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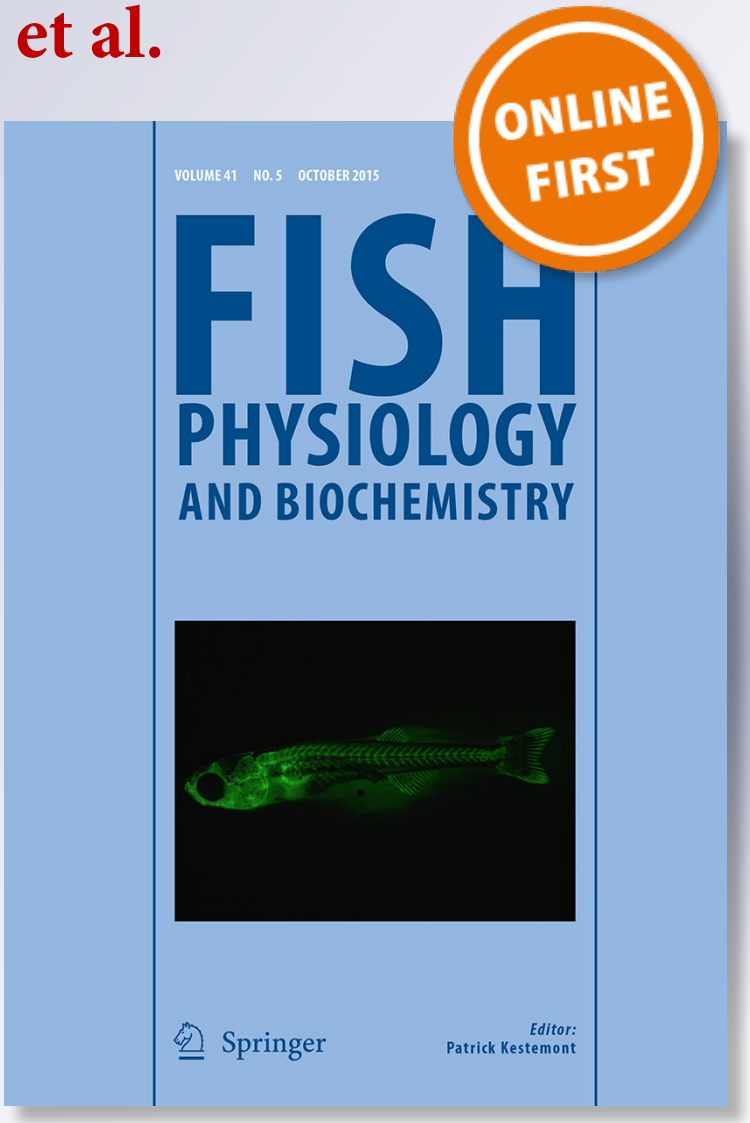

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Effect of diets enriched with rutin on blood parameters, oxidative biomarkers and pituitary hormone expression in silver catfish (*Rhamdia quelen*)

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Abstract The effects of adding rutin to the diet (0, 0.15 or 0.30 %) of silver catfish for 21 days on blood parameters, oxidative stress biomarkers and pituitary hormones expression were investigated. Fish that received the diet containing 0.15 % rutin exhibited reduced plasma cortisol levels. The levels of lipid peroxidation were lowered in the all tissues of animals receiving the diet containing rutin. Rutin increased the activity of the superoxide dismutase (SOD), catalase (CAT), nonprotein thiols (NPSH), ascorbic acid content (AA) and total reactive antioxidant potential (TRAP) in the brain; glutathione S-transferase (GST) activity and TRAP in the gills; SOD, CAT and GST activity, NPSH, AA levels and TRAP in the liver; CAT and GST activity and TRAP levels in the kidneys; and glutathione peroxidase activity, NPSH, AA levels and TRAP in the muscle. There were no changes regarding the expression of growth hormone, prolactin and somatolactin in fish fed with the diet containing rutin when compared with the control. The supplementation of rutin to the diet of fish is beneficial because it increases the antioxidant responses of tissues.

Keywords Fish · Flavonoid · Antioxidant defenses · Oxidative stress · Gene expression

Introduction

Several factors influence the success and high productivity in the practice of fish farming, such factors include environmental conditions, management and

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susceptibility to diseases. Fish health is the focus of industry and improves the performance of species, ensuring the well-being. Substances derived from plants are already being used as functional dietary supplements in commercial fish (Zheng et al. 2009).

The silver catfish (*Rhamdia quelen*) has great potential for fish farming in the South of Brazil; it is a species with rapid growth rates in low temperatures and reproduces almost every month of the year. Like other species in aquaculture environments, the silver catfish is subject to constant factors inherent to aquaculture such as handling, transport, induced spawning, egg and sperm collection, poor water quality and high stocking density, which lead to stress and could affect the growth and feed efficiency (Barcellos et al. 2003). These factors can also trigger oxidative stress (OS) and a predisposition to diseases, thus leading to yield losses. When an imbalance between the concentration of reactive oxygen species (ROS) and antioxidant defenses occurs, OS may cause damage to the cellular components and tissues. However, there are distinct cellular defense strategies against ROS-mediated processes that include enzymatic defenses such as superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPx) and glutathione S-transferase (GST), among other non-enzymatic defenses such as glutathione, ascorbic acid (vitamin C), α-tocopherol (vitamin E), carotenoids and flavonoids (Lushchak and Bagnyukova 2006).

The fish pituitary hormones, growth hormone (GH), prolactin (PRL) and somatolactin (SL) regulate the synthesis and secretion of many hormones that control other endocrine glands, and they are originated from a common ancestral molecule (Arukwe 2001; Sudo et al. 2012). They also involved in stress responses, as SL and PRL. PRL is able to prevent loss of ions in fish gills when confronted by stressors (Manzon 2002). Different studies have demonstrated the involvement of these hormones in physiological processes such as reproduction, growth, metabolism and immune responses (Kaneko 1996; Manzon 2002; Sakamoto and McCormick 2006; Fukamachi and Meyer 2007; Benedet et al. 2008).

These pituitary hormones play a key role in regulating homeostasis of several physiological processes in response to challenges (Kaneko 1996).

Diets containing antioxidant compounds may inhibit the onset of various diseases and enhance the resistance of fish. This is important because the development of intensive livestock farming elevates the risk of disease caused by opportunistic microorganisms in the water, thus resulting in losses in production and the quality of the fish (Hirsch et al. 2006). Studies with the addition of natural antioxidants of vegetable origin in the diet of fish have been conducted to improve the growth performance, antioxidant activity (Zheng et al. 2009), storage and delay deterioration of meat (Álvarez et al. 2012).

Moreover, new antioxidant strategies using phenolipids, as rutin laurate and rutin palmitate, to protect food (as fish) against lipid peroxidation, have been developed. Sorensen et al. (2012) demonstrated that rutin laurate has better antioxidant action than original rutin, whereas rutin palmitate performed slightly better as antioxidant compared with original rutin, indicating that the cutoff effect exists in relation to the alkyl chain length (at least below 16 carbon atoms) with respect to optimal antioxidant activity in fish oil-enriched milk emulsion.

Rutin (quercetin-3-rutinoside) belongs to a group of natural substances with variable phenolic structures and is found in many plants, particularly *Fagopyrum esculentum* Moench and *F. tataricum* (L.) Gaertn., which are species of the Polygonaceae (buckwheat) family (Kim et al. 2005). Besides antioxidant, it has several activities, among them, anti-inflammatory, neuroprotective, anti-viral and anti-carcinogenic, which have been demonstrated in different animal models (Aron and Kennedy 2008). These properties are potentially beneficial in preventing diseases and protecting the stability of the genome. Rutin acts as a scavenger of ROS by donating hydrogen atoms to peroxyl radicals, superoxide ($O2^{-}$), singlet oxygen and hydroxyl radicals ($OH^{\cdot}$); it also acts as a terminator and chelating agent of metal ions that are able to oxidize lipid peroxidation (LPO) (Afanas'ev et al. 1995; Yang et al. 2008). According to Afanas'ev et al. (1995) who investigated the antioxidant activity of rutin, this flavonoid has a therapeutic effect in diseases involving free radicals, is not toxic and non-oxidizable compound. Rutin may have an advantage as a therapeutic agent over other flavonoids such as quercetagetin, myricetin and delphinidin, since unlike them, rutin does not catalyze oxygen radical production, not manifesting prooxidant properties.

Thus, considering that the use of natural antioxidants such as rutin can be an alternative to improve animal welfare, this study aimed to investigate the

effects of adding 0.15 and 0.30 % rutin to the diet of silver catfish on the blood parameters, oxidative stress biomarkers in different tissues and pituitary hormones expression. Such concentrations were chosen based on reports found in the literature using quercetin in the diet of other fish species (Awad et al. 2013; Zhai and Liu 2013).

Materials and methods

Fish and culture conditions

The experiments were conducted in a recirculating aquaculture system in the Fish Physiology Laboratory at the Federal University of Santa Maria (UFSM), Rio Grande do Sul (RS), Brazil. Silver catfish (244.05 ± 1.62 g, 27.36 ± 0.37 cm) were obtained from a fish culture sector of UFSM. The animals were randomly distributed in nine plastic boxes (40 L), four fish per box, and acclimated to the laboratory conditions for 2 weeks. Water parameters were checked daily (temperature, total ammonia, nitrite and dissolved oxygen) or weekly (alkalinity, total hardness and pH). The experimental protocol was approved by the Committee on Animal Experimentation of UFSM under registration no (077/2013).

Chemicals

Rutin ($C_{27}H_{30}O_{16}$) was obtained from the Sichuan Yabao Guangtai Pharmaceutical Co., Ltd. (Chengdu, Sichuan Province, China). All of the other reagent-grade chemicals were obtained from Sigma (St. Louis, Missouri, USA).

Diets and experimental design

Three diets were formulated based on the study of Saccol et al. (2013). The different concentrations of rutin (0, 0.15 and 0.30 % rutin) were added to the mixture together with rice bran (Table 1). Water was then added to the diets, and a drying process was performed in a forced air circulation oven for 24 h (55 °C). The fish received the experimental diets until apparent satiation once a day (9 a.m.) for 21 days. The experimental design resulted in three groups (performed in triplicate).

Table 1 Formulation (%) of the experimental diet

Ingredients	(%)		
Soybean meal	30		
Meat and bone meal	35		
Rice bran	12		
Corn	15		
Canola oil	3		
Salt	1		
Vitamins and minerals (premix)[a]	3		
Phosphate dicalcium	1		
Analyzed proximate composition	0	0.15 %	0.30 %
Dry matter content	99.84	99.60	99.61
Protein	38.94	38.51	37.50
Ether extract	0.98	0.99	0.99
Mineral matter	1.69	1.60	1.61
Neutral detergent fiber	13.11	13.10	13.49

[a] Vitamin and mineral mixture (security levels per kilogram of product)—folic acid: 250 mg, pantothenic acid: 5000 mg, antioxidant: 0.60 g, biotin: 125 mg, cobalt: 25 mg, copper: 2000 mg, iron: 820 mg, iodo: 100 mg, manganese: 3750 mg, niacin: 5000 mg, selenium: 75 mg, vitamin A: 1,000,000 UI, vitamin B1: 1250 mg, vitamin B12: 3750 mcg, vitamin B2: 2500 mg, vitamin B6: 2485 mg, vitamin C: 28,000 mg, vitamin D3: 500,000 UI, vitamin E: 20,000 UI, vitamin K: 500 mg, zinc: 17,500 mg

The total phenolic compounds were determined in the diets according to the Folin–Ciocalteau procedure (Singleton et al. 1999). The absorbance of the resulting blue color was measured at 765 nm. Gallic acid was used as a standard, and the results were expressed as gallic acid equivalents (mg GAE) per 100 g of diet. The reaction was conducted in triplicate.

Sample collection and chemical analysis

After 21 days, blood samples were collected from the fish and biochemical analyses were performed. The blood sampling was performed from the caudal vein with heparinized sterile syringes. The fish were then euthanized by sectioning the spinal cord and brain, and the gills, liver, kidneys, muscle and pituitary were removed and immediately frozen in liquid nitrogen. The tissues were stored at −80 °C for further analysis.

Blood analysis

The blood was utilized for different analyses. The plasma cortisol levels were quantified using an enzyme-linked immunosorbent assay (ELISA) kit (Diagnostics Biochem Canada Inc., Canada). The samples were measured in duplicate, and the absorbance was determined in a spectrophotometer at 450 nm. The inter- and intra-assay variation coefficients were 5.15 ± 0.53 and 4.13 ± 0.67 %, respectively. The results are presented as ng/mL. One aliquot of whole blood was subsequently transferred to microcentrifuge tubes and centrifuged at 3000×*g* for 10 min (Centrifuge 5804 R) for the determination of hematocrit using microhematocrit methods. The hemoglobin concentration was obtained using the Drabkin reagent (Kamper and Zijlstra 1964), read spectrophotometrically at 540 nm and expressed as g/dL blood. The mean cell hemoglobin concentration (MCHC) was calculated using the equation [Hb] ∗ 100/Hct and expressed as g/dL. The levels of glucose (GLU), lactate dehydrogenase (LDH), triglycerides (TRI), cholesterol (CHO), low-density lipoprotein cholesterol (LDL), high-density lipoprotein cholesterol (HDL) and urea (URE) in the plasma were determined using commercial kits (Labtest, Minas Gerais, Brazil) and expressed as mg/dL.

Oxidative measurements

For the measurement of the OS biomarkers, each tissue was homogenized in 154 mmol/L KCl containing 1 mmol/L phenylmethylsulfonyl fluoride and centrifuged at 3000×*g* for 10 min at 4 °C to discard the nuclei and cell debris. The supernatant fraction obtained was frozen at −80 °C for further measurements. The protein content was measured using the method of Lowry et al. (1951), and the results are reported as mg/mL.

The LPO levels were estimated using the thiobarbituric acid-reactive substance (TBARS) assay (Buege and Aust 1978) and by determining the lipid hydroperoxides (LOOH) (Södergren et al. 1998). The results are reported as nmol/mg protein.

The SOD, CAT, GPx and GST activities were measured similarly to the study of Saccol et al. (2013). The nonprotein thiols (NPSH) content, an indirect measure of GSH, was evaluated using the method of Ellman (1959) and reported in nmol/mg protein. The ascorbic acid (AA) was measured according to the method of Roe and Kuether (1942). The standard curve was prepared by using different concentrations of ascorbic acid, and the slope was used to express the amount of ascorbic acid as μmol/mg protein. The total reactive antioxidant potential (TRAP) was determined by a chemiluminescence assay with 2,2′-azobis (2-aminodipropane) dihydrochloride and luminol, with the results expressed as μmol trolox/mg protein (Evelson et al. 2001).

Pituitary hormones expression

RNA Extraction and cDNA synthesis

Total RNA was extracted from samples using Trizol reagent (Invitrogen) according to the manufacture's instructions. Total RNA quantity and purity were assessed by NanoDrop (Thermo Scientific, Delaware, USA; Abs 260/280 nm ratio) spectrophotometer. Ratios above 1.7 were used, and samples below this threshold were discarded. Total RNA (1 μg) was treated with DNase (Invitrogen) at 37 °C for 5 min to digest any contaminating DNA. The reverse transcriptase reaction was performed with iScript cDNA synthesis kit (Bio-Rad) in a final volume of 20 μL.

mRNA expression of GH, PRL and SL

The mRNA expression was analyzed through qRT-PCR, using the StepOnePlusTM RT-PCR system (Applied Biosystems) with Power SYBR Green PCR Master Mix (Applied Biosystems). The primers used for the amplification of specific genes were based on previous studies (Baldisserotto et al. 2014; Dolci et al. 2014) and were designed using the Primer Express software 3.3 (Applied Biosystems) (Table 2). The results were normalized to the expression of the constitutive gene β-actin. The calculation of relative expression was performed as recommended by Pfaffl (2001).

Statistical analysis

The results were analyzed using a one-way analysis of variance (ANOVA) test followed by Tukey's test. The level of statistical significance was set at $P < 0.05$. All analyses were performed using Statistica Software (StatSoft, Inc.), version 7.0. The results are presented as the mean ± standard error (SEM).

Table 2 Primers design for amplification of β-actin, GH, PRL and SL genes based on the sequences of these genes based on Dolci et al. (2014)

Gene	Sequences
β-actin	
Forward	5′- CGA ATG CCA GGG TAC ATG GT -3′
Reverse	5′- CCA CCT TCA ACT CCA TCA TTGA A -3′
GH	
Forward	5′- TTG ACA GTC TTG GTG CTG CTT T -3′
Reverse	5′- GAG CGA CTG CGT TGT TGA AG -3′
PRL	
Forward	5′- ACC AGA GAC AGG AGC TCG TTC T -3′
Reverse	5′- AGC TCA TGA GAC CGT CCA TGT -3′
SL	
Forward	5′- CGA GGC CAG GAC TTT GTT TG -3′
Reverse	5′- GAC GCG CAC AAG GTT TGA T -3′

Results

Water quality parameters

The water quality parameters remained stable throughout the experimental period. The temperature was maintained at 22.05 ± 0.06 °C, the pH at 7.41 ± 0.03 and the dissolved oxygen at 6.84 ± 0.14 mg/L. The mean of the other parameters was as follows: hardness (21.6 ± 1.2 mg/L $CaCO_3$), alkalinity (23.4 ± 1.2 mg/L $CaCO_3$), nitrite (0.91 ± 0.09 mg/L), total ammonia (2.61 ± 0.3 mg/L) and non-ionized ammonia (0.06 ± 0.006 mg/L).

Determination of total phenolic compounds

The samples displayed different concentrations of phenolic compounds (Fig. 1), which were 40 and 21 % higher on 0.15 and 0.30 % dietary rutin, respectively, when compared with the control ($P < 0.05$).

Blood analysis

The plasma cortisol levels were 34 % lower in the fish fed diets containing 0.15 % rutin when compared with the control animals (Fig. 2). The diets containing the rutin concentrations did not exert any significant effect on the HCT, HGB and MCHC in whole blood. Compared with the control, the plasma levels of GLU, LDH, TRI, CHO, LDL, HDL and URE did not differ among the experimental groups (Table 3).

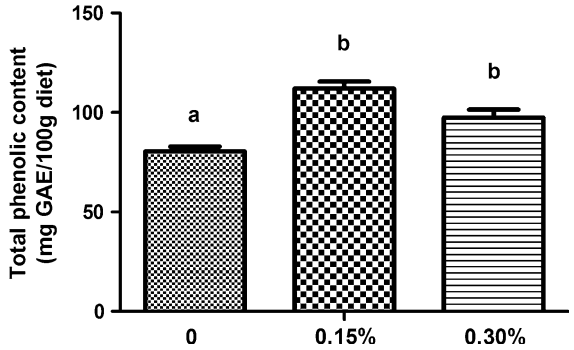

Fig. 1 Total phenolic content of diets containing different concentrations of rutin. The data appear as the mean ± SEM ($n = 3$). Different letters denote that the data are significantly different from the control at $P < 0.05$. *GAE* gallic acid equivalents

Biomarkers of oxidative stress in the brain

The protein contents did not differ among the experimental groups. The LPO levels, determined from the TBARS in the brain, were 40 % and 43 % lower in the fish receiving 0.15 and 0.30 % rutin, respectively, than in the control; this was also true for the LOOH measurements, which were 54 and 62 % lower in the silver catfish fed with 0.15 and 0.30 % rutin, respectively. The SOD activity was 145 and 103 % higher in the fish receiving 0.15 and 0.30 % rutin than in the control, respectively. The CAT activity was 38 % higher in the fish receiving 0.15 % rutin than in the

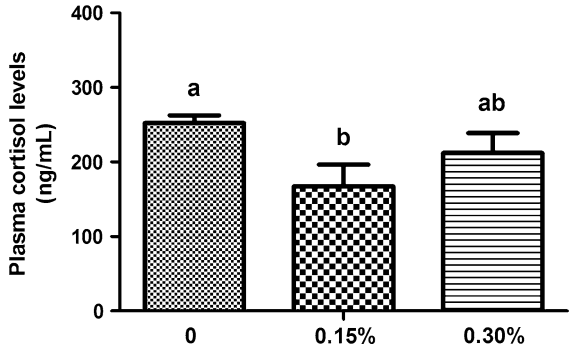

Fig. 2 Plasma cortisol levels of *Rhamdia quelen* fed diets containing different concentrations of rutin. The data appear as the mean ± SEM ($n = 12$). Different letters denote that the data are significantly different from the control at $P < 0.05$

Table 3 Hematological and biochemical parameters of the silver catfish *Rhamdia quelen* fed with diets containing different concentrations of rutin

	Diets (% rutin)		
	0	0.15	0.30
HCT[a]	23.04 ± 0.71	24.08 ± 0.90	22.71 ± 1.97
HGB[b]	5.44 ± 0.05	5.43 ± 0.11	5.77 ± 0.17
MCHC[c]	13.12 ± 0.55	13.98 ± 0.19	13.39 ± 0.65
GLU[d]	33.65 ± 1.55	39.54 ± 3.37	36.90 ± 3.06
LDH[e]	1494.33 ± 245.35	1208.58 ± 438.33	1290.14 ± 399.97
TRI[f]	185.17 ± 27.52	209.11 ± 39.35	150.29 ± 37.11
CHO[g]	203.12 ± 1.04	200.61 ± 6.11	212.94 ± 10.47
LDL[h]	38.93 ± 8.44	24.66 ± 6.08	39.79 ± 10.39
HDL[i]	160.98 ± 11.25	149.34 ± 14.30	134.43 ± 4.29
URE[j]	50.69 ± 6.31	50.13 ± 4.90	57.91 ± 9.89

[a] Hematocrit (%), [b] Hemoglobin (g/dL), [c] Mean cell hemoglobin concentration (g/dL), [d] Glucose, [e] Lactate dehydrogenase (U/L), [f] Triglycerides, [g] Cholesterol, [h] Low-density lipoprotein cholesterol, [i] High-density lipoprotein cholesterol, [j] Urea are mg/dL

Data are presented as the mean ± SEM ($n = 12$). Means obtained showed no significant difference ($P > 0.05$)

control. The non-enzymatic antioxidant measurements determined from the NPSH content were 15 and 13 % higher in the fish receiving 0.15 and 0.30 % rutin than in the control, respectively. The content of AA was 101 % higher in the fish receiving 0.15 % rutin than in the control. The TRAP was 97 % higher in the fish receiving 0.15 and 0.30 % rutin than in the control. By contrast, the GPx and GST activity did not differ among the various experimental groups (Table 4).

Biomarkers of oxidative stress in the gills

The protein contents did not differ among the experimental groups. The LPO levels, determined from the TBARS in the gills, were 19 and 25 % lower in the fish receiving 0.15 and 0.30 % rutin, respectively, than in the control; this was also true for the LOOH measurements, which were 57 and 32 % lower in the silver catfish fed with 0.15 and 0.30 % rutin, respectively. The GST activity was 175 and 58 % higher in the silver catfish receiving 0.15 and 0.30 % rutin than in the control, respectively. The TRAP was 138 % higher in the fish receiving 0.30 % rutin than in the control. By contrast, the SOD, CAT, and GPx activity and the NPSH and AA content did not differ among the experimental groups (Table 4).

Biomarkers of oxidative stress in the liver

The protein contents did not differ among the experimental groups. The LPO levels, determined from the TBARS in the liver, were 36 and 32 % lower in the fish receiving 0.15 and 0.30 % rutin, respectively, than in the control; this was also true for the LOOH measurements, which were 42 and 26 % lower in the silver catfish fed with 0.15 and 0.30 % rutin, respectively. The SOD activity was 139 and 172 % higher in the fish receiving 0.15 and 0.30 % rutin than in the control, respectively. The CAT activity was 99 % higher in the fish receiving 0.15 % rutin than in the control. The GST activity was 100 % higher in the silver catfish receiving 0.15 % rutin than in the control. The non-enzymatic antioxidant measurements determined from the NPSH content were 14 % higher in the fish receiving 0.15 and 0.30 % rutin than in the control, respectively. The AA content was 100 and 88 % higher in the fish receiving 0.15 and 0.30 % rutin than in the control, respectively. The TRAP was 65 and 59 % higher in the fish receiving 0.15 and 0.30 % rutin than in the control, respectively. By contrast, the GPx activity did not differ among the various experimental groups (Table 4).

Table 4 Biomarkers of oxidative stress in tissues of *Rhamdia quelen* fed diets containing different concentrations of rutin

	Diets (% rutin)		
	0	0.15	0.30
Brain			
PROTEIN[a]	5.32 ± 0.08^{A}	4.92 ± 0.27^{A}	4.92 ± 0.29^{A}
TBARS[b]	2.84 ± 0.10^{A}	1.70 ± 0.08^{B}	1.62 ± 0.09^{B}
LOOH[c]	11.18 ± 0.66^{A}	5.13 ± 0.31^{B}	4.27 ± 0.37^{B}
SOD[d]	1.15 ± 0.09^{A}	2.82 ± 0.17^{B}	2.34 ± 0.11^{B}
CAT[e]	0.26 ± 0.01^{A}	0.36 ± 0.02^{B}	0.26 ± 0.01^{AB}
GPx[f]	18.03 ± 3.84^{A}	29.34 ± 4.06^{A}	35.00 ± 2.63^{A}
GST[g]	0.47 ± 0.02^{A}	0.38 ± 0.03^{A}	0.48 ± 0.04^{A}
NPSH[h]	5.80 ± 0.10^{A}	6.70 ± 0.25^{B}	6.60 ± 0.15^{B}
AA[i]	71.50 ± 7.74^{A}	144.20 ± 12.27^{B}	117.60 ± 19.05^{AB}
TRAP[j]	1.80 ± 0.30^{A}	3.54 ± 0.24^{B}	3.55 ± 0.40^{B}
Gills			
PROTEIN[a]	10.77 ± 0.45^{A}	11.79 ± 0.33^{A}	11.64 ± 0.77^{A}
TBARS[b]	0.64 ± 0.03^{A}	0.52 ± 0.02^{B}	0.48 ± 0.03^{B}
LOOH[c]	6.58 ± 0.33^{A}	2.85 ± 0.37^{B}	4.45 ± 0.57^{B}
SOD[d]	0.20 ± 0.07^{A}	0.31 ± 0.04^{A}	0.26 ± 0.05^{A}
CAT[e]	0.31 ± 0.03^{A}	0.32 ± 0.02^{A}	0.40 ± 0.06^{A}
GPx[f]	8.00 ± 0.43^{A}	10.90 ± 0.94^{A}	13.50 ± 2.13^{A}
GST[g]	0.72 ± 0.05^{A}	1.98 ± 0.08^{B}	1.14 ± 0.08^{B}
NPSH[h]	5.88 ± 0.20^{A}	6.85 ± 0.19^{A}	6.45 ± 0.36^{A}
AA[i]	20.0 ± 1.63^{A}	16.9 ± 2.23^{A}	21.0 ± 2.42^{A}
TRAP[j]	0.83 ± 0.13^{A}	1.60 ± 0.13^{AB}	1.98 ± 0.29^{B}
Liver			
PROTEIN[a]	12.27 ± 0.33^{A}	11.90 ± 0.28^{A}	11.79 ± 0.30^{A}
TBARS[b]	0.25 ± 0.01^{A}	0.16 ± 0.01^{B}	0.17 ± 0.01^{B}
LOOH[c]	5.57 ± 0.27^{A}	3.22 ± 0.32^{B}	4.12 ± 0.23^{B}
SOD[d]	0.43 ± 0.03^{A}	1.03 ± 0.03^{B}	1.17 ± 0.08^{B}
CAT[e]	1.55 ± 0.12^{A}	3.09 ± 0.08^{B}	2.60 ± 0.06^{AB}
GPx[f]	13.30 ± 0.96^{A}	16.09 ± 0.74^{A}	15.34 ± 1.75^{A}
GST[g]	0.08 ± 0.01^{A}	0.16 ± 0.01^{B}	0.12 ± 0.01^{AB}
NPSH[h]	2.70 ± 0.08^{A}	3.10 ± 0.07^{B}	3.10 ± 0.05^{B}
AA[i]	7.23 ± 1.81^{A}	14.50 ± 1.01^{B}	13.60 ± 0.94^{B}
TRAP[j]	1.84 ± 0.15^{A}	3.04 ± 0.17^{B}	2.93 ± 0.15^{B}

[a] Protein content (mg/mL), [b] Thiobarbituric acid-reactive substances (μmol/mg protein), [c] Lipid hydroperoxide (nmol/mg protein), [d] Superoxide dismutase (SOD units/mg protein), [e] Catalase (ρmol/min/mg protein), [f] Glutathione peroxidase (nmol/min/mg protein), [g]Glutathione S-transferase (ρmol/min/mg protein), [h] Nonprotein thiols (nmol/mg protein), [i] Ascorbic acid (μmol/mg protein), [j] Total reactive antioxidant potential (μMTrolox/mg protein)

Data are reported as the mean ± SEM ($n = 12$). Values within the same row having different superscripts are significantly different ($P < 0.05$)

Biomarkers of oxidative stress in the kidneys

The LPO levels, determined from the LOOH in the kidneys, were 42 and 52 % lower in fish fed diets containing 0.15 and 0.30 % rutin than in the control, respectively. The CAT activity was 42 % higher in the fish receiving 0.15 % rutin than in the control. The GST activity was 100 % higher in the fish receiving 0.15 % rutin than in the control. There was also a significant increase (86 %) in comparison with the diet of 0.30 % rutin. The TRAP was 75 and 65 % higher in the fish receiving 0.15 and 0.30 % rutin than in the control, respectively. By contrast, the TBARS levels, the GPx activity, and the NPSH and AA content did not differ among the various experimental groups (Table 5).

Biomarkers of oxidative stress in the muscle

The protein contents did not differ among the experimental groups. The LPO levels, determined from the

Table 5 Biomarkers of oxidative stress in the kidneys and muscle of *Rhamdia quelen* fed diets containing different concentrations of rutin

	Diets (% rutin)		
	0	0.15	0.30
Kidneys			
PROTEIN[a]	19.41 ± 0.42[A]	18.77 ± 0.44[A]	20.03 ± 0.45[A]
TBARS[b]	0.67 ± 0.01[A]	0.70 ± 0.01[A]	0.69 ± 0.02[A]
LOOH[c]	5.00 ± 0.22[A]	2.9 ± 0.25[B]	2.4 ± 0.34[B]
CAT[e]	0.98 ± 0.06[A]	1.39 ± 0.09[B]	1.18 ± 0.09[AB]
GPx[f]	6.08 ± 0.37[A]	8.52 ± 1.58[A]	6.64 ± 0.67[A]
GST[g]	0.14 ± 0.01[A]	0.28 ± 0.03[B]	0.15 ± 0.01[AC]
NPSH[h]	3.86 ± 0.13[A]	3.85 ± 0.15[A]	3.87 ± 0.24[A]
AA[i]	13.52 ± 1.74[A]	15.41 ± 5.17[A]	19.32 ± 2.12[A]
TRAP[j]	0.77 ± 0.05[A]	1.35 ± 0.04[B]	1.27 ± 0.05[B]
Muscle			
PROTEIN[a]	10.07 ± 0.34[A]	10.61 ± 0.40[A]	10.55 ± 0.74[A]
TBARS[b]	0.37 ± 0.02[A]	0.26 ± 0.01[B]	0.29 ± 0.01[B]
LOOH[c]	5.81 ± 0.42[A]	3.79 ± 0.24[B]	2.70 ± 0.32[B]
SOD[d]	3.40 ± 0.20[A]	3.86 ± 0.18[A]	4.14 ± 0.31[A]
GPx[f]	9.17 ± 0.74[A]	19.54 ± 1.23[B]	16.06 ± 2.54[B]
GST[g]	0.62 ± 0.04[A]	0.69 ± 0.05[A]	0.72 ± 0.07[A]
NPSH[h]	4.37 ± 0.25[A]	4.90 ± 0.29[AB]	6.04 ± 0.41[B]
AA[i]	3.37 ± 0.87[A]	5.37 ± 1.10[AB]	9.41 ± 1.51[B]
TRAP[j]	1.49 ± 0.12[A]	2.80 ± 0.15[B]	2.84 ± 0.21[B]

[a] Protein content (mg/mL), [b] Thiobarbituric acid-reactive substances (μmol/mg protein), [c] Lipid hydroperoxide (nmol/mg protein), [d] Superoxide dismutase (SOD units/mg protein), [e] Catalase (ρmol/min/mg protein), [f] Glutathione peroxidase (nmol/min/mg protein), [g] Glutathione S-transferase (ρmol/min/mg protein), [h] Nonprotein thiols (nmol/mg protein), [i] Ascorbic acid (μmol/mg protein), [j] Total reactive antioxidant potential (μMTrolox/mg protein)

Data are reported as the mean ± SEM ($n = 12$). Values within the same row having different superscripts are significantly different ($P < 0.05$)

TBARS in the muscle, were 30 and 22 % lower in the fish receiving 0.15 and 0.30 % rutin, respectively, than in the control; this was also true for the LOOH measurements, which were 35 and 54 % lower in the silver catfish fed with 0.15 and 0.30 % rutin, respectively. The GPx activity was 113 and 81 % higher in the fish receiving 0.15 and 0.30 % rutin, respectively, than in the control. The non-enzymatic antioxidant level determined from the NPSH content was 38 % higher in the fish receiving 0.30 % rutin than in the control. The AA content was 179 % higher in the fish receiving 0.30 % rutin than in the control. The TRAP was 87 and 90 % higher in the fish receiving 0.15 and 0.30 % rutin than in the control, respectively. By contrast, the SOD and GST activity did not differ among the various experimental groups (Table 5).

Pituitary hormones expression

No differences in the expression of pituitary hormones were observed after 21 days of food with rutin (Fig. 3).

Discussion

Recent studies have demonstrated that the addition of antioxidants to the diets of fish of commercial interest can significantly increase productivity and improve fish health (Kütter et al. 2014; Li et al. 2014). Rutin is a flavonol glycoside composed of flavonol quercetin and disaccharide rutinose, which exhibited the capacity of limiting LPO, scavenging superoxide anion and

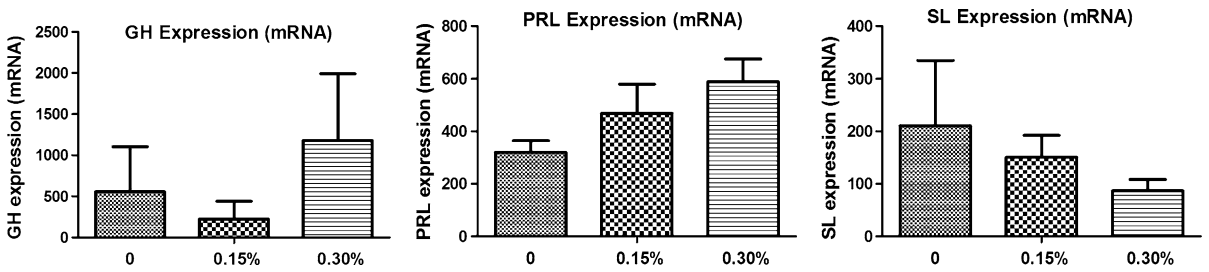

Fig. 3 Growth hormone (GH), prolactin (PRL) and somatolactin (SL) expression in the pituitary of *Rhamdia quelen* fed diets containing different concentrations of rutin. For better visualization of results, the data were multiplied by 1000. The data appear as the mean ± SEM ($n = 8$). The data are not significantly different from the control at $P > 0.05$

chelating metal ions such as ferrous cations, responsible for catalyzing the formation of ROS (Cos et al. 1998). The antioxidant properties of flavonoids have been attributed to the presence of phenolic compounds, which act as radical scavengers and occasionally as metal chelators (Hotta et al. 2002). However, little is known about the antioxidant effects of natural phenolic compounds in fish, and no studies have yet established the relationship of dietary fish rutin as an antioxidant. Our results showed that diets containing rutin presented high levels of phenolic compounds. In this study, we demonstrated that rutin showed positive effects by an improved response to antioxidants in the tissues of silver catfish, which may be attributed to the high content of phenolic compounds observed in the diets containing rutin.

In the plasma cortisol levels, there was a decrease in fish fed diets containing 0.15 % rutin compared with the control. Several reports in the scientific literature have described observations of differing cortisol responses among fish of different species (Adamante et al. 2008; Fagundes and Urbinati 2008). The blood parameters (HCT, HGB and MCHC in whole blood and plasma levels of GLU, LDH, TRI, CHO, LDL, HDL and URE) were not influenced by diets containing rutin compared with the control, which demonstrates that the use of diets containing rutin for 21 days is not harmful to the fish, considering that hematological variations can be caused by any agent stressor and constitute an index of health status in a number of fish species (Aubin et al. 2001).

The brain is particularly vulnerable to oxidative damage due to its high oxygen consumption, high content of polyunsaturated fatty acids and the presence of transition metals as Fe and Cu, which can lead to ROS formation via the Fenton reaction (Mieiro et al. 2011). The addition of rutin to the silver catfish's diet caused a reduction in the LPO levels in the brain, as measured by the TBARS and LOOH levels. Menezes et al. (2014b) demonstrated that the addition of 3.0 mg/kg of diphenyl diselenide in the diet of *Cyprinus carpio* during 60 days also reduced the TBARS in the brain. Similar to other animals, fish have antioxidant defense mechanisms that help to maintain their health and prevent oxidation lesions. The CAT and SOD are important antioxidant enzymes (Halliwell and Gutteridge 1999; Tocher et al. 2002). The SOD exhibited higher activity in the brain of silver catfish fed with 0.15 and 0.30 % rutin compared with the control. The CAT activity was higher in the brain of silver catfish fed with 0.15 % rutin compared with the control. The CAT and SOD are scavengers of ROS and act on H_2O_2 and O_2^-, respectively (Tocher et al. 2002).

Moreover, the NPSH content, as an indirect measure of GSH, was also higher in the brain of silver catfish fed with any of the diets supplemented with rutin than those fed with the control diet. GSH, an important non-enzymatic antioxidant that plays a central role in second-line antioxidant defenses, can reduce ROS and is a substrate for GPx and GST activities (Elia et al. 2011). Ascorbic acid has several functions in the central nervous system, including its action as an important antioxidant that is able to scavenge oxygen and nitrogen radical species (Harrison and May 2009). The AA content was higher in the brain of silver catfish fed with 0.15 % rutin compared with the control. Furthermore, the TRAP was higher in the brain of fish fed with any of the diets supplemented with rutin than those fed with the control diet. This type of defense includes a variety of compounds bearing different reactive centers

(phenols, thiols) with widely different hydrophobicities that allow the trapping of both hydrophobic and hydrophilic radicals. In this regard, there is great interest in the evaluation of the TRAP (Evelson et al. 2001).

The gills of fish are a multifunctional organ and perform vital functions such as respiration, osmoregulation, acid–base balance, and nitrogenous waste excretion (Evans et al. 2005). The addition of rutin to the silver catfish's diet caused a reduction in the LPO levels and increased the GST activity and TRAP (only diet 0.30 %). The LPO levels have decreased because of the ability of rutin to scavenge free radicals, regulate endogenous antioxidants status, chelate metal catalysts and maintain a pro-oxidant/antioxidant balance. Monserrat et al. (2014) demonstrated that the addition of 6000 mg/kg of lipoic acid in the diet (fed over 8 days) also resulted in decreased LPO levels and unlike our study, decrease in the GST activity in the gills of *Jenynsia multidentata.*

The liver is the main organ of various key metabolic pathways and the most frequently studied tissue regarding OS. The addition of rutin to the silver catfish's diet caused a reduction in LPO levels and an increase in the SOD activity in the liver. The CAT activity was higher in the liver of silver catfish fed with 0.15 % rutin. The enhanced activity of SOD and CAT in the liver in response to rutin supplementation may be responsible for the decrease in hepatic LPO levels. SOD is considered as the first line of defense against the deleterious effects of oxygen radicals in the cells, and it scavenges ROS by catalyzing the dismutation of superoxide to H_2O_2. CAT acts as a preventive antioxidant and plays an important role against the harmful effects of LPO. Thus, an increase in these enzymes can prevent the damage caused by ROS. These data are in agreement with those reported by Elia et al. (2011) that supplemented the diet of *Cyprinus carpio* with 1.0 mg/kg selenium during 8 weeks and with Li et al. (2014), who also reported that LPO levels were reduced with the addition of 25, 50, 100, 200 and 400 mg/kg of vitamin E in the diet of *Ctenopharyngodon idellus* during 8 weeks. The GST activity was higher in the silver catfish receiving diets containing 0.15 % rutin than in the control. These findings are in agreement with those of Monserrat et al. (2014).

The levels of the non-enzymatic antioxidants (NPSH and AA) were higher in the liver of silver catfish receiving diets containing rutin than those fed with the control diet. Elia et al. (2011) also reported increases in NPSH content. Moreover, Menezes et al. (2013) reported that the addition of 3.0 mg/kg diphenyl diselenide in the diet of *Cyprinus carpio* during 60 days also increased the levels of NPSH and AA in the liver. The TRAP was higher in the fish receiving the two concentrations of rutin compared with the control.

The kidneys play a vital role in the maintenance of an organism's internal environment and are key to the extracellular fluid volume, composition and acid–base balance regulation, presenting an additional function related to hematopoiesis (Oliveira et al. 2008). In the kidneys, the LPO levels, determined from the LOOH, were reduced by rutin supplementation. The CAT and GST activity was higher in the fish receiving 0.15 % rutin; the GST activity was also higher in the fish fed with the diet of 0.30 % rutin. Zhao et al. (2013) also demonstrated decrease in LPO measured of TBARS in the kidneys of *Cyprinus carpio* fed diet containing 7.0, 9.5, 11.9, 13.9 and 16.9 g/kg isoleucine for 60 days; and increased activities of CAT and GST (13.9 g/kg isoleucine). The TRAP was higher in the fish receiving 0.15 and 0.30 % rutin. The interest in this type of determination is because they can provide information regarding the system's capacity to withstand OS unbalances (Evelson et al. 2001).

LPO is an important cause of the deterioration of muscle. The fish muscle is particularly susceptible to lipid oxidation because of the coexistence of highly oxidizable long-chain polyunsaturated fatty acids (PUFAs) and pro-oxidant substances with the ability to catalyze lipid oxidation, such as redox-active metals and hemeproteins (Erickson 2002). The LPO levels were lower in the muscle of silver catfish receiving diets containing rutin. The GPx activity was higher in the fish receiving 0.15 and 0.30 % rutin. These results are similar to Elia et al. (2011) findings. GPx catalyzes the conversion of both H_2O_2 and organic hydroperoxides to less reactive products, employing GSH in its reduced form as an electron donor (Lackner 1998).

Moreover, NPSH, AA content and TRAP were higher in the muscle of fish receiving 0.30 % rutin than in the control. Similar to our results, Menezes et al. (2013) also showed increased levels of NPSH and AA in muscle tissue.

Tissues and organs have different rates of metabolic activity and oxygen consumption, and their levels of

antioxidants are different. In general, the susceptibility of a given organ to LPO is determined by different predispositions. For example, for xenobiotic accumulation, characteristic antioxidant basal levels, adaptation capacity and consequent antioxidant activation, and metabolic rates increase the potential to produce ROS and challenge the respective defenses (Oliveira et al. 2008).

In addition to these antioxidant effects of rutin on the tissues of silver catfish, it was also observed that the expression of pituitary hormones did not change, demonstrating that this flavonoid did not cause hormonal dysfunctions in the pituitary axis.

The dysfunction of these hormones is well characterized when the fish face stressful situations. The increase in PRL was observed when silver catfish were exposed to manganese (Dolci et al. 2014). GH has also been associated with stress (Rotllant et al. 2000). Menezes et al. (2014c) observed lower values of GH expression in the fed silver catfish under high stocking density group, although its values were enhanced in the food-deprived fish under low stocking density group. Laiz-Carrión et al. (2009) observed an increase in SL expression levels in gilthead sea bream maintained under lower food conditions and high density.

Thus, the fish fed with diet containing rutin were able to maintain hyposmotic environmental acclimatization. Furthermore, no increase in stress levels was demonstrated by the results too, suggesting that the diet probably also would not change the osmoregulation, reproduction and intermediary metabolism of silver catfish, but more studies are needed to highlight these mechanisms.

In the present study, the addition of flavonoid rutin to the silver catfish's diet caused a reduction in the LPO levels of most tissues and was occasionally accompanied by the modulation of the antioxidant defense. Thus, rutin exerts an antioxidant effect on lipid membranes. This effect is attributed to its anti-free radical properties, mainly directed at the superoxide and hydroxyl radicals, which are highly reactive species involved in the initiation of the LPO chain (Afanas'ev et al. 1989). Furthermore, both rutin concentrations added to the diet were beneficial, resulting in an increased antioxidant response. Apart from being a natural product with low cost to the producer, it may be an important tool to minimize physiological changes and improve animal welfare. This finding is in agreement with the results found in the phenolic compounds, which were present in larger amounts in the lower concentration of rutin in the diet.

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