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Rhamdia quelen (Quoy & Gaimard, 1824), submitted to a stressful condition: effect of dietary addition of the essential oil of *Lippia alba* on metabolism, osmoregulation and endocrinology

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The aim of this study was to evaluate the effect of the essential oil of *Lippia alba* (EOLA) as a feed additive on ionoregulatory and metabolic parameters and pituitary hormones expression in silver catfish, *Rhamdia quelen*, submitted to a stressful condition (stocking density of 10.6 kg m^{-3} and limited space). Fish were fed with different concentrations of EOLA (0.0 – control, 0.25 and 0.50 mL kg $food^{-1}$) for 20 days. Metabolic parameters were not affected by the diet, with the exception of alanine aminotransferase, which was higher in the liver of fish fed 0.50 mL EOLA kg $food^{-1}$. Plasma ions and activity of H^+-ATPase did not change, but fish fed 0.25 mL EOLA kg $food^{-1}$ presented higher Na^+/K^+-ATPase activity. Somatolactin expression in the pituitary was higher in the fish fed 0.25 mL EOLA kg food $^{-1}$, but the expression of growth hormone and prolactin did not change. Therefore, dietary EOLA does not exert a protective effect in *R. quelen* submitted to a stressful situation because it did not alter most measured parameters. The use of 0.25 mL EOLA kg $food^{-1}$ seems to be more suitable than 0.50 mL EOLA kg $food^{-1}$ since the latter may be related to liver damage.

O objetivo deste estudo foi avaliar o efeito do óleo essencial de *Lippia alba* (OELA) como aditivo em rações na ionoregulação, parâmetros metabólicos e expressão de hormônios hipofisários em jundiás, *Rhamdia quelen*, submetidos a uma situação estressante (densidade de estocagem de 10,6 kg m^{-3} e espaço limitado). Os peixes foram alimentados com diferentes concentrações de OELA (0,0 - controle, 0,25 e 0,50 mL kg de $ração^{-1}$) durante 20 dias. Parâmetros metabólicos não foram afetados pela dieta, com a exceção da alanina aminotransferase, que foi mais elevada no fígado dos peixes alimentados com 0,50 mL de OELA kg de $ração^{-1}$. Íons plasmáticos e a atividade da H^+-ATPase não apresentaram nenhuma alteração, mas os peixes alimentados com 0,25 mL OELA kg de $ração^{-1}$ apresentaram maior atividade da Na^+/K^+-ATPase. A expressão da somatolactina na hipófise de peixes alimentados com 0,25 mL OELA kg de $ração^{-1}$ aumentou, porém a expressão do hormônio de crescimento e da prolactina não mudou. Portanto, a adição do OELA na ração não tem um efeito protetor em jundiás submetidos a uma situação estressante, pois não influiu na maioria dos parâmetros medidos. O uso de 0,25 mL OELA kg de $ração^{-1}$ parece ser mais adequado que 0,50 mL OELA kg de $ração^{-1}$, uma vez que este nível de inclusão pode estar relacionado a danos hepáticos.

Keywords: Cortisol, Enzymatic activity, Growth hormone, Somatolactin.

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Introduction

Addition of herbal extracts in fish feed is increasingly seen as a safe and practical alternative to synthetic pharmaceuticals. Some studies showed that dietary addition of plants has several advantages. The addition of 0.5% of *Massa medicata*, *Crataegi fructus*, *Artemisia capillaries* or *Cnidium officinale* to the diet led to better use of lipids in stress and recovery of *Pagrus major* (Ji *et al*., 2009). The use of the oregano essential oil (*Origanum heracleoticum*) as a supplement in the food improved growth, antioxidant status and resistance against *Aeromonas hydrophila* in channel catfish (*Ictalurus punctatus*) (Zheng *et al.,* 2009) and the extract of *Allium sativum* in the diet also promoted growth and reduced mortality rate of Nile tilapia (*Oreochromis niloticus*) challenged with *Aeromonas hydrophila* (Shalaby *et al.,* 2006).

The plant *Lippia alba* (Verbenaceae) is found in South and Central America and tropical areas of Africa (Terblanche & Kornelius 1996). In *Rhamdia quelen* (Quoy & Gaimard 1824), the essential oil of *L. alba* (EOLA) is an effective anesthetic (Cunha *et al.,* 2010) and sedative for transport (Azambuja *et al.,* 2011; Becker *et al.,* 2012), can delay lipid peroxidation (LPO) during frozen storage of fillets (Veeck *et al.,* 2013) and decreases LPO and increases tissue antioxidant response when added to the diet (Saccol *et al.,* 2013). The EOLA is also an effective anesthetic for the sea horse (*Hippocampus reidi*) (Cunha *et al.,* 2011).

The pituitary hormones control several physiological processes. Growth hormone (GH) is related to growth, metabolism, stress (Laiz-Carrión *et al.,* 2009; Sinha *et al.,* 2012), and osmoregulation (Sakamoto & McCormick 2006). Prolactin (PRL) also participates in the control of growth, stress and osmoregulation (Sakamoto & McCormick 2006; Laiz-Carrión *et al.,* 2009), while somatolactin (SL) is apparently related to energy balance, acid-base equilibrium (Kakizawa *et al.,* 1996; Furukawa *et al.,* 2010), stress (Laiz-Carrión *et al.,* 2009) and metabolism (Company *et al.,* 2001). Cortisol, produced by the interrenal cells, is the main glucocorticoid and mineralocorticoid steroid in fish, and is a good indicator for assessing primary stress (Mommsen *et al.,* 1999).

Rhamdia quelen can be found from Argentina to southern Mexico (Perdices *et al.,* 2002), and is the most raised native species in South Brazil (Baldisserotto, 2009). A recent study demonstrated that feeding *R. quelen* with different levels of dietary EOLA for 60 days does not affect growth, but alters some metabolic parameters and improves the antioxidant status (Saccol *et al.,* 2013). However, it is not known whether the effect of EOLA on anesthesia and antioxidant status may induce any protective effect in *R. quelen* exposed to non-optimal culture situations, as at a stocking density of 10.6 kg m^{-3}, which is considered stressful for this species when fish are maintained in a limited space (tanks or cages around up to 300-400 L) (Barcellos *et al*., 2004; Menezes *et al*., 2015). Consequently, the aim of this study was to evaluate metabolic, osmoregulatory and endocrinological parameters of *R. quelen* fed with a diet containing different levels of EOLA and submitted to a stressful situation.

Material and Methods

Essential oil. The plant *L. alba* was cultivated in the Centro de Educação Superior do Norte (CESNORS-UFSM) - Frederico Westphalen *Campus*. A voucher specimen (SMDB 10050) was deposited in the herbarium of the Department of Biology (UFSM). Botanical identification was made by Gilberto Dolejal Zanetti (Department of Industrial Pharmacy, UFSM). The EOLA was obtained from fresh leaves of *L. alba* by hydrodistillation in a Clevenger apparatus for 2 h (Council of Europe (COE), 2007) and stored at -20°C until use. The composition of the EOLA was the same as that described by Saccol *et al.* (2013): the major components of the EO were linalool (55.26%), 1,8-cineole (7.85%),γ-muurolene (4.63%), β-caryophyllene (3.15%) and *E*-carveol (2.79%).

Experimental design. The experiment was conducted through January and February 2013 (summer), natural photoperiod (15 h light, 9 h darkness) in a continuously aerated recirculation system at the Laboratory of Fish Physiology, Universidade Federal de Santa Maria (UFSM). Adult *R. quelen* (132.74 ± 10.24 g, 24.05 ± 0.55 cm, voucher number UFRGS 19612, Ichthyology Laboratory, Universidade Federal do Rio Grande do Sul) of both sexes were obtained from the Fish Culture sector (UFSM) and placed in 60-L tanks (4 fish/tank) at a stocking density of 10.6 kg m^{-3}.

The animals were fed to satiation once a day (15:00) with a diet (34% crude protein) formulated according to Lazzari *et al.* (2007) (Table 1). Three concentrations of the EOLA in the diet (0-control, 0.25 and 0.50 mL kg $food^{-1}$) were added to the ingredients together with canola oil. The EOLA concentrations were the lowest concentrations that improved antioxidant status in *R. quelen* (Saccol *et al.,* 2013). The amount of feed offered and the unconsumed remains were weighed to determine feed intake. The fish (n=10/dietary EOLA concentration) were fed with the control diet for one week prior to the experiment for acclimation to the system and to the diet, and then for additional 20 days with the treatment diets. Uneaten food and feces were siphoned 30 minutes after feeding, and the water removed in this process was replaced with water under the same conditions and proportions found in the system.

The experimental protocol was approved by the Committee on Animal Experimentation - UFSM, under the registration number 46/2010.

Table 1. Formulation of the experimental diets. [a]Vitamin and mineral mixture (security levels per kilogram of product)-folic acid: 250 mg, pantothenic acid: 5000 mg, antioxidant: 060 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: 1000000 UI, vitamin B1: 1250 mg, vitamin B12: 3750 mcg, vitamin B2: 2500 mg, vitamin B6: 2485 mg, vitamin C: 28000 mg, vitamin D3: 500000 UI, vitamin E: 20000 UI, vitamin K: 500 mg, zinc: 17500 mg.

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		
Analysis of the feed (%)	0.00 mL kg^{-1}	0.25 mL kg^{-1}	0.50 mL kg^{-1}
Dry matter	95.43	95.48	95.56
Ashes	14.97	15.15	15.58
Crude protein	38.96	38.98	39.07
Fat	10.42	9.92	10.08

Water sampling and analyses. The water parameters measured daily during the experimental period and their respective values were: dissolved oxygen 6.85 ± 0.12 mg L^{-1} and temperature 24.0 °C ± 0.2 (oxygen meter Y5512; YSI Inc., Yellow Springs, OH, USA); pH 7.15 ± 0.06 (DMPH-2 pH meter, Digimed, São Paulo, SP, Brazil); total ammonia nitrogen levels 0.85 ± 0.11 mg L^{-1} (American Public Helth Association (APHA), 2005) and un-ionized ammonia (NH_3) levels 0.007 ± 0.001 mg L^{-1} (Colt, 2002). Alkalinity 29.5 ± 1.0 mg L^{-1} $CaCO_3$ (Boyd & Tucker, 1992) and water hardness 26.0 ± 1.4 mg L^{-1} $CaCO_3$ (EDTA titrimetric method) were determined weekly.

Sample collection. After being fasted for 24 h, fish were anesthetized with 50 mg L^{-1} eugenol and blood was collected by caudal puncture with heparinized 5 mL syringes. The samples were centrifuged at 1000 xg for 5 min and the plasma was stored at -20°C until analyses. The fish were then euthanized and the pituitary gland, gills, liver and muscle were excised and stored in a -80° C freezer.

RNA Extraction and cDNA synthesis. Total RNA was extracted from pituitary using Trizol reagent (Invitrogen) according to manufacture 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 considered pure and samples below this threshold were discarded. Total RNA (1µg) 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.

Pituitary expression of GH, PRL and SL mRNA. The mRNA expression was analyzed through qRT-PCR, using the StepOnePlus™ RT-PCR system (Applied Biosystems) with Power SYBR Green PCR Master Mix (Applied Biosystems). The sequences used to design all the primers were according to Baldisserotto *et al.* (2014) using the Primer Express software 3.3 (Applied Biosystems). The results were normalized to the expression of the constitutive gene β-actin according to Baldisserotto *et al.* (2014). The calculation of relative expression was performed as recommended by Pfaffl (2001).

Cortisol. Plasma cortisol was determined in duplicates using an enzyme-linked immunosorbent assay (ELISA) kit (Diagnostics Biochem Canada Inc., Canada). Absorbance was determined in spectrophotometer at 450 nm, and the inter- and intra-assay variation coefficients were 5.15±0.53% and 4.13±0.67% respectively. The specificity of the test was evaluated by comparing the standard curve and serial dilutions of the plasma samples. The curve obtained using serial dilutions of *R. quelen* plasma ran parallel to the curve constructed with human standards provided by the kit.

Biochemical measurements. The protein content in liver and muscle was measured according to Lowry *et al.* (1951) using bovine serum albumin as standard. Plasma glucose and lactate were measured with Labtest kits (Lagoa Santa, MG, Brazil). Glycogen and glucose in the liver and muscle were determined according to Dubois (1956). Lactate in the muscle was determined as in Harrower & Brown (1972) and total lipids were determined in the liver and muscle by the method of Bligh & Dyer (1959). Activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in plasma and liver were determined colorimetrically according to Reitman & Frankel (1957).

Ionoregulatory measurements. Plasma Cl^- levels were determined according to Zall *et al.* (1956) and Na^+ and K^+ with a flame spectrophotometer B262 (Micronal, São Paulo, Brazil). Standard solutions were made with analytical reagent grade (Vetec Merck) dissolved in deionized water, and standard curves for each ion were tested at five different concentrations. The activities of Na^+/K^+-ATPase and H^+-ATPase were assessed in the gills as described by Gibbs & Somero (1989).

Statistical Analyses. A Levene test was conducted to evaluate the homogeneity of variances. The data were compared using one-way analysis of variance (ANOVA) followed by the Tukey's test. The data regarding GH were not homocedastic and were compared using the Kruskal-Wallis test followed by the multiple comparison of mean ranks for all groups. All analyzes were performed with the software Statistica 7.0 (Stat Soft, Tulsa, OK). The minimum level of significance was $P < 0.05$. The results were expressed as the mean ± standard error of the mean (SEM).

Results

Feed consumed and metabolic parameters. The amount of feed consumed per day was similar in the different treatments (g feed kg fish^{-1}): 1.70 ± 0.1 g kg^{-1} for control, 1.32 ± 0.1 g kg $^{-1}$ for those fed 0.25 mL EOLA kg food^{-1} and 1.36 ± 0.2 g kg^{-1} for those fed 0.50 mL EOLA kg food^{-1}. Glycogen (liver and muscle), glucose (plasma, liver and muscle), lactate (plasma and muscle), protein, total lipids, plasma cortisol (Fig. 1 and Table 2) and AST (plasma and liver) (Fig. 2A) were not affected by the dietary EOLA. Hepatic ALT of fish fed 0.50 mL EOLA kg food^{-1} was significantly higher compared to the other treatments (Fig. 2B).

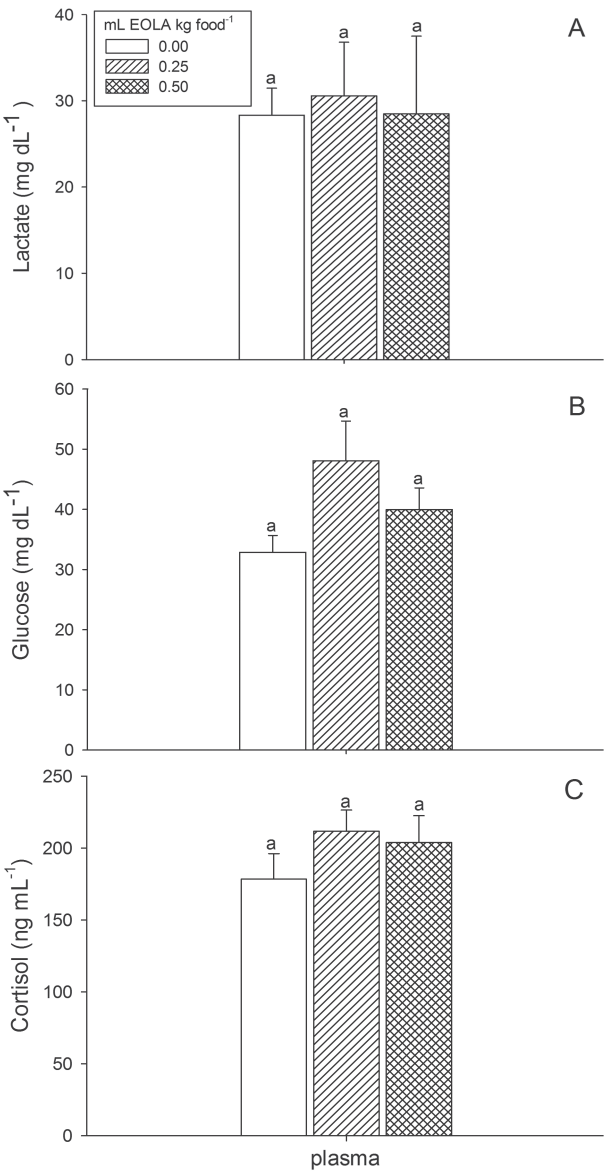

Fig. 1. Plasma lactate (A), glucose (B) and cortisol (C) of *Rhamdia quelen* fed with different concentrations of dietary essential oil of *Lippia alba* (EOLA). Mean ± SEM. Different letters indicate significant differences (P < 0.05) between treatments.

Table 2. Biochemical parameters in liver and muscle of *Rhamdia quelen* fed with diets containing different concentrations of the essential oil (EO) of *Lippia alba*. Glycogen (µmol g tissue^{-1}), glucose (mg dL^{-1}), lactate (mg dL^{-1}), protein (mg g^{-1}), total lipids (% fat). Values are reported as mean ± ES (n=10). Significantly different from control group (P<0.05).

	Diet (mL EO kg food^{-1})		
	0	0.25	0.50
LIVER			
Glycogen	7.24 ± 0.91	8.44 ± 1.10	9.57 ± 1.05
Glucose	276.80 ± 17.54	206.65 ± 26.50	222.58± 28.66
Protein	283.37 ±49.89	234.11 ± 16.65	301.86 ± 20.80
Total lipids	15.79 ±4.08	15.25 ± 0.23	17.61 ± 2.75
MUSCLE			
Glycogen	1.48 ± 0.23	1.11 ± 0.19	1.23 ± 0.10
Glucose	73.62 ± 12.33	103.04 ± 6.53	101.33 ± 5.60
Lactate	14.03 ± 1.21	13.68 ± 2.34	13.68 ± 1.24
Protein	162.11 ± 8.73	191.78 ± 17.39	184.25 ± 9.53
Total lipids	16.74 ±1.53	18.98 ± 5.61	22.90 ± 3.66

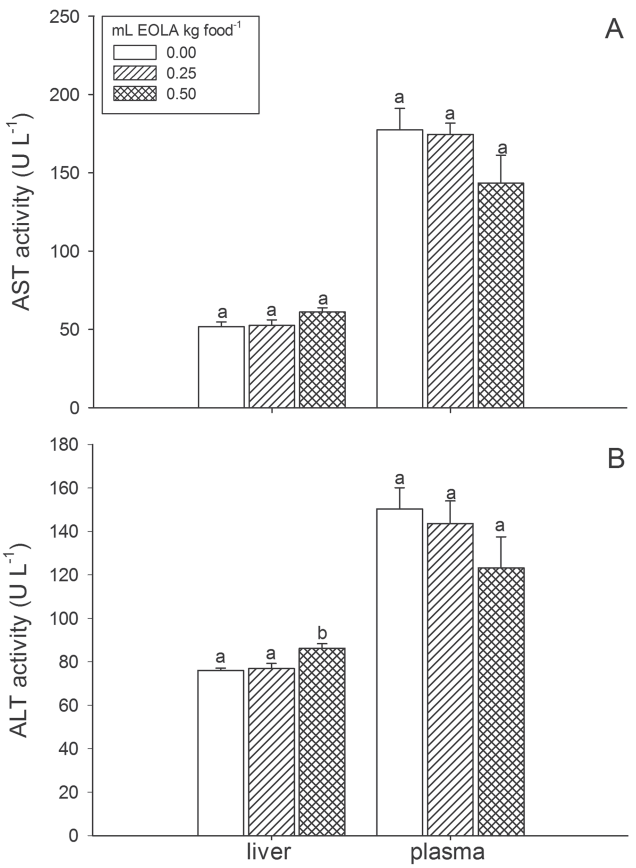

Fig. 2. Plasma and hepatic activities of aspartate aminotransferase (AST) (A) and alanine aminotransferase (ALT) (B) of *Rhamdia quelen* fed with different concentrations of dietary essential oil of *Lippia alba* (EOLA). Mean ± SEM. Different letters indicate significant differences (P < 0.05) between treatments.

Ions and enzyme activities. Fish fed 0.25 mL EOLA kg food^{-1} presented significantly higher Na^+/K^+-ATPase activity compared to the other treatments, but plasma Na^+, K^+ and Cl^- levels, as well as H^+-ATPase activity, were not significantly affected by the treatments (Fig. 3).

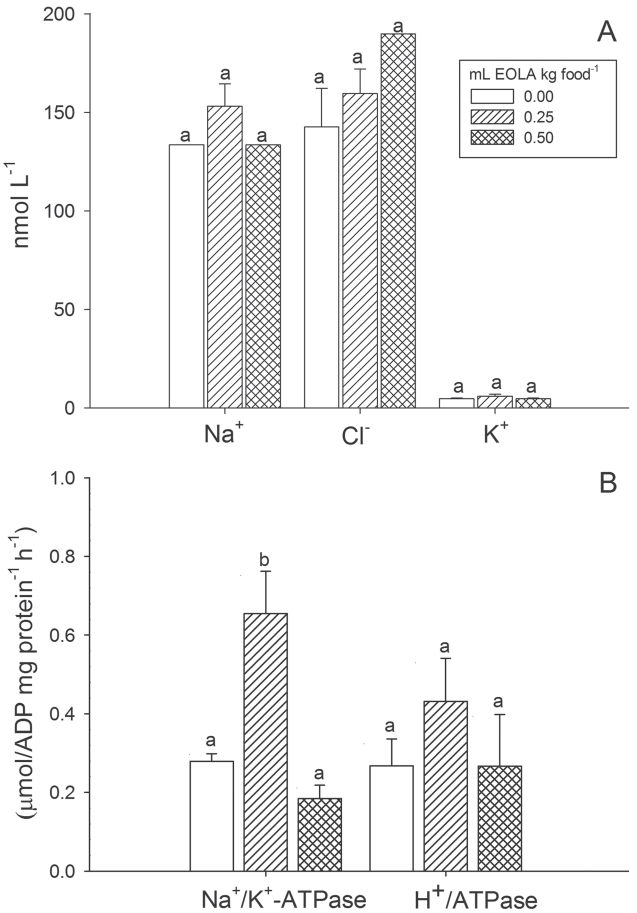

Fig. 3. Osmoregulatory parameters of *Rhamdia quelen* with different concentrations of dietary essential oil of *Lippia alba* (EOLA). A) plasma ion levels and B) gill Na^+/K^+-ATPase and H^+/ATPase activities. Mean ± SEM. Different letters indicate significant differences ($P < 0.05$) between treatments.

Hormones expression. Pituitary GH and PRL expressions did not change significantly between groups, but SL expression was higher in the group treated with 0.25 mL EOLA kg food^{-1} compared to the others (Fig. 4).

Discussion

High plasma cortisol levels were observed in the control group, demonstrating that the stocking density and the limited space of the 60 L tanks of the present experiment was stressful for *R. quelen* as previously indicated by Barcellos *et al*. (2004) and Menezes *et al*. (2015). According to Barcellos *et al*. (2001) plasma cortisol values for non-stressed adult *R. quelen* maintained in 100 m^3 ponds with stocking density of 0.8 kg m^3 are 15-30 ng mL^{-1}. Immersion anesthesia with EOLA prevented plasma cortisol increase in *R. quelen* subjected to handling (Cunha *et al.,* 2010), but dietary EOLA did not change the levels of such hormone, indicating that it did not have an effect upon stress parameters when administered through this route.

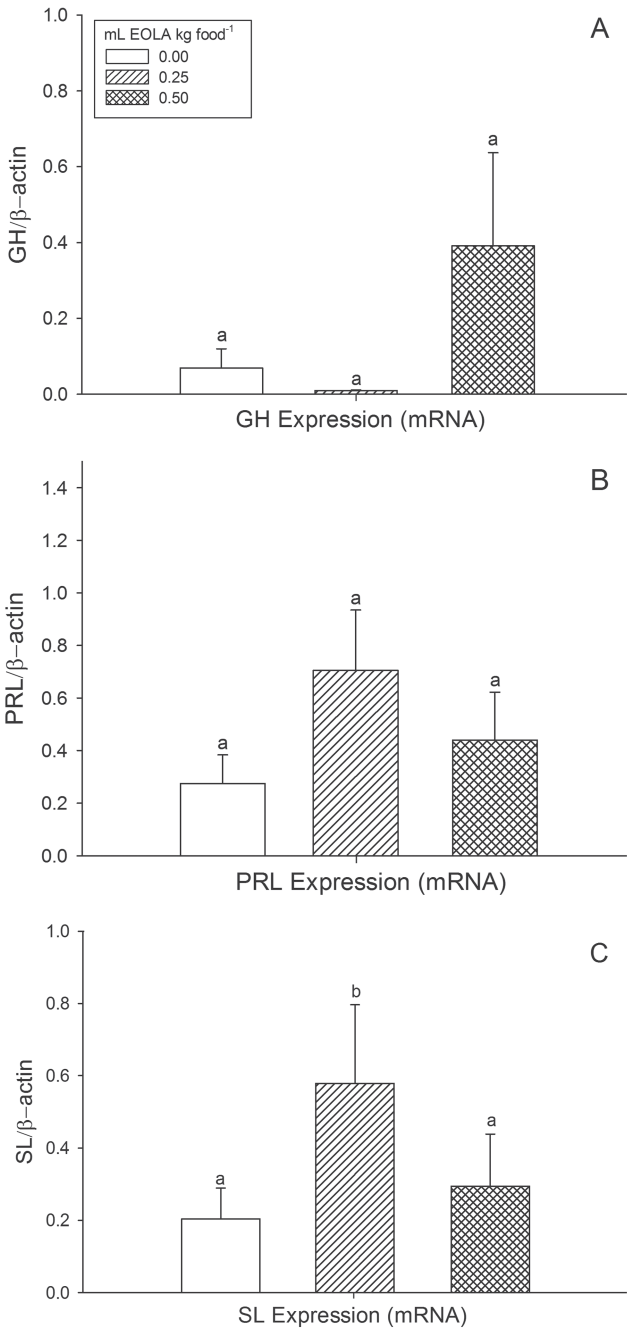

Fig. 4. Expression of pituitary hormones of *Rhamdia quelen* fed with different concentrations of dietary essential oil of *Lippia alba* (EOLA). A) growth hormone (GH), B) prolactin (PRL) and C) somatolactin (SL). Mean ± SEM. Different letters indicate significant differences ($P < 0.05$) between treatments.

Lactate, glycogen, glucose, protein and lipids are biochemical parameters commonly used to assess the metabolic state of fish tissues (Pretto *et al.,* 2014). Apparently, dietary addition of both EOLA concentrations for 20 days did not change *Rhamdia quelen* metabolism since these parameters were not affected. *R. quelen* fed with dietary EOLA for 60 days also did not show significant alteration in most of these biochemical parameters in the plasma, but the liver and muscle glycogen increased and hepatic glucose decreased in *R. quelen* fed 0.50 mL EOLA kg food^{-1} (Saccol *et al.,* 2013). Therefore, probably dietary EOLA only change *R. quelen* metabolism after a long-term treatment.

The enzymes AST and ALT are mainly used as biomarkers to assess liver damage, although they are also found in organs such as skeletal muscle, heart, pancreas and kidneys (Gharaei & Ghaffari, 2010). The increased ALT activity in the liver of silver catfish fed with the highest dietary EOLA concentration suggests that there was hepatocyte damage. However, the lack of alteration in plasma ALT and in plasma and liver AST indicates that the observed increase in hepatic ALT did not cause any serious damage. In agreement with this hypothesis, *R. quelen* fed with up to 2.0 mL EOLA kg food^{-1} for 60 days only decreased the glucose levels in the liver, resulting in an increase in the glycogen and lactate reserves in the liver (Saccol *et al.,* 2013).

The addition of EOLA to the water of transport reduced the net Na^+, K^+ and Cl^- losses in *R. quelen* (Becker *et al.,* 2012), and immersion anesthesia with this oil increased gill Na^+/K^+-ATPase and H^+/ATPase activities in this species (Toni *et al.,* 2014), indicating an osmoregulatory effect. However, the only osmoregulatory effect of dietary EOLA observed in the present experiment was the increase in gill Na^+/K^+-ATPase activity in fish fed with 0.25 mL EOLA kg food^{-1}. The expression of SL also increased in *R. quelen* fed with that concentration of EOLA, indicating that both effects may be related. Previous studies verified a negative relationship between expression of SL and gill Na^+/K^+-ATPase activity in chum salmon (*Oncorhynchus keta*) migrating from the ocean to the rivers (Onuma *et al.*, 2010) and in Atlantic salmon (*Salmo salar*) sampled in the later weeks of smoltification compared to those at the beginning (O'Keeffe *et al.*, 2008). However, both studies compared fish with different reproductive stages, which may have altered expression of SL. The Na^+/K^+-ATPase plays a major role in fish osmoregulation (Duarte *et al.,* 2013), while SL seems to be involved with acid-base regulation in rainbow trout (*Oncorhynchus mykiss*) (Kakizawa *et al.,* 1996) and correction of plasma osmotic balance in Mozambique tilapia (*Oreochromis mossambicus*) exposed to acidic freshwater (Furukawa *et al.,* 2010). The absence of significant difference in gill Na^+/K^+-ATPase activity and SL expression in the fish fed with 0.50 mL EOLA kg food^{-1} may be because at such concentration the effect was faster and the osmotic and/or acid-base equilibrium was fully reestablished.

Maintenance of GH expression is in accordance with the findings by Saccol *et al.* (2013), which demonstrated that dietary EOLA did not change *R. quelen* growth. Prolactin is considered one of the most important hormones related to freshwater adaptation, and it is essential for ion uptake as well as reduction in ion and water permeability of osmoregulatory surfaces (Sakamoto & McCormick, 2006). Its unaltered expression in *R. quelen* fed with dietary EOLA is probably related to the unchanged plasma ion levels.

In conclusion, dietary EOLA does not have a protective effect in *R. quelen* submitted to a stressful situation because it did not alter most measured parameters. The use of 0.25 mL EOLA kg food^{-1} may be more suitable than 0.50 mL EOLA kg food^{-1} since the latter increased ALT, indicating a possible liver damage.

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