INFLUENCE OF DIETARY PURPLE CORN EXTRACT ON PRODUCTION, REDOX PARAMETERS AND MYOGENESIS IN RAINBOW TROUT

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Authorization to Submit Dissertation

This dissertation of Alejandro Villasante, submitted for the degree of Doctor of Philosophy with a Major in Animal Physiology and titled ″INFLUENCE OF DIETARY PURPLE CORN EXTRACT ON PRODUCTION, REDOX PARAMETERS AND MYOGENESIS IN RAINBOW TROUT," has been reviewed in final form. Permission, as indicated by the signatures and dates below, is now granted to submit final copies to the College of Graduate Studies for approval.

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Abstract

An *in vivo* experiment was conducted to determine the effects of dietary intake of purple corn extract (PCE) supplementation (5%), an ingredient rich in anthocyanins, on growth performance, plasma antioxidant status and *n*-3 PUFA levels in muscle and plasma of rainbow trout. Results suggest dietary intake of PCE may protect against lipid peroxidation by increasing the antioxidant capacity in plasma as well as by up-regulating the expression of the antioxidant enzyme *gpx1* in erythrocytes in fish. A significantly lower total fat content in fish body implying reduced fat substrate prone to lipid peroxidation was observed. In agreement with this statement we detected a trend toward significantly lower levels of lipid-peroxidation marker (MDA) in plasma of fish fed the PCE supplemented diet. We observed no differences in *n*-3 PUFA levels in muscle, however, significantly higher levels of *n*-3 PUFA in plasma of fish fed the PCE supplemented diet were detected. In addition, an *in vitro* experiment was conducted to determine the effects of three doses (treatments A, B and C; 1x, 2.5x and 10x, respectively) of a mixture of three types of anthocyanidins (peonidin, cyanidin and pelargonidin chloride) on the expression of genes involved in myogenic program, the Notch signaling and antioxidant enzymes in primary myogenic cells from skeletal muscle of juvenile rainbow trout after 24 hours treatment. Anthocyanidins enhance *gpx1* expression in myogenic cells, thereby boosting the skeletal muscle tissue antioxidant defense in fish. For its part, anthocyanidins appears to delay myogenic differentiation in myocytes by up-regulating the expression of *pax7* and decreasing *pax7/myoD* ratio. This effect seems to be mediated by Notch signaling since we observed an up-regulation of two Notch signaling target genes (*her6* and *hey2*), similarly to what has been observed in mammalian C2C12 myoblasts. A second *in vitro* experiment was conducted to determine the effects of same anthocyanidin mixture doses on myogenic program after 24 and 36 hours treatment in early-induced and non-induced myogenic cells from rainbow trout. The effect of anthocyanidins on myogenic program appears to differ between time of exposition to anthocyanidin doses in early-induced myogenic cells by boosting myogenic differentiation signaling after 24 hours treatment while pausing differentiation after 36 hours treatment. These findings demonstrate that anthocyanidins modulate myogenic program in fish myogenic primary cells, thereby potentially affecting somatic growth in fish fed plant-derived extracts rich in this type of

polyphenols. Further research related to *in vivo* protection against lipid peroxidation and modulation of lipid metabolism related to decreasing adiposity in fish fed diets supplemented with additives rich in plant-derived biocompounds is warranted.

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Dedication

To my father and mother for believing in me no matter what...

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CHAPTER 1

Overview

In the past decade the agriculture industry worldwide witnessed sustained fluctuations in both food prices and food supplies. The situation became even more volatile due to global climate changes and its impacts on land productivity, fresh water reservoirs and epidemiologic patterns that have stressed crops and animal industry production. Furthermore, other factors such as the escalation of oil prices, resulting in higher freight cost, and the increased use of agricultural products as feedstock (i.e. cereal grains) for production of biofuels have further complicated this equation (Alexandratos and Bruinsma, 2012; Rana et al., 2009). Therefore, the combination of these economic and environmental pressures leads us to the inexorable question; how will humankind feed a growing human population, expected to reach 9.15 billion in 2050, with finite natural resources and without increasing undernourishment?

It is expected that changes in human diets characterized by increases in the consumption of livestock products (i.e. meat and milk) as well as social-demographic structure, lifestyle and per capita consumption will take place alongside the growth of the human population in the next three decades. However, growth of world food production necessary to meet the increased demand for livestock will be lower than in the past (Alexandratos and Bruinsma, 2012). Although transition to livestock-based diets have largely been completed in most of the developed countries, it will be slower in some major developing countries such as China and India (Alexandratos and Bruinsma, 2012).

Fish is a valuable source of high quality nutrients in human nutrition such as proteins, polyunsaturated long chain fatty acids, vitamins and minerals. Fish derived nutrients could play an important role in structural dietary changes, particularly in developing countries (Bondad-Reantaso et al., 2005; Shim et al., 2009). In 2011, for example, fish accounted for 16.7 % of the global population's intake of animal proteins and 6.5 % of all proteins consumed, highlighting the potential of fish to expand further as a major animal protein source in human nutrition (FAO, 2012). In this regard, among the main forces that influence changes in consumer dietary behavior are urbanization, lifestyle and dietary habits (Popkin,

1999). However, when comparing developing and developed countries different factors may influence fish consumption. In developing countries, for example, increasing income and urbanization are the leading forces behind the increase in demand for fish and meat (Delgado, 2003). To the contrary, in developed countries, the increasing demand for fish is be driven by increased consumer awareness of the health and nutritional benefits of seafood, product quality, standardization, availability and safety, particularly absence of pollutants, antibiotics and carcinogens (Chakraborty et al., 2014; Lubchenco, 2003). The trend worldwide is toward increased urbanization, implying less income gap between developed and developing countries, and an increase in the per capita income estimated to be 1.8 fold higher than the present one by 2050 (Alexandratos and Bruinsma, 2012; Rana et al., 2009).

Over the past two decades, capture fisheries productivity has reached maximum capacity level, and cannot expand further due to the full exploitation of wild fish stocks (FAO, 2010; Rana et al., 2009). Moreover, total global capture fisheries production (91.3 million tonnes) showed a decrease of 2.6 % in 2012 compared with the previous year. Thus, with stagnating global capture fisheries production, aquaculture, the farming and husbandry of freshwater and marine organisms, is the only option to address both the shortfall in capture fisheries and to increase global fish supply. Although, aquaculture has been practiced in various forms and degrees of intensity for more than 2500 years, it has only become an important animal protein industry over the last two decades. Indeed, during this period, aquaculture has shown a remarkable annual average growth rate of 6%, becoming the fastest-growing animal food-producing sector worldwide (FAO, 2012; Reverter et al., 2014; Wang et al., 2008). This dynamic growth has been made possible by the intensification and diversification (i.e. marine species) of the industry, which produces half of the fish and shellfish consumed by humans worldwide (Naylor et al., 2009). Intensification of aquaculture systems has been possible due to the implementation of breeding programs, with genetic selection toward faster growth and disease resistance, higher culture densities (Gjedrem, 1985; Gjedrem et al., 2012; Gjerde, 1986; Overturf and Gaylord, 2009), optimization in the aquafeed industry (i.e. formulation and manufacture technologies) (Barrows et al., 2007; Rana et al., 2009) and improvement in veterinary drugs (i.e. prophylactic and therapeutic) (Reverter et al., 2014; Rico et al., 2013). Additionally, urbanization has been a major driving force in aquaculture diversification due to changes in consumer lifestyle and health concerns (Rana et al., 2009). Regardless of

whether the increasing production in the fed-aquaculture industry is due to an increase in production of low-value species (i.e. herbivorous and/or omnivorous species) or high-value species (i.e. carnivorous species), both scenarios will impact the demand for feed ingredients such as grains and oils of plant origins as well as fishmeal and fish oil, respectively. Furthermore, the direct consequences of this increased demand on feed ingredients can be observed in the escalation of their current prices, stressing the need for the aqua-feed industry to search for more economical alternatives, mainly in carnivorous species aquaculture (Naylor et al., 2009). Moreover, if aquaculture growth continues as predicted over the next few decades, there will be a shortfall of fishmeal needed to supply animal protein to feed carnivorous finfish species (Naylor et al., 2000). Fishmeal, due to its high protein content, excellent amino acid profile, high nutrient digestibility, lack of anti-nutrients and until recently its relative cost-effectiveness has been used as the major protein source for carnivorous species (Gatlin et al., 2007). However, in the midst of an era of volatile commodity prices and supplies, and increasing consumer preferences toward aquatic products high in long-chain omega-3 fish oil content, aquaculture faces the challenge of lowering the aggregate level of fishmeal and fish oil inputs in aquafeeds to pursue more sustainable production as well as to alleviate pressure on fisheries in the near future. Alternative ingredients to substitute fishmeal and fish oil in the aquafeed industry should be nutritionally suitable, readily available, economically viable (cost-effective), consistent with fish growth performance and fish health and environmentally friendly. Substitutes for fishmeal and fish oil replacement in finfish diets include terrestrial plant-based proteins, terrestrial plant-based lipids, single-cell protein and oil, rendered terrestrial animal products, seafood by-products and krill (Naylor et al., 2009).

In the past, research in fish nutrition was mainly focused on nutrient requirement determination of species with an aquaculture potential. However, nutritional research has changed its approach toward evaluation of the effect of different ingredients and their dietary inclusion percentages, in physiology and productive traits of fish, mainly carnivorous species. In this regard, research has been intensively conducted to evaluate the effects of total or partial replacement of fishmeal and fish oil with terrestrial plant-based proteins and oil in growth, reproduction performance, disease resistance and product quality (Burel et al., 2000; Gomes et al., 1995; Izquierdo et al., 2001; Kaushik et al., 2004; Kaushik et al., 1995).

Suitable plant meal alternatives for fishmeal replacement in carnivorous fish diets have been evaluated following nutritional criteria such as digestibility, protein concentration, amino acid profile and anti-nutritional factors (Halver and Hardy, 2002). Overall, carnivorous fish growth performance is reduced by 10% or more when fed diets are substituted with plant protein in lieu of fishmeal, regardless of similar protein concentration, energy density and micronutrient concentration (Barrows et al., 2008; Dias et al., 1997; Kaushik et al., 2004; Overturf and Gaylord, 2009; Pierce et al., 2008). Although, it has been suggested that low feed intake could be a major reason behind poor growth of fish fed fishmeal-free, plant protein-based diets, differences in experimental design when comparing results across studies must be taken into account (Davalos et al., 2006; Dias et al., 1997; Gomes et al., 1995; Kaushik et al., 2004). Factors such as the type of ingredient and the inclusion percentage, ingredient digestibility, anti-nutritional compounds, nutrient supplementation, intestinal inflammatory response and ingredient processing have been suggested to contribute to the reduced growth performance observed in fish fed fishmeal-free, plant protein-based diets (Aksnes et al., 2006; Barrows, et al., 2008; Carter and Hauler, 2000; Cheng et al., 2003; Gatlin et al., 2007; Glencross et al., 2005; Gomes et al., 1995; Heikkinen et al., 2006; Kaushik et al., 1995; Romarheim et al., 2008; Sugiura et al., 2001; Vielma et al., 2000; Yamamoto et al., 2008). The increased inclusion of plant-based feedstuff in fish nutrition implies a greater amount of phytochemicals in finfish diets. Phytochemicals are large plant-derived compounds commonly found in fruits, vegetables, grains and plant-derived beverages (i.e. tea and wine) (Arts and Hollman, 2005). These phytochemical compounds provide protection to plants against microbes, insects and higher animals, and thus, they may exert anti-nutritional effects in animal physiology when present in feeds (anti-nutritive factors) (Krogdahl et al., 2010). In this regard, some of these harmful effects include reduced palatability, less efficient utilization of feed nutrients, altered nutrient balance in diets, inhibition of growth, intestinal dysfunction, altered gut microflora, immune modulation, pancreatic hypertrophy and liver damage (Krogdahl et al., 2010). Among the most relevant anti-nutritive factors in nutrition of carnivorous fish species mentioned are lectins, saponins, insoluble fibres, phytic acid, glucosinolates, phytosterols and oligosaccharides (Krogdahl., 2010). From these, lectins and saponins are of particular interest in carnivorous fish species fed plant meal-based diet including soybean meal since these phytocompounds have been

identified as potential etiological factors in distal enteritis induced by diet observed in theses fish species (Hedrera et al., 2013; Krogdahl et al., 2003; Krogdahl et al., 2010). On the contrary, phytochemicals such as alkaloids, terpenoids, tannins, glycosides, flavonoids, phenolics, steroids and essential oils have been demonstrated to exert beneficial effects such as appetite stimulation, promotion of weight gain, immunostimulation as well as providing bacterial and parasitic resistance in fish (Chakraborty et al., 2014; Reverter et al., 2014). Most of the researches concerning the effects of phytocompounds in fish have been conducted in relation to anti-nutrient activity. Therefore expanding our understanding with regard to the effects of plant-derived bioactive compounds that potentially could exert beneficial effects in fish physiology, productive traits and product quality is an important aspect of future aquaculture research.

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intestinal conditions in rainbow trout *Oncorhynchus mykiss* (Walbaum). *Aquacult Res,* 39, 986-994.

CHAPTER 2

Literature Review

2.1 Introduction

In the past decades, a recurrent practice in the terrestrial animal feed industry (i.e. livestock and poultry) was to include feed additives, such as antibiotics, in sub-therapeutic doses in feeds to promote animal growth. However, recent evidence highlights the strong association between this practice and the development of antibiotic-resistance populations of bacteria, including resistance to antibiotics used to treat human diseases (Hashemi and Davoodi, 2011). Because of the implicit risk of this practice to human health, the European Union and the United States have restricted the use of antibiotics for non-therapeutic uses in animal feeds (Hashemi and Davoodi, 2011). Similarly, the aquaculture industry worldwide has been challenged to look for solutions to address consumer concerns regarding quality and safety of aquaculture products, emphasizing the absence of pollutants, antibiotics and carcinogenic compounds (Chakraborty and Hancz, 2011). In addition, consumers have shown an increased interest toward organic foods and environmentally friendly foods produced in an environmentally friendly manner in the last decade. A promising strategy to reduce the use of drugs and therapeutants without jeopardizing the productive performance in aquaculture systems is to incorporate plant extracts or herbal plants as a source of phytochemicals to evoke beneficial biological effects in aquafeeds (Reverter et al., 2014). The main advantage of this strategy is to use natural products as sources of active compounds, prophylactically or therapeutically, in finfish aquaculture systems. This approach minimizes any threat to fish health and human health (i.e. bacterial resistance, toxicity and carcinogens) as well as being environmentally friendly, since natural products tend to be more biodegradable than synthetic drugs (Reverter et al., 2014).

Phytochemicals are organic compounds derived from plant metabolic processes that provide pigmentation for light energy harvest, protection against radiation, attract pollinators and provide a defense-type mechanism against microbes, insects and higher animals in plants (Krogdahl et al., 2010). There is a plethora of different phytochemical groups such as alkaloids, polyphenols (i.e. flavonoids and phenolics), terpenoids, steroids and essential oils

categorized by their chemical structure and properties. Phytochemicals have been reported to evoke beneficial effects such as anti-oxidation, appetite stimulation, promotion of weight gain, antibacterial and anti-parasitic effects in fish (Chakraborty et al., 2014; Reverter et al., 2014).

Optimization of fish growth performance while reducing production costs is a major goal in the finfish aquaculture industry. Research has been conducted to measure beneficial effects such as growth promotion from the intake of diets supplemented with plant extracts in fish (Chakraborty and Hancz, 2011; Chakraborty et al., 2014; Reverter et al., 2014). Shalaby and colleagues (2006), for example, showed that supplementing fish diets with garlic (*Allium sativum*), increased growth rate and final weight when compared to a control group in Nile tilapia (*Oreochromis niloticus*). Ji et al. (2007a) demonstrated that an herbal mixture of *Massamedicata fermentata*, *Crataegi fructus*, *Artemisia capillaris* and *Cnidium officiale* (2:2:1:1), promoted weight gain in olive flounder (*Paralichthys olivaceus*). Furthermore, the authors observed an increase in the total content of unsaturated fatty acids and a decrease in the percentage of saturated fatty acids in fish fed the plant mixture supplemented diet (0.3%, 0.5% and 1%), indicating a shift in fatty acid metabolism with an apparent protein sparing effect of saturated fatty acids. Moreover, Oskoii et al. (2012) observed a greater final weight and specific growth rate as well as a significantly reduced feed conversion ratio in rainbow trout fingerlings fed diets supplemented with 0.25% and 0.5% of *Echinacea purpurea* compared to a control group. The authors also detected significant increases in hematological parameters such as red blood cell packed volume, white blood cell count, hemoglobin, lymphocyte and neutrophil percentage in fish fed the test diets.

The promotion of growth observed in fish fed diets supplemented with plant extracts is likely to be a consequence of the interactions between the effects of phytochemicals in different contexts such as redox homeostasis, myogenesis, gut microflora, endocrine signaling and immunostimulation (Chakraborty et al., 2014). In this chapter, the topic of redox homeostasis and its influence on myogenesis will be discussed with an emphasis on fish and how phytochemicals, particularly polyphenols, can be used to promote muscle growth.

2.2 Redox Homeostasis in Teleost

Cell redox homeostasis is the balance between the rate of generation and the rate of clearance of pro-oxidant molecules (i.e. free radicals intermediated and non-radicals oxidants) in cells (Figure 1). Free radicals are low molecular weight, diffusible and highly reactive molecules containing one or more unpaired electrons in their atomic electron configuration (Jones, 2008). In contrast, non-radical oxidants, derived from radical molecules, are low reactive molecules with no unpaired electrons. Pro-oxidant molecules such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) are normally produced by cellular metabolism in eukaryotic cells (Trachootham et al., 2008; Valko et al., 2007). Reactive oxygen species can be classified into free radicals such as superoxide anion (O_2^-) and hydroxyl radical (OH^-), as well as, non-radicals such as hydrogen peroxidase (H_2O_2), hypochlorous acid (HCLO) and ozone (O_3) (Diehn et al., 2009). At low to moderate concentrations, within a physiological range, the reactive nature of ROS and RNS allows them to reversibly interact with the structure of biomolecules. These reversible oxidative modifications can modulate biological activity of bio-molecules, mainly proteins, which play crucial roles in the regulation of cellular function such as differentiation, maturation and trafficking of intracellular vesicles (Dröge, 2002; Trachootham et al., 2008). Moreover, protein function can be modulated by the redox system via regulation of their expression, post-translational modifications and stability (Trachootham et al., 2008).

Physiological concentrations of ROS are mainly produced non-enzymatically via redox-reactive compounds, such as semi-ubiquinone, during the mitochondrial electron transfer process in cells where a molecule of oxygen is the last electron acceptor (Dröge, 2002). In this process, a small proportion of electrons “leak” to the oxygen molecule, producing superoxide anion (Cadenas and Sies, 1998; Valko et al., 2007). Additionally, ROS can be generated as bioproducts of reactions catalyzed by enzymes such as NADPH oxidases, xanthine oxidase, lipoxygenases and cycloxygenases. Other sources of ROS are the metabolism of exogenous compounds (i.e. drugs or toxins) via reactions catalyzed by enzymes such as cytochrome p450 and mono-oxygenases. Exposure to environmental factors such as UV radiation promote ROS production as well (Masella et al., 2005). Additionally, transition metal ions such as iron and copper, in their free form, can spontaneously catalyze single electron transfer reactions (i.e. Fenton reaction) thereby giving rise to different free

radical products, mainly ROS. Therefore, animals employ synthesized proteins such as ferritin, transferrin and metallothionein as defense mechanisms to bind such metal ions and reduce their concentrations to trace levels in animal tissues (Hamre, 2011; Valko et al., 2007).

Orthologues of ROS producing enzyme systems found in mammals, bacteria and fungi have been identified in teleost fish species. Members of the NADPH oxidase family members are enzymes that transfer electrons across biological membranes, where the electron acceptor is an oxygen molecule and the product of this process is superoxide anion and hydrogen peroxide (Bedard and Krause, 2007). These are enzymes that are part of the respiratory burst found in phagocytes such as macrophages and neutrophils, which generate ROS as part of an anti-pathogen defense mechanism (Masella et al., 2005). Previous studies have shown the existence of both NADPH oxidase and xanthine oxidase in different teleost fish species, denoting an evolutionary conservation of these enzymes across taxa. In teleost macrophages, for example, expression of NADPH oxidase, as part of the respiratory burst mechanism where ROS are produced to kill pathogens, has been described (Boltaña et al., 2013; Secombes et al., 1992).

Xanthine oxidase is the terminal enzyme of purine catabolism, catalyzing both the hydroxylation of hypoxanthine to xanthine with the production of hydrogen peroxide, and the hydroxylation of xanthine to uric acid, a nitrogen waste product of protein catabolism in mammals and birds (Bedard and Krause, 2007). Terrestrial vertebrates convert ammonia, a direct product of amino acids deamination, into either urea or uric acid to avoid a build-up in ammonia blood concentrations However, teleosts do not need to expend energy converting ammonia to urea or uric acid, since ammonia can be efficiently excreted across the gills into the aquatic environment (NRC, 2011). Recently, it has been shown that xanthine oxidase transcription and enzymatic activity is up-regulated by ammonia-induced oxidative stress in the liver of rainbow trout (*Oncorhynchus mykiss*), goldfish (*Carassius auratus*) and common carp (*Cyprinus carpio*) (Sinha et al., 2014). Lipoxygenases are a family of non-heme iron containing enzymes which catalyze the deoxygenation of polyunsaturated fatty acids, with the production of hydroperoxides as byproducts (Nelson and Seitz, 1994). German et al. (1986) detected a 12-lipoxygenase-like activity in a cytosolic fraction from gill tissue in rainbow trout. Similar, a 12-lipoxygenase activity was observed in thrombocytes from rainbow trout via measuring of 12-lipoxygenase activity products: such as 12- hydroxyeicosatetraenoic acid

(12-HETE) and 12-hydroxyeicosapentaenoic acid (12-HEPE) (Hill et al.,1999). Recently, Ganga et al. (2011) demonstrated that cortisol secretion is regulated through cyclooxygenase and lipoxygenase derivatives from eicosapentaenoic acid (EPA), arachidonic acid (ARA) and docosahexaenoic acid (DHA) in sea bream (*Sparus aurata*).

The cytochrome p450-dependent mono-oxygenases are hemoproteins, highly conserved in all domains, which catalyze the oxidation of a variety of xenobiotics and endogenous compounds such as drugs, carcinogens, fatty acids and hormones, producing ROS as byproducts of these reactions (Chen et al., 1995). Hepatic cytochrome p450 mono-oxygenase activity was identified and compared between different species; brown trout (*Salmo trutta*), leopard frog (*Rana pipiens*) and garter snake (*Thamnophis elegans*) (Schwen and Mannering, 1982). Melancon et al. (1987) detected an increase in hepatic microsomal mono-oxygenase activity when rainbow trout were exposed to PCBs. Moreover, hepatic microsomal cytochrome p450 mono-oxygenase activity has been demonstrated to show sexual dimorphism during the reproductive cycle in rainbow trout (Forlin and Haux, 1990).

Higher organisms produce nitric oxide radicals ($NO^{-\cdot}$) through the activity of nitric oxide enzymes (NOSs) which catalyze the oxidation of one of the terminal guanine nitrogen atoms of L-arginine residues (Palmer et al., 1988). Nitric oxide can be converted further to other types of nitrogen oxygen species such as nitrosonium cation (NO^{+}), nitroxyl anion (NO^{-}) or peroxynitrite ($ONOO^{-}$) (Dröge, 2002). In mammals, three isoforms of NOS; NOS 1 or nNOS (neuronal NOS), NOS 2 or iNOS (inducible NOS) and NOS 3 or eNOS (endothelial NOS) have been identified. The three isoforms catalyze the same reaction; however they differ in their regulation, expression and physiological role (Martinez and Andriantsitohaina, 2009). Both nNOS and eNOS are constitutively expressed, predominantly in neuronal and blood vessel endothelial tissue (regulating smooth muscle activity), respectively (Liu and Huang, 2008). Similarly, NOS has been detected in several tissues such as brain, peripheral nervous system, retina, gut and kidney in fish (Ebbesson et al., 2005). The activity of nNOS was observed via a non-specific indicator of NOS activity (NADPH-diaphorase staining) and through NOS immunochemical staining in the brain of Atlantic salmon (Holmqvist, et al., 1994; Øyan et al., 2000). Furthermore, iNOS was observed to be highly inducible in the gill tissue of rainbow trout post challenge with *Renibacterium salmoninarum* (Campos-Perez, et al., 2000). Saeij et al. (2000) molecularly and functionally characterized iNOS from an

activated carp phagocyte cDNA library. However, eNOS homologues and their activity have not yet been reported in fish (Cox et al., 2001). Recently, nNOS and iNOS-like molecules were detected, via positive immunoprecipitation, both in germ cells and interstitial cells in recrudescing and fully mature catfish (*Clarias batrachus*) testis (Lal and Dubey, 2013).

The clearance rate of pro-oxidant molecules depends both in the concentration of antioxidant compounds and the activity of intracellular and extracellular antioxidant enzymes. Antioxidant enzymes and compounds (endogenous or exogenous) with antioxidant activity either delay or inhibit the oxidation of cellular substrates. The antioxidant enzyme battery includes the superoxide dismutase family (SODs), glutathione peroxidase family (GPx) and catalase (Dröge, 2002). Isoforms of the superoxide dismutase (SODs) family are present in different cellular compartments; SOD1 (Cu-ZnSOD) is found in the cytoplasm, mitochondrial intermembrane space, nucleus, and lysosomes, SOD2 (MnSOD) and SOD3 are found in the mitochondria and extracellular matrix, respectively. All SOD isoforms catalyze the dismutation of superoxide anion into non-radical species such as hydrogen peroxide (H_2O_2) (Trachootham et al., 2008; Zelko et al., 2002). Catalase (CAT), a heme-based enzyme normally found in peroxisomes, catalyzes the conversion of H_2O_2 into H_2O and O_2. Alternatively, isoforms of the GPx selenoprotein family interact with the glutathione system, catalyzing the conversion H_2O_2 into O_2 via oxidation of reduced glutathione (GSH) into oxidized glutathione (GSSG). Previous studies measuring activity, characterization and gene expression have identified isoforms of the above-mentioned antioxidant enzymes in several teleost species. Briefly, Desrochers and Hoffert (1983) compared SOD activity in retina, liver and brain tissue between rainbow trout, frogs and rats. Sutton (1983) used the electro-focusing pattern of SOD from red blood cells to identify different species within the Salmonidae family. Similarly, Scott and Harrington (1989) compared activity of SOD and CAT in erythrocytes between several salmon species, where authors observed differences in the electrophoretic bands among salmon species. Morales et al. (2004) detected an increased enzymatic activity for SOD, CAT and GPx in liver tissue from common dentex (*Dentex dentex*) after five weeks of starvation. Tovar-Ramírez et al. (2009) measured activity and expression of GPx and SOD in European seabass (*Dicentrarchus labrax*) larvae fed a diet supplemented with live yeast *Debaryomyces hansenii*. Yamashita et al. (2012) purified and characterized GPx1 in the red muscle of albacore tuna (*Thunnus orientalis*). Pacitti<1 et al.

(2013) characterized three isoforms for both GPx1 (GPX1a, 1b1 and 1b2) and GPx4 (Gpx4a1, a2 and b) in 18 different tissues from rainbow trout.

On the other hand, a primary intracellular non-enzymatic antioxidant defense mechanism is the glutathione system, composed of a reduced form (GSH) and two oxidized species: glutathione disulphide (GSSG) and glutathione mixed disulphide with protein thiols (GS-R) (Meister and Anderson, 1983). The oxidized species, GSSG and GS-R, can recover their reduced status (GSH) enzymatically, through the activity of NADPH-dependent glutathione reductase and the thioredoxin/glutaredoxin system, respectively (Filomeni et al., 2002). Although, GSH functions as an intracellular redox buffer, it also modulates the activity of thiol-dependent enzymes, with crucial cysteine residues sensitive to redox changes (Finkel, 2000; Klatt and Lamas, 2000). Additionally, GSH, the most abundant peptide in cells, perpetrates a plethora of functions. These include direct scavenging of $HO^{\cdot}$, singlet oxygen, and regeneration of other antioxidants such as vitamin C and E to their active forms (Trachootham et al., 2008).

The glutathione system and its related enzymes has been identified and analyzed in teleost fish species. Lange et al. (2002) measured the effect of single and/or combined exposition to cadmium and zinc in the hepatic concentration of glutathione in rainbow trout. Nagai et al. (2002) purified and characterized glutathione peroxidase from liver of Japanese sea bass (*Lateolabrax japonicas*). Glutathione, glutathione transferase, glutathione peroxidase and glutathione reductase were identified and their activities measured in liver, kidney and gills from Nile tilapia (*Oreochromis niloticus*), African sharptooth catfish (*Claris lazera*) and common carp (*Cyprinus carpio*) (Hamed et al., 2004). Hansen et al. (2007) analyzed the mRNA concentrations of SOD, CAT, GPx and GR in gills, liver and kidney of *Salmo trutta* (brown trout) from three different populations exposed to different metal concentrations in their natural environment. Rosety et al. (2005) determined the activities of several antioxidant enzymes such as SOD, CTA and GPx in gill from *Sparus aurata* (sea bream) toward malathion (an organophosphate insecticide) exposure. Further, Malandrakis et al. (2014) cloned and characterized four full-length GPx cDNAs from the liver of sea bream. Additional endogenous non-enzymatic antioxidant defense mechanisms include free amino acids, peptides and proteins. Although, these compounds have relatively low specific antioxidant activity on a molar basis, when present at high concentrations they show

significant antioxidant activity (Dröge, 2002; Medina-Navarro, et al., 2010). In animals, another important antioxidant defense mechanism includes exogenous antioxidants obtained directly from the diet. These compounds can exert their antioxidant activity directly or indirectly by derived metabolites. Dietary antioxidants show a wide variety of chemical structures; fat-soluble compounds such as tocopherols (i.e. vitamin E) and carotenes (i.e. vitamin A precursors), water-soluble vitamins such as ascorbic acid (vitamin C), trace minerals (i.e. selenium, zinc, manganese) and polyphenols (Sies, 1993; Valko et al., 2007). These compounds (i.e. vitamins and minerals) are essential to animals since they play important roles other than antioxidants, for example, as coenzymes and cofactors in the metabolism of macronutrients such as carbohydrates, lipids and amino acids as well as precursors of metabolites with important role in the animal physiology (i.e. retinoic acid) (Halver and Hardy, 2002).

In animals including fish, endogenous and exogenous anti-oxidant defenses are crucial for the maintenance of the cellular redox balance and ultimately cell homeostasis (Trachootham et al., 2008). However, under certain circumstances including an increase in pro-oxidant production or a decrease in pro-oxidant-scavenging capacity, pro-oxidant concentrations build up, disrupting the redox equilibrium and causing oxidative stress in cells (Jones, 2008; Valko et al., 2007). Oxidative stress induces oxidative damage to macromolecules, such as lipids (lipid peroxidation), proteins and nucleic acids, which can affect cellular signaling processes, causing for example, aberrant cell death or contributing to disease development (Trachootha et al., 2008). Lipid peroxidation, the process where oxidants such as free radicals or non-radical species attack lipid containing carbon-carbon double bond(s) (unsaturation), mainly PUFAs and HUFAs, is considered one of the most prevalent mechanisms of cell damage (Halliwell and Chirico, 1993). An interchange of hydrogen with an oxygen atom in a carbon atom occurs during oxidative attacks, resulting in the production of lipid peroxyl radicals and hydroperoxides, which can further attack any subsequent lipid molecules, and thus propagate as a chain reaction (Yin et al., 2011). Oxidative damage of the polypeptide backbone or protein peroxidation is initiated by the hydroxyl-dependent extraction of the α-hydrogen atom of an amino acid residue to form a carbon-centered radical which can react with oxygen, resulting in an alkylperoxyl radical intermediate. Through a series of reactions, the alkylperoxyl intermediate by interacting with ROS and/or iron or copper, can form

alkylperoxide followed by formation of an alkoxyl radical, which may be converted into a hydroxyl protein radical. Alkoxyl radicals can initiate the cleavage stage of a peptide bond (oxidative cleavage of proteins) which can result in direct oxidation of lysine, arginine, proline and threonine residues yielding protein carbonyls (Berlett and Stadtman, 1997). For its part, oxidative damage to DNA occurs when free radicals such as ROS react with the bases or sugar-phosphate backbone, causing either crosslinks or hydroxylation and base fragmentation (Núñez, et al., 1999).

Aquatic organisms are exposed to a wide variety of environmental stressors such as fluctuations in water temperature and in oxygen partial pressure, ultra-violet radiation and anthropogenic pollutants (Aurousseau, 2002; Dabrowski et al., 2004; Farmen et al., 2010; Finne et al., 2008; Jeffries, et al., 2014; Krumschnabel et al., 2005; Olsvik et al., 2013; Vinagre et al., 2012). In commercial intensive fish culture systems, the use of high stocking densities can predispose oxidative stress in fish, for example, through oxygen partial pressure depletion and overall water quality deterioration (Ashley, 2007; North et al., 2006; Turnbull et al., 2005). Oxygenation of the rearing water to increase biomass and production has become a common practice in recirculation intensive systems. However, this practice has the underlying risk of causing hyperoxia, which can induce respiratory acidosis and gill oxidative cell damage in cultured fish (Olsvik et al., 2006).

Similarly, cold water species must deal with high dissolved oxygen levels, since oxygen partial pressure varies inversely with water temperature (Egginton and Sidell, 1989). Furthermore, cold water fish species maintain high concentrations of intracellular lipids, mainly polyunsaturated, as a mechanism to ensure optimal cell membrane fluidity in low environmental temperature habitats (Hulbert, 2003; Raynard and Cossins, 1991). However, enhanced intracellular contents of PUFAs and highly unsaturated fatty acids (HUFA) can aggravate lipid peroxidation in these fish species in an oxygen rich environment. As a consequence, cold water fish species, such as salmon and trout, deposit high concentrations of lipid-soluble antioxidants (i.e. vitamin E and astaxanthin) in their tissues; mainly skeletal muscle and liver to protect unsaturated lipids against lipid peroxidation (Hamre, 2011; Nakano et al., 1999). Therefore, *in vivo* lipid peroxidation is an important aspect in nutrition of carnivorous cold water fish species, particularly because these are species with a high content of unsaturated fatty acids in their tissues. Lipids and proteins from the diet are

important components in carnivorous fish nutrition, since they supply most of the energy requirement of such fish. In this regard, the aquafeed industry produces diets with relatively high levels of lipids based on ¨the protein sparing effect of lipids¨ to ensure a cost effective growth performance, thereby saving dietary protein for muscle growth in fish (Naylor et al., 2009). However, fish fed high fat diets tend to exhibit an undesirable increase in visceral fat content (Arzel et al., 1994; De la higuera, et al., 1977; Heinen, et al., 1995; Reinitz et al., 1978). In the past, fish oil has been the lipid source of choice in aquafeeds because of its excellent fatty acid profile, rich in PUFAs and HUFAs from the omega-3 series (Hamre et al., 2001; Hung et al., 1981; Koshio at al., 1994). Carnivorous fish (mainly marine species) are unable to synthesize sufficient amounts of HUFAs, making it necessary to obtain them via the diet (Sargent et al., 1999). Thus, fish fed high fish oil diets under intensive culture conditions are prone to lipid peroxidation, due to the increase in visceral fat content, as well as, increment in PUFA and HUFA concentrations in their tissues. Furthermore, lipid peroxidation can be aggravated in carnivorous fish species fed plant meals/vegetable oils based diets due to a higher risk of hepatic oxidative stress, greater adiposity and greater plasma lipids concentration as well (Benedito-Palos et al., 2008; Naylor et al., 2009; Olsvik et al., 2011; Torstensen et al., 2011) (Figure 2). Hence, feeding fish a proper diet with adequate amounts of dietary antioxidants (i.e. vitamins and trace minerals) is of vital importance to promote health in fish under intensive culture operations (Nakano et al., 1999; Oliva-Teles, 2012).

2.3 Dietary Antioxidants in Fish

As previously mentioned, dietary antioxidants including carotenoids (i.e. vitamin A or retinol), vitamin E (α-tocopherol), vitamin C, trace minerals (i.e. selenium, cooper and zinc) and polyphenols are a crucial part in the antioxidant defense mechanisms in fish (Halver and Hardy, 2002).

Carotenoids are a vast class of naturally occurring fat-soluble pigments that are synthesized by algae and plants (Figure 3). Although, animals including fish do not synthesize carotenoids *de novo*, they are able to either directly accumulate them from food or partly modify them through metabolic reactions (Maoka, 2013). Although, carotenoids are well-described antioxidant compounds, protecting against the oxidative damage induced by

free radicals (Burton 1989; Krinsky and Yeum, 2003), they serve as precursors to vitamin A (retinoic acid), a key regulatory molecule of cell signaling in the epithelium as well as during embryonic development in vertebrates (Bowles et al., 2006; Kawakami et al., 2005; Vermot and Pourquie, 2005). In addition, carotenoids may improve resistance against pathogens by increasing either the production of immunoglobulin or the proliferation of immune cells (Bendich, 1989; Jyonouchi et al., 1994; McGraw and Ardia, 2003). Moreover, during sexual maturation in fish, carotenoids provide external pigmentation to increase the chances to attract mates (Garner et al., 2010). In fish, carotenoids are mainly accumulated in their integument (i.e. skin) and gonads. However during growth phase (pre-sexual maturation), salmonids species are able to accumulate carotenoids like astaxanthin or canthanxanthin in the skeletal muscle, which are relocated in skin and developing eggs during sexual maturation (Garner et al., 2010; Maoka, 2011).

Due to their hydrophobic nature carotenoid digestion, absorption and transport mechanism are associated with lipids. In fish, most dietary carotenoids are lost in the feces due to reduced gut absorption, thus affecting the ability to utilize dietary carotenoids in fish (Bjerkeng, 2008). Most carotenoid intestinal absorption takes place with no previous conversion at the enterocyte brush border along the proximal intestine (García-Chavarría and Lara-Flores, 2013). Upon intestinal cell uptake, dietary precursors of vitamin A (pro-A carotenoids) are enzymatically converted into retinal, which is further reduced to its alcohol form by NAD dependent dehydrogenases, which together with the retinol derived from diet, is converted to retinyl esters (Zempleni and Daniel, 2003). Retinyl esters are incorporated into chylomicrons that are released into the lymph. The subsequently uptake of retinyl esters by the liver follows an ester-catalyzed hydrolysis that yields retinol that binds with cytoplasmic retinol-binding protein. A fraction of the all-trans-retinol in hepatocyte binds with a specific retinol-binding protein that together with transthyretin in secreted into the plasma and distributed to other tissues. (Zempleni and Daniel, 2003). In fish, carotenoids are transported by high-density lipoproteins (HDL) and albumin to peripheral tissues and to a lesser extent by low-density lipoproteins (LDL) (Ando et al., 1985; García-Chavarría and Lara-Flores, 2013). Additionally, during sexual maturation, both HDL and vitellogenin are associated with carotenoid mobilization from muscle to integument and from muscle to ovaries, in salmonids species (Ando and Hatano, 1988).

Vitamin E is another group of fat-soluble compounds (i.e. tocopherols and tocotrienols) with an important role as dietary antioxidants in animals including fish (Hamre, 2011). In addition, vitamin E exerts other biological roles including biological membrane stabilization, modulation in eicosanoid synthesis and disease resistance by immune response modulation (Hamre, 2011). Furthermore, it has been reported that vitamin E protects unsaturated fatty acids in cell membrane against lipid peroxidation, positioning its phytyl chain within the hydrophobic inner part of cell membranes, while exposing the chromanol ring (with the reactive polar hydroxyl group) close to the membrane surface (Kagan et al. 1993; Wang and Quinn 2000; Quinn 2004). Thereby, vitamin E protects PUFAs by donating hydrogen atoms to the lipid peroxyl radicals, and thus breaking the chain reaction involved in lipid auto-oxidation (Hamre, 2011). However, the oxidation of vitamin E results in the formation of tocopheroxyl radical, which can promote lipid peroxidation if it is not reduced back into vitamin E. In this regard, ascorbic acid has been demonstrated to regenerate vitamin E by reducing the tocopheroxyl radical in liposome model membranes (Niki, 1987) and organelle preparations (Wefers and Sies, 1988). Similarly, it has long been reported that vitamin C has the ability to regenerate and/or spare vitamin E in fish such as Atlantic salmon (Hamre et al., 1997), gilthead sea bream (*Saprus aurata*) (Montero et al., 1999), juvenile lake sturgeon (*Acipenser fulvescens*) (Moreau et al., 1999b) and channel catfish *(Ictalurus puncattus)* (Lim et al., 2000). Sealey and Gatlin (2002) observed a significant interaction effect between vitamin E and Vitamin C in weight gain, feed efficiency and mortality in juvenile hybrid striped bass. The authors suggest that the significant interaction detected may be due to the ability of vitamin C to regenerate vitamin E to its functional form and/or since the ability of vitamin E to spare vitamin C to some extent. Similarly, Lee and Dabrowski (2003) detected a significant interaction between vitamin C and vitamin E over weight gain, feed intake, feed efficiency in yellow perch (*Perca flavescens*). Moreover, the authors reported that liver vitamin E concentration was increased in fish fed vitamin E-deficient diets supplemented with vitamin C (250 mg/kg) compared with fish fed the same diet with no vitamin C supplementation. These results support the thesis that vitamin C can regenerate or spare vitamin E *in vivo*. In agreement with these studies, Yildirim-Aksoy and coauthors (2008) observed an increase in vitamin E liver concentration by supplementing the diet with vitamin C in channel catfish. Similar, Gao and collaborators (2014) found liver vitamin E

concentration was significantly augmented with increasing dietary levels of vitamin C in juvenile flounder (*Paralichthys olivaceus*), again indicating a sparing effect of vitamin C on liver vitamin E in fish.

Selenium as part of the active site (selenocysteine residues) in glutathione peroxidase enzymes contributes with vitamin E regeneration as well. The significant interaction between selenium and vitamin E observed in fish is evidenced when selenium deficiency leads to reduced levels of vitamin E in tissues (Hamre, 2011). Therefore, the dietary vitamin E requirement can vary based upon dietary composition (Hamre, 2011). Feeding fish, for example, diets with oxidized oil increases vitamin E requirement due to a higher consumption of tocopherols. This is especially true in cold water fish species, since the vitamin E requirement seems to vary inversely with temperature (Cowey et al., 1984; Tacon, 1996). Moreover, the vitamin E requirement can be increased in fish fed diets with low inclusion levels of vitamin C and selenium (Hamre, 2011). In fish diets, tocopherol is used as an ester since it shown to be more stable than its free form under storage conditions. The tocopherol ester is hydrolyzed into the free alcohol form for further absorption in the gut (Halver and Hardy, 2002). Although, vitamin E absorption mechanism has been poorly described in both mammals and fish, it appears to be protein-mediated with kinetics shown to be similar those observed in protein-mediated transporters (i.e. fast, saturable and temperature dependent) (Hamre, 2011). Similar to vitamin A, most part of the tocopherols is incorporated into chylomicrons that pass into the lymph and ultimately transported to the liver. However, a lower fraction of the chylomicrons containing vitamin E goes to peripheral tissues such as muscle and adipose tissue where are catabolized by lipoprotein lipases (Traber et al. 1985; Rigotti 2007). The vitamin E homeostasis in fish has been suggested to be similar to mammals (Hamer, 2011). Upon liver uptake, vitamin E can be either excreted in the bile or transported to the blood stream via very low-density lipoproteins (VLDL). For its part, VLDL can be either delipidated in the circulation and returned to the liver or converted into low-density lipoprotein (LDL). Low-density lipoprotein can transport vitamin E toward peripheral tissues such as adipose and skeletal muscle. However, fish under certain situations require an increase of vitamin E, for instance, when fish are fed a diet rich in PUFAs and/or HUFAs (Trenzado et al., 2009), vitamin E will be reallocated from adipose tissue toward liver for further metabolization.

As previously mentioned, vitamin C is a water-soluble compound essential for fish since they cannot synthesize it, and thus fish require vitamin C from the diet (Dabrowski, 2001). Upon dietary intake, vitamin C is absorbed by the enterocytes via an active transport system in species that cannot synthesize it such us teleost species. However, vitamin C is absorbed via a passive transport in species that are able to synthesize this vitamin (Zempleni and Daniel, 2002). Upon gut uptake, vitamin C is transported by albumins to tissues with an active metabolism including brain, liver and head kidney in fish (Dabrowski, 2001), possibly due to vitamin C providing an effective antioxidant mechanism in these tissues with high mitochondrial activity, and thus with high ROS production. However, environmental stimuli including changes in both salinity and temperature, exposition to heavy metals and fuel oils cause changes in vitamin C concentration in fish tissues, many of them in a tissue-specific manner (Dabrowski, 2001). Although, vitamin C has long been described as an antioxidant compound in humans and animals including fish, ascorbic acid is essential for collagen formation, wound healing, hematopoiesis and detoxification of compounds (Dabrowski, 2001). Moreover, it has been reported that vitamin C stimulates serum hemolytic complement activity, proliferation of immune cells, phagocytosis, the release of signal substances and antibody production in salmonids species (Verlhac and Gabaudan, 1997). The antioxidant potential of vitamin C synergistically acts with vitamin E and selenium to maintain the activity of glutathione peroxidase and superoxide dismutase (Halver and Hardy, 2002) (Figure 4).

Previous works have been conducted to evaluate the antioxidant potential of vitamin C in fish under different environmental pro-oxidant conditions. For example, Dabrowski et al, (2004) reported that hyperoxia (180% oxygen saturation) increased the growth rate of juvenile rainbow trout fed diets supplemented with the highest ascorbic acid dose (1000 mg/kg) compared with fish under hypoxia and normoxia fed diets supplemented with lower doses 10 and 100 mg/kg of vitamin C, respectively. The authors suggest the significant effects found in growth rate by supplementing high dietary levels of vitamin C in fish under hyperoxia may be due to the ability of vitamin C to scavenge free radicals generated by oxygen supersaturating environment. Gao et al, (2013) observed enhanced antioxidant protection as well as an improvement in growth performance in red sea bream juveniles fed diets containing oxidized fish oil supplemented with 400 mg and 800 mg of vitamin C per kg

of diet. Recently, Mozhdeganloo et al, (2015) reported that vitamin C (17.2 μg/L) protects against oxidative damage induced by methylmercury in liver isolated from rainbow trout. Interestingly, Passi et al. (2004) suggested that the increase *in vivo* concentrations of oxidative damage biomarkers associated with aging in rainbow trout could be linked to an age-dependent drop in the concentration of antioxidants such as vitamin E and vitamin C in skeletal muscle tissue, rather than a decline in antioxidant enzyme activities and GSH concentration.

Another important dietary antioxidant is selenium (Se), an essential trace mineral that is crucial involved in the active site of enzymes with antioxidant functions including glutathione peroxidases (Hilton et al., 1980). In terrestrial animals, glutathione peroxidase activity in erythrocytes, plasma and other tissues shows a direct linear association to selenium dietary intake (Halver and Hardy, 2002). Dietary selenium supplementation has been shown to improve growth and antioxidant status in rainbow trout under crowding conditions. Indeed, concentrations of lipid peroxidation biomarkers such as malondialdehyde (MDA) and 8-isoprostane showed inverse linear associations with selenium intake in the serum and muscle of fish. Moreover, activity of GPx in trout serum increased linearly in relation to selenium intake. Interestingly, it appears that the bioefficiency of Se depends on the conjugated form through which it is being given in the diet. Küçükbay et al. (2009) observed that the organic form of selenium supplementation (selenomethionine) was more effective at exerting beneficial effects than the inorganic form (sodium selenite) in rainbow trout. Similarly, Han et al. (2011) observed a direct linear association between hepatic GPx activity and selenium hepatic concentration with selenium dietary intake in gibel carp (*Carassius auratus gibelio*).

Although vitamins E and C, carotenoids, and some trace minerals are the most studied antioxidant compounds, currently, polyphenols and their metabolites are widely recognized as strong antioxidant compounds as well (Scalbert et al., 2005a; Scalbert et al., 2005b). These phytochemicals, secondary plant metabolites, have a molecular structure characterized by having several hydroxyl groups on aromatic rings. Based upon the number of phenol rings contained in their structures and the elements that bind these rings to one another, polyphenols can be classified into different groups such as phenolic acids, flavonoids, stilbenes and lignans (Manach, et al., 2004). Furthermore, polyphenols may be associated with various types of carbohydrates as well as with organic acids. Most of the research

pertinent to polyphenol biological activity has been conducted in cultured cells or isolated tissues with their aglycones1 or glycosides conjugated (Manach et al., 2004). Polyphenols, for example, may boost the synergism between vitamin C and vitamin E (i.e. oxidation regeneration cycle) by acting at the water-lipid interfaces based on their intermediate hydrophobicity nature (Manach et al., 2005). However, evidence suggests that polyphenols or their metabolites may exert their antioxidant protection by acting at several points such as neutralizing ROS and RNS or transition metals (chelation) that produce pro-oxidants oxidizing GSH. Additional antioxidant protection exerted by polyphenols includes decreasing the activity of enzymes such as NADPH oxidase and NOS, suppressing inflammatory signaling cascades like ASK1 and MAPK downstream, and activating transcription factors such as Nrf2 that regulates the transcriptional activity of antioxidant enzymes (i.e. SOD, GPx, heme oxygenase (HO)-1 and γ-glutamate-cysteine ligase catalytic subunit, which is the rate-limiting step for GSH synthesis) (Chuang and McIntosh, 2011). Therefore, polyphenols can modulate the cellular redox status and redox-dependent reactions either by interacting with cellular receptors or enzymes involved in signaling transduction, or promoting growth via enhancing cell survival and cell/tissue function in fish (Chakraborty et al., 2014; Scalbert et al., 2005). A plethora of cellular processes rely on redox balance, pointing out the importance of maintaining pro-oxidants (i.e. ROS and RNS) at low to moderate concentrations in cells (Virgili and Marino, 2008). Nitric oxide and superoxide anions at moderate concentrations play important roles as regulatory mediators in signaling processes involved in a wide variety of physiological functions such as regulation of vascular tone, monitoring of oxygen tension in the control of ventilation, erythropoietin production, signal transduction from membrane receptors, defense against pathogens, activation of mitogenic response and the regulation of skeletal muscle myogenesis (Barbieri and Sestili, 2012; Dröge, 2002; Valko et al., 2007).

In the past years, emerging evidence suggests that polyphenols may evoke a major part of their beneficial effects either by polyphenol-derived metabolites from the gut microbiota or by polyphenol-derived metabolites from both enterocyte and hepatocyte phase I and phase II metabolism in humans and mammalian models (Cardona et al., 2013; Dueñas et al., 2015). A fraction of dietary polyphenols is deconjugated (i.e. deglycolyzation) in the small intestinal lumen by enzyme activity. Subsequently, these compounds are absorbed by enterocyte and successively undergo intestinal and liver phase I and phase II metabolism. On the other hand,

a second fraction of dietary polyphenols passes directly into the lumen of the colon where microbial metabolization takes place. Then the microbial-derived metabolites are absorbed by colonocytes, and transported to the liver for further phase I and phase II metabolism. Whether similar processes occur in teleost species fed diets supplemented with additives rich in polyphenols has not yet been addressed, and thus further research in this field is warranted.

2.4 Myogenesis in Teleost

Skeletal muscle is a complex tissue matrix of muscle fibers, myogenic precursor cells (MPCs), nerves, connective tissues, fibroblasts, skeletal osteocytes, adipocytes and capillary endothelial cells (Johnston et al., 2011). In fish, skeletal muscle (striated muscle) can be discretely divided into two main fibers types. The fast-twitch fibers (white fibers) are supported by anaerobic metabolism (phosphocreatine hydrolysis and glycolysis) and constitute nearly 90 – 95% of all muscle tissue in most teleost fish species, with the exception of sustained swimming species. The slow-twitch fibers (red fibers) are mainly aerobic muscle fibers with high oxidative capacity and thus mitochondrial density being predominant in the steady swimming species myotomal mass (Johnston et al., 2011). The main function of myotomal mass is to serve as a locomotion mechanism, providing enough propulsion power for the functional demands of swimming in fish.

Skeletal muscle development and growth are important components of the animal growth process. Muscle growth involves the production of new muscle fibers (hyperplasia or recruitment) and the subsequent enlargement of existing muscle fibers (hypertrophy and elongation) in teleost species. Myogenesis is the process of new muscle fiber generation that is common to all vertebrates, and consists of several events that occur in a sequential manner including the specification of undetermined stem cells into myogenic lineage, the determination and activation of quiescent satellite cells, proliferation of myoblast, myoblast commitment toward differentiation into myocytes and ultimately progression toward terminal differentiation with the formation of myotubes or multinucleated muscle fibers. In teleosts, skeletal muscle can be divided into three main phases that exhibit differences in the gene expression patterns of myogenic transcription factors as well as in the expression of structural muscle protein encoding genes (myogenic waves) (Valente et al., 2013). The first phase is embryonic myogenesis, which is driven by the maternal mRNA transcripts (maternal effects).

This maternal effect orchestrates the development until mid-blastula transition when zygotic transcription is initiated. In this phase, activated MPCs, which express myogenic regulatory factors (MRFs) such as *myoD* are activated, proliferate, and become committed to a myogenic fate at the end of gastrulation (Johnston, 2006). The second phase of myogenesis is stratified hyperplasia, involving the production of muscle fibers on discrete germinal zones in fish embryo. This process is the main mechanism producing new fibers during late embryonic and early post-embryonic growth. Furthermore, the appearance of intermediate fibers, which are muscle fibers with an intermediated aerobic capacity and contraction speed as well as a high anaerobic glycolysis capacity found between fast and low muscle layers, occurs during stratified hyperplasia in most teleost species (Valente et al., 2013). The final phase of myogenesis is mosaic hyperplasia involving the proliferation of MPCs present in the myotome that subsequently fuse to form myotubes on the scaffold of existing fibers, forming a mosaic appearance of fiber diameters. This process continues during juvenile stage in fish, contrary to mammals and birds where hyperplasia stops shortly after birth (Valente et al., 2013). Mosaic hyperplastic growth is responsible for the expansion of the major part of fast fibers during juvenile and adult stages as well as strongly influences growth rate and maximum size in the vast majority of teleost species (Johnston, 2006; Valente et al., 2013). The largest and fastest growing fish generally exhibit greater hyperplasia than slow growing fish (Valente et al., 2013). However, depending on whether the teleost species in question exhibits determinate-like growth or indeterminate-like growth during adult life, skeletal muscle growth relies on the increase in length and diameter of existing muscle fibers (hypertrophy) or in a combination of both hyperplasia and hypertrophy of muscle fibers, respectively (Johnston, 2006; Johnston et al., 2011).

Molecular mechanisms involved in embryonic myogenesis are well conserved across vertebrates. However, differences such as progenitor cells commitment to myogenic lineage via *MyoD* expression, occurs earlier (before the onset of somitogenesis) in fish embryos, compared to birds and mammal embryonic myogenesis tempo (Johnston et al., 2011). This is an evolutionary advantage that provides swimming propulsion to fish larvae necessary to evade predation immediately after hatching. On the contrary, amniote vertebrates have little need for skeletal musculature until after birth (Johnston et al., 2011). In teleost species, inductive signals coming from adjacent tissues such as the neural tube, notochord, and the

dorsal and lateral ectoderm play a decisive role in coordinating the activation of internal signaling during embryonic myogenesis. During embryonic development, the growth processes are genetically regulated in all vertebrates. However, in the case of ectotherms such as teleost species, environmental cues such as temperature, oxygen availability as well as photoperiod also play important roles in determining the rate and duration of myogenesis (Duan, 1998; Johnston, 2006; Johnston, 2011). In this regard, in juvenile and adult fish, somatic growth and the number and size distribution of muscle fibers are influenced by thermal imprinting occurring earlier during embryo development (epigenetic effect). Therefore, cellular commitment during teleost embryo development is regulated by the interplay of intrinsic regulators, morphogenetic signals and environmental factors.

During development, myogenic specification begins with the formation of embryonic somites (somitogenesis). Somites are cellular compartments observed from head to tail of the embryo, which are derived from pairwise condensation of paraxial mesoderm (Rescan et al., 2001; Rescan, 2001; Rossi and Messina, 2014). Spatiotemporal somitogenesis occurs because of the combined effects of the expression of genes with different timing, such as genes from the Notch and Wnt signaling pathways as well as the existence of concentration gradients of Wnt, Fgf and retinoic acid signaling at different embryonic segments (morphogen gradients) (Bentzinger et al., 2012). In amniote organisms (birds and mammals), cartilage and bone cell precursors derived from the mesenchymal sclerotome that comes from the ventral somitogenic compartment of the embryo. For its part, the dorsal part of the somite remains epithelial, becoming *a posteriori* the embryonic dermomyotome (Bentzinger et al., 2012). In non-amniote embryos, the epithelial cells from the anterior region of the somite become the external cell layer (ECL), with similarities to the amniote dermomyotome (Johnston et al., 2011). Therefore, the dermomyotome is an ancient well-conserved embryonic structure across vertebrate species (amniote and non-amniote) (Devoto et al., 2006; Stellabotte and Devoto, 2007).

During myogenesis, MRFs are activated in response to inductive signals that are interpreted by their associated regulatory elements in different precursor cells (Carvajal and Rigby, 2010; Johnston et al., 2011). These transcription factors regulate the expression of muscle structural genes by triggering a cascade of transcription factors, and thus begin the specification process in muscle progenitor cells. Embryonic progenitor cells undergo

commitment and specifications into a myogenic lineage following the orchestration of instructions given by a broad spectrum of signaling molecules from an extracellular and/or intracellular origin in the somite (Bentzinger et al., 2012). Indeed, the activation of cell surface receptors by the extracellular signals induces intracellular pathways that regulate the activity of specific transcription and chromatin remodeling factors. This action ultimately ends in the translation of the external signals into the gene and microRNA expression program, following a spatiotemporally induced hierarchical gene expression network (Bentzinger et al., 2012). The surrounding tissues of the somite release external signaling factors that exert a modulatory effect over morphogenesis during development. Neural tubes, for example, release *sonic hedgehog* (*Shh*), bone morphogenetic proteins (BMPs), and members of the wingless-type MMTV integration site family of proteins (Wnt). Similarly, the ectoderm releases BMPs and Wnt (Bentzinger et al., 2010).

Internal myogenic signaling factors such as members of the paired box protein family (i.e. Pax3 and Pax7) are important regulatory factors of the progress of myogenesis during development as well. Pax proteins regulate a plethora of cellular process, such as cell proliferation, self-renewal, apoptosis, migration and differentiation. Pax3 and Pax7, for example, play important roles in myogenesis and neurogenesis in the neural crest (Koblar et al., 1999). The role of Pax3 and Pax7 during embryonic myogenesis is more evident when considering the existence of a cell population in the dermomyotome or ECL. These embryonic progenitor cells are also characterized by low expression of the basic helix-loop-helix transcription factor Myf5 ($Pax3^{+}$/ $Pax7^{+}$/ $Myf5^{-}$) (Bentzinger et al., 2012). The $Pax3^{+}$/ $Pax7^{+}$/ $Myf5^{-}$ cells undergo proliferation, continuing with their transition into a subset of muscle committed satellite cells ($Pax3^{-}$/ $Pax7^{+}$/ $Myf5^{+}$) within the primary myotome, which are characterized by the expression of Myf5 and MyoD, a member of the basic helix-loop-helix transcription factors as well (Bentzinger et al., 2012). The onset of early myogenesis during embryonic development follows a molecular hierarchy of these myogenic regulators where Pax3 controls embryonic myogenesis via regulation of the canonical MRFs, while cells in the somite become committed to the myogenic lineage forming the myotome. However, despite the existence of a hierarchy of myogenic regulators, such as the case of MyoD which functions downstream from Pax3 and Pax7 in muscle progenitor cells, Myf5 can also act in parallel with Pax transcription factors in muscle progenitor cells (Bentzinger et al., 2010).

Myf5 and MyoD regulate the commitment of mesodermal cells into muscle cell lineage. Furthermore, MyoD and Myf5 are both considered to be markers of terminal specification of muscle lineage (Pownall et al., 2002; Francetic and Li, 2011).

During embryonic myogenesis, Pax3 is first expressed in both the paraxial mesoderm and the early somite. Subsequently, Pax3 expression is restricted to the developing dermomyotome, marking the early stages of myogenic specification (Ridgeway and Skerjanc, 2001). Pax3 expression is required for the delamination and migration of muscle progenitor cells to sites where the primary myotome will form (Kuang et al., 2006). Furthermore, Pax3 activity was reported to be crucial in skeletal muscle development by regulating the expression of the somite pattering transcription factors including Mox1, Gli2, Six1 and Eya2 prior to the expression of MRFs (i.e. MyoD and myogenin) (Ridgeway and Skerjanc 2001). In agreement with these findings, deletion of Pax3 results in the absence of diaphragm and limb muscles, which highlights the essentiality of Pax3 activity in fetal and embryonic development (Bober et al., 1994; Goulding et al., 1994). Despite Pax3 is expressed in a novel myogenic population that is distinct from the sublaminar satellite cell lineage in specific muscles (Relaix et al., 2006; Conboy and Rando, 2002; Kuang et al., 2006), the role of Pax3 in adult myogenesis has not yet been elucidated in adult mammals. On the contrary, emerging evidence suggests Pax3 may play a crucial role in the hyperplasic type of growth in teleost species with indeterminate growth including giant danio (*Devario aequipinnatus*) during juvenile and adult life. The ability of these species to promote muscle growth by recruiting new myofibers appears to be founded in the existence of a MPCs population with an "embryonic-like" nature and with a largely Pax7–independent proliferation, similar to fetal myoblasts exhibit during secondary myogenesis in mammals (Froehlich et al., 2013).

Pax7 is expressed later in the central dermomyotome, playing a pivotal role during juvenile and adult myogenesis (Young and Wagers, 2010). The importance of Pax7 in the regulation of the expansion and differentiation of satellite cells during adult myogenesis has previously been demonstrated in mammalian models (Kuang et al., 2006; Zammit et al., 2006; von Maltzahn et al., 2013). Although deletion of Pax7 expression does not affect muscle formation at embryonic stages, it provokes a reduction in post-natal muscle growth in mice, most likely by depleting the myogenic precursor cells pool at later ontogenetic stages (Seale et al., 2000). In agreement with this, it has been reported that Pax7-null mice have

significant numbers of satellite cells at birth, but the population is progressively depleted as a result of cell cycle defects and increased apoptosis (Oustanina et al., 2004; Relaix et al., 2006; Kuang et al., 2006). Furthermore, Von Maltzahn and associates (2013) observed inactivation of Pax7 in adult satellite cells markedly impaired muscle regeneration, causing a genetically engineered ablation of satellite cells-like phenotype. In addition, Olguín and Pisconti (2012) detected that $Pax7^{-/-}$ mice have no gross defects in muscle formation; however, when adults, their skeletal muscles appear devoid of satellite cells. Collectively, these studies suggest that Pax7 expression is required for maintenance and survival of satellite cells, thus being critical for the normal function of satellite cells in myogenesis in adult skeletal muscle in mammals.

As previously mentioned, the activation of cell surface receptors by the extracellular signals induces intracellular pathways that regulate the activity of specific transcription factors such as MRFs. Proteins from the Wnt family, for example, indirectly interact with MRF members via binding to cellular Frizzled (Fzd) receptors. This ligand-receptor interaction triggers either a canonical (β-catenin/TCF transcriptional complex) or a non-canonical pathway based on the type of Fzd receptor activated (van Amerongen and Nusse, 2009). Expression of Wnt1, for example, strongly induced Myf5 levels, whereas Wnt6 or Wnt7a induced expression of MyoD in explant cultures of mouse presomitic mesoderm (Tajbakhsh et al., 1998). Other external signaling factors, which indirectly interact with MRFs during embryonic myogenesis, are members of the Hedgehog family proteins such as Shh, which is released from the notochord and floor plate of the neural tube. These proteins are essential for the maturation of dermomyotomal cells into committed myotomal cells.

Bone Morphogenetic Proteins (BMP) constitute another relevant signaling pathway in myogenesis regulation during development. Despite the crucial regulatory role that BMP exert in both formation and repair in bone and cartilage, BMP signaling inhibits myogenic differentiation to prevent ectopic myogenesis in the lateral plate mesoderm as well as promotes osteogenic differentiation. However, BMP signaling must be inhibited to permit the onset of myogenesis in the dermomyotome (Ono et al., 2011; Ruschke et al., 2012). During embryonic myogenesis, BMP signaling seems to expand the pool of myogenic progenitors before commitment is initiated in the somites, via inhibition of the expression of Myof5 and MyoD via fostering Pax3 expression (Bentzinger et al., 2012). However, BMP signaling is not solely restricted to embryonic myogenesis. This is especially true since previous work has

demonstrated BMP signaling has a proliferative effect on satellite cells during adult muscle regeneration. BMP signaling prevents satellite cells differentiation via inhibition of MyoD, thus promoting myogenic precursor cells proliferation (Ono et al., 2011; Ruschke et al., 2012).

Another crucial external signaling pathway is Notch, a highly conserved cell signaling mechanism which plays a pivotal role in metazoan development, including regulation of vertebrate myogenesis. Notch signaling activation exerts different biological outcomes depending on the cell/tissue context as well as the ontogenetic stage of the organism. In myogenesis, for example, Notch signaling act as a check point system during muscle regeneration by inhibiting the progress to differentiation commitment in quiescent satellite cells, however, Notch signaling promotes proliferation in committed myoblast, therefore blocking the progress toward terminal differentiation into myocytes and subsequently into myotubes (Buas and Kadesch, 2010). In addition, Notch signaling plays critical roles in cell-cell communication and cell fate regulation during tissue morphogenesis, homeostasis, and regeneration (Kopan and Ilagan, 2009). In this regard, Notch mediates cell-cell communication by engaging with Delta and Jagged ligands on neighboring cells. Only dermomyotomal cells that transiently make contact with Delta1-expressing cells undergo myogenesis during development. Moreover, Notch signaling suppresses MyoD activity when interacting with the DNA-binding protein RBP9-J and the transcriptional repressor Hes1 (Bentzinger et al., 2012). Therefore, Notch appears to promote proliferation of myogenic precursors while preventing differentiation during myogenesis. In agreement with this, disruption of Notch signaling is associated with myogenesis progression, while sustained Notch activity is correlated with maintenance of the undifferentiated cell state. This is especially true since myogenic progenitor cells expressing Numb, an inhibitor ligand of Notch signaling, were found to express pro-myogenic molecular markers, such as Myf5 and Desmin, but not Pax3. In myogenic progenitor cells where Numb expression was down-regulated, the opposite expression pattern was detected, thus promoting the proliferation of undifferentiated cells (Buas and Kadesch, 2010). Interestingly, upon mitosis, Numb can be asymmetrically distributed into newly formed daughter cells, giving rise to asymmetric Notch activity and distinct fate choices for the two daughter cells (Buas and Kadesch, 2010).

Although the Notch signaling pathway plays crucial roles in tissue morphogenesis via regulation of cell fate decisions during development, Notch is involved in maintaining adult skeletal muscle homeostasis as well (Bjornson et al., 2012; Brack et al., 2008; Lin et al., 2013; Parker et al., 2012). In past years, it has been shown that Notch signaling is a key regulatory factor in adult skeletal muscle regeneration via regulation of recruitment, proliferation and cell fate determination of muscle stem cells (satellite cells) (Artavanis-Tsakonas et al., 1999; Luo, et al., 2005). Indeed, the asymmetric division of satellite cells that either advance toward myogenic differentiation or return to the quiescent state are mainly regulated by Notch signaling (Kassar-Duchossoy et al., 2004; Kuang et al., 2007; Shinin et al., 2006). Additionally, Lin et al. (2013) found that blocking Notch signaling in muscle stem cells results in a dystrophic phenotype and impaired muscle regeneration. The authors concluded that the Notch signaling pathway controls processes that are critical to regeneration in muscle dystrophy. Indeed, Notch signaling is required for maintaining cells in the quiescent state, and for muscle stem cell homeostasis via regulation of self-renewal and differentiation during normal adult myogenesis (Bjornson et al., 2012; Mourikis et al., 2012). In addition, Notch signaling appears to be essential in maintaining proliferation of stem cells at the base of intestinal crypts as well. However, such an effect in quiescent hematopoietic stem cells was not observed (Mourikis et al., 2012).

Notch activity is under tight regulation via regulatory proteins and protein complexes that either enhance or inhibit Notch signaling through regulation of protein processing, localization activity and stability (Luo et al., 2005). Overall, evidence suggests that activation of Notch appears to inhibit myoblast differentiation, while Notch inhibition impairs adult skeletal muscle regeneration (Buas and Kadesch, 2010; Conboy et al., 2003). In this regard, activated satellite cells up-regulate Delta, a Notch ligand, which triggers Notch activation after muscle injury. Notch signaling promotes satellite cell
progeny proliferation as well as the expansion of an intermediate progenitor cell populations (pre-myoblast population), which is characterized by a remarkable proliferative potential (Luo et al., 2005). During adult skeletal muscle regeneration, the interaction between MRFs and Notch signaling appears to be a critical one. This is especially true since Wen et al. (2012) demonstrated that Pax7 up-regulation promoted self-renewal, and acquirement of the quiescent state in skeletal muscle satellite cells via constitutive Notch signaling activation. In

this regard, Pax7 activity in adult satellite cells was shown to be essential for skeletal muscle regeneration (Günther et al., 2013; von Maltzahn et al., 2013). These findings are in agreement with previous work that showed Pax7 up-regulation was involved in the acquisition of the quiescent, undifferentiated state in satellite cells, (Mourikis et al., 2012; Olguin and Olwin, 2004; Rocheteau et al., 2012; Sacco et al., 2008). In mammalian-derived cell lines and mammalian animal models, Pax7 was demonstrated to be required for the specification of myogenic satellite cells (Seale et al., 2000). Moreover, it appears that Pax7 and myogenin expression are mutually exclusive during differentiation via reciprocal regulatory interactions. In this context satellite cells evade differentiation and exit the cell cycle when Pax7 expression is up-regulated (Olguin and Olwin, 2004). Although Zammit et al. (2006) observed that Pax7 was involved in maintaining proliferation and preventing precocious differentiation, the authors observed no stimulation toward re-acquirement of the quiescence state. Despite this conflicting point, Pax7 has been shown to be necessary for maintenance of satellite cells during perinatal and juvenile life in mammals (Rossi and Messina, 2014). Indeed, von Maltzanhn et al. (2013) demonstrated Pax7 was essential to regulate proliferation and differentiation of satellite cells during both neonatal and adult myogenesis. Moreover, the authors reported that inactivation of Pax7 in satellite cells markedly impairs adult muscle regeneration, and increases adipose tissue deposition. Furthermore, Seale et al. (2000) suggested that the inducible expression of Pax7 in muscle-derived stem cells promotes satellite cell differentiation by restricting alternate developmental programs, thus being required for the differentiation of myogenic satellite cells. In agreement with this hypothesis, Brack et al. (2008) observed that a temporal balance between the Notch signaling pathway and Wnt signaling, both signaling pathways being involved in the regulation of developmental programs, orchestrates the progression of muscle precursor cells throughout myogenesis via proliferation and differentiation during normal adult myogenesis. It appears that a nodal point represented by GSK3β, connects Notch and Wnt signaling regulating the progress during myogenesis. This is especially true since the canonical Wnt pathway was found to inactivate the kinase GSK3β, yet Notch signaling was shown to sustain the activated form of this protein (Buas and Kadesch, 2010). For its part, LeGrand et al. (2009) found that the Wnt7 protein regulates the maintenance of the satellite cell pool by modulating the increase in satellite stem cell expansion during regenerative myogenesis and

that planar cell polarity (PCP) pathway basal levels are insufficient to maintain the satellite cell pool during adult regenerative myogenesis.

Interestingly, the existence of a cooperative mechanism has been suggested between Foxo1, a member of the Forkhead box O transcription factors family that governs metabolism and cellular differentiation, and Notch signaling. This interaction may integrate environmental cues through Notch with metabolic signaling via Foxo1 to regulate cell maintenance and differentiation in adult myogenesis (Kitamura et al., 2007; Tuteja and Kaestner, 2007a; 2007b). Therefore, this cooperative mechanism via Foxo1 activation would keep committed progenitor cells from differentiation in response to developmental signals in the absence of growth factors. On the other hand, quiescent satellite cells would be activated and terminally differentiate via Foxo1 inhibition through the interaction of Notch ligand with hormonal/nutritional cues (Kitamura et al., 2007).

Contrary to what is observed in amniote vertebrates, where skeletal muscle growth is determined according to a fixed body size, postembryonic skeletal muscle growth is indeterminate in the vast majority of teleost species. However, some teleost species such as zebrafish exhibit determinate growth (Rossi and Messina, 2014). The indeterminate-like growth is a unique feature to fish species, which results from the combination of a hyperplastic and a hypertrophic type of growth. Both hypertrophy (enlargement of existing fiber size) and hyperplasia (genesis of new muscle fibers) contribute to muscle growth, but their relative importance varies at different life stages (Johnston, 2011). On the other hand, determinant-like growth is mostly due to hypertrophy, since the increase in the number of fibers stop shortly after embryonic development, limiting growth potential and body size (Rescan et al., 2001).

In indeterminate growth, muscle fiber hyperplasia is characterized by increasing muscle fiber number (muscle fiber recruitment). The new muscle fibers are formed from the fusion of multiples myocytes derived from myoblasts committed to differentiation. On the other hand, hypertrophic type of growth is based on increasing the length and diameter of the newly formed muscle fibers achieved by myonuclear accretion and protein synthesis in order to maintain the nuclear to cytoplasm ratio (Valente et al., 2013). Interestingly, the myoblasts that contribute to new fiber production and nuclear accretion are likely to be phenotypically

distinct. However, whether myoblast fate arises early in development or is specified later in response to external and local signaling remains largely unknown (Valente et al., 2013).

The combined effect of hyperplasic and hypertrophic growth continues throughout the life resulting in the indeterminate growth in fish (Bower and Johnston, 2010; Mommsen, 2001). However the contribution of either of these two types of growth varies according with the fish life stage. In juveniles, for example, most muscle growth is based on hyperplasia, and to a lesser extent on hypertrophy, however, the opposite occurs during adult life (Johnston et al., 2011).

The hyperplasic and hypertrophic type of growth as well as the remarkable capacity of the skeletal muscle for regeneration in response to injury or disease depends on adult mononucleated muscle satellite cells or MPCs originating from the ECL during embryonic development. The postembryonic skeletal muscle growth model suggests the existence of a resident quiescent satellite cell population characterized by the expression of Pax7, c-Met receptor (receptor for HGF) and M-cadherin protein (Seale et al., 2000).

Teleost species retain an epithelial layer of $Pax7^{+}$ –undifferentiated cells into their early juvenile period, contributing to post-larval muscle growth. A subset of proliferative MPC migrates through the somite to give rise to the lateral fast muscle fibers in the late embryo and larval stages. A second subset of MPC is a resident and self–renewing myogenic stem cell population. Upon activation, cells undergo an asymmetric division, giving rise to quiescent myogenic stem cells (phase G_0 of the cell cycle), and activated myogenic stem cells. This quiescent satellite cell pool provides a source of future myogenic precursor cells (myoblast) which will be required for the formation of new muscle fiber in fish (Johnston et al., 2011).

In early larval life, satellite cells provide myonuclei for skeletal muscle growth. Later, during adult life, mononucleated muscle satellite cells are the progenitors of the myogenic cells that play important roles during adult growth, repair, and maintenance of skeletal muscle mass. In mature adult muscle, satellite cells are predominantly mitotically quiescent. However, upon the appropriate stimuli, satellite cells are activated, enter the cell cycle, and proliferate. Proliferating satellite cells or myoblasts withdraw from the cell cycle to either differentiate or self-renew. The state in which satellite cells are found can be readily distinguished by the expression of MRFs, for example, Pax7 $^{+}$/ MyoD $^{-}$, Pax7 $^{+}$ / MyoD $^{+}$, Pax7 $^{+}$ / MyoD $^{-/+}$ and Pax7 $^{-}$ / MyoD $^{+}$ for quiescent, proliferating, self-renewing and

differentiating satellite cells, respectively (Wen et al., 2012). Furthermore, satellite cells are activated in response to both physiological stimuli (i.e. exercise) and pathological conditions (e.g. injury, degenerative diseases) to generate a committed population of myoblasts capable of fusion and differentiation (Luo et al., 2005). Indeed, the progeny of the activated satellite cells, the myogenic precursors cells or myoblasts, undergo mitotic divisions before fusion with new or pre-existing myofibers. The molecular mechanism involved in satellite cell activation, proliferation and cell fate decision of satellite cells progeny, as well as, the differentiation of myoblasts population are complex and intricate (Luo et al., 2005). Most nuclei within adult skeletal muscles come from the myogenic cells derived from satellite cells. In the adult musculoskeletal system, maintaining, repairing and regulation of muscle mass depends upon the activation of a subset of mitotically quiescent satellite cells which have the ability to respond to diverse external signals (Rhoads et al., 2009). Once activated, satellite cells co-express Pax7, MyoD and Myf5 before entering into the S-phase of the cell cycle. Upon entering into the proliferation phase, a sub-set of satellite cells undergoes a self-renewal fate, maintaining Pax7 expression while losing MyoD (increase in Pax7/MyoD ratio). These cells withdraw from both cell cycle and myogenic differentiation to return to quiescent status. On the other hand a second sub-set of activated satellite cells will down-regulate Pax7 while up-regulating MyoD (Zammit et al., 2006). This subset of daughter, satellite cells becomes myogenic precursor cells committed to terminal differentiation, which can follow one of two fates: they either undergo hypertrophy and are absorbed into growing muscle fibers as they expand in length and diameter or they undergo hyperplasia and fuse together to form additional short myotubes on the surface of an existing muscle fiber to form multinucleated myotubes (fiber recruitment or hyperplasia) (Johnston et al., 2011). This subset of daughter, satellite cells becomes myogenic precursor cells committed to terminal differentiation, which can follow one of two fates: they either undergo hypertrophy and are absorbed into growing muscle fibers as they expand in length and diameter or they undergo hyperplasia and fuse together to form additional short myotubes on the surface of an existing muscle fiber to form multinucleated myotubes (fiber recruitment or hyperplasia) (Johnston et al., 2011).

As defined by biological and biochemical criteria, satellite cells are composed of a population of mono-potential stem cells that are distinct from their daughter myogenic

precursors cells (Seale et al., 2000). A proposed model for the Pax7/MRF protein ratios, and satellite cell fate decision in mammals is as follows; quiescent satellite cells exhibit a $Pax7^{+}$ / $MyoD^{-}$ / myogenin $^{-}$ ratio (Olguín and Pisconti, 2012). Following external stimuli, satellite cells become activated and committed to proliferate, up-regulating the expression of MyoD and down regulating Pax7 expression ($Pax7^{-/+}$ / $MyoD^{+}$ / myogenin $^{-}$). Following terminal differentiation commitment, up-regulation of myogenin down-regulates Pax7 expression ($Pax7^{-}$ / $MyoD^{+}$ / $myogenin^{+}$). Finally, expression of the terminal differentiation genes, required for the fusion of myocytes and the formation of myotubes, are regulated by both myogenin and MRF4 (Bentzinger et al., 2012) (Figure 5). Additionally, a subset of undifferentiated and quiescent cells are characterized by down-regulation of Pax7 expression, which result in MyoD down-regulation, blocking myogenin induction (Olguín and Pisconti, 2012). In fish, muscle growth follows a similar myogenic expression wave, where quiescent myogenic cells express markers such as Pax7. Committed and activated cells express MRFs such as Myf5 and MyoD. After activation, myogenic cells proliferate and subsequently differentiate, expressing myogenin, MRF4, and myocyte enhancer factor-2 (Mef2). Finally, at the later stages of myogenic cell differentiation, several genes encoding for structural muscle proteins which contribute to sarcomeric assemblies such as myosin light chain (Mlc), actin, and myosin heavy chain (Mhc) are up-regulated (Fuentes et al., 2013). Interestingly, the Pax7/MyoD ratio has been used to monitor cell fate decisions when evaluating satellite cell differentiation, whether into myocytes, self-renewal to repopulate satellite cell pool or apoptosis in fish, particularly in response toward nutritional challenge (Chapalamadugu et al., 2009).

Additionally, growth factors such as insulin-like growth factor (IGF), fibroblast growth factor (FGF) and hepatocyte growth factor as well as inhibitors such as myostatin are examples of external signaling factors that exert a regulatory action on muscle satellite cells, mainly in an endocrine or paracrine manner (Rhoads et al., 2009). The IGF/IGF binding protein system recognizes two ligands, the IGF-I and II, their corresponding cellular receptors, and a set of isoforms of the IGF binding protein family. Both IGF isoforms are structurally related to insulin, and are produced mainly in the liver under the regulation of Growth Hormone (GH). They have the ability to regulate satellite cell activity, although their effect depends on the developmental stage of myoblasts. IGF-I, for example, can stimulate

either satellite cell proliferation or differentiation. However, the regulatory function of IGF-II appears to be solely related to satellite cell differentiation (Rhoads et al., 2009). Skeletal muscle is the main target tissue of both IGF isoforms, where they stimulate muscle cell proliferation, differentiation and hypertrophy while inhibiting muscle atrophy. On the other hand, IGF binding proteins serve as a carrier both to transport IGFs as well as to increase their half-life in the bloodstream (Fuentes et al., 2013). Upon this ligand-receptor interaction, a myogenic intracellular signaling transduction response can be activated, promoting muscle cell proliferation and terminal differentiation (hyperplasic growth) via the MAPK/ERK signaling pathway. Alternatively, the activation of the PI3K/AKT signaling pathway can stimulate protein synthesis (hypertrophic growth) via regulation of the target of rapamycin (TOR). TOR activity can be also regulated by nutrient availability (i.e. amino acids) and cellular energy status (i.e. AMP/ATP) (Fuentes et al., 2013). In fish, similarly to other vertebrates such as mammals and birds, the growth hormone insulin-like growth factor system has been well described (Björnsson et al., 2002; Castillo et al., 2002; Fuentes, et al., 2013; Pérez-Sánchez et al., 2002; Reinecke et al., 2005; Weil et al., 2011). Similarly, in higher vertebrates such as mammals and birds, members of the fibroblast growth factor family have been shown to be involved in the regulation of myogenic cell proliferation and differentiation, where the Ras/MAP kinase signaling pathway seems to play a central role in satellite cell responsiveness to FGFs in regulating cell proliferation and differentiation (Rhoads, et al., 2009). Expression of FGFs members have been described in teleost fish species as well (Hata et al., 1997; Lepilina et al., 2006; Shin et al., 2007). However, most of the research has been related to elucidating its biological role during development and cardiac muscle regeneration in teleosts. Therefore, whether members of the FGF family are involved in regulating satellite cell proliferation and/or differentiation in teleost fish species remains to be elucidated. For its part, the hepatocyte growth factor, which was originally identified exerting a stimulatory effect in hepatocyte proliferation, was found to be the main factor in regulating the remarkable regenerative capacity of the liver (Nakamura and Mizuno, 2010). Additionally, hepatic growth factor (HGF) has been shown to promote skeletal muscle quiescent satellite cell activation (entering into cell cycle) by increasing satellite cell DNA synthesis in a dose-dependent manner in mice and C2C12 myoblasts as well as exerting an inhibitory effect of satellite cell differentiation via suppressing basic helix-loop-helix E

protein complexes, and thus inhibiting the transactivation of MyoD and myogenin in chickens and turkeys (Rhoads et al., 2009). However, whether HGF plays similar regulatory role in skeletal muscle satellite cells in teleost species remains to be elucidated.

Myostatin, a transforming growth factor-β family member, negatively regulates the process of myogenesis, playing a crucial role in skeletal muscle mass homeostasis (Dominique and Gérard, 2006). In mammals, most of the knowledge regarding the biological role of myostatin comes from the observation of cases where expression of the myostatin gene (*mstn*) was either artificially (gene knock out or inhibition of protein activity) or naturally (mutation) suppressed. Suppression of *mstn* expression suppression leads to a substantial increase in skeletal muscle mass, a phenotype known as "double muscling". Well-established examples of these cases are genetically modified mice, and the naturally occurring mutant cattle breeds Piedmontese and Belgian blue (Rhoads et al., 2009). However, whether the increase in skeletal muscle mass observed in myostatin deficient animals is solely due to a hyperplasic or hypertrophic type of growth or a combination of both, is not well understood, mainly because of differences in experimental approaches (Dominique and Gérard, 2006). From a mechanistic point of view, it appears that myostatin exerts part of its effect via negative regulation of satellite cell activation, proliferation and differentiation (Rhoads et al., 2009). In this regard, it has been observed that myostatin inhibition of myoblast proliferation is through control of cell cycle progression, particularly via up-regulation of *p21* expression and decreasing the hyperphosporylated form of Rb in C2C12 cells. Additionally, *in vitro* studies conducted in C2C12 cell lines suggest myostatin inhibits myoblast terminal differentiation as well. Similarly, the myostatin gene has also been detected in teleost fish species such as zebrafish and medaka, where it seems to exert a similar biological role to that observed in higher vertebrates (Terova et al., 2013; Xu, et al., 2003). This is especially true since studies using transgenic *mstn* deficient zebrafish and medaka showed the expression of this gene inhibits skeletal muscle growth in fish as well. However, recently, it has been proposed that myostatin does not exert the exact biological role in fish when compared to mammals; this since it appears that myostatin is not restricted solely to skeletal muscle, but rather functions as a general inhibitor of cell proliferation and cell growth to control tissue mass by being express in non-muscle tissues in fish (Gabillard et al., 2013).

In some teleost species such as rainbow trout and Atlantic salmon, the existence of two distinct genes of myostatin in their genome has been detected, probably as result of paralogue retention originating from the whole-genome duplication event that occurred at the base of teleosts evolution (Jaillon et al., 2004). Interestingly, the two myostatin genes observed in salmonid species are differentially expressed in different somatic tissues. In Atlantic salmon, for example, the myostatin I isoform was identified in white muscle, intestine, brain, gills and eyes, whereas the myostatin II isoform was detected in red skeletal muscle, heart, spleen, and ovarian tissue (Østbye et al., 2001; Rescan et al., 2001). Therefore, myostatin isoforms could play differential regulatory roles over somatic tissue development as well as on growth rate between white and red muscle in teleosts (Dominique and Gérard, 2006).

Thus, the teleost fish musculoskeletal system shows remarkable plasticity toward both environmental cues and fish physiological systems. Research on the effects of environmental conditions, such as nutritional stimuli and/or the impact of the redox balance in the functional output of the MPCs during adult myogenesis in fish under intensive culture is warranted. Furthermore, genotype-environmental interactions appear to exert an important effect over the mechanism involved in muscle growth and muscle plasticity in fish as well. To achieve this goal it is necessary to increase our knowledge regarding signaling mechanism such as Pax family members, MRFs, Notch, Wnt, BMPs, Hedgehog and other transcription factors in MPC physiology during adult myogenesis in fish. Evidence suggests that distinct myogenic regulatory pathways control embryonic development, juvenile myogenesis and adult regeneration of skeletal myofibers in mammals (Young and Wagers, 2010).

2.5 The Regulatory Role of the Cellular Redox Balance in Skeletal Muscle Homeostasis

Many physiological functions are regulated by redox-responsive signaling pathways including redox regulated production of NO, ROS production by phagocytic NADPH oxidase (oxidative burst), ROS production by NADPH oxidases in non-phagocytic cells and regulation of vascular tone by NO. Additionally, ROS serve as sensor for oxygen concentration changes, redox regulation of cell adhesion and immune response as well as ROS-induced apoptosis based upon their hormetic nature (Dröge, 2002). Indeed, clear evidence shows that ROS, within certain concentrations, are useful signaling molecules regulating cellular processes related to homeostasis and adaptation of skeletal muscle cells

(Barbieri and Sestili 2012). Whether ROS triggers a beneficial or detrimental cellular response depends on the coexistence of extrinsic and intrinsic factors such as ROS concentration and time exposure with ROS as well as the antioxidant status of cells. This implies the crucial role of the cellular REDOX environment as a regulatory factor to major signaling pathways in skeletal muscle tissue. Other factors that will influence the cellular response toward ROS are mechanisms of ROS generation, cellular DNA repair capacity and the differentiation stage of muscle cells (satellite cell, myocytes or mature myotubes) (Barbieri and Sestili 2012). In this regard, low concentrations/short durations of ROS elicit positive effects on muscle physiology by activating specific key molecules such as PGC-1α, AMPK and MAPK, which control cellular mechanisms involved in muscle adaptation, e.g., oxidative metabolism, mitochondrial biogenesis and mitochondrial functionality. Irrcher et al. (2009) found that ROS produced by skeletal muscle cells is important for the maintenance of PGC-1α expression levels within a normal physiological range. Indeed, the PGC-1 family of coactivators (PGC-1α, PGC-1β and PRC) plays a central role in the regulation of cellular metabolism via coordinating the activity of several transcription factors implicated in the mitochondrial biogenesis process (Yoboue and Devin, 2012). Promotion of mitochondrial biogenesis has been shown to be a determining factor in myoblast differentiation, and thus provides an essential role in myogenesis progress in adult skeletal muscle (Barbieri and Sestili, 2012). PGC-1α coactivator mediates many aspects of oxidative metabolism such as mitochondrial adaptation, insulin sensitivity via up-regulation of target genes involved in fatty acid β- oxidation, glucose transport (GLUT 4), and oxidative phosphorylation (Barbieri and Sestili, 2012). Furthermore, the PGC-1 coactivators have been shown to exert part of their mitochondrial biogenesis promotional effect by binding to other transcription factors such as NRF1 and NRF2 (members of the nuclear respiratory factors family that regulate expression of respiratory genes) and to the estrogen-related receptor-α (ERR- α) (Yoboue and Devin, 2012). It appears that AMPK and SIRT1, two metabolic sensors, directly affect PGC-1α activity through phosphorylation and de-acetylation, respectively, to orchestrate the regulatory network for metabolic homeostasis (Cantó and Auwerx, 2009; Jäger et al., 2007). Higashida et al. (2013) suggested that the mechanism by which SIRT1 regulates mitochondrial biogenesis is by inhibiting PGC-1α coactivators activity, resulting in a decrease in mitochondria. Therefore, the mitochondrial biogenesis process seems to be

regulated, in part, by the activity of PGC-1, which in turn appears to be regulated by the coordinated activity of AMPK (stimulatory) and SIRT1 (inhibitory), and thus exerts a crucial role over myoblast differentiation and adult myogenesis. Additionally, low ROS concentrations appear to promote the initiation of the differentiation program by increasing activity of CDK-2 activity, which is positively correlated with MyoD1 activity (Matés et al., 2008).

The ERK MAP kinase pathway is another major-signaling pathway that regulates energy substrate uptake, mitochondrial respiration and biogenesis, and which is regulated by ROS concentration as well (Barbieri and Sestili 2012). ERK MAP kinase activity is under regulation of the ERR*α* receptor, a member of the estrogen-related receptor family. The activity of members of this family of the nuclear receptor superfamily of transcription factors (ERRα, ERRβ and ERRγ) is regulated via interactions with coregulatory proteins such as PGC-1α and PGC-1β, and thus, on the basis of this interaction, it is possible that ROS exerts an indirect regulatory role over ERRs activity. However, the nature of their natural ligand remains to be elucidated.

Murray and Huss (2011) detected overexpression of the ERR*α* receptor accelerated differentiation in C2C12 myocytes, whereas the loss of ERR*α* receptor function delayed myogenesis and resulted in myotubes with fewer mitochondria and disorganized sarcomeres, suggesting a regulatory role of ERR*α* in myogenic differentiation. Thus, the authors concluded that ERR*α* receptor activity is required for normal skeletal myocyte differentiation via modulation of MPA kinase signaling. Furthermore, in a later study, Murray et al. (2013) detected that a loss of function of ERR*γ* caused impaired myogenesis due to metabolic defects mediated by oxidative stress. Collectively, these data suggest that part of the ERRs myogenic regulatory role could be via indirect regulation of cellular REDOX balance, by maintaining low concentrations of ROS such as H_2O_2. Moreover, ROS at low concentrations promotes expression of antioxidant enzymes that also regulate intracellular levels of pro-oxidants molecules within muscle cells, and thus ensure the adequate REDOX intracellular environment for the optimal function of the network of signaling pathways involved in myogenesis regulation. Another important regulatory effect of low ROS concentrations is the up-regulation of the expression of growth factors such as IGF-1, which regulates skeletal muscle mass via modulation of protein turnover (protein synthesis *vs.* protein breakdown),

and supports oxidative metabolism. Low concentrations of ROS promote IGF-1 signaling by inducing phosphorylation on specific tyrosine residues of insulin receptor (IR) and IR substrate (IRS) proteins (Bashan, et al., 2009). However, it appears ROS elicits a regulatory role over IGF-1 at the transcriptional level as well. Previous works has demonstrated the existence of positive and negative interactions between ROS and IGF-1 synthesis mediated by changes in mRNA IGF-1 expression in cells (Delafontaine and Ku, 1997; Handayaningsih, et al., 2011). Although low concentrations of ROS enhance IGF-1 signaling, mildly toxic ROS concentrations down regulate mRNA IGF-1 expression in C2C12 cells. Although Delafontaine and Ku (1997) found that ROS up-regulated mRNA expression of IGF-1 in VSMCs cells when treated either with H_2O_2 or XO, the authors suggested that this could be related to an autocrine regulatory role of IGF-1 in VSMCs cells. Additionally, slight increases in ROS concentrations cause the inhibition of phosphatases and promotion of a phosphorylation state of proteins involved in the muscle signaling response. On the other hand, a sustained increment in ROS concentrations suggests the disruption of the REDOX balance (oxidative stress), causing oxidative damage to proteins, lipids and nucleic acids involved in signaling pathways in the cell. Recently, it has been suggested that increased ROS concentrations inhibit IGF-1 signaling cascades, thus inducing insulin resistance and its pathological consequences (Bashan et al., 2009). Additionally, under elevated ROS concentrations, most cells are exposed to stress, increasing intracellular Ca^{+2} concentrations, which activate signaling cascades for apoptosis or autophagy via NF-kB or FoxO paths, respectively (Barbieri and Sestili, 2012). Siu et al. (2009) observed that H_2O_2 (4mM) causes apoptosis-induced oxidative stress, both via caspase-dependent and caspase-independent mechanisms in C2C12 myotubes. This suggests a detrimental impact of oxidative stress over post-mitotic skeletal muscular cells, thus potentially affecting the maintenance of adult skeletal muscle mass. Moreover, increased ROS concentrations negatively affect the myogenic differentiation process in myoblasts thus impairing skeletal muscle regeneration as well (Figure 6). Hansen et al. (2007) observed that addition of pro-oxidant H_2O_2 (25 μM) caused a sustained intracellular oxidizing redox potential causing defective terminal muscle differentiation in C2C12 myoblasts. This observation was supported by the significant down regulation in myogenin expression in C2C12 cells treated with H_2O_2. Additionally, an enhancement in C2C12 differentiation when treated with a ROS trapping agent (phenyl-N-

tert-butylnitrone, PBN) was observed. Thus, the authors concluded that oxidative intracellular environments impair myoblast differentiation while reducing myogenesis. In support of this concept, Sestili et al. (2009) detected that treatment with H2O2 (0.3 mM) caused a drop in mRNA concentration of MyoD, MRF4 and myogenin, causing an inhibition of the myogenic differentiation progress in C2C12 cells.

The NF-kB transcription factor family is a well-conserved group of signaling molecules that play a role in the pathophysiology of skeletal muscle disorders. These signaling molecules are also involved in muscle remodeling physiology (Mourkioti and Rosenthal, 2008). It appears that NF-kB mostly acts as a negative regulator of myogenic differentiation in myoblasts. NF-kB inhibits myogenesis progress either by promoting myoblast to enter cell cycle (mitogenic activity) via expression of cyclin D1 or by inhibiting the synthesis of MyoD (Guttridge et al., 1999; Guttridge et al., 2000).

During the inflammatory response, several pro-inflammatory cytokines such as tumor necrosis factor alpha (TNFα), interferon gamma (IFNγ) and interleukin-1β are released into the extracellular environment to coordinate the repair of tissue damage, including elimination of pathogens and restoration of homeostasis. In teleost fish species the existence of similar pro-inflammatory cytokines, as well as, the presence of cells from a myeloid lineage have been described (Grayfer and Belosevic, 2012). Cellular components of the inflammatory response such as infiltrating neutrophils release ROS as defense mechanisms. However, an exacerbated increase in ROS concentrations can trigger oxidative stress causing oxidative damage to differentiating myoblasts and myotubes, thus delaying the regeneration and repair of skeletal muscle tissue (Barbieri and Sestili, 2012). TNFα has been observed to enhance ROS production thus inducing oxidative stress. TNFα-induced-oxidative stress and mitochondrial dysfunction have been observed in several pathophysiological states (Mariappan et al., 2007). The role of TNFα in skeletal muscle homeostasis and the pathogenesis of related diseases have been studied, mainly in mammalian models. Langen et al. (2004) reported that TNFα inhibits myogenic differentiation by destabilizing the MyoD protein in a NF-kB-dependent manner, potentially interfering with skeletal muscle regeneration. Moreover, in a different study, cell fusion as well as muscle differentiation was inhibited when human recombinant TNF-α was added (25 ng) to proliferating myoblasts (Miller et al., 1988). The authors found that TNF-α inhibition of myogenesis was mediated by

repressed synthesis of MyoD at the post-transcriptional level. Ardite et al. (2004) observed ROS reduced MyoD expression via sustained NF-kB activation, and thus impairing myogenesis in C2C12 cells. In agreement with these findings, NF-κB activation and degradation of MyoD protein was observed in C2C12 myoblasts treated with a TNF-like weak inducer of apoptosis (TWEAK) (Mourkioti and Rosenthal, 2008). Although, ROS can promote NF-kB activation and subsequent gene expression, it has been observed that its DNA binding activity is inhibited by the oxidized form of NF-κB. This evidence suggests ROS may also inhibit NF-κB transcriptional activity, and whether NF-κB will become active or not depends on the type, concentration and time of persistence of ROS in the cell (Powers et al., 2010). Additionally, Alter et al. (2008) found that TNF-α promotes myoblast proliferation through JNK1, a stress responsive pathway, and prevents myoblast differentiation via JNK-mediated secretion of leukemia inhibitor factor (LIF).

Willkomm et al. (2014) found that lactate treatment induced expression of early differentiation markers (i.e. Pax7 and Myf5), whereas it down-regulated expression of late differentiation markers such as myogenin and MHC in a time and dose-dependent manner in C2C12 cells. Furthermore, the authors found that lactate increased ROS concentration which was correlated with an increase in the activated form of Casp-3, providing further evidence of a link between oxidative stress and the cellular stress response. It is worth considering that lactate-dependent inhibition of the late differentiation phase in C2C12 myoblasts appears to be related to activation of Casp-3, leading to actin-myosin degradation and myonuclear apoptosis (Powers et al., 2010). This synchronization allows myoblasts to avoid wasting protein sources to produce myosin chains when proteolytic degradation of myosin chains is already in action.

Therefore, intracellular redox potential seems to be critical in several vital cellular process including DNA synthesis, transcriptional activation, enzyme kinetics and protein folding, all processes with fundamental roles in cellular differentiation. However, the effect of the intracellular redox potential during the differentiation process appears to depend on the particular cellular lineage under study. A reducing redox environment promotes muscle differentiation, whereas an oxidizing redox environment induces differentiation in adipocytes (Hansen et al., 2007). Whether differences in intracellular redox potential sensitivity between different phases of myogenesis exists remains to be elucidated.

2.6 Phytochemicals and their Potential Benefits in Fish Growth Physiology

Phytochemicals are chemical compounds that occur naturally in plants, providing different biological functions based on their chemical nature (Arts and Hollman, 2005). It is well known that phytochemicals can exert beneficial effects in different aspects of the physiology and health in humans and animals. Some of the biological activities of these phytocompounds are antibacterial, antiviral, antifungal, antioxidant, anti-inflammatory, anti-stress and immunostimulation. However, many of these beneficial effects have been observed in *in vitro* studies, and thus whether similar effects can be replicated *in vivo*, implying an economic importance for the animal industry, has not yet been addressed.

In the livestock industry (i.e. swine and poultry), plant-derived products such as herbs spices, essential oils and oleoresins have been used as feed additives to improve growth and reproductive performance, disease resistance as well as the quality of animal-derived products for human consumption (Hashemi and Davoodi, 2011; Windisch et al., 2008). As an alternative to veterinary drugs, which are used for prophylaxis and therapeutic purposes for limited time periods, feed additives are products applied via the diet to healthy animals for nutritional purposes within a defined chronological window of the growth phase (Windisch et al., 2008). In relation to this, one of the main driving forces behind the use of phyto-additives in the livestock industry is the increased restrictions in the use of antibiotics as growth promoters as well as the use of disinfectants to prevent and control diseases in the United States and European Union (Hashemi and Davoodi, 2011). This is especially true due to the increased concerns regarding the risk of developing antibiotic resistant pathogens, environmental pollution and accumulation of chemical residues in animal products.

Although the benefits to health, growth and reproductive performance and final product quality observed in various terrestrial animals species fed phyto-additive supplemented diets have been studied, the potential effects of these phytochemicals has received little attention in fish farming. These antecedents along with the fact that consumers are becoming more aware about quality and safety of fish products, create a fertile context for fish nutrition research, and provide a driving force for the aquaculture industry to increase the use of these plant-derived additives in aquafeeds. Indeed, several phytochemicals found in plant extracts such as alkaloids, terpenoids, tannins, saponins, glycosides, polyphenols (i.e. flavonoids and phenolics), steroids and essential oils have been shown to exert beneficial effects in fish, such

as appetite stimulation, promotion of weight gain, immunostimulation as well as antibacterial and anti-parasitic effects (Chakraborty et al., 2014).

As previously mentioned, polyphenols are a group of phytochemicals with multiple biological activities such as antioxidant, anti-apoptosis, anti-aging, anticarcinogen, anti-inflammation, anti-atherosclerosis, cardiovascular protection, improvement of the endothelial function, as well as, inhibition of angiogenesis and cell proliferation activity (Han et al., 2007). Most of these biological activities have been attributed to their intrinsic reducing/antioxidant properties, and their free radical scavenging capabilities. It is well established that oxidative stress plays an important role in the pathogenesis of several degenerative/inflammatory age-related diseases in humans (Scalbert et al., 2005a; Scalbert et al., 2005b). However, increasing evidence suggests that the mode of action of these compounds goes beyond antioxidation. Polyphenols most likely interact with cell receptors or enzyme systems involved in signal transduction pathways, resulting in modification or the cellular REDOX balance, and thus activating redox-dependent reactions (Scalbert et al., 2005a; Salbert et al., 2005b). Indeed, many of these redox-dependent reactions may be related to endogenous defense systems, cell energy metabolism and cell differentiation process. Some of the signaling transduction pathways that are responsive to polyphenols include nuclear factor-kappa B activation, activator protein-1 (AP-1) DNA binding, glutathione biosynthesis, phosphoinositide 3 (PI3)-kinase/protein kinase B (Akt) pathway, mitogen-activated protein kinase (MAPK) proteins [extracellular signal-regulated protein kinase (ERK), c-jun N-terminal kinase (JNK) and P38 activation, and the translocation into the nucleus of nuclear factor erythroid 2 related factor 2 (Nrf2) (Han et al., 2007; Scalbert et al., 2005a; Scalbert et al., 2005b).

The capacity to promote regeneration in damaged skeletal muscle post-injury or in those afflicted with muscular dystrophy has been another important biological activity attributed to polyphenols (Dorchies et al., 2006; Kruger and Smith, 2012; Myburgh et al., 2012; Nakae et al., 2012). Most studies addressing this field of research have been conducted in mammalian models. Dorchies et al, (2006) and Nakae et al, (2012), for example, demonstrated that green tea extract, rich in polyphenols, improve several parameters associated with muscle dystrophy in *mdx* dystrophic mice. Nakazato et al. (2010) observed significantly higher glutathione-S-transferase α1 mRNA levels in rats fed a diet supplemented with apple polyphenols

compared to the non-supplemented group. The authors observed significantly lower concentrations of markers of oxidative damage to lipids and proteins such as TBARS and protein carbonyl, respectively, in rats fed a diet supplemented with apple polyphenols. The authors concluded that dietary apple polyphenols provided a protective effect against lengthening contraction-induced muscle injury in rats. Similarly, it has been reported that muscle fiber regeneration was faster due to enhanced activation of satellite cells and an anti-inflammatory response in skeletal muscle in rats fed a diet supplemented with grape seed-derived pro-anthocyanidolic oligomers compared to control group (Kruger and Smith, 2012; Myburgh et al., 2012). Interestingly, it appears that oxidative stress plays an important part in the pathogenesis of skeletal muscle disorders or inflammatory response after muscle injury by causing alterations in calcium homeostasis, redox imbalance and inflammatory response. The oxidative stress-regulated redox-sensitive inflammatory cascade has been shown to be involved in muscle damage (Cooper et al., 2002; Ji, 1999). Free radical generation by infiltrating immune cells such as macrophages and neutrophils accelerates the progression of muscle pathologies as previously demonstrated (Best et al., 1999; Halter et al., 2010; Ji et al., 2009; Nguyen et al., 2005). An increase in the concentrations of oxidative damage markers such as protein carbonyls TBARS and 8-hydroxy-2′-deoxyguanosine (DNA oxidative damage marker) have been detected after exercise in human and animals (Bloomer et al., 2005; Bloomer et al., 2006; Ji, 1999; Liu et al., 2000). In this regard, several polyphenols species were evaluated to determine their capacity to regulate skeletal muscle regeneration. One such polyphenol is resveratrol, which has long been reported to elicit a promotional effect over the skeletal muscle differentiation process, particularly in C2C12-derived myoblast cells. The mechanism of action is still not fully understood as well as whether other types of polyphenols such as anthocyanins can cause a similar pro-differentiation effect. However, it appears that this biological activity is related to the ability of resveratrol to interact with a wide range of molecules from miRNA expression up to signaling transductions factors. Kaminski et al. (2012) reported that resveratrol promoted terminal differentiation in C2C12 myoblasts by becoming myotube. This phenotypic observation was supported by up-regulation of muscular pro-differentiation markers and transcription factors such as *myogenin* and *Scrp3*, with an increase in heavy chain myosin content as well. Additionally, resveratrol increases serum response factor (*Srf*) transcript level, which leads to

stimulation of myoblast differentiation. The up-regulation of *Srf* appears to have been caused via down-regulation of miRNA-133b, which inhibits the transcription of *Srf*. In agreement with these findings, Lagouge et al. (2006) reported a 40% increase of mitochondrial DNA (mtDNA) in muscle fibers of rats fed resveratrol supplemented diets, which implies an increase in the number of mitochondria, and thus oxidative capacity for energy generation in myotubes. Moreover, the authors observed that resveratrol exerted the pro-differentiation effect on C2C12 skeletal muscle cells via modulation of the transcription of miRNAs such as miR-133, miR-20b, miR-149 and miR-21. Furthermore, the same authors in a second study observed resveratrol slightly decreased proliferation, while up-regulating the expression of PGC-1α, a transcription factor that regulates the expression of metabolism related genes in C2C12 myoblasts. Therefore, it appears that the pro-myoblast differentiation effect of resveratrol is mediated by stimulating mitochondrial biogenesis via activation of PGC-1α, which through its interaction with NRF-1 promotes the expression of mitochondrial biogenesis-related genes such as mitochondrial transcription factor A (TFAM), a pivotal transcription factor for mtDNA synthesis. In agreement with this hypothesis, a recent study by Davinelli et al. (2013) demonstrated that a co-administration of resveratrol and equol, a type of isoflavandiol, increased mitochondrial mass and mitochondrial DNA concentration. Moreover, the authors detected that the combined bioactive compounds enhanced SIRT1 activity and induced mitochondrial biogenesis factors including PGC-1α, TFAM and NRF-1 in human endothelial cells (HUVEC). Therefore, whether or not resveratrol stimulates mitochondrial biogenesis via the same mechanisms shown in C2C12 myoblasts needs to be elucidated for potential therapeutic use or drug development for the treatment of age-related muscle pathologies. Interestingly, resveratrol appears to have a dual capacity, particularly, inducing differentiation in undifferentiated myoblasts (early differentiation phase) as well as promoting hypertrophy in late differentiation phase C2C12 neo-formed myotubes via modulation of cytoskeletal proteins expression by activating the IGF-1 signaling pathway (AKT and ERK 1/2 protein activation), as observed by Montesano et al. (2013).

Anthocyanins, a major flavonoid-polyphenol subclass that provides red, blue and purple color to vegetables and fruits has gained nutritional and therapeutic relevance in human nutrition in recent years (Wang and Stoner, 2008) (Figures 7 and 8). A plethora of health benefits such as antioxidation, cardio-protection, anti-inflammatory and anti-carcinogen from

dietary intake of food rich in anthocyanins have been observed in human and mammalian models (Galvano et al., 2004; Kaspar et al., 2011; Poudyal et al., 2010; Vennat et al., 1994; Whitehead et al., 1995). Guo et al. (2012), for example, demonstrated that intake of C3G lowered fasting plasma glucose concentrations, improved insulin sensitivity and alleviated hepatic steatosis in both obese and genetically diabetic *db/db* mice fed a high-fat diet. Moreover, dietary anthocyanin-rich bilberry extracts diminished hyperglycemia and insulin insensitivity via AMP activity in white adipose tissue, skeletal muscle and liver from diabetic mice. This was further accompanied by up-regulation of the glucose transporter GLUT 4 in white adipose tissue and skeletal muscle from the same mice (Takikawa et al., 2010). The intake of purple carrot juice, rich in C3G, was linked either to a reduction or reversal of alterations in the cardiovascular system, and liver structure and function related to metabolic syndrome in rats fed a high carbohydrate-high fat diet (Poudyal et al., 2010). Rojo et al. (2011) detected that oral administration of anthocyanins isolated form *Aristotelia chilensis* improved fasting blood glucose concentration and glucose tolerance in hyperglycemic obese C57BL/6J mice fed a high fat diet. Furthermore, anthocyanins decreased glucose production and enhanced the insulin-mediated down-regulation of the gluconeogenic enzyme, glucose-6-phosphatase, in H4IIE rat liver cells. It has been suggested that anthocyanins could be used as a nutritional therapy for nonalcoholic fatty liver disease by reducing hepatic lipid accumulation (steatosis) via activation of PPAR, which induces lipolysis and reduces lipogenesis (Valenti et al., 2013). Additionally, Zhang et al. (2013) detected that anthocyanins from Chinese bayberry extract negatively regulated oxidative stress-induced autophagy in β cells via activation of Nrf2.

Therefore, the study of beneficial effects on fish health, growth performance and final product quality either from the intake of food ingredients or plant extracts rich in anthocyanins in aquaculture species is warranted. Some examples of anthocyanins-rich food are purple potatoes, purple carrots, purple corn, black soybean and purple beans (Ha et al., 2010; Hwang et al., 2011; Poudyal et al., 2010; Ramos-Escudero et al., 2012; Zhang et al., 2013). The list of anthocyanins-rich food with potential as ingredients in animal feed industry can further increase if we consider waste products derived from the human feed industry such as the wine waste industry and vegetable processing industry (Bhuiyan et al., 2012; Choung, et al., 2001; Kim et al., 2012; Lee et al., 2009; Makris et al., 2007; Soto et al., 2012).

Based upon this background, an *in vivo* experiment was conducted to determine whether dietary supplementation of a purple corn extract, a natural source of anthocyanins, enhanced plasma and erythrocyte antioxidant defenses and protected PUFA and HUFA from lipid peroxidation in rainbow trout. Additionally, an *in vitro* experiment was conducted to determine whether a mixture of anthocyanidins enhanced the enzymatic antioxidant defenses and promoted myogenic differentiation in late differentiated primary myoblasts isolated from white skeletal muscle of rainbow trout. An additional experiment was carried out to determine whether an anthocyanidin mixture and/or time of treatment promoted myogenic differentiation processes in early-differentiated primary myoblasts isolated from white skeletal muscle of rainbow trout. Finally, the major goal of this series of experiments was to contribute to fish nutrition science and ultimately to the aquaculture industry by generating experimental evidence, which expands our knowledge regarding the potential beneficial effects of polyphenol-rich ingredients in fish of interest to aquaculture.

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2.8 Figures

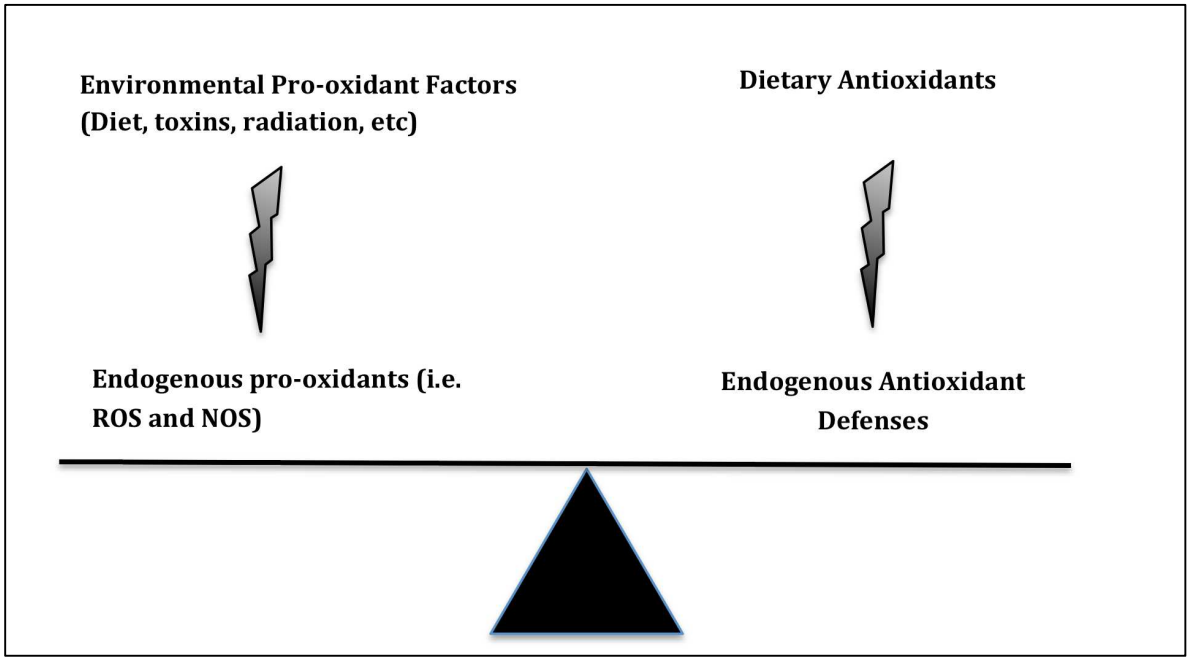


Figure 2.8.1. REDOX balance in an organism.

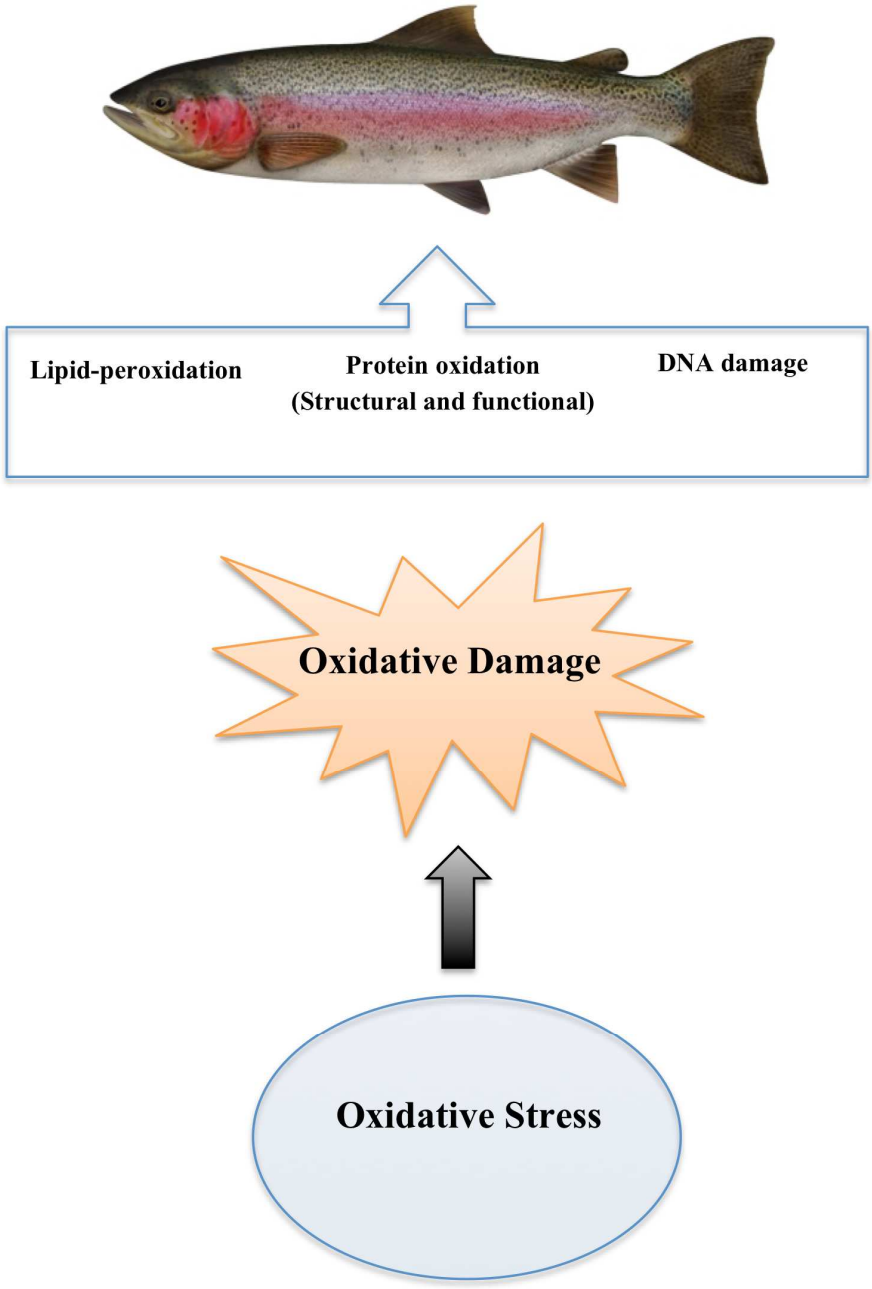


Figure 2.8.2 Scheme of oxidative stress and oxidative damage on bio-molecules in fish (Fish picture, copyright notice © State of New South Wales through NSW Department of Industry).

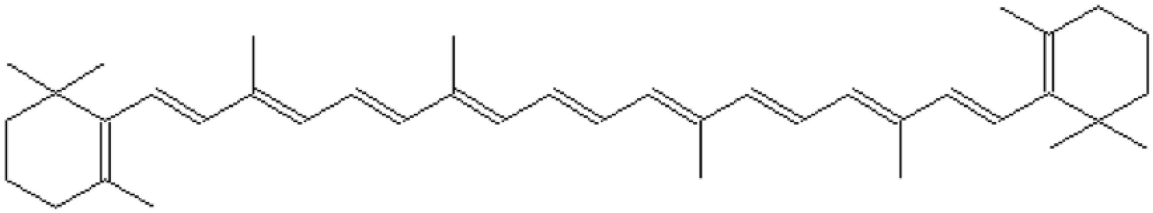


Figure 2.8.3. Molecular structure of carotenoids.

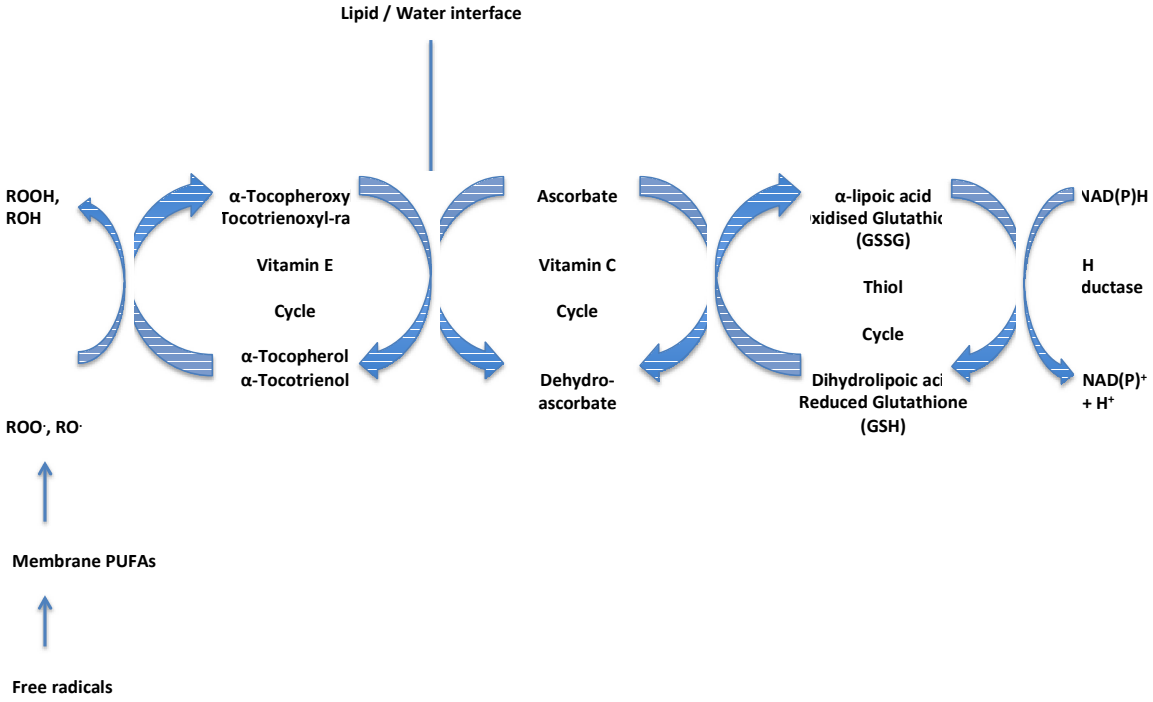


Figure 2.8.4. Interaction between cycles of antioxidant molecules in fish.

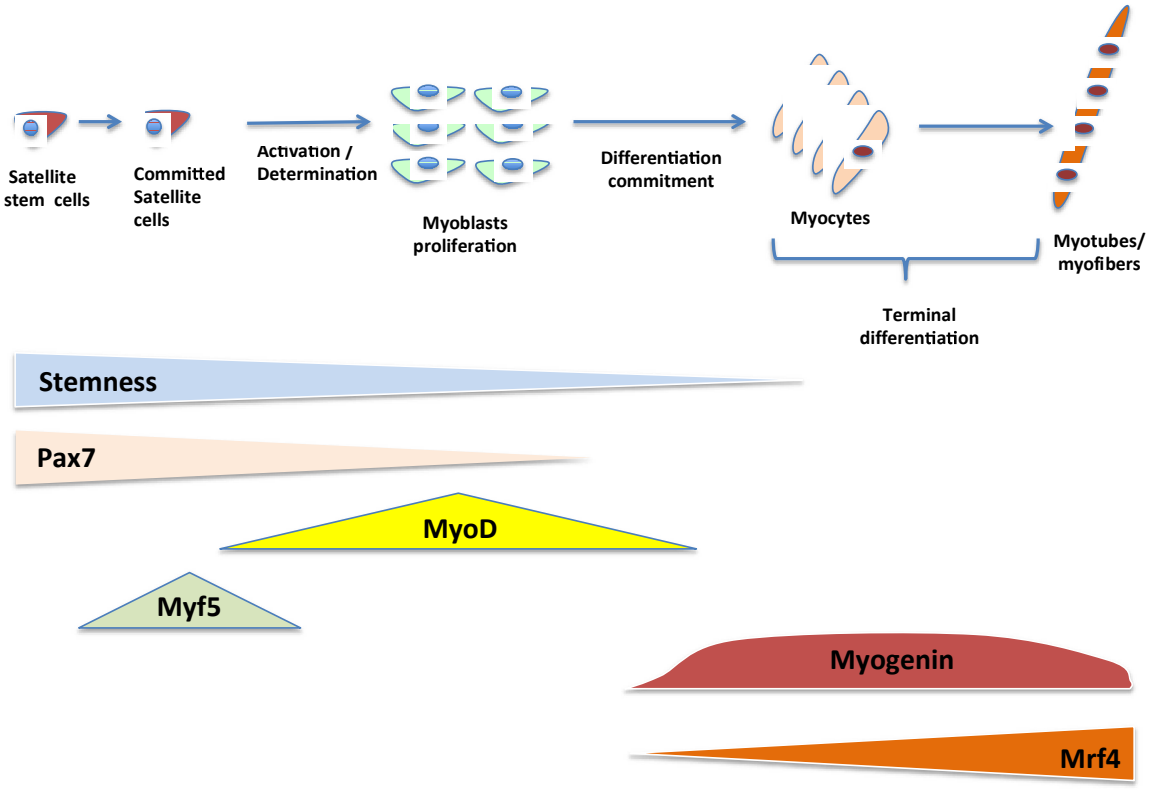


Figure 2.8.5. Dynamic of gene expression of factors regulating progression through the myogenic lineage during postnatal myogenesis.

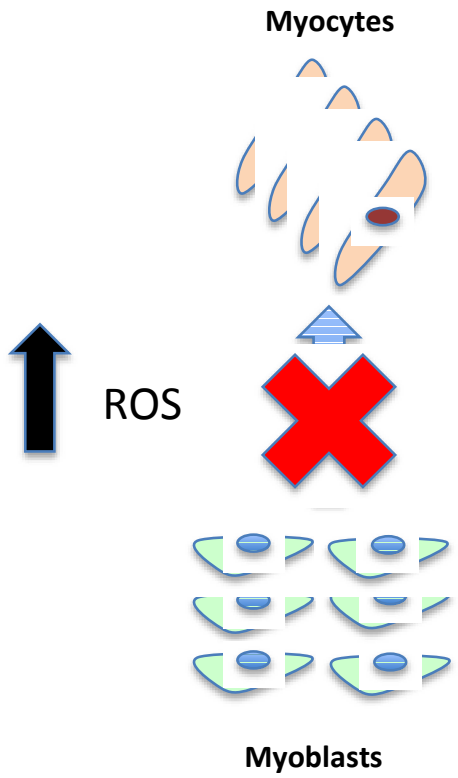
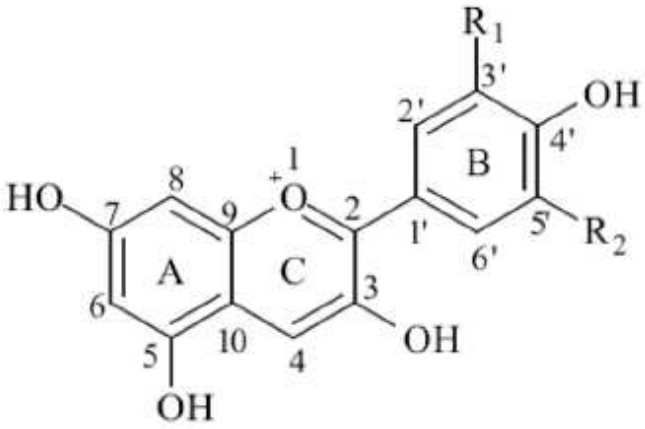


Figure 2.8.6. Disruption of myogenic differentiation progress by increasing ROS concentration.



Name	R1	R2
Delphinidin	**OH**	**OH**
Petunidin	**OCH_3**	**H**
Cyanidin	**OH**	**H**
Pelargonidin	**H**	**H**
Peonidin	**OCH_3**	**H**
Malvidin	**OCH_3**	**OCH_3**

Figure 2.8.7. Chemical structures of anthocyanidins (Hou et al., 2004).

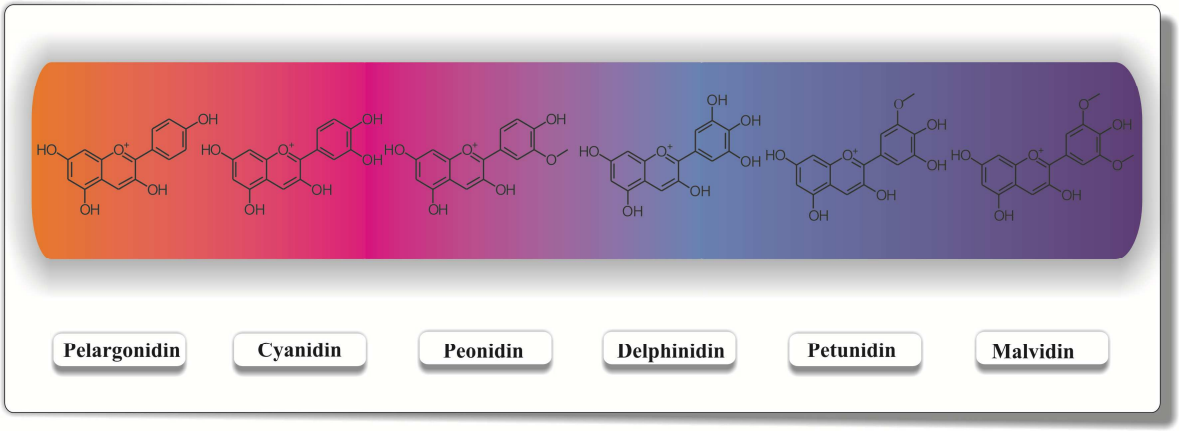


Figure 2.8.8. Visible color range of common anthocyanidins (Ananga et al., 2013)

CHAPTER 3

Dietary Intake of Purple Corn Extract Reduces Fat Body Content and Improves Antioxidant Capacity and *n*-3 PUFA Profile in Plasma of Rainbow Trout, *Oncorhynchus mykiss*

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3.1 Abstract

A study was conducted to determine the benefits of the intake of purple corn extract (PCE), a natural source of anthocyanins, in rainbow trout, Oncorhynchus mykiss. A control or test diet supplemented with 5% PCE were fed to triplicate tanks with 25 fish each for eight weeks. The concentrations of three anthocyanins: cyanidin-3-glucoside (C3G), pelargonidin-3-glucoside (Pel3G) and peonidin-3-glucoside (Peo3G), were measured in PCE. The chemical composition and fatty acid profiles of fish, as well as, the fatty acid profiles in plasma, liver and muscle were determined. The antioxidant capacity (TAC) and the concentration of oxidative damage biomarkers, e.g., protein carbonyls, 8-hydroxydeoxyguanosine (8-OHdG) and thiobarbituric acid reactive substances (TBARS), were determined in plasma. Transcription of two antioxidant enzymes, glutathione peroxidase 1 (*gpx1)* and superoxide dismutase 1 (*sod1*), were measured in erythrocytes. Significant lower adiposity, and significant higher percentage of total *n*-3 and total *n*-6 PUFA in the body of fish fed the test diet were detected. Significant higher plasma percentage of total *n*-3 PUFA, significant higher plasma TAC and significant higher expression of *gpx1* in erythrocytes in fish fed the test diet were measured. Overall, our results suggest potential protection against in vivo lipid peroxidation in fish fed the PCE supplemented diet. This is especially true, due to the detection of an enhanced antioxidant protection in plasma and erythrocytes in fish, the reduced adiposity and greater proportion of total *n*-3 and *n*-6 PUFA in fish body, as well as, a tendency toward lower TBARS plasma concentration in fish fed the test diet when compared to the control group.

3.2 Introduction

In intensive finfish aquaculture systems, high rearing density, poor water quality, water temperature fluctuations and hyperoxia can trigger oxidative stress in fish (Aurousseau 2002; Dabrowski et al. 2004; Krumschnabel et al. 2005; Finne et al. 2008; Farmen et al. 2010; Vinagre et al. 2012). Oxidative stress is caused by the imbalance between the generation rate of free radicals, such as reactive oxygen species (ROS) and reactive nitrogen species (RNS), and the removal rate through the antioxidant mechanisms (Valko et al. 2007; Jones 2008). Although free radicals in high concentrations are cytotoxic, they are required in low concentrations to regulate signaling processes in cells due to their hormetic nature (Virgili and

Marino 2008). The antioxidant defense mechanisms of an organism are based on both enzymatic and non-enzymatic antioxidant systems. Glutathione reductase, superoxide dismutase, catalase and peroxidase are the main enzymatic antioxidant systems. On the other hand, micronutrients such as vitamins (i.e. A, C and E), and minerals (i.e. selenium, zinc, manganese) are examples of non-enzymatic antioxidant defenses (Sies 1993; Birben et al. 2012).

Oxidative damage to macromolecules, such as lipids (lipid peroxidation), proteins and nucleic acids, are direct consequences of oxidative stress. Polyunsaturated fatty acids (PUFA) and highly unsaturated fatty acids (HUFA), as part of cell membrane phospholipids structures, are highly susceptible to lipid peroxidation. An increase in the concentration of lipid peroxidation products, such as lipid peroxyl radicals and malondialdehyde (MDA), can cause deleterious effects in cell structure and function (Poli et al. 1987; Kanazawa 1991; Kanazawa 1993; Gutteridge 1995; Mylonas and Kouretas 1999). In fish, secondary vitamin E/antioxidant deficiency, hepatotoxicity, depressed immune function and reduced growth performance are examples of detrimental effects of lipid peroxidation (Hamre et al. 1994; Nakano et al. 1999; Hamre et al. 2001; Peng et al. 2009; Oliva-Teles 2012; Yun et al. 2013).

Lipids and proteins are the main sources of energy for carnivorous fish species. Fishmeal and fish oil have been used as the main sources of protein and lipids in carnivorous fish diets, respectively (Naylor et al. 2009; Leaver et al. 2008). Due to the increase in the demand and prices of fishmeal, previous studies tested the protein sparing effects of lipids by feeding carnivorous fish high fat diets (Ogino et al. 1976; de la Higuera et al. 1977; Takeuchi et al. 1978b; Takeuchi et al. 1978a). However, fish fed high fat diets showed an increase in visceral fat content (higher adiposity) (de la Higuera et al. 1977; Reinitz et al. 1978; Arzel et al. 1994; Heinen et al. 1995). A higher adiposity could impose lipid peroxidation on carnivorous fish fed high fish oil diets rich in PUFA and/or HUFA. On the other hand, the search for alternatives to fish oil and meal such as vegetable oils and plant proteins have been a priority for the aqua-feed industry to develop more sustainable marine aquaculture. However, evidence suggests that combined plant protein and vegetable oil replacement of fish-based marine feed ingredients promotes hepatic oxidative stress, as well as, increases adiposity and plasma lipid concentrations in carnivorous species (Benedito-Palos et al. 2008; Olsvik et al. 2011; Torstensen et al. 2011). Therefore, the use of cost effective, naturally occurring, dietary

antioxidant compounds, which could protect carnivorous fish against lipid peroxidation, can be beneficial to the aquaculture industry.

Anthocyanins are a class of flavonoids that impart blue, red and purple color to fruits and vegetables (Tonon et al. 2010). It has long been reported that anthocyanins evoke antioxidant, cardio-protective, anti-inflammatory and anti-carcinogenic effects in mammals (Lietti et al. 1976; Vennat et al. 1994; Kamei et al. 1995; Whitehead et al. 1995; Kendall et al. 1998; Galvano et al. 2004; Garcia-Alonso et al. 2004; Cooke et al. 2006; Toufektsian et al. 2008; Wang and Stoner 2008). A previous study demonstrated the intake of C3G lowered fasting plasma glucose concentrations, improved insulin sensitivity and alleviated hepatic steatosis in both obese and genetically diabetic *db/db* mice fed a high fat diet (Guo et al. 2012). Furthermore, the intake of purple carrot juice, rich in C3G, was linked either to a reduction or reversion of alterations in the cardiovascular system, and liver structure and function related to metabolic syndrome in rats fed a high carbohydrate-high fat diet (Poudyal et al. 2010).

However, the potential benefits of the use of dietary sources of anthocyanins have been poorly explored in fish (Perez-Escalante et al. 2012). Purple corn, a grain with high anthocyanin concentrations (1.6 g per 100 g), can be used as a natural anthocyanins source with the potential to enhance the antioxidant status and increase the proportions of PUFA and/or HUFA in fish reared in intensive culture systems (Cevallos-Casals and Cisneros-Zevallos 2003).

Therefore, considering these antecedents, we conducted a study to test whether 1) supplementation of PCE in fish diet would increase the antioxidant defenses in fish plasma and 2) supplementation of PCE in fish diets would protect PUFA and HUFA from lipid peroxidation *in vivo*. We measured the antioxidant activity of plasma as well as the concentration of plasma biomarkers of oxidative damage to lipids, proteins and DNA. Because nucleated erythrocytes have been previously used to monitor the response toward oxidative stress in fish (Gwoździński et al. 1992; Roche and Boge 1993; Fedeli et al. 2004; Trenzado et al. 2009; Fedeli et al. 2010), we quantified the expression of glutathione peroxidase 1 (*gpx1*) and superoxide dismutase 1 (*sod1*) in these cells. Finally, fatty acid profiles of plasma, whole-body, muscle and liver were determined as well as the chemical composition of whole fish.

3.3 Materials and Methods

3.3.1 Fish, Rearing Conditions and Feeding Trial

Eggs from a commercial strain of rainbow trout were obtained from TroutLodge (Sumner, WA, USA) and reared at the Hagerman Fish Culture Experimental Station of University of Idaho (Hagerman, ID, USA) for the trial period. Fish were fed a commercial diet (Nelsons and Sons Inc., Murray, UT, USA) until the start of the feeding trial. A total of 150 fish (130 ± 0.9 g) were evenly stocked into six 140-litre fiberglass tanks. The tanks were supplied with constant temperature well-aerated spring water (15 C, 10 L/min). Fish were exposed to a constant photoperiod (14 h light/10 h dark). During the eight-week feeding trial, fish were fed by hand twice per day to apparent satiation, six days-per-week. Fish in each tank were bulk weighed at the beginning of the trial and every four weeks after 48 h of feed deprivation during the trial. The study was carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee at the University of Idaho.

3.3.2 Experimental Diets

Purple corn extract was obtained from Nutricargo, LLC (Belleville, NJ, USA) and stored in darkness at -20 C until use. The control or test diets were isonitrogenous, isolipidic and isoenergetic as well as met NRC (2011) requirements for rainbow trout (Table 3.1). Briefly, diets contained the same ingredients at the same proportions with the exception of PCE, which was included (5%) at the expense of gelatinized wheat starch in the test diet. The percentage of PCE inclusion was chosen to evaluate a nutritionally and economically feasible dose. A previous study conducted in rats showed a 5% inclusion level was the highest dose tested with no toxic effects in the experimental animals (Nabae et al. 2008). Diets were prepared by cold pelleting and stored in a dry atmosphere at 4 C. Control or test diets were randomly assigned to six tanks with three replicates per treatment (n = 3).

3.3.3 Determination of Anthocyanins in PCE and Total Antioxidant Activity in the Experimental Diets

The concentration of cyanidin-3-glucoside (C3G), pelargonidin-3-glucoside (Pel3G) and peonidin-3-glucoside (Peo3G) in PCE, as well as, total hydrophilic antioxidant activity in the

control or test diet were analyzed at the Bioactives Research Lab of the School of Food Science at Washington State University as previously described (Fuleki and Francis 1968; Brand-Williams et al. 1995; Lee et al. 2005).

3.3.4 Sampling

At the beginning of the trial six fish were collected from the initial population for whole-body proximate analysis. At the end of the feeding trial, three fish per tank were sacrificed six hours postprandially with a sharp blow to the head, and blood, liver and muscle samples were collected. Blood was centrifuged at 1,000 g for 5 min at 4 C and plasma aliquots were stored at -80 C pending determination of antioxidant capacity, oxidative damage and plasma fatty acids analysis. The erythrocyte fraction was recovered after discarding the buffy coat, frozen and stored at -80 C until RNA isolation. Liver and muscle samples were frozen in liquid nitrogen and kept at -80 C until determination of fatty acids and total lipid. Additionally, four fish per tank were euthanized 48 h postprandially with an overdose (200 mg/L) of MS-222 (tricainemethanesulfonate, Argent Laboratories, Redmond, WA), weighed and kept at -20 C until chemical and fatty acid profile analyses of the whole-body.

3.3.5 Determination of Growth Parameters

Specific growth rate was calculated as [100 x (ln (final mean body weight)-ln (initial mean body weight))/days]. Feed intake was estimated as the total amount of ingested food (g as fed) divided by the number of fish and the number of days of the trial. Feed conversion ratio was calculated as (feed intake/wet weight gain). Protein and lipid retention were calculated as [100 x (final body weight x % final whole-body nutrient content) – (initial body weight x % initial whole-body nutrient content)/nutrient intake]. Hepatosomatic index was estimated as (100 x (liver weight / body weight)).

3.3.6 Chemical Analyses

Chemical analyses of diets and fish proximate composition were conducted in duplicates following AOAC methods (1990). Briefly, dry matter was determined by drying samples overnight (12 h) in an oven (105 C) to a constant weight. Crude protein content was

determined (total nitrogen x 6.25) using a nitrogen analyzer (TruSpec N, LECO Instruments, St. Joseph, MI, USA). Crude fat content was determined with an ANKOM XT 15 extractor (ANKOM Technology, Macedon, NY, USA) using petroleum ether as the extracting solvent. Ash content was determined by incineration (600 C) for four hours. Total energy content was determined using an adiabatic bomb calorimeter (Parr 6300, Instrument Co., Moline, IL, USA).

3.3.7 Total Lipids and Fatty Acid Analyses

Determination of total lipids was conducted gravimetrically in duplicates by following the extraction method of Folch as modified by Clark et al. (1982). Fatty acids of whole-body, muscle and liver were trans-esterified to fatty acid methyl esters (FAME) following the two-step methylation procedure of Kramer et al. (1997). FAME were separated using a Shimadzu GC-17A (Shimadzu Corp., Nakagyo-ku, Kyoto, Japan) operated in splitinjection mode with a flame ionization detector, a silica capillary column Omegawax 320 (30 m x 0.32 mm x 0.25 µm, SIGMA, St. Louis, MO, USA) and helium as the carrier gas. Analysis conditions were as follows: injection temperature, 250 C; detector temperature, 280 C; initial oven temperature set at 69 C, hold for 1.4 min, programmed to increase to 170 C at 50 C/min and hold for 8 min, increase again to 220 C at 3 C/min, and hold for 20 min for a total of 48 min run time. Heptadecanoic acid (17:0) was used as internal standard. FAME in samples were identified using the peak retention times of certified FAME standards (Supelco, Bellefonte, PA and Nu-Chek Prep Inc., Elysian, MN, USA), normalized and expressed as % of total FAME. Fatty acid analysis of plasma was conducted following the method 991.39 of AOAC (2007). Plasma samples were dried at 50 C under constant nitrogen, saponified in 0.5 N NaOH, methylated with boron trifluoride-methanol solution (14% w/v) and further separated in a hexane phase. FAME were identified with a GC-MS (Shimadzu GC-17 A), which was operated in split mode with a Zebron ZB-Wax column (Phenomenex, Torrance, CA, USA). The MS (Shimadzu GCMS-QP5050A) unit was operated in scan mode (50-400m/z). Internal standard 17:0 and the Shimadzu Lab Solutions software were used for quantification of each FAME.

3.3.8 Plasma Antioxidant Capacity and Oxidative Damage Biomarkers Analyses

Plasma TAC (Cayman Chemical, Ann Arbor, MI, USA) and 8-OHdG (Bioxytech 8-OHdG-EIA kit, Oxis Health Products, Portland, OR, USA) were measured by ELISA. The protein carbonyl concentration in plasma was measured using a colorimetric assay (Cayman Chemical, Ann Arbor, MI, USA). Plasma lipid peroxidation was analyzed by measuring TBARS as previously described (Uchiyama and Mihara 1978).

3.3.9 RNA Isolation, cDNA Synthesis and Gene Expression Analyses

Total RNA isolation was carried out using TRizol according to the manufacture´s recommendations (Invitrogen, Rockville, MD, USA). Total RNA concentrations were determined spectrophotometrically using an Eppendorf Biophotometer (Eppendorf AG, Hamburg, Germany). RNA was further purified using DNase I (RNase-free) according to manufacturer's indications (Ambion, Austin, TX, USA). Reverse transcription of RNA was carried out using the High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor following manufacturer recommendations (Invitrogen, Rockville, MD, USA). Determination of gene expression was carried out by real-time quantitative PCR on an AB 7500 Fast Real Time Quantitative PCR System using Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). The concentration of cDNA loaded was 30 ng in a 20 µl PCR reaction. Nuclease-free water was used as negative control. Each reaction was carried out in duplicate. PCR reaction cycle conditions were 95 C for 30 s followed by 60 C for 3 min over 40 cycles with an initial denaturation step of 95 C for 2 min. Primers for the genes of interest and reference genes were designed using Primer Express 3.0 (Applied Biosystems, Foster City, CA) (Table 3.2).

From three putative references genes that were tested - ribosomal protein L11 (*rpl11*), ribosomal protein S15 (*rps15*) and alpha tubulin (*aTub*) - *rps15* was observed to be the most stable for normalization purposes. Amplification efficiency of qPCR reactions for each gene was determined using a standard curve with five different concentration points (0.0015 ng to 75 ng/µl). Primer concentrations used in this study for *rps15*, *sod1* and *gpx1* were 300 nM, 200 nM and 400 nM respectively. Transcriptional data of *sod1* and *gpx1* were analyzed following the formula $R_0 = 1/(E+1)^{Ct}$, where R_0 is the target mRNA quantity, E is the mean of

amplification efficiency and Ct is the number of amplification cycles needed to reach the selected threshold fluorescence (Cikos et al. 2007). Finally data were normalized against *rps15* and transformed to the square root.

3.4. Statistical Analysis

Data were analyzed for normality (Kolmogorov–Smirnov test) and homoscedasticity (Levene's test). Dependent variables were analyzed using Student's t-test with a 5% of significance level (STATISTICA 6.0, StatSoft®, Tulsa, OK, USA).

3.5. Results

The concentrations of Peo3G, C3G and Pel3G in purple corn extract were 64.8 ± 2.2 mg/g, 21.5 ± 4.3 mg/g and 18.7 ± 1.7 mg/g, respectively. Total antioxidant activity was 0.1 ± 0.0 mg/ml and 0.02 ± 0.0 mg/ml of Trolox equivalent for the test and the control diet, respectively. Chemical composition and fatty acid profiles of both diets were equivalent (data not shown).

Growth performance, feed intake and feed conversion ratio of fish fed the control or test diets were not different at the end of the trial (Table 3.3). Regarding nutrient retention, lipid retention efficiency was significantly (P = 0.016) higher in the control diet compared to the test diet. However, no significant differences in protein and energy retention efficiency, survival and hepatic somatic index were observed between fish fed either diet.

Whole-body proximate composition of fish fed the control diet was significantly higher in dry matter (P = 0.007), crude fat (P = 0.011) and gross energy (P = 0.025) (Table 3.4) compared to fish fed the test diet. No significant difference in crude protein content between fish fed both diets was observed.

The fatty acid profile analyses of whole-body, muscle and plasma showed significant differences between fish fed the control or test diet (Table 3.5 and 3.6). However, no differences were detected in the hepatic fatty acid profiles of fish fed either diet. In whole-body, fish fed the test diet showed significantly higher proportions of linoleic acid and docosapentaenoic acid (DPA) (P = 0.004 and P = 0.027, respectively) than fish fed the control diet. Similarly, the proportion of total PUFA, total *n*-3 and total *n*-6 fatty acids were significantly (P = 0.02; P = 0.04 and P = 0.004, respectively) higher in whole-body of fish fed

the test diet compared to fish fed the control diet. In muscle, fish fed the test diet showed significant higher percentages of linoleic acid and total *n*-6 fatty acids (P = 0.003 and P = 0.001, respectively) compared to those detected in fish fed the control diet. In plasma, DHA and total *n*-3 fatty acids were significantly (P = 0.029 and P = 0.016, respectively) higher in fish fed the test diet than the percentages observed in fish fed the control diet (Table 3.6). Significantly (P = 0.04) higher proportion of oleic acid in whole-body of fish fed the control diet when compared to fish fed the test diet was detected. Similarly, total mono unsaturated fatty acids (total MUFA) were significantly (P = 0.019) higher in whole-body of fish fed the control diet compared to fish fed the test diet. However, no differences were detected in the percentages of total saturated fatty acid (total SFA) between the experimental groups. Fish fed the control diet showed significantly (P = 0.022) higher proportion of palmitic acid in muscle, compared to fish fed the test diet. Similarly, total SFA was significantly higher (P = 0.006) in muscle of fish fed the control diet than the percentage detected in fish fed the test diet. No differences in the ratio *n*-3/*n*-6 in the muscle of fish fed either diet were observed. Total lipids in the liver of fish fed the control or test diets did not show significant differences between groups, 6.5 ± 1.3 % and 6.1 ± 0.6 %, respectively. Similarly total lipids in the muscle of fish fed the control or test diets did not show significant differences (P = 0.074) compared to the control diet, 4.5 ± 0.4 % and 4.4 ± 0.3 %, respectively.

The TAC in plasma of fish fed the test diet was 0.04 ± 0.0 mM of Trolox equivalent. However, TAC in the plasma of fish fed the control diet was below detection level (Table 3.7). No differences in plasma concentrations of protein carbonyls and 8-OHdG between fish fed either diet were detected. Despite no significant difference in the concentrations of TBARS between fish fed either diet, a tendency (P = 0.075) toward higher plasma concentrations in the fish fed the control diet was observed. Transcriptional analysis of erythrocytes showed significantly (P = 0.008) higher concentrations of mRNA of *gpx1* in fish fed the test diet. However, no significant difference in the level of mRNA of *sod1* between fish fed the control or test diet was observed (Table 7).

3.6. Discussion

Lipids are the main source of energy for most fish species. Fish oil has been used as the main source of lipids and HUFA in diets for carnivorous fish, mainly marine species (Leaver

et al. 2008). HUFA are highly susceptible to lipid peroxidation, and thus, carnivorous fish fed fish oil based diets are prone to lipid peroxidation (Mourente et al. 2002). The main dietary antioxidants that provide protection against oxidative stress to fish are vitamin C, vitamin E, carotenoids (i.e. retinol and astaxanthin) and trace elements such as Mn, Cu, Zn and Se (Nakano et al. 1999; Oliva-Teles 2012). Research has also been conducted to determine the beneficial effects of dietary intake of polyphenols on antioxidant protection in different animal species (Gladine et al. 2007; Thawonsuwan et al. 2010; Surai 2014). For example, the intake of anthocyanins, a type of polyphenol, exerts antioxidant, anti-inflammatory and anti-obesity effects in humans, and other mammals such as rat and mice (Kondo et al. 1996; Mazur et al. 1999; Heim et al. 2002; Poudyal et al. 2010b; Guo et al. 2012).

This study was conducted to test the effect of dietary supplementation of PCE as a natural source of anthocyanins in antioxidant capacity and plasma concentration of oxidative damage markers as well as the proportion of PUFA and HUFA in whole-body, muscle, liver and plasma of fish. Furthermore, the effect of PCE supplementation on transcriptional activity of *sod1* and *gpx1* in red blood cells of fish was analyzed. The effect of PCE supplementation on fatty acid profiles in whole-body, muscle, liver and plasma of fish was determined.

Cevallos-Casals & Cisneros-Zevallos (2003) found that C3G was the most abundant of the anthocyanins in the purple corn analyzed, whereas, we found that Peo3G was the main type of anthocyanin in the PCE tested in our study. Several factors can influence the anthocyanin profiles in fruits and vegetables, such as agronomic conditions, genetic background and the section of the plant from which it is obtained (Cevallos-Casals and Cisneros-Zevallos 2003; Routray and Orsat 2011). Therefore, different profiles of anthocyanins could account for variability on the biological effect caused by the intake of the same vegetable or fruit.

Although there were no differences in diet composition or feed intake between the experimental groups, fish fed test diet had lower fat deposition than the control group. This observation is in agreement with the findings of Tsuda et al. (2003) who observed lower body adiposity and hepatic triacylglycerol deposition in mice fed a high fat diet supplemented with C3G-rich purple corn color (PCC). The authors observed the intake of PCC down-regulated the transcription of enzymes involved in fatty acid and triacylglycerol synthesis as well as the sterol regulatory element binding protein-1 (SREBP-1) in white adipose tissue in the experimental mice. Similarly, Poudyal et al. (2010) observed a reduction in abdominal fat

pads of rats fed a high carbohydrate/high fat diet supplemented with purple carrot juice rich in anthocyanins. Furthermore, Vendrame et al. (2014) showed intake of a diet enriched with wild blueberry, a natural source of anthocyanins, improved lipid profiles in dyslipidaemic obese Zucker rats (an animal model of metabolic syndrome) such as a reduction in triglycerides and plasma total cholesterol. The authors also detected a reduction in the expression of enzymes involve in the synthesis of lipids such as SREBP-1, fatty acid synthase and ATP-binding cassettes transporter 1 both in liver and abdominal adipose tissue. Since no differences in the amount of lipids both in liver and muscle between the experimental groups were observed in our study (data not shown), we suggest that the differences detected on fat content in the body of fish between the experimental groups, were due to differences in the amount of fat in viscera and/or subcutaneous tissue.

A potential mechanism of anthocyanins in the modulation of hepatic lipogenesis has been observed in mammals. Guo et al. (2011) showed that C3G suppressed hepatic *de novo* lipid synthesis by preventing translocation of the enzyme controlling the first step of triacylglycerol synthesis, mitochondrial acyl-CoAglycerol-sn-3-phosphate acyltransferase 1, from the endoplasmic reticulum toward the outer mitochondrial membrane. This mechanism could have been involved in the low fat deposition observed by Tsuda et al. (2003) and Poudyal et al. (2010) in their test groups. Hence, in order to determine whether anthocyanins activate similar mechanism, reducing *de novo* lipid synthesis in fish, further studies conducted either in primary hepatocyte cell culture or hepatoma cell lines are warranted.

It has long been reported anthocyanins promote health by acting as antioxidants (radical-scavenging) and/or increasing the expression of genes associated with antioxidant defenses. For example, Tsuda et al. (1996) demonstrated the antioxidant activity of three types of anthocyanins-C3G, P3G, and D3G-derived from *Phaseolus vulgaris* and their respective aglycons by reducing the formation of malondialdehyde in an *in vitro* liposomal system irradiated with UV-B. Subsequently, Tsuda et al. (1998) showed that serum from rats fed a diet supplemented with C3G was significantly less susceptible to lipid peroxidation provoked by 2,2'-azobis (2-amidinopropane) hydrochloride or Cu^{+2} than that of the control group. Furthermore, Ramos-Escudero et al. (2012) found that a phenolic extract obtained from purple corn significantly reduced lipid peroxidation and increased endogenous antioxidant enzymes such as catalase, total peroxidase and SOD in isolated organs from mouse. In our

study, an increase of total antioxidant capacity in plasma of fish fed the diet supplemented with PCE diet was observed. We analyzed the expression of genes coding for antioxidant enzymes in fish erythrocytes, since they have been previously used to monitor cellular oxidative stress response toward environmental and nutritional cues (Gwoździński et al. 1992; Roche and Boge 1993; Fedeli et al. 2004, Trenzado et al. 2009; Fedeli et al. 2010). Erythrocytes transport oxygen in blood via a reversible association to hemoglobin, and as results they are exposed to ROS and consequently to oxidative stress (Saltman 1989). Although no differences in the expression of catalase and *sod1* were observed, an up-regulation in the transcription of *gpx1* in fish fed the PCE supplemented diet was detected. Experimental evidence suggests that polyphenols can promote the expression of enzymes with antioxidant role via activation of the nuclear factor (erythroid-derived 2)-like 2 (*nrf2*) (Rahman et al. 2006).

Additionally, other beneficial effect described from dietary intake of polyphenols is the increase in *n*-3 and *n*-6 PUFA proportions in plasma of humans and mammalian models such as mice. De Lorgeril et al. (2008), for example, reported increased concentrations of EPA and DHA in plasma of red wine drinkers. Similarly, di Giuseppe et al. (2009) found that alcohol intake was associated with higher plasma and erythrocyte concentration of *n*-3 PUFA. These studies suggested components of red wine such as polyphenols might have been responsible of the observed effects. In agreement with these findings, Cazzola & Cestaro (2011) found that red wine polyphenols significantly protected *n*-3 PUFA and to a lesser extent n-6 PUFA in plasma from lipid peroxidation when compared to the control group. Similarly, Toufektsian et al. (2011) found the dietary intake of a genetically modified corn rich in anthocyanins induced an increase in plasma very long-chain (n-3) PUFA percentage in rats. Toufektsian et al. (2011) proposed a potential mechanism of anthocyanins on the biosynthesis pathway of EPA and DHA from their precursor α-linolenic acid (ALA), in a similar approach to di Giuseppe et al. (2009). However, the authors did not test the proposed mechanism. A significant increase of total PUFA, *n*-3 and *n*-6 in the whole-body and a higher total *n*-3 proportion in the plasma of fish fed the PCE supplemented diet were measured in our study. Although we detected small differences in plasma fatty acids, these changes were similar in proportions to the differences in plasma fatty acids percentages observed by di Giuseppe et al. (2009), Toufektsian et al. (2011) and Cazzola & Cestaro (2011). Taking into consideration the

increase in the antioxidant capacity, the up-regulation of *gpx1* and the observed tendency towards lower concentrations of markers of lipid peroxidation in plasma of fish fed the PCE supplemented diet, we suggest that an *in vivo* protection against oxidative stress could be provided by the intake of dietary PCE. Finally our results demonstrated dietary intake of PCE exerted beneficial effects, such as enhanced plasma antioxidant potential, and up regulation in the expression of the antioxidant enzyme *gpx1* in fish erythrocytes. Additionally, dietary intake of PCE decreased the fat body content in fish, as well as, increased the proportions of *n*-3 PUFA in plasma and body of fish. These findings suggest potential benefits of dietary natural sources of polyphenols in cold fish species physiology, contributing to the welfare of these species under intensive aquaculture conditions, particularly at low water temperature, and/or under pro-oxidative stress conditions. Moreover, within the framework of sustainable aquaculture, further research regarding *in vivo* protection against lipid peroxidation, and decreasing adiposity in fish fed diets supplemented with natural sources of polyphenols is warranted.

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Table 3.1. Ingredients and chemical composition of the experimental diets and PCE.

Ingredients (%)	Control Diet (No PCE)	Test Diet (5% PCE)
PCE[1]	0.0	5.0
Fish meal, sardine[2]	26.3	26.3
Soybean meal[2]	20.0	20.0
Soy protein concentrate[2]	15.0	15.0
Wheat gluten meal[2]	7.0	7.0
Wheat starch, gelatinized[3]	14.0	9.0
Choline chloride[2]	0.6	0.6
Vitamin premix[2, 4]	0.8	0.8
Stable C (35%) vitamin[2]	0.2	0.2
Dicalcium phosphate[5]	2.0	2.0
Trace mineral mix[5, 6]	0.1	0.1
Fish oil (Alaska pollock)[7]	14.0	14.0
Chemical composition (%, DM)		
Crude protein	46.8	46.9
Fat	18.4	18.8
Ash	10.3	10.5
Gross energy (Kcal/kg)	5015	4992

[1]PCE, Purple corn extract. Forestrx.com, Belleville, NJ, USA.

[2]Skretting USA, Tooele, UT, USA.

[3]GemGel 50, Manildra Group USA, Shawnee Mission, KA, USA.

[4]Vitamin premix supplied the following per kg diet: vitamin A, 8000 IU; vitamin D, 6000 IU; vitamin E, 400 IU; vitamin K as menadione sodium bisulfite, 20 µg; thiamin as thiamin mononitrate, 32 mg; riboflavin, 64 mg; pyridoxine as pyridoxine-HCl, 64 mg; pantothenic acid as Ca-d-pantothenate, 192 mg; niacin as nicotinic acid, 240 mg; biotin, 0.56 mg; folic acid, 12 mg; vitamin B_{12}, 50 µg; and inositol as meso-inositol, 400 mg.

[5]Rangen Inc., Buhl, ID, USA.

[6]US Fish and Wildlife Service Trace Mineral Premix #3. It supplied the following (mg/kg diet): Zn (as $ZnSO_4.7H_2O$), 75; Mn (as $MnSO_4$), 20; Cu (as $CuSO_4.5H_2O$), 1.54; I (as KIO_3), 10.

[7]Fishery Industrial Technology Center, University of Alaska at Fairbanks, Kodiak, AK, USA.

Table 3.2. Primer sequences used in real-time PCR.

Gene	Accession N°	Forward 5'-3' and Reverse 5'-3'
		F: ACAGAGGTGTGGACCTGGAC
rps15	BT074197.1	R: AGGCCACGGTTAAGTCTCCT
		F: GTGGAGCTAAGGCTGAGGTG
rpl11	BT074162.1	R: CCCAGTGTCGGAGAAGTTGT
		F: ACTGGTCTCCAGGGCTTCTT
aTub	AY150303.1	R: ACGGAGAGACGTTCCATCAG
		F: CGCCCACCCACTGTTTGT
gpx1	NM_001124525.1	R: GCTCGTCGCTTGGGAATG
		F: ACTCTATCATCGGCAGGACCAT
sod1	AF469663.1	R: GCCTCCTTTTCCCAGATCATC

Table 3.3. Growth parameters, nutrients and energy utilization, and hepatosomatic index of rainbow trout fed the experimental diets for eight weeks[1].

	Control diet		Test diet	
	Mean	SEM	Mean	SEM
Initial body weight (g)	134.3	0.6	134.6	0.6
Final body weight (g)	334.6	5.9	343.4	9.5
Specific growth rate (%/d)[2]	1.6	0.0	1.7	0.0
Feed intake rate (g/fish/day)[3]	4.6	0.1	4.8	0.2
Feed conversion ratio[4]	1.2	0.0	1.3	0.0
Protein retention (% protein intake)[5]	28.9	1.9	29.7	1.5
Lipid retention (% lipid intake)[5]	73.7*	2.7	61.5	2.7
Energy retention (% gross energy intake)[5]	40.9	2.0	38.6	1.5
Hepatosomatic index (%)[6]	1.4	0.1	1.4	0.1

[1]Mean values with their standard error for three tanks per group, twenty-five fish each. Mean values marked with

a * were significantly different between the experimental diets ($P < 0.05$).

[2]Specific growth rate was calculated as [100 x (ln final mean body weight-ln initial mean body weight)/initial mean

body weight].

[3]Feed intake was estimated as the total amount of ingested food (g as fed) divided by the number of fish and the number

of days of the trial.

[4]Feed conversion ratio was calculated as (feed intake/wet weight gain).

[5]Nutrient and energy retention were calculated as [100 x ((final body weight x final body nutrient content) –

(initial body weight x initial body nutrient content))/nutrient intake].

[6]Hepatosomatic index was estimated as (100 x (liver weight / body weight)).

Table 3.4. Chemical composition of the whole-body of rainbow trout fed the experimental diets for eight weeks[1].

	Control diet		Test diet	
Chemical composition (%)	Mean	SEM	Mean	SEM
Dry matter	33.6*	0.2	32.6	0.1
Crude protein	17.5	0.1	17.6	0.2
Crude fat	14.1*	0.3	12.5	0.3
Ash	2.0	0.1	2.1	0.1
Gross energy (Kcal/Kg)	2293*	23.8	2197	0.0

*Mean values were significantly different between the experimental diets ($P < 0.05$).

[1]Mean values with their standard error for three tanks per group; four fish pooled from each tank.

Table 3.5. Fatty acid profiles of whole-body and muscle of rainbow trout fed the experimental diets for eight weeks (mean values with their standard error for three tanks per group. Whole-body of four fish and muscle of three fish analyzed per tank)[1].

	Whole-Body				Skeletal Muscle			
	Control diet		Test diet		Control diet		Test diet	
Fatty acid (%)	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Myristic	4.3	0.0	4.4	0.0	3.8	0.0	3.8	0.1
Palmitic	18.8	0.0	18.4	0.2	20.2*	0.1	19.8	0.0
Stearic	4.2	0.0	4.1	0.1	4.5	0.0	4.5	0.1
Oleic	25.8*	0.0	25.1	0.1	21.9	0.3	21.3	0.9
Linoleic	4.3	0.1	4.7**	0.0	3.5	0.0	3.8**	0.0
α-Linolenic	0.8	0.0	0.8	0.0	0.7	0.0	0.7	0.0
Arachidonic	0.4	0.0	0.5	0.0	0.6	0.0	0.6	0.0
EPA	4.9	0.1	5.3	0.2	6.1	0.0	6.4	0.2
DPA	1.7	0.0	1.8*	0.0	1.6	0.0	1.7	0.1
DHA	9.3	0.0	9.5	0.2	15.2	0.4	15.9	1.0
Total SFA	27.8	0.0	27.4	0.2	29.6**	0.0	28.9	0.1
T MUFA	47.9*	0.0	47.2	0.0	34.7	0.2	33.6	1.1
T. PUFA	24.3	0.0	25.4*	0.0	30.1	0.4	31.8	1.3
Total (*n*-3)	19.1	0.1	19.8*	0.3	25.2	0.3	26.5	1.3
Total (*n*-6)	5.3	0.0	5.7**	0.0	4.4	0.0	4.7**	0.0
n-3/*n*-6	3.6	0.0	3.5	0.0	5.8	0.0	5.7	0.3

EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; T. SFA, total saturated fatty acids; T. MUFA, total monounsaturated fatty acids; T. PUFA, total polyunsaturated fatty acids. [1]Mean values were significantly different between diets: * $P < 0.05$, ** $P < 0.01$.

Table 3.6. Fatty acid profiles of the liver and plasma of rainbow trout fed the experimental diets for eight weeks (mean values with their standard error for three tanks per group with three fish pooled per tank)[1].

	Liver				Plasma			
	Control diet		Test diet		Control diet		Test diet	
Fatty acid (%)	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Myristic	1.8	0.1	1.9	0.2	1.7	0.1	1.5	0.1
Palmitic	17.9	0.4	17.8	0.7	18.6	0.4	18.2	0.3
Stearic	7.0	0.7	7.6	0.4	4.5	0.1	4.5	0.4
Oleic	28.5	2.9	27.4	1.6	16.5	0.2	16.0	0.9
Linoleic	1.7	0.1	1.9	0.2	2.1	0.1	2.1	0.2
α-Linolenic	0.2	0.0	0.2	0.0	0.3	0.0	0.3	0.0
Arachidonic	1.5	0.6	0.9	0.1	1.3	0.1	1.2	0.1
EPA	3.9	0.3	4.1	0.3	7.8	0.2	8.3	0.5
DPA	1.4	0.1	1.5	0.2	2.5	0.1	2.3	0.2
DHA	17.4	3.0	18.6	1.1	29.8	0.1	30.9*	0.0
Total SFA	27.2	1.2	27.7	0.9	25.0	0.4	24.4	0.4
T MUFA	40.2	3.9	38.4	1.6	28.9	0.2	28.4	0.4
T. PUFA	27.3	3.6	28.8	2.0	46.0	0.5	47.2	0.4
Total (*n*-3)	23.4	3.3	25.3	1.6	41.0	0.0	42.3*	0.0
Total (*n*-6)	3.9	0.6	3.7	0.3	4.9	0.2	4.7	0.5
n-3/*n*-6	6.1	0.7	6.8	0.1	8.3	0.3	9.0	1.1

EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid, Total SFA, total saturated fatty acids; Total MUFA, total monounsaturated fatty acids, Total PUFA, total polyunsaturated fatty acids. [1]Mean values were significantly different between diets: * $P < 0.05$.

Table 3.7. Antioxidant activity, oxidative damage markers in plasma and gene expression in erythrocytes of rainbow trout fed the experimental diets for eight weeks[1].

	Control diet		Test diet	
	Mean	SEM	Mean	SEM
Antioxidant activity				
TAC (mM of Trolox)[2]	ND	ND	0.04	0.0
Oxidative damage				
Protein carbonyls (nmol/mL)	31.5	7.4	24.9	8.9
8-OHdG (ng/mL)	11.9	1.4	12.5	0.7
TBARS (μM)[3]	13.8	4.2	4.3	0.4
Relative mRNA quantity[4]				
gpx1	1.42	0.0	1.52*	0.0
sod1	0.59	0.0	0.62	0.0

TAC, total antioxidant capacity; 8-OHdG, 8-hydroxydeoxyguanosine; TBARS, thiobarbituric acid reactive substances.

*Mean values were significantly different between the experimental diets ($P < 0.05$).

[1]Mean values with their standard error for three tanks per group; three fish analyzed each.

[2]ND, not detectable. *P* value not calculated.

[3] Mean values were not significantly different ($P = 0.075$).

[4]Arbitrary units in square root.

CHAPTER 4

Effects of Anthocyanidins on Myogenic Differentiation and Antioxidant Defense in Primary Myogenic Cells Isolated From Rainbow Trout (*Oncorhynchus mykiss*)

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4.1 Abstract

There is increasing interest in using plant-derived extracts to promote growth and health in finfish species in recent years. Elucidating the effects of plant secondary metabolites on skeletal muscle growth signaling will contribute to an improved understanding of the effects of feeding carnivorous fish diets supplemented with plant extracts on fish somatic growth. Dietary intake of anthocyanins, a type of flavonoid widely distributed in plants, has long been associated with beneficial effects in both human and animal health; however, their effects in finfish are largely unknown. We conducted an experiment to test the effect of three doses (treatments A, B and C; 1x, 2.5x and 10x, respectively) of a mixture of three types of anthocyanidins (peonidin, cyanidin and pelargonidin chloride) on the expression of several genes in primary myogenic cells isolated from the skeletal muscle of rainbow trout (*Oncorhynchus mykiss*) after 24 hours of treatment. The genes of interest analyzed are involved in myogenic programing (*pax7*, *myoD* and *myogenin*), Notch signaling (*her6* and hey2) and antioxidant enzymes (*sod1*, *cat* and *gpx1*). Significantly greater expression of *pax7* in cells under treatment B compared with the untreated cells was detected. Although no differences in expression of myogenic regulatory factors, *myoD* and *myogenin* between test groups or the control were detected, a trend toward significantly lower expression in all groups tested compared with the control group was observed. Moreover, significantly higher expression levels of *her6* and *hey2* in cells under treatments A and B compared with untreated cells were detected. Although no significant differences in the expression of *cat* and *sod1*, significantly greater expression in *gpx1* in all treated groups compared with the control group was detected. Collectively, we demonstrated anthocyanidins enhance the expression of *gpx1* in primary myogenic cells, thereby contributing to skeletal muscle tissue defense against oxidative stress in finfish species. Further, anthocyanidins appear to delay myogenic differentiation in primary myogenic cells by up-regulating the expression of *pax7* while decreasing myogenic regulatory factors in a Notch signaling-dependent interaction. Whether this effect results a reduced growth performance and/or an increase in feed conversion ratio in fish fed diets supplemented with plants extracts rich in anthocyanins or anthocyanidins needs further study, and the need to better define the potential effects of different polyphenol classes in myogenic differentiation on primary myogenic cells from carnivorous fish is warranted.

Keywords: anthocyanidins, rainbow trout, skeletal muscle, myogenic cells, myogenesis, antioxidant

4.2 Introduction

During the last several decades, research in nutrition of carnivorous teleost species has been predominantly focused on the effects of feeding fish either total or partial plant ingredient-based diets on growth, health and product quality in fish (Burel et al., 2000; Gomes et al., 1995; Kaushik, et al., 1995; Kaushik et al., 2004; Overturf and Gaylord, 2009; Snyder et al., 2012; Wacyk et al., 2012). This is especially true due to the steady increase in prices of marine-derived aquafeed ingredients, fishmeal and fish oil (Naylor et al., 2009). As inclusion levels of plant ingredients in carnivorous fish diets increase, it is necessary to understand the effects of feeding diets containing phytocompounds in these species. In this regard, most of the research in this field has been to alleviate the detrimental effects of phytocompounds acting as anti-nutritional factors in finfish species, mainly carnivorous species (Krogdahl et al., 2010). Nevertheless, recent evidence has demonstrated phytochemicals including flavonoids, alkaloids, terpenoids, tannins, glycosides, steroids and essential oils, elicit a plethora of beneficial effects in finfish species (Bennetau-Pelissero et al., 2001; Chakraborty et al., 2014; Leiro et al., 2004; Perez-Escalante et al., 2012; Reverter et al., 2014; Saito et al., 2002). Therefore, there is growing interest in the potential use of plant-derived extracts for disease control as an alternative to chemical treatments as well as their use as promoters of appetite and growth performance in finfish species (Reverter et al., 2014).

Anthocyanins, a flavonoid-polyphenol subclass, are found in several vegetables such as purple potatoes, purple carrots, purple corn, black soybean and purple beans (Ha et al., 2010; Hwang et al., 2011; Poudyal et al., 2010; Ramos-Escudero et al., 2012; Zhang et al., 2012). Previous studies have reported potential health benefits, such as antioxidant, cardio-protective, anti-inflammatory and anti-carcinogenic effects, from dietary intake of anthocyanins in humans and other mammals (Galvano et al., 2004; Vennat et al., 1994; Wallace and Giusti, 2013; Whitehead et al., 1995). We recently demonstrated beneficial effects such as higher plasma antioxidant potential and greater gene expression of glutathione peroxidase 1 (*gpx1*) in erythrocytes of trout fed a diet supplemented with purple corn extract, a natural source of anthocyanins (Villasante et al., 2015). Additionally, in a previous study,

Perez-Escalante et al. (2012) observed significant improvement in biometric parameters such as higher specific growth rate and lower feed conversion ratio as well as higher survival in goldfish (*Carassius auratus*) fed a diet supplemented with roselle anthocyanin extract in comparison to a control group. Whether this growth promotion observed in fish fed the anthocyanin extract was due to a stimulatory effect on myogenesis needs to be further explored in fish species of aquaculture importance. In this regard, previous studies have reported polyphenols including resveratrol and (-)-epicatechin promote myogenic differentiation in mammalian-derived C2C12 myoblasts by up-regulating the expression of several myogenic regulatory factors including *myf5*, *myoD*, *myogenin* and *mef2* (Gutierrez-Salmean et al., 2014; Kaminski et al., 2012; Lançon et al., 2012; Montesano et al., 2013). In agreement with this statement Myburgh et al. (2013) observed an accelerated skeletal muscle recovery after *in vivo* administration of grape-derived proanthocyanidolic oligomers in rats with contusion-induced damage. The authors observed that an accelerated activation and proliferation of satellite cells as well as the earlier expression of the fetal isoform of myosin heavy chain (MHC_f) contributed to the faster recovery effect observed in rats fed the polyphenol supplemented diet compared to the control. However, recent evidence suggests that pro-differentiation effect of polyphenols such as resveratrol depends on the dose and the reductive-oxidative balance status of the myogenic cell. A low resveratrol dose promoted *in vitro* muscle regeneration and attenuated the impact of reactive oxygen species (ROS), while high doses reduced plasticity and metabolism induced by oxidative stress in C2C12 myoblasts (Bosutti and Degens, 2015). The mechanism involved appears to be intricate and complex, involving the role of both free radicals acting as signalling molecules and miRNAs including miR-133, miR-20b and miR-149 regulating the expression of pro-myogenic genes (Kaminski et al., 2012; Lançon et al., 2012; Gutierrez-Salmean et al., 2014; Montesano et al., 2013). However, the potential modulatory effect of polyphenols including flavonoids such as anthocyanins or their aglycon forms (anhtocyanidins) in myogenic differentiation in fish species of aquaculture interest has not yet been addressed.

The paired box protein 7 (Pax7) is a member of the paired box transcription factor family, which plays a crucial role during proliferation of and maintenance of an effective satellite cell pool (myogenic progenitor cells) essential for growth, repair, and maintenance of skeletal muscle in juvenile and adult mammals (Bentzinger et al., 2012; von Maltzahn et al., 2013;

Zammit et al., 2006). Myogenic regulatory factors (MRF) including *myoD* and *myogenin* exhibit different expression patterns during myogenesis. *MyoD* is up-regulated during recruitment and determination of satellite cells as well as proliferation of myoblasts while *myogenin* is expressed during myoblast terminal differentiation into myocytes, regulating the expression of myotube specific genes (Olguín and Pisconti, 2012; Pownall et al., 2002). Determination of *pax7/myoD* ratio is an important indicator of satellite cell fate, identifying progression toward differentiation into myoblasts or promotion of satellite cell self-renewal (Olguín et al., 2007; Chapalamadugu et al., 2009; Olguín and Pisconti, 2012).

The Notch signaling pathway plays a crucial role during development (Artavanis-Tsakonas et al., 1999) but its biological importance goes well beyond that. Notch signaling activation is shown to be crucial in avoiding certain muscular dystrophic phenotypes and promotes muscle regeneration in mice (Lin et al., 2013). Bjornson et al. (2012) demonstrated that Notch signaling promotes both self-renewal of skeletal muscle satellite cells and maintenance of normal adult myogenesis in mice. Constitutive activation of Notch signaling is known to induce self-renewal of skeletal muscle satellite cells via up-regulation of *pax7* in C2C12 myoblasts (Wen et al., 2012). Considering the above-mentioned, we analyzed the effect of three type of anthocyanidins (peonidin, cyanidin and pelargonidin chloride) which are the aglycons (non-glycoside form) of anthocyanins, on the expression of genes involved in cell antioxidant defense, namely catalase (*cat*), superoxide dismutase 1 (*sod1*), glutathione peroxidase 1 (*gpx1*) and the nuclear factor (erythroid-derived 2)-like 2 (*nrf2*) and genes associated with myogenic differentiation including *pax7*, *myoD* and *myogenin* and two target genes of the Notch signaling pathway, namely Hairy/enhancer-of-split related with YRPW motif protein (*hey2*) and Hairy/enhancer-of-split related 6 (*her6*), an ortholog of mammalian *hes1* (Davis and Turner, 2001; Liu et al., 2006). The findings of this study provide novel insight with regard to the potential modulatory role of anthocyanidins in myogenic program in primary myogenic cells isolated from carnivorous fish. Although polyphenols including anthocyanins and anthocyanidins are found in several vegetables and fruits that are not common ingredients for aquafeeds, the use of extracts derived from low-cost agroindustry by-products rich in these compounds could offer a cost-effective option to include functional ingredients in aquafeeds that could contribute to improve growth, health and final product quality in finfish species under intensive culture.

4.3 Material and Methods

4.3.1 Anthocyanidin Mixture Preparation

An anthocyanidin mixture of three types of commercial anthocyanidins aglycons, peonidin chloride (A385015M005, Fisher Scientific, Houston, TX, USA), cyanidin chloride (79457, Sigma-Aldrich, St. Louis, MO) and pelargonidin chloride (P1659, Sigma-Aldrich, St. Louis, MO) was prepared using nanopure water as the solvent. The final stock solution concentrations of peonidin chloride, cyanidin chloride and pelargonidin chloride were 50 mM, 20 mM and 15 mM respectively. The anthocyanidin proportions were similar to that measured in a sample of purple corn extract analysed previously in our lab (Villasante et al., 2015).

4.3.2 Cell Culture

4.3.2.1 Myogenic Cell Isolation

All experimental procedures were conducted following the guidelines of the Institutional Animal Care and Use Committee at the University of Idaho. Primary cultures of muscle cells were obtained from rainbow trout stocked at the Hagerman Fish Culture Experiment Station of the University of Idaho (Hagerman, ID, USA). Myogenic cells were isolated as previously described by Cleveland and Weber (2010) with some modifications. Briefly, muscle tissue without skin was removed from the lateral dorsal muscle of juvenile rainbow trout (5-7 g) and collected in ice-cold suspension media (DMEM, 9 mM $NaHCO_3$, 20 mM HEPES, 100 U/ml penicillin, and 100 µg/ml streptomycin). Muscle tissue was minced and resuspended in suspension media and centrifuged (300 g, 5 min, 4°C). The supernatant was discarded and the resultant pellet was resuspended in 0.2% collagenase (C9891, Sigma-Aldrich, St. Louis, MO) in suspension media and gently agitated at 22°C for 1 h. This suspension was centrifuged (900 g, 20 min, 4°C), after which the supernatant was discarded. The resultant pellet was resuspended in 0.1% trypsin (T9935, Sigma-Aldrich, St. Louis, MO) in suspension media and gently agitated for 45 min at 22°C. This mixture was diluted 1:4 with additional suspension media and further centrifuged (900 g, 25 min, 4°C). After removing the supernatant, the resultant pellet was resuspended in suspension media. This cell suspension was filtered through three cell strainers (100 µm, 70 µm and 40 µm) followed by a cell

collection via centrifugation (900 g, 10 min, 4°C). The final pellet containing myogenic cells was resuspended in growth media (suspension media with 10% FBS), and the cells were counted and diluted to a desired density. Cells were plated on a six-well plate previously coated with poly-L lysine/laminin to a concentration between 1.8 and 2 x 10^6 cells/well. After 16 h, wells were gently washed with Hanks` buffered salt saline (HBSS), and the adhered myogenic cells were covered with fresh growth media.

4.3.2.2 Culture Conditions

Culture conditions followed Cleveland and Weber (2010). Plate wells were prepared with 100 µg/ml poly-L-lysine (P4832, Sigma-Aldrich, St. Louis, MO) for 3 h at 18°C. After two washes with sterile nanopure water, wells were layered with 5 µg/ml laminin (L2020, Sigma-Aldrich, St. Louis, MO) in PBS and incubated overnight at 18°C. Laminin solution was discarded and wells further washed with PBS. After cells were plated, they were incubated at 18°C under normal atmospheric condition; thereafter media was changed every other day. A control group with no anthocyanin mixture added, and three treatments with different anthocyanin mixture concentrations (Treatment A: 50 µM of peonidin chloride, 20 µM of cyanidin chloride and 15 µM of pelargonidin chloride, Treatment B: 120 µM of peonidin chloride, 50 µM of cyanidin chloride and 40 µM of pelargonidin chloride and Treatment C: 500 µM of peonidin chloride, 200 µM of cyanidin chloride and 150 µM of pelargonidin chloride) were added to 5 day old cells for 24 h at 18°C. We followed a 24 h treatment since a similar time was used to test the effect of anthocyanins or other polyphenols on cell cultures in previous studies (Boussouar et al., 2013; Davalos et al., 2006; Hemdan et al., 2009; Wang et al., 2012; Zhang et al., 2013). The lowest doses tested in this study was calculated from the estimated anthocyanins intake in a previous study conducted in rainbow trout fed a diet supplemented with purple corn extract as natural source of this type of polyphenols (Villasante eta l., 2015). The three concentrations of anthocyanidin mixture were tested to determine a potential dose response in the analysed dependent variables. In order to determine whether there is a cytotoxic effect of either treatment we analysed the expression level of B-cell lymphoma 2 (*bcl2*) as a marker for apoptotic signaling. Each treatment was performed in triplicate. Each experimental group was replicated in three wells per experiment. The experiment was conducted a total of three independent times (n = 3).

4.3.3 Bioinformatics

Sequences for primer development of *cat*, *hey2, her6* and *nrf2* genes were identified using the Basic Local Alignment Search Tool (BLAST) based searches against the rainbow trout expressed sequence transcript (EST) database from The Gene Index Project (COMPBIO). Sequences for the genes of interest (GOI), *myoD*, *pax7*, *myogenin*, *gpx1*, *sod1*, *cat* and *bcl2* and reference genes *rps15*, *elf1α*, *gapdh*, were identified using sequences found in the GenBank (NCBI). Primers were designed and analyzed using the PrimerQuest and OligoAnalyzer tool available at the web page of Integrated (IDT).

4.3.4 RNA Extraction and cDNA Synthesis

After removing the treatment medium, wells were washed twice with HBSS. Total RNA was isolated from early differentiated myogenic cells using 1 ml/well of TRIzol (Invitrogen, Carlsbad, CA, USA) following the manufacturer`s instructions. Purity and quantity of RNA was determined using a Nanodrop® ND-1000 UV-Vis Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). Subsequently, 5 µg of total RNA was DNAse treated according to manufacturer's methods (Ambion, Austin, TX, USA), 2 µg of which was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor following the manufacturer's recommendations (Invitrogen, Rockville, MD, USA).

4.3.5 Quantification of Gene Expression by real-time Quantitative PCR

Determination of the expression of the genes of interest was carried out by real-time quantitative PCR on an AB 7500 Fast Real Time Quantitative PCR System using Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). The concentration of cDNA was 18 ng for each 20 µl PCR reaction. Nuclease-free water was used as negative control. Each reaction was carried out in duplicate. PCR reaction cycle conditions were 95°C for 30 s followed by 60°C for 3 min over 40 cycles with an initial denaturation step of 95°C for 2 min. Primers sequences, RT-PCR reaction concentrations and accession numbers are shown in Table 4.1. The annealing temperature for both forward and reverse primers of the GOI and reference genes were between 61ºC and 62.5ºC, with the exception of *bcl2*, which was 56.2ºC

for the forward primer and 58.2ºC for the reverse primer. From three putative references genes that were tested – elongation factor 1 alpha (*elf1α*), glyceraldehyde 3-phosphate dehydrogenase (*gapdh*) and ribosomal protein subunit 15 (*rps15*) – *gapdh* showed to be the most stable putative internal control gene against treatments (variations lower than 0.5 Ct values). Further, Gapdh has previously been used as internal control for normalization purpose in study conducted on myogenic cells (Cleveland and Weber, 2010; Günter et al., 2013; Mourikis et al., 2012; Olguín, 2011; Sriram et al., 2011). Amplification efficiency of qPCR reactions for each gene was determined using a standard curve with four different concentration points (4.3 ng to 43 ng/µl). Gene expression data were analyzed following the formula $R_0 = 1/(E+1)^{Ct}$, where R_0 is the target mRNA quantity, E is the mean of amplification efficiency and Ct is the number of amplification cycles needed to reach the selected threshold fluorescence (Cikos et al., 2007). Finally, data were normalized against *gapdh*.

4.4. Statistical Analysis

Data were analyzed for normality (Shapiro-Wilk's Test) and homoscedasticity (Bartlett's test). Natural log transformation of the data was performed when required. Dependent variables were analyzed using one-way analysis of variance (ANOVA) at a 5% level of significance ($P \leq 0.05$). *Post-hoc* tests (Tukey's HSD Test) were performed to identify treatments that differed significantly. Statistical analysis was conducted using R statistical software (R Foundation for Statistical Computing, Vienna, Austria). Mean ± S.E.M. of relative mRNA expression quantity for each treatment ($n = 3$) were graphically reported using Microsoft Office Excel software.

4.5. Results

4.5.1 Effects of Anthocyanidins on Myogenic Programming in Primary Myogenic Cells Isolated from Skeletal Muscle of Rainbow Trout

A mixture of anthocyanidins was shown to modulate the expression of *pax7* after a 24 h treatment in primary myogenic cells isolated from white skeletal muscle of rainbow trout (Fig. 4.1). Treatment B showed mRNA expression of *pax7* to be significantly greater than for

both treatment C and the control ($p = 0.003$ and $p = 0.008$ respectively). No differences in the relative mRNA transcription of *pax7* between treatments A/B, A/C, as well as A/control and C/control were observed. Similarly, no differences in the relative mRNA expression quantity of *myoD* and *myogenin* were observed between the experimental groups (Fig. 4.2). The ratio of *pax7/myoD* was calculated since it has been previously suggested as a parameter likely to indicate myoblast cell fate (Chapalamadugu et al., 2009; Olguin et al., 2007). The *pax7/myoD* ratio was significantly higher ($p = 0.042$) in treatment B than in the control. However, no differences in the *pax7/myoD* ratio between treatments A, B and C as well as between treatments A, C and control were observed (Fig. 4.3).

4.5.2 Effect of Anthocyanidins on the Notch Signaling Pathway in Primary Myogenic Cells Isolated from Skeletal Muscle of Rainbow Trout

The relative mRNA expression of *her6* was significantly higher in both treatments A and B than that observed in the control ($p = 0.002$ and $p = 0.048$ respectively). No differences in the relative mRNA expression of *her6* between treatment C/control as well as between treatments A/B and B/C were observed. The relative mRNA expression of *hey2* were significantly higher in both treatment A and B than observed in the control group and treatment C ($p = 0.005$, $p = 0.017$, $p = 0.005$ and $p = 0.017$, respectively). However, no difference in the relative mRNA expression of *hey2* between treatment C and the control was observed (Fig. 4.4).

4.5.3 Effect of Anthocyanidins on Antioxidant Defenses in Primary Myogenic Cells Isolated from Skeletal Muscle of Rainbow Trout

The relative mRNA expression of *gpx1* was significantly greater in treatment C compared with the control and treatments A and B ($p < 0.0001$, $p = 0.012$ and $p = 0.017$ respectively). However, no difference in the relative mRNA expression of *gpx1* between groups A and B was detected (Fig 4.5). No differences in the relative mRNA expression of *nrf2, cat* and *sod1* between the experimental groups were detected. However, we observed a trend ($p = 0.09$) toward greater expression levels of *nrf2* in the anthocyanidin-tested groups compared with the control (Fig. 4.6).

4.5.4 Effect of Anthocyanidins in the Apoptotic Pathway in in Primary Myogenic Cells Isolated from Skeletal Muscle of Rainbow Trout

Relative mRNA expression quantity of *bcl2* was used determined as an apoptotic marker in order to evaluate potential cytotoxic effects of the highest experimental treatment dose in myoblasts (Hasnan et al., 2010; Porebska et al., 2006). No differences in the relative mRNA expression quantity of *bcl2* between the experimental groups were observed (Fig. 4.7).

4.6 Discussion

Two main objectives were evaluated in the present study. The first was to determine whether a mixture of three types of anthocyanidins, the anthocyanin sugar-free forms, promote the antioxidant defense in primary myogenic cells isolated from skeletal muscle of juvenile rainbow trout. The three doses of anthocyanidins caused an up-regulation in the expression of *gpx1* compared to the control in myogenic cells after 24 hrs of treatment. Moreover, we detected a significantly greater expression of *gpx1* in myogenic cells exposed to the highest concentration compared to myogenic cells exposed to the lowest and middle anthocyanidins concentration. However, no effect of either of the anthocyanidin mixture concentrations on expression of *sod1* and *cat* between either experimental groups were detected. Up-regulation in the expression of *gpx1* has been reported to provide protection against oxidative stress in the rat dopaminergic pheochromocytoma cell line PC12 by protecting against 6-hydroxydopamine and hydrogen peroxide toxicities (Gharib et al., 2013). Similarly, McLean et al. (2005) demonstrated a dose-dependent protection against hydrogen peroxide-induced oxidative stress in primary neuronal culture obtained from mouse fetuses over-expressing human *gpx1* compared to wild types of the same genetic background. It has long been reported anthocyanins enhance the antioxidant enzyme expression and/or activity in several mammalian tissues (Chuang and McIntosh, 2011; Fiander and Schneider, 2000; Ross and Kasum, 2002; Zhang et al., 2013). Nevertheless, whether anthocyanins and/or anthocyanidins evoke similar effects in skeletal muscle of a finfish species has not yet been addressed. We demonstrated anthocyanidins enhanced the expression of *gpx1* in primary myogenic cells from juvenile rainbow trout, thus conferring protection from oxidative stress in skeletal muscle in fish. Similarly, previous works have demonstrated the antioxidant role of

polyphenols in cells other than myogenic cells in rainbow trout. Fedeli et al. (2004) reported tannins, including tanic, gallic and ellagic acid, at low concentrations (10 and 30 μM) protect erythrocytes against DNA breakage caused by hydrogen peroxide induced oxidative stress. However, tannins might exert a genotoxic effect at high concentrations (100 μM) in rainbow trout. Thawonsuwan et al., 2010 demonstrated that dietary supplementation of epigallocatechin-3-gallate (EGCG) exerts a potent anti-oxidant and immunostimulant effect in rainbow trout. The authors observed an increase in the bioavailability of vitamin E and lower levels of lipid hydroperoxide (lipoperoxidation markers) in the liver of fish fed diet supplemented with 100 mg of EGCG per kilogram of diet. In addition, we analyzed the expression of *nrf2* to determine whether the anthocyanidin-induced *gpx1* up-regulation was associated with an increase in *nrf2* expression. Nrf2 is considered to be a master regulator of the antioxidant response by binding to the cis-acting antioxidant response element, thus promoting the expression of antioxidant genes (Jaiswal, 2004; Li et al., 2004; Nguyen et al., 2000; Rahman et al., 2006; Zhang et al., 2013). It has been suggested that polyphenols including anthocyanins promote the expression of antioxidant genes by activating the expression of *nrf2* (Wallace and Giusti, 2013). We observed no effect of anthocyanidins in the expression of *nrf2* between either of the experimental groups. However the anthocyanidin-induced *gpx1* up-regulation might have been consequence of increasing Nrf2 stability via promoting the dissociation of the Kelch-like ECH-associated protein 1 (Keap1)-Nrf2 complex as reported (Eades et al., 2011; Jaiswal, 2004; Lee and Johnson, 2004; Motohashi and Yamamoto, 2004; Nguyen et al., 2003; Zhang and Hannink, 2003). Differences in both the effect and the potency between different types of anthocyanidins as well as between the glycoside and the non-glycoside form of these types of polyphenols are expected to occur, thus accounting for differences in the biological effects analyzed in cells (Kähkönen and Heinonen, 2003). Further research for a better understanding of the mechanism by which polyphenols including their glycoside and non-glycoside forms activate the antioxidant genes response in cells from fish tissues is warranted.

The second goal was to determine whether anthocyanidins promote myogenic differentiation in primary myogenic cells obtained from juvenile rainbow trout. Previous studies have demonstrated that polyphenols promote myogenic differentiation as well as muscle regeneration in mammalian models. (Bosutti and Degens, 2015; Gutierrez-Salmean et

al., 2014; Kaminski et al., 2012; Lançon et al., 2012; Montesano et al., 2013; Myburgh et al., 2013). However, whether polyphenols including anthocyanidins exert similar effects in myogenic cells from fish remains largely unknown. Despite myogenic cells were not induced to differentiate, the detected expression pattern of *pax7, myoD* and *myogenin* demonstrated that myogenic cells after 5 days of culture were at the end of differentiation commitment by becoming myocytes, thus progressing through myogenic differentiation toward terminal differentiation (Betzinger et al., 2012; Olguín et al., 2007; Olguín and Pisconti, 2012). The high confluence (>90%) achieved by the high seed density used in our study most likely promoted myogenic differentiation as reported in previous studies where high seed density *per se* triggered myogenic differentiation in C2C12 myoblasts (Angelis et al., 1998; Kaspar et al., 2005; Lindon et al., 2001; Tanaka et al., 2011).

Our data suggest the middle dose of anthocyanidin mixture caused a break pedal-like effect by delaying the progression of myogenesis toward terminal differentiation. This is likely to be true since we detected significantly greater expression of *pax7* and a significantly greater *pax7/myoD* ratio in myogenic cells exposed to the middle dose of anthocyanidins compared to the control. It has been reported that the up-regulation of *pax7* expression inhibits MyoD activity, thereby inhibiting progression of myogenic differentiation in myoblasts (Olguín et al., 2007: Olguín and Pisconti, 2012). In addition, it has been described a greater *pax7/myoD* ratio promotes self-renewal of satellite cells (Chapalamadugu et al., 2009; Olguin et al., 2007; Olguín and Pisconti, 2012). However, m*yogenin* expression inhibits the cell cycle by committing myogenic cells to terminal differentiation (Valente et al., 2013). Previous research has demonstrated that Pax7 is incapable of inhibiting muscle differentiation and myoblast progress toward terminal differentiation when expressed after *myogenin* induction (Zammit et al., 2006; Olguín et al., 2007). Therefore, in the present study, myogenic cells exhibiting an up-regulation of *pax7*, greater *pax7/myoD* ratio along with a co-expression of *myogenin* were most likely progressing to terminal differentiation. Previous work demonstrated constitutive Notch activation induces *pax7* up-regulation mediated inhibition of C2C12 myoblast differentiation (Buas and Kadesch, 2010; Wen et al., 2012). In addition, Sun et al. (2008) suggested that expansion of Pax7-positive cells observed after stimulation of the Notch pathway may be a consequence of decreased *myoD* and *myogenin* expression. We detected an up-regulation in the expression of two Notch target genes, *her6* and *hey2*, in myocytes

expressing greater levels of *pax7* exposed to the middle dose of anthocyanidins compared to the control, suggesting similar association between Notch signaling and *pax7* expression in fish myogenic cells. It has been described that Notch signaling inhibits the commitment to differentiation in favor of self-renewal in satellite cells while Notch activity promotes cell proliferation in committed myoblasts, thus blocking their progress to terminal differentiation (Buas and Kadesch, 2010; Wen et al., 2012). Additionally, it has also been demonstrated that activation of Notch signaling favors survival in differentiated cells by interacting with mitochondrial remodeling proteins (Perumalsamy et al., 2009). Therefore, the biological outcome of the activation of the Notch signaling pathway differs based upon the differentiation stage of cells.

Collectively, our data suggest the middle dose of anthocyanidins modulates myogenesis progress by inducing a gene expression pattern in accordance with a delay toward terminal differentiation, most likely in favor of cell survival in fish myocytes. This is especially true since myogenic cells were committed to terminal differentiation by expressing the myogenic regulatory factor *myogenin*. The biological outcome of Notch signaling activation in myocytes committed to terminal differentiation in juvenile and adult fish remains largely unknown and requires further research.

Overall, the results from this study give new insight with regard the potential effects of using plant-derived extract rich in phytocompounds including anthocyanidins on antioxidant defense and somatic growth in fish species of aquaculture interest. The study of the effects of plant-derived secondary metabolites in growth physiology and immune system of fish as well as the quality of the final product is a field with potential to understand plant raw materials impact on metabolism of fish species of aquaculture interest. The use of low cost agroindustry by-products rich in these compounds could be a cost-effective option to include functional ingredients in aquafeeds that could contribute to improve growth, health and final product quality in finfish species under intensive culture.

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Table 4.1. Primer sequences used in real-time PCR.

Gene	Sequence 5'-3'	Accession Number
rps15	F: ACAGAGGTGTGGACCTGGAC	BT074197.1[a]
	R: AGGCCACGGTTAAGTCTCCT	
elfα	F: GGTCACCACCTACATCAAGAAG	AF498320.1[a]
	R: CCCTTGAACCAGCCCATATT	
gapdh	F: CATGAAGGGATACGTGGGATAC	AF027130.1[a]
	R: CAAAGTTGTCGTTGAAGGAGATG	
myoD	F: CCAACTGCTCTGATGGAATGA	Z46924.1[a]
	R: TTGGAGTCTCGGCGAAATAAG	
pax7	F: TGAGGCTTCATCTGTGAGTTC	JQ303311.1[a]
	R: TTCTCCGTCTTCATCCTTCTTATC	
myogenin	F: TGAGAAGAGGAGGCTGAAGA	Z46912.1[a]
	R: GCCTCTCAATGTACTGGATGG	
gpx1	F: CGCCCACCCACTGTTTGT	NM_001124525.1[a]
	R: GCTCGTCGCTTGGGAATG	
sod1	F: ACTCTATCATCGGCAGGACCAT	AF469663.1[a]
	R: GCCTCCTTTTCCCAGATCATC	
cat	F: GGCTTTGCAGTTAAGTTCTACAC	TC185820[b]
	R: AGCATTGCGTCCCTGATAAA	
hey2	F: CAGCGACATGGATGAAACTATTG	TC208370[b]
	R: CTTGGGTTGTTGTTGTTGGG	
her6	F: TGCCACAGACGGACAATTC	TC180436[b]
	R: GTTGACCTGGTTCGCATACA	
nrf2	F: GCACCCTCTCAAGTCATACAG	TC193607[b]
	R: GTCTCAGTTGCCTCTACCAAAG	
bcl2	F: TGCATCCTGAAACTCTGTGTC	EZ771692.1[a]
	R: CCGAGTCCCCAGGTTGTG	

[a] NCBI,

[b] TIGR.

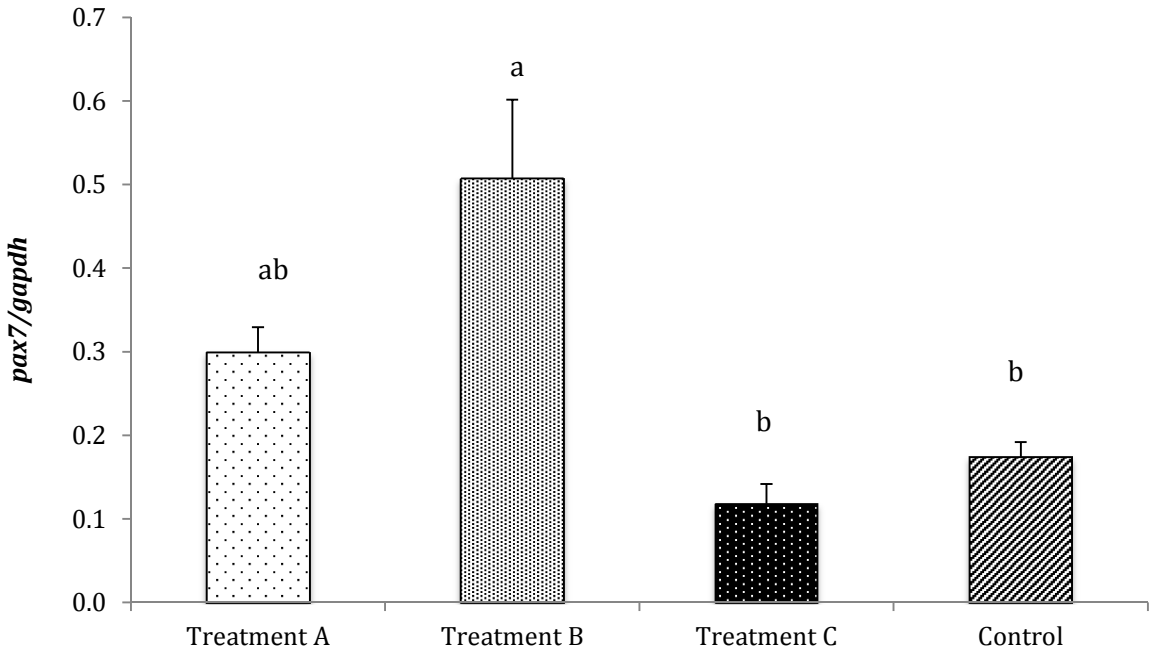

Figure 4.1. Relative mRNA expression quantity of *pax7* after 24 h of treatment of different concentrations of an anthocyanidin mixture in primary myogenic cells from white skeletal muscle of rainbow trout. Treatment A: 50 μM of peonidin chloride, 20 μM of cyanidin chloride and 15 μM of pelargonidin chloride, Treatment B: 120 μM of peonidin chloride, 50 μM of cyanidin chloride and 40 μM of pelargonidin chloride and Treatment C: 500 μM of peonidin chloride, 200 μM of cyanidin chloride and 150 μM of pelargonidin chloride. Bars represent the mean ± S.E.M. of relative mRNA expression normalized against *gapdh*. Treatments that differed significantly at $p < 0.05$ are indicated by different letters (Tukey's test). Each experiment was conducted three independent times ($n = 3$).

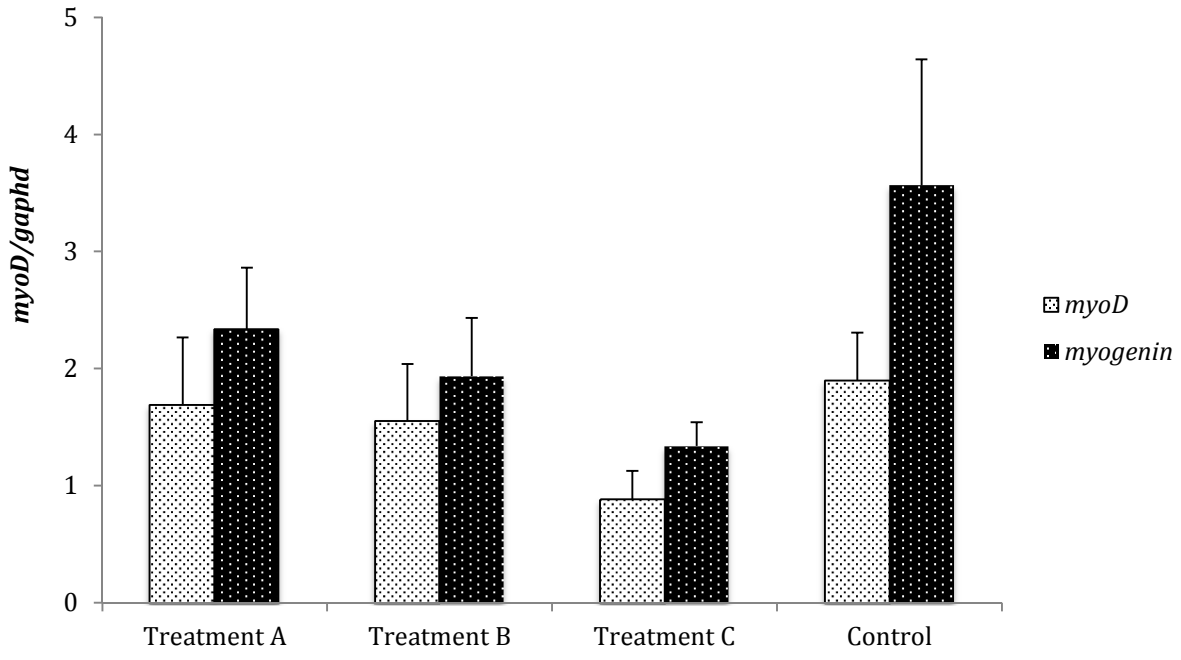

Figure 4.2. Relative mRNA expression quantities of *myoD* and *myogenin* after 24 h of treatment of different concentrations of an anthocyanidin mixture in primary myogenic cells from white skeletal muscle of rainbow trout. Treatment A: 50 μM of peonidin chloride, 20 μM of cyanidin chloride and 15 μM of pelargonidin chloride, Treatment B: 120 μM of peonidin chloride, 50 μM of cyanidin chloride and 40 μM of pelargonidin chloride and Treatment C: 500 μM of peonidin chloride, 200 μM of cyanidin chloride and 150 μM of pelargonidin chloride. Bars represent the mean ± S.E.M. of relative mRNA expression normalized against *gapdh*. Each experiment was conducted three independent times (n = 3).

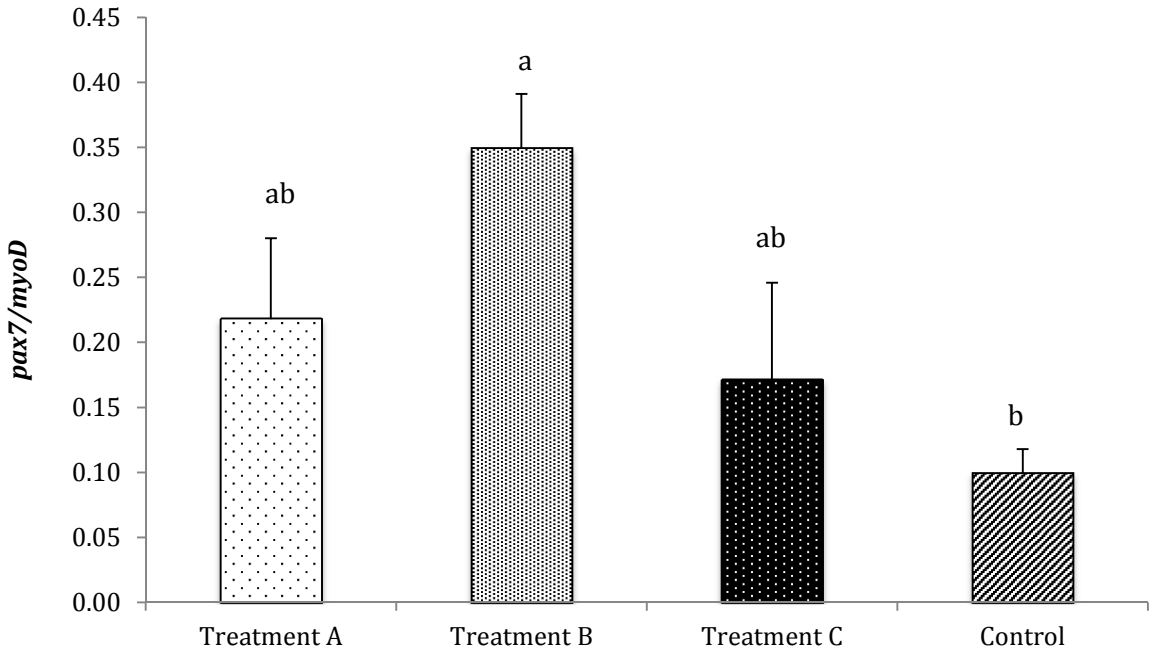

Figure 4.3. *Pax7/myoD* ratio after 24 h of treatment of different concentrations of an anthocyanidin mixture in primary myogenic cells from white skeletal muscle of rainbow trout. Treatment A: 50 μM of peonidin chloride, 20 μM of cyanidin chloride and 15 μM of pelargonidin chloride, Treatment B: 120 μM of peonidin chloride, 50 μM of cyanidin chloride and 40 μM of pelargonidin chloride and Treatment C: 500 μM of peonidin chloride, 200 μM of cyanidin chloride and 150 μM of pelargonidin chloride. Bars represent the mean ± S.E.M. of relative mRNA expression normalized against *gapdh*. Treatments that differed significantly at $p < 0.05$ are indicated by different letters (Tukey's test). Each experiment was conducted three independent times ($n = 3$).

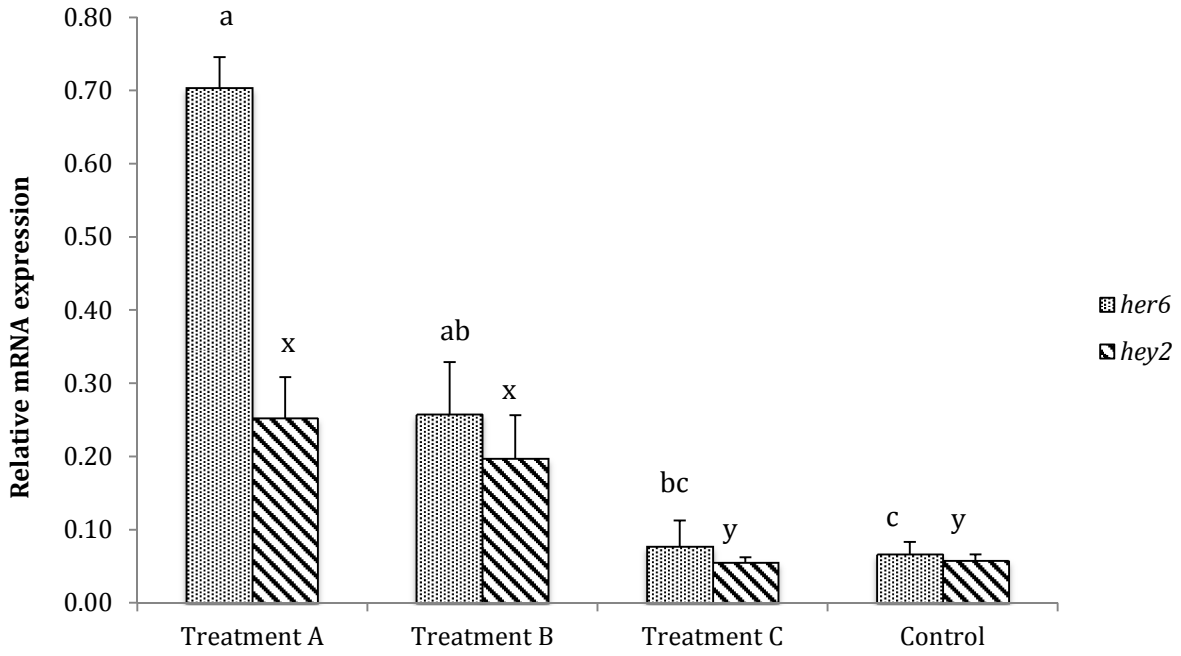

Figure 4.4. Relative mRNA expression quantities of *her6* and *hey2* after 24 h of treatment of different concentrations of an anthocyanidin mixture in primary myogenic cells from white skeletal muscle of rainbow trout. Treatment A: 50 μM of peonidin chloride, 20 μM of cyanidin chloride and 15 μM of pelargonidin chloride, Treatment B: 120 μM of peonidin chloride, 50 μM of cyanidin chloride and 40 μM of pelargonidin chloride and Treatment C: 500 μM of peonidin chloride, 200 μM of cyanidin chloride and 150 μM of pelargonidin chloride. Bars represent the mean ± S.E.M. of relative mRNA expression normalized against *gapdh*. Treatments that differed significantly at $p < 0.05$ are indicated by different letters (Tukey's test). Each experiment was conducted three independent times ($n = 3$).

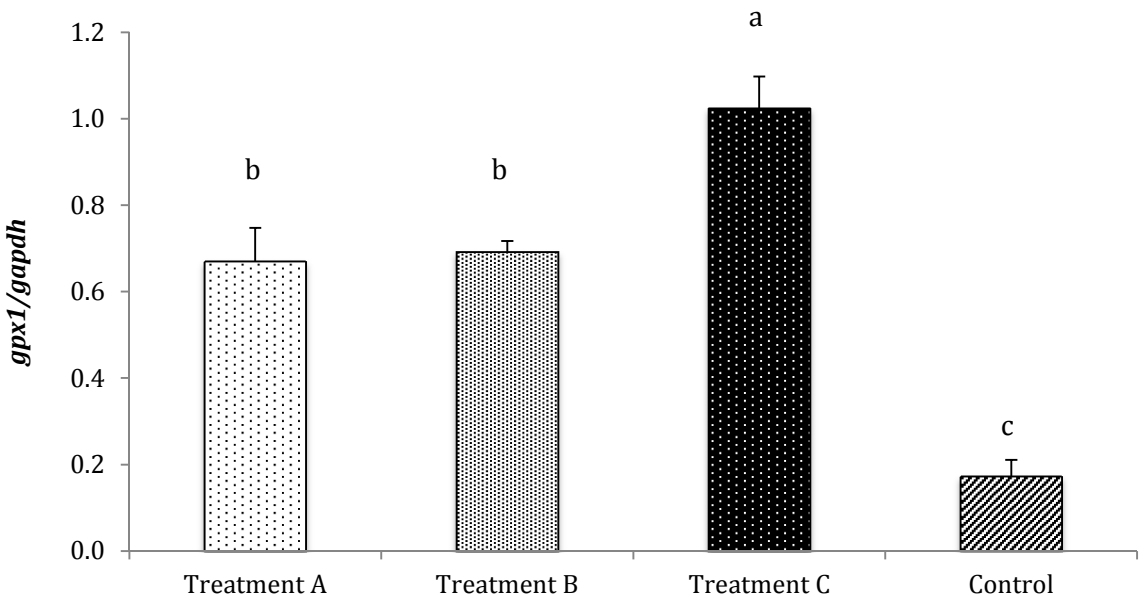

Figure 4.5. Relative mRNA expression quantity of *gpx1* after 24 h of treatment of different concentrations of an anthocyanidin mixture in primary myogenic cells from white skeletal muscle of rainbow trout. Treatment A: 50 μM of peonidin chloride, 20 μM of cyanidin chloride and 15 μM of pelargonidin chloride, Treatment B: 120 μM of peonidin chloride, 50 μM of cyanidin chloride and 40 μM of pelargonidin chloride and Treatment C: 500 μM of peonidin chloride, 200 μM of cyanidin chloride and 150 μM of pelargonidin chloride. Bars represent the mean ± S.E.M. of relative mRNA expression normalized against *gapdh*. Treatments that differed significantly at $p < 0.05$ are indicated by different letters (Tukey's test). Each experiment was conducted three independent times ($n = 3$).

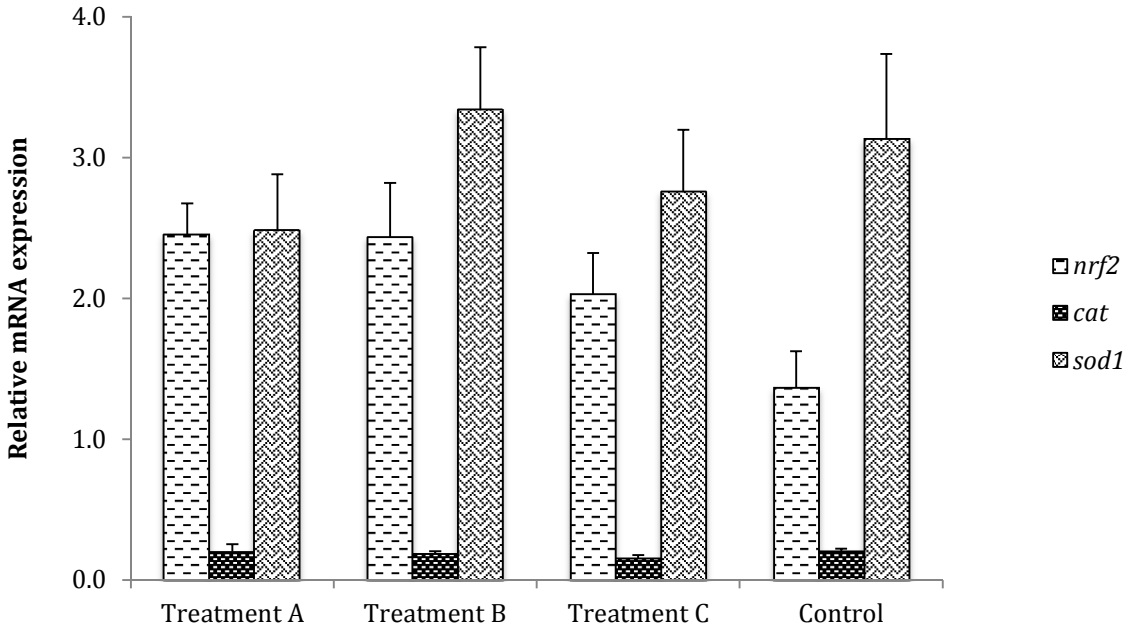

Figure 4.6. Relative mRNA expression quantities of *nrf2, cat and sod1* after 24 h of treatment of different concentrations of an anthocyanidin mixture in primary myogenic cells from white skeletal muscle of rainbow trout. Treatment A: 50 μM of peonidin chloride, 20 μM of cyanidin chloride and 15 μM of pelargonidin chloride, Treatment B: 120 μM of peonidin chloride, 50 μM of cyanidin chloride and 40 μM of pelargonidin chloride and Treatment C: 500 μM of peonidin chloride, 200 μM of cyanidin chloride and 150 μM of pelargonidin chloride. Bars represent the mean ± S.E.M. of relative mRNA expression normalized against *gapdh*. Each experiment was conducted three independent times (n = 3).

CHAPTER 5

Effect of Anthocyanidins on Myogenic Differentiation in Induced and non-Induced Primary Myogenic Cells from Rainbow Trout (*Oncorhynchus mykiss*)

Alejandro Villasante[1]*, Madison S. Powell[1], Gordon K. Murdoch, Ken Overturf, Kenneth Cain, Ronald W. Hardy[1]

5.1 Abstract

A study was conducted to test whether an anthocyanidin mixture (peonidin, cyanidin and pelargonidin chloride) modulates myogenesis in both induced and non-induced myogenic cells from juvenile rainbow trout (*Oncorhynchus mykiss*). We evaluated three different anthocyanidin concentrations (1X, 2.5X and 10X) at two sampling times (24 and 36 hours). To test for the effect of the treatments, we analyzed the expression of *myoD* and *pax7* as well as two target genes of the Notch signaling pathway, *hey2* and *her6.* In induced myogenic cells, the lowest and middle anthocyanidin doses caused significantly greater expression of *myoD* after 24 hours of treatment compared to the control. Although *pax7* expression was unaffected by anthocyanidin treatments after 24 hours, a significantly higher expression of this gene in cells exposed to either anthocyanidin treatment during 36 hours when compared to the control was observed. Similarly, the *pax7/myoD* ratio was significantly lower in cells exposed to the lowest anthocyanidin doses during 24 hours compared to control. No significant effect of anthocyanidin treatments on the expression of *hey2* and *her6* at either sampling point when compared to control was observed. In non-induced cells, we observed no effect of anthocyanidins on *myoD* expression, and a significant down-regulation on *pax7* expression in cells exposed to either anthocyanidin treatments after both 24 hours and 36 hours of treatment when compared to control. The *pax7/myoD* ratio was significantly lower in cells exposed to either anthocyanidin doses at both sampling time. In the non-induced condition, the highest anthocyanidin dose provoked significantly greater expression of *hey2* after 24 hours of treatment compared to the control. We detected no such effect in non-induced cells exposed to the lowest anthocyanidin doses during 24 hours of treatment

compared to the control. The expression of *her6* was unaffected by anthocyanidin treatments at either sampling time or dose when compared to the control. Collectively, these findings provide evidence that anthocyanidins modulate specific components of the myogenic programming in fish, thereby potentially affecting somatic growth in fish fed plant-derived extracts rich in this type of polyphenols. Moreover, in early differentiating myogenic cells, the anthocyanidin effect on myogenic programming appears to differ based upon the exposure time and the differentiation stage of the myogenic cells by boosting myogenic differentiation signaling after 24 hours treatment while pausing differentiation, potentially favoring cell survival after 36 hours treatment. Further research to determine whether plant-derived secondary metabolites including alkaloids, terpenoids, tannins, saponins, glycosides, flavonoids, phenolics, steroids and essential oils can modulate myogenic programming in myogenic cells isolated from finfish species is warranted.

5.2 Introduction

In the last two decades aquaculture production has grown by 6% per year, being the fastest-growing animal food-producing sector worldwide. Increased fish production from aquaculture is the only solution to increase the supply of fisheries products for the future; capture fisheries have declined over the past two decades and cannot expand further (FAO, 2012). Aquaculture production has become more intensive and this has increased productivity and reduced costs in culture systems. However, this has increased the demand for fish feed and associated primary ingredients; fishmeal and fish oil, raising their prices and making them less economical for the aquafeed industry (Naylor et al., 2009). Hence, the search for cost-effective and sustainably produced feed ingredients such as plant meals and plant oils to replace fishmeal and fish oil is a priority for the aquaculture industry (Burel et al., 2000; Gomes et al.,1995; Kaushik et al., 2004; Kaushik et al., 1995; Pierce et al., 2008). However, the increased use of plant ingredients in fish feeds results in greater amounts of phytochemicals in feeds, which makes it necessary to understand the potential effects of these compounds on fish physiology, productive traits and product quality, particularly in carnivorous fish species.

In the search for more environmentally friendly and less risky compounds to human health (i.e. antibiotic resistant bacteria strains and the presence of residual antibiotics in

the muscle of commercialized fish) either to prevent or treat disease outbreaks as well as to promote growth of fish in intensive culture, there has been an increased interest in the use of plant extracts in finfish diets (Chakraborty, et al., 2014; Reverter et al., 2014). Several phytochemicals found in plant extracts such as alkaloids, terpenoids, tannins, saponins, glycosides, flavonoids, phenolics, steroids and essential oils have been shown to exert beneficial effects in fish, such as appetite stimulation, promotion of weight gain as well as resistance to bacteria and parasites (Chakraborty et al., 2014; Reverter et al., 2014).

Anthocyanins and anthocyanidins, a type of flavonoids, provide the red, blue and purple pigmentation to vegetables and fruits (Wang and Stoner, 2008). Several health benefits such as antioxidant, cardio-protective, anti-inflammatory and anti-carcinogenic effects have been observed from the intake of anthocyanins both in human and mammalian models (Galvano et al., 2004; Vennat et al., 1994; Whitehead et al., 1995). However, much less is known about the potential beneficial effects from dietary intake of plant extracts rich in anthocyanins or anthocyanidins on fish grown in intensive culture systems, particularly carnivorous species. Differences in both the effect and the potency between different types of anthocyanidins as well as between the glycoside and the non-glycoside form of these types of polyphenols are expected to occur, thus accounting for differences in the biological effects analyzed in cells (Kähkönen and Heinonen, 2003). Improvement in growth performance is a major goal in the finfish aquaculture industry. Hence, the study of potential stimulatory effects from the intake of phytochemicals in fish growth physiology will contribute to improving the productive performance of finfish aquaculture.

We conducted an *in vitro* study to test whether an anthocyanidin mixture modulates primary myogenic cell programming via transcriptional regulation of genes involved in stem cell determination and differentiation, such as *pax7* and *myoD* in early-induced and non-induced primary muscle cells isolated from white skeletal muscle of rainbow trout, a representative model of indeterminate muscle growth. In addition, we analyzed the transcription of two target genes of the canonical Notch signaling pathway such as Hairy/enhancer-of-split related with YRPW motif protein (*hey2*) and Hairy/enhancer-of-split related 6 (*her6*), an orthologue of mammalian *hes1* (Davis and Turner, 2001; Liu et al., 2006). Notch is a highly conserved cell signaling mechanism, which plays a crucial role in metazoan development (Artavanis-Tsakonas et al., 1999). However, the biological importance of Notch

signaling pathway goes beyond its role in the developmental biology of an organism. Notch signaling is involved in adult muscle homeostasis in human and mammalian models (Bjornson et al., 2012; Brack et al., 2008; Lin et al., 2013; Parker et al., 2012). Moreover, it has been shown that Notch signaling induces self-renewal of skeletal muscle satellite cells via up regulation of *pax7* (Wen et al., 2012). The role of the Notch pathway in signaling involved in juvenile and adult muscle growth physiology remains largely unknown in finfish species.

5.3 Materials and methods

5.3.1 Anthocyanidin Mixture Preparation

An anthocyanidin stock solution containing three types of commercial anthocyanidins peonidin chloride (A385015M005, Fisher Scientific, Houston, TX, USA), cyanidin chloride (79457, Sigma-Aldrich, St. Louis, MO) and pelargonidin chloride (P1659, Sigma-Aldrich, St. Louis, MO) was prepared using nanopure water as the solvent. The final stock solution concentrations of peonidin chloride, cyanidin chloride and pelargonidin chloride were 50 mM, 20 mM and 15 mM, respectively. The anthocyanin proportions were similar to that measured in a sample of purple corn extract analysed previously in our laboratory.

5.3.2 Cell culture

5.3.2.1 Myogenic Cell Isolation

All experimental procedures were approved in advance by the University of Idaho Institutional Animal Care and Use Committee. Primary cultures of muscle cells were obtained from rainbow trout stocked at the Hagerman Fish Culture Experiment Station of the University of Idaho (Hagerman, ID, USA). Primary myogenic cells were isolated as previously described by Cleveland and Weber (2010) with some modifications. Briefly, muscle tissue was removed from the latero-dorsal muscle of juvenile rainbow trout (5-7 g) and collected in ice-cold suspension media (DMEM, 9 mM $NaHCO_3$, 20 mM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin). Muscle tissue was minced and resuspended in suspension media and centrifuged (300 g, 5 min, 4°C). The supernatant was discarded, and the resultant pellet was resuspended in 0.2% collagenase (C9891, Sigma-Aldrich, St. Louis,

MO) in suspension media and gently agitated at 22°C for 1 h. This suspension was centrifuged (700 g, 20 min, 4°C) after which the supernatant was discarded. The resultant pellet was resuspended in 0.1% trypsin (T9935, Sigma-Aldrich, St. Louis, MO) in suspension media and gently agitated for 45 min at 22°C. This mixture was diluted 1:4 with additional suspension media and further centrifuged (700 g, 25 min, 4°C). After removing the supernatant, the resultant pellet was resuspended in media. This cell suspension was filtered through three passages using cell strainers (100 μm, 70 μm and 40 μm) following by a cell collection via centrifugation (700 g, 10 min, 4°C). The final pellet containing the myosatellite cells was resuspended in growth media (suspension media with 10% FBS), and the cells were counted and diluted to a desired density. Cells were plated on a six-well plate coated with poly-L lysine/laminin to a concentration near to 9 x 10^5 cells/well to achieve a low confluence level (< 50%). Cell counting was performed using the TC20™ automated cell counter prototype according to manufacturer´s instructions (Bio-Rad Laboratories Inc., Hercules, CA, USA). After 16 h, wells were gently washed with Hanks` buffered salt saline (HBSS), and the adhered myosatellite cells were covered with fresh growth media.

5.3.2.2 Culture conditions

Culture conditions followed Cleveland and Weber (2010). Wells were prepared with 100 μg/ml poly-L-lysine (P4832, Sigma-Aldrich, St. Louis, MO) for 3 h at 18°C. After washes with sterile nanopure water, wells were layered with 5 μg/ml laminin (L2020, Sigma-Aldrich, St. Louis, MO) in PBS and incubated overnight at 18°C. Laminin solution was discarded and wells further washed with PBS. Cells were randomly assigned into "induced" (treated with differentiation media) and "not induced" (not treated with differentiation media) groups after 24 h of incubation. In the induced group, sub confluent (< 50%) myoblasts were switched from growth medium to differentiation medium (suspension media with 0.5% fetal bovine serum) in order to induce differentiation. In the non-induced group, sub confluent (< 50%) myoblasts were kept under proliferation medium to delay differentiation. Five hours post-segregation a "Time zero" sampling in both groups, induced and not induced, was carried out, followed by the administration of the anthocyanidin treatments. The "Time zero" was used as the calibrator against which the treatment groups were compared when gene transcription

analysis was conducted. A control group and three different anthocyanin concentrations (Control with no anthocyanin, Treatment A: 50 μM of peonidin chloride, 20 μM of cyanidin chloride and 15 μM of pelargonidin chloride, Treatment B: 120 μM of peonidin chloride, 50 μM of cyanidin chloride and 40 μM of pelargonidin chloride and Treatment C: 500 μM of peonidin chloride, 200 μM of cyanidin chloride and 150 μM of pelargonidin chloride) were supplied for 24 h and 36 h to either group after six hours from the segregation point. The three concentrations were tested to determine if a dose response was observable in the analysed dependent variables. Transcriptional analyses of B-cell lymphoma 2 (*bcl2*) (anti-apoptotic gene) and bcl-2-associated X protein (*bax*) (proapoptotic gene) were analyzed in the group that received treatment C, in order to determine a potential cytotoxic effect of the highest dose applied. Treatments were performed in triplicate a total of three independent times ($n = 3$).

5.3.3 Bioinformatics

Complementary DNA (cDNA) sequences for primers development of *hey2, her6* and *bcl2* were identified using the Basic Local Alignment Search Tool (BLAST) based searches against the rainbow trout expressed sequence transcript (EST) database from The Gene Index Project (COMPBIO). Sequences were verified based upon e-values and the percentage of similarity and identical sequence against homologues and similar sequences obtained from other vertebrate species by similarity research sequences (> 88%). Sequences for primers development of *elf1α*, *myoD*, *pax7* and *bax* were identified using sequences found in the GenBank (NCBI) (Table 5.1).

5.3.4 RNA Extraction, cDNA Synthesis and Quantification of Gene Expression by Real-Time Quantitative PCR

After removing the treatment medium, wells were washed twice with HBSS. Total RNA was isolated from cells using 1 ml/well of Trizol (Invitrogen, Carlsbad, CA, USA) following the manufacturer`s instructions. Purity and quantity of RNA was determined using a Nanodrop® ND-1000 UV-Vis Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). Subsequently, 1.2 μg of total RNA were DNase treated with RQ1 RNase-Free DNase

according to manufacturer's methods (Promega, Madison, WS, USA). A total of 60 ng of DNase treated total RNA were used in the reverse transcription reaction, which were followed by the real-time quantitative PCR reactions for gene expression determination in a final volume of 15 µl. Both reactions were performed in the same tube, following the one-step protocol of the Verso™ 1-Step QRT-PCR ROX Kit according to the manufacture's recommendations (Fisher Scientific, Pittsburgh, PA, USA) on a AB 7900 Fast Real Time Quantitative PCR System (Applied Biosystems, Foster City, CA, USA). Nuclease-free water was used as a negative control and each sample reaction was performed in duplicate. Reverse transcription reaction conditions were 50°C for 2 min followed by 60°C for 30 min. The PCR cycling conditions were an initial denaturation step of 95°C for 5 min and then followed by 40 cycles of 95°C for 20 sec and 62°C for 1 min. Primers and probes for both for the genes of interest (GOI) and reference genes were designed and analyzed using the PrimerQuest and OligoAnalyzer tool available at the web page of Integrated DNA Technologies (IDT). Primers sequences and accession numbers are shown in Table 1. Amplicon sizes for primers and probes were between 91 and 130 bps. Amplification efficiency of qPCR reactions for each gene was determined using a standard curve. For all of the genes analyzed, samples Ct values were within the standard curve. Gene expression data were analyzed following the model ($2^{-\Delta\Delta Ct}$) reported by Pfaffl (2001).

5.4. Statistical analysis

Data were analyzed for normality (Shapiro-Wilk's Test) and homoscedasticity (Bartlett's test). Dependent variables within each group, induced and non-induced were blocked by time and subsequently were analyzed using a one-way analysis of variance (ANOVA). *Post-hoc* tests (Tukey's HSD Test) were performed to identify anthocyanin treatments that differed significantly within time sampling. When blocked by anthocyanidin treatment, data were analyzed using Student's t-test to identify significant differences between time sampling, and between Treatment C and Control group at 36 hours for *bax* and *bcl2* transcription analysis. A 5% level of significance level was used for all statistical tests ($P \leq 0.05$). Statistical analysis was conducted using R statistical software (R Foundation for Statistical Computing, Vienna, Austria). Mean ± S.E.M. of relative mRNA expression quantity for each treatment ($n = 3$) were graphically reported using Microsoft Office Excel software.

5.5. Results

5.5.1 Effect of Anthocyanidins in Myogenic Programming in Early-Induced Myogenic Cells

The relative mRNA expression quantities of *myoD* were significantly greater in both treatment A and B ($P = 0.0009$ and $P = 0.042$, respectively) compared to the control after 24 hours of treatment (Fig. 5.1). However, no difference between treatment C and the control group after 24 hours was detected. Although, no differences between treatment A when compared to treatment B was observed, a significant ($P = 0.017$) greater expression of *myoD* in treatment A compared to treatment C was observed. No differences in *myoD* expression between treatments and the control group after 36 hours were detected. However, expression of *myoD* was significantly ($P = 0.0085$) greater in treatment A after 24 hours compared to same treatment after 36 hours. No differences in *myoD* relative mRNA expression in treatment B and C between 24 hours and 36 hours were detected. The relative mRNA expression quantities of *pax7* between treatments A, B and C when compared to each other and to the control group after 24 hours were not significantly different (Fig. 5.2). However, after 36 hours of treatment, significantly greater expression of *pax7* in treatments A, B and C ($P = 0.014$, $P = 0.023$ and $P = 0.033$, respectively) compared to the control group were observed. However, no differences among treatments A, B and C were detected. Significantly ($P = 0.022$) greater expression of *pax7* in treatment A after 36 hours when compared to the same treatment after 24 hours was observed. No differences between treatments B and C when compared to the same treatment between 24 hours and 36 hours were detected. The ratio of *pax7/myoD* was calculated since it has been previously suggested as a parameter likely to indicate satellite cell fate (Chapalamadugu et al., 2009; Olguin et al., 2007). The *pax7/myoD* ratio was significantly ($P = 0.021$) greater in the control group compared to treatment A after 24 hours (Fig. 5.3). However, no significant differences between the control group and treatment B and C were detected after 24 h ours of treatment. In addition, no differences in the *pax7/myoD* ratio between treatments and the control group after 36 hours were detected. No differences in the *pax7/myoD* ratio between treatment A, B and C were observed. Both treatment A and B showed significantly ($P = 0.0031$ and $P < 0.001$ and $P = 0.032$, respectively) greater ratio after 36 hours when compared to the same treatment after 24 hours. No differences for treatment C between 24 hours and 36 hours were observed.

5.5.2 Effect of Anthocyanidins in Cells Myogenic Programming in Non-Induced Myogenic cells

The relative mRNA expression quantities of *myoD* were not significantly different between treatments and the control group after 24 hours (Fig. 5.4). Similarly, *myoD* mRNA expression quantities were not significantly different between treatments and the control group after 36 hours. However, treatment C showed a significantly ($P = 0.046$) greater *myoD* expression after 36 hours when compared to the same treatment after 24 hours. No significant differences in treatment A and B after 24 hours when compared with the respective treatment after 36 hours were detected. The relative mRNA expression quantities of *pax7* were significantly greater in the control group when compared to treatments A, B and C after 24 hours ($P = 0.014$, $P = 0.036$ and $P = 0.009$, respectively) (Fig. 5.5). Similarly, *pax7* was significantly greater expressed in the control group when compared to treatments A, B and C after 36 hours ($P = 0.0001$, $P < 0.0001$ and $P < 0.0001$, respectively).

We detected significantly lower *pax7/myoD* ratio in non-induced myoblasts exposed to treatments A, B and C at 24 hours compared to the control ($P = 0.005$, $P = 0.001$, $P = 0.005$, respectively). Similarly, we detected significantly lower *pax7/myoD* ratio in non-induced myoblasts exposed to treatments A, B and C at 36 hours compared to the control ($P < 0.001$, $P < 0.001$, $P < 0.001$, respectively). Pax7/myoD ratio was significantly higher in non-induced myoblasts exposed to treatments A and B at 24 hours compared to non-induced myoblasts exposed to same treatments at 36 hours ($P = 0.013$, $P = 0.028$, respectively) (Fig. 5.6).

5.5.3 Effect of Anthocyanidins in the Notch Signaling Target Genes in Early-Induced Myogenic Cells

The relative mRNA expression of *hey2* was not significantly affected by anthocyanidin concentrations or the timing of treatments (Fig. 5.7). The relative mRNA expression of *her6* was unaffected by anthocyanidin concentrations after 24 hours of treatment compared to the control group (Fig. 5.8). No significant differences between treatments and the control group after 36 hours were detected. There was a significantly ($P = 0.041$) greater level of expression in treatment A after 36 hours compared to the same treatment after 24 hours.

5.5.4 Effect of Anthocyanidins in the Notch Signaling Target Genes in Non-Induced Myogenic Cells

The relative mRNA expression of *hey2* was significantly ($P = 0.021$) greater in treatment C compared to control group after 24 hours of treatment (Fig. 5.9). However, no significant differences between treatment A and B compared to control group was observed. No dose-response in the expression level of *hey2* after 24 hours was measured. Although the relative mRNA expression of *hey2* was unaffected by anthocyanidin concentrations compared to the control group after 36 hours of treatment, a trend ($P = 0.053$) toward greater expression level in treatment A when compared to the control was observed. No significant differences between treatment A, B and C were detected. With regard to the relative mRNA expression of *her6,* no significant differences between anthocyanidin concentrations and the control group after 24 hours were detected (Fig. 5.10). No significant differences between anthocyanidin treatments were observed as well. Similarly, the expression level of *her6* was unaffected by anthocyanidin concentrations when compared to the control group after 36 hours of treatment. There was significantly ($P = 0.024$) greater expression of *her6* in treatment A after 36 hours compared to the same treatment after 24 hours. No differences between treatment B and C after 24 hours compared to the same treatment after 36.

5.5.5 Effect of Anthocyanidins in the Apoptotic Pathway in Early-Induced and Non-Induced Myogenic Cells

We determined relative mRNA expression of *bax,* a pro-apoptotic signaling protein, and *bcl2,* an anti-apoptotic signaling protein, to examine potential cytotoxic effects of the highest doses of anthocyanidin used in our experimental treatment in both in induced and non-induced myoblasts cells (Hasnan, et al., 2010; Pawlowski and Kraft, 2000). The expression level of both *bax* and *bcl2* remained unaffected by the highest dose of anthocyanidin (Treatment C) after 36 hours of treatment, in both induced and non-induced cells (Fig. 5.11, 5.12, 5.13, 5.14).

5.6 Discussion

We conducted an *in vitro* study to test whether an anthocyanidin mixture of peonidin,

cyanidin and pelargonidin chloride modulated myogenic programming in primary muscle cells at two different myogenic stages (induced and non-induced to differentiate) after 24 and 36 hours of treatment. Our findings demonstrate that anthocyanidins modulation on myogenesis differs with time of exposure and doses in myogenic cells induced to differentiate. An exposure to the lower doses of anthocyanidins during 24 hours triggers a gene expression pattern in accordance with a promotion of myogenic differentiation by up-regulating *myoD* expression with no effect on *pax7* expression. This concept is illustrated by the significant decrease in *pax7/myoD* mRNA ratio after 24 hours observed in cells exposed to the low anthocyanidin doses compare to the control. The *pax7*/myoD ratio is a determinant factor in myogenic cell fate where a lower ratio indicates myogenic cells progress toward terminal differentiation (Chapalamadugu et al., 2009; Olguín et al., 2004, Olguín et al., 2007; Olguín and Pisconti, 2012). On the contrary, an exposure to either anthocyanidins doses after 36 hours induced up-regulation of *pax7* and a trend toward higher *myoD* level, thus causing an intermediate *pax7/myoD* ratio. This gene expression pattern is accordance with a promotion of proliferation and survival in committed myoblasts (Chapalamadugu et al., 2009; Olguin et al., 2007). Therefore, it appears that long exposure to anthocyanidins caused a disruption in myogenic differentiation progress favoring proliferation and survival of committed myoblasts. Committed myoblast could remain responsive to environmental stimuli in order to determine cell fate between progressing toward terminal differentiation and keeping the cell poised to rapidly differentiate (Chapalamadugu et al., 2009; Olguín et al., 2004, Olguín and Pisconti, 2012). Previous studies have reported that polyphenols including resveratrol can either promote or inhibit myogenic differentiation in a dose-dependent manner in C2C12 myoblasts (Abdulla et al., 2013; Bosutti and Degens, 2015; Kaminski et al., 2012; Montesano et al., 2012). Low resveratrol concentrations (10 to 25 μM) have been shown to stimulate myoblast cell cycle arrest and favor further commitment toward terminal differentiation in C2C12 myoblasts (Bosutti and Degens, 2015; Kaminski et al., 2012; Montesano et al., 2012). On the contrary, higher concentrations (above 40 μM) have been shown to inhibit myogenic differentiation in myoblast cells (Bosutti and Degens, 2015; Abdulla et al., 2013). Although we observed no such dose-dependent effect on myogenic cell fate in induced myoblasts to differentiate, anthocyanidins caused similar double effects in a time-dependent manner in cells maintained in differentiation media. Singularities in the

chemical structures of different polyphenols could account for the detection of differences in both their biological effect and potency (dose-response) *in vivo* and/or *in vitro* studies (Abdulla et al., 2013; Pandey and Rizvi, 2009; Heim et al., 2002).

The Notch pathway plays a pivotal role in adult skeletal myogenesis by regulating expression of *pax7* and promyogenic genes (Bjornson et al., 2012; Buas and Kadesch, 2010; Sun et al., 2008; Wen et al., 2012). Previous work has reported that Notch signaling promotes *pax7* expression by reducing the expression and activity of MyoD, thus causing inhibition of myogenic differentiation (Kopan et al., 1994; Shawber et al., 1996; Kuroda et al., 1999; Wen et al., 2012; Wilson-Rawls et al., 1999). Here we analysed the transcriptional response of two Notch target genes, *her6* and *hey2*, to anthocyanidin treatments in both induced and non-induced myogenic cells after 24 and 36 hours of treatment. We failed to detect significant changes in these genes that could support a potential role of this pathway in the anthocyanidin-dependent changes of myogenic programming including *pax7* and *myoD* expression observed in induced cells. However, whether these changes are detectable at the protein level remains to be elucidated. This is especially true since transcription of mRNA for *hes1* is regulated by a negative feedback loop with an oscillatory cycle of 2 hours in several mammalian-derived cell lines (Hirata et al., 2002). The authors proposed that alterations of synthesis and degradation rates (i.e. Hes1 turn-over) by external cues should change the oscillatory cycle length directly affecting the activity of the Notch-dependent target cellular functions. Thus, whether anthocyanidin or other plant-derived secondary metabolites can modulate the turnover rate of Hes1 protein, and potentially directly affect the dynamic of the negative feedback loop, requires further research.

In non-induced cells, anthocyanidins appear to promote differentiation by down regulating *pax7* expression at either dose tested after both 24 and 36 hours of treatment. This statement was further corroborated by the significantly reduced *pax7/myoD* ratio detected in these groups (Chapalamadugu et al., 2009; Olguín et al., 2007; Olguín and Pisconti, 2012). Contrary to what we expected, anthocyanidins provoked a significant up-regulation of *hey2* expression in non-induced myoblasts exposed to the highest anthocyanidin dose as well as a strong trend toward higher expression in non-induced cells exposed to the lowest and middle anthocyanidin doses after 24 hour of treatment compared to the control. The mechanisms by which Notch signaling exerts the inhibitory effect on myogenic differentiation are still poorly

understood, and whether such mechanisms are the same in both the embryonic and adult myogenesis regulation remains elusive (Buas and Kadesch, 2010). It has long been reported hairy-related proteins members including Hes and Hey repressor proteins families can form both heterodimers and homodimers. Heterodimers between Hes and Hey proteins appear to be more stable than the corresponding homodimers (Fischer and Gessler, 2007). Moreover, hairy-related proteins have also been shown to interact with lineage-specific basic helix-loop-helix proteins (bHLH) including the muscle-specific factor MyoD, thus disrupting MyoD activity by counteracting the formation of the functional MyoD/E47 heterodimer during myogenic differentiation (Buas and Kadesch, 2010; Sasai et al., 1992; Sun et al., 2001). However, the hairy-related proteins Hey1 and Hes1 were constitutively expressed in most of these studies, therefore making unclear whether similar dimerization will occur under physiological conditions (Buas and Kadesch, 2010). In addition, it has been reported that Hey2 is not expressed in proliferating myoblasts (Sun et al., 2001). Considering these antecedents, we propose that in the non-induced myogenic cells, which are under active proliferation, the anthocyanidin-induced up-regulation of *hey2* could have caused an increase in Hey2 levels, thus potentially favoring its dimerization with Her6 which is expressed in non-induced myoblasts. An increase in Her6/Hey2 heterodimers will result in increased MyoD/E47 dimers, thus inducing down-regulation in *pax7* expression that we detected in these cells and favoring myogenic differentiation. This is especially likely due to the existence of a Pax7-MyoD reciprocal inhibitory action (Olguín et al., 2007; Wen et al., 2012). This statement is in agreement with Sun et al. (2008) who suggested that *pax7* is not directly regulated by the Notch target genes but rather indirectly by a Notch-dependent modulation of the expression and activity of *myoD* and *myogenin*.

Overall, we demonstrated that plant-derived compounds, e.g. anthocyanidins, modulate myogenesis by promoting differentiation in primary myogenic cells at two different myogenic stages, induced and non-induced. Moreover, we demonstrated that myogenic cells respond in a different manner regarding time of exposure to anthocyanidin in induced myogenic cells. In addition, the expression patterns of the two Notch target genes to anthocyanidin treatments differ between induced and non-induced myogenic cells. Contrary to mammalian species, the role of the Notch signaling pathway regulating skeletal muscle homeostasis in juvenile and adult finfish species remains largely unknown. Further research to increase our understanding

with regards the role of Notch signaling pathway and the potential crosstalk with other pathways in myogenic differentiation in finfish species is warranted. Finally, whether difference in the effect on Notch signaling and the implications of this modulation on myogenesis between glycoside and non-glycoside form of polyphenols needs to be addressed.

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Table 5.1. Primer sequences used in real-time PCR.

Gene	Sequence 5'-3'	Accession Number
elf1α	F: ACAGAGGTGTGGACCTGGAC	BT074197.1[a]
	R: AGGCCACGGTTAAGTCTCCT	
myoD	F: CCAACTGCTCTGATGGAATGA	Z46924.1[a]
	R: TTGGAGTCTCGGCGAAATAAG	
pax7	F: TGAGGCTTCATCTGTGAGTTC	JQ303311.1[a]
	R: TTCTCCGTCTTCATCCTTCTTATC	
hey2	F: CAGCGACATGGATGAAACTATTG	TC208370[b]
	R: CTTGGGTTGTTGTTGTTGGG	
her6	F: TGCCACAGACGGACAATTC	TC180436[b]
	R: GTTGACCTGGTTCGCATACA	

[a] NCBI,

[b] TIGR

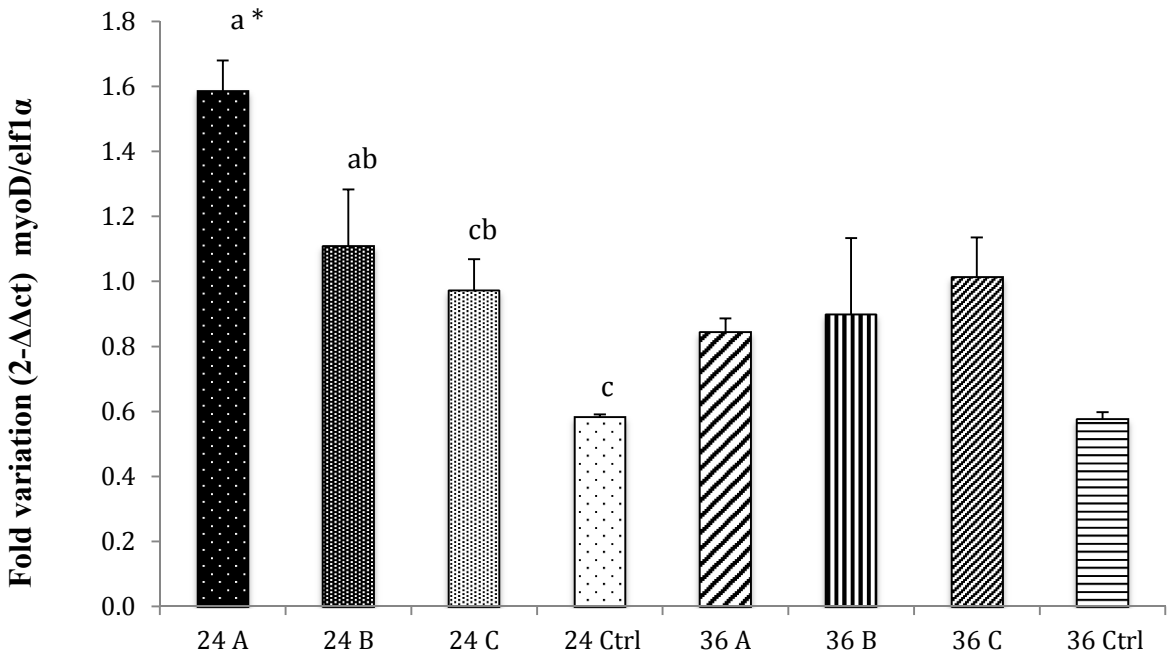

Figure 5.1. Relative mRNA expression quantity of *myoD* after 24 and 36 h of treatment of different concentrations of an anthocyanidins mixture in induced myogenic cells from white skeletal muscle of rainbow trout. Treatment A: 50 µM of peonidin chloride, 20 µM of cyanidin chloride and 15 µM of pelargonidin chloride, Treatment B: 120 µM of peonidin chloride, 50 µM of cyanidin chloride and 40 µM of pelargonidin chloride and Treatment C: 500 µM of peonidin chloride, 200 µM of cyanidin chloride and 150 µM of pelargonidin chloride. Bars represent the mean ± S.E.M. of the relative mRNA expression. Gene expression data were analyzed following the model ($2^{-\Delta\Delta Ct}$). Each experiment was conducted three independent times (n = 3). Differences between anthocyanidins levels are compared within each Time level (one-factor ANOVA, Tukey's HSD test), values without a common letter are different ($p < 0.05$). Differences between Time levels are compared within each anthocyanidins level (T-test), and indicated by * ($p < 0.05$).

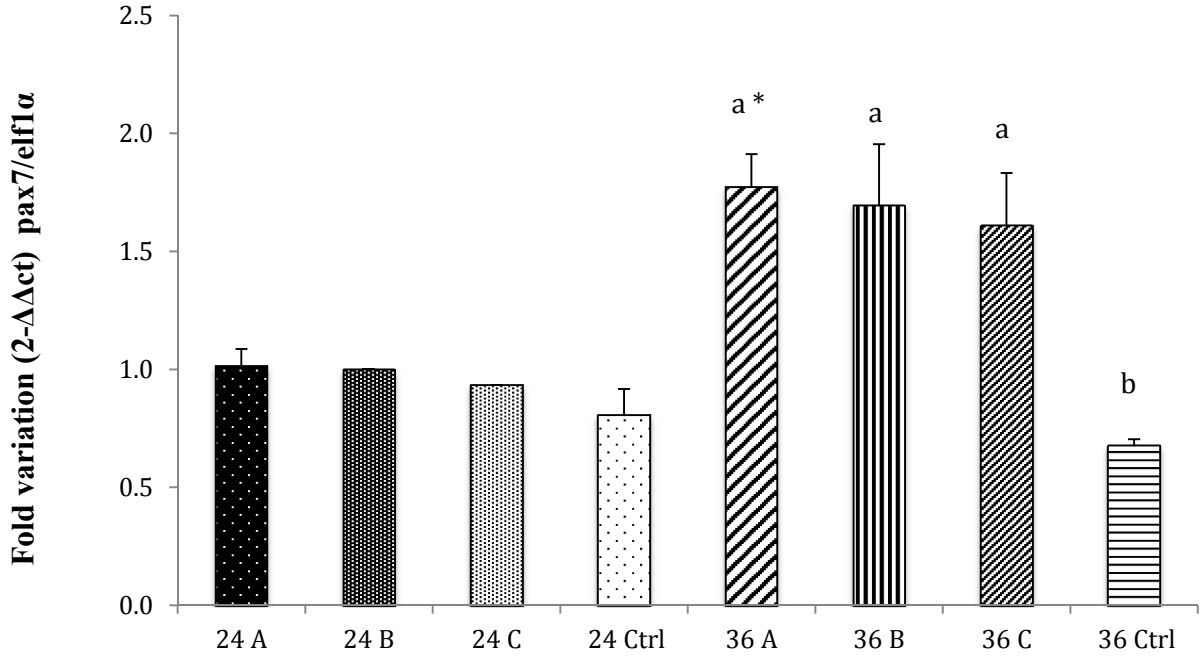

Figure 5.2. Relative mRNA expression quantity of *pax7* after 24 and 36 h of treatment of different concentrations of an anthocyanidins mixture in induced myogenic cells from white skeletal muscle of rainbow trout. Treatment A: 50 µM of peonidin chloride, 20 µM of cyanidin chloride and 15 µM of pelargonidin chloride, Treatment B: 120 µM of peonidin chloride, 50 µM of cyanidin chloride and 40 µM of pelargonidin chloride and Treatment C: 500 µM of peonidin chloride, 200 µM of cyanidin chloride and 150 µM of pelargonidin chloride. Bars represent the mean ± S.E.M. of the relative mRNA expression. Gene expression data were analyzed following the model ($2^{-\Delta\Delta Ct}$). Each experiment was conducted three independent times (n = 3). Differences between Time levels are compared within each anthocyanidins level (T-test), and indicated by * ($p < 0.05$).

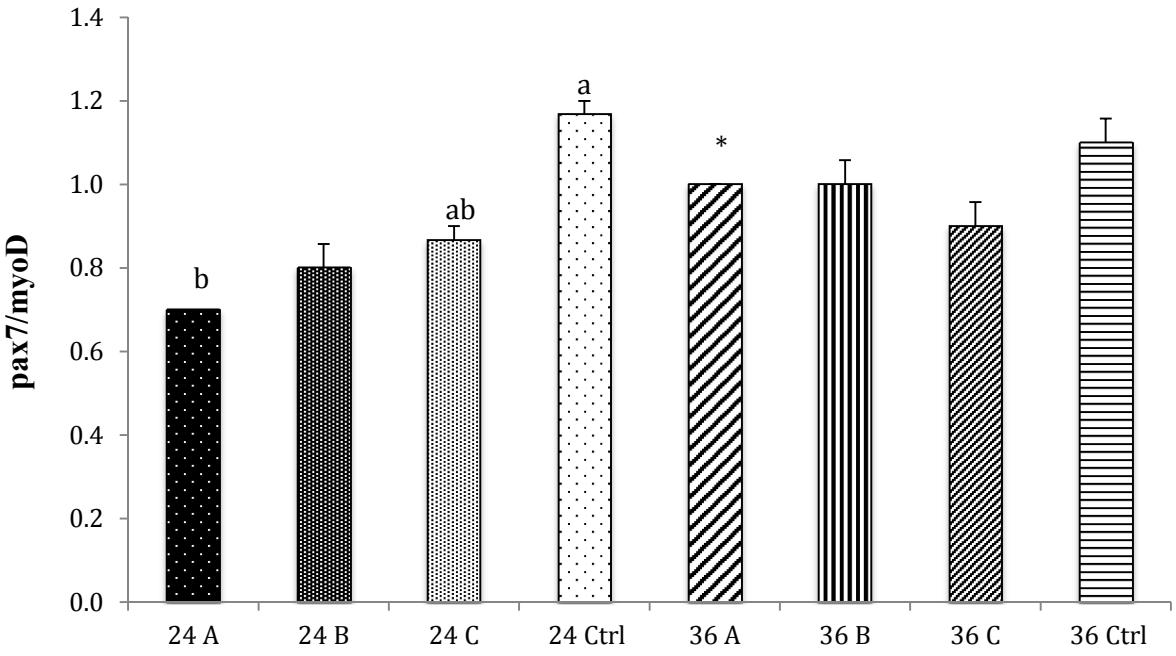

Figure 5.3. Relative mRNA expression quantity of *pax7/myoD* after 24 and 36 h of treatment of different concentrations of an anthocyanidins mixture in induced myogenic cells from white skeletal muscle of rainbow trout. Treatment A: 50 µM of peonidin chloride, 20 µM of cyanidin chloride and 15 µM of pelargonidin chloride, Treatment B: 120 µM of peonidin chloride, 50 µM of cyanidin chloride and 40 µM of pelargonidin chloride and Treatment C: 500 µM of peonidin chloride, 200 µM of cyanidin chloride and 150 µM of pelargonidin chloride. Bars represent the mean ± S.E.M. of the relative mRNA expression. Gene expression data were analyzed following the model ($2^{-\Delta\Delta Ct}$). Each experiment was conducted three independent times ($n = 3$). Differences between anthocyanidins levels are compared within each Time level (one-factor ANOVA, Tukey's HSD test), values without a common letter are different ($p < 0.05$). Differences between Time levels are compared within each anthocyanidins level (T-test), and indicated by * ($p < 0.05$).

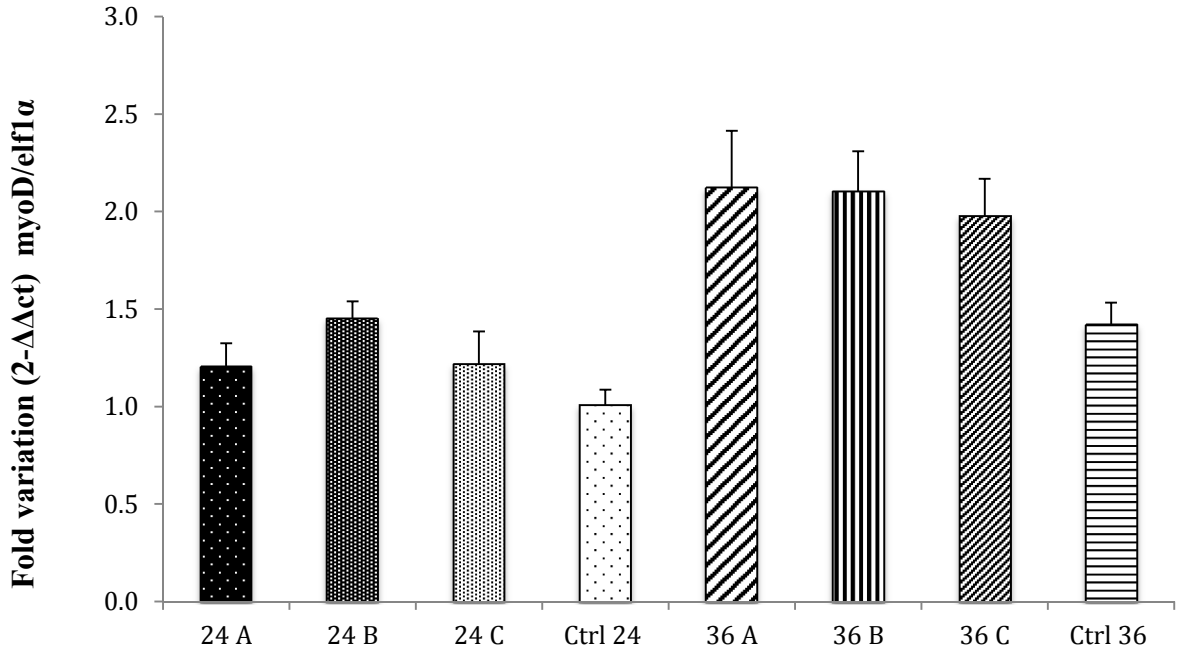

Figure 5.4. Relative mRNA expression quantity of *myoD* after 24 and 36 h of treatment of different concentrations of an anthocyanidins mixture in not induced myogenic cells from white skeletal muscle of rainbow trout. Treatment A: 50 μM of peonidin chloride, 20 μM of cyanidin chloride and 15 μM of pelargonidin chloride, Treatment B: 120 μM of peonidin chloride, 50 μM of cyanidin chloride and 40 μM of pelargonidin chloride and Treatment C: 500 μM of peonidin chloride, 200 μM of cyanidin chloride and 150 μM of pelargonidin chloride. Bars represent the mean ± S.E.M. of the relative mRNA expression. Gene expression data were analyzed following the model ($2^{-\Delta\Delta Ct}$). Each experiment was conducted three independent times (n = 3). Differences between Time levels are compared within each anthocyanidins level (T-test), and indicated by * ($p < 0.05$).

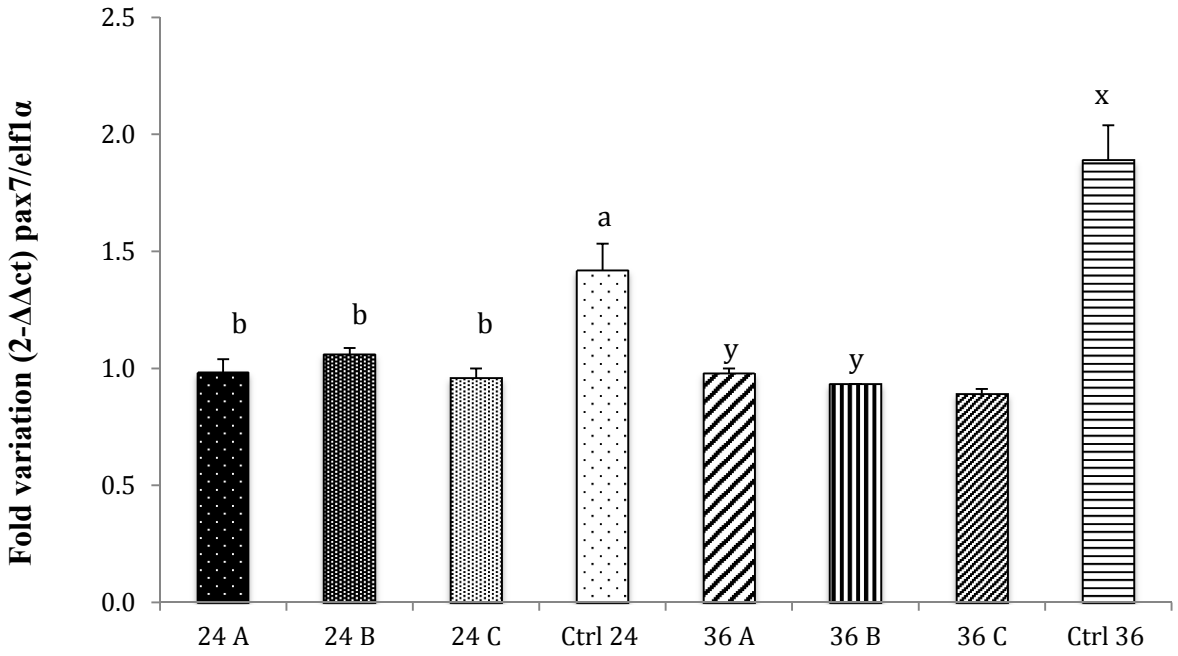

Figure 5.5. Relative mRNA expression quantity of *pax7* after 24 and 36 h of treatment of different concentrations of an anthocyanidins mixture in not induced myogenic cells from white skeletal muscle of rainbow trout. Treatment A: 50 μM of peonidin chloride, 20 μM of cyanidin chloride and 15 μM of pelargonidin chloride, Treatment B: 120 μM of peonidin chloride, 50 μM of cyanidin chloride and 40 μM of pelargonidin chloride and Treatment C: 500 μM of peonidin chloride, 200 μM of cyanidin chloride and 150 μM of pelargonidin chloride. Bars represent the mean ± S.E.M. of the relative mRNA expression. Gene expression data were analyzed following the model ($2^{-\Delta\Delta Ct}$). Each experiment was conducted three independent times ($n = 3$). Differences between anthocyanidins levels are compared within each Time level (one-factor ANOVA, Tukey's HSD test), values without a common letter are different ($p < 0.05$).

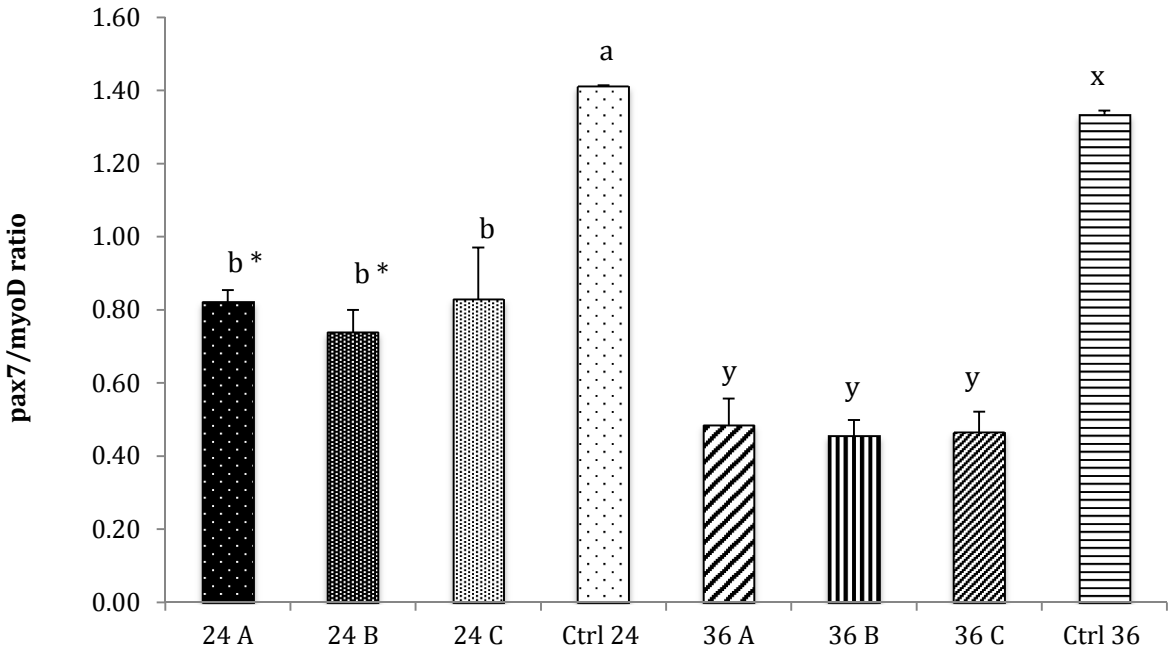

Figure 5.6. Relative mRNA expression quantity of *pax7/myoD* after 24 and 36 h of treatment of different concentrations of an anthocyanidins mixture in not induced myogenic cells from white skeletal muscle of rainbow trout. Treatment A: 50 µM of peonidin chloride, 20 µM of cyanidin chloride and 15 µM of pelargonidin chloride, Treatment B: 120 µM of peonidin chloride, 50 µM of cyanidin chloride and 40 µM of pelargonidin chloride and Treatment C: 500 µM of peonidin chloride, 200 µM of cyanidin chloride and 150 µM of pelargonidin chloride. Bars represent the mean ± S.E.M. of the relative mRNA expression. Gene expression data were analyzed following the model ($2^{-\Delta\Delta Ct}$). Each experiment was conducted three independent times (n = 3). Differences between anthocyanidins levels are compared within each Time level (one-factor ANOVA, Tukey's HSD test), values without a common letter are different ($p < 0.05$).

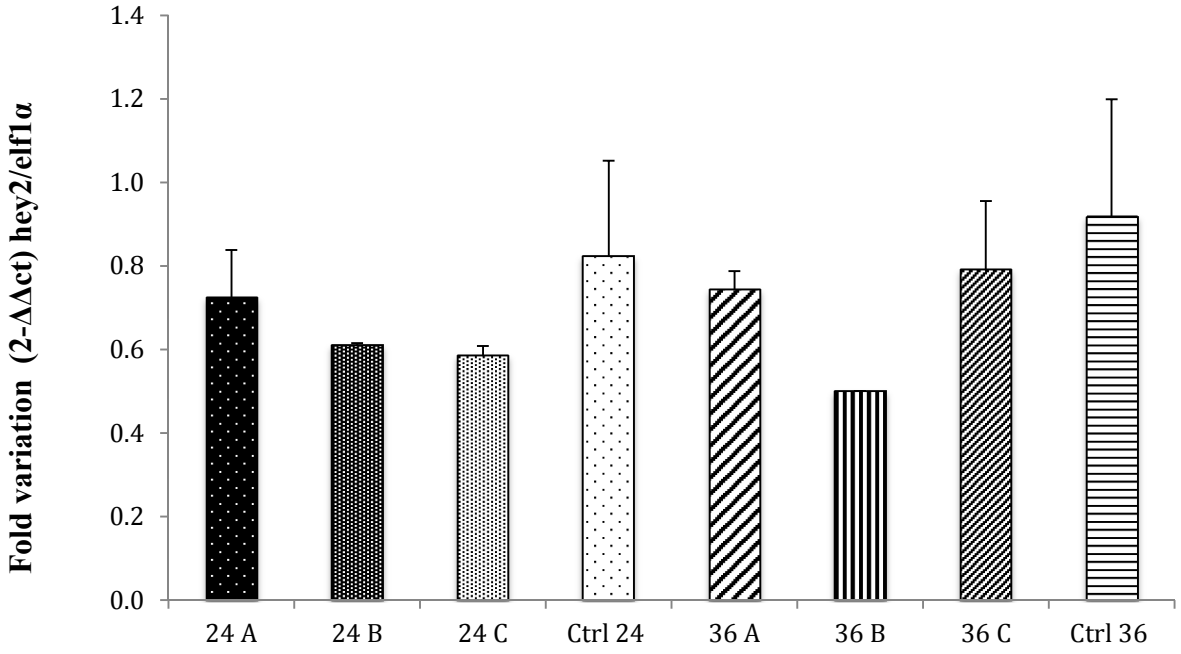

Figure 5.7. Relative mRNA expression quantity of *hey2* after 24 and 36 h of treatment of different concentrations of an anthocyanidins mixture in induced myogenic cells from white skeletal muscle of rainbow trout. Treatment A: 50 µM of peonidin chloride, 20 µM of cyanidin chloride and 15 µM of pelargonidin chloride, Treatment B: 120 µM of peonidin chloride, 50 µM of cyanidin chloride and 40 µM of pelargonidin chloride and Treatment C: 500 µM of peonidin chloride, 200 µM of cyanidin chloride and 150 µM of pelargonidin chloride. Bars represent the mean ± S.E.M. of the relative mRNA expression. Gene expression data were analyzed following the model ($2^{-\Delta\Delta Ct}$). Each experiment was conducted three independent times ($n = 3$).

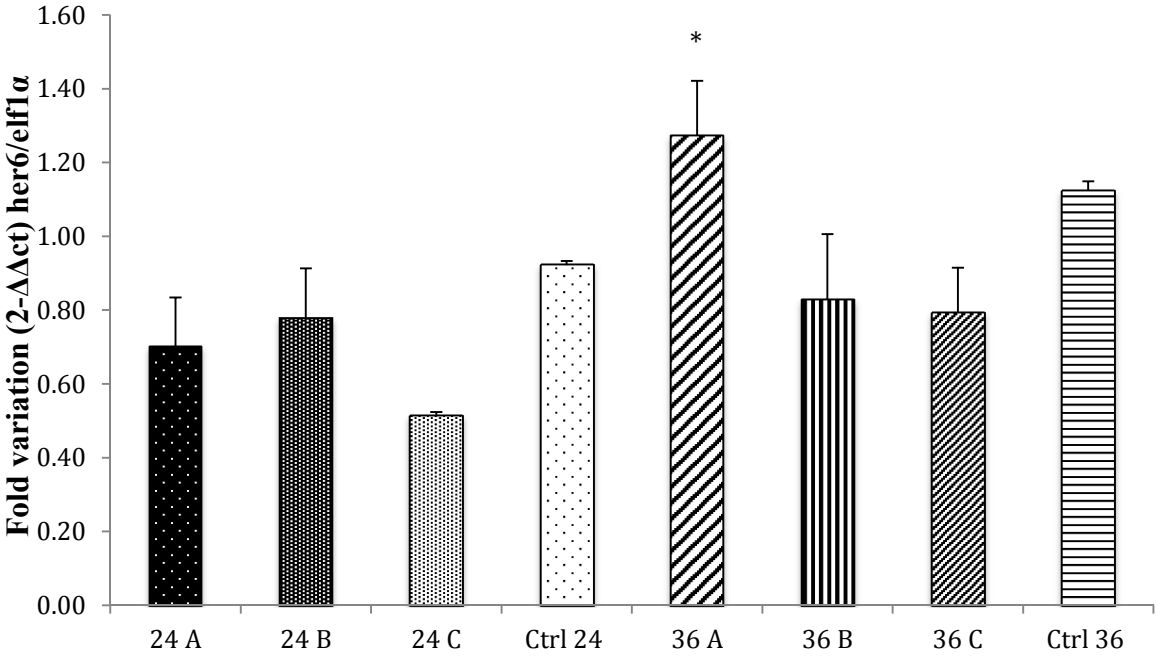

Figure 5.8. Relative mRNA expression quantity of *her6* after 24 and 36 h of treatment of different concentrations of an anthocyanidins mixture in induced myogenic cells from white skeletal muscle of rainbow trout. Treatment A: 50 µM of peonidin chloride, 20 µM of cyanidin chloride and 15 µM of pelargonidin chloride, Treatment B: 120 µM of peonidin chloride, 50 µM of cyanidin chloride and 40 µM of pelargonidin chloride and Treatment C: 500 µM of peonidin chloride, 200 µM of cyanidin chloride and 150 µM of pelargonidin chloride. Bars represent the mean ± S.E.M. of the relative mRNA expression. Gene expression data were analyzed following the model ($2^{-\Delta\Delta Ct}$). Each experiment was conducted three independent times (n = 3). Differences between Time levels are compared within each anthocyanidins level (T-test), and indicated by * ($p < 0.05$).

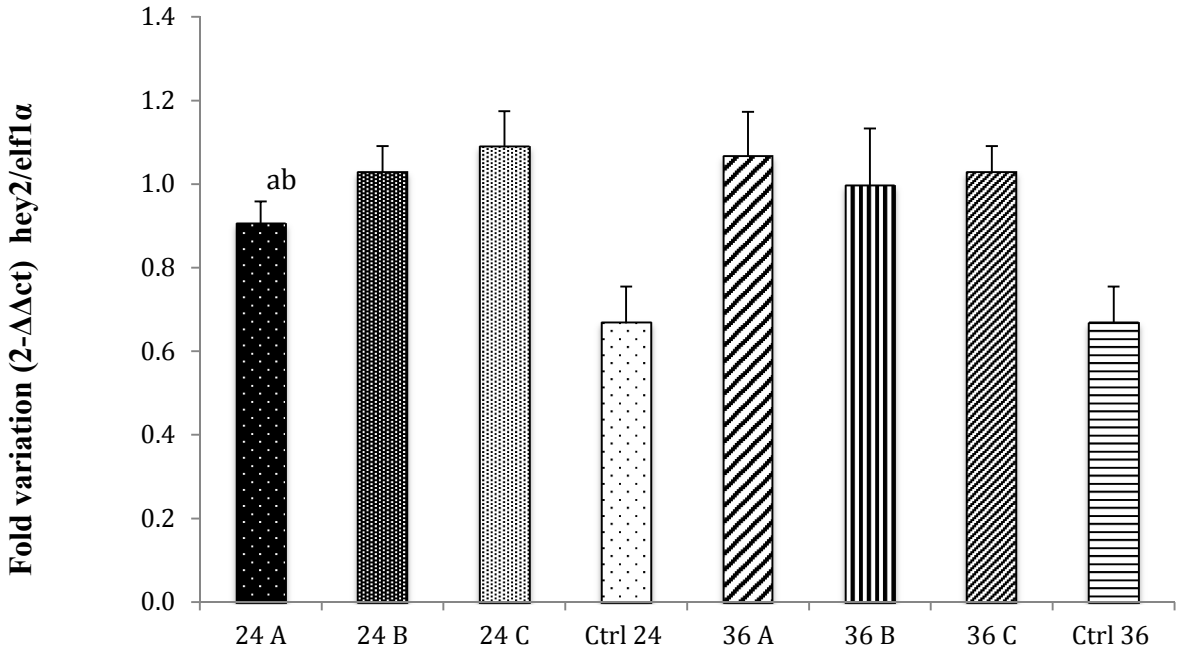

Figure 5.9. Relative mRNA expression quantity of *hey2* after 24 and 36 h of treatment of different concentrations of an anthocyanidins mixture in not induced myogenic cells from white skeletal muscle of rainbow trout. Treatment A: 50 µM of peonidin chloride, 20 µM of cyanidin chloride and 15 µM of pelargonidin chloride, Treatment B: 120 µM of peonidin chloride, 50 µM of cyanidin chloride and 40 µM of pelargonidin chloride and Treatment C: 500 µM of peonidin chloride, 200 µM of cyanidin chloride and 150 µM of pelargonidin chloride. Bars represent the mean ± S.E.M. of the relative mRNA expression. Gene expression data were analyzed following the model ($2^{-\Delta\Delta Ct}$). Each experiment was conducted three independent times (n = 3). Differences between anthocyanidins levels are compared within each Time level (one-factor ANOVA, Tukey's HSD test), values without a common letter are different ($p < 0.05$).

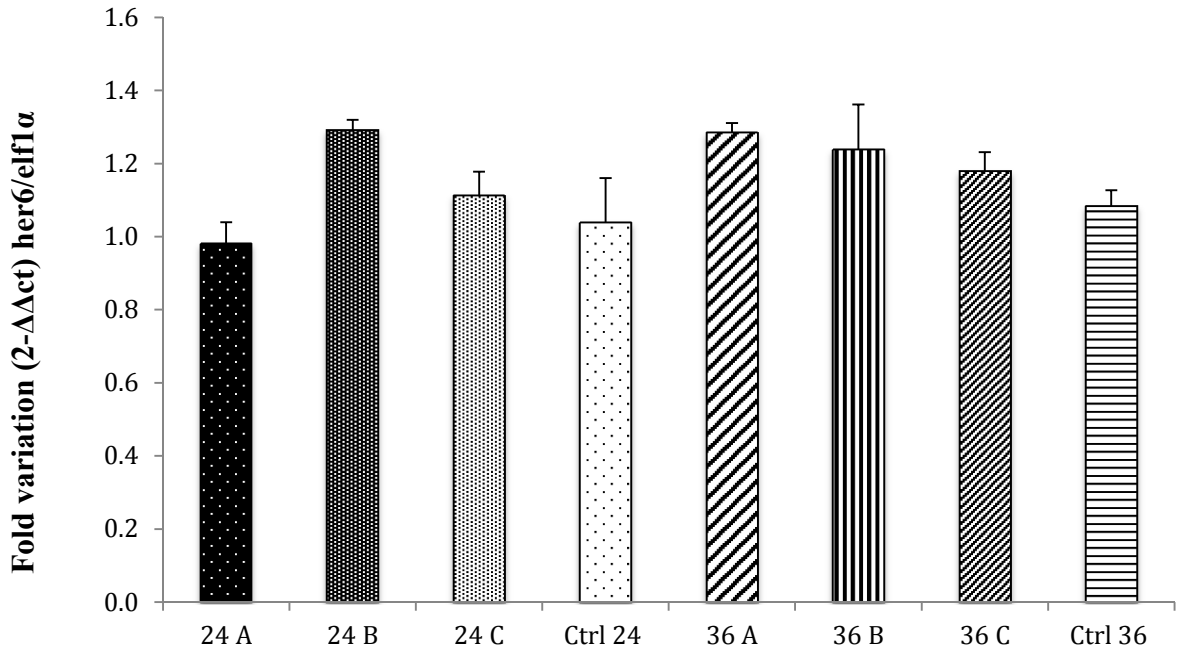

Figure 5.10. Relative mRNA expression quantity of *her6* after 24 and 36 h of treatment of different concentrations of an anthocyanidins mixture in not induced myogenic cells from white skeletal muscle of rainbow trout. Treatment A: 50 µM of peonidin chloride, 20 µM of cyanidin chloride and 15 µM of pelargonidin chloride, Treatment B: 120 µM of peonidin chloride, 50 µM of cyanidin chloride and 40 µM of pelargonidin chloride and Treatment C: 500 µM of peonidin chloride, 200 µM of cyanidin chloride and 150 µM of pelargonidin chloride. Bars represent the mean ± S.E.M. of the relative mRNA expression. Gene expression data were analyzed following the model ($2^{-\Delta\Delta Ct}$). Each experiment was conducted three independent times (n = 3). Differences between Time levels are compared within each anthocyanidins level (T-test), and indicated by * ($p < 0.05$).

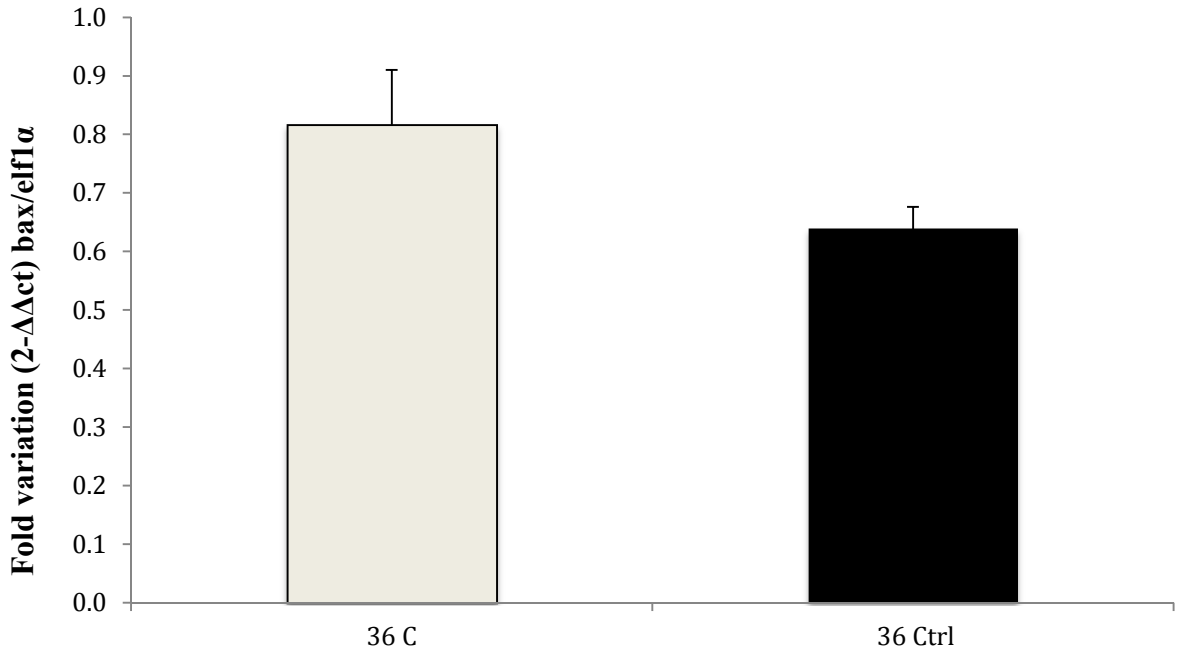

Figure 5.11. Relative mRNA expression quantity of *bax* after 36 h of Treatment C: 500 μM of peonidin chloride, 200 μM of cyanidin chloride and 150 μM of pelargonidin chloride of different concentrations in induced myogenic cells from white skeletal muscle of rainbow trout. Bars represent the mean ± S.E.M. of the relative mRNA expression. Gene expression data were analyzed following the model ($2^{-\Delta\Delta Ct}$).

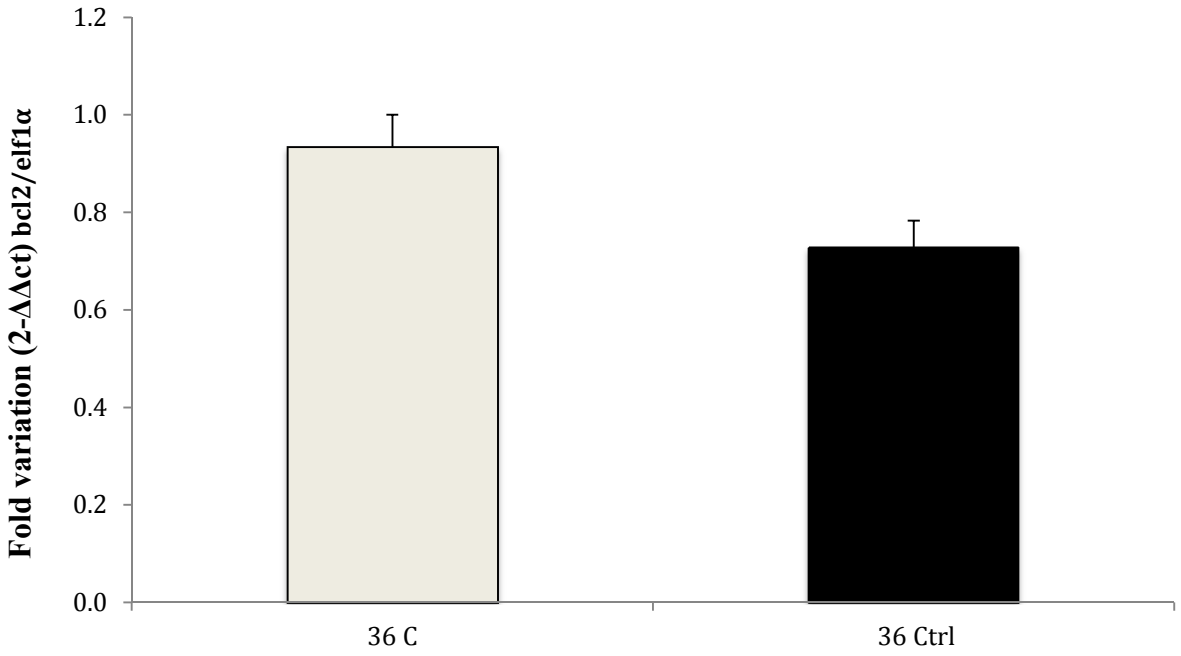

Figure 5.12. Relative mRNA expression quantity of *bcl2* after 36 h of treatment of Treatment C: 500 µM of peonidin chloride, 200 µM of cyanidin chloride and 150 µM of pelargonidin chloride of different concentrations in induced myogenic cells from white skeletal muscle of rainbow trout. Bars represent the mean ± S.E.M. of the relative mRNA expression. Gene expression data were analyzed following the model ($2^{-\Delta\Delta Ct}$).

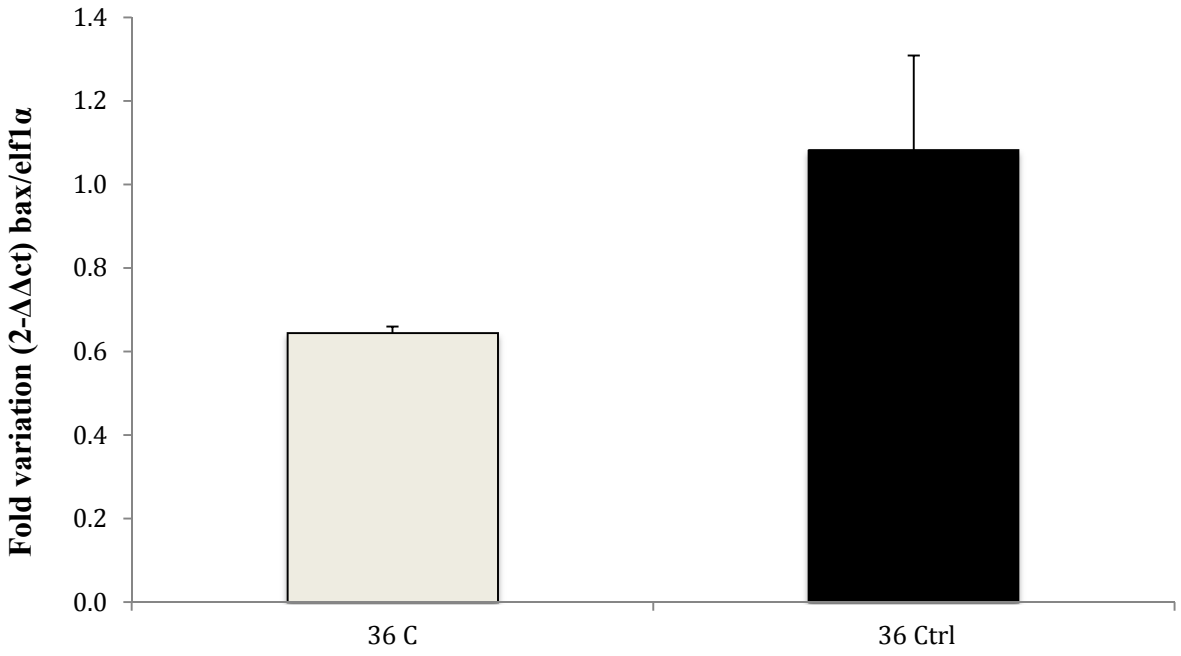

Figure 5.13. Relative mRNA expression quantity of *bax* after 36 h of treatment of Treatment C: 500 μM of peonidin chloride, 200 μM of cyanidin chloride and 150 μM of pelargonidin chloride of different concentrations in not induced myogenic cells from white skeletal muscle of rainbow trout. Bars represent the mean ± S.E.M. of the relative mRNA expression. Gene expression data were analyzed following the model ($2^{-\Delta\Delta Ct}$).

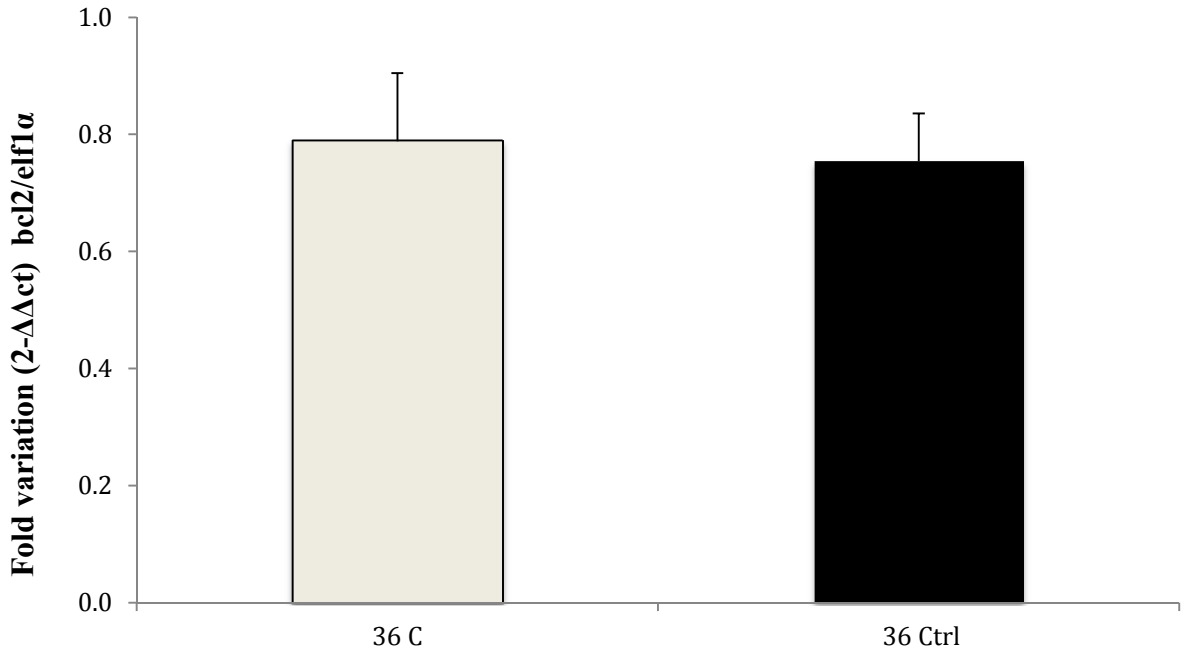

Figure 5.14. Relative mRNA expression quantity of *bcl2* after 36 h of treatment of Treatment C: 500 µM of peonidin chloride, 200 µM of cyanidin chloride and 150 µM of pelargonidin chloride of different concentrations in not induced myogenic cells from white skeletal muscle of rainbow trout. Bars represent the mean ± S.E.M. of the relative mRNA expression. Gene expression data were analyzed following the model ($2^{-\Delta\Delta Ct}$).

CHAPTER 6

Research Summary

Worldwide capture fisheries production reached its maximum in 1996 and has slowly declined since (FAO, 2010; Rana et al., 2009). In contrast aquaculture production has increased at an annual average rate of 6% and has become the fastest-growing animal food-producing sector worldwide (FAO, 2012; Reverter et al., 2014; Naylor et al., 2009). Hence, aquaculture is the only industry to increase fish supply to a growing human population estimated to reach 9.2 billion in 2050. The aquaculture industry grows to meet the increasing demand for aquaculture-derived products. Fast development and intensification of farming systems to cope with the increased food demand of a growing human population will be achieved through implementing selection breeding programs to improve growth rate, feed utilization as well as resistance to diseases and stress in finfish species fed diets formulated to contain relatively low levels of marine-derived ingredients. However, improving performance of fish through selective breeding requires at least three or four generations. Therefore, short-term approaches such as research in fish nutrition and fish immunology are necessary to contribute with fish farming productivity in the near future. In this regard, research is needed to evaluate cost-effective alternative ingredients, such as terrestrial plant-based proteins and lipids, single-cell protein and oil, rendered terrestrial animal products, seafood by-products and krill (Naylor et al., 2009). In addition, the development of strategies to prevent and control diseases as well as to permit fish to thrive under stressful or marginal environmental conditions associated with intensive fish farming is warranted.

Recently, attention has being focused on the use of plant-derived products for disease control and growth promotion in finfish aquaculture (Gabor et al., 2012; Reverter et al., 2014). The use of low cost, plant-derived extracts including residual products rich in phytocompounds such as alkaloids, polyphenols (i.e. anthocyanins and anthocyanidins), terpenoids, steroids and essential oils may prove useful to promote growth and health in fish under intensive culture conditions as well as to improve final product quality (Chakraborty et al., 2014; Reverter et al., 2014). However, further improvements in quality control points as

well as technical aspects during the entire production process to obtain homogenized commercial plant-derived extracts feasible for the aquaculture industry are required.

Polyphenols are a vary class of phytocompounds abundant in vegetables and fruits as well as in agro-industry by-products, which dietary intake has long been associated with a variety of beneficial effects on animal and human health (Chuang and McIntosh, 2011; Han et al., 2007; Manach et al., 2003; Teixeira et al., 2014). Among polyphenols, anthocyanins, a class of flavonoids, have long been reported as health promoters by acting as antioxidant, cardio-protectors, anti-inflammatory, and anti-carcinogenic in mammals (Cooke et al., 2006; Lietti et al. 1976; Galvano et al. 2004; Garcia-Alonso et al. 2004; Kamei et al. 1995; Kendall et al. 1998; Toufektsian et al. 2011; Vennat et al. 1994; Wang and Stoner 2008; Whitehead et al. 1995). In addition, previous studies have reported that polyphenols including resveratrol promote myogenic differentiation in C2C12 myoblasts by inducing the up-regulation of myogenic regulatory factors, i.e. *myf5, myoD, myogenin* and *mef2* (Gutierrez-Salmean et al., 2014; Kaminski et al., 2012; Lançon et al., 2012; Montesano et al., 2013). However, the potential beneficial effect on growth, health and final product quality including total omega-3 fatty acids portion in the fillet of feeding plant-derived extracts rich in polyphenols (i.e. anthocyanins and anthocyanidins) to finfish species under intensive culture conditions has received less attention.

6.1 First study: *In vivo* experiment

An *in vivo* experiment was conducted to evaluate whether dietary intake of purple corn extract (PCE), an ingredient rich in anthocyanins, exerts beneficial effects on health, product quality and growth performance in rainbow trout. Dietary PCE intake increased the antioxidant capacity and the expression of the antioxidant enzyme *gpx1* in plasma and erythrocyte, respectively. In addition, dietary PCE intake reduced fat body content in 1.6% in fish, thus reducing the amount of substrate prone to *in vivo* lipid peroxidation in fish. A strong trend ($P = 0.075$) toward significantly lower concentration of TBARS, a biomarkers of lipid peroxidation in plasma of fish fed the PCE supplemented diet compared to the control group was measured. These findings demonstrated that dietary intake of PCE may protect fish against lipid peroxidation. Flavonoids are likely to act as antioxidants when found between the aqueous phase and the phospholipid bilayer of cells due to their hydrophilicity nature, by

trapping chain-initiating radicals at the interface of the membranes. Hence preventing the progression of the radical chain reaction associated with lipid peroxidation (Ross and Kasum, 2002). In agreement with our findings, the intake of red wine anthocyanins was shown to increase antioxidant capacity in human plasma (Garcia-Alonso et al., 2009). In addition, previous studies have demonstrated the protective role of different types of flavonoids from oxidative stress in red blood cells in human and animal species including fish. Youdim et al. (2000) demonstrated *in vitro* incubation with anthocyanins or hydroxycinnamic acid (0.5 and 0.05 mg/ml) significantly enhanced human red blood cells resistance to hydrogen peroxide (100 μM) induced ROS production. Tedesco et al. (2001) reported that fractions of aged red wine containing anthocyanins reduced ROS and methemoglobin production in human erythrocytes treated with H2O2. The authors also described a protective effect of anthocyanins in red blood cells deprived of catalase activity. In rainbow trout, Fedeli et al., (2004) reported tannins, including tanic, gallic, and ellagic acid, at low concentrations (10 and 30 μM) protect red blood cells from DNA breakage caused by hydrogen peroxide induced oxidative stress. However, tannins, at high concentrations (100 μM) might exert a genotoxic effect in fish red blood cells.

Although we observed no significant differences in the proportion of omega-3 fatty acids in muscle of fish fed the test diets compared to the control, a higher proportion of total omega-3 fatty acids in both plasma and whole-body in fish fed the test diet was detected. In order to observe potential increase in the proportion of omega-3 fatty acids in muscle from fish fed the PCE supplemented diet a longer trial period might be required. Although, it has been demonstrated flavonoids induce increase in omega-3 fatty acids in human plasma, the mechanism involved remains controversial. It has been proposed flavonoids including anthocyanins might promote the biosynthesis of both EPA and DHA from their precursor α -linolenic acid (di Giuseppe et al., 2009; Toufektsian et al., 2011). The other mechanism proposes polyphenols might protect PUFA and HUFA from oxidation by acting as antioxidants (Cazzola and Cestaro, 2011; Gladine et al., 2007). Although, we measured no expression and activity of delta 6 desaturase, our data is in agreement with the second mechanism since we detected an increase in both total omega-3 and DHA levels in plasma along with higher plasma total antioxidant activity, with no differences in the proportions of hepatic α -linolenic acid in fish fed the PCE supplemented diet.

The other relevant finding from this study is the reduced total fat content detected in the body of fish fed the PCE supplemented diet. In mammalian models including mice, potential mechanisms associated with the modulatory effect on hepatic lipogenesis of anthocyanins, and the implications on body adiposity have been described. Guo et al. (2011), for example, showed that Cyanidin-3-glucoside (C3G) suppressed hepatic *de novo* lipid synthesis by preventing translocation of the enzyme controlling the first step of triacylglycerol synthesis, mitochondrial acyl-CoAglycerol-sn-3-phosphate acyltransferase 1, from the endoplasmic reticulum toward the outer mitochondrial membrane. Further, Guo et al. (2012) detected C3G enhanced phosphorylation of *Akt* and *FoxO1*, after which *FoxO1* was translocated from nucleus to cytoplasm, thus reducing its transcriptional regulatory function. This effect was supported by down-regulation of glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate kinase (PEPCK), causing a decrease in glycaemia and thus reducing hepatic glucose and lipid accumulation in both *db/db* mice and high fat diet mice. Similarly, Tsuda et al. (2003) observed lower body adiposity and lower hepatic triacylglycerol content in mice fed a high fat diet supplemented with C3G-rich purple corn color (PCC). The authors observed intake of PCC down-regulated the transcription of enzymes involved in fatty acid and triacylglycerol synthesis as well as the sterol regulatory element binding protein-1 (SREBP-1) in white adipose tissue in mice. In a different study, dietary intake of purple carrot juice, rich in C3G, was associated to a reduction in abdominal fat pads in rats fed a high carbohydrate/high fat diet (Poudyal et al., 2010). In addition, the authors observed attenuation in the alterations of the cardiovascular system and liver structure and function related to metabolic syndrome in rats fed a high carbohydrate-high fat diet supplemented with purple carrot juice (Poudyal et al., 2010). Therefore, whether the use of plant-derived extracts rich in biocompounds including polyphenols exert similar effects by reducing fat deposition in fish requires further research. The reduced amount of fat in fish body, mainly visceral fat contributes 1) to decrease the amount of substrate prone to lipid peroxidation and 2) to optimize body composition to yield the maximal dressing percentage. Carnivorous fish fed high-digestible carbohydrates or all plant meal/plant oil based diets exhibit deregulation in lipid homeostasis evidenced by increased visceral fat, development of fatty liver and hepatic oxidative stress (Aksnes, 1995; Benedito-Palos et al., 2008; Hemre et al., 2002; Moon, 2001; Olsvik et al., 2011; Torstensen et al., 2011). Therefore, studying the potential role of phytocompounds in

the modulation of glucose and lipid homeostasis as well as reducing adiposity and hepatic oxidative stress in carnivorous fish fed plant-derived based diets, will contribute for better understanding plant-derived secondary metabolites impact on metabolism of fish grown in intensive culture conditions.

6.1.2 Limitations of this Study

This study provides potentially useful information and evidence that a dietary anthocyanin supplementation in the form of PCE evokes certain antioxidant responses and reduces whole body adiposity in fish like in mammals. Further investigation is warranted to understand whether the fat lowering mechanism of anthocyanins observed in human hepatoma cell lines or the mice liver model applies to fish mesenteric adipose tissue as well, even when fish are fed diets very high in energy (lipid) such as those being used by the salmon and trout farming industry. However, the results obtained from this study are preliminary. This is especially true since only one supplementation level of PCE against a control diet (un-supplemented) was tested. Hence a potential dose-response effect was not established in this study. Although we analyzed total antioxidant activity as well as the concentration of markers of oxidative damage in lipid, protein and DNA in plasma, no parameters such as activity of enzymes /SOD, CAT and GPX) or markers of oxidative status in liver in fish were evaluated. Moreover, fish were not exposed to conditions promoting oxidative stress. Such conditions would be necessary to detect any protective effect of PCE against oxidative damage and should be an element of future experiments designed to test antioxidant activity of PCE in fish.

6.2 Second Study: *In vitro* Experiment (1)

Measuring the effects of plant secondary metabolites on skeletal muscle growth signaling will improve our understanding of the effects of feeding carnivorous fish diets supplemented with plant-derived extracts on fish somatic growth. Thus, we conducted an *in vitro* study to evaluate the effects of 24 hour exposure to three different doses of a mixture of purified anthocyanidins, the non-glycoside form of anthocyanins, in primary myogenic cells isolated from skeletal muscle of rainbow trout. The treatments were: (A) 50 μM of peonidin chloride, 20 μM of cyanidin chloride and 15 μM of pelargonidin chloride; (B) 120 μM of peonidin

chloride, 50 μM of cyanidin chloride and 40 μM of pelargonidin chloride; and (C) 500 μM of peonidin chloride, 200 μM of cyanidin chloride and 150 μM of pelargonidin chloride.

The findings of this study demonstrated that the anthocyanidin mixture enhances the expression of the antioxidant enzyme glutathione peroxide 1 (*gpx1*) in primary myogenic cells, thereby contributing to the antioxidant defense system in skeletal muscle. Although we observed no significant differences in the expression of a nuclear factor (erythroid-derived 2)-like 2 (*nrf2*), a trend ($P = 0.09$) toward higher expression levels in both groups compared to the control was detected, suggesting a potential role of this transcriptional factor in promoting the up-regulation of *gpx1*. Further, the anthocyanidin-induced *gpx1* up-regulation might have been consequence of increasing Nrf2 stability via promoting the dissociation of the Kelch-like ECH-associated protein 1 (Keap1)-Nrf2 complex as reported (Eades et al., 2011; Jaiswal, 2004; Lee and Johnson, 2004; Motohashi and Yamamoto, 2004; Nguyen et al., 2003; Zhang and Hannink, 2003). In addition, this study demonstrates that anthocyanidins modulate myogenic programming in fish primary myogenic cells. The intermediate anthocyanidin concentration appeared to delay the progress of myogenic differentiation by up-regulating *pax7* expression, causing a higher ratio of *pax7/myoD* in fish myogenic cells. A higher *pax7/myoD* ratio has been suggested to be a critical factor promoting myogenic cells self-renewal (Chapalamadugu et al., 2009; Olguin et al., 2007; Olguin and Pisconti, 2012). MyoD is a myogenic regulatory factor promoting the progress of myogenesis as well as the expression of *myogenin*. Furthermore, *myoD* expression has been reported to increase during activation and proliferation of myoblasts, and to decrease while progressing to terminal differentiation. Pax7 up-regulation has been shown to inhibit myogenesis by inhibiting MyoD expression and activity in C2C12 cells (Olguin and Olwin, 2004; Olguin et al., 2007). In addition, myogenin regulates the differentiation process via triggering the expression of myotube-specific genes, thereby increasing its protein level while progressing toward myogenic terminal differentiation. Therefore, the trend toward down-regulation in the expression of *myoD* and *myogenin* observed in myogenic cells exposed to the intermediate dose of anthocyanidins compared to the control further support the above-mentioned idea of the “brake-like pedal effect” on myogenic differentiation caused by the intermediate dose of anthocyanidin in fish myogenic cells.

Notch signaling activation has been shown to inhibit myoblast differentiation (Buas and Kadesch, 2010) and recent evidence showed that constitutive Notch activation induces *pax7* up-regulation, thus promoting self-renewal of skeletal muscle satellite cells (Wen et al., 2012). Therefore, the expression of two target genes of the Notch signaling pathway, *hey2* and *her6*, was measured to determine whether the observed up-regulation of *pax7* was potentially associated with Notch signaling activation. We detected an up-regulation of both Notch target genes in myogenic cells exposed to the low and intermediate dose of the anthocyanidin mixture compared to the control. In agreement with our findings, Sun et al. (2008) demonstrated that Notch signaling activation increased Pax7 protein levels and decreased MyoD and myogenin protein levels in adult myoblast and satellite cell-derived cell lines in culture. Additionally, it has also been demonstrated that activation of Notch signaling favors survival in differentiated cells by interacting with mitochondrial remodeling proteins (Perumalsamy et al., 2009). Although, a higher *pax7/myoD* ratio has previously been suggested to be indicative of the maintenance of an undifferentiated progenitor state in myosatellite cells (Olguin et al., 2007; Chapalamadugu et al., 2009), we suggest a greater *pax7/myoD* ratio may be indicative of promoting survival in *myogenin* expressing myogenic cells committed to terminal differentiation to myotubes. Therefore, our data suggest the intermediate dose of anthocyanidins modulates myogenesis progress by inducing a gene expression pattern in accordance with a delay toward terminal differentiation, most likely in favor of cell survival in fish myocytes. This is especially true since myogenic cells were committed to terminal differentiation by expressing the myogenic regulatory factor *myogenin*, thus being unable to go backwards toward a quiescent satellite cells phenotype. We suggest the capacity to delay the progress to terminal differentiation most likely favoring cell survival provides greater plasticity for muscle growth in response to environmental factors in fish. Allowing myogenic cells to modulate the progress toward terminal differentiation in fish under particular external conditions (i.e. adverse conditions) by redirecting energy to metabolic functions may be necessary for cell survival.

6.2.1 Limitations of this Study

Overall, this study provides new insights that contribute to understand the potential impact of using plant-derived extracts rich in biocompounds including flavonoids on fish somatic

growth cellular mechanisms. It is important to consider differences in the effects caused by either the sugar-free (aglycon) or the sugar-conjugated forms of flavonoids are expected to occur in cells (Kähkönen and Heinonen, 2003). In this study we tested only the effects of a mixture of anthocyanidins, making it impossible to elucidate whether single anthocyanidin forms exert the same effect as well as whether is there are synergistic or antagonistic effects between them. Although myogenic cells were most likely induced to differentiate by the high confluence resulting from the high seed density used in this study (Angelis et al., 1998; Kaspar et al., 2005; Lindon et al., 2001; Tanaka et al., 2011) a differentiation media to ensure a homogenous induction to terminal differentiation would have provided less variance in the data. Therefore, we designed a third experiment to determine whether differences in the myogenic cellular stage (i.e. induced and non-induced myogenic cells) constitute a factor that accounts for differences in the respond to anthocyanidins treatments.

6.3 Third Study: *In vitro* Experiment (2)

A second *in vitro* study was conducted to determine whether an anthocyanidin mixture promotes myogenic differentiation in two different myogenic cellular stages, induced and non-induced primary myogenic cells. We evaluated the same three different concentrations (1X, 2.5X and 10X) tested in the previous *in vitro* study at two sampling times (24 and 36 hrs.). We measured expression levels of *myoD, pax7 hey2* and *her6*.

In induced myogenic cells, the lowest and intermediate anthocyanidin doses caused a gene expression pattern in accordance with a pro-differentiation effect by up-regulating *myoD* expression with no effect on *pax7* expression. This concept is supported by the significant decrease in *pax7/myoD* ratio observed in induced myogenic cells exposed to the low and intermediate anthocyanidin doses after 24 hours compare to the control. It has been suggested that a low *pax7/myoD* ratio indicates myogenic cell progressing to terminal differentiation (Chapalamadugu et al., 2009; Olguín et al., 2007; Olguín and Pisconti, 2012). To the contrary, we detected an up-regulation of *pax7*, a trend to higher *myoD* expression level and an intermediate *pax7/myoD* ratio which indicates a stimulatory effect on proliferation and survival in committed myoblasts cells. Thus, it appears that a long exposure to anthocyanidins caused a change in myogenic cell fate decision by disrupting differentiation progress in favor

of proliferation and survival of committed myoblasts. We detected no effect of anthocyanidins in the two Notch target genes, *her6* and *hey2*, after 24 and 36 hours of treatment.

In non-induced cells, anthocyanidins evoke a stimulatory effect on myogenic differentiation by down-regulating *pax7* expression at either doses tested after 24 and 36 hours of treatment. This concept was further supported by the significantly reduced *pax7/myoD* ratio detected in these groups. Previous work has reported that Notch signaling promotes *pax7* expression by reducing the expression and activity of MyoD, thus causing inhibition of myogenic differentiation (Kopan et al., 1994; Shawber et al., 1996; Kuroda et al., 1999; Wen et al., 2012; Wilson-Rawls et al., 1999). Contrary to what we expected, a significant up-regulation of *hey2* expression in non-induced myoblasts exhibiting a down-regulation in *pax7* expression exposed to the highest anthocyanidin dose was detected. We observed similar trend to higher expression in non-induced myoblast exposed to the lowest and intermediate dose of anthocyanidins after 24 hours of treatment compare to the control ($P = 0.19$ and $P = 0.05$, respectively). However, we observed no effect of either anthocyanidin treatment in the expression of *myoD* in non-induced cells after 24 hours of treatment. Similarly, despite no significant differences in *hey2* expression, a strong trend toward greater levels in all anthocyanidin treatments after 36 hours compared to the control was detected ($P = 0.05$). Collectively, the effect of anthocyanidins on myogenic programming appears to differ based upon the exposure time in induced cells. Anthocyanidins appears to boost differentiation signaling after 24 hours treatment by up-regulating *myoD* expression and causing a significant decrease in the *pax7/myoD* ratio which indicates progression toward terminal differentiation. However, prolonged exposure to anthocyanidins induces both up-regulation of *pax7* and a trend toward higher *myoD* levels causing an intermediate *pax7/myoD* ratio, which indicates a shift in myogenic cell fate choice promoting proliferation and cell survival in committed myoblasts.

6.3.1 Limitations of this Study

This study provides new evidence of the effects of anthocyanidins on myogenic programming in primary myogenic cells. We evaluated 1) whether the effects of anthocyanidins in myogenic programming differ based upon the myogenic stage of the cells (induced *versus* non-induced) and 2) whether the effects of anthocyanidins in myogenic

programming differ based upon time of exposure to treatments. Although, the genes analyzed in this study play crucial roles in myogenesis, analysis of other genes involved in this process including *myf5, mfr4*, *myogenin* and *myostatin* would have provide more data to support more robust conclusions. However, the low yield of total mRNA obtained from the low cell seed density used to keep cells from differentiation limited analysis to only a few genes of interest in the rqPCR analysis. Although the transcriptional analysis of genes provides knowledge with regard the response of an organism or cells to changes in either internal or external conditions, the regulatory mechanism involved in such responses are more complex, implying the interaction of several regulatory levels from the expression of the information contained in the genetic code up to the conformation of the phenotype of the organism/cell (Zempleni and Daniel, 2003).

6.4 Future Research

Myogenesis is a well-described physiological process that has received great attention in both mammalian and teleost species in the past. Extensive research has been conducted on the regulatory mechanisms involved in embryonic, postnatal and adult myogenesis as well as how mechanisms are modulated by external cues in fish. In this regard, environment and nutrition play crucial roles in fish myogenesis by determining the rate of myogenesis, the sub-cellular organelles, patterns of gene expression, the number and size of muscle fibers and influence protein turn-over and the efficiency of protein degradation (Valenete et al., 2013). However, there still are significant gaps concerning optimal nutritional and culture conditions affecting the survival rates and growth potential of larvae of finfish species (Valenete et al., 2013). Conducting further research to determine the effect of phytocompounds as nutraceutics by promoting growth, modulating the immune system and enhancing fish antioxidant defense against oxidative stress will provide empirical evidence to either support or refute the value of using of plant-derived extracts rich in these compounds in finfish aquafeeds. Some of the plant-derived active principles reported to exert beneficial effects in fish species include alkaloids, terpenoids, tannins, saponins, glycosides, flavonoids, phenolics, steroids and essential oils (Reverter et al., 2014). Moreover, differences in both the effect and the potency between different types of phytocompounds as well as between the glycoside and the non-

glycoside form of the active principles are expected to occur, thus accounting for differences in the effects in cells.

Previous research reported several aspects of the pharmacokinetics of anthocyanins and anthocyanidins in mammalian species including human (He and Giusti, 2010). Anthocyanins exhibit low postprandial plasma concentrations, suggesting an apparent low bioavailability of these compounds in mammalian species (Fang, 2014; Marczylo et al., 2009; Czank et al., 2013). However, emerging evidence suggests that the observed low apparent bioavailabilities of anthocyanins could be the result of an extensive pre-systemic metabolism occurring at the gastrointestinal mucus layer as well as at the level of the gut microbiota community, rather than low absorption from the gastrointestinal lumen (Fang, 2014). Close to 90% of polyphenols persist into the colon where they are metabolized via esterase, glucosidase, demethylation, dehydroxylation and decarboxylation activities of bacteria. The results of these transformations are metabolites including phenolic acids and short-chain fatty acids, which are absorbed across the intestinal mucosa (Etxeberria et al., 2013). Previous studies have reported the absorption of anthocyanins takes place in the stomach, small intestinal and colon in mammalian species (Fang, 2014; He and Giusti, 2010). Although the mechanism of anthocyanins absorption in the gastrointestinal tract remains controversial, it has been suggested that anthocyanins absorption requires the organic anion membrane carrier bilitranslocase in the stomach (Passamonti et al. 2002). Further, anthocyanin tissue distribution has recently been evaluated in rat and pig models. Among the main tissues exhibiting anthocyanin concentrations are the stomach, jejunum, kidney, liver, eyes and brain (He and Giusti, 2010). Further, gut absorption mechanisms, microbiota and intestinal tissue metabolism and tissue distribution of plant-derived secondary metabolites including anthocyanins has not yet been examined in fish. Therefore, further research is warranted in these areas to elucidate how these compounds interact with the physiology of fishes and whether there are differences among teleost species from different trophic levels (i.e.. herbivorous, omnivorous and carnivorous).

Skeletal muscle homeostasis integrates signaling regulating myogenic cellular fate (commitment, proliferation/activation and differentiation) and cellular mechanisms involved in protein turnover rate (**protein synthesis-protein degradation balance**). Teleost fish exhibit a higher rate of protein deposition in skeletal muscle than mammals (Mommsen, 2001;

Kaushik and Seliez, 2010). Therefore, protein degradation systems play a more prominent role in somatic growth. There are three major mechanism for protein degradation: **1)** the lysosomal system characterized by acidic proteases namely chatepsins and other hydrolases (Mitch et al., 1999); **2)** the ubiquitin-proteasome system, which occurs in two consecutives steps; first, the covalently attachment of multiple ubiquitin molecules in the target protein and second, the proteasome degradation of these ubiquitin tagged proteins yielding peptides of 7-9 amino acid residues (Goldberg 1995; Voges et al., 2000); and **3)** calpeins, a calcium-dependent cysteine proteases family. Although the ATP-dependent ubiquitin-proteosome pathway is considerate to be the main protein degradation system of skeletal muscle in mammals, conflicting data regarding its actual importance in skeletal muscle homeostasis in fish exist (Kaushik and Seliez, 2010). The calpain calcium-dependent system seems to be involved in muscle protein degradation related to stress such as imbalances in dietary factors (Lie et al., 2011). Therefore, in fish, the bulk of the protein degradation appears to depend on the activity of the autophagy/lysosomal system (Lie et al., 2011). The autophagy/lysosomal system mostly degrades aging proteins as well as redundant or damaged cellular structures by sequestering portions of the cytoplasm and cell organelles through vacuole formation, namely autophagosomes. The autophagosomes fuse with lysosomes for further digestion of the vacuoles content by the activity of lysosomal hydrolases (Lum et al., 2005). However, in order to avoid self-digestion of the cell, the autophagic/lysosomal system needs to be under a negative regulation, particularly the target of rapamycin complex (mTOR) (Jung et al., 2010). Therefore, external factors modulating the mTOR signaling cascade may exert a direct effect over autophagy/lysosomal system, thereby affecting protein turnover rate in fish.

Emerging evidence demonstrates polyphenols including anthocyanins can induce autophagy as a mechanism promoting cell survival against apoptosis (Kim et al., 2012; Longo et al., 2008). In agreement with these findings, Lee and colleagues (2010) reported that anthocyanins exert their suppressive tumor growth effect by activating AMPKα1, an inhibitor of the mTOR pathway cascade. Surprisingly this regulatory effect of polyphenols has not yet been investigated in fish, thereby making necessary to determine whether stimulation of the autophagic/lysosomal system by polyphenols occurs in skeletal muscle of finfish species as described in mammals. Therefore, conducting research to 1) elucidate the modulatory effects of polyphenols in determining myogenic cellular fate, and 2) determine the potential

modulatory effect on protein turnover rate of polyphenols in skeletal muscle, will improve our understating regarding the *in vivo* effect on fish biometric parameters of feeding diets supplemented with plant extracts rich in polyphenols, mainly in carnivorous finfish species.

Research conducted to evaluate the health and growth promoting effects of feeding farm animals ingredients rich in polyphenols has been scarce (Barrenetxe et al., 2006; Chamorro et al., 2013). Recently, Fiesel et al. (2014) reported feeding dietary polyphenol-rich plant products from grapes or hops improves gain: feed ratio and lowers expression of various pro-inflammatory genes in the duodenum, ileum and colon in weaned piglets. Emerging evidence suggests the potential use of functional food rich in polyphenols as natural and efficient therapeutants to mitigate the inflammatory process and redox imbalance associated with the pathogenesis of intestinal inflammatory disorders including intestinal bowel disease and colitis in humans and mammalian models (Shapiro et al., 2007; Romier et al., 2008; Romier et al., 2009; Sergent et al., 2010; Biasi et al., 2011; Piberger et al., 2011; Sánchez-Fidalgo et al., 2013). These intestinal inflammatory disorders exhibit similar intestinal mucosa alterations to those detected in fish with distal enteritis induced by diet, particularly carnivorous fish fed diets rich in legume meals such as of soybean meal (SBM) (Dale et al., 2009). Intestinal mucosa alterations include shorter primary and secondary mucosal folds, increase in the number of goblet cells and the infiltration of macrophages, neutrophils, lymphocytes, eosinophils, immunoglobulin M (IgM) and T cells into the lamina propria (Krogdahl et al., 2003; Heikkinen et al., 2006; Knudsen et al., 2008; Urán et al., 2008; Venold et al., 2012). The potential use of low-cost phyto-additives rich in bioactive compounds as gut health promoters in carnivorous fish fed plant meal-based diets has not been explored, hence further research in this field is warranted. Agroindustry by-products such as those from the wine processing industry have been reported to contain high concentrations of bioactive compounds including polyphenols (Arnanitoyannis et al., 2006; Teixeira et al., 2014). Considering the production scale of the wine industry worldwide, the use of low cost winery by-products rich in polyphenols in favoring growth, health and final product quality in fish, constitute a promising strategy to support sustainable aquaculture.

Understanding the effects of plant-derived extracts rich in polyphenols on glucose and lipid metabolism in carnivorous fish fed plant ingredient-based diets needs further research as well. Evidence demonstrates carnivorous fish including salmonid species fed high-digestible

carbohydrate diets (30% or more) exhibit a glucose intolerant phenotype evoking metabolic disturbances (Polakof et al., 2012) similar to those observed in humans and mammalian models with non-alcoholic fatty liver diseases, metabolic syndrome or diabetes mellitus type 2 (Han et al., 2007; Chuang and McIntosh, 2011; Valenti et al., 2013). Some of the metabolic disturbances observe in carnivorous fish fed high-digestible carbohydrate diets include prolonged post-prandial hyperglycemia, fatty liver and increased visceral fat deposition. These negative effects are associated with sub-optimal growth compared to fish fed optimal dietary carbohydrate levels (10-15% CHO), thereby constituting a dietary inclusion limit in carnivorous fish diets. It has long been reported that polyphenols elicit beneficial health effects by reducing metabolic disturbances such as glucose intolerance, fatty liver disease and obesity associated with either non-alcoholic fatty liver diseases, metabolic syndrome or diabetes mellitus type 2 in humans and mammalian models (Han et al., 2007; Chuang and McIntosh, 2011; Valenti et al., 2013). Whether dietary plant-derived extracts rich in polyphenols may reduce the metabolic syndrome-like disturbances detected in carnivorous fish such as salmonids species fed diets high in digestible carbohydrate levels needs further research.

Finally, the use of plant-derived extracts as natural sources of phytocompounds could become a feasible strategy to increase aquaculture productivity by promoting growth rates, feed utilization, resistance to stress and diseases as well as final product quality (i.e. pigmentation). Further research is required to improve our understanding of the effects of these compounds on metabolism of specific tissues, somatic growth and overall physiology in carnivorous fish. Additionally, the search for plant-derived extracts should not be restricted solely to terrestrial plants or agroindustry by-products but also should be further expanded toward potential use of algae-derived extracts since algae produce secondary bioactive metabolites including carotenoids, phenolic compounds and phycobiliportein pigments, among others. These phytocompounds have been reported to exert a variety of biological activities including antioxidant, anti-cancer and antimicrobial (Shalaby, 2011). Further, recent work has demonstrated antiviral activity against infectious salmon anemia virus (ISA virus) in serum from salmon fed a diet supplemented with 1 and 10% of a lyophilized of *Gracilaria chilensis* (red macroalgae) (Lozano et al., 2015). However, the potential effects on nutrient

metabolism, myogenesis and product quality in carnivorous fish fed diets supplemented with algae-derived extracts remains largely unknown.

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Appendix A

Standard Operating Procedure:

Title: Rainbow Trout Myosatellite Isolation and Primary Culture Protocol

Purpose: To detail a procedure for the isolation and primary culture of myosatellite cells from rainbow trout.

Important notes:

1. This is the procedure of Cleveland, B. M. and Weber, G.M. (2010) (Please refer to reference Chapter4 and Chapter 5).
2. All of the procedures should take place on ice or at 4ºC for this protocol unless it is stated differently.
3. This is a sterile procedure and precautions should be taken not to contaminate the cells and reagents or equipments that come in direct contact with the cells.

Reagents:

- Sterile, Nanopure Water
- DMEM:
  - Sigma Cat#D7777-high glucose 4.5 g/liter and phenol red **OR**
  - **For sex steroid use only**: Sigma Cat# 2902-no phenol red, low glucose. (Need to add additional 3.5 g/liter of glucose.)
- Glucose: Sigma Cat#G5767
- HEPES: Sigma Cat#H3784
- Sodium Bicarbonate: Fisher Cat#S-233
- Fetal Bovine Serum: Invitrogen Cat#16000-044
- Pen-Strep: 10,000 units/ml and 10,000 µg/ml, respectively. Invitrogen Cat#15140-122
- Fungizone (Amphotericin B): Sigma Cat#A9528
- Sterile 1x PBS
- Trypan Blue stain: Sigma Cat#T8154

- Poly-L-Lysine: Sigma Cat#P4832
- Laminin: Sigma Cat#L2020-1 mg
- Collagenase: Sigma Cat#C9891
- Trypsin: Sigma Cat#T9935

Materials:

- 6-well tissue culture plates (Falcon #35-3046)
- Hemocytometer
- Weigh boats
- Scalpel
- Razor blade
- Cutting board
- 50 ml conical tubes
- Table top centrifuge
- 20° C incubator
- Nutator rotating, rocking mixer
- Ice buckets
- Cell Strainers 100 µm, 70µm and 40µm (BD #s 352-360, 352-2350, 352-2340, respectively.

Procedures

Plate Preparation (begin at least the day before isolation)

1. Add 0.5 ml poly-L-lysine to each well of a 6 well tissue culture plate. Swirl to cover. Incubate at 18° C for 3 hours.
2. Remove excess lysine and wash wells with sterile nanopure water. Dry under UV light.
3. Dilute laminin to 100 µg/24 ml PBS (100 µl) and pipette 1 ml/well (enough for 4 plates).

4. Incubate at 18°C overnight.
5. Wrap with parafilm and store at -80°C until use.
6. Before use, aspirate laminin solution and wash wells with PBS.

Media Preparation

To 1 L of sterile, nanopure water add:

1. 1 jar of DMEM (+ 3.5 g/liter of glucose, if doing sex steroid assays)
2. 4.76 g HEPES
3. 0.75 g Sodium Bicarb
4. 5 ml Pen-Strep and 0.5 ml 1000 x fungizone
5. Sterile Filter

For complete medium add:

1. 450 ml base medium (above)
2. 50 ml FBS

Anesthesia/Euthanasia

Submerge 30 x 5-8 g (less if fish are larger) fish in an anesthetic bath containing 100 mg MS-222/liter of water for a few minutes, until the fish are euthanized. Place on ice and transfer to lab.

Tissue Harvest

While working in ice:

1. Warm 2 x 150 ml DMEM to RT for preparation of dissociating enzymes.
2. Remove 5-5.6 g of white muscle tissue into 25 ml of Cold DMEM media (x 2).
3. Remove tissue from media and mince quickly with a razor blade (pieces of 1-3 mm) aseptically under the hood. Place tissue into a conical tubes containing 25 ml cold fresh DMEM for washing it. Repeat for remaining tissue.
4. Centrifuge at 300 **g** for 5 minutes at 4**°C**. Meanwhile prepare 0.2% collagenase by adding 0.1 g into 50 ml RT DMEM. Discard supernatant.

Add 25ml 0.2% collagenase to each tube and dissociate the pellet by passing it through 10ml pipettes. Incubate at 22°C for 1 hr while gently rotating.

5. Centrifuge at 900 **g** for 20 minutes at 4**°C**. Discard supernatant.
   To each tube add 25 ml 0.1% trypsin. Dissociate the pellet by passing it through 10ml pipette. Incubate at 22°C for 45 min while gently rotating.
6. Divide each cell-trypsin solution into two conical tubes. Add DMEM to a total of 50 ml.
7. Centrifuge tubes 900 **g** for 25 minutes at 4**°C**.
8. Remove supernatant. Resuspend each pellet in 20 ml media. Combine two suspensions for a total of 40 ml/vial and pass through a 5ml pipette five times.
9. Strain cells sequentially through 100, 70 and 40 micron cell strainers.
10. Centrifuge cells at 900 **g** for 10 minutes at 4**°C**.
11. Dissociate cell pellet in DMEM w/10% FBS. Combine resuspended pellets and bring volume to a total of 10 ml.
12. Count cells.
13. Dilute cells to desired density→1.4x 10^6 cells/well
14. Place 2 ml/well of a poly-L lysine/laminin-coated 6 well TC plate.
15. After 16 hrs perform media exchange (before the exchange wash each well with approx. 2ml of warm HBSS).
16. Replace with fresh complete media every second day.

***Cell Counting*:**

1. Clean hemocytometer with ethanol.
2. To a 1.5 ml microfuge tube add 130µl of PBS, 20µl of trypan blue, and 50µl of cell suspension. This is a 1:4 dilution. Calculations will need to be adjusted for any other dilution.
3. Load 10µl into each side of the hemocytometer.
4. Count the 4 corners on each side of the hemocytometer. Average the counts.
5. Average count x 10^4=#cells/ml media. Multiply the total number of mls to arrive at final cell count.