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

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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 was fed to triplicate tanks with 25 fish each for 8 wk. The concentrations of three anthocyanins, cyanidin-3-glucoside, pelargonidin-3-glucoside, and peonidin-3-glucoside, 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 total antioxidant capacity (TAC) and the concentration of oxidative damage biomarkers, for example, protein carbonyls, 8-hydroxydeoxyguanosine, and thiobarbituric acid reactive substances (TBARS), were determined in plasma. Transcription of two antioxidant enzymes, glutathione peroxidase 1 (*gpx1*) and superoxide dismutase 1 (*sod1*), was measured in erythrocytes. Significant lower adiposity and significant higher percentage of total n-3 and total n-6 polyunsaturated fatty acids (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 of 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 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 the fish body, as well as a tendency toward lower TBARS plasma concentration in fish fed the test diet when compared to the control group.

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

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rates of free radicals, such as reactive oxygen species (ROS) and reactive nitrogen species, 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, is a direct consequence of oxidative stress. Polyunsaturated fatty acids (PUFAs) and highly unsaturated fatty acids (HUFAs), as part of cell membrane phospholipids structures, are very 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, 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, 2001; Nakano et al. 1999; Peng et al. 2009; Oliva-Teles 2012; Yun et al. 2013).

Lipids and proteins are the main sources of energy for carnivorous fish species. Fish meal and fish oil have been used as the main sources of protein and lipids in carnivorous fish diets, respectively (Leaver et al. 2008; Naylor et al. 2009). Due to the increase in the demand and prices of fish meal, 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. 1978a, 1978b). 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 has 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 and 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 that the intake of cyanidin-3-glucoside (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/100 g), can be used as a natural anthocyanin 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 purple corn extract (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, 2010; Trenzado et al. 2009), 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 as well as the chemical composition of the whole fish were determined.

Materials and Methods

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-L 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 8-wk feeding trial, fish were fed by hand twice per day to apparent satiation, 6 d/wk. Fish in each tank were bulk weighed at the beginning of the trial and every 4 wk 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.

Experimental Diets

PCE 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 and also met National Research Council (2011) requirements for rainbow trout (Table 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 on rats showed that 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 ($n = 3$).

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

The concentration of 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).

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 6 h postprandially with a sharp blow to the head, and blood, liver, and muscle samples were collected. Blood was centrifuged at 1000 *g* for 5 min at 4 C and plasma aliquots were stored at −80 C pending determination of antioxidant capacity, oxidative damage, and

TABLE 1. *Ingredients and chemical composition of the experimental diets.*

Ingredients (%)	Control diet (no PCE)	Test diet (5% PCE)
PCE[a]	0.0	5.0
Fish meal, sardine[b]	26.3	26.3
Soybean meal[b]	20.0	20.0
Soy protein concentrate[b]	15.0	15.0
Wheat gluten meal[b]	7.0	7.0
Wheat starch, gelatinized[c]	14.0	9.0
Choline chloride[b]	0.6	0.6
Vitamin premix[h, d]	0.8	0.8
Stable C (35%) vitamin[b]	0.2	0.2
Dicalcium phosphate[e]	2.0	2.0
Trace mineral mix[e, f]	0.1	0.1
Fish oil (Alaska pollock)[g]	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

PCE = purple corn extract.

[a]Forestrx.com, Belleville, NJ, USA.

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

[c]GemGel 50, Manildra Group USA, Shawnee Mission, KS, USA.

[d]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; thiamine as thiamine 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.

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

[f]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.

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

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, USA), weighed, and kept at −20 C until chemical and fatty acid profile analyses of the whole body.

Determination of Growth Parameters

Specific growth rate was calculated as [100 × (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 × (final body weight × % final whole-body nutrient content) − (initial body weight × % initial whole body nutrient content)/nutrient intake]. Hepatosomatic index was estimated as (100 × (liver weight/body weight)).

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 × 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 4 h. Total energy content was determined using an adiabatic bomb calorimeter (Parr 6300, Instrument Co., Moline, IL, USA).

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 (FAMEs) following the two-step methylation procedure of Kramer et al. (1997). FAMEs were separated using a Shimadzu GC-17A (Shimadzu Corp., Kyoto, Japan) operated in split injection mode with a flame ionization detector, a silica capillary column Omegawax 320 (30 m × 0.32 mm × 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. FAMEs in samples were identified using the peak retention times of certified FAME standards (Supelco, Bellefonte, PA, USA, 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. FAMEs 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 (50–400 m/z). Internal standard 17:0 and the Shimadzu Lab Solutions software were used for quantification of each FAME.

Plasma Antioxidant Capacity and Oxidative Damage Biomarkers Analyses

Plasma total antioxidant capacity (TAC; Cayman Chemical, Ann Arbor, MI, USA) and 8-hydroxydeoxyguanosine (8-OHdG, Bioxytech 8-OHdG-EIA kit; Oxis Health Products, Portland, OR, USA) were measured by enzyme-linked immunosorbent assay. The protein carbonyl concentration in plasma was measured using a colorimetric assay (Cayman Chemical). Plasma lipid peroxidation was analyzed by measuring thiobarbituric acid reactive substances (TBARS) as previously described (Uchiyama and Mihara 1978).

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 the 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 the manufacturer's recommendations (Invitrogen). Determination of gene expression was carried out by real-time quantitative polymerase chain reaction (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. Nuclease-free water was used as negative control. Each reaction was carried out in duplicate. PCR cycle conditions were 95 C for 30 sec 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) (Table 2).

TABLE 2. *Primer sequences used in real-time polymerase chain reaction.*

Gene	Accession number	Forward 5′–3′ and Reverse 5′–3′
rps15	BT074197.1	ACAGAGGTGTGGACCTGGAC
		AGGCCACGGTTAAGTCTCCT
rpl11	BT074162.1	GTGGAGCTAAGGCTGAGGTG
		CCCAGTGTCGGAGAAGTTGT
aTub	AY150303.1	ACTGGTCTCCAGGGCTTCTT
		ACGGAGAGACGTTCCATCAG
gpx1	NM_001124525.1	CGCCCACCCACTGTTTGT
		GCTCGTCGCTTGGGAATG
sod1	AF469663.1	ACTCTATCATCGGCAGGACCAT
		GCCTCCTTTTCCCAGATCATC

From the 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 qPCRs 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, 200, and 400 nM, respectively. Transcriptional data of

TABLE 3. *Growth parameters, nutrients and energy utilization, and hepatosomatic index of rainbow trout fed the experimental diets for 8 wk.*[a]

	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)[b]	1.6	0.0	1.7	0.0
Feed intake rate (g/fish/d)[c]	4.6	0.1	4.8	0.2
Feed conversion ratio[d]	1.2	0.0	1.3	0.0
Protein retention (% protein intake)[e]	28.9	1.9	29.7	1.5
Lipid retention (% lipid intake)[e]	73.7*	2.7	61.5	2.7
Energy retention (% gross energy intake)[e]	40.9	2.0	38.6	1.5
Hepatosomatic index (%)[f]	1.4	0.1	1.4	0.1

[a]Mean values with their SE for three tanks per group, 25 fish each. Mean values marked with * were significantly different between the experimental diets ($P < 0.05$).

[b]Specific growth rate was calculated as [100 × (ln final mean body weight − ln initial mean body weight)/initial mean body weight].

[c]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.

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

[e]Nutrient and energy retention were calculated as [100 × ((final body weight × final body nutrient content) − (initial body weight × initial body nutrient content))/nutrient intake].

[f]Hepatosomatic index was estimated as (100 × (liver weight/body weight)).

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.

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).

Results

The concentrations of Peo3G, C3G, and Pel3G in PCE were 64.8 ± 2.2, 21.5 ± 4.3, and 18.7 ± 1.7 mg/g, respectively. Total antioxidant activity was 0.1 ± 0.0 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). 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

TABLE 4. *Chemical composition of the whole body of rainbow trout fed the experimental diets for 8 wk.*[a]

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

[a]Mean values with their SE for three tanks per group; four fish pooled from each tank.

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

TABLE 5. *Fatty acid profiles of whole-body and muscle of rainbow trout fed the experimental diets for 8 wk (mean values with their SE for three tanks per group. Whole body of four fish and muscle of three fish analyzed per tank).*[a]

	Whole-body				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
T. 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

DHA = docosahexaenoic acid; DPA = docosapentaenoic acid; EPA = eicosapentaenoic acid; T. MUFA = total monounsaturated fatty acids; T. PUFA = total polyunsaturated fatty acids; T. SFA = total saturated fatty acids.

[a]Mean values were significantly different between diets.

*$P < 0.05$; **$P < 0.01$.

dry matter ($P = 0.007$), crude fat ($P = 0.011$), and gross energy ($P = 0.025$) (Table 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 (Tables 5 and 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 ($P = 0.004$ and $P = 0.027$, respectively) than fish fed the control diet. Similarly, the proportions 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 significantly 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, docosahexaenoic acid (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 6). A significantly ($P = 0.04$) higher proportion of oleic acid was detected in the whole body of fish fed the control diet when compared to fish fed the test diet. Similarly, total monounsaturated fatty acids (total MUFA) were significantly ($P = 0.019$) higher in the 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 (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 n-3 : n-6 ratio 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 $6.1 \pm 0.6\%$, respectively. Similarly,

TABLE 6. *Fatty acid profiles of the liver and plasma of rainbow trout fed the experimental diets for 8 wk (mean values with their SE for three tanks per group with three fish pooled per tank).*[a]

	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
T. 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

DHA = docosahexaenoic acid; DPA = docosapentaenoic acid; EPA = eicosapentaenoic acid; T. MUFA = total monounsaturated fatty acids, T. PUFA = total polyunsaturated fatty acids; T. SFA = total saturated fatty acids.

[a]Mean values were significantly different between diets.

*$P < 0.05$

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 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).

TABLE 7. *Antioxidant activity, oxidative damage markers in plasma, and gene expression in erythrocytes of rainbow trout fed the experimental diets for 8 wk.*[a]

	Control diet		Test diet	
	Mean	SEM	Mean	SEM
Antioxidant activity				
TAC (mM of Trolox)[b]	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)[c]	13.8	4.2	4.3	0.4
Relative mRNA quantity[d]				
gpx1	1.42	0.0	1.52[e]	0.0
sod1	0.59	0.0	0.62	0.0

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

[a]Mean values with their SE for three tanks per group; three fish analyzed each.

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

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

[d]Arbitrary units in square root.

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

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). HUFAs 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 antiobesity 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. 2010; 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 and 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 that the intake of PCC downregulated 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 that intake of a diet enriched with wild blueberry, a natural source of anthocyanins, improved lipid profiles in dyslipidemic 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 involved 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. Because 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 for 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 on primary hepatocyte cell culture or hepatoma cell lines are warranted.

It has long been reported that 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 MDA 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 TAC 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, as 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, 2010, Trenzado et al. 2009). Erythrocytes transport oxygen in blood via a reversible association to hemoglobin, and as a result 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 upregulation 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 that components of red wine such as polyphenols might have been responsible for the observed effects. In agreement with these findings, Cazzola and 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 that 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, 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 acid percentages observed by di Giuseppe et al. (2009), Cazzola and Cestaro (2011), and Toufektsian et al. (2011). Taking into consideration the increase in the antioxidant capacity, the upregulation of *gpx1*, and the observed tendency toward 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 upregulation 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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