

Liver histology and hematological parameters of female *Rhamdia quelen* fed different lipid sources

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ABSTRACT - The objective with this study was to evaluate the liver histology and hematological parameters of female *Rhamdia quelen* fed diets supplemented with different oils: 5% marine fish oil, 5% refined palm oil, 5% soybean oil, and a combination of the three. The lipid vacuolization (steatosis) in the liver was analyzed according to a score of vacuolization when: 0 = absence, 1 = reduced, 2 = intermediate, and 3 = intense. At the end of the experiment, females (n = 10) were selected from each treatment (two per cage), and the blood was collected for erythrocyte and biochemical analysis. Lower vacuolization indices in the liver were observed at the beginning of the experiment. However, with time, the presence of vacuoles was more evident but presented similar morphology among all treatments. The blood parameters were also not influenced by the different diets, except for glucose levels, which was higher in the treatment with the mixture of oils. The absence of differences regarding liver morphology (steatosis) and hematological parameters indicates that the replacement of fish oil by vegetable oils could be performed without damage to the health of *R. quelen* females.

Keywords: blood, fatty acids, fish, nutrition

Introduction

In fish farming, the diet represents the highest cost (Babalola and Apatá, 2012), especially when its composition is derived from animal source, such as meal and fish oil. However, due to scarcity and high cost of such resources, studies are being developed with its partial or total replacement by vegetable-origin products (Ng and Wang, 2011; Ng, 2004; Ayisi et al., 2018).

Among the lipid sources employed in the replacement of fish oil, soybean and palm oil (Turchini et al., 2009) can be highlighted. Soybean oil represents 19 to 30% of oleic acid (C18:1n-9); 44 to 62% of linoleic acid (C18:2n-6), the main representative of the omega-6 fatty acids series; and 4 to 11% of linolenic acid (C18:3n-3), the main representative of the omega-3 series (Brasil, 1999). Palm oil, on the other hand, is considered a superior source of energy because it contains a large amount of saturated fatty acids (48%), represented mainly by palmitic acid (C16:0), and monounsaturated acids (42%), represented by oleic acid (C18:1n-9) (Ng, 2004; Ng and Wang, 2011). Therefore, vegetable oils can be used for partial or complete replacement of fish oil in fish diets (Babalola et al., 2011; Ayisi et al., 2018; Ayisi and Zhao, 2014; Ng, 2002).

Histological changes in the liver are easily detectable when the feedings are not suitable (Tacon, 1992), and some studies show that the replacement of fish oil by vegetable oils can induce the occurrence

of steatosis in fish (Tessaro et al., 2014; Caballero et al., 2004). Consequently, the analysis of liver histology is essential in such types of study, which can be used as an indicator of the nutritional status of the individuals (Caballero et al., 2004; Raskovic et al., 2011). Additionally, lipid changes in the diet could also influence blood parameters, an indicator of the physiological equilibrium of the animal (Babalola et al., 2009).

The silver catfish, *Rhamdia quelen* (Quoy and Gaimard, 1824), are classified as an omnivorous species (Hernández et al., 2009), and some previous studies have demonstrated that this species can efficiently convert C18 into polyunsaturated fatty acids when feed diets contain vegetable oils (Vargas et al., 2015). Therefore, the objective of this study was to histologically analyze the liver and the blood parameters of *Rhamdia quelen* females fed different lipid sources.

Material and Methods

The experiment was carried out in Toledo, Paraná, Brazil (24°46'48.31" S; 53°43'25.77" W) from May 2013 to April 2014. All the procedures involving animals were conducted in accordance with the approved protocol of institutional committee on animal use (case number 6728/12).

Four isoproteic diets were formulated, containing 38% crude protein and 5% lipids: 5% of marine fish oil, 5% palm oil, 5% soybean oil, and a combination of the three (Mix) (Table 1). The experimental diets were extruded using an Ex-micro (Exteec) and dried under forced ventilation at 55 °C for 12 h. After weighing, the oil was added with a rotation mixer for 30 min, resulting in pellets of 4 mm.

Table 1 - Formulation and mean values of the composition of experimental diets

Item	Experimental diet			
	Soybean oil	Palm oil	Fish oil	Mix
Ingredient (g kg ⁻¹)				
Soybean meal	300.00	300.00	300.00	300.00
Maize (ground grain)	255.60	255.60	255.60	255.60
Salmon meal	200.00	200.00	200.00	200.00
Wheat gluten	93.60	93.60	93.60	93.60
Dicalcium phosphate	75.71	75.71	75.71	75.71
Soybean oil	50.00	-	-	16.60
Refined palm oil	-	50.00	-	16.60
Fish oil	-	-	50.00	16.60
Premix ¹	20.00	20.00	20.00	20.00
Salt (NaCl)	5.00	5.00	5.00	5.00
BHT (antioxidant)	0.20	0.20	0.20	0.20
Fungicide (propionic acid)	0.01	0.01	0.01	0.01
Nutrient (%) ²				
Dry matter	95.62	95.11	93.49	96.35
Crude protein	38.46	38.51	37.92	38.84
Ether extract	6.36	7.74	7.20	6.07
Crude fibre	1.72	2.61	2.19	2.27
Ash	12.93	12.93	12.81	13.20
Nitrogen-free extract	36.15	33.32	33.37	35.97

¹ Basic composition: vitamin A, 1,000,000 IU kg⁻¹; vitamin D3, 500,000 IU kg⁻¹; vitamin E, 20,000 IU kg⁻¹; vitamin K3, 500 mg kg⁻¹; vitamin B1, 1900 mg kg⁻¹; vitamin B2, 2000 mg kg⁻¹; vitamin B6, 2400 mg kg⁻¹; vitamin B12, 3500 mcg kg⁻¹; vitamin C, 50 g kg⁻¹; niacin, 5000 mg kg⁻¹; pantothenic acid, 4000 mg kg⁻¹; folic acid, 200 mg kg⁻¹; biotin, 40 mg kg⁻¹; manganese, 7500 mg kg⁻¹; zinc, 25 g kg⁻¹; iron, 12.5 g kg⁻¹; copper sulphate, 2000 mg kg⁻¹; iodine, 200 mg kg⁻¹; selenium, 70 mg kg⁻¹; BHT, 300 mg kg⁻¹.

² Weende analysis.

The experimental design was completely randomized with four treatments and five replicates. Two thousand fish were distributed in 20 concrete tanks with a total area of 12 m². Each tank received 100 juveniles of *R. quelen* with initial mean weight of 24.50±0.78 g. The fish remained in this system and were fed the experimental diets for five months until sex identification was possible. In October 2013, 20 females (total = 400) from each concrete tank were selected and transferred to twenty cages (4×2×1 m). Feeding rate was established to be 2% of body weight.day⁻¹, and fish were fed twice a day, scheduled at 10:00 and 16:00 h.

Water temperature was monitored twice daily, in the morning (25.5±1.59 °C) and in the afternoon (27.2±1.16 °C) with a YSI 550 A. Dissolved oxygen (YSI 550 A) and pH (Tecnal® Tec 5) were recorded weekly, determined at 06.00 h (5.36±1.64 mg L⁻¹ and 7.18±0.33) and at 16.00 h (7.64±2.73 mg L⁻¹ and 8.60±0.93), respectively. Ammonia levels were checked monthly (0.03±0.02) by the colorimetric method (Koroleff, 1976).

Liver sampling (central portion) was performed in October 2013, December 2013, February 2014, and April 2014, after five, seven, nine, and eleven months of feeding, respectively. Two females were randomly collected from each cage, euthanized (0.75 mg L⁻¹ benzocaine), and dissected for removal of the liver. Fragments of the organ (~0.5 cm) were fixed in Bouin solution for 24 h. Afterwards, the samples were washed in water and maintained in 70% ethanol until processing. Paraffin blocks were cut into 5-µm sections and stained with PAS (Periodic acid-Schiff) (Tolosa et al., 2003).

The lipid vacuolization (steatosis) in the liver was analyzed according to a score of vacuolization (0 = lack of vacuoles, 1 = reduced, 2 = intermediate, 3 = intense), according to Caballero et al. (2004) and Tessaro et al. (2014), with the use of a light microscope (400X) (Leica, DM 2500).

At the end of the experiment (April 14), two females were collected from each cage (10 females/treatment), anesthetized (75 mg L⁻¹ of benzocaine), and the blood collected (1.5 mL) by a caudal puncture with the aid of a syringe (without coagulant). Afterwards, 0.5 mL of the blood was poured into a tube containing 25 µL of anticoagulant (EDTA 10%) for erythrocyte analyses: erythrocyte count in Neubauer chamber; hematocrit determination, using the micro-hematocrit method; and hemoglobin measurements using the cyanomethemoglobin method.

An additional 1 mL of the blood was poured into a tube without coagulant and centrifuged at 2060 × g (Baby I 206 BL FANEM®). The serum was sent for biochemical analysis (total cholesterol, triglycerides, total proteins, and glucose), performed by spectrophotometry using a commercial kit (Analisa®).

Data were checked for homogeneity using Levene's test (Brown and Forsythe, 1974), and for normality using the Cramer-von Mises test (Darling, 1957). Afterwards, the data were analyzed by ANOVA, followed by Tukey's test using the software STATISTICA 7.0. Significance was considered at P = 0.05.

Results

At the beginning of the analysis, lower indices of vacuolization were observed (Table 2). With time, the presence of vacuoles was more evident but occurred homogeneously in all experimental diets (Table 2, Figure 1). In the blood parameters (Table 3), no difference among treatments were observed

Table 2 - Histological alterations of the liver of *R. quelen* females fed different lipid sources

Vacuolization	Diet			
	Soybean oil	Palm oil	Fish oil	Mix
October (five months)	0-2	0-2	1-2	0-2
December (seven months)	1-2	1-2	1-2	1-2
February (nine months)	1-3	1-3	1-3	2-3
April (eleven months)	1-3	1-3	1-3	1-2

Vacuolization score: 0 = not observed, 1 = reduced, 2 = intermediate, 3 = severe.

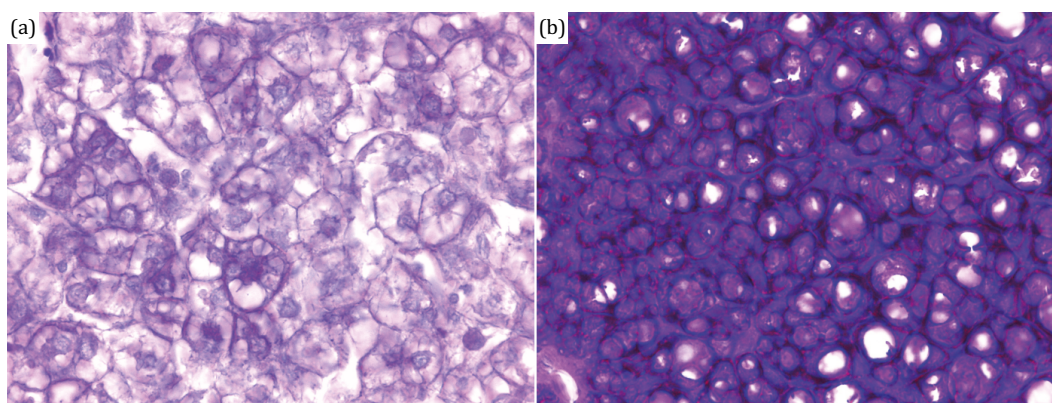


Figure 1 - Small change in cell size (vacuolization score = 1), palm oil treatment/October 2013 (A); hepatocytes-like adipocytes; smaller vacuoles (vacuolization score = 2), mix treatment/December (B). (PAS, 400X).

Table 3 - Mean \pm standard error of the erythrocyte and biochemical variables of the blood of *R. quelen* females fed different lipid sources

Parameter	Diet				P-value
	Soybean oil	Palm oil	Fish oil	Mix	
Erythrocytes ($10^6 \mu\text{L}^{-1}$)	1.78 \pm 0.15	1.54 \pm 0.12	1.59 \pm 0.06	1.64 \pm 0.12	0.53
Hematocrit (%)	41.73 \pm 1.47	41.67 \pm 2.30	39.27 \pm 1.31	41.73 \pm 1.63	0.65
Hemoglobin (g dL $^{-1}$)	7.93 \pm 0.28	7.86 \pm 0.51	7.73 \pm 0.34	7.80 \pm 0.50	0.99
Total proteins (g dL $^{-1}$)	4.71 \pm 0.20	4.86 \pm 0.09	4.86 \pm 0.16	4.78 \pm 0.19	0.91
Cholesterol (mg dL $^{-1}$)	306.81 \pm 27.66	298.14 \pm 29.35	288.76 \pm 21.70	311.17 \pm 30.82	0.94
Triglycerides (mg dL $^{-1}$)	369.62 \pm 73.08	330.66 \pm 61.88	384.93 \pm 69.48	361.27 \pm 64.08	0.95
Glucose (mg dL $^{-1}$)	67.93 \pm 4.55ab	51.17 \pm 3.82a	68.68 \pm 6.33ab	74.46 \pm 4.81b	0.03

Distinct letters in the same row indicate significant difference (ANOVA, $P < 0.05$).

for erythrocytes ($P = 0.53$), hematocrit ($P = 0.65$), hemoglobin ($P = 0.99$), total proteins ($P = 0.91$), cholesterol ($P = 0.94$), and triglycerides ($P = 0.95$). However, for glucose levels, lower values ($P = 0.03$) were observed for palm oil treatment ($51.17 \pm 3.82 \text{ mg dL}^{-1}$), especially when compared with the mix treatment ($74.46 \pm 4.81 \text{ mg dL}^{-1}$).

Discussion

In the present study, it is observed that vegetable oils do not significantly interfere with the histology and hematological parameters of female *Rhamdia quelen*. The vacuolization observed in the current study were homogenous in fish fed all experimental diets and does not indicate an intense pattern of steatosis. Therefore, such injury cannot be attributed to the inclusion of 5% of vegetable oils in the diet. However, other authors, such as Tessaro et al. (2014) with females *R. quelen* and Bombardelli et al. (2009) with Nile tilapia, observed an increased level of vacuolization with high levels of energy associated with vegetable oils.

Regardless of the oil source, digestible energy levels must be considered in diet formulation. The results of this study showed that until 5% of replacement of fish oil for soybean oil and palm oil, it does not cause significant changes in liver histology. The steatosis could be characterized by the excess of lipid ingestion that exceeds the oxidation capacity of the liver, which results in the deposition of lipid in the hepatocytes (vacuoles) (Caballero et al., 2004). Therefore, the results suggest that different diets using vegetable oils do not overload the liver.

The results of blood parameters evaluated also indicated a normal pattern expected for the species, presenting no significant influence of the diets. Mature erythrocytes transport oxygen and carbon dioxide

through hemoglobin (Ranzani-Paiva et al., 2013). In the present study, the diets did not influence the number of erythrocytes, and the values are within the normal range established by Tavares-Dias et al. (2002), 1.55 to $2.92 \times 10^6/\mu\text{L}$, for *R. quelen*. As hemoglobin, the hematocrit is employed to categorize anemia when the values are below the normal pattern. The diets also did not influence the values of hemoglobin and hematocrit; however, there were increased values of hematocrits in relation to other reports for *R. quelen* (Tavares-Dias et al., 2002), as well as when evaluating diets containing different protein levels (Camargo et al., 2005; Melo et al., 2006).

Among the biochemical variables analyzed, only a difference in glucose levels was observed, which was significantly lower in females fed diets containing palm oil. Similar results were observed by Babalola et al. (2009), who tested four sources of saturated fat (palm oil, karite butter, swine fat, and poultry fat) and two sources of oil, from animal (cod liver oil) and vegetable (sunflower oil) origin, in catfish (*Heterobranchus longifilis*) juveniles. The authors observed that lower levels of glucose were observed for fish fed diets containing fish oil, poultry fat, and karite butter, followed by palm oil. Therefore, as glucose is commonly used as an indicator of stress in fish (Martínez-Porchas et al., 2009), this specific aspect needs to be evaluated in the future.

Conclusions

Diets using vegetable oils or their mixture do not interfere with the steatosis and hematological parameters of *R. quelen* females, making them an interesting source to replace fish oil.

Author Contributions

Conceptualization: C.C. Hilbig, L.S.O. Nakaghi and R.A. Bombardelli. Data curation: A.C.S. Campos and L.F. Martins. Formal analysis: C.C. Hilbig, N.F. Nascimento, A.C.S. Campos, L.F. Martins, A.S. Ventura and L.S.O. Nakaghi. Funding acquisition: L.F. Martins, A.S. Ventura, L.S.O. Nakaghi and R.A. Bombardelli. Investigation: C.C. Hilbig, N.F. Nascimento, A.C.S. Campos, L.F. Martins and A.S. Ventura. Methodology: C.C. Hilbig, N.F. Nascimento, A.C.S. Campos, L.F. Martins and A.S. Ventura. Project administration: C.C. Hilbig, A.C.S. Campos, L.F. Martins, A.S. Ventura, L.S.O. Nakaghi and R.A. Bombardelli. Resources: N.F. Nascimento, L.S.O. Nakaghi and R.A. Bombardelli. Supervision: C.C. Hilbig, N.F. Nascimento, L.S.O. Nakaghi and R.A. Bombardelli. Visualization: L.S.O. Nakaghi. Writing-original draft: C.C. Hilbig, N.F. Nascimento and L.S.O. Nakaghi. Writing-review & editing: N.F. Nascimento, A.C.S. Campos, L.F. Martins, A.S. Ventura, L.S.O. Nakaghi and R.A. Bombardelli.

Conflict of Interest

The authors declare no conflict of interest.

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