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Effects of anthocyanidins on myogenic differentiation and antioxidant defense in primary myogenic cells isolated from rainbow trout (*Oncorhynchus mykiss*)

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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; 1×, 2.5× and 10×, 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 h 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 that 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 plant 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.

Statement of relevance: The study contributes to increase our understanding regarding the effect of plant-derived secondary metabolites such as anthocyanidins on myogenic program and antioxidant enzyme defense in differentiating myogenic cells from carnivorous fish. We have demonstrated that anthocyanidins may delay the progress of the myogenic differentiation process and promote antioxidant defense expression in myogenic cells.

1. 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;

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Kaushik et al., 1995, 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 that 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., 2013a). 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 that 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; Laçon; et al., 2012; Montesano et al., 2013). In agreement with this statement Myburgh et al. (2012) 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 (MHCf) 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 signaling molecules and miRNAs including miR-133, miR-20b and miR-149 regulating the expression of pro-myogenic genes (Kaminski et al., 2012; Laçon 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 (Olguin 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 abovementioned, we analyzed the effect of three types 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.

2. Material and methods

2.1. 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 analyzed previously in our lab (Villasante et al., 2015).

2.2. Cell culture

2.2.1. 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 × 106 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.

2.2.2. 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., 2013a, 2013b). 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 et al., 2015). The three concentrations of anthocyanidin mixture were tested to determine a potential dose response in the analyzed dependent variables. In order to determine whether there is a cytotoxic effect of either treatment we analyzed 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 ($n = 3$).

2.3. 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).

2.4. 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).

2.5. 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 1. The annealing temperature for both forward and reverse primers of the GOI and reference genes were between 61 °C and 62.5 °C, with the exception of *bcl2*, which was 56.2 °C for the forward primer and 58.2 °C 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

Table 1
Primer sequences used in real-time PCR.

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

[a] NCBI.
[b] TIGR.

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 $R0 = 1 / (E + 1)Ct$, where R0 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*.

3. 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. Results

4.1. Effects of anthocyanidins on myogenic programing 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. 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. 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. 3).

4.2. 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* was significantly higher in both treatments 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).

4.3. 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. 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. 6).

4.4. Effect of anthocyanidins in the apoptotic pathway in primary myogenic cells isolated from skeletal muscle of rainbow trout

Relative mRNA expression quantity of *bcl2* was used determined as an anti-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. 7).

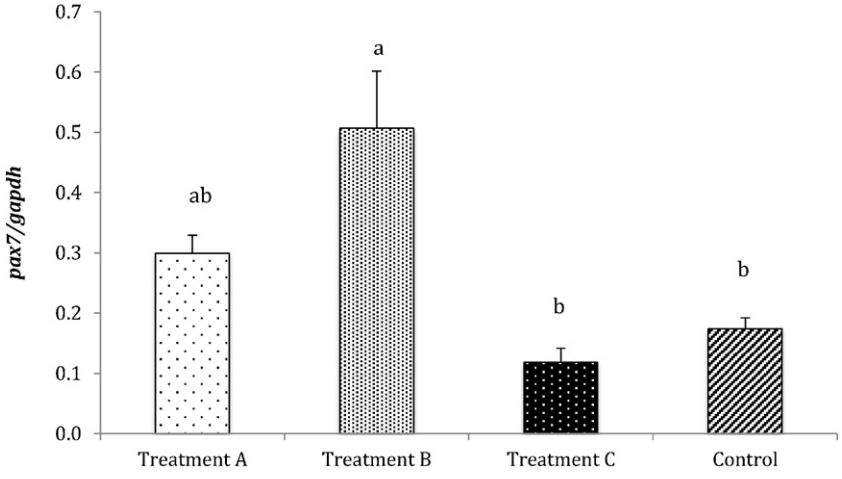

Fig. 1. Relative mRNA expression quantity of *pax7* after 24 h of treatment of different concentrations of an anthocyanidin mixture in primary myogenic cells from white skeletal muscle of rainbow trout. Treatment A: 50 μM of peonidin chloride, 20 μM of cyanidin chloride and 15 μM of pelargonidin chloride, 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 ($n = 3$).

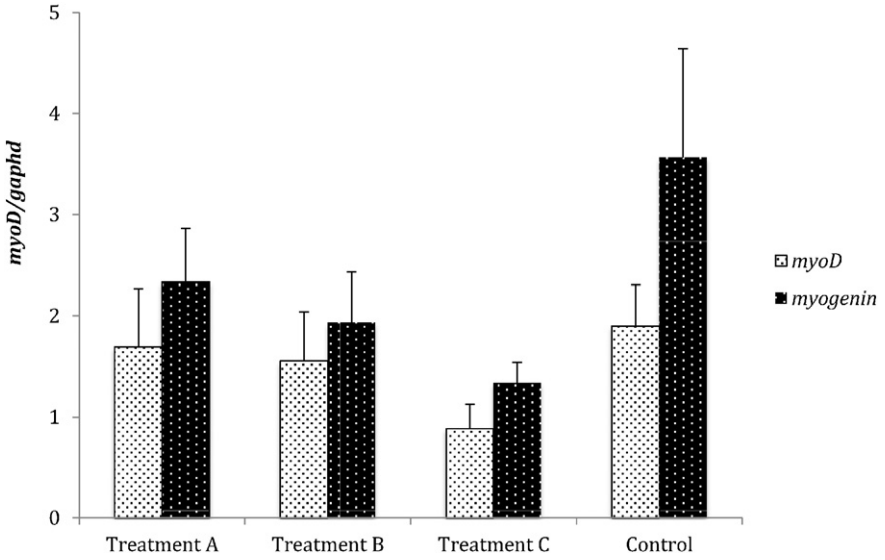

Fig. 2. Relative mRNA expression quantities of *myoD* and *myogenin* after 24 h of treatment of different concentrations of an anthocyanidin mixture in primary myogenic cells from white skeletal muscle of rainbow trout. Treatment A: 50 μM of peonidin chloride, 20 μM of cyanidin chloride and 15 μM of 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 ($n = 3$).

5. 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 h 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 effects 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 (Gharabi 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., 2013a, 2013b). 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.

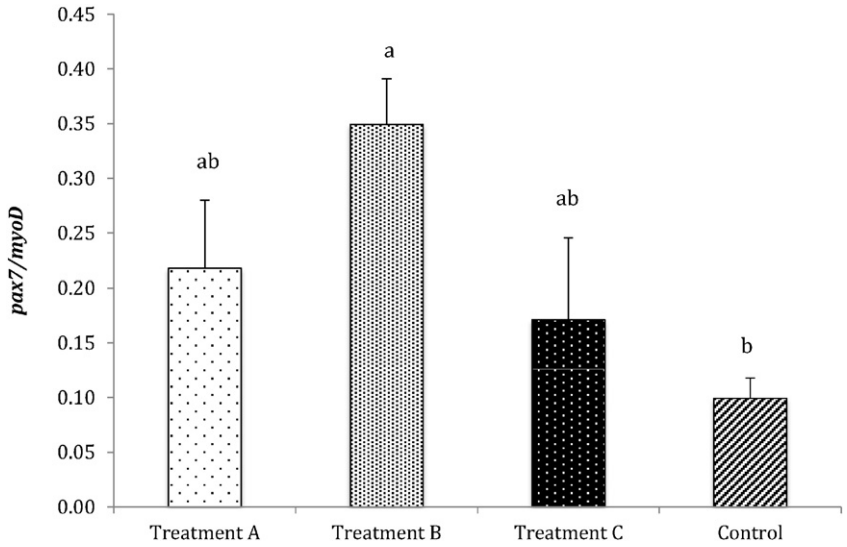

Fig. 3. *Pax7*/*myoD* ratio after 24 h of treatment of different concentrations of an anthocyanidin mixture in primary myogenic cells from white skeletal muscle of rainbow trout. Treatment A: 50 μM of peonidin chloride, 20 μM of cyanidin chloride and 15 μM of pelargonidin chloride, Treatment B: 120 μM 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 ($n = 3$).

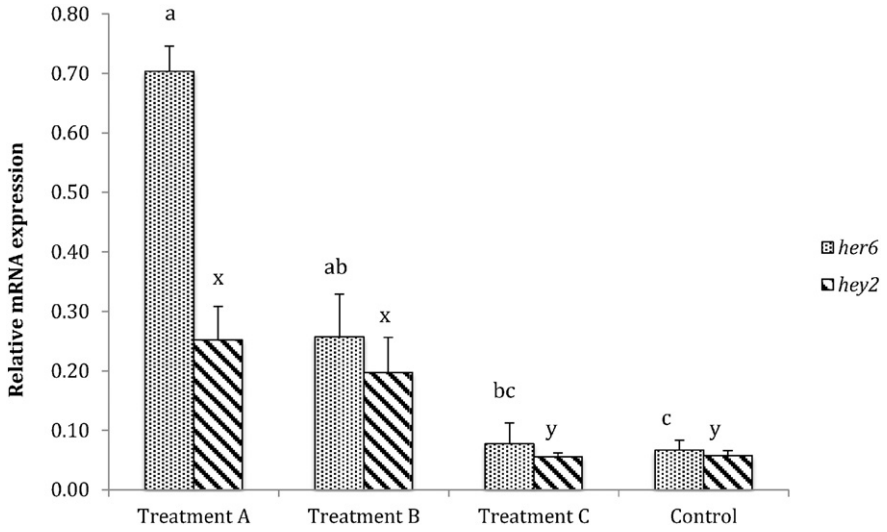

Fig. 4. Relative mRNA expression quantities of *her6* and *hey2* after 24 h of treatment of different concentrations of an anthocyanidin mixture in primary myogenic cells from white skeletal muscle of rainbow trout. Treatment A: 50 μM of peonidin chloride, 20 μM of cyanidin chloride and 15 μM of pelargonidin 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 ($n = 3$).

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.

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; Gutierez-Salmean et al., 2014; Kaminski et al., 2012; Lançon et al., 2012; Montesano et al., 2013; Myburgh et al., 2012). 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 (Bentzinger et al., 2012; Olguin et al., 2007; Olguín and Pisconti, 2012). The high confluence (>90%) achieved by the high seed

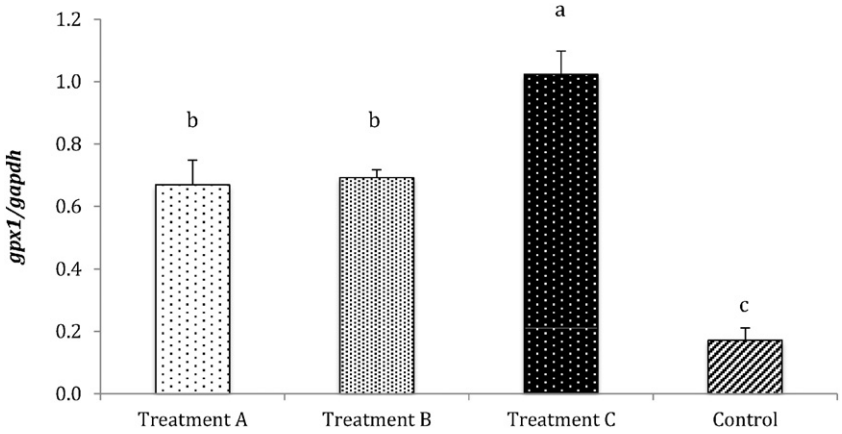

Fig. 5. Relative mRNA expression quantity of *gpx1* after 24 h of treatment of different concentrations of an anthocyanidin mixture in primary myogenic cells from white skeletal muscle of rainbow trout. Treatment A: 50 μM of peonidin chloride, 20 μM of cyanidin chloride and 15 μM of pelargonidin chloride, 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 ($n = 3$).

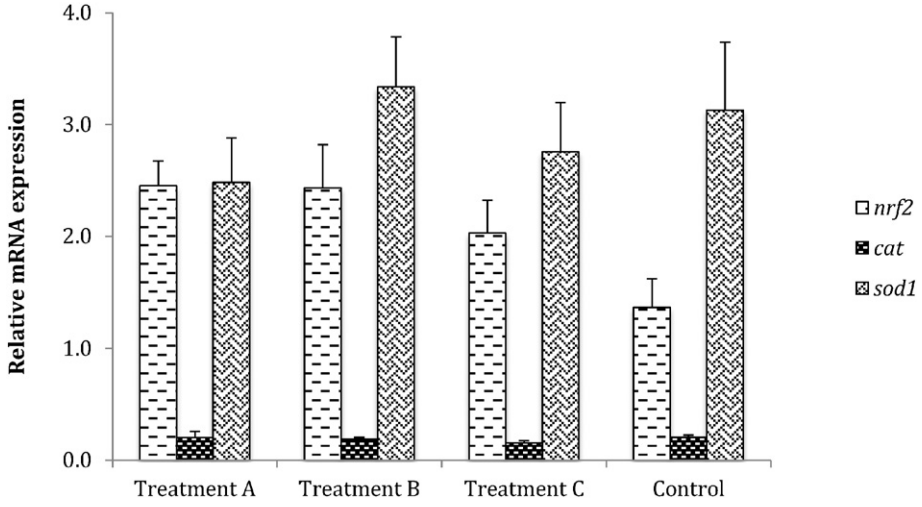

Fig. 6. Relative mRNA expression quantities of *nrf2*, *cat and sod1* after 24 h of treatment of different concentrations of an anthocyanidin mixture in primary myogenic cells from white skeletal muscle of rainbow trout. Treatment A: 50 μM of peonidin chloride, 20 μM of cyanidin chloride and 15 μM of 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 ($n = 3$).

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 (Olguin 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, *myogenin* 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; Olguin 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 and Zolkiewska (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.

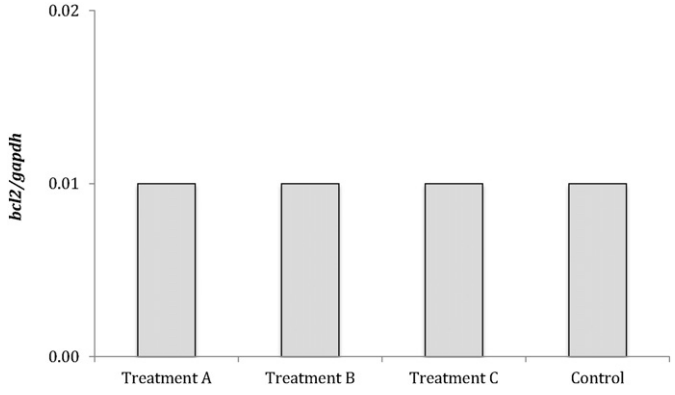

Fig. 7. Relative mRNA expression quantity of *bcl2* after 24 h of treatment of different concentrations of an anthocyanidin mixture in primary myogenic cells from white skeletal muscle of rainbow trout. Treatment A: 50 μM of peonidin chloride, 20 μM of cyanidin chloride and 15 μM of pelargonidin chloride, 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 the relative mRNA expression normalized against *gapdh*. Each experiment was conducted three independent times ($n = 3$).

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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