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Effect of anthocyanidins on myogenic differentiation in induced and non-induced primary myoblasts from rainbow trout (*Oncorhynchus mykiss*)

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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 (1×, 2.5× and 10×) at two sampling times (24 and 36 h). To test for treatment effects, 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 h of treatment compared to control. A significantly higher expression of *pax7* in cells exposed to either anthocyanidin treatment during 36 h compared was observed. Similarly, the *pax7*/*myoD* ratio was significantly lower in cells exposed to the lowest anthocyanidin doses during 24 h compared to control. No significant effect of anthocyanidin treatments on the expression of *hey2* and *her6* at either sampling point was detected. In non-induced cells, we observed no effect of anthocyanidins on *myoD* expression and significant down-regulation on *pax7* expression in cells exposed to either anthocyanidin mixture concentrations after 24 and 36 h of treatment compared to control. Further, the *pax7*/*myoD* ratio was significantly lower in cells exposed to either anthocyanidin doses at both sampling time. In non-induced cells, the highest anthocyanidin dose provoked significantly greater expression of *hey2* after 24 h of treatment compared to control. We detected no such effect in non-induced cells exposed to the lowest and middle anthocyanidin doses during 24 h of treatment. The expression of *her6* was unaffected by anthocyanidin treatments at either sampling time or doses compared to 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 h treatment while pausing differentiation, potentially favoring cell survival after 36 h 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.
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1. Introduction

In the last two decades aquaculture production has grown in average by 6% per year, being the fastest-growing animal food-producing sector worldwide (FAO, 2012). Increased fish production from aquaculture is the only solution to increase the supply of fisheries products for a growing human population. This is especially true since capture fisheries have declined over the past two decades and cannot expand further (FAO, 2012). Therefore, aquaculture production has become more intensive to increase productivity and reduce costs in culture systems. However, the industrial aquaculture growth has increased the demand for aquafeed causing a concomitant increase in marine-derived ingredients prices including fishmeal and fish oil (Naylor, Hardy, Bureau, Chiu, Elliott, Farrell, Forster, Gatlin, Goldburg, Hua, Nichols, 2009). Hence, the

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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, Boujard, Kaushik, Boeuf, Van der Geyten, Mol, Kuhn, Quinsac, Krouti, Ribaillier, 2000; Gomes, Rema, Kaushik, 1995; Kaushik, Coves, Dutto, Blanc, 2004; Kaushik, Cravedi, Lalles, Sumpter, Fauconneau, Laroche, 1995; Pierce, Palti, Silverstein, Barrows, Hallerman, Parsons, 2008). However, the greater use of plant ingredients in fish feeds implies several nutritional challenges including greater amounts of phytochemicals in aquafeeds. This scenario requires a better understanding of the 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 potential use of plant-derived extracts in finfish diets in the past years (Chakraborty, Horn, Hancz, 2014; Reverter, Bontemps, Lecchini, Banaigs, Sasal, 2014). Several phytochemicals found in plant extracts such as alkaloids, terpenoids, tannins, saponins, glycosides, flavonoids, phenolics, steroids and essential oils have been reported to exert beneficial effects over fish physiology including appetite stimulation, growth promotion and increasing resistance against bacteria and parasites diseases (Chakraborty, Horn, Hancz, 2014; Reverter, Bontemps, Lecchini, Banaigs, Sasal, 2014).

Anthocyanins and anthocyanidins, a type of flavonoids, provide the red, blue and purple pigmentation to vegetables and fruits (Wang, Stoner, 2008). Several health benefits such as antioxidant, cardio-protective, anti-inflammatory and anti-carcinogenic effects have been observed from the dietary intake of food rich in anthocyanins in both human and mammalian models (Galvano, La Fauci, Lazzarino, Fogliano, Ritieni, Ciappellano, Battistini, Tavazzi, Galvano, 2004; Vennat, Bos, Pourrat, Bastide, 1994; Whitehead, Robinson, Allaway, Syms, Hale, 1995). However, much less is known about the potential beneficial effects from dietary intake of plant-derived extracts rich in anthocyanins or anthocyanidins on fish physiology and growth. Recently, we reported that a mixture of anthocyanidins enhances the expression of genes associated with anti-oxidant mechanisms (i.e. glutathione peroxidase 1) and triggers a myogenic gene expression pattern in accordance with a delay in myogenic progression in myocytes obtained from rainbow trout (Villasante et al., 2016). However, whether the anthocyanidin effect on myogenic progress varies regarding the myogenic stage of the myogenic cells have not yet been addressed.

We conducted an in vitro study to determine the potential effect of an anthocyanidin mixture in myogenic programming by modulating the transcription of genes involved in determination and initial differentiation, such as *pax7* and *myoD*, in-induced and non-induced myoblast cells isolated from white skeletal muscle of juvenile rainbow trout. In addition, we analyzed the transcription of two target genes of the 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, Turner, 2001; Liu, Sun., Wang, Wang, Zhu, 2006). Notch is a highly conserved cell signaling mechanism, which plays a crucial role in metazoan development (Artavanis-Tsakonas, Rand, Lake, 1999). However, the biological importance of Notch signaling pathway goes beyond its role in the developmental biology of an organism. Notch signaling is also involved in adult muscle homeostasis in human and mammalian models (Bjornson, Cheung, Liu, Tripathi, Steeper, Rando, 2012; Brack, Conboy, Conboy, Shen, Rando, 2008; Lin, Shen, Jin, Gu, Chen, Cao, Hu, Keller, Pear, Wu, 2013; Parker, Loretz, Tyler, Duddy, Hall, Olwin, Bernstein, Storb, Tapscott, 2012). Moreover, it has been shown that Notch signaling induces self-renewal of skeletal muscle satellite cells via up regulation of *pax7* (Wen, Bi, Liu, Asakura, Keller, Kuang, 2012). However, whether Notch signaling plays similar role in juvenile and adult muscle growth physiology remains largely unknown in finfish species.

The potential use of extracts derived from low-cost agroindustry by-products rich in polyphenols including anthocyanins and anthocyanidins that could promote growth, health and final product quality in finfish species under intensive culture, constitute a cost-effective option to the aquafeed industry. The findings of this study provide novel insight with regard to the potential modulatory role of anthocyanidins in myogenic programming in primary fish myoblasts at two different myogenic stages.

2. Materials and methods

2.1. 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 analyzed previously in our laboratory.

2.2. Cell culture

2.2.1. 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 9×10^5 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.

2.2.2. 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. The laminin solution was discarded and wells further washed with PBS. Cells were seeded in wells and after 24 h of incubation, they were randomly assigned into "induced" (treated with differentiation media) and "not induced" (not treated with differentiation media) group. 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 (Seiliez et al., 2010). 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 (three wells *per* group), 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 experimental groups were compared to carried out the relative gene expression analysis. 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 6 h from the segregation point. The three concentrations were tested to determine if a dose response was observable in the analyzed 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 ($n = 3$).

2.2.3. 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, the percentage of similarity sequence against homologues sequences obtained from other vertebrate species. Sequences for primers development of *elf1α*, *myoD*, *pax7* and *bax* were identified using sequences found in the GenBank (NCBI) (Table 1).

2.2.4. 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 were determined using a Nanodrop® ND-1000 UV–Vis Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). Subsequently, 1.2 μg of total RNA was 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 was 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 s and 62 °C for 1 min. Primers and probes for both for the genes of interest (GOI) and reference gene (elongation factor 1 alpha; *elf1α*) 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).

Table 1
Primer sequences used in real-time PCR.

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

[a] NCBI.
[b] TIGR.

3. 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 h 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.

4. Results

4.1. Effect of anthocyanidins in myogenic programming in early-induced myogenic cells

The relative mRNA quantities of *myoD* were significantly greater in both treatments A and B (low and middle doses, respectively) ($P = 0.0009$ and $P = 0.042$, respectively) compared to control after 24 h of treatment in early-induced myogenic cells (Fig. 1). No difference between treatment C (higher dose) and control group after 24 h of treatment was detected. Further, a significantly ($P = 0.017$) greater expression of *myoD* in treatment A compared to treatment C after 24 h of treatment was detected. We observed no differences in *myoD* expression between either anthocyanidin doses compared to control group after 36 h of treatment. Further, the expression of *myoD* was significantly ($P = 0.008$) greater in treatment A after 24 h compared to cells exposed to same treatment during 36 h. No differences in *myoD* relative expression in cells under either treatment B or C between 24 and 36 h of treatment were detected. We observed no differences in *pax7* expression between treatments A, B and C when compared to control group after 24 h of treatment (Fig. 1). However, significantly greater expression of *pax7* in treatments A, B and C ($P = 0.014$, $P = 0.023$ and $P = 0.033$, respectively) compared to control after 36 h of treatment

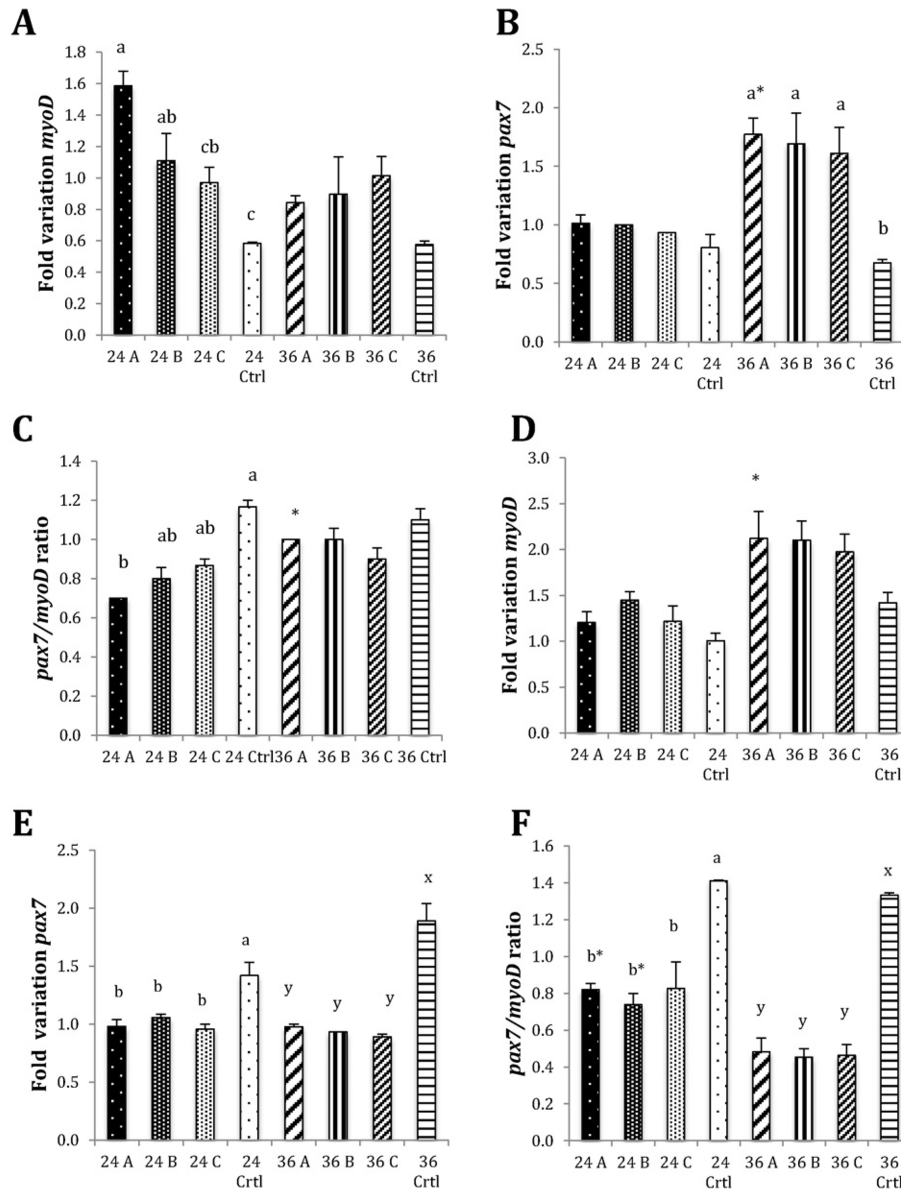

Fig. 1. Relative mRNA expression of A) *myoD* and B) *pax7*, respectively in induced myogenic cells, and of D) *myoD* and E) *pax7*, respectively in non-induced myogenic cells from white skeletal muscle of rainbow trout exposed to different concentrations of an anthocyanidins mixture during 24 or 36 h. *Pax7*/*myoD* ratio in C) induced and F) non-induced myogenic cells, respectively exposed to different concentrations of an anthocyanidins mixture during 24 or 36 h. 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 normalized against *elf1α* and 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 * ($P < 0.05$).

was observed. We detected significantly ($P = 0.022$) higher expression of *pax7* in cells under treatment A during 36 h compared to cells exposed to same treatment during 24 h. We detected no differences in *pax7* expression in cells exposed to either treatment B or C during 24 h compared to cells under same treatments during 36 h. The ratio of *pax7*/*myoD* was analyzed 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 ($P = 0.021$) higher in the control group compared to cells under treatment A during 24 h (Fig. 1). However, no differences were observed between the control group and cells exposed to either treatment B or C during 24 h. Similarly, no differences in the *pax7*/*myoD* ratio between experimental groups after 36 h were detected. However, within treatment level, treatments A and B exhibited significantly ($P = 0.0031$ and $P < 0.001$ and $P = 0.032$, respectively) higher *pax7*/*myoD* ratio after 36 h of treatment versus cells under same treatments during 24 h.

4.2. Effect of anthocyanidins in cells myogenic programming in non-induced myogenic cells

The relative mRNA expression of *myoD* was not significantly different in cells under either anthocyanidin treatments during 24 h compared to cells exposed to same treatments during 36 h (Fig. 1). However, in treatment C, the expression level of *myoD* was significantly ($P = 0.046$) higher after 36 h compared to cells exposed to same treatment during 24 h. The relative mRNA expression of *pax7* was significantly higher in the control group compared to cells exposed to treatments A, B and C in both sampling time ($P = 0.014$, $P = 0.036$ and $P = 0.009$, respectively and $P = 0.0001$, $P < 0.0001$ and

$P < 0.0001$, respectively) (Fig. 1). We detected significantly lower *pax7*/*myoD* ratio in non-induced myoblasts exposed to treatments A, B and C during 24 h compared to control group ($P = 0.005$, $P = 0.001$, $P = 0.005$, respectively) (Fig. 1). Similar pattern was observed in *pax7*/*myoD* ratio in non-induced myoblasts exposed to treatments A, B and C during 36 h compared to control group ($P < 0.001$, $P < 0.001$, $P < 0.001$, respectively). Within treatment level, *pax7*/*myoD* ratio was significantly higher ($P = 0.013$, $P = 0.028$, respectively) after 24 h compared to 36 h of treatment in cells exposed to treatments A and B.

4.3. Effect of anthocyanidins in the Notch signaling target genes in early-induced myogenic cells

The relative mRNA expression of *hey2* and *her6* was not affected neither by the anthocyanidin concentrations nor the length of the treatment in early-induced myogenic cells (Fig. 2). However, within treatment level, we observed a significantly ($P = 0.041$) higher of expression of *her6* in myogenic cells exposed to the lowest dose during 36 h compared to cells under same treatment during 24 h.

4.4. Effect of anthocyanidins in the Notch signaling target genes in non-induced myogenic cells

The relative mRNA expression of *hey2* was significantly ($P = 0.021$) higher in treatment C after 24 h of treatment compared to control (Fig. 2). Although the relative expression of *hey2* was unaffected by anthocyanidin concentrations after 36 h of treatment, a trend ($P = 0.053$) toward greater expression in cells under treatment A compared to control was observed. We observed no significant differences between either anthocyanidin doses compared to the control group at either sampling time (Fig. 2). Within treatment level, we observed significantly ($P = 0.024$) higher expression of *her6* in non-induced cells exposed to treatment A during 36 h compared to cells under same treatment during 24 h. However, no such effect was detected in cells exposed to either treatments B or C when compared between sampling time.

4.5. Effect of anthocyanidins in the apoptotic pathway genes in early-induced and non-induced myogenic cells

We quantified relative mRNA levels of *bax*, a pro-apoptotic signaling gene, and *bcl2*, an anti-apoptotic signaling gene, to examine potential cytotoxic effects of the highest doses of anthocyanidin used in our experimental treatment in both induced and non-induced myoblasts cells (Hasnan, Yusof, Damitri, Faridah, Adenan, Norbaini, 2010; Pawlowski, Kraft, 2000). The expression level of *bax* and *bcl2* remained unaffected in either early-induced or non-induced cells exposed to the highest dose of anthocyanidins (Treatment C) during 36 h of treatment.

5. 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 differentiation) after two different treatment times, 24 and 36 h. We provide evidences in regard to anthocyanidins modulating the expression of genes involved in the regulation of myogenesis progress in primary myogenic cells

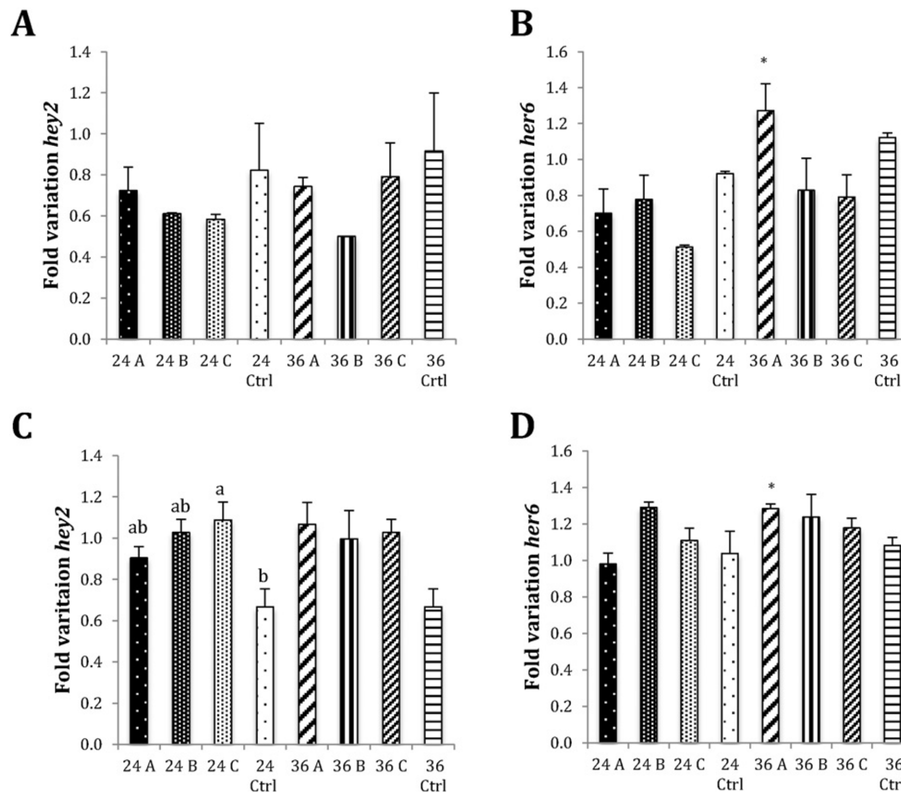

Fig. 2. Relative mRNA expression of A) *hey2* and B) *her6*, respectively in induced myogenic cells, and of C) *hey2* and D) *her6*, respectively in non-induced myogenic cells from white skeletal muscle of rainbow trout exposed to different concentrations of an anthocyanidins mixture during 24 or 36 h. 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 normalized against *elf1α* and 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 * ($P < 0.05$).

from fish. Further, this effect appears to differ with time of exposure to treatment and doses in myogenic cells induced to differentiation. However, no such effect was detected in non-induced myogenic cells. An exposure to the lower doses of anthocyanidins during 24 h triggers a gene expression pattern in accordance with a promotion of myogenic differentiation by up-regulating *myoD* expression with no effect on *pax7* expression. The significant decrease in *pax7*/*myoD* mRNA ratio observed in cells exposed to the lower anthocyanidin doses during 24 h compared to control group further reinforces this statement. The *pax7*/myoD ratio is a determinant factor in myogenic cell fate where a low ratio indicates myogenic cells progress toward terminal differentiation (Chapalamadugu et al., 2009; Olguín et al., 2004, Olguín et al., 2007; Olguín and Pisconti, 2012). In the present study an exposure to either anthocyanidin concentrations during 36 h induced an up-regulation of *pax7* expression as well as a trend toward higher *myoD* levels, thus causing an intermediate *pax7*/*myoD* ratio in early-induced cells. This gene expression pattern is in accordance with a promotion of proliferation and survival in committed myoblasts cells as described (Chapalamadugu et al., 2009; Olguin et al., 2007). Therefore, it appears that prolong exposure (i.e. 36 h) to anthocyanidins disrupt myogenic differentiation progress in favor of 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., 2013). Low resveratrol concentrations (10 to 25 μM) have been reported 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., 2013). 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, anthocyanidins caused similar dual effects (i.e. promoting differentiation and disrupting differentiation) 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). Further research to better understand these differences in the context of fish physiology is warranted.

The Notch pathway plays a pivotal role in adult skeletal myogenesis by regulating expression of *pax7* and pro-myogenic 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 both the transcription and activity of MyoD and 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 analyzed 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 h of treatment. We detect no effect in the expression of 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, these anthocyanidin-dependent changes could have occurred at the protein level. This is especially true since it has been shown that transcription of *hes1* mRNA is regulated by a negative feedback loop with an oscillatory cycle of 2 h in several mammalian-derived cell lines as described by 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 targets genes and 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 seem to promote differentiation by down regulating *pax7* expression at either dose tested after 24 and 36 h of treatment. This statement was supported 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 trend toward higher expression in non-induced cells exposed to the lowest and middle anthocyanidin doses compared to control after 24 h of treatment. 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 (Buas and Kadesch, 2010). In addition, it has been reported that Hey2 protein is not expressed in proliferating myoblasts (Sun et al., 2001). As intent to explain the gene expression pattern observed in our study, we hypothesize the anthocyanidin-induced up-regulation of *hey2* could have resulted in increased Hey2 protein concentrations, thus promoting its dimerization with Her6 in non-induced myoblast cells. The increase in Her6/Hey2 heterodimers would have favored MyoD/E47 dimerization indirectly, thus inducing down-regulation in *pax7* expression and further promoting myogenic differentiation in non-induced cells. This is especially likely to occur due to the existence of a Pax7-MyoD reciprocal inhibitory action (Olguín et al., 2007; Wen et al., 2012). Furthermore, Sun et al. (2008) 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*. However, this concept has not yet been addressed, and further analyses of protein expression pattern as well as the protein dimerization process to demonstrate this statement is warranted.

We recently demonstrated that an anthocyanidin mixture triggers a gene expression pattern in accordance with a "delay-like effect" in myogenic differentiation progress in myocytes from fish after 24 h of treatment (Villasante et al., 2016). Therefore, anthocyanidins appears to exert a differential effect on myogenic differentiation based upon the myogenic stage of cells by promoting differentiation in both early-induced and non-induced myoblasts and delaying differentiation in myocytes committed to terminal differentiation after 24 h treatment. Overall, we demonstrated that plant-derived compounds, e.g. anthocyanidins, modulate the expression of specific genes of myogenesis, 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 levels 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 regard to 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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