skeletal muscle mass. *Cell. Signal.* **23:** 1896–906

Goodwin GW & Taegtmeyer H (2000) Improved energy homeostasis of the heart in the metabolic state of exercise. *Am. J. Physiol. Heart Circ. Physiol.* **279:** H1490-501

Goodyear LJ & Kahn BB (1998) Exercise, glucose transport, and insulin sensitivity. *Annu. Rev. Med.* **49:** 235–261

Gordon BS, Kelleher AR & Kimball SR (2013) Regulation of muscle protein synthesis and the effects of catabolic states. *Int. J. Biochem. Cell Biol.* **45:** 2147–57

Gore A V., Monzo K, Cha YR, Pan W & Weinstein BM (2012) Vascular Development in the Zebrafish. *Cold Spring Harb. Perspect. Med.* **2:** a006684–a006684

Goyal A, Neill T, Owens RT, Schaefer L & Iozzo R V (2014) Decorin activates AMPK, an energy sensor kinase, to induce autophagy in endothelial cells. *Matrix Biol.* **34:** 46–54

Guerci A, Lahoute C, Hébrard S, Collard L, Graindorge D, Favier M, Cagnard N, Batonnet-Pichon S, Précigout G, Garcia L, Tuil D, Daegelen D & Sotiropoulos A (2012) Srf-dependent paracrine signals produced by myofibers control satellite cell-mediated skeletal muscle hypertrophy. *Cell Metab.* **15:** 25–37

Guimarães-Camboa N, Stowe J, Aneas I, Sakabe N, Cattaneo P, Henderson L, Kilberg MS, Johnson RS, Chen J, McCulloch AD, Nobrega MA, Evans SM & Zambon AC (2015) HIF1α Represses Cell Stress Pathways to Allow Proliferation of Hypoxic Fetal Cardiomyocytes. *Dev. Cell* **33:** 507–521

Guinamard R, Bouvagnet P, Hof T, Liu H, Simard C & Sallé L (2015) TRPM4 in cardiac electrical activity. *Cardiovasc. Res.* **108:** 21–30

Gurd BJ, Yoshida Y, McFarlan JT, Holloway GP, Moyes CD, Heigenhauser GJF, Spriet L & Bonen A (2011) Nuclear SIRT1 activity, but not protein content, regulates mitochondrial biogenesis in rat and human skeletal muscle. *AJP Regul. Integr. Comp. Physiol.* **301:** R67–R75

Gwinn DM, Shackelford DB, Egan DF, Mihaylova MM, Mery A, Vasquez DS, Turk BE & Shaw RJ (2008) AMPK Phosphorylation of Raptor Mediates a Metabolic Checkpoint. *Mol. Cell* **30:** 214–226

Habchi M, Duvillard L, Cottet V, Brindisi M-C, Bouillet B, Beacco M, Crevisy E, Buffier P, Baillot-Rudoni S, Verges B & Petit J-M (2014) Circulating apelin is increased in patients with type 1 or type 2 diabetes and is associated with better glycaemic control. *Clin. Endocrinol. (Oxf).* **81:** 696–701

Haffter P, Granato M, Brand M, Mullins MC, Hammerschmidt M, Kane DA, Odenthal J, van Eeden FJ, Jiang YJ, Heisenberg CP, Kelsh RN, Furutani-Seiki M, Vogelsang E, Beuchle D, Schach U, Fabian C & Nüsslein-Volhard C (1996) The identification of genes with unique and essential functions in the development of the zebrafish, Danio rerio. *Development* **123:** 1–36

Hamilton KL, Powers SK, Sugiura T, Kim S, Lennon S, Tumer N & Mehta JL (2001) Short-term exercise training can improve myocardial tolerance to I/R without elevation in heat shock proteins. *Am. J. Physiol. Heart Circ. Physiol.* **281:** H1346-52

Han P, Zhou X-H, Chang N, Xiao C-L, Yan S, Ren H, Yang X-Z, Zhang M-L, Wu Q, Tang B, Diao J-P, Zhu X, Zhang C, Li C-Y, Cheng H & Xiong J-W (2014) Hydrogen peroxide primes heart regeneration with a derepression mechanism. *Cell Res.* **24:** 1091–1107

Hannan R, Jenkins A, Jenkins A & Brandenburger Y (2003) Cardiac hypertrophy: A matter of translation. *Clin. Exp. Pharmacol. Physiol.* **30:** 517–527

Hardie DG, Ross FA & Hawley SA (2012) AMPK: a nutrient and energy sensor that maintains energy homeostasis. *Nat. Rev. Mol. Cell Biol.* **13:** 251–62

Hashmi S & Al-Salam S (2012) Hypoxia-inducible factor-1 alpha in the heart: a double agent? *Cardiol. Rev.* **20:** 268–73

Hasumura T & Meguro S (2016) Exercise quantity-dependent muscle hypertrophy in adult zebrafish (Danio rerio). *J. Comp. Physiol. B.*

Hawley JA, Hargreaves M, Joyner MJ & Zierath JR (2014) Integrative Biology of Exercise. *Cell* **159:** 738–749

He W, Xia W, Cao Z-D & Fu S-J (2013) The effect of prolonged exercise training on swimming performance and the underlying biochemical mechanisms in juvenile common carp (Cyprinus carpio). *Comp. Biochem. Physiol. A. Mol. Integr. Physiol.* **166:** 308–15

Hecksteden A, Wegmann M, Steffen A, Kraushaar J, Morsch A, Ruppenthal S, Kaestner L & Meyer T (2013) Irisin and exercise training in humans - results from a randomized controlled training trial. *BMC Med.* **11:** 235

Hein SJ, Lehmann LH, Kossack M, Juergensen L, Fuchs D, Katus HA & Hassel D (2015) Advanced echocardiography in adult zebrafish reveals delayed recovery of heart function after myocardial cryoinjury. *PLoS One* **10:** e0122665

Heineke J & Molkentin JD (2006) Regulation of cardiac hypertrophy by intracellular signalling pathways. *Nat. Rev. Mol. Cell Biol.* **7:** 589–600

Henningsen J, Rigbolt KTG, Blagoev B, Pedersen BK & Kratchmarova I (2010) Dynamics of the skeletal muscle secretome during myoblast differentiation. *Mol. Cell. Proteomics* **9:** 2482–96

Herbert SP, Huisken J, Kim TN, Feldman ME, Houseman BT, Wang RA, Shokat KM & Stainier DYR (2009) Arterial-venous segregation by selective cell sprouting: an alternative mode of blood vessel formation. *Science* **326:** 294–8

Hinits Y & Hughes SM (2007) Mef2s are required for thick filament formation in nascent muscle fibres. *Development* **134:** 2511–9

Hiscock N, Chan MHS, Bisucci T, Darby IA & Febbraio MA (2004) Skeletal myocytes are a source of interleukin-6 mRNA expression and protein release during contraction: evidence of fiber type specificity. *FASEB J.* **18:** 992–4

Hoage T, Ding Y & Xu X (2012) Quantifying cardiac functions in embryonic and adult zebrafish. *Methods Mol. Biol.* **843:** 11–20

Hoier B & Hellsten Y (2014) Exercise-induced capillary growth in human skeletal muscle and the dynamics of VEGF. *Microcirculation* **21:** 301–14

Hong CC, Peterson QP, Hong J-Y & Peterson RT (2006) Artery/vein specification is governed by opposing phosphatidylinositol-3 kinase and MAP kinase/ERK signaling. *Curr. Biol.* **16:** 1366–72

Hotamisligil GS (2006) Inflammation and metabolic disorders. *Nature* **444:** 860–867

Howe K, Clark MD, Torroja CF, Torrance J, Berthelot C, Muffato M, Collins JEJ, Humphray S, McLaren K, Matthews L, McLaren S, Sealy I, Caccamo M, Churcher C, Scott C, Barrett JC, Koch R, Rauch G-J, White S, Chow W, et al (2013) The zebrafish reference genome sequence and its relationship to the human genome. *Nature* **496:** 498–503

Hruscha A, Krawitz P, Rechenberg A, Heinrich V, Hecht J, Haass C & Schmid B (2013) Efficient CRISPR/Cas9 genome editing with low off-target effects in zebrafish. *Development* **140:** 4982–7

Hsieh PCH, Segers VFM, Davis ME, MacGillivray C, Gannon J, Molkentin JD, Robbins J & Lee RT (2007) Evidence from a genetic fate-mapping study that stem cells refresh adult mammalian cardiomyocytes after injury. *Nat. Med.* **13:** 970–974

Hu N, Yost HJ & Clark EB (2001) Cardiac morphology and blood pressure in the adult zebrafish. *Anat. Rec.* **264:** 1–12

Huang DW, Sherman BT & Lempicki RA (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat. Protoc.* **4:** 44–57

Huang Y, Harrison MR, Osorio A, Kim J, Baugh A, Duan C, Sucov HM, Lien C-L, Li F, Wang X, Capasso J, Gerdes A, Pasumarthi K, Field L, Amerongen M van, Engel F, Walsh S, Ponten A, Fleischmann B, Jovinge S, et al (2013) Igf Signaling is Required for Cardiomyocyte Proliferation during Zebrafish Heart Development and Regeneration. *PLoS One* **8:** e67266

Huh JY, Mougios V, Kabasakalis A, Fatouros I, Siopi A, Douroudos II, Filippaios A, Panagiotou G, Park KH & Mantzoros CS (2014) Exercise-induced irisin secretion is independent of age or fitness level and increased irisin may directly modulate muscle metabolism through AMPK activation. *J. Clin. Endocrinol. Metab.* **99:** E2154-61

Huh JY, Panagiotou G, Mougios V, Brinkoetter M, Vamvini MT, Schneider BE & Mantzoros CS (2012) FNDC5 and irisin in humans: I. Predictors of circulating concentrations in serum and plasma and II. mRNA expression and circulating concentrations in response to weight loss and exercise. *Metabolism.* **61:** 1725–38

Ibarz A, Felip O, Fernández-Borràs J, Martín-Pérez M, Blasco J & Torrella JR (2011) Sustained swimming improves muscle growth and cellularity in gilthead sea bream. *J. Comp. Physiol. B.* **181:** 209–217

Ibrahim M, Siedlecka U, Buyandelger B, Harada M, Rao C, Moshkov A, Bhargava A, Schneider M, Yacoub MH, Gorelik J, Knöll R & Terracciano CM (2013) A critical role for Telethonin in regulating t-tubule structure and function in the mammalian heart. *Hum. Mol. Genet.* **22:** 372–83

Icardo J (2012) The Teleost Heart: A Morphological Approach. In *Ontogeny and Phylogeny of the Vertebrate Heart*, Sedmera D & Wang T (eds) pp 35–53.

Icardo JM & Colvee E (2011) The Atrioventricular Region of the Teleost Heart. A Distinct Heart Segment. *Anat. Rec. Adv. Integr. Anat. Evol. Biol.* **294:** 236–242

Iemitsu M, Miyauchi T, Maeda S, Tanabe T, Takanashi M, Irukayama-Tomobe Y, Sakai S, Ohmori H, Matsuda M, Yamaguchi I, Aoyama T, Peters J, Iritani N, Nakajima T, Furihata K, Hashimoto T, Gonzalez F, Barger P, Kelly D, Bass A, et al (2002) Aging-induced decrease in the PPAR-α level in hearts is improved by exercise training. *Am. J. Physiol. - Hear. Circ. Physiol.* **283:** 5678–5684

Inoki K, Zhu T & Guan K-L (2003) TSC2 mediates cellular energy response to control cell growth and survival. *Cell* **115:** 577–90

Itou J, Oishi I, Kawakami H, Glass TJ, Richter J, Johnson A, Lund TC & Kawakami Y (2012) Migration of cardiomyocytes is essential for heart regeneration in zebrafish. *Development* **139:** 4133–42

Jackson HE & Ingham PW (2013) Control of muscle fibre-type diversity during embryonic development: the zebrafish paradigm. *Mech. Dev.* **130:** 447–57

Jäger S, Handschin C, St-Pierre J & Spiegelman BM (2007) AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1alpha. *Proc. Natl. Acad. Sci. U. S. A.* **104:** 12017–22

Jayakumar J, Suzuki K, Sammut IA, Smolenski RT, Khan M, Latif N, Abunasra H, Murtuza B, Amrani M & Yacoub MH (2001) Heat shock protein 70 gene transfection protects mitochondrial and ventricular function against ischemia-reperfusion injury. *Circulation* **104:** I303-7

Je HS, Yang F, Ji Y, Nagappan G, Hempstead BL & Lu B (2012) Role of pro-brain-derived neurotrophic factor (proBDNF) to mature

BDNF conversion in activity-dependent competition at developing neuromuscular synapses. *Proc. Natl. Acad. Sci. U. S. A.* **109:** 15924–9

Jean MJ, Deverteuil P, Lopez NH, Tapia JD & Schoffstall B (2012) Adult zebrafish hearts efficiently compensate for excessive forced overload cardiac stress with hyperplastic cardiomegaly. *Biores. Open Access* **1:** 88–91

Jessen N & Goodyear LJ (2005) Contraction signaling to glucose transport in skeletal muscle. *J. Appl. Physiol.* **99:** 330–7

Jiang L, Romero-Carvajal A, Haug JS, Seidel CW & Piotrowski T (2014) Gene-expression analysis of hair cell regeneration in the zebrafish lateral line. *Proc. Natl. Acad. Sci. U. S. A.* **111:** E1383-92

Jibb LA & Richards JG (2008) AMP-activated protein kinase activity during metabolic rate depression in the hypoxic goldfish, Carassius auratus. *J. Exp. Biol.* **211:** 3111–22

Johansen KA, Sealey WM & Overturf K (2006) The effects of chronic immune stimulation on muscle growth in rainbow trout. *Comp. Biochem. Physiol. Part B Biochem. Mol. Biol.* **144:** 520–531

Johnston IA, Bower NI & Macqueen DJ (2011) Growth and the regulation of myotomal muscle mass in teleost fish. *J. Exp. Biol.* **214:** 1617–28

Johnston IA, Lee H-T, Macqueen DJ, Paranthaman K, Kawashima C, Anwar A, Kinghorn JR & Dalmay T (2009) Embryonic temperature affects muscle fibre recruitment in adult zebrafish: genome-wide changes in gene and microRNA expression associated with the transition from hyperplastic to hypertrophic growth phenotypes. *J. Exp. Biol.* **212:** 1781–1793

Jonsdottir IH, Schjerling P, Ostrowski K, Asp S, Richter EA & Pedersen BK (2000) Muscle contractions induce interleukin-6 mRNA production in rat skeletal muscles. *J. Physiol.* **528 Pt 1:** 157–63

Jopling C, Sleep E, Raya M, Martí M, Raya A & Izpisúa Belmonte JC (2010a) Zebrafish heart regeneration occurs by cardiomyocyte dedifferentiation and proliferation. *Nature* **464:** 3–8

Jopling C, Sleep E, Raya M, Martí M, Raya A & Izpisúa Belmonte JC (2010b) Zebrafish heart regeneration occurs by cardiomyocyte dedifferentiation and proliferation_Supplementary. *Nature* **464:** 606–9

Jopling C, Suñé G, Faucherre A, Fabregat C & Izpisua Belmonte JC (2012a) Hypoxia induces myocardial regeneration in zebrafish. *Circulation* **126:** 3017–27

Jopling C, Suñé G, Morera C & Belmonte JCI (2012b) p38a MAPK regulates myocardial regeneration in zebrfish. *Cell Cycle* **11:** 1195–1201

Jørgensen LH, Petersson SJ, Sellathurai J, Andersen DC, Thayssen S, Sant DJ, Jensen CH & Schrøder HD (2009) Secreted protein acidic and rich in cysteine (SPARC) in human skeletal muscle. *J. Histochem. Cytochem.* **57:** 29–39

Kahn BB, Alquier T, Carling D & Hardie DG (2005) AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. *Cell Metab.* **1:** 15–25

Kaitetzidou E, Crespo D, Vraskou Y, Antonopoulou E & Planas J V (2012) Transcriptomic response of skeletal muscle to lipopolysaccharide in the gilthead seabream (Sparus aurata). *Mar. Biotechnol. (NY).* **14:** 605–19

Kajstura J, Urbanek K, Perl S, Hosoda T, Zheng H, Ogórek B, Ferreira-Martins J, Goichberg P, Rondon-Clavo C, Sanada F, D'Amario D, Rota M, Del Monte F, Orlic D, Tisdale J, Leri A & Anversa P (2010) Cardiomyogenesis in the adult human heart. *Circ. Res.* **107:** 305–15

Kantidakis T, Ramsbottom BA, Birch JL, Dowding SN & White RJ (2010) mTOR associates with TFIIIC, is found at tRNA and 5S rRNA genes, and targets their repressor Maf1. *Proc. Natl. Acad. Sci. U. S. A.* **107:** 11823–8

Kanzleiter T, Rath M, Görgens SW, Jensen J, Tangen DS, Kolnes AJ, Kolnes KJ, Lee S, Eckel J, Schürmann A & Eckardt K (2014) The myokine decorin is regulated by contraction and involved in muscle hypertrophy. *Biochem. Biophys. Res. Commun.* **450:** 1089–94

Karinch AM, Pan M, Lin CM, Strange R & Souba WW (2001) Glutamine metabolism in sepsis and infection. *J. Nutr.* **131:** 2535S–8S; discussion 2550S–1S

Karra R, Knecht AK, Kikuchi K & Poss KD (2015) Myocardial NF-κB activation is essential for zebrafish heart regeneration. *Proc. Natl. Acad. Sci.* **112:** 13255–13260

Kawahara G, Karpf JA, Myers JA, Alexander MS, Guyon JR & Kunkel LM (2011) Drug screening in a zebrafish model of Duchenne muscular dystrophy. *Proc. Natl. Acad. Sci.* **108:** 5331–5336

Kawanishi N, Yano H, Yokogawa Y & Suzuki K (2010) Exercise training inhibits inflammation in adipose tissue via both suppression of macrophage infiltration and acceleration of phenotypic switching from M1 to M2 macrophages in high-fat-diet-induced obese mice. *Exerc. Immunol. Rev.* **16:** 105–18

Keller C, Steensberg A, Pilegaard H, Osada T, Saltin B, Pedersen BK & Neufer PD (2001) Transcriptional activation of the IL-6 gene in human contracting skeletal muscle: influence of muscle glycogen content. *FASEB J.* **15:** 2748–50

Keller P, Vollaard NBJ, Gustafsson T, Gallagher IJ, Sundberg CJ, Rankinen T, Britton SL, Bouchard C, Koch LG & Timmons JA (2011) A transcriptional map of the impact of endurance exercise training on skeletal muscle phenotype. *J. Appl. Physiol.* **110:** 46–59

Kelly M, Gauthier M-S, Saha AK & Ruderman NB (2009) Activation of AMP-activated protein kinase by interleukin-6 in rat skeletal muscle: association with changes in cAMP, energy state, and endogenous fuel mobilization. *Diabetes* **58:** 1953–60

Kemi OJ, Ceci M, Wisloff U, Grimaldi S, Gallo P, Smith GL, Condorelli G & Ellingsen O (2008) Activation or inactivation of cardiac Akt/mTOR signaling diverges physiological from pathological hypertrophy. *J. Cell. Physiol.* **214:** 316–21

Kemi OJ, Haram PM, Loennechen JP, Osnes J-B, Skomedal T, Wisløff U & Ellingsen Ø (2005) Moderate vs. high exercise intensity: Differential effects on aerobic fitness, cardiomyocyte contractility, and endothelial function. **67:** 161–172

Kemi OJ, Haram PM, Wisløff U & Ellingsen Ø (2004) Aerobic fitness is associated with cardiomyocyte contractile capacity and endothelial function in exercise training and detraining. *Circulation* **109:** 2897–904

Kikuchi K, Gupta V, Wang J, Holdway JE, Wills AA, Fang Y & Poss KD (2011a) tcf21+ epicardial cells adopt non-myocardial fates during zebrafish heart development and regeneration. *Development* **138:** 2895–902

Kikuchi K, Holdway JE, Major RJ, Blum N, Dahn RD, Begemann G & Poss KD (2011b) Retinoic acid production by endocardium and epicardium is an injury response essential for zebrafish heart regeneration. *Dev. Cell* **20:** 397–404

Kikuchi K, Holdway JE, Werdich A a, Anderson RM, Fang Y, Egnaczyk GF, Evans T, Macrae C a, Stainier DYR & Poss KD (2010) Primary contribution to zebrafish heart regeneration by gata4(+) cardiomyocytes. *Nature* **464:** 601–5

Kim J, Wende AR, Sena S, Theobald HA, Soto J, Sloan C, Wayment BE, Litwin SE, Holzenberger M, LeRoith D & Abel ED (2008) Insulin-Like Growth Factor I Receptor Signaling Is Required for Exercise-Induced Cardiac Hypertrophy. *Mol. Endocrinol.* **22:** 2531–2543

Kim J, Wu Q, Zhang Y, Wiens KM, Huang Y, Rubin N & Shimada H (2010) PDGF signaling is required for epicardial function and blood vessel formation in regenerating zebra fi sh hearts.

Koch LG, Pollott GE, Britton SL, Albert F, Carlborg O, Plyusnina I, Besnier F, Hedwig D, Lautenschlager S, Lorenz D, McIntosh J, Neumann C, Richter H, Zeising C, Kozhemyakina R, Shchepina O, Kratzsch J, Trut L, Teupser D, Thiery J, et al (2013) Selectively bred rat model system for low and high response to exercise training. *Physiol. Genomics* **45:** 606–14

Kokkinos P & Myers J (2010) Exercise and physical activity: clinical outcomes and applications. *Circulation* **122:** 1637–48

Kopp R, Köblitz L, Egg M, Pelster B, Adams V, Linke A, Krankel N, Erbs S, Gielen S, Mobius-Winkler S, Gummert J, Mohr F, Schuler G, Hambrecht R, Alpadi K, Magupalli V, Käppel S, Köblitz L, Schwarz K, Seigel G, et al (2011) HIF signaling and overall gene expression changes during hypoxia and prolonged exercise differ considerably. *Physiol. Genomics* **43:** 506–516

Kozàkovà M, Galetta F, Gregorini L, Bigalli G, Franzoni F, Giusti C & Palombo C (2000) Coronary vasodilator capacity and epicardial vessel remodeling in physiological and hypertensive hypertrophy. *Hypertens. (Dallas, Tex. 1979)* **36:** 343–9

Kubica N, Bolster DR, Farrell PA, Kimball SR & Jefferson LS (2005) Resistance exercise increases muscle protein synthesis and translation of eukaryotic initiation factor 2Bepsilon mRNA in a mammalian target of rapamycin-dependent manner. *J. Biol. Chem.* **280:** 7570–80

Kukidome D, Nishikawa T, Sonoda K, Imoto K, Fujisawa K, Yano M, Motoshima H, Taguchi T, Matsumura T & Araki E (2006) Activation of AMP-activated protein kinase reduces hyperglycemia-induced mitochondrial reactive oxygen species production and promotes mitochondrial biogenesis in human umbilical vein endothelial cells. *Diabetes* **55:** 120–7

Kuschel A, Simon P & Tug S (2012) Functional regulation of HIF-1α under normoxia--is there more than post-translational regulation? *J. Cell. Physiol.* **227:** 514–24

Laaksonen DE, Lindström J, Lakka TA, Eriksson JG, Niskanen L, Wikström K, Aunola S, Keinänen-Kiukaanniemi S, Laakso M, Valle TT, Ilanne-Parikka P, Louheranta A, Hämäläinen H, Rastas M, Salminen V, Cepaitis Z, Hakumäki M, Kaikkonen H, Härkönen P, Sundvall J, et al (2005) Physical activity in the prevention of type 2 diabetes: the Finnish diabetes prevention study. *Diabetes* **54:** 158–65

Laplante M & Sabatini DM (2012) mTOR signaling in growth control and disease. *Cell* **149:** 274–93

Lavie CJ, Arena R, Swift DL, Johannsen NM, Sui X, Lee D, Earnest CP, Church TS, O'Keefe JH, Milani R V. & Blair SN (2015) Exercise and the Cardiovascular System. *Circ. Res.* **117:** 207–219

Lawler PR, Filion KB & Eisenberg MJ (2011) Efficacy of exercise-based cardiac rehabilitation post-myocardial infarction: a systematic review and meta-analysis of randomized controlled trials. *Am. Heart J.* **162:** 571–584.e2

Lee HJ, Lee JO, Kim N, Kim JK, Kim HI, Lee YW, Kim SJ, Choi J-I, Oh Y, Kim JH, Suyeon-Hwang, Park SH & Kim HS (2015a) Irisin, a Novel Myokine, Regulates Glucose Uptake in Skeletal Muscle Cells via AMPK. *Mol. Endocrinol.* **29:** 873–81

Lee I-M, Shiroma EJ, Lobelo F, Puska P, Blair SN & Katzmarzyk PT (2012a) Effect of physical inactivity on major non-communicable diseases worldwide: an analysis of burden of disease and life expectancy. *Lancet* **380:** 219–29

Lee K, Ochi E, Song H & Nakazato K (2015b) Activation of AMP-activated protein kinase induce expression of FoxO1, FoxO3a, and myostatin after exercise-induced muscle damage. *Biochem. Biophys. Res. Commun.* **466:** 289–94

Lee L, Genge CEC, Cua M, Sheng X, Rayani K, Beg MF, Sarunic MVM, Tibbits GGF, Steinhausen M, Sandblom E, Eliason E, Verhille C, Farrell A aJD, Gollock M, Currie S, Petersen L, Gamperl A, Somero G, Paajanen V, Vornanen M, et al (2016) Functional Assessment of Cardiac Responses of Adult Zebrafish (Danio rerio) to Acute and Chronic Temperature Change Using High-Resolution Echocardiography. *PLoS One* **11:** e0145163

Lee P, Linderman JD, Smith S, Brychta RJ, Wang J, Idelson C, Perron RM, Werner CD, Phan GQ, Kammula US, Kebebew E, Pacak K, Chen KY & Celi FS (2014) Irisin and FGF21 are cold-induced endocrine activators of brown fat function in humans. *Cell Metab.* **19:** 302–9

Lee S-J, Huynh T V, Lee Y-S, Sebald SM, Wilcox-Adelman SA, Iwamori N, Lepper C, Matzuk MM & Fan C-M (2012b) Role of satellite cells versus myofibers in muscle hypertrophy induced by inhibition of the myostatin/activin signaling pathway. *Proc. Natl. Acad. Sci. U. S. A.* **109:** E2353-60

Lee S-J, Lee Y-S, Zimmers TA, Soleimani A, Matzuk MM, Tsuchida K, Cohn RD & Barton ER (2010) Regulation of muscle mass by follistatin and activins. *Mol. Endocrinol.* **24:** 1998–2008

Lee SJ & McPherron AC (2001) Regulation of myostatin activity and muscle growth. *Proc. Natl. Acad. Sci. U. S. A.* **98:** 9306–11

Léger B, Cartoni R, Praz M, Lamon S, Dériaz O, Crettenand A, Gobelet C, Rohmer P, Konzelmann M, Luthi F & Russell AP (2006) Akt signalling through GSK-3beta, mTOR and Foxo1 is involved in human skeletal muscle hypertrophy and atrophy. *J. Physiol.* **576:** 923–33

Lehnen AM, Leguisamo NM, Pinto GH, Markoski MM, De Angelis K, Machado UF & Schaan B (2010) The beneficial effects of exercise in rodents are preserved after detraining: a phenomenon unrelated to GLUT4 expression. *Cardiovasc. Diabetol.* **9:** 67

Leite CF, Lopes CS, Alves AC, Fuzaro CSC, Silva MV, Oliveira LF de, Garcia LP, Farnesi TS, Cuba MB de, Rocha LB, Rodrigues V, Oliveira CJF de & Dias da Silva VJ (2015) Endogenous resident c-Kit cardiac stem cells increase in mice with an exercise-induced, physiologically hypertrophied heart. *Stem Cell Res.* **15:** 151–164

Leiter JRS, Peeler J & Anderson JE (2011) Exercise-induced muscle growth is muscle-specific and age-dependent. *Muscle Nerve* **43:** 828–38

LeMoine CMR, Craig PM, Dhekney K, Kim JJ & McClelland GB (2010a) Temporal and spatial patterns of gene expression in skeletal muscles in response to swim training in adult zebrafish (Danio rerio). *J. Comp. Physiol. B.* **180:** 151–60

LeMoine CMR, Lougheed SC & Moyes CD (2010b) Modular Evolution of PGC-1α in Vertebrates. *J. Mol. Evol.* **70:** 492–505

Lennon SL, Quindry J, Hamilton KL, French J, Staib J, Mehta JL & Powers SK (2004) Loss of exercise-induced cardioprotection after cessation of exercise. *J. Appl. Physiol.* **96:** 1299–305

Lepilina A, Coon AN, Kikuchi K, Holdway JE, Roberts RW, Burns CG & Poss KD (2006) A Dynamic Epicardial Injury Response Supports Progenitor Cell Activity during Zebrafish Heart Regeneration. *Cell* **127:** 607–619

Li X-N, Song J, Zhang L, LeMaire SA, Hou X, Zhang C, Coselli JS, Chen L, Wang XL, Zhang Y & Shen YH (2009) Activation of the AMPK-FOXO3 pathway reduces fatty acid-induced increase in intracellular reactive oxygen species by upregulating thioredoxin. *Diabetes* **58:** 2246–57

Liao J, Li Y, Zeng F & Wu Y (2015) Regulation of mTOR Pathway in Exercise-induced Cardiac Hypertrophy. *Int. J. Sports Med.* **36:** 343–50

Lien C-L, Schebesta M, Makino S, Weber GJ & Keating MT (2006) Gene expression analysis of zebrafish heart regeneration. *PLoS Biol.* **4:** e260

Lieschke GJ & Currie PD (2007) Animal models of human disease: zebrafish swim into view. *Nat. Rev. Genet.* **8:** 353–367

Lin J, Wu H, Tarr PT, Zhang C-Y, Wu Z, Boss O, Michael LF, Puigserver P, Isotani E, Olson EN, Lowell BB, Bassel-Duby R & Spiegelman BM (2002) Transcriptional co-activator PGC-1 alpha drives the formation of slow-twitch muscle fibres. *Nature* **418:** 797–801

Lipina C, Kendall H, McPherron AC, Taylor PM & Hundal HS (2010) Mechanisms involved in the enhancement of mammalian target of rapamycin signalling and hypertrophy in skeletal muscle of myostatin-deficient mice. *FEBS Lett.* **584:** 2403–8

Liu CC, Li L, Lam YW, Siu CW & Cheng SH (2016) Improvement of surface ECG recording in adult zebrafish reveals that the value of this model exceeds our expectation. *Sci. Rep.* **6:**

Liu Y, Cao Z-D, Fu S-J, Peng J-L & Wang Y-X (2009) The effect of exhaustive chasing training and detraining on swimming performance in juvenile darkbarbel catfish (Peltebagrus vachelli). *J. Comp. Physiol. B* **179:** 847–855

Louis E, Raue U, Yang Y, Jemiolo B & Trappe S (2007) Time course of proteolytic, cytokine, and myostatin gene expression after acute exercise in human skeletal muscle. *J. Appl. Physiol.* **103:** 1744–51

Love NR, Chen Y, Ishibashi S, Kritsiligkou P, Lea R, Koh Y, Gallop JL, Dorey K & Amaya E (2013) Amputation-induced reactive oxygen species are required for successful Xenopus tadpole tail regeneration. *Nat. Cell Biol.* **15:** 222–228

Lumeng CN, Bodzin JL & Saltiel AR (2007) Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *J. Clin. Invest.* **117:** 175–184

Lundby C, Gassmann M & Pilegaard H (2006) Regular endurance training reduces the exercise induced HIF-1α and HIF-2α mRNA expression in human skeletal muscle in normoxic conditions. *Eur. J. Appl. Physiol.* **96:** 363–369

Luquet S (2003) Peroxisome proliferator-activated receptor controls muscle development and oxydative capability. *FASEB J.* **17:** 2299–2301

Ma X, Fu Y, Xiao H, Song Y, Chen R, Shen J, An X, Shen Q, Li Z, Zhang Y, Peter P, Brady J, Yan L, Chen W, Engelhardt S, Wang Y, Kwak H, Kim J, Joshi K, Yeh A, et al (2015) Cardiac Fibrosis Alleviated by Exercise Training Is AMPK-Dependent. *PLoS One* **10:** e0129971

Macaluso F & Myburgh KH (2012) Current evidence that exercise can increase the number of adult stem cells. *J. Muscle Res. Cell Motil.* **33:** 187–198

Magnoni LJ, Crespo D, Ibarz A, Blasco J, Fernández-Borràs J & Planas J V (2013a) Effects of sustained swimming on the red and white muscle transcriptome of rainbow trout (Oncorhynchus mykiss) fed a carbohydrate-rich diet. *Comp. Biochem. Physiol. A. Mol. Integr. Physiol.* **166:** 510–21

Magnoni LJ, Felip O, Blasco J & Planas J V. (2013b) Metabolic Fuel Utilization During Swimming: Optimizing Nutritional Requirements for Enhanced Performance. In *Swimming Physiology of Fish* pp 203–235. Berlin, Heidelberg: Springer Berlin Heidelberg

Magnoni LJ, Palstra AP & Planas J V (2014) Fueling the engine: induction of AMP-activated protein kinase in trout skeletal muscle by swimming. *J. Exp. Biol.* **217:** 1649–52

Magnoni LJ, Roher N, Crespo D, Krasnov A & Planas J V (2015) In Vivo Molecular Responses of Fast and Slow Muscle Fibers to Lipopolysaccharide in a Teleost Fish, the Rainbow Trout (Oncorhynchus mykiss). *Biology (Basel).* **4:** 67–87

Magnoni LJ, Vraskou Y, Palstra AP & Planas J V (2012) AMP-activated protein kinase plays an important evolutionary conserved role in the regulation of glucose metabolism in fish skeletal muscle cells. *PLoS One* **7:** e31219

Mahmoud AI, O'Meara CC, Gemberling M, Zhao L, Bryant DM, Zheng R, Gannon JB, Cai L, Choi W-Y, Egnaczyk GF, Burns CE, Burns CG, MacRae CA, Poss KD & Lee RT (2015) Nerves Regulate Cardiomyocyte Proliferation and Heart Regeneration. *Dev. Cell* **34:** 387–399

Maillet M, van Berlo JH & Molkentin JD (2013) Molecular basis of physiological heart growth: fundamental concepts and new players. *Nat. Rev. Mol. Cell Biol.* **14:** 38–48

Maki N, Suetsugu-Maki R, Tarui H, Agata K, Del Rio-Tsonis K & Tsonis PA (2009) Expression of stem cell pluripotency factors during regeneration in newts. *Dev. Dyn.* **238:** 1613–6

Makinde AO, Kantor PF & Lopaschuk GD (1998) Maturation of fatty acid and carbohydrate metabolism in the newborn heart. *Mol. Cell. Biochem.* **188:** 49–56

Manceau M, Gros J, Savage K, Thomé V, McPherron A, Paterson B & Marcelle C (2008) Myostatin promotes the terminal differentiation of embryonic muscle progenitors. *Genes Dev.* **22:** 668–81

Manio MCC, Inoue K, Fujitani M, Matsumura S & Fushiki T (2016) Combined pharmacological activation of AMPK and PPARδ potentiates the effects of exercise in trained mice. *Physiol. Rep.* **4:**

Mann N & Rosenzweig A (2012) Can exercise teach us how to treat heart disease? *Circulation* **126:** 2625–35

Marini M, Falcieri E, Margonato V, Treré D, Lapalombella R, di Tullio S, Marchionni C, Burattini S, Samaja M, Esposito F & Veicsteinas A (2008) Partial persistence of exercise-induced myocardial angiogenesis following 4-week detraining in the rat. *Histochem. Cell Biol.* **129:** 479–87

Marín-Juez R, Rovira M, Crespo D, van der Vaart M, Spaink HP & Planas J V (2015) GLUT2-mediated glucose uptake and availability

are required for embryonic brain development in zebrafish. *J. Cereb. Blood Flow Metab.* **35:** 74–85

Marino M, Scuderi F, Provenzano C & Bartoccioni E (2011) Skeletal muscle cells: from local inflammatory response to active immunity. *Gene Ther.* **18:** 109–116

Marques IJ, Leito JTD, Spaink HP, Testerink J, Jaspers RT, Witte F, van den Berg S & Bagowski CP (2008) Transcriptome analysis of the response to chronic constant hypoxia in zebrafish hearts. *J. Comp. Physiol. B.* **178:** 77–92

Mathers CD & Loncar D (2006) Projections of global mortality and burden of disease from 2002 to 2030. *PLoS Med.* **3:** e442

Mathieu-Costello O, Agey PJ, Wu L, Hang J & Adair TH (1996) Capillary-to-fiber surface ratio in rat fast-twitch hindlimb muscles after chronic electrical stimulation. *J. Appl. Physiol.* **80:** 904–9

Matsakas A, Macharia R, Otto A, Elashry MI, Mouisel E, Romanello V, Sartori R, Amthor H, Sandri M, Narkar V & Patel K (2012) Exercise training attenuates the hypermuscular phenotype and restores skeletal muscle function in the myostatin null mouse. *Exp. Physiol.* **97:** 125–40

Matsui T, Nagoshi T & Rosenzweig A (2003) Akt and PI 3-kinase signaling in cardiomyocyte hypertrophy and survival. *Cell Cycle* **2:** 220–3

Matthews VB, Aström M-B, Chan MHS, Bruce CR, Krabbe KS, Prelovsek O, Akerström T, Yfanti C, Broholm C, Mortensen OH, Penkowa M, Hojman P, Zankari A, Watt MJ, Bruunsgaard H, Pedersen BK & Febbraio MA (2009) Brain-derived neurotrophic factor is produced by skeletal muscle cells in response to contraction and enhances fat oxidation via activation of AMP-activated protein kinase. *Diabetologia* **52:** 1409–18

Mauro A (1961) Satellite cell of skeletal muscle fibers. *J. Biophys. Biochem. Cytol.* **9:** 493–5

Maybaum S, Ilan M, Mogilevsky J & Tzivoni D (1996) Improvement in ischemic parameters during repeated exercise testing: A possible model for myocardial preconditioning. *Am. J. Cardiol.* **78:** 1087–1091

Mayer C, Zhao J, Yuan X & Grummt I (2004) mTOR-dependent activation of the transcription factor TIF-IA links rRNA synthesis to nutrient availability. *Genes Dev.* **18:** 423–34

McCarthy JJ, Mula J, Miyazaki M, Erfani R, Garrison K, Farooqui AB, Srikuea R, Lawson BA, Grimes B, Keller C, Van Zant G, Campbell KS, Esser KA, Dupont-Versteegden EE & Peterson CA (2011) Effective fiber hypertrophy in satellite cell-depleted skeletal muscle. *Development* **138:** 3657–66

McClelland GB, Craig PM, Dhekney K & Dipardo S (2006) Temperature- and exercise-induced gene expression and metabolic enzyme changes in skeletal muscle of adult zebrafish (Danio rerio). *J. Physiol.* **577:** 739–51

McCroskery S, Thomas M, Maxwell L, Sharma M & Kambadur R (2003) Myostatin negatively regulates satellite cell activation and self-renewal. *J. Cell Biol.* **162:** 1135–47

McElroy CL, Gissen SA & Fishbein MC (1978) Exercise-induced reduction in myocardial infarct size after coronary artery occlusion in the rat. *Circulation* **57:** 958–62

McGee SL & Hargreaves M (2010) AMPK-mediated regulation of transcription in skeletal muscle. *Clin. Sci. (Lond).* **118:** 507–18

McMullen JR, Shioi T, Huang W-Y, Zhang L, Tarnavski O, Bisping E, Schinke M, Kong S, Sherwood MC, Brown J, Riggi L, Kang PM & Izumo S (2004) The insulin-like growth factor 1 receptor induces physiological heart growth via the phosphoinositide 3-kinase(p110alpha) pathway. *J. Biol. Chem.* **279:** 4782–93

McPherron AC, Lawler AM & Lee S-J (1997) Regulation of skeletal muscle mass in mice by a new TGF-p superfamily member. *Nature* **387:** 83–90

McPherron AC & Lee SJ (1997) Double muscling in cattle due to mutations in the myostatin gene. *Proc. Natl. Acad. Sci. U. S. A.* **94:** 12457–61

Menke AL, Spitsbergen JM, Wolterbeek APM & Woutersen RA (2011) Normal anatomy and histology of the adult zebrafish. *Toxicol. Pathol.* **39:** 759–75

van der Meulen T, Schipper H, van den Boogaart JGM, Huising MO, Kranenbarg S & van Leeuwen JL (2006) Endurance exercise differentially stimulates heart and axial muscle development in zebrafish (Danio rerio). *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **291:** R1040-8

Michaelides AP, Andrikopoulos GK, Oikonomou E V, Psomadaki ZD, Richter DJ, Dilaveris PE, Exadaktylos NI, Stefanadis CI & Toutouzas PK (2003) Improved myocardial performance during repetitive exercise testing: The role of extracellular superoxide dismutase activity in a model of exercise-induced myocardial preconditioning. *Am. Heart J.* **146:** 160–167

Mifune M, Ohtsu H, Suzuki H, Frank GD, Inagami T, Utsunomiya H, Dempsey PJ & Eguchi S (2004) Signal transduction of betacellulin in growth and migration of vascular smooth muscle cells. *Am. J. Physiol. Cell Physiol.* **287:** C807-13

Miura T, Kishioka Y, Wakamatsu J, Hattori A, Hennebry A, Berry CJ, Sharma M, Kambadur R & Nishimura T (2006) Decorin binds myostatin and modulates its activity to muscle cells. *Biochem. Biophys. Res. Commun.* **340:** 675–80

Miyazaki M & Esser KA (2009) Cellular mechanisms regulating protein synthesis and skeletal muscle hypertrophy in animals. *J. Appl. Physiol.* **106:** 1367–73

Montarras D, L'honoré A & Buckingham M (2013) Lying low but ready for action: the quiescent muscle satellite cell. *FEBS J.* **280:** 4036–50

Mooren FC ed. (2012) Encyclopedia of Exercise Medicine in Health and Disease Berlin, Heidelberg: Springer Berlin Heidelberg

Morales Fénero CI, Colombo Flores AA & Câmara NOS (2016) Inflammatory diseases modelling in zebrafish. *World J. Exp. Med.* **6:** 9–20

Morris CA, Morris LD, Kennedy AR & Sweeney HL (2005) Attenuation of skeletal muscle atrophy via protease inhibition. *J. Appl. Physiol.* **99:** 1719–27

Mouisel E, Relizani K, Mille-Hamard L, Denis R, Hourde C, Agbulut O, Patel K, Arandel L, Morales-Gonzalez S, Vignaud A, Garcia L, Ferry A, Luquet S, Billat V, Ventura-Clapier R, Schuelke M & Amthor H (2014) Myostatin is a key mediator between energy metabolism and endurance capacity of skeletal muscle. *AJP Regul. Integr. Comp. Physiol.* **307:** R444–R454

Mousavi K & Jasmin BJ (2006) BDNF is expressed in skeletal muscle satellite cells and inhibits myogenic differentiation. *J. Neurosci.* **26:** 5739–49

Muller LMAJ, Gorter KJ, Hak E, Goudzwaard WL, Schellevis FG, Hoepelman AIM & Rutten GEHM (2005) Increased Risk of Common Infections in Patients with Type 1 and Type 2 Diabetes Mellitus. *Clin. Infect. Dis.* **41:** 281–288

Muñoz-Cánoves P, Scheele C, Pedersen BK & Serrano AL (2013) Interleukin-6 myokine signaling in skeletal muscle: a double-edged sword? *FEBS J.* **280:** 4131–48

Murry CE, Jennings RB & Reimer KA (1986) Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* **74:** 1124–36

Nachlas M, Tsou K, De Souza E, Cheng C & Seilgman A (1957) Cytochemical demonstration of succinic dehydrogenase by the use of a new p-nitrophenyl substituted ditetrazole. *J. Histochem. Cytochem.* **5:** 420–36

Nagaraju K (2001) Immunological capabilities of skeletal muscle cells. *Acta Physiol. Scand.* **171:** 215–23

Narkar VA, Downes M, Yu RT, Embler E, Wang Y-X, Banayo E, Mihaylova MM, Nelson MC, Zou Y, Juguilon H, Kang H, Shaw RJ & Evans RM (2008) AMPK and PPARδ Agonists Are Exercise Mimetics. *Cell* **134:** 405–415

Nedachi T, Fujita H & Kanzaki M (2008) Contractile C2C12 myotube model for studying exercise-inducible responses in skeletal muscle. *Am. J. Physiol. Endocrinol. Metab.* **295:** E1191-204

Neri Serneri GG, Boddi M, Modesti PA, Cecioni I, Coppo M, Padeletti L, Michelucci A, Colella A & Galanti G (2001) Increased cardiac sympathetic activity and insulin-like growth factor-I formation are associated with physiological hypertrophy in athletes. *Circ. Res.* **89:** 977–82

Nichol KE, Poon WW, Parachikova AI, Cribbs DH, Glabe CG & Cotman CW (2008) Exercise alters the immune profile in Tg2576 Alzheimer mice toward a response coincident with improved cognitive performance and decreased amyloid. *J. Neuroinflammation* **5:** 13

Nicoli S, Standley C, Walker P, Hurlstone A, Fogarty KE & Lawson ND (2010) MicroRNA-mediated integration of haemodynamics and Vegf signalling during angiogenesis. *Nature* **464:** 1196–200

Nielsen AR, Hojman P, Erikstrup C, Fischer CP, Plomgaard P, Mounier R, Mortensen OH, Broholm C, Taudorf S, Krogh-Madsen R, Lindegaard B, Petersen AMW, Gehl J & Pedersen BK (2008) Association between interleukin-15 and obesity: interleukin-15 as a potential regulator of fat mass. *J. Clin. Endocrinol. Metab.* **93:** 4486–93

Nielsen AR, Mounier R, Plomgaard P, Mortensen OH, Penkowa M, Speerschneider T, Pilegaard H & Pedersen BK (2007) Expression of interleukin-15 in human skeletal muscle effect of exercise and muscle fibre type composition. *J. Physiol.* **584:** 305–12

Nielsen S & Pedersen BK (2008) Skeletal muscle as an immunogenic organ. *Curr. Opin. Pharmacol.* **8:** 346–51

Norheim F, Langleite TM, Hjorth M, Holen T, Kielland A, Stadheim HK, Gulseth HL, Birkeland KI, Jensen J & Drevon CA (2014) The effects of acute and chronic exercise on PGC-1α, irisin and browning of subcutaneous adipose tissue in humans. *FEBS J.* **281:** 739–49

Novoa B, Bowman T V, Zon L & Figueras A (2009) LPS response and tolerance in the zebrafish (Danio rerio). *Fish Shellfish Immunol.* **26:** 326–31

Nygaard H, Slettaløkken G, Vegge G, Hollan I, Whist JE, Strand T, Rønnestad BR & Ellefsen S (2015) Irisin in blood increases transiently after single sessions of intense endurance exercise and heavy strength training. *PLoS One* **10:** e0121367

Nystrom G, Pruznak A, Huber D, Frost RA & Lang CH (2009) Local insulin-like growth factor I prevents sepsis-induced muscle atrophy. *Metabolism.* **58:** 787–97

O'Neil TK, Duffy LR, Frey JW & Hornberger TA (2009) The role of phosphoinositide 3-kinase and phosphatidic acid in the regulation of mammalian target of rapamycin following eccentric contractions. *J. Physiol.* **587:** 3691–701

Ochi H & Westerfield M (2007) Signaling networks that regulate muscle development: Lessons from zebrafish. *Dev. Growth Differ.* **49:** 1–11

Olesen J, Biensø RS, Meinertz S, van Hauen L, Rasmussen SM, Gliemann L, Plomgaard P & Pilegaard H (2015) Impact of training status on LPS-induced acute inflammation in humans. *J. Appl. Physiol.* **118:** 818–29

Olesen J, Kiilerich K & Pilegaard H (2010) PGC-1α-mediated adaptations in skeletal muscle. *Pflügers Arch. - Eur. J. Physiol.* **460:** 153–162

Ookawara T, Suzuk K, Haga S, Ha S, Chung K-S, Toshinai K, Hamaoka T, Katsumura T, Takemasa T, Mizuno M, Hitomi Y, Kizaki T, Suzuki K & Ohno H (2002) Transcription regulation of gene expression in human skeletal muscle in response to endurance training. *Res. Commun. Mol. Pathol. Pharmacol.* **111:** 41–54

Otto A, Collins-Hooper H, Patel A, Dash PR & Patel K (2011) Adult Skeletal Muscle Stem Cell Migration Is Mediated by a Blebbing/Amoeboid Mechanism. *Rejuvenation Res.* **14:** 249–260

Paik EJ & Zon LI (2010) Hematopoietic development in the zebrafish. *Int. J. Dev. Biol.* **54:** 1127–37

Palstra AP, Beltran S, Burgerhout E, Brittijn SA, Magnoni LJ, Henkel C V, Jansen HJ, van den Thillart GEEJM, Spaink HP & Planas J V (2013) Deep RNA sequencing of the skeletal muscle transcriptome in swimming fish. *PLoS One* **8:** e53171

Palstra AP, Rovira M, Rizo-Roca D, Torrella JR, Spaink HP & Planas J V (2014) Swimming-induced exercise promotes hypertrophy and vascularization of fast skeletal muscle fibres and activation of myogenic and angiogenic transcriptional programs in adult zebrafish. *BMC Genomics* **15:** 1136

Palstra AP, Tudorache C, Rovira M, Brittijn SA, Burgerhout E, van den Thillart GEEJM, Spaink HP & Planas J V. (2010) Establishing zebrafish as a novel exercise model: Swimming economy, swimming-enhanced growth and muscle growth marker gene expression. *PLoS One* **5:**

Park I, Qian D, Kiel M, Becker MW, Pihalja M, Weissman IL, Morrison SJ & Clarke MF (2003) Bmi-1 is required for maintenance of adult self-renewing haematopoietic stem cells. *Nature* **423:** 302–305

Parra VM, Macho P, Sánchez G, Donoso P & Domenech RJ (2015) Exercise preconditioning of myocardial infarct size in dogs is triggered by calcium. *J. Cardiovasc. Pharmacol.* **65:** 276–81

Pate RR, Pratt M, Blair SN, Haskell WL, Macera CA, Bouchard C, Buchner D, Ettinger W, Heath GW & King AC (1995) Physical activity and public health. A recommendation from the Centers for Disease Control and Prevention and the American College of Sports Medicine. *JAMA* **273:** 402–7

Paul PK, Gupta SK, Bhatnagar S, Panguluri SK, Darnay BG, Choi Y & Kumar A (2010) Targeted ablation of TRAF6 inhibits skeletal muscle wasting in mice. *J. Cell Biol.* **191:** 1395–411

Pedersen BK (2012) Muscular interleukin-6 and its role as an energy sensor. *Med. Sci. Sports Exerc.* **44:** 392–6

Pedersen BK & Febbraio MA (2008) Muscle as an endocrine organ: focus on muscle-derived interleukin-6. *Physiol. Rev.* **88:** 1379–406

Pedersen BK & Febbraio MA (2012) Muscles, exercise and obesity: skeletal muscle as a secretory organ. *Nat. Rev. Endocrinol.* **8:** 457–65

Pedersen BK, Steensberg A, Fischer C, Keller C, Keller P, Plomgaard P, Febbraio M & Saltin B (2003) Searching for the exercise factor: is IL-6 a candidate? *J. Muscle Res. Cell Motil.* **24:** 113–9

Pekkala S, Wiklund PK, Hulmi JJ, Ahtiainen JP, Horttanainen M, Pöllänen E, Mäkelä KA, Kainulainen H, Häkkinen K, Nyman K, Alén M, Herzig K-H & Cheng S (2013) Are skeletal muscle FNDC5 gene expression and irisin release regulated by exercise and related to health? *J. Physiol.* **591:** 5393–400

Pelster B (2002) Developmental plasticity in the cardiovascular system of fish, with special reference to the zebrafish. *Comp. Biochem. Physiol. A. Mol. Integr. Physiol.* **133:** 547–53

Pelster B, Sänger a M, Siegele M & Schwerte T (2003) Influence of swim training on cardiac activity, tissue capillarization, and mitochondrial density in muscle tissue of zebrafish larvae. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **285:** R339-47

Petersen AMW & Pedersen BK (2005) The anti-inflammatory effect of exercise. *J. Appl. Physiol.* **98:** 1154–62

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **29:** e45

Philibert C, Bouillot S, Huber P & Faury G (2012) Protocadherin-12 deficiency leads to modifications in the structure and function of arteries in mice. *Pathol. Biol. (Paris).* **60:** 34–40

Philp A, Hamilton DL & Baar K (2011) Signals mediating skeletal muscle remodeling by resistance exercise: PI3-kinase independent activation of mTORC1. *J. Appl. Physiol.* **110:** 561–568

Pierce JR, Maples JM & Hickner RC (2015) IL-15 concentrations in skeletal muscle and subcutaneous adipose tissue in lean and obese humans: local effects of IL-15 on adipose tissue lipolysis. *Am. J. Physiol. Endocrinol. Metab.* **308:** E1131-9

Pietretti D & Wiegertjes GF (2014) Ligand specificities of Toll-like receptors in fish: indications from infection studies. *Dev. Comp. Immunol.* **43:** 205–22

Pillon NJ, Bilan PJ, Fink LN & Klip A (2013) Cross-talk between skeletal muscle and immune cells: muscle-derived mediators and metabolic implications. *Am. J. Physiol. Endocrinol. Metab.* **304:** E453-65

Pipalia TG, Koth J, Roy SD, Hammond CL, Kawakami K & Hughes SM (2016) Cellular dynamics of regeneration reveals role of two distinct Pax7 stem cell populations in larval zebrafish muscle repair. *Dis. Model. Mech.* **9:** 671–84

Platt C, Houstis N & Rosenzweig A (2015) Using Exercise to Measure and Modify Cardiac Function. *Cell Metab.* **21:** 227–236

Plaut I & Gordon MS (1994) SWIMMING METABOLISM OF WILD-TYPE AND CLONED ZEBRAFISH BRACHYDANIO RERIO. *J. Exp. Biol.* **194:** 209–23

Plyley MJ, Olmstead BJ & Noble EG (1998) Time course of changes in capillarization in hypertrophied rat plantaris muscle. *J. Appl. Physiol.* **84:** 902–7

Poliner LR, Dehmer GJ, Lewis SE, Parkey RW, Blomqvist CG & Willerson JT (1980) Left ventricular performance in normal subjects: a comparison of the responses to exercise in the upright and supine positions. *Circulation* **62:** 528–34

Porrello ER, Mahmoud AI, Simpson E, Hill JA, Richardson JA, Olson EN & Sadek HA (2011) Transient regenerative potential of the neonatal mouse heart. *Science* **331:** 1078–80

Poss KD, Wilson LG & Keating MT (2002) Heart regeneration in zebrafish. *Science* **298:** 2188–90

Potente M, Gerhardt H & Carmeliet P (2011) Basic and therapeutic aspects of angiogenesis. *Cell* **146:** 873–87

Potthoff MJ & Olson EN (2007) MEF2: a central regulator of diverse developmental programs. *Development* **134:** 4131–40

Powers SK, Demirel HA, Vincent HK, Coombes JS, Naito H, Hamilton KL, Shanely RA & Jessup J (1998) Exercise training improves myocardial tolerance to in vivo ischemia-reperfusion in the rat. *Am. J. Physiol.* **275:** R1468-77

Prior BM, Yang HT & Terjung RL (2004) What makes vessels grow with exercise training? *J. Appl. Physiol.* **97:** 1119–28

Puente BN, Kimura W, Muralidhar SA, Moon J, Amatruda JF, Phelps KL, Grinsfelder D, Rothermel BA, Chen R, Garcia JA, Santos CX, Thet S, Mori E, Kinter MT, Rindler PM, Zacchigna S, Mukherjee S, Chen DJ, Mahmoud AI, Giacca M, et al (2014) The Oxygen-Rich Postnatal Environment Induces Cardiomyocyte Cell-Cycle Arrest through DNA Damage Response. *Cell* **157:** 565–579

Puri PL, Iezzi S, Stiegler P, Chen TT, Schiltz RL, Muscat GE, Giordano A, Kedes L, Wang JY & Sartorelli V (2001) Class I histone deacetylases sequentially interact with MyoD and pRb during skeletal myogenesis. *Mol. Cell* **8:** 885–97

Quindry JC, Hamilton KL, French JP, Lee Y, Murlasits Z, Tumer N & Powers SK (2007) Exercise-induced HSP-72 elevation and cardioprotection against infarct and apoptosis. *J. Appl. Physiol.* **103:** 1056–62

Quindry JC, Miller L, McGinnis G, Kliszczewicz B, Irwin JM, Landram M, Urbiztondo Z, Nanayakkara G & Amin R (2012) Ischemia reperfusion injury, KATP channels, and exercise-induced cardioprotection against apoptosis. *J. Appl. Physiol.* **113:** 498–506

Quinn LS, Anderson BG, Conner JD & Wolden-Hanson T (2013) IL-15 overexpression promotes endurance, oxidative energy metabolism, and muscle PPARδ, SIRT1, PGC-1α, and PGC-1β expression in male mice. *Endocrinology* **154:** 232–45

Quinn LS, Anderson BG, Drivdahl RH, Alvarez B & Argilés JM (2002) Overexpression of Interleukin-15 Induces Skeletal Muscle Hypertrophy in Vitro: Implications for Treatment of Muscle Wasting Disorders. *Exp. Cell Res.* **280:** 55–63

Quinn LS, Strait-Bodey L, Anderson BG, Argilés JM & Havel PJ (2005) Interleukin-15 stimulates adiponectin secretion by 3T3-L1 adipocytes: evidence for a skeletal muscle-to-fat signaling pathway. *Cell Biol. Int.* **29:** 449–57

Rahimi M, Shekarforoush S, Asgari AR, Khoshbaten A, Rajabi H, Bazgir B, Mohammadi MT, Sobhani V & Shakibaee A (2015) The effect of high intensity interval training on cardioprotection against ischemia-reperfusion injury in wistar rats. *EXCLI J.* **14:**

237–46

Ransom DG, Haffter P, Odenthal J, Brownlie A, Vogelsang E, Kelsh RN, Brand M, van Eeden FJ, Furutani-Seiki M, Granato M, Hammerschmidt M, Heisenberg CP, Jiang YJ, Kane DA, Mullins MC & Nüsslein-Volhard C (1996) Characterization of zebrafish mutants with defects in embryonic hematopoiesis. *Development* **123:** 311–9

Raschke S, Elsen M, Gassenhuber H, Sommerfeld M, Schwahn U, Brockmann B, Jung R, Wisløff U, Tjønna AE, Raastad T, Hallén J, Norheim F, Drevon CA, Romacho T, Eckardt K & Eckel J (2013) Evidence against a beneficial effect of irisin in humans. *PLoS One* **8:** e73680

Rasmussen P, Brassard P, Adser H, Pedersen M V, Leick L, Hart E, Secher NH, Pedersen BK & Pilegaard H (2009) Evidence for a release of brain-derived neurotrophic factor from the brain during exercise. *Exp. Physiol.* **94:** 1062–9

Razeghi P, Young ME, Abbasi S & Taegtmeyer H (2001) Hypoxia in Vivo Decreases Peroxisome Proliferator-Activated Receptor α-Regulated Gene Expression in Rat Heart. *Biochem. Biophys. Res. Commun.* **287:** 5–10

Relaix F & Zammit PS (2012) Satellite cells are essential for skeletal muscle regeneration: the cell on the edge returns centre stage. *Development* **139:** 2845–56

Remick DG, Newcomb DE, Bolgos GL & Call DR (2000) Comparison of the mortality and inflammatory response of two models of sepsis: lipopolysaccharide vs. cecal ligation and puncture. *Shock* **13:** 110–6

Renehan AG & Howell A (2005) Preventing cancer, cardiovascular disease, and diabetes. *Lancet (London, England)* **365:** 1449–51

Rescan P-Y, Montfort J, Fautrel A, Rallière C & Lebret V (2013) Gene expression profiling of the hyperplastic growth zones of the late trout embryo myotome using laser capture microdissection and microarray analysis. *BMC Genomics* **14:** 173

Riechman SE, Balasekaran G, Roth SM & Ferrell RE (2004) Association of interleukin-15 protein and interleukin-15 receptor genetic variation with resistance exercise training responses. *J. Appl. Physiol.* **97:** 2214–9

Rinnov A, Yfanti C, Nielsen S, Akerström TCA, Peijs L, Zankari A, Fischer CP & Pedersen BK (2014) Endurance training enhances skeletal muscle interleukin-15 in human male subjects. *Endocrine* **45:** 271–8

Ritchie DG (1990) Interleukin 6 stimulates hepatic glucose release from prelabeled glycogen pools. *Am. J. Physiol.* **258:** E57-64

Robson-Ansley PJ, Blannin A & Gleeson M (2007) Elevated plasma interleukin-6 levels in trained male triathletes following an acute period of intense interval training. *Eur. J. Appl. Physiol.* **99:** 353–60

Roher N, Samokhvalov V, Díaz M, MacKenzie S, Klip A & Planas J V (2008) The proinflammatory cytokine tumor necrosis factor-alpha increases the amount of glucose transporter-4 at the surface of muscle cells independently of changes in interleukin-6. *Endocrinology* **149:** 1880–9

Rommel C, Bodine SC, Clarke BA, Rossman R, Nunez L, Stitt TN, Yancopoulos GD & Glass DJ (2001) Mediation of IGF-1-induced skeletal myotube hypertrophy by PI(3)K/Akt/mTOR and PI(3)K/Akt/GSK3 pathways. *Nat. Cell Biol.* **3:** 1009–1013

Roostalu U & Strähle U (2012) In Vivo Imaging of Molecular Interactions at Damaged Sarcolemma. *Dev. Cell* **22:** 515–529

Rosenblatt S, Clowes GH, George BC, Hirsch E & Lindberg B (1983) Exchange of amino acids by muscle and liver in sepsis. *Arch. Surg.* **118:** 167–75

Ross R, Janssen I, Dawson J, Kungl A-M, Kuk JL, Wong SL, Nguyen-Duy T-B, Lee S, Kilpatrick K & Hudson R (2004) Exercise-induced reduction in obesity and insulin resistance in women: a randomized controlled trial. *Obes. Res.* **12:** 789–98

Rossi A, Kontarakis Z, Gerri C, Nolte H, Hölper S, Krüger M & Stainier DYR (2015) Genetic compensation induced by deleterious mutations but not gene knockdowns. *Nature* **524:** 230–3

De Rossi M, Bernasconi P, Baggi F, de Waal Malefyt R & Mantegazza R (2000) Cytokines and chemokines are both expressed by human myoblasts: possible relevance for the immune pathogenesis of muscle inflammation. *Int. Immunol.* **12:** 1329–35

Rotllant J, Liu D, Yan Y-L, Postlethwait JH, Westerfield M & Du S-J (2008) Sparc (Osteonectin) functions in morphogenesis of the pharyngeal skeleton and inner ear. *Matrix Biol.* **27:** 561–72

Ruas JL, White JP, Rao RR, Kleiner S, Brannan KT, Harrison BC, Greene NP, Wu J, Estall JL, Irving BA, Lanza IR, Rasbach KA, Okutsu M, Nair KS, Yan Z, Leinwand LA & Spiegelman BM (2012) A PGC-1α isoform induced by resistance training regulates skeletal muscle hypertrophy. *Cell* **151:** 1319–31

Ruyra A, Cano-Sarabia M, García-Valtanen P, Yero D, Gibert I, Mackenzie SA, Estepa A, Maspoch D & Roher N (2014) Targeting and stimulation of the zebrafish (Danio rerio) innate immune system with LPS/dsRNA-loaded nanoliposomes. *Vaccine*

Rytkönen KT, Prokkola JM, Salonen V & Nikinmaa M (2014) Transcriptional divergence of the duplicated hypoxia-inducible factor alpha genes in zebrafish

Sage H, Vernon RB, Decker J, Funk S & Iruela-Arispe ML (1989) Distribution of the calcium-binding protein SPARC in tissues of embryonic and adult mice. *J. Histochem. Cytochem.* **37:** 819–29

Saini A, Al-Shanti N, Nasser A-S & Stewart CEH (2006) Waste management - cytokines, growth factors and cachexia. *Cytokine Growth Factor Rev.* **17:** 475–86

Saleem A, Adhihetty PJ & Hood DA (2009) Role of p53 in mitochondrial biogenesis and apoptosis in skeletal muscle. *Physiol. Genomics* **37:** 58–66

Sallin P, de Preux Charles A-S, Duruz V, Pfefferli C & Jaźwińska A (2014) A dual epimorphic and compensatory mode of heart regeneration in zebrafish. *Dev. Biol.*

Sancak Y, Thoreen CC, Peterson TR, Lindquist RA, Kang SA, Spooner E, Carr SA & Sabatini DM (2007) PRAS40 is an insulin-regulated inhibitor of the mTORC1 protein kinase. *Mol. Cell* **25:** 903–15

Sanchez AMJ, Csibi A, Raibon A, Cornille K, Gay S, Bernardi H & Candau R (2012) AMPK promotes skeletal muscle autophagy through activation of forkhead FoxO3a and interaction with Ulk1. *J. Cell. Biochem.* **113:** 695–710

Sandri M (2008) Signaling in muscle atrophy and hypertrophy. *Physiology (Bethesda).* **23:** 160–70

Sandri M, Sandri C, Gilbert A, Skurk C, Calabria E, Picard A, Walsh K, Schiaffino S, Lecker SH & Goldberg AL (2004) Foxo transcription factors induce the atrophy-related ubiquitin ligase atrogin-1 and cause skeletal muscle atrophy. *Cell* **117:** 399–412

Sanger AM (1992) Effects of training on axial muscle of two cyprinid species: Chondrostoma nasus (L.) and Leuciscus cephalus (L.). *J. Fish Biol.* **40:** 637–646

Sänger AM & Stoiber W (2001) Muscle Fiber Diversity and Plasticity. In *Muscle development and growth*, Johnston I (ed) pp 187–250. San Diego, California, USA: Academic Press

Santabárbara-Ruiz P, López-Santillán M, Martínez-Rodríguez I, Binagui-Casas A, Pérez L, Milán M, Corominas M, Serras F, Finkel T, Bigarella C, Liang R, Ghaffari S, Moreira S, Stramer B, Evans I, Wood W, Martin P, Niethammer P, Grabher C, Look A, et al (2015) ROS-Induced JNK and p38 Signaling Is Required for Unpaired Cytokine Activation during Drosophila Regeneration. *PLOS Genet.* **11:** e1005595

van der Sar AM, Spaink HP, Zakrzewska A, Bitter W & Meijer AH (2009) Specificity of the zebrafish host transcriptome response to acute and chronic mycobacterial infection and the role of innate and adaptive immune components. *Mol. Immunol.* **46:** 2317–2332

Sartori R, Milan G, Patron M, Mammucari C, Blaauw B, Abraham R & Sandri M (2009) Smad2 and 3 transcription factors control muscle mass in adulthood. *Am. J. Physiol. Cell Physiol.* **296:** C1248-57

Sartori R, Schirwis E, Blaauw B, Bortolanza S, Zhao J, Enzo E, Stantzou A, Mouisel E, Toniolo L, Ferry A, Stricker S, Goldberg AL, Dupont S, Piccolo S, Amthor H & Sandri M (2013) BMP signaling controls muscle mass. *Nat. Genet.* **45:** 1309–18

Schiaffino S, Dyar KA, Ciciliot S, Blaauw B & Sandri M (2013) Mechanisms regulating skeletal muscle growth and atrophy. *FEBS J.* **280:** 4294–314

Schindler YL, Garske KM, Wang J, Firulli BA, Firulli AB, Poss KD & Yelon D (2014) Hand2 elevates cardiomyocyte production during zebrafish heart development and regeneration. *Development* **141:** 3112–3122

Schnabel K, Wu C-C, Kurth T & Weidinger G (2011) Regeneration of cryoinjury induced necrotic heart lesions in zebrafish is associated with epicardial activation and cardiomyocyte proliferation. *PLoS One* **6:** e18503

Schönherr E, Sunderkötter C, Iozzo R V & Schaefer L (2005) Decorin, a novel player in the insulin-like growth factor system. *J. Biol. Chem.* **280:** 15767–72

Sciarretta S, Volpe M & Sadoshima J (2014) Mammalian target of rapamycin signaling in cardiac physiology and disease. *Circ. Res.* **114:** 549–64

Sedmera D, Reckova M, deAlmeida A, Sedmerova M, Biermann M, Volejnik J, Sarre A, Raddatz E, McCarthy RA, Gourdie RG & Thompson RP (2003) Functional and morphological evidence for a ventricular conduction system in zebrafish and Xenopus hearts. *Am. J. Physiol. Heart Circ. Physiol.* **284:** H1152-60

Senyo SE, Steinhauser ML, Pizzimenti CL, Yang VK, Cai L, Wang M, Wu T-D, Guerquin-Kern J-L, Lechene CP & Lee RT (2012) Mammalian heart renewal by pre-existing cardiomyocytes. *Nature* **493:** 433–436

Sergeeva IA & Christoffels VM (2013) Regulation of expression of atrial and brain natriuretic peptide, biomarkers for heart development and disease. *Biochim. Biophys. Acta - Mol. Basis Dis.* **1832:** 2403–2413

Serrano AL, Baeza-Raja B, Perdiguero E, Jardí M & Muñoz-Cánoves P (2008) Interleukin-6 is an essential regulator of satellite cell-

mediated skeletal muscle hypertrophy. *Cell Metab.* **7:** 33–44

Seth A, Stemple DL & Barroso I (2013) The emerging use of zebrafish to model metabolic disease. *Dis. Model. Mech.* **6:** 1080–1088

Seyoum B, Fite A & Abou-Samra AB (2011) Effects of 3T3 adipocytes on interleukin-6 expression and insulin signaling in L6 skeletal muscle cells. *Biochem. Biophys. Res. Commun.* **410:** 13–8

El Shafey N, Guesnon M, Simon F, Deprez E, Cosette J, Stockholm D, Scherman D, Bigey P & Kichler A (2016) Inhibition of the myostatin/Smad signaling pathway by short decorin-derived peptides. *Exp. Cell Res.* **341:** 187–95

Shah BR & Hux JE (2003) Quantifying the Risk of Infectious Diseases for People With Diabetes. *Diabetes Care* **26:** 510–513

Shefer G, Rauner G, Stuelsatz P, Benayahu D & Yablonka-Reuveni Z (2013) Moderate-intensity treadmill running promotes expansion of the satellite cell pool in young and old mice. *FEBS J.* **280:** 4063–73

Shefer G, Rauner G, Yablonka-Reuveni Z & Benayahu D (2010) Reduced satellite cell numbers and myogenic capacity in aging can be alleviated by endurance exercise. *PLoS One* **5:** e13307

Shephard RJ (2010) Development of the discipline of exercise immunology. *Exerc. Immunol. Rev.* **16:** 194–222

Simoes FC, Peterkin T & Patient R (2011) Fgf differentially controls cross-antagonism between cardiac and haemangioblast regulators. *Development* **138:** 3235–3245

Sipola P, Heikkinen J, Laaksonen DE, Kettunen R, Wolfe LA, Cunningham DA, Rechnitzer PA, Nichol PM, Wieling W, Borghols EA, Hollander AP, Danner SA, Dunning AJ, Fagard R, Aubert A, Lysens R, Staessen J, Vanhees L, Amery A, Haykowsky M, et al (2009) Influence of 12 weeks of jogging on magnetic resonance-determined left ventricular characteristics in previously sedentary subjects free of cardiovascular disease. *Am. J. Cardiol.* **103:** 567–71

Smart N, Bollini S, Dubé KN, Vieira JM, Zhou B, Davidson S, Yellon D, Riegler J, Price AN, Lythgoe MF, Pu WT & Riley PR (2011) De novo cardiomyocytes from within the activated adult heart after injury. *Nature* **474:** 640–644

Smith HK & Merry TL (2012) Voluntary resistance wheel exercise during post-natal growth in rats enhances skeletal muscle satellite cell and myonuclear content at adulthood. *Acta Physiol. (Oxf).* **204:** 393–402

Smith SC, Benjamin EJ, Bonow RO, Braun LT, Creager MA, Franklin BA, Gibbons RJ, Grundy SM, Hiratzka LF, Jones DW, Lloyd-Jones DM, Minissian M, Mosca L, Peterson ED, Sacco RL, Spertus J, Stein JH & Taubert KA (2011) AHA/ACCF secondary prevention and risk reduction therapy for patients with coronary and other atherosclerotic vascular disease: 2011 update: a guideline from the American Heart Association and American College of Cardiology Foundation endorsed by the Worl. *J. Am. Coll. Cardiol.* **58:** 2432–46

Song H, Guan Y, Zhang L, Li K & Dong C (2010) SPARC interacts with AMPK and regulates GLUT4 expression. *Biochem. Biophys. Res. Commun.* **396:** 961–6

Song HK, Hong S-E, Kim T, Kim DH, Hunter J, Chien K, Catalucci D, Latronico M, Ellingsen O, Condorelli G, Ueno S, Ohki R, Hashimoto T, Takizawa T, Takeuchi K, Zhao M, Chow A, Powers J, Fajardo G, Bernstein D, et al (2012a) Deep RNA Sequencing Reveals Novel Cardiac Transcriptomic Signatures for Physiological and Pathological Hypertrophy. *PLoS One* **7:** e35552

Song MS, Salmena L & Pandolfi PP (2012b) The functions and regulation of the PTEN tumour suppressor. *Nat. Rev. Mol. Cell Biol.* **13:** 283

Stainier DY, Fouquet B, Chen JN, Warren KS, Weinstein BM, Meiler SE, Mohideen MA, Neuhauss SC, Solnica-Krezel L, Schier AF, Zwartkruis F, Stemple DL, Malicki J, Driever W & Fishman MC (1996) Mutations affecting the formation and function of the cardiovascular system in the zebrafish embryo. *Development* **123:** 285–92

Starkie R, Ostrowski SR, Jauffred S, Febbraio M & Pedersen BK (2003) Exercise and IL-6 infusion inhibit endotoxin-induced TNF-alpha production in humans. *FASEB J.* **17:** 884–6

Starkie RL, Rolland J, Angus DJ, Anderson MJ & Febbraio MA (2001) Circulating monocytes are not the source of elevations in plasma IL-6 and TNF-alpha levels after prolonged running. *Am. J. Physiol. Cell Physiol.* **280:** C769-74

Steensberg A, Febbraio MA, Osada T, Schjerling P, van Hall G, Saltin B & Pedersen BK (2001) Interleukin-6 production in contracting human skeletal muscle is influenced by pre-exercise muscle glycogen content. *J. Physiol.* **537:** 633–9

Steensberg A, Fischer CP, Keller C, Møller K & Pedersen BK (2003) IL-6 enhances plasma IL-1ra, IL-10, and cortisol in humans. *Am. J. Physiol. Endocrinol. Metab.* **285:** E433-7

Steensberg A, van Hall G, Osada T, Sacchetti M, Saltin B & Klarlund Pedersen B (2000) Production of interleukin-6 in contracting human skeletal muscles can account for the exercise-induced increase in plasma interleukin-6. *J. Physiol.* **529 Pt 1:** 237–42

Steensberg A, Keller C, Starkie RL, Osada T, Febbraio MA & Pedersen BK (2002) IL-6 and TNF-alpha expression in, and release from,

contracting human skeletal muscle. *Am. J. Physiol. Endocrinol. Metab.* **283:** E1272-8

Steiner JL & Lang CH (2015) Sepsis attenuates the anabolic response to skeletal muscle contraction. *Shock* **43:** 344–51

Stoletov K, Fang L, Choi S-H, Hartvigsen K, Hansen LF, Hall C, Pattison J, Juliano J, Miller ER, Almazan F, Crosier P, Witztum JL, Klemke RL & Miller YI (2009) Vascular lipid accumulation, lipoprotein oxidation, and macrophage lipid uptake in hypercholesterolemic zebrafish. *Circ. Res.* **104:** 952–60

Stouthard JM, Romijn JA, Van der Poll T, Endert E, Klein S, Bakker PJ, Veenhof CH & Sauerwein HP (1995) Endocrinologic and metabolic effects of interleukin-6 in humans. *Am. J. Physiol.* **268:** E813-9

Strasser B (2013) Physical activity in obesity and metabolic syndrome. *Ann. N. Y. Acad. Sci.* **1281:** 141–159

Sun JZ, Tang XL, Park SW, Qiu Y, Turrens JF & Bolli R (1996) Evidence for an essential role of reactive oxygen species in the genesis of late preconditioning against myocardial stunning in conscious pigs. *J. Clin. Invest.* **97:** 562–76

Sun L, Lien C-L, Xu X, Shung KK, Aristizabal O, Christopher DA, Foster FS, Turnbull DH, Cannata JM, Ritter TA, Chen W, Silverman RH, Shung KK, Chen JN, Haffter P, Odenthal J, Vogelsang E, Brand M, Eeden FJM van, Furutani-Seiki M, et al (2008) In vivo cardiac imaging of adult zebrafish using high frequency ultrasound (45-75 MHz). *Ultrasound Med. Biol.* **34:** 31–9

Sun X, Hoage T, Bai P, Ding Y, Chen Z, Zhang R, Huang W, Jahangir A, Paw B, Li Y-G & Xu X (2009) Cardiac hypertrophy involves both myocyte hypertrophy and hyperplasia in anemic zebrafish. *PLoS One* **4:** e6596

Suwa M, Nakano H, Radak Z & Kumagai S (2008) Endurance exercise increases the SIRT1 and peroxisome proliferator-activated receptor gamma coactivator-1alpha protein expressions in rat skeletal muscle. *Metabolism.* **57:** 986–98

Suzuki K, Kishioka Y, Wakamatsu J & Nishimura T (2013) Decorin activates Akt downstream of IGF-IR and promotes myoblast differentiation. *Anim. Sci. J. = Nihon chikusan Gakkaihō* **84:** 669–74

Suzuki K, Murtuza B, Sammut IA, Latif N, Jayakumar J, Smolenski RT, Kaneda Y, Sawa Y, Matsuda H & Yacoub MH (2002) Heat shock protein 72 enhances manganese superoxide dismutase activity during myocardial ischemia-reperfusion injury, associated with mitochondrial protection and apoptosis reduction. *Circulation* **106:** I270-6

Swain P, Nayak SK, Nanda PK & Dash S (2008) Biological effects of bacterial lipopolysaccharide (endotoxin) in fish: a review. *Fish Shellfish Immunol.* **25:** 191–201

Taba Y, Sasaguri T, Miyagi M, Abumiya T, Miwa Y, Ikeda T & Mitsumata M (2000) Fluid shear stress induces lipocalin-type prostaglandin D(2) synthase expression in vascular endothelial cells. *Circ. Res.* **86:** 967–73

Taegtmeyer H (2002) Switching metabolic genes to build a better heart. *Circulation* **106:** 2043–5

Tamura Y, Watanabe K, Kantani T, Hayashi J, Ishida N & Kaneki M (2011) Upregulation of circulating IL-15 by treadmill running in healthy individuals: is IL-15 an endocrine mediator of the beneficial effects of endurance exercise? *Endocr. J.* **58:** 211–5

Teufel A, Malik N, Mukhopadhyay M & Westphal H (2002) Frcp1 and Frcp2, two novel fibronectin type III repeat containing genes. *Gene* **297:** 79–83

Thalacker-Mercer A, Stec M, Cui X, Cross J, Windham S & Bamman M (2013) Cluster analysis reveals differential transcript profiles associated with resistance training-induced human skeletal muscle hypertrophy. *Physiol. Genomics* **45:** 499–507

Ticho BS, Stainier DY, Fishman MC & Breitbart RE (1996) Three zebrafish MEF2 genes delineate somitic and cardiac muscle development in wild-type and mutant embryos. *Mech. Dev.* **59:** 205–18

van Tol BAF, Huijsmans RJ, Kroon DW, Schothorst M & Kwakkel G (2006) Effects of exercise training on cardiac performance, exercise capacity and quality of life in patients with heart failure: a meta-analysis. *Eur. J. Heart Fail.* **8:** 841–50

Trendelenburg AU, Meyer A, Rohner D, Boyle J, Hatakeyama S & Glass DJ (2009) Myostatin reduces Akt/TORC1/p70S6K signaling, inhibiting myoblast differentiation and myotube size. *Am. J. Physiol. Cell Physiol.* **296:** C1258-70

Tsuchiya Y, Ando D, Takamatsu K & Goto K (2015) Resistance exercise induces a greater irisin response than endurance exercise. *Metabolism.* **64:** 1042–50

Tudorache C, de Boeck G & Claireaux G (2013) Forced and Preferred Swimming Speeds of Fish: A Methodological Approach. In *Swimming Physiology of Fish* pp 81–108. Berlin, Heidelberg: Springer Berlin Heidelberg

Tulotta C, He S, Chen L, Groenewoud A, van der Ent W, Meijer AH, Spaink HP & Snaar-Jagalska BE (2016) Imaging of Human Cancer Cell Proliferation, Invasion, and Micrometastasis in a Zebrafish Xenogeneic Engraftment Model. *Methods Mol. Biol.* **1451:** 155–69

Ullum H, Haahr PM, Diamant M, Palmø J, Halkjaer-Kristensen J & Pedersen BK (1994) Bicycle exercise enhances plasma IL-6 but does not change IL-1 alpha, IL-1 beta, IL-6, or TNF-alpha pre-mRNA in BMNC. *J. Appl. Physiol.* **77:** 93–7

Valiente-Alandi I, Albo-Castellanos C, Herrero D, Arza E, Garcia-Gomez M, Segovia JC, Capecchi M & Bernad A (2015) Cardiac Bmi1 + cells contribute to myocardial renewal in the murine adult heart. *Stem Cell Res. Ther.* **6:** 205

Varela M, Dios S, Novoa B & Figueras A (2012) Characterisation, expression and ontogeny of interleukin-6 and its receptors in zebrafish (Danio rerio). *Dev. Comp. Immunol.* **37:** 97–106

Vary TC & Kimball SR (1992) Regulation of hepatic protein synthesis in chronic inflammation and sepsis. *Am. J. Physiol.* **262:** C445-52

Vaynman S, Ying Z & Gomez-Pinilla F (2004) Hippocampal BDNF mediates the efficacy of exercise on synaptic plasticity and cognition. *Eur. J. Neurosci.* **20:** 2580–90

van der Velden YU, Wang L, Zevenhoven J, van Rooijen E, van Lohuizen M, Giles RH, Clevers H & Haramis A-PG (2011) The serine-threonine kinase LKB1 is essential for survival under energetic stress in zebrafish. *Proc. Natl. Acad. Sci. U. S. A.* **108:** 4358–4363

Vozzi C, Dupont E, Coppen SR, Yeh HI & Severs NJ (1999) Chamber-related differences in connexin expression in the human heart. *J. Mol. Cell. Cardiol.* **31:** 991–1003

Vukusic K, Asp J, Henriksson HB, Brisby H, Lindahl A & Sandstedt J (2015) Physical exercise affects slow cycling cells in the rat heart and reveals a new potential niche area in the atrioventricular junction. *J. Mol. Histol.* **46:** 387–398

Wackerhage H (2014) Molecular exercise physiology: an introduction 1st ed. Wackerhage H (ed) New York, USA

Wagers AJ & Conboy IM (2005) Cellular and molecular signatures of muscle regeneration: current concepts and controversies in adult myogenesis. *Cell* **122:** 659–67

Wahl P, Brixius K & Bloch W (2009) Exercise-induced stem cell activation and its implication for cardiovascular and skeletal muscle regeneration. *Minim. Invasive Ther. Allied Technol.*

Wang J, Panáková D, Kikuchi K, Holdway JE, Gemberling M, Burris JS, Singh SP, Dickson AL, Lin Y-F, Sabeh MK, Werdich A a, Yelon D, Macrae C a & Poss KD (2011) The regenerative capacity of zebrafish reverses cardiac failure caused by genetic cardiomyocyte depletion. *Development* **138:** 3421–30

Wang W, Zhang H, Xue G, Zhang L, Zhang W, Wang L, Lu F, Li H, Bai S, Lin Y, Lou Y, Xu C & Zhao Y (2014) Exercise Training Preserves Ischemic Preconditioning in Aged Rat Hearts by Restoring the Myocardial Polyamine Pool. *Oxid. Med. Cell. Longev.* **2014:** 1–14

Wang Y, Wisloff U & Kemi OJ (2010) Animal models in the study of exercise-induced cardiac hypertrophy. *Physiol. Res.* **59:** 633–44

Wang Y-X, Zhang C-L, Yu RT, Cho HK, Nelson MC, Bayuga-Ocampo CR, Ham J, Kang H & Evans RM (2004) Regulation of Muscle Fiber Type and Running Endurance by PPARδ. *PLoS Biol.* **2:** e294

Waring CD, Henning BJ, Smith AJ, Nadal-Ginard B, Torella D & Ellison GM (2015) Cardiac adaptations from 4 weeks of intensity-controlled vigorous exercise are lost after a similar period of detraining. *Physiol. Rep.* **3:**

Waring CD, Vicinanza C, Papalamprou A, Smith AJ, Purushothaman S, Goldspink DF, Nadal-Ginard B, Torella D & Ellison GM (2014) The adult heart responds to increased workload with physiologic hypertrophy, cardiac stem cell activation, and new myocyte formation. *Eur. Heart J.* **35:** 2722–31

Weatherley AH, Gill HS & Lobo AF (1988) Recruitment and maximal diameter of axial muscle fibres in teleosts and their relationship to somatic growth and ultimate size. *J. Fish Biol.* **33:** 851–859

Weihs D (1973) Optimal Fish Cruising Speed. *Nature* **245:** 48–50

Weiner RB, Wang F, Berkstresser B, Kim J, Wang TJ, Lewis GD, Hutter AM, Picard MH & Baggish AL (2012) Regression of "gray zone" exercise-induced concentric left ventricular hypertrophy during prescribed detraining. *J. Am. Coll. Cardiol.* **59:** 1992–4

Werlang-Coelho C, Westphal GA, Dal-Pizzol F, Streck EL & Silva E Protective effects of exercise against sepsis-induced energy metabolism dysfunction in skeletal muscle of rats. *Fisioter. e Pesqui.* **22:** 133–141

White FC, Bloor CM, McKirnan MD & Carroll SM (1998) Exercise training in swine promotes growth of arteriolar bed and capillary angiogenesis in heart. *J. Appl. Physiol.* **85:** 1160–8

White JA, Boffa MB, Jones B & Petkovich M (1994) A zebrafish retinoic acid receptor expressed in the regenerating caudal fin. *Development* **120:** 1861–72

Wiendl H, Hohlfeld R & Kieseier BC (2005) Immunobiology of muscle: advances in understanding an immunological microenvironment. *Trends Immunol.* **26:** 373–80

Wilson MG, Ellison GM & Cable NT (2015) Basic science behind the cardiovascular benefits of exercise. *Heart* **101:** 758–765

Winbanks CE, Chen JL, Qian H, Liu Y, Bernardo BC, Beyer C, Watt KI, Thomson RE, Connor T, Turner BJ, McMullen JR, Larsson L, McGee SL, Harrison CA & Gregorevic P (2013) The bone morphogenetic protein axis is a positive regulator of skeletal muscle mass. *J. Cell Biol.* **203:** 345–57

Winder WW & Hardie DG (1996) Inactivation of acetyl-CoA carboxylase and activation of AMP-activated protein kinase in muscle during exercise. *Am. J. Physiol.* **270:** E299-304

Wisløff U, Ellingsen Ø & Kemi OJ (2009) High-intensity interval training to maximize cardiac benefits of exercise training? *Exerc. Sport Sci. Rev.* **37:** 139–46

Wisløff U, Loennechen JP, Falck G, Beisvag V, Currie S, Smith G & Ellingsen O (2001) Increased contractility and calcium sensitivity in cardiac myocytes isolated from endurance trained rats. *Cardiovasc. Res.* **50:** 495–508

World Health Organization (2008) The global burden of disease: 2004 update Geneva, Switzerland: World Health Organization

Worlh Health Organization (2009) Global Health risks. Mortality and burden of disease attributable to selected major risks. Geneva, Switzerland

Wrann CD, White JP, Salogiannnis J, Laznik-Bogoslavski D, Wu J, Ma D, Lin JD, Greenberg ME & Spiegelman BM (2013) Exercise Induces Hippocampal BDNF through a PGC-1α/FNDC5 Pathway. *Cell Metab.* **18:** 649–659

Wu C-C, Kruse F, Vasudevarao MD, Junker JP, Zebrowski DC, Fischer K, Noël ES, Grün D, Berezikov E, Engel FB, van Oudenaarden A, Weidinger G, Bakkers J, Bergmann O, Bhardwaj RD, Bernard S, Zdunek S, Barnabe-Heider F, Walsh S, Zupicich J, et al (2016) Spatially Resolved Genome-wide Transcriptional Profiling Identifies BMP Signaling as Essential Regulator of Zebrafish Cardiomyocyte Regeneration. *Dev. Cell* **36:** 36–49

Wu H, Kanatous SB, Thurmond FA, Gallardo T, Isotani E, Bassel-Duby R, Williams RS, Williams RS, Wang H, Hiatt WR, Barstow TJ, Brass EP, Hood DA, Williams RS, Salmons S, Newsholme EA, Kaufman RE, Mellor J, Pette D, Vrbova G, et al (2002) Regulation of mitochondrial biogenesis in skeletal muscle by CaMK. *Science* **296:** 349–52

Wu H, Rothermel B, Kanatous S, Rosenberg P, Naya FJ, Shelton JM, Hutcheson KA, DiMaio JM, Olson EN, Bassel-Duby R, Williams RS, Bigard X, Sanchez H, Zoll J, Mateo P, Rousseau V, Veksler V, Ventura-Clapier R, Black B, Olson E, et al (2001) Activation of MEF2 by muscle activity is mediated through a calcineurin-dependent pathway. *EMBO J.* **20:** 6414–6423

Wyatt HL & Mitchell J (1978) Influences of physical conditioning and deconditioning on coronary vasculature of dogs. *J. Appl. Physiol.* **45:**

Xiao H, Ma X, Feng W, Fu Y, Lu Z, Xu M, Shen Q, Zhu Y & Zhang Y (2010) Metformin attenuates cardiac fibrosis by inhibiting the TGFbeta1-Smad3 signalling pathway. *Cardiovasc. Res.* **87:** 504–13

Xiao J, Chen P, Qu Y, Yu P, Yao J, Wang H, Fu S, Bei Y, Chen Y, Che L & Xu J (2016) Telocytes in exercise-induced cardiac growth. *J. Cell. Mol. Med.* **20:** 973–979

Xiao J, Xu T, Li J, Lv D, Chen P, Zhou Q & Xu J (2014) Exercise-induced physiological hypertrophy initiates activation of cardiac progenitor cells. *Int. J. Clin. Exp. Pathol.* **7:** 663–9

Xu C, Wu G, Zohar Y & Du S-J (2003) Analysis of myostatin gene structure, expression and function in zebrafish. *J. Exp. Biol.* **206:** 4067–79

Xu S, Tsao PS & Yue P (2011) Apelin and insulin resistance: another arrow for the quiver? *J. Diabetes* **3:** 225–31

Xu Z, Li E, Xu C, Gan L, Qin JG & Chen L (2016) Response of AMP-activated protein kinase and energy metabolism to acute nitrite exposure in the Nile tilapia Oreochromis niloticus. *Aquat. Toxicol.* **177:** 86–97

Yamaguchi Y, Mann DM & Ruoslahti E (1990) Negative regulation of transforming growth factor-beta by the proteoglycan decorin. *Nature* **346:** 281–4

Yamashita N, Hoshida S, Otsu K, Asahi M, Kuzuya T & Hori M (1999) Exercise provides direct biphasic cardioprotection via manganese superoxide dismutase activation. *J. Exp. Med.* **189:** 1699–706

Yan Z, Lira VA & Greene NP (2012) Exercise training-induced Regulation of Mitochondrial Quality. *Exerc. Sport Sci. Rev.* **40:** 159–164

Yang H, Zhao L, Zhang J, Tang C-S & Qi Y-F (2015) Effect of Treadmill Running on Apelin and APJ Expression in Adipose Tissue and Skeletal Muscle in Rats Fed a High-fat Diet. *Int. J. Sports Med.* **36:** 535–541

Yin VP, Lepilina A, Smith A & Poss KD (2012) Regulation of zebrafish heart regeneration by miR-133. *Dev. Biol.* **365:** 319–327

Yogev O, Williams VC, Hinits Y & Hughes SM (2013) eIF4EBP3L acts as a gatekeeper of TORC1 in activity-dependent muscle growth

by specifically regulating Mef2ca translational initiation. *PLoS Biol.* **11:** e1001679

You T & Nicklas BJ (2008) Effects of exercise on adipokines and the metabolic syndrome. *Curr. Diab. Rep.* **8:** 7–11

Young PS & Cech Jr. JJ (1994) Optimum Exercise Conditioning Velocity for Growth, Muscular Development, and Swimming Performance in Young-of-the-Year Striped Bass (*Morone saxatilis*). *Can. J. Fish. Aquat. Sci.* **51:** 1519–1527

Yue P, Jin H, Aillaud M, Deng AC, Azuma J, Asagami T, Kundu RK, Reaven GM, Quertermous T & Tsao PS (2010) Apelin is necessary for the maintenance of insulin sensitivity. *Am. J. Physiol. Endocrinol. Metab.* **298:** E59-67

Zhang H & Anderson JE (2014) Satellite cell activation and populations on single muscle-fiber cultures from adult zebrafish (Danio rerio). *J. Exp. Biol.*

Zhao L, Borikova AL, Ben-Yair R, Guner-Ataman B, MacRae CA, Lee RT, Burns CG & Burns CE (2014) Notch signaling regulates cardiomyocyte proliferation during zebrafish heart regeneration. *Proc. Natl. Acad. Sci. U. S. A.* **111:** 1403–8

Zhao W-W, Pang X, Peng J-L, Cao Z-D & Fu S-J (2012) The effects of hypoxia acclimation, exercise training and fasting on swimming performance in juvenile qingbo (Spinibarbus sinensis). *Fish Physiol. Biochem.* **38:** 1367–1377

Zhou T, Wang L, Zhu K-Y, Dong M, Xu P-F, Chen Y, Chen S-J, Chen Z, Deng M & Liu TX (2011) Dominant-negative C/ebpα and polycomb group protein Bmi1 extend short-lived hematopoietic stem/progenitor cell life span and induce lethal dyserythropoiesis. *Blood* **118:** 3842–52

Ziaaldini MM, Koltai E, Csende Z, Goto S, Boldogh I, Taylor AW & Radak Z (2015) Exercise training increases anabolic and attenuates catabolic and apoptotic processes in aged skeletal muscle of male rats. *Exp. Gerontol.* **67:** 9–14

Zimmerman A & Lowery M (1999) Hyperplastic development and hypertrophic growth of muscle fibers in the white seabass (Atractoscion nobilis). *J. Exp. Zool.* **284:** 299–308

Zimmers TA, Davies M V, Koniaris LG, Haynes P, Esquela AF, Tomkinson KN, McPherron AC, Wolfman NM & Lee S-J (2002) Induction of cachexia in mice by systemically administered myostatin. *Science* **296:** 1486–8

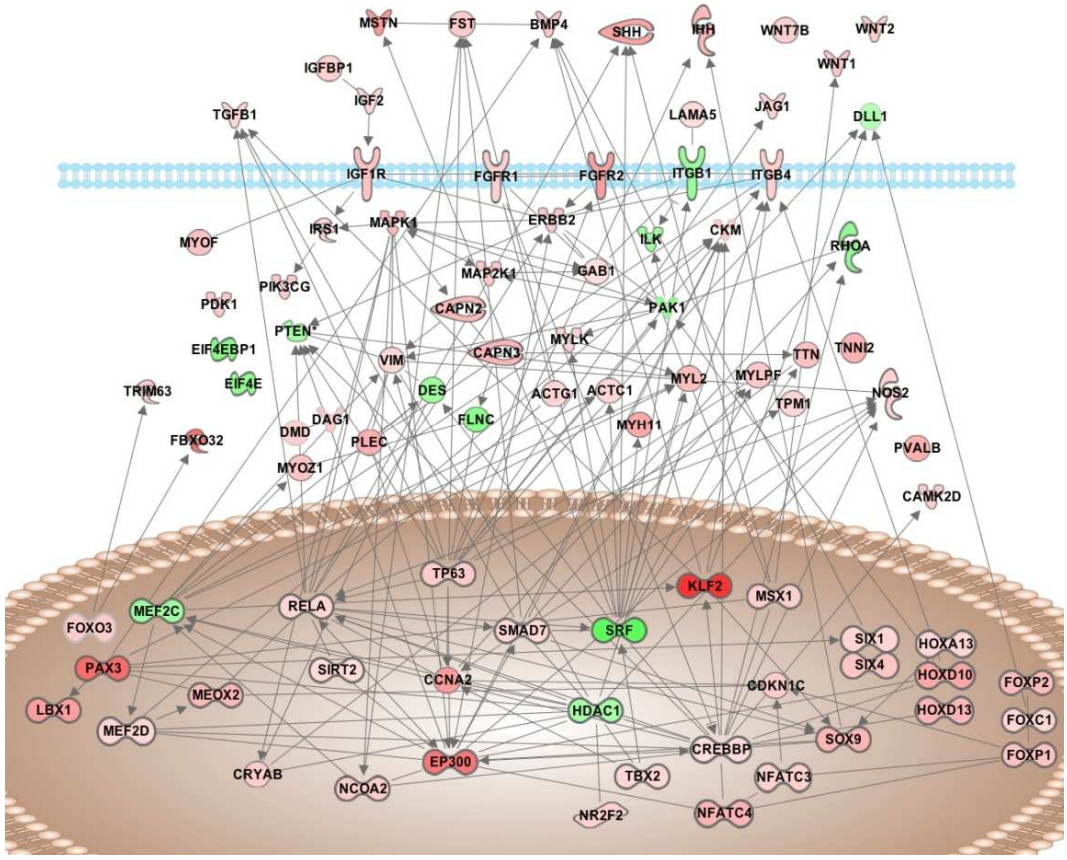
Zingman L V, Zhu Z, Sierra A, Stepniak E, Burnett CM-L, Maksymov G, Anderson ME, Coetzee WA & Hodgson-Zingman DM (2011) Exercise-induced expression of cardiac ATP-sensitive potassium channels promotes action potential shortening and energy conservation. *J. Mol. Cell. Cardiol.* **51:** 72–81

Zoico E & Roubenoff R (2002) The role of cytokines in regulating protein metabolism and muscle function. *Nutr. Rev.* **60:** 39–51

APPENDIX

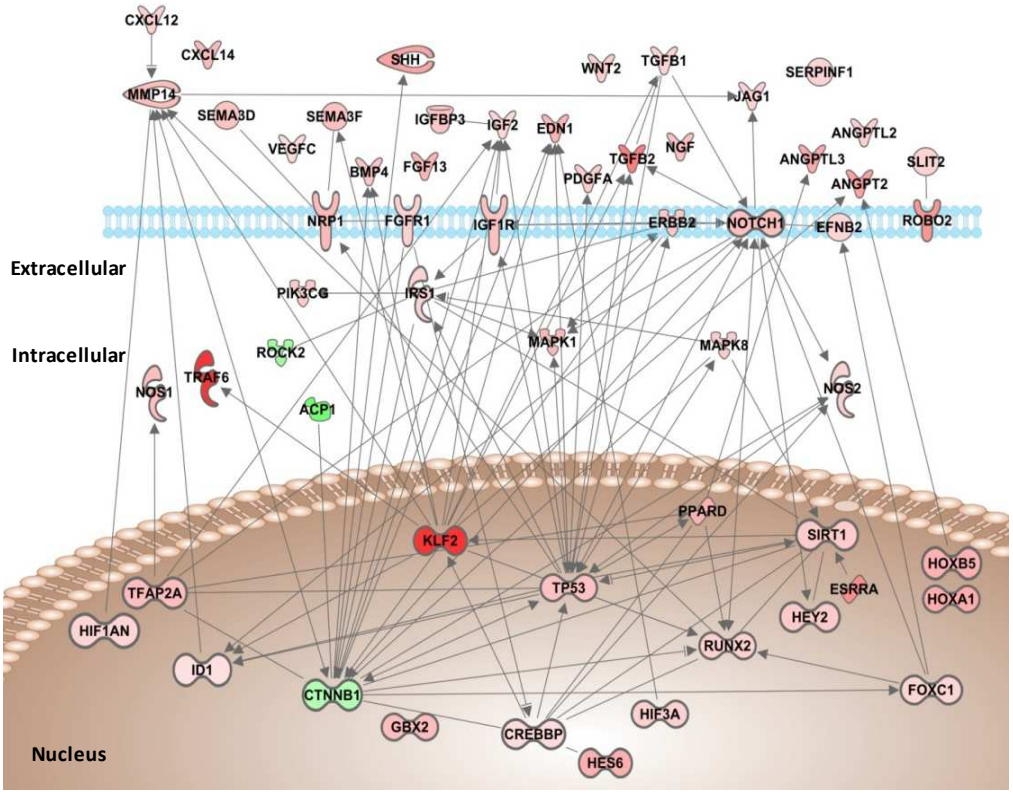
Additional Figures and Tables

ADDITIONAL FIGURE A.1



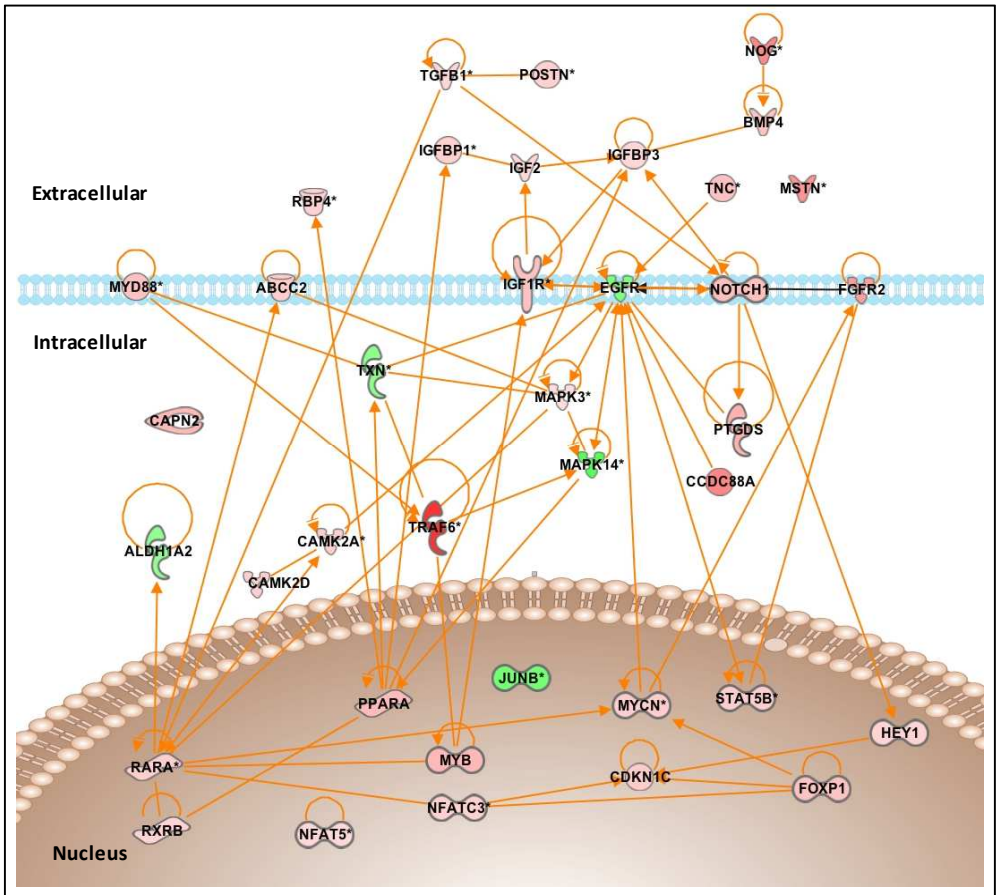
Additional Figure A.1. IPA-based network generated from molecules involved in muscle development and myogenesis that are differentially expressed in fast muscle of exercised adult zebrafish. The shapes of the genes correlate with the functional classification symbolized in the legend. Arrows represent the direct relationship between molecules. Color intensity correlates to transcription value, calculated as log2ratio (exercised/non-exercised); green represents molecules with repressed transcription (negative log2ratio); red represents molecules with enhanced transcription (positive log2ratio).

ADDITIONAL FIGURE A.2



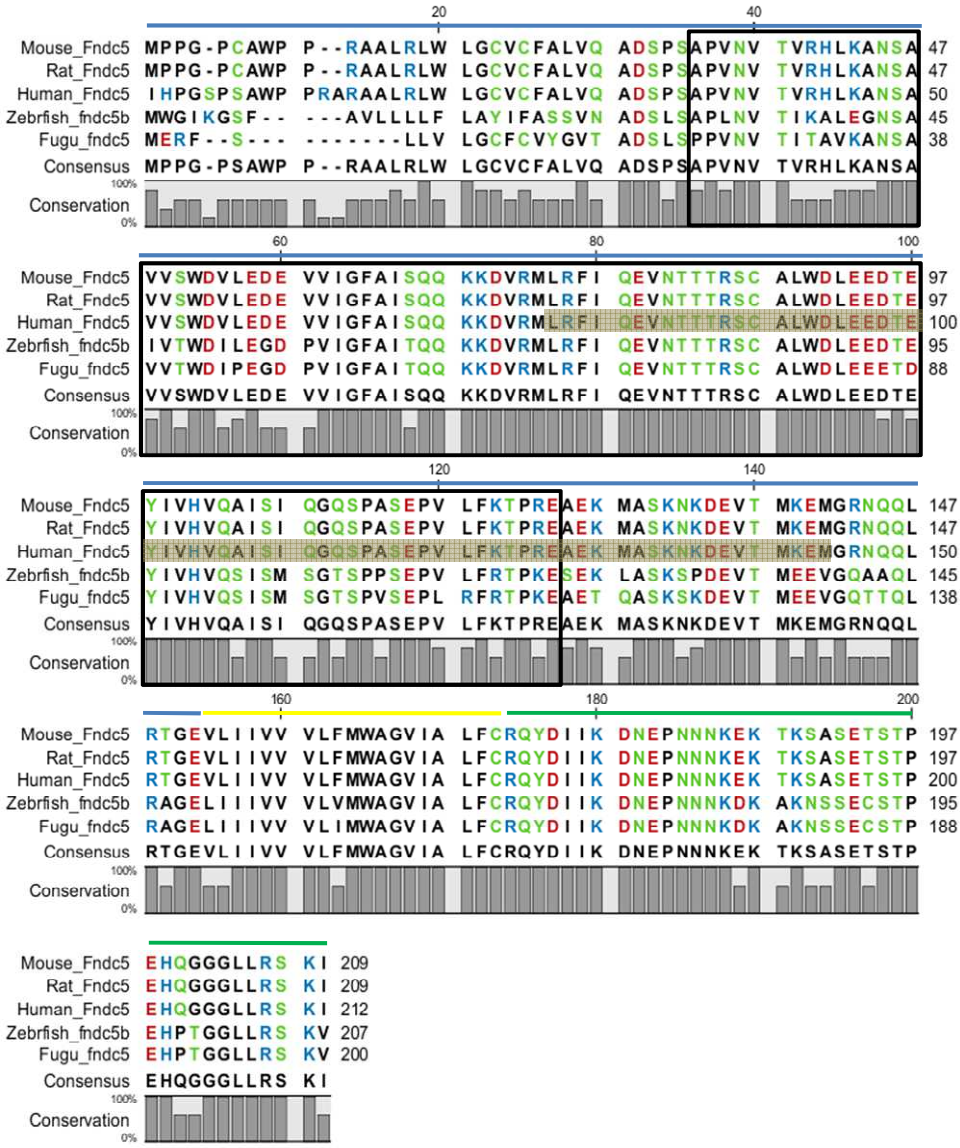
Additional Figure A.2. IPA-based network generated from molecules involved in angiogenesis that are differentially expressed in fast muscle of exercised adult zebrafish. The shapes of the genes correlate with the functional classification symbolized in the legend. Arrows represent the direct relationship between molecules. Color intensity correlates to transcription value, calculated as log2ratio (exercised/non-exercised); green represents molecules with repressed transcription (negative log2ratio); red represents molecules with enhanced transcription (positive log2ratio).

ADDITIONAL FIGURE A.3



Additional Figure A.3. IPA-based network generated from molecules involved in cell proliferation that are differentially expressed in fast muscle of exercised adult zebrafish. The shapes of the genes correlate with the functional classification symbolized in the legend. Arrows represent the direct relationship between molecules. Color intensity correlates to transcription value, calculated as log2ratio (exercised/non-exercised); green represents molecules with repressed transcription (negative log2ratio); red represents molecules with enhanced transcription (positive log2ratio).

ADDITIONAL FIGURE A.4



Additional Figure A.4. Protein sequence alignment of FNDC5. Upper lines indicate *Phobious* domain predictions in different colors: non-cytoplasmatic (blue), transmembrane (yellow) and cytoplasmatic (green). *ScanProsite* fibronectin type III (FNIII) domain prediction as a black frame. Polarity of the residues are indicated in different colours. Sequence recognized by FNDC5 antibody is highlighted in brown in human sequence. Mouse (*Mus musculus*) FNDC5, NP_081678.1, rat (*Rattus norvegicus*) NP_001257910.1, human (*Homo sapiens*) FNDC5 NP_001165411.2, zebrafish (*Danio rerio*) FNDC5b NP_001037802.1 and fugu (*Takifugu rubripes*) XP_003962368.

ADDITIONAL FIGURE A.5

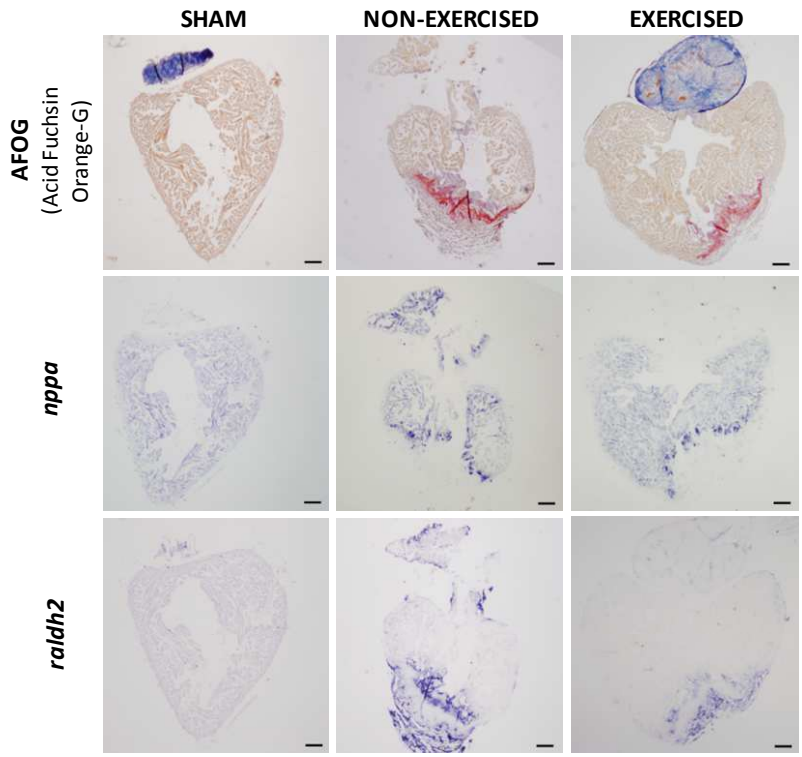


Figure Additional A.5. Expression at 7dpi of genes reported to be expressed at the injury area. **A-C** Acid Fucsin Organge G stained sagital sections to reveal the lesioned area in sham, control and exercised cryoinjured ventricles at 7dpi. **D-F** In situ hybridization of *nppa*. **G-I** In situ hybridization of *raldh2*. Scales bar represent 100 µm.

ADDITIONAL TABLE A.1

Additional Table A.1. Equation parameters for the log-normal regression of the fiber cross-sectional area histograms in the fast muscle of zebrafish.

	Non-exercised	Exercised
Percentage of muscle fibers		
FCSA <1.200 μm^2	47.3 ± 9.1	21.3 ± 4.6 *
1.200≤ FCSA ≤2.400 μm^2	50.4 ± 8.2	70.1 ± 3.3 *
FCSA >2.400 μm^2	2.3 ± 1.1	8.6 ± 2.6 *

Values are mean ± SEM. *P<0.05.

ADDITIONAL TABLE A.2

Additional Table A.2. Quantitative real-time PCR (qPCR) validation of fast muscle microarray results from selected genes.

Ensmbl ID	Gene name	Regulation	Microarray (FC)	qPCR (FC)
ENSG00000164434	fabp7	Down-regulated	-3.96	-2.31
ENSDARG00000055216	tuba1b	Down-regulated	-4.13	-3.20
ENSDARG00000012234	psme3	Down-regulated	-3.11	-3.16
ENSDARG00000003526	psma5	Down-regulated	-2.43	-2.40
ENSDARG00000013804	capns1	Down-regulated	-1.29	1.39
ENSG00000127418	fgfrl1	Up-regulated	4.32	1.81
ENSDARG00000019150	foxa1	Up-regulated	4.22	2.71

Data shown represent mean fold change (FC) and real-time PCR data are shown as the mean ± S.E.M.

ADDITIONAL TABLE A.3

Additional Table A.3. Zebrafish *il6* promoter binding sites found by MatInspector software analysis.

Transcription factor		Position	Sequence	Score
vertebrate TATA binding protein factor	TATA	109 to 125	atgtaTAAAaagcactg	1.0
Nuclear factor kappa B	NFKB	138 to 152	tctggagtTTCCatt	0.983
Nuclear factor of activated T-cells	NFAT	348 to 366	aatGGAAatttcaaatcta	0.912
Activating protein 1	AP1	409 to 421	aagtGAGTaaacc	0.905
Myocyte-specific enhancer binding factor	MEF2	492 to 514	tgacttgtTAAAagtaaaccccat	0.802
Ccaat/Enhancer Binding Protein	CEBPB	524 to 538	aatatttaGAAAgtg	0.953
Human and murine ETS 1 factor	ETS1	594 to 614	ccgagaggGGAAataaataga	0.961
cAMP-responsive element binding protein	CREB	729 to 749	taattcTGACttcaactgtat	0.998
GATA binding factor	GATA	746 to 758	ggggGATAataat	0.939
Nuclear factor of activated T-cells	NFAT	806 to 824	caaGGAAatttcacagta	0.906
GATA binding factor	GATA	826 to 838	gtctGATAatatt	0.964
Glucocorticoid response element	GRE	840 to 854	tttctcctGGAGaaa	0.872
Glucocorticoid response element and related elements	GREF	969 to 987	taatgatggttTGTTctgt	0.903

Basepairs in red are in a position with a high conservation profile. Basepairs in capital letters indicate the core sequence used by MatInspector software. Score indicates software matrix similarity, where a perfect match to the matrix gets a socre of 1.00 and a "good" match usually has a similarity of >0.80.

ADDITIONAL TABLE A.4

AdditionalTable A.4. Quantitative-real time PCR (qPCR) validation of heart microarray results from selected genes.

Ensmbl ID	Gene name	Regulation	Microarray (FC)	qPCR (FC)
ENSDART00000039551	*mef2ca*	Up- regulated	1.55	1.49
ENSDART00000003042	*mdk b*	Down-regulated	-1.94	-1.71
ENSDART00000077197	*tmsb*	Up- regulated	2.07	1.76
ENSDART00000017098	*postnb*	Up- regulated	2.83	2.60
ENSDART00000060839	*tgfb1a*	Up- regulated	1.81	1.69
ENSDART00000121456	*fstl1b*	Up- regulated	1.61	1.99

Data shown represent mean fold change (FC) and real-time PCR data are shown as the mean ± S.E.M.

ADDITIONAL TABLE A.5

Additional Table A.5 Quantitative real-time PCR (qPCR) validation of lps/pbs challenge of fast muscle microarray results from selected genes.

	Ensmbl ID	Gene name	Regulation	Microarray (FC)	qPCR (FC)
Non-exercised LPS	ENSDARG00000068458	nrg1	Down-regulated	-2.316	-1.64
	ENSDARG00000032639	cd36	Down-regulated	-2.170	-1.75
	ENSDARG00000052139.5	notch3	Down-regulated	-2.155	-1.07
	ENSDARG00000056627	cxcl14	Up-regulated	1.956	1.88
	ENSDARG00000059282	lck	Up-regulated	2.383	5.08
	ENSDARG00000057789	lyzl2	Up-regulated	8.983	11.14
Exercised LPS	ENSDARG00000093440	tnfaip6	Down-regulated	-15.528	-14.93
	ENSDARG00000010425	scara5	Down-regulated	-5.709	-2.57
	ENSDARG00000012066	dcn	Down-regulated	-3.317	-4.29
	ENSDARG00000039576	fstl1	Down-regulated	-3.120	-3.96
	ENSDARG00000024746	hsp90aa1	Down-regulated	-3.127	-3.27
	ENSDARG00000005616	cfb	Up-regulated	4.037	7.35
	ENSDARG00000043154	ucp2	-	4.180	-1.84

Microarray analysis was succesfully validated by qPCR. Results showed the same regulation trend for both techniques. Data shown represent mean fold change (FC) and real-time PCR data are shown as the mean ± S.E.M.

RESUM DE LA TESI

INTRODUCCIÓ

1.- L'exercici i els efectes beneficiosos sobre la salut

L'exercici o l'activitat física té un gran impacte en la salut humana. De fet, hi ha evidències que indiquen que la pràctica d'activitat física regularment està associada a un menor risc de mortalitat i prevalença de malalties cròniques com ara diabetis, càncer, obesitat i síndrome metabòlica, malalties que, a la vegada, sovint comprometen el sistema cardiovascular.

Per tant, les malalties cròniques són preocupants per a la salut pública i hi ha una necessitat urgent per la seva prevenció i tractament. En aquest sentit, hi ha diversos factors de risc que milloren amb la pràctica de l'exercici regular com ara reducció del pes corporal i acumulació de greix, millora de la proporció HDL/LDL, reducció de la pressió sanguínia o hipertensió, reducció de la hiperglucèmia augmentant la sensibilitat a la insulina, reducció de la inflamació sistèmica, millora de la funció endotelial, millora del flux sanguini coronari i de la funció cardíaca. Tot i que per avaluar els efectes beneficiosos de l'exercici en condicions de salut i de malaltia cal considerar el tipus, durada i intensitat de l'exercici físic, és evident que la inactivitat física és un factor important en l'augment de la incidència de les malalties cròniques. De fet, la inactivitat física és el quart factor de risc de mortalitat en tot el món, després de l'alta pressió sanguínia, tabaquisme i hiperglucèmia. Alguns dels canvis fisiològics rellevants de la pràctica de l'exercici tenen lloc al múscul esquelètic, com a principal òrgan responsable de la locomoció. Per exemple, l'augment de la utilització de carbohidrats com a combustible energètic (degut a l'augment de glicogenòlisis o gluconeogènesis al fetge) i l'augment de captació de glucosa i glicogenòlisis al múscul esquelètic són canvis beneficiosos particularment en pacients amb malalties metabòliques com la diabetis. A més, a mesura que les reserves de glicogen s'esgoten amb l'exercici, el metabolisme lipídic augmenta com a resultat de la captació d'àcids grassos a les cèl·lules musculars. Alguns dels efectes fisiològics durant la pràctica de l'exercici es representen a la Figura I.1.

Al 2004, l'Organització Mundial de la Salut va classificar les malalties cardiovasculars(CVD) com la primera causa de mort a nivell mundial, representant aproximadament el 29% de morts globals, i està previst ser la causa principal de mortalitat pel 2030.CVD generalment inclou hipertensió, infart de miocardi (isquèmia o malaltia coronària), ictus (malalties cerebrovasculars), insuficiència cardíaca, malaltia vascular perifèrica, malaltia de cor congènita o cardiomiopaties. Juntament amb altres recomanacions, l'exercici és un element clau dins programes de rehabilitació cardíacs supervisats per professionals pels pacients amb malalties cardiovasculars. Anàlisis de recopilació d'estudis clínics, evidencienque la pràctica d'exercici,sota recomanacions i directrius clíniques establertes, resulta en un augment de la qualitat de vida, reducció de la mortalitat i disminució de la probabilitat de recaiguda en pacients amb infart de miocardi o insuficiència cardíaca. Creixement cardíac, contractilitat del miocardi, augment del flux de sang coronari, reducció de infart de miocardi en cors susceptiblesi possiblement renovació dels cardiomiòcits, són alguns dels efectes de l'exercici en el cor observats en diferents estudis.

En conjunt, la recerca ha establert que la funció de l'exercici promou el benestar físic ja sigui com a prevenció primària o secundària. No obstant, els mecanismes pels quals l'exercici promou beneficis saludables encara no es coneixen completament. A més, la recerca en l'àmbit de la fisiologia de l'exercici també és determinant per tal d'explicar quins són els mecanismes moleculars que promouen la prevenció de malalties.

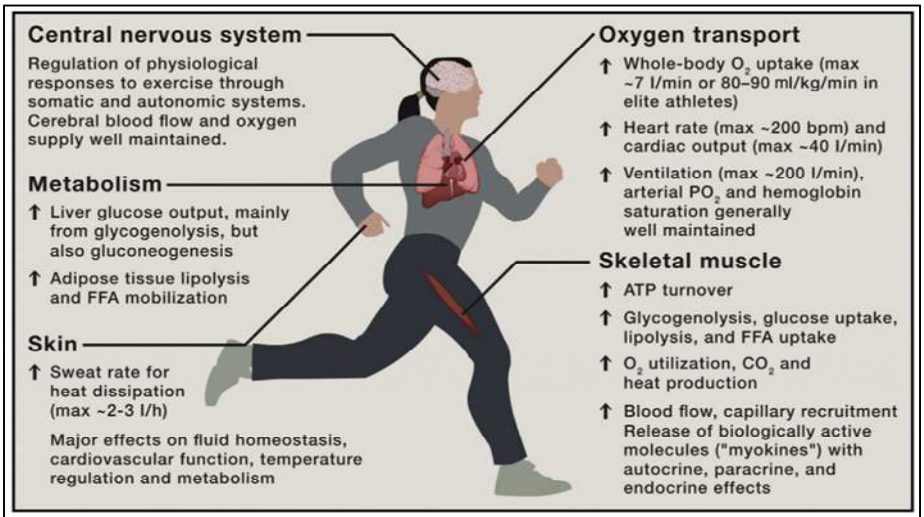


Figura I.1. Effectes fisiològics de l'exercici en humans. Hawley *et al*. (2014)

2.- El múscul esquelètic i l'exercici en mamífers

2.1.- Adaptació del múscul esquelètic a l'exercici

Les contraccions musculars s'inicien pels estímuls nerviosos de les neurones motores que innerven les cèl·lules musculars a través de les unions neuromusculars. La generació del potencial d'acció de l'activació neuronal resulta en la despolarització de la membrana cel·lular de la fibra muscular (o sarcolemma) i la generació de tensió mecànica, que provoca les vies de senyalització intracel·lular dirigides a regular la contracció muscular i programes transcripcionals rellevants per a l'adaptació fisiològica del múscul esquelètic per a l'exercici. La durada i intensitat de l'activitat contràctil induïda per l'exercici provoca un conjunt determinat d'adaptacions fisiològiques per ajustar el fenotip de la cèl·lula muscular a l'entrenament físic. L'exercici és, en línies generals, classificat en dos tipus: exercici aeròbic i l'exercici de resistència (o força), que donen lloc a diferents fenotips de músculs. L'exercici aeròbic implica una alta freqüència i baixa potència de càrrega de les contraccions musculars i es caracteritza per adaptacions a llarg termini que inclouen augments en el contingut mitocondrial i vascularització, així com la transformació del tipus de fibra. En canvi, l'exercici de resistència consisteix en exercicis de baixa freqüència i muscular elevada potència de càrrega de les contraccions musculars i es caracteritza per una hipertròfia de les fibres per tal d'augmentar la força muscular. Aquests dos modes extrems d'exercici estan regulats per diferents mecanismes moleculars (Figura 2).

A més de generar la contracció muscular, les concentracions de calci intracel·lulars transitòries dins la cèl·lula muscular com a conseqüència de la potencial d'acció, també juguen un paper en la plasticitat musculo-esquelètica. En efecte, l'augment del calci intracel·lular activa vies de senyalització com ara proteïnes calci/calmodulina-dependent (CAMKs) i dependent de

calci/calmodulina fosfatasa (calcineurina), responsables d'una resposta transcripcional en cèl·lules de múscul esquelètic. S'ha demostrat que les vies de senyalització transcripcionals dependents de calci modulen la transformació del tipus de fibra i la capacitat oxidativa de les cèl·lules musculars.

A banda de les vies dependents de calci, els canvis de l'estat d'energia cel·lular en les cèl·lules musculars en contractació també promou respostes adaptatives a través de la proteïna activada per AMP (AMPK) (Figura 2). AMPK s'activa per l'augment de les ràtios AMP/ATP i creatina/fosfocreatina que reflecteixen un estat d'esgotament d'energia dins la cèl·lula i, al seu torn, estimula adaptacions metabòliques que augmenten els processos de generació d'ATP com captació o lípids oxidació de glucosa.

Com a quinasa, l'AMPK modula l'activitat de diversos factors com són PGC1α per fosforilació directa i, per tant controlar la transcripció dels gens oxidatius, però AMPK també activa indirectament PGC1α a través de desacetilació mitjançant sirtuin 1 (SIRT1), augmentant els nivells NAD+ intracel·lular. A més, AMPK regula l'expressió de determinats gens modulant l'activitat de factors de transcripció com PPARs, NRF1, MEF2 i HDACs. AMPK s'activa en resposta a la contracció muscular l'exercici aeròbic i, encara que el seu paper en l'exercici de resistència encara no ha estat àmpliament explorat, AMPK ha demostrat ser activat de manera dependent a la intensitat i la durada de l'exercici. D'altra banda, l'exercici activa la via MAPK p38 que estimula l'expressió de PGC1α a través de ATF2 i MEF2, dos factors de transcripció que s'uneixen a PGC1α. A més, laproteïna supressora de tumors p53, també s'ha proposat per modular la capacitat oxidativa cel·lular durant l'exercici. La pèrdua de p53 afecta la respiració mitocondrial en els músculs esquelètics, perjudica el rendiment de l'exercici i s'ha suggerit que s'activi per AMPK i/o p38 MAPK.

Una adaptació freqüent a l'exercici de resistència o de força és l'augment de l'àrea de la fibra muscular (o hipertròfia de la cèl·lula muscular) per proporcionar la força. L'augment de la mida de la fibra es produeix per un increment en la síntesi de proteïnes sobre una reducció en la degradació de la proteïna en la cèl·lula muscular. L'activació del complex sensible a la rapamicina (mTORC), que existeix en dos complexos diferents anomenats mTORC1 i mTORC2, és conegut per ser la principal via de senyalització que promou la síntesi de proteïnes. A més, la via de mTOR integra moltes senyals intracel·lulars procedents de l'acció del factor de creixement, l'estrès, estat d'energia cel·lular i aminoàcids per controlar processos anabòlics (síntesi de proteïnes i lípids), cicle cel·lular i degradació (autofàgia). Particularment, l' activació de mTORC1 (compost per mTOR, Raptor, GβL i DEUTOR), promou la síntesi de proteïnes mitjançant la fosforilació de dues proteïnes que intervenen en el control de la traducció: proteïna d'unió del factor eucariota d'iniciació a la traducció 4E (4E-BP1) i la proteïna ribosomal S6-cinasa (p70S6K) (Figura 2).

La regulació de mTORC1 és essencial en condicions d'estrès energètic. Una regulació negativa està mediada per l'heterodímer complex d'esclerosi tuberosa (TSC1 i TSC2), que inactiva la GTPasa Rheb, activadora de mTORC1 (Figura 2). Per tant, estímuls que activen la via mTOR com factors de creixement, fosforil·len i inactiven el complex TSC a través d'Akt, un factor ascendent a mTORC1. AKT també activa directament a mTORC1 inhibint l'activitat de PRAS40. Un altre mecanisme per regular l'activitat de mTOR és a través d'AMPK. De fet, AMPK pot regular l'activitat mTORC1 ja sigui per fosforilació de TSC2 i, per tant, inactivant Rheb o fosforilant directament la subunitat Raptor del complex mTORC1, regulant negativament l'activitat de mTORC1 (Figura 2). Per contra, sota estats nutricionals amb alts nivells d'aminoàcids, l'activitat d'AMPK és suprimida mentre que l'activitat de mTOR s'accentua en les cèl·lules musculars fet que suggereix una regulació creuada entre aquestes dues vies de senyalització que encara està insuficientment entesa.

La contribució de cèl·lules mare musculars (cèl·lules satèl·lit) també ha estat informada en hipertròfia muscular en adults, encara que el seu paper ha estat força discutit. Les cèl·lules satèl·lit. lit (SC) són cèl·lules residents al múscul esquelètic adult, situades entre la membrana plasmàtica i la làmina basal que envolta cada fibra muscular. SC tot expressar el factor de transcripció Pax7, que s'utilitza com a marcador per a la identificació de SC, i romanen inactives en els músculs esquelètics adults i s'activen en resposta a una lesió o un estímul de creixement. Un cop activades, SC expressen factors reguladors miogènics(MRFs), especialment MyoD, migren a la superfície de la fibra muscular, proliferen i es diferencien (o renoven). La funció de les SC durant la hipertròfia muscular és encara incerta.

2.2.-Mioquines i senyalització entre el múscul esquelètic i altres òrgans

L'existència d'un factor humoral alliberat sota condicions d'exercici va ser assenyalat per M.Goldstein el 1961, a partir de les observacions que la contracció muscular promou hipoglucèmia de manera independent de la insulina. Anys més tard, el terme "myokine" va aparèixer en la literatura per definir "citoquines i altres pèptids expressats, produïts i/o alliberats per fibres musculars que exerceixen efectes autocrins, paracrins o endocrins" i el múscul esquelètic posteriorment va ser suggerit per ser un òrgan endocrí. En condicions d'exercici, certes mioquines expressades en el múscul esquelètic en contractació poden actuar localment en el teixit muscular mentre que altres són alliberades a la circulació per funcionar a nivell sistèmic, influint en el metabolisme i en la funció de diversos òrgans com el fetge, el teixit adipós i els vasos sanguinis. En aquest sentit, es creu que les mioquines poden ser responsables, almenys en part, d'alguns dels efectes beneficiosos de l'exercici. Les mioquines identificades actualment són nombroses i probablement en sorgiran més amb el temps. Algunes de les myokines es caracteritzen millor que altres, i encara hi ha manca d'evidències experimentals per a algunes. La mioquina més rellevant per a aquest estudi són: IL-6, IL-15, miostatina, SPARC, decorin, apelin, irisin i BDNF.

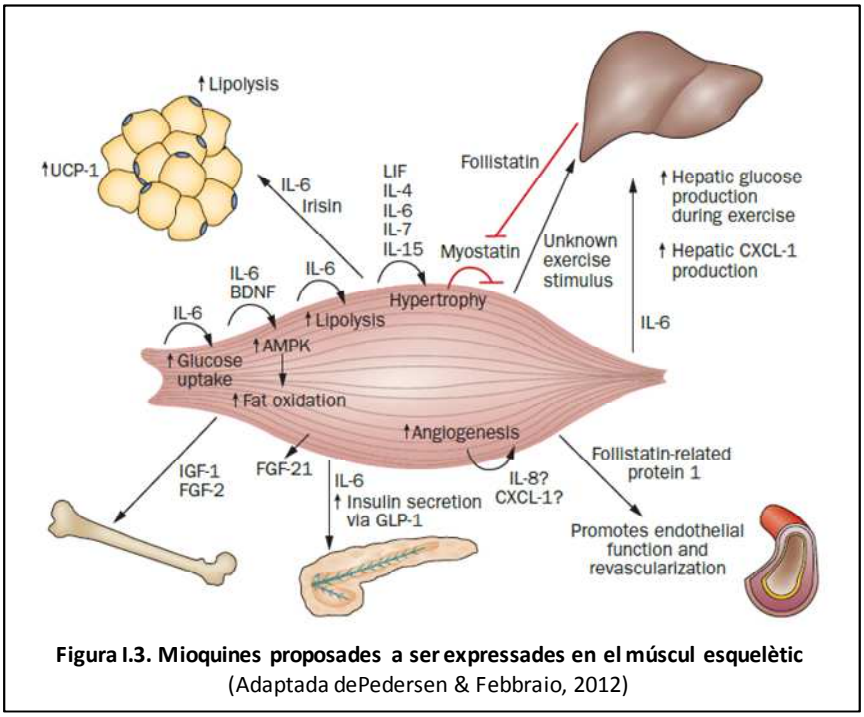


Figura I.3. Mioquines proposades a ser expressades en el múscul esquelètic
(Adaptada dePedersen & Febbraio, 2012)

2.3.- L'exercici, el múscul esquelètic i el sistema immunitari

Ja cap al 1893, es va informar d'un augment del nombre de glòbuls blancs en circulació (leucocitosi) després d'un episodi agut d'exercici i va ser només uns anys més tard que l'àmbit de "immunologia de l'exercici" va ser concebut durant la dècada de 1990. Probablement els efectes més notables o més ben estudiats de l'exercici s'observen en aquelles malalties cròniques que es caracteritzen per una inflamació sistèmica de baix grau. Tals patologies inclouen diabetis mellitus tipus 2, síndrome metabòlica, obesitat, malalties cardiovasculars i fins i tot trastorns d'edat neurodegeneratius com la malaltia de Alzheimer. Aquestes malalties s'han caracteritzat per la presència de citocines pro-inflamatòries circulants, incloent-hi la interleuquina 6 (IL-6), factor de necrosi tumoral alfa (TNF-α) i la proteïna C reactiva (PCR) entre d'altres marcadors d'inflamació, així com amb un nombre elevat de monòcits inflamatoris circulants. D'altra banda, els pacients amb malalties metabòliques com la diabetis tipus 1 o tipus 2 tenen una major susceptibilitat a infeccions agudes que subjectes sans.

Des d'estudis realitzats en humans i en models animals, es creu que l'efecte protector de l'exercici sobre la funció immunitària recau sobre l'entorn antiinflamatori que es produeix en cada període d'exercici i diversos possibles mecanismes s'han postulat en la literatura. En primer lloc, la pràctica d'exercici regular pot reduir la massa de greix corporal, especialment visceral i abdominal i, en conseqüència, redueix els nivells de citocines inflamatòries alliberades pel teixit adipós (adipoquines), reduint els nivells sistèmics de la inflamació. En segon lloc, la producció de màquines com IL-6 des de la contracció muscular, pot contribuir a reduir els nivells de citocines inflamatòries circulants. De fet, IL-6 estimula la producció de les citocines antiinflamatòries circulants IL-1RA, un inhibidor de la senyalització de IL-1, i IL-10. Aquesta última és una citocina antiinflamatòria que inhibeix la producció de diverses citocines com IL-1β i TNF-α i regula l'activació de cèl·lules immunitàries. En vista d'això, Petersen et al (2005) proposa que els augments de IL-6, IL-1ra i IL-10 circulants induïdes per l'exercici produeixen accions antiinflamatòries que poden ser beneficioses per a la resistència a l'obesitat i la insulina. En tercer lloc, l'exercici redueix la infiltració dels macròfags en el teixit adipós, possiblement per inhibició de l'alliberament de quemocines i la disminució de molècules d'adhesió. D'altra banda, s'ha demostrat que l'exercici per alterar el fenotip de macròfags infiltrats en el teixit adipós de ratolins obesos, induint un canvi fenotípic dels macròfags pro-inflamatoris predominants (coneguts com tipus M1) a antiinflamatori (coneguts com tipus M2), per tant reduint l'estat inflamatori en el teixit adipós.

D'altra banda, els efectes immunomoduladors de l'exercici també han estat investigats durant la sèpsia. La sèpsia és una complicació greu d'una infecció que, entre altres conseqüències, provoca debilitat muscular i està relacionada amb un augment de catabolisme muscular que condueix a la pèrdua muscular quan persisteix la resposta inflamatòria sistèmica. En particular, durant la fase inicial d'infecció, els aminoàcids alliberats del teixit muscular constitueixen una font de combustible per les cèl·lules immunològiques per a la gluconeogènesi en altres teixits o per la síntesi de proteïnes de fase aguda del fetge. Diferents models de la sèpsia s'utilitzen experimentalment per injecció de toxines bacterianes/patògens (com per exemple, lipopolisacàrid o LPS), administració de patògens viables o alteració de la barrera de defensa de l'organisme. Per cert, el perfil de citoquines després de l'administració de LPS no és tan fort com els altres models de sèpsia. No obstant això, el primer assaig clínic en humans amb un bol

de LPS de *Escherichia coli* per induir la inflamació de baix grau, va demostrar que en els subjectes prèviament exercitats, l'augment de TNF-α va ser atenuat en comparació amb el grup control. L'augment de nivells circulants de IL-6 en els subjectes exercitats va produir resultats similars a l'administració de IL-6 humana recombinant. Per tant, aquest estudi va suggerir un possible paper en humans de IL-6 induïda per exercici.

No obstant això, els mecanismes exactes pel qual l'exercici provoca els efectes beneficiosos proposats sobre la funció immune tant en condicions de salut i com de malaltia, així com la determinació de la quantitat d'exercici necessari, segueixen sent desconeguts. En aquest sentit, el múscul esquelètic ha rebut l'atenció de la comunitat científica perquè s'han observat que les cèl·lules musculars poden expressar factors immunològics que contribueixin a la funció immunitària i, per tant, participar en els efectes immunomodulador d'exercici. De fet, s'ha descobert que les cèl·lules musculars expressen molècules normalment presents en les cèl·lules dendrítiques, monòcits o cèl·lules B que funcionen com a cèl·lules presentadores d'antígens (APCs). Les APCs són responsables de la presentació d'antígens a les cèl·lules T per orientar o estimular la resposta immunitària d'altres cèl·lules immunes. Especialment, molècules del complex principal d'histocompatibilitat (MHC), d'adhesió, citocines, o molècules co-estimulatories s'han vist expressades en cèl·lules de múscul esquelètic en mamífers. Per exemple, les cèl·lules mioblasts humanes expressen molècules del MHC després d'una estimulació *in vitro* amb citocines inflamatòries (com IFN-γ, TNF-α , IL-1a o IL-1b), així com *invivo* en miopaties inflamatòries. D'altra banda, com s'ha esmentat prèviament, les cèl·lules musculars en cultiu disposen de la maquinària de processament d'antígensnecessària per a presentar antígens de fonts intracel·lulars o extracel·lulars. Diferents receptors de tipus *toll*, que són els principals receptors de la resposta immunitària innata en reconèixer molècules associades a patògens (PAMPs) i que s'expressen normalment en macròfags i cèl·lules dendrítiques, també s'han trobat expressats en les cèl·lules musculars. Aquestes observacions han estat particularment interessants en relació a les vacunes d'ADN intramuscular basades en la evidència que les cèl·lules musculars poden processar antígens i presentar-les a les APCs, un cop han estat reclutades en resposta a un procés inflamatori. Per tant, les cèl·lules musculars, en condicions inflamatòries poden desencadenar la producció de citoquines, quimoquines i molècules d'adhesió per reclutar o estimular els leucòcits. Així, a més de la capacitat de presentació d'antígens, les cèl·lules musculars podrien funcionar com a cèl·lules APCs no professionals per tal de modular la resposta immune innata i adaptativa. No obstant això, moltes d'aquestes observacions s'han aconseguit en condicions *invitro* i és necessària més recerca per confirmar el seu rol en condicions *invivo*.

Per tant, tenint en compte el paper del múscul esquelètic en modular la resposta adaptativa de l'exercici, les propietats moleculars de les cèl·lules musculars sota un entorn inflamatori i les patologies associades a una inflamació, cal definir el paper del múscul esquelètic en els efectes cardioprotectors de l'exercici, així com en les patologies relacionades amb alteracions inflamatòries.

3.- El múscul cardíac i l'exercici en mamífers

L'exercici regular inicia canvis cardiovasculars i respiratoris per subministrar i lliurar l'oxigen i els nutrients per contraure els músculs i com també a altres teixits. Durant l'exercici, pot

augmentar el flux de sang als músculs en contracció en un 60%. Així, la despesa cardíaca s'incrementa (producte del volum sistòlic i la freqüència cardíaca) i el flux sanguini és redistribuït des dels òrgans o teixits (sistema digestiu) inactius cap al múscul esquelètic. No obstant això, a part de les respostes cardíaques agudes (p. ex. una major despesa cardíaca) per tal de proporcionar l'augment enèrgic requerit, l'exercici regular provoca adaptacions cardíaques i efectes protectors al cor o el sistema cardiovascular. D'altra banda, diverses evidències apunten a un efecte beneficiós de l'actuació de l'exercici en pacients que han patit una malaltia cardiovascular, com ara un infart de miocardi o insuficiència cardíaca, així com en els models animals d'infart de miocardi o isquèmia/reperfusió (I/ R). És intrigant, que l'exercici ha estat relacionat amb un efecte de precondicionament del cor, amb efectes similars que un precondicionament isquèmic, protegint així el cor de lesions cardíaques posteriors. No obstant això, els mecanismes responsables de la cardioprotecció de l'exercici en condicions saludables i malaltia encara no es coneixen. Per exemple, l'òxid nítric, proteïnes de xoc tèrmic (HSPs), canals de potassi dependents de ATP (KATP), antioxidants, espècies reactives d'oxigen (ROS) o AMPK, són alguns dels mecanismes proposats en la literatura de l'exercici en medicar els efectes en el cor.

L'exercici regular produeix canvis morfològics en el cor que poden resultar en creixement cardíac. La hipertròfia (o augment de la massa cardíaca) és una resposta adaptativa del cor per tal de satisfer un estrès hemodinàmic com per exemple, l'increment de despesa cardíaca durant l'exercici aeròbic o en malalties cardiovasculars com la hipertensió. No obstant això, en aquests casos, quan l'estrès mecànic és persistent o crònic, la hipertròfia cardíaca ja no pot ser favorable o cardioprotectora i evoluciona per ser maladaptativa. Això es tradueix en un augment enl'expressió gènica de gens fetals (p. ex. augment del pèptid natriurètic, cadena pesant de miosina-β i disminució de la cadena pesant de miosina-α i proteïnesdependents del calci com ara SERCA2a), un augment de la mort delscardiomiòcits seguit per un augment de fibrosi, deteriorament cardíac, augment de la utilització de glucosa sobre l'oxidació dels àcids grassos i un augment d'àrea delscardiomiòcits que en última instància condueix a la disfunció cardíaca, elevant el risc d'insuficiència cardíaca. En canvi, cap d'aquests efectes perjudicials són presents en un procés d'hipertròfiacom durant la hipertròfia en el desenvolupament, exercici o l'embaràs, processos en que l'augment de massa cardíaca és reversible i els cardiomiòcits augmenten de manera proporcional en longitud i amplada i millorant (o no) la funció cardíaca.

4.- El peix zebra com a un model rellevant en fisiologia

4.1.- Introducció al model del peix zebra

El peixo teleostis peix zebra (*Daniorerio*) es va descobrir com a prometedor model experimental per George Streisinger (Universitat d'Oregon) en la dècada de 1970, i actualment és utilitzat per molts laboratoris arreu del món. Camps de recerca combiologia del desenvolupament, cardiovascular, immunitat, càncer, envelliment, metabolisme o regeneració inclouen el peix zebra com a model d'investigació. Els principals avantatges d'aquest vertebrat inclouen la fecundació externa, desenvolupament ràpid i embrions translúcids que permeten unamanipulació fàcil dels embrions durant totes les etapes del desenvolupament. Ja que un gran nombre d'òvuls fecundats poden ser recollits fàcilment, els embrions poden ser manipulats genèticament en l'estadi d'una cèl·lula i visualitzar o analitzar el fenotip resultant. Els embrions translúcids han permès desenvolupar el peix zebra transgènic, expressant constitutivamentproteïnes fluorescents (p. ex. la GFP) per tal de traçar processos de desenvolupament *invivo*a nivell d'una sola cèl·lula, òrgans o estructures sub-cel·lularsinclús

després de 5 dies de fecundació. El peix zebra ofereix un avantatgeaddicional en models genètics ja que es pot induir la seva mutagènesi amb substàncies químiques o amb elements inserció en el genoma en adults i embrions.

4.1.1.- El múscul esquelètic en peixos

El múscul esquelètic en peixos, incloent-hi el peix zebra, comprèn aproximadament un 60-80% del total de la massa i, a més de la seva funció en la locomoció, contribueix a mantenir la homeòstasi metabòlica com en la resta de vertebrats superiors. Els peixos teleostis, com els mamífers, el múscul esquelètic es compon de dos tipus de fibres musculars distingibles anatòmicament: fibres de contracció lentes (oxidatives o vermelles) i de contracció ràpida (glicolítiques o blanques) amb diferents propietats funcionals i una expressió diferent de les isoformes de miosina. A diferència dels mamífers, els diferentstipus de fibra muscular en peixos estan anatòmicament separats com s'il·lustra en figura I.4. Entre les fibres lentes i ràpides, en algunes espècies de peix com el peix zebra, s'ha trobat un tercer tipus de fibra amb característiques intermèdies. Les fibres lentes i les ràpides estan especialitzades per diferents demandes durant la locomoció: les fibres lentes s'utilitzen en velocitats reduïdes i sostingudes mentre que les fibres ràpides s'utilitzen a velocitats altes per com escapar d'un depredador.

Les fibres de contracció lenta estan situades al llarg de la línia lateral (figura I.4) i constitueixen menys del 10% de la musculatura. Estan altament vascularitzades, tenen elevat contingut de glicogen i abundants mitocondris. La concentració de mioglobina és elevada i són més eficients en l'ús d'oxigen per generar ATP utilitzant lípids i hidrats de carboni com a combustible energètic (metabolisme aeròbic). Tenen un diàmetre petit i el terme "lentes" es refereix a la seva velocitat de contracció més baixa en comparació amb les fibres de contracció ràpida.

En canvi, fibres de contracció ràpida és el tipus de fibra muscular més abundant i constitueixen més que el 70% de la musculatura en peixos. Estan poc vascularitzades (per això el terme *blanques*), tenen un baix contingut en glucogen i un nombre reduït de mitocondris. Els nivells de mioglobina i lípids són baixos i l'energia es produeix a partir de metabolisme anaeròbic, principalment a partir de la degradació del glicogen. Tenen un diàmetre més gran i per les seves propietats metabòliques (glicolític) subministren energia (ATP) ràpidament però són menys resistents a la fatiga.

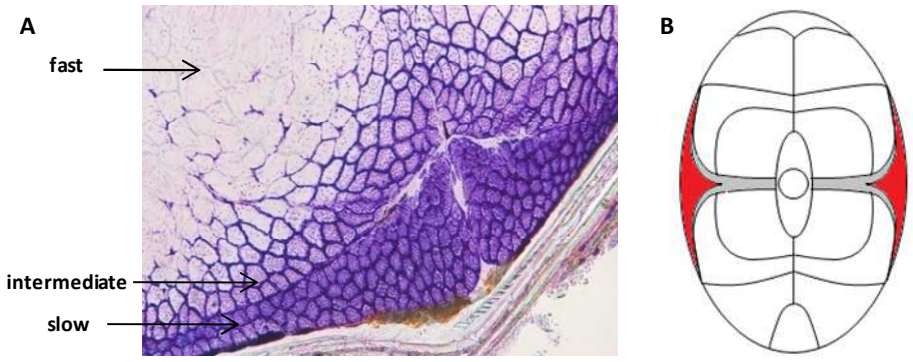


Figure I.4. Fiber type distribution in teleost fish. A Succinate dehydrogenase activity assay showing different fiber types from a tail section of an adult muscle zebrafish. Arrows indicate the three fiber types in fish: fast, intermediate, and slow. Staining method according to Nachlas *et al* (1957) performed in the laboratory. **B** Schematic representation of a tail section. Coloured areas represent fast (white), intermediate (grey) and slow (red) muscle fibers.

4.1.2.- El cor en peixos

El cor en els peixos teleostis està situat en el sac pericardial, anterior a la cavitat corporal i ventral a l'esòfag, entre l'opercle i l'os de l'aleta pectoral (figura I.5). El cor dels peixos teleostis està format per un únic ventricle i un sol atri. El ventricle mostra variabilitat anatòmica entre les espècies de teleostis, que pot estar constituït peruna capa més compacte, per una absència de vasos coronarisal miocardi o per ventricles completament trabècules (sense capa compacta). El cor està format per tres capes de teixit especialitzat (com en altres vertebrats): l'epicardi, el miocardi i l'endocardi. L'epicardi, la capa exterior del cor (o capa interna del pericardi), és una capa fina i elàstica que protegeix el cor de la fricció i composta de cèl·lules epitelials. El miocardi es compon de cardiomiòcits, cèl·lules de múscul esquelètic especialitzat responsable de la contractilitat cardíaca. L'endocardi, la capa interna del cor, està formada per les cèl·lules endotelials i proporciona una superfície llisa en contacte amb el flux de sang. A més de cardiomiòcits, existeixen altres cèl·lules a l'atri i ventricle com fibroblasts (manteniment de la matriu extracel·lular).

El sistema circulatori en peixos difereix de mamífers en que la sang flueix en una única direcció. La sang provinent dels teixits (baixa en oxigen) flueix cap a l'atri a través del *sinusvenosus* (majoritàriament de teixit connectiu) del qual està separat per la vàlvula *sinus valve*. Aquest vàlvula conté les cèl·lules marcapassos responsables per iniciar la contracció. L'atri bombeja la sang cap al ventricle a través de la vàlvula atri ventricular i després, la sang és bombejada al *bulbusarteriosus*. El *bulbusarteriosus* és una cambra elàstica, composta principalment d'elastina i una capa externa col·lagen que s'expandeix durant l'ejecció ventricular per reduir la pressió sistòlica generada pel ventricle i així evitar possibles danys de la vasculatura branquial. Finalment, la sang rica en oxigen flueix cap als teixits. Curiosament, en peixos la pressió arterial entre l'aorta dorsal i l'aorta ventral es redueix (en un 40% en el peix zebra adult) a causa de la resistència exercida per les bràncquies. En mamífers, la sang rica en oxigen irriga el cor a través del vasos coronaris que procedeixen de l'aorta (ventricle esquerre), mentre que en els peixos, la circulació coronària irriga la capa compacta i s'origina a les bràncquies (a pressió reduïda). Curiosament, els cardiomiòcits del miocardi trabeculat del peix zebra contenen abundants mitocondris per la manca de subministrament coronària.

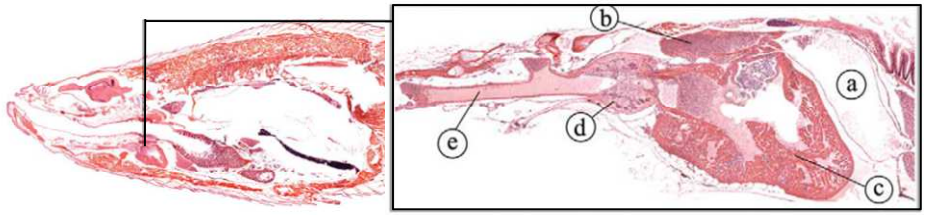


Figure I.5. Zebrafish heart and structures. a, sinus venosus; b, atrium; c, ventricle; d, bulbus arteriosus; e, ventral aorta. Adapted from Menke *et al*. (2011).

4.2.1.- El múscul esquelètic i el peix zebra com a model

Després d'aproximadament dues dècades com un model de recerca, el peix zebra ha fet importants contribucions al coneixement actual del múscul esquelètic degut a que el múscul esquelètic de peix zebra té moltes característiques moleculars com, una xarxa transcripcional per a la regulació de la miogènesi altament conservada (és a dir, factors reguladors de miogènesi), així com les característiques histològiques i ultraestructurals que són molt similars a les dels mamífers. Especialment, el peix zebra ha rebut una atenció especial a l'estudi de la miogènesi en vertebrats ja que, com en altres peixos teleostis, el desenvolupament muscular

es produeix en diferents etapes miogèniques, presenta diferents poblacions de cèl·lules progenitores, es produeix la formació d'un miòtom, signatures moleculars conservats de confinament cel·lular i la diferents tipus de fibres musculars amb diferents propietats contràctils. No obstant això, hi ha algunes diferències en comparació amb mamífers, com ara un creixement muscular continuat o la presència de cèl·lules adaxials mentre que en els mamífers, que la determinació d'una fibra de tipus lenta depèn de senyals moleculars i no s'ha identificat cap població miogènica de fibres lentes.

4.2.2.- Regeneració cardíaca i el model de zebrafish

Últimament, el peix zebra ha guanyat l'atenció en el camp de la regeneració cardíaca. En particular, el descobriment que el peix zebra pot regenerar aproximadament un 20% del ventricle ha suscitat l'interès de la comunitat científica en aquest petit teleosti.

Diversos models de lesió cardíaca s'han desenvolupat en el peix zebra adult: resecció de l'àpex ventricular, crio lesió i ablació genètic dels cardiomiòcits. L'amputació o resecció va ser la primera tècnica utilitzada i la més utilitzada des de llavors. De manera similar que en l'amputació de la cua d'urodels, la resecció de l'àpex ventricular produeix un coàgul de sang que és segellat pel creixement del teixit nou. En canvi, la crio lesió també s'utilitza en els mamífers com a alternativa a la lligació de les artèries coronàries, un model d'isquèmia de miocardi. La crio lesió provoca la destrucció del teixit cardíac i condueix a una resposta apoptòtica més severa que en el model de resecció, que s'estén a vasos coronàries i la vasculatura del voltant de la zona lesionada. D'altra banda, un tret característic de la crio lesió és el desenvolupament d'una cicatriu fibròtica prominent, poc present en els models d'amputació, i que s'assimila a les conseqüències patològiques després d'un infart de miocardi. El teixit fibròtic format per fibrina i col·lagen és substituït completament per teixit cardíac intacte després d'aproximadament 130 dies post lesió (dpi) encara que la recuperació funcional s'ha demostrat ser reduïda. La resecció ventricular, que produeix menor deposició de fibrina especialment en col·lagen, només està localitzada a la frontera amputada i s'elimina ràpidament en comparació amb el model de crio lesió. D'altra banda, l'ablació genètic dels cardiomiòcits no desenvolupa cicatrius (o només ocasionalment segons els autors) i és una metodologia no invasiva. Per contra als models anteriors, l'ablació genètica produeix una gran pèrdua muscular (o de cardiomiòcits) d'aproximadament el 60% de l'àrea total ventricle i elevada mort cel·lular així com una proliferació dispersa per tot el ventricle i l'atri. A causa de l'ablació específic del teixit muscular i del baix rendiment de natació observat en comparació amb el model de resecció, l'ablació genètica dels cardiomiòcits ha estat associada a la patologia d'insuficiència cardíaca. A més, l'índex de proliferació de cardiomiòcits augmenta massivament a aproximadament un 42% en comparació amb els models de resecció i crio lesió. Malgrat les diferències entre els tres models de lesió cardíaca, tots ells evidencien una capacitat notable del peix zebra per regenerar completament el cor, un procés encara poc conegut.

4.3.- El peix zebra com a un nou model d'exercici

La recerca en exercici o l'exercici induït per la natació en peixos s'ha centrat especialment en les espècies migratòries per entendre el comportament migratori, la maduració reproductiva, així com el creixement del múscul esquelètic, amb especial atenció a les espècies comercialment rellevants per a la indústria aqüícola. En peixos, se sap que l'exercici té efectes beneficiosos en augmentar la taxa de creixement i la taxa de supervivència, en reduir el comportament agressiu i millorar la resistència a la fatiga, el rendiment cardíac i la capacitat de càrrega d'oxigen, encara que els efectes poden variar segons el protocol d'exercici i

l'espècie utilitzada. Com a mètode de estandardització, la velocitat de natació en peixos es mesura en longituds de cos per segon (BL/s) ja que la longitud dels peixos indica una mesura de la distància que és relatiu a l'etapa del desenvolupament del peix per a una determinada espècie. La velocitat òptima (U_{opt}) es defineix com la velocitat a la qual l'eficiència energètica és major i el cost de transport (és a dir, consum d'oxigen per unitat de distància recorreguda) és més baix. El nostre grup ha definit l'economia de natació del peix zebra adult i ha determinat la U_{opt} a 13 BL/s, una velocitat que és sorprenentment alta en comparació amb el que s'ha determinat en peixos salmònids (p. ex. 1-2 BL/s), indicant que peix zebra és capaç de sostenir altes velocitats de la natació sense senyals de fatiga.
Encara que en el nostre estudi previ va ser el primer a investigar els efectes fisiològics de la natació a U_{opt} en el peix zebra adult, altres estudis han utilitzat el peix zebra (principalment a estadis larvaris) en condicions d'exercici, establint aquest peix teleosti com a model emergent en la fisiologia de l'exercici, que comprèn àrees de creixement muscular i desenvolupament, metabolisme i també recerca de l'envelliment. Notablement, tot i que s'han realitzat diversos estudis sobre la fisiologia de l'exercici utilitzant el peix zebra, hi ha molt poca investigació que estudiï els efectes de l'exercici en el múscul cardíac i no s'estudia la regeneració del cor.

L'ús de peix zebra com a model d'exercici podria millorar el coneixement sobre els efectes beneficiosos (encara no ben caracteritzades) de l'exercici en vertebrats en condicions de salut i malaltia. El múscul esquelètic és el principal òrgan responsable de la locomoció i, encara que s'ha realitzat una àmplia investigació en aquest teixit, hi ha un considerable interès en esclarir el paper i els mecanismes moleculars de l'activitat muscular en contracció per a una aplicació en patologies humanes i també durant el desenvolupament. D'altra banda, l'exercici ha demostrat ser beneficiós en malalties cardiovasculars i per tant, hi ha una necessitat d'estudiar el paper del paper cardioprotector de l'exercici en mamífers, establint el peix zebra com a un model adequat per a complementar els estudis de mamífers i humans. Aprofitant el model del peix zebra i continuant en nostres estudis previs en exercici induït a partir de la natació en el peix zebra adult, l'objectiu del present treball és millorar el coneixement sobre els efectes cel·lulars i moleculars de l'exercici en el múscul esquelètic i el múscul cardíac en aquest prometedor model experimental.

RESUMS DELS CAPÍTOLS

CAPÍTOL 1:

El present treball descriu els mecanismes adaptatius cel·lulars i moleculars que són responsables de la plasticitat del múscul esquelètic ràpid sota una activitat contràctil induïda per exercici. Aquí, hem adoptat la natació del peix zebra adult com a model d'activitat muscular i hem demostrat, per primera vegada en el peix zebra adult, que l'exercici induït per natació sostingudaresulta en la hipertròfia de les fibres musculars ràpides. La nostra hipòtesi és que això pot explicar, almenys en part, l'estimulació del creixement muscular en peix zebra adult que prèviament hem informat amb les mateixes condicions experimentals. Per tant, al igual que en mamífers i en altres espècies de peix, l'exercici promou el creixement en el peix zebra adult per augmentar la massa muscular com a conseqüència de la hipertròfia de les fibres musculars.

Mitjançant tècniques histològiques hem determinat que els individus sotmesos a condicions d'exercici han incrementant significativament l'àrea i el perímetre de les fibres musculars.A més, mitjançant la tècnica del microarray, hem realitzat un anàlisi transcriptòmic del múscul esquelètic per veure diferències d'expressió gènica entre els individus sotmesos a natació i els individus que van estar en condicions control (no exercitats), que dóna evidències a nivell molecular d'una activació de programes de miogènesi i creixement. Per exemple, aquest anàlisi transcriptòmic mostra per primera vegada en el peix zebra que l'activitat contràctil estimulada per l'exercici en el múscul ràpid ha induït canvis significatius en l'expressió de vies moleculars rellevants per la regulació de la renovació proteica com la via anabòlica IGF-1/PI3K/Akt/mTOR que promou la síntesi de proteïnes. Aquestes observacions reforcen la idea que l'acreció de proteïnes miofibril·lars és un contribuent important pel creixement muscular en peixos i suggereix que la miogènesi de les fibres musculars pot ser estimulada per l'activitat contràctil induïda per exercici en peix zebra adult. En suport a aquesta hipòtesi, l'augment de l'agregació de les proteïnes en el múscul ràpid de truita per l'activitat de natació induïda s'ha associat amb l'activació transcripcional d'un gran conjunt de gens implicats en la biosíntesi de proteïnes i en la contracció muscular i el desenvolupament, incloent components de l'estructura sarcomèrica del múscul esquelètic. Curiosament, en l'estudi actual l'exercici també incrementa els nivells d'expressió ARNm dels coneguts reguladors d'atròfia en múscul esquelètic. Aquestes observacions suggereixen que gens implicats en la regulació de la degradació de proteïnes del múscul esquelètic (atrogens), a més d'un gran conjunt de gens de la via d'ubiquitinització del proteasoma o altres sistemes proteolítics (p. ex. calpaines), podran també participar en la resposta hipertròfica del múscul ràpid de peix zebra induïda per l'activitat contràctil de l'exercici, possiblement per facilitar el manteniment de la renovació proteica muscular durant l'entrenament a llarg termini. A nivell transcriptòmic també hem observat l'activació de gairebé tots els gens de la superfamília de senyalització TGFβ, coneguda per regular la massa del múscul esquelètic en mamífers (p. ex. miostatina, folistatina, receptors i molècules de senyalització) i components de la via de senyalització de BMP fet que suggereix, per primer cop, implicació de la via BMP en la hipertròfia muscular induïda per l'exercici. La nostra hipòtesi és que l'augment de massa muscular associat amb el fenomend'hipertròfia en el peix zebra adult en resposta a l'exercici induït podria haver resultat, almenys en part, d'alteracions en l'equilibri normal entre reguladors negatius (és a dir, MSTN) i positius (és a dir, FST, BMPs) del creixement del múscul esquelètic. A més, l'expressió de diferents factors de diferenciació miogènica com els factors de transcripció de la família Mef2 també es veu alterada en resposta a l'exercici en el múscul ràpid de peix zebra. Aquest

estudi també proporciona proves moleculars per a suggerir que l'exercici en peix zebra adult pot activar un programa de miogènesi resultant de l'activació de les cèl·lules satèl·lit (cèl·lules precursores musculars amb propietats de cèl·lules mare). Es troben diferencialment expressats reguladors de proliferació i diferenciació i marcadors moleculars de cèl·lulessatèl·lit (*pax3*, *pax7* i components de la via Notch i Wnt). A nivell proteic, també hem trobat un augment en el marcador les cèl·lules satèl·lit Pax7 durant les quatre setmanes d'exercici i un augment del marcador de proliferació cel·lular fosfo-histona 3 des de la segona setmana fins al final del període d'exercici. Per tant, el nostre estudi també presenta evidències per suggerir que l'exercici en peix zebra adult ha pogut activar un programa molecular de miogènesi resultant de l'activació de les cèl·lulessatèl·lit, ja que és sabut que poden contribuir de manera notable en el creixement post natal i regeneració muscular després d'una lesió. Fins ara, el paper exacte de les cèl·lules satèl·lit en l'activitat muscular induïda per exercici en múscul esquelètic o si l'activitat contràctil de les fibres musculars esquelètiques pot modificar l'estat de quiescència de les cèl·lules satèl·lit i promoure la seva activació en el múscul adult, no està suficientment clar. Per tant, serà important per investigar en futur estudis si exercici pot promoure la proliferació i/o activació de les cèl·lules satèl·lit en el múscul ràpid de peix zebra per a adults.

Un altre descobriment important en aquest estudi és que la natació induïda estimula l'activació de la proteïna-quinasa activada per AMP (AMPK) en el peixzebra adult. L'AMPK regula l'expressió de gens metabòlics implicats amb processos de generació d'ATP com la glucòlisi o l'oxidació d'àcids grassos per restablir els nivells d'energia de les cèl·lules i, de fet, el perfil transcriptòmic del múscul ràpid de peix zebra adult mostra un enriquiment de gens implicats en processos regulats per AMPK. Tant l'activitat enzimàtica com l'expressió de ARNm de les seves diferents subunitats es troben significativament elevades en el múscul ràpid de peix zebra exercitat. L'avaluació de l'activació de AMPK en resposta a l'exercici en peixos també ha estat avaluada en un altre estudi previ, fet que concorda amb el nostre estudi. Notablement, la inducció de l'activitat d'AMPK després de quatre setmanes d'entrenament en peix zebra, va coincidir amb una sobre-regulació de de *pgc1α*i, en menor mesura, del nivell de proteïna. PGC1α és un regulador co-transcriptional important que controla l'expressió de gens implicats en el metabolisme oxidatiu i biogènesi mitocondrial i és fosforilat per AMPK en mamífers. Estudis previs han demostrat que els nivells de l'ARNm de *pgc1α* poden augmentar després de la natació induïda en peixos encara que s'ha suggerit que PGC1α potser no té un paper regulador en la biogènesi mitocondrial en peixos a diferència dels mamífers. Basat en els resultats presentats aquí, podem establir la hipòtesi que l'adaptació del múscul ràpid induïda per exercici en el peix zebra, inclou una remodelació metabòlica afavorint rutes metabòliques oxidatives i que aquests canvis poden estar mediats, almenys en part, per AMPK, com ha estat prèviament suggerit en truita.

Paral·lelament, i per primer cop en aquesta espècie, s'ha observat un augment significatiu de la capil·laritat en resposta a l'exercici en el múscul ràpid de peix zebra adult. Per tant, a més de la hipertròfia de les fibres musculars ràpides, l'exercici ha augmentat la vascularització d'aquest teixit en el peix zebra adult. Això és consistent amb el conegut augment en nombre de capil·lars que acompanya la hipertròfia de les fibres en els humans i els models experimentals de mamífers i també amb els informes previs que indiquen que la natació induïda augmenta la capil·laritat muscular en diverses espècies de peixos, incloent les larves de peix zebra. Cal destacar que el perfil transcriptòmic del múscul ràpid exercitat de peix zebra adult evidencia clarament l'activació de la majoria de vies de senyalització conegudes en mamífers i peix zebra per regular l'angiogènesi i identifica per primera vegada els programes moleculars responsables de l'augment devascularització d'aquest teixit per l'exercici. Els mecanismes per la qual s'inicia l'angiogènesi en condicions normals en conseqüència de la remodelació adaptativa de l'exercici, són complexes i no totalment caracteritzats, fins i tot en

els éssers humans. Des del nostre coneixement, aportem evidència per primer cop que l'exercici en el peix zebra activa un programa transcripcional complex en el múscul ràpid amb múltiples vies de senyalització (per exemple, VEGF, HSI, TGFβ, efrina-B, PDGF, angiopoietina) conegudes per participar en la inducció i la regulació de l'angiogènesi, resultant en un important augment de vascularització d'aquest teixit. Per tant, hem formulat la hipòtesi que, com en mamífers, l'augment de capil·laritat en resposta a l'exercici pot potenciar l'intercanvi de gasos respiratoris, substrats i els metabòlits entre la sang i el múscul ràpid en el peix zebra adult. En conseqüència, per augmentar la capacitat de bescanvi d'oxigen i la capacitat oxidativa subsegüent, l'exercici pot induir un fenotip més aeròbic en el múscul ràpid en el peix zebra, d'acord amb estudis previs que mostren que la natació incrementa la capacitat aeròbica del múscul ràpid incrementant l'expressió de gens respiratoris en el peix zebra adult, com l'eritropoetina i la mioglobina. En suport a un canvi cap a un fenotip més aeròbic de les fibres musculars ràpides, hem observat un augment en l'expressió gènica d'un gran conjunt de gens que participen en el metabolisme oxidatiu en els mitocondris (és a dir, cicle TCA i fosforilació oxidativa) i de la mioglobina, proteïna de transport d'oxigen. Malgrat el fet que l'expressió dels gens mitocondrials fou poc alterat pel període de formació al llarg quatre setmanes, l'augment de capil·larització de les fibres ràpides induït per exercici en relació amb la seva àrea i el perímetre (és a dir, CCA i CCP índexs) ofereix més suport de la hipòtesi d'augmentar la capacitat oxidativa mitocondrial de fibres musculars ràpides en el peix zebra adults sotmès a la formació de l'exercici aeròbic.

CAPÍTOL 2:

En aquest treball s'ha investigat la regulació de l'expressió de les màquines descrites en mamífers en el múscul esquelètic ràpid de peix zebra adult després de quatre setmanes d'exercici induït per natació. Una de les conclusions més rellevants és la major expressió de *il6*, coneguda com la mioquina prototip en mamífers. Aquests resultats mostren clarament que activitat contràctil en el peix zebra induïda perl'exercici augmenta l'expressió d'ARNm de *il6* en múscul esquelètic com ocorre en mamífers.

Encara que no tenim cap evidència per a la producció i secreció de la proteïna IL-6 ni per l'activitat biològica de Il-6 en el peix zebra múscul ràpid, més suport per a un possible paper per *il6* en la resposta adaptativa del múscul ràpid en el peix zebra a la natació pot suggerir-se des de diverses observacions. En primer lloc, la presència de la seqüència de MEF2 en el promotor de il6 en el peix zebra, juntament amb l'augment de l'expressió dels nivells de MEF2 de mARN i les proteïnes en el múscul ràpid de peix zebra exercitat (capítol 1), recolza la idea que l'augment de ARNm de il6 pot estar associat amb un programa de miogènesi en resposta a la natació induïda en el múscul ràpid. En segon lloc, l'augment en nivells d'expressió il6 ARNm pot associar amb un augment en els nivells d'activitat d'AMPK i d'expressió en el múscul ràpid d'exercici peix zebra (capítol 1) que és coherent amb la coneguda mediació d'AMPK de diverses accions de IL-6 en el múscul esquelètic en mamífers, especialment la potenciació d'oxidació de greixos. En suport a la possibilitat que la producció de Il-6 induïda per exercici podria haver resultat en l'activació d'AMPK i de les seves dianes metabòliques, l'anàlisi transcriptòmic del múscul ràpid de peix zebra exercitat evidencia l'activació de vies catabòliques com la β-oxidació d'àcids grassos i la glicòlisi (capítol 1). En tercer lloc, encara que IL-6 ha demostrat que augmenta la fosforilació de p38 MAPK al múscul dels mamífers, el nostre estudi demostra que l'augment en nivells d'ARNm de *il6* després de 1 setmana d'entrenament és acompanyada per una disminució de l'activitat de p38 MAPK. Aquests resultats podrien conduir a la proposta que la natació no potencia la diferenciació de múscul

esquelètic (és a dir, un procés dependent de p38 MAPK) sobre la proliferació cel·lular muscular, recolzat en el nostre estudi per dades moleculars i l'augment en nivells H3-fosforol·lada (capítol 1). Per altra banda, l'augment de l'expressió de l'ARNm de reguladors positius i negatius de la massa muscular com són*igf1*, *decorin* i *miostatina* després de 1 setmana de natació suggereixen que la regulació del creixement muscular en resposta a l'exercici pot implicar una senyalització complex entre diferents factors de creixement i creiem que la regulació temporal en el nivell de proteïna o sota condicions de cultiu cel·lular podrà aclarir la interacció d'aquests factors en el peix zebra adult.

A més, hem analitzat per primera vegada en una espècie de teleosti l'expressió de *fndc5b*, l'ortòlog en mamífers de FNDC5 (que codifica per irisin). El paper de FNDC5 en mamífers va ser descrit per Bostrom et al (2012), suggerint que la contracció muscular induïda per l'exercici estimula la producció d'irisin per promoure desenvolupament de teixit adipós marró i termogènesi (anomenat *browning*) mitjançant un mecanisme de amb la participació de PGC1α. Tanmateix, els peixos teleostis (incloent-hi el peix zebra) són ectoterms (i el teixit adipós marró, responsable de la termogènesi i de la despesa energètica en els mamífers, no ha estat identificat en aquest grup de vertebrats. Per tant, en lloc de tenir una funció de *browning* del teixit adipós, Fndc5 seria més probable que exercís tingués un efecte metabòlic induint l'activitat d'AMPK i la captació de glucosa localment en les cèl·lules musculars, tal i com s'ha descrit en mamífers. Donada la inducció de *fndc5b*, *pgc1α* il'activitat d'AMPK al múscul ràpid de peix zebra per l'exercici en el peix zebra adult, el possible paper metabòlic de Fndc5 al múscul ràpid de peix zebra requereix més investigació.

El mecanisme molecular pel qual cada una de les màquines esmentades realitza els efectes en els mamífers és encara matèria d'investigació. No obstant això, diverses màquines com IL-6, FNDC5, miostatina, decorin, apelin, BDNF o SPARC s'ha observat que modulen l'activitat d'AMPK i influencien el metabolisme en les cèl·lules musculars. Per tant, seria d'interès en futurs estudis explorar els mecanismes moleculars de les màquines identificades en el peix zebra i el seu impacte en el metabolisme cel·lular del múscul en el nostre model d'exercici.

CAPÍTOL 3:

Els resultats descrits en el present Capítol aborden la qüestió dels possibles efectes immunomoduladors de l'exercici en el peix zebra adult. Al nostre entendre, la regulació molecular de la funció del múscul esquelètic en resposta a LPS en condicions d'exercici no ha estat encara estudiada en els peixos. Ja que el múscul esquelètic és el principal teixit implicat en la resposta adaptativa a l'exercici durant l'exercici i les cèl·lules musculars són capaces d'expressar molècules relacionades amb el sistema immunitari en peixos, hem investigat la resposta transcriptòmic del múscul ràpid exercitat i no exercitat a l'LPS, un component de la capa externa dels bacteris gram negatius habitualment utilitzat per a simular els efectes d'una infecció bacteriana.

El múscul ràpid exercitat i estimulat amb LPS en el peix zebra, va mostrar una major representació de gens relacionats amb la funció immunitària que els peixos no exercitats. Per exemple, l'expressió augmentada dels factors del sistema del complement, així com gens relacionats amb activació leucocitària o limfocitària suggereixen un major activació de la resposta immunitària en l'exercici als músculs. Tanmateix, som conscients que molts dels gens de immune relacionats identificats en el múscul esquelètic de peix zebra en aquest estudi, sovint estan expressats en cèl·lules immunitàries o al fetge (en el cas dels factors del sistema del complement). Això planteja la qüestió de si els gens relacionats amb immunitat identificats

en aquest estudi són en realitat expressats per cèl·lules de múscul esquelètic o per cèl·lules immunitàries infiltrades en el teixit muscular. D'altra banda, és sabut que les cèl·lules musculars dels mamífers, així com el teixit muscular té la capacitat d'expressar diverses molècules immunes, incloent-hi les molècules de presentació d'antígens, els receptors *toll*, citocines i quimosines, quan és estimulat amb agents inflamatoris (incloent LPS) i es creu que contribueixen a una resposta immunitària sistèmica. L'exercici s'ha relacionat amb un efecte antiinflamatori, per exemple, reduint els nivells de citoquines pro-inflamatòries després d'un desafiament immunològic agut i millorar l'estatus dels trastorns associats amb elevat nivells de molècules inflamatòries. No obstant això, la resposta immunitària del múscul esquelètic en peixos en condicions d'exercici ha estat poc investigada. Aquest estudi representa, en el millor del nostre coneixement, el primer intent d'investigar la relació entre exercici i la resposta immunològica en els múscul esquelètic en peixos.

Un fenomen comú durant una infecció sistèmica, com la induïda per l'administració LPS, és una resposta catabòlica en el múscul, descrita com a combinació de la disminució de la síntesi de proteïnes, un augment de la degradació proteica i possiblement apoptosi. En l'estudi actual, la regulació gènica observada en l'expressió de gens implicats en el metabolisme de les proteïnes, especialment en el múscul del peix zebra exercitat, suggereix que això pot ser possiblement un mecanisme necessari per respondre a una infecció aguda amb LPS i, per tant, no podem descartar la implicació addicional d'atròfia muscular en aquest procés com s'ha demostrat en altres espècies de peixos. De totes maneres, degut a que el mostreig es va fer al cap de 72 hores de l'estimulació amb LPS, la supressió dels gens relacionats amb degradació proteica suggereix que caldria analitzar a temps més tempra'ns per veure majors diferències.En considerar la resposta a LPS en el múscul ràpid exercitat, és temptador especular que, a causa d'una elevada proporció d'elements estructurals i contràctils resultants de la hipertròfia de la fibra muscular induïda per l'exercici (capítol 1), el peix zebra hauria de fer front a la demanda energètica de la cèl·lula davant d'una infecció immune i en detriment de la despesa de creixement muscular induïda per l'exercici que concordaria amb la regulació de diferents processos de degradació cel·lular i gens relacionats amb creixement i desenvolupament muscular presents en l'anàlisi transcriptòmic. Per contra, en els músculs no-exercitats, la resposta transcripcional a LPS no ha estat tan robusta com en els músculs exercitats, suggerint que no sigui necessari fer tants ajustos estructurals en la cèl·lula per fer front a l'administració LPS en comparació als músculs que han estat exercitats.

En contrast amb les diferències observades en la resposta transcriptòmica del múscul ràpid a l'administració de LPS entre el peix zebra exercitat i no exercitat, l'exercici induït per la natació no va millorar supervivència a una infecció *invivo*per *Pseudomonasaeruginosa*, un patogen oportunista. Per tant, ens preguntem si els efectes immunomoduladors de l'exercici en múscul esquelètic poden ser prou estimulants com per resoldre una infecció aguda i – en definitiva - letal com s'ha realitzat en aquest estudi. Davant la manca dels efectes de la natació en la supervivència davant d'una infecció letal, la nostra hipòtesi és que és possible que els efectes immunomoduladors de l'exercici podrien ser més rellevantsenmodels crònics d'inflamació de baix grau, mitjançant, per exemple, infeccions cròniques o subletals de patògens o utilitzant models de peix zebra d'obesitat que tenenels nivells de molècules inflamatòries elevats. Per tant, el peix zebra podria ser utilitzat com a model per estudiar l'impacte de l'exercici en condicions d' inflamació crònica de baix grau, característic de malalties com la T2D o artritis reumatoide en els mamífers.

CAPÍTOL 4:

Hem descrit la resposta d'exercir en el cor de peix zebra adult a nivell transcriptòmic i hem demostrat per primera vegada, utilitzant el nostre protocol de formació d'exercici, que quatre setmanes de natació promou la proliferació de cardiomiòcits. El potencial per a la cardiomiogènesi del cor del peix zebra per regenerar d'una lesió cardíaca és ben documentat que, almenys en gran mesura, es produeix a través d'un procés de desdiferenciació i proliferació de cardiomiòcits preexistents, amb només un estudi que assenyala una possible contribució de cèl·lules no diferenciades. Atès que l'exercici i lesió són diferents estímuls, no podem descartar qualsevol possibilitat sobre el llinatge o font cel·lular dels nous cardiomiòcits de peix zebra que hem observat en el nostre estudi. Altres estudis en peixos han vist un augment de l'expressió de marcadors de proliferació cel·lular com *pcna* però no s'ha determinat el tipus cel·lular. De manera semblant en els mamífers, s'ha observat que l'exercici indueix la proliferació de cardiomiòcits en ratolins tot i la baixa activitat mitòtica del cor adult en mamífers. Per tant, l'exercici podria ser un mecanisme conservat a vertebrats per estimular la proliferació de cardiomiòcits.Possibles factors moleculars que podrien induir la proliferació dels cardiomiòcits en el nostre estudi inclou la disminució de l'activitat de p38 MAPK, quinasa que en mamífers s'ha relacionat inversament amb creixement cardíac durant el desenvolupament i és un supressor de la proliferació de cardiomiòcits en mamífers i també en peix zebra; o possiblement mitjançant la inducció de neuroregulina (nrg1) que, tot i no ser significativa, presenta una tendència a estar sobreexpressada i ha estat relacionada amb proliferació de cardiomiòcits en mamífers i peix zebra.
A continuació, hem observat un lleuger augment de la capa compacta del ventricle i miocardi total després de quatre setmanes d'exercici que s'assemblaria a un procés d'hipertròfia similar al que succeeix durant l'entrenament a llarg termini en els mamífers, durant la qual un creixement uniforme del cor és aconseguit per un augment proporcional de les cambres del cor i les parets del miocardi. En peixos teleostis hi ha resultats equívocs sobre les respostes de creixement cardíac a la natació. La hipertròfia cardíaca induïda per l'exercici en mamífers ha demostrat ser dependent del règim d'exercici segons el tipus (aeròbic o exercici anaeròbic),la durada o la intensitat que afectenal creixement cardíac. No obstant això, encara que la resposta transcriptòmica del cor no ha resultat en un nombre tan gran de gens expressats diferencialment com en el múscul ràpid, la regulació de l'expressió de gens que codifiquen per elements contràctils i factors del desenvolupament muscular observats en l'anàlisi transcriptòmic, té semblança amb el perfil transcriptòmic associat amb hipertròfia del múscul ràpid anteriorment descrit en el capítol 1. A més, l'augment significatiu de mTOR a nivell proteic també podria suggerir un augment de la síntesi de proteïnes ja que la hipertròfia cardíaca s'ha correlacionat amb una increment de acumulació de proteïnes derivades d'un increment net de la síntesi de proteïnes sobre la degradació de les proteïnes.Per tant, encara que calen més mesures per avaluar a nivell cel·lular la hipertròfia cardíaca, les nostres dades suggereixen que s'ha produït una resposta hipertròfica en el ventricle del peix zebra adult.

Aquest estudi és el primer en analitzar la funció cardíaca en el peix zebra adult després d'un protocol de natació mitjançant l'ecocardiografia. Els nostres resultats indiquen que no hi ha diferències significatives en la fracció d'ejecció (capacitat contràctil del cor) o ritme cardíac que suggereix que una adaptació de la funció cardíaca no és present després de quatre setmanes d'entrenament en el cor de peix zebra adult. Per tant, és plausible que els augments modestos del miocardi compacte (o paret del ventricle) i mida del cor en aquest estudi no haurien estat suficients per augmentar el volum de contractilitat i el volum d'ejecció i, per tant, la fracció d'ejecció. D'altra banda, la funció cardíaca inalterada en aquest estudi suggereix que el nostre

protocol d'exercici a U_{opt} mantindria els nivells de lliurament d'oxigen als músculs esquelètics, possiblement perquè es compensa per l'augment de capil·laritat en múscul esquelètic ràpid com s'ha descrit prèviament en el capítol 1.

Important, Aquests resultats concorden amb el concepte emergent que exercici pot ser una estratègia alternativa per a la teràpia cardíaca estimulant la proliferació dels cardiomiòcits de manera endògena. A més s'ha demostrat que l'exercici indueix un precondicionament del miocardi protegint així el cor de futures lesions cardíaques com ara un infart de miocardi. Per tant, com hem proposat, podem reforçar l'ús de peix zebra adult com a model per a l'estudi de les adaptacions cardiovasculars en vertebrats. Tanmateix, malgrat molts avenços en el camp, cal més recerca per determinar els mecanismes responsables de la cardioprotecció o precondicionament de l'exercici.

Important, els nostres resultats estan d'acord amb el concepte emergent que exercici pot ser una estratègia alternativa per a la teràpia cardíaca per proliferació endogen estimulant de manera fisiològica. Per tant, com hem proposat, podem reforçar l'ús de peix zebra per a adults com un model d'exercici a l'estudi dels vertebrats adaptacions cardiovasculars d'exercir. Tanmateix, malgrat molts avenços en el camp, més recerca, cal aclarir els mecanismes responsables de la cardioprotecció o precondicionament de l'exercici. De fet, una consideració important és la determinació de quant exercici és necessari per aconseguir els efectes beneficiosos de l'exercici i en el cas nostre estudi, com vols millorar aquests efectes. Creiem que l'anàlisi actual després de quatre setmanes d'entrenament podria estendre a punts de temps anteriors per identificar la rellevància temporal dels mecanismes de regulació específics en l'adaptació.

CAPÍTOL 5:

Els efectes beneficiosos d'exercici regular sobre la salut s'han informat extensament en la literatura i la pràctica de l'exercici regular es recomana com a prevenció per a malalties cardiovasculars en humans. No obstant això, la qüestió de si es mantenen els efectes cardio-moduladors de l'exercici després d'un període de desentrenament (per exemple, aquest estudi) és objecte de debat com ho demostra la literatura disponible de mamífers. Malgrat els resultats aparentment contradictoris, aquests informes indiquen que les adaptacions cardiovasculars a l'exercici (p. ex. hipertròfia cardíaca, capil·laritat cardíaca, pressió sanguínia, contractilitat dels cardiomiòcits) són processos altament dinàmics i que podria ser dependent del protocol d'exercici realitzat així com el temps de desentrenament. Cal esmentar que els estudis en peixos examinant els efectes a llarg termini de l'exercici i el desentrenament (amb o senselesió cardíaca) són limitats.

Aquí, hem investigat els efectes d'exercici com a factor de precondicionament del cor de peix zebra adult, en la recuperació d'una lesió cardíaca després del període d'exercici. Hem trobat que cors exercitats milloren funcionalment i augmenten la proliferació dels cardiomiòcits en aproximadament un mes després de la darrera sessió de formació, en comparació amb els cors lesionats però que no va ser prèviament exercitats. Per tant, tenint en compte el paper de l'exercici proposat en mamífers, inclosos els humans, com a precondicionador enfront a una lesió cardíaca (imitant el fenomen de precondicionament isquèmic descrit en mamífers) i tenint en compte la demostració per Gamperl et al (2001) que el cor en peixos pot ser precondicionat enfront a un dany cardíac (però sotmetent-los en condicions anòxiques), els nostres resultats suggereixen que el cor exercitat del peix zebra d'adult pot haver

adquiritcaracterístiques adaptatives gràcies a l'exercici que proporcionen precondicionament, millorant la regeneració cardíaca després de 28 dies de la crio lesió. L'augment de la proliferació de cardiomiòcits s'acompanya d'una tendència a disminuir el contingut de fibrina després 28 dies post-lesió. Aquestes observacions suggereixen que les cèl·lules cardiomiòcits responen a l'exercici amb una capacitat proliferativa, de la mateixa manera que al capítol anterior i en un context de lesió cardíaca. També hem volgut analitzar l'efecte de l'exercici durant el procés de regeneració cardíaca en el cor del peix zebra després de 7 i 14 dies de la lesió que corresponen a 5 i 12 dies de natació. En aquest cas, l'exercici també ha provocat un augment en la proliferació de cardiomiòcits en els ventricles del peix zebra adult a 15 dies post-lesió i aquesta resposta cel·lular de l'exercici concorda amb marcades diferències en l'expressió gènica en el ventricle, com mostra el nostre anàlisi transcriptòmic. Concretament, hem realitzat l'anàlisi transcriptòmic de ventricles en regeneració de peixos zebra exercitats contra no exercitats per tal d'identificar els gens expressats diferencialment que són rellevants per a la resposta de l'exercici. Notablement, l'exercici ha regulat l'expressió dels factors de transcripció *hand2*, *nfkb2* i *hif1aa*, relacionats en la literatura amb la resposta proliferativa dels cardiomiòcits en el cor de peix zebra adult durant la regeneració cardíaca i també ha regulat diferencialment l'expressió de membres de la família FGF com *fgf17b*, relacionat amb el reclutament de cèl·lules de l'epicardi per la neovascularització del cor en regeneració. A més a més, l'expressió de bmi1 en aquest estudi, un factor recentment identificat com a possible marcador de cèl·lules mare residents en el cor de ratolins, podria indicar la implicació d'una població no identificada de cèl·lules mare al cor de peix zebra adult en resposta a l'exercici. Els factors relacionats amb la potenciació de la proliferació dels cardiomiòcits descrits anteriorment, juntament amb l'expressió diferencial de gens relacionats amb el cicle cel·lular, donen suport a l'augment de proliferació dels cardiomiòcitsobservat en resposta l'exercici en ventricles en regeneració del peix zebra adult. Seria interessant examinar entre els factors identificats en el nostre perfil transcriptòmic quins poden afectar a la capacitat regenerativa del cor de peix zebra i, a més, que podrien tenir implicacions en la investigació cardiovascular en humans. Tanmateix, calen més investigacions per a identificar els factors o mecanismes subjacents (encara no ben descrits en mamífers) per caracteritzar completament la resposta cardioprotectora o precondicionadora de l'exercici en el cor de peix zebra en regeneració, ja que no cada modalitat d'exercici ha de ser necessàriament beneficiosa.

CONCLUSIONS

1. Quatre setmanes d'exercici en el peix zebra adult augmenta la mida de la fibra muscular (hipertròfia) i la vascularització. La resposta hipertròfica a l'exercici en el múscul ràpid es pot regular, almenys en part, per la via de senyalització de mTOR i possiblement també per un de la augment proliferació.
2. L'exercici incrementa l'activitat d'AMPK i l'expressió de pgc1α al múscul ràpid després de quatre setmanes d'entrenament. Aquests resultats suggereixen un paper important per AMPK en la modulació de la resposta anabòlica (és a dir, hipertròfia) i oxidativa proposades en aquest estudi.
3. L'augment de la vascularització en el múscul ràpid juntament amb l'expressió diferencial de gens oxidatius (i.e. metabolisme oxidatiu i pgc1α) i angiogènics suggereixen un canvi cap a un fenotip més aeròbic en el múscul ràpid del peix zebra adult després de quatre setmanes d'entrenament.
4. Els nivells d'expressió de mRNA de il6, il6ra, decorin, miostatina, bdnf, sparc, apelin i els receptors són regulats dinàmicament durant quatre setmanes d'entrenament en el múscul ràpid de peix zebra. En mamífers, aquests factors es descriuen com a factors musculars secretats o *myokines* que influeixen en el metabolisme muscular i el creixement durant l'exercici i que poden promour els efectes beneficiosos per a la salut atribuïts a l'exercici. Hem caracteritzat per primera vegada la regulació de l'expressió de diverses *myokines* en el muscul ràpid del peix zebra adult que podrien tenir un paper rellevant en la mediació els efectes fisiològics de la natació en el peix zebra, de la mateixa manera que s'ha proposat en mamífers.
5. L'exercici promou respostes transcripcionals específiques al múscul ràpid del peix zebra adult exposat a un repte immune. Un repte immune amb lipopolisacàrid bacterià provoca una resposta transcriptòmica en el múscul ràpid, que està particularment enriquida de factors immunològics, desenvolupament muscular i factors de creixement en el múscul ràpid de peix zebra exercicitat en comparació amb múscul ràpid no exercitat. De la mateixa manera que les cèl·lules musculars en mamífers, el múscul ràpid del peix zebra adult podria modular la resposta immune, almenys localment, en condicions d'exercici.
6. El protocol d'exercici establert en el peix zebra adult no va millorar la supervivència després un repte immunològic amb el patogen oportunista *P. aeruginosa* després de quatre setmanes d'entrenament.
7. Quatre setmanes d'entrenament estimula la proliferació de cardiomiòcits i una resposta transcriptòmica al cor de peix zebra que està acompanyada d'una tendència cap a un augment en el creixement cardíac. No obstant això, no s'han observat canvis en la funció cardíaca, concretament de la fracció d'ejecció desrpés de quatre setmanes d'exercici.
8. L'entrenament previ de quatre setmanes millora la funció cardíaca i augmenta la proliferació de cardiomiòcits després de dpi 28 dies post-lesió en el ventricle del peix zebra adult. Aquests resultats suggereixen que l'exercici facilita una millor recuperació després d'una lesió cardíaca provocant un efecte de precondicionament semblant al que s'ha observat en mamífers.
9. L'exercici durant el procés de regeneració després de una lesió cardíaca, estimula la proliferació dels cardiomiòcits després de 11 dies post-lesió (14 dpi) que concorda amb un

perfil transcriptòmic diferencial. L'exercici durant la regeneració regula l'expressió de gens prèviament coneguts a ser expressats durant la regeneració del cor en el peix zebra adult, així com nous gens, suggerint que l'exercici ha induït una resposta molecular concreta durant la regeneració cardíaca i que alguns dels gens identificats podrien ser potencialment interessants per a millorar la regeneració cardíaca en el peix zebra, fet que podria tenir un potencial en medicina clínica.