Revista Brasileira de Zootecnia
Brazilian Journal of Animal Science

ISSN 1806-9290
www.sbz.org.br

Full-length research article

R. Bras. Zootec., 47:e20160234, 2018
https://doi.org/10.1590/rbz4720160234

Aquaculture

Metabolic and histologic responses of pacu (*Piaractus mesopotamicus*) fed diets supplemented with increasing concentrations of ractopamine

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ABSTRACT - An experiment was conducted during 60 days with forty pacu males fed diets supplemented with increasing concentrations of ractopamine (0.00, 11.25, 22.50, 33.75, and 45.00 mg kg^{-1}). Eight fish were evaluated for each experimental diet. Performance and survival rate of the fish were measured. At the end of the experiment, blood was collected to determine the levels of cortisol, triacylglycerol, and protein. Moreover, the liver was collected to determine the activities of glucose-6-phosphate dehydrogenase and malic enzymes. The fillets were collected to determine chemical composition, and histologic cuts were analyzed to verify muscle growth and deposition of adipose tissue between muscle fibers. Increasing concentrations of dietary ractopamine did not change feed intake, fillet yield, fillet content of protein and ash, and frequency of relative distribution of muscle fibers. By increasing the dietary ractopamine concentration, the serum cortisol level was elevated. Ractopamine supplementation (45.00 mg kg^{-1}) increased serum levels of triacylglycerol and protein and reduced the activity of hepatic lipogenic enzymes and the survival rate of the fish, probably in response to the high concentration of circulating cortisol. In addition, the higher level of ractopamine supplementation evaluated in this research impaired the weight gain and feed conversion. However, 11.25 mg kg^{-1} ractopamine reduced the ether extract level determined in the fillet and the fat deposition between muscle fibers, improving the nutritional quality of meat.

Key Words: beta-adrenergic agonist, enzyme, metabolism, stress

Introduction

Aquaculture is an activity of great importance and productive potential worldwide (FAO, 2014). In many countries of South America, such as in Brazil, the captive production of tropical native fish is increasing and pacu (*Piaractus mesopotamicus*, Holmberg, 1887) is one of the species of interest (Boscolo et al., 2011; Barbieri and Bondioli, 2015; Venturini et al., 2015). Pacu has great potential for intensive fish farming because of its basic management, good growth rates, and relatively easy artificial reproduction (Jomori et al., 2005). However, pacu has a higher content of body fat mainly in the finishing rearing phase (Bicudo et al., 2010). As this is an undesirable feature by most consumers, it is necessary to establish strategies to reduce fat deposition in the carcasses of *P. mesopotamicus* (Oliveira et al., 2014).

Ractopamine is a beta-adrenergic agonist that alters nutrient metabolism and may promote greater muscle protein deposition, reducing fat content in the carcass (Ferreira et al., 2013). For pigs and cattle, ractopamine use as a feed additive is authorized in countries such as Brazil, United States, Canada, and South Korea (Niño et al., 2017), and there are many studies reporting that dietary ractopamine improves meat quality and animal performance (Andretta et al., 2012; Boler et al., 2012; Gerlemann et al., 2014; Kill et al., 2015). However, the European Union, China, Taiwan, and Russia have banned the use of this substance based on the lack of conclusive results on their safety for animal and human health (Niño et al., 2017). As there is no global consensus on ractopamine use in meat production, the scientific community needs to generate more information about the safety and animal performance effectiveness of this beta-adrenergic agonist. In this context, there are few studies about the use of ractopamine

Received: July 6, 2016
Accepted: February 22, 2018
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in diets for pacu (Bicudo et al., 2012; Oliveira et al., 2014), signaling the need for further work in this area.

The effect of ractopamine on the animal metabolism occurs by its binding to specific β-adrenergic receptors; however, the body responses can be influenced by the concentration and duration of the dietary ractopamine (Edmonds and Baker, 2010). Previous reports regarding effects of ractopamine on muscle growth in fish are contradictory (Haji-abadi et al., 2010; Bicudo et al., 2012; Devens et al., 2012), and none evaluated the hyperplasic and hypertrophic growth of pacu fed diets supplemented with ractopamine. Moreover, it is still controversial whether ractopamine reduces fat in the carcass due to lipogenesis inhibition or by lipolysis stimulation (Ferreira et al., 2013). Therefore, this study was conducted with pacu in the finishing phase to determine the effects of diets supplemented with increasing concentrations of ractopamine on performance, survival rate, serum parameters, and specific activity of hepatic lipogenic enzymes, as well as the yield, chemical composition, and histologic parameters of their fillets.

Material and Methods

Forty *P. mesopotamicus* were randomly distributed into five dietary treatments; therefore, eight fish were evaluated for each experimental diet. Each tank housed one fish for a total period of 70 days. For the first 10 days, the fish were fed a basal diet to allow them to adapt to the working environment; for the remaining 60 days, the fish received the experimental diets. Initially, one basal diet without ractopamine was formulated to meet the nutritional requirements of pacu in the finishing phase (Table 1) in accordance with Boscolo et al. (2011), except for the crude protein level which was increased to 312.8 g kg^{-1} because animals fed diet containing ractopamine can exhibit increased protein synthesis (Mitchell et al., 1990). This basal diet was then supplemented with four increasing concentrations of ractopamine (11.25, 22.50, 33.75, and 45.00 mg kg^{-1}) (Ractosuin®, OuroFino Animal Health, Cravinhos, São Paulo, Brazil).

All five diets were then pelleted (industrial pelletizer CPM 2000®, São Paulo, Brazil), dried in a forced-ventilation oven (40 °C; 24 h), hermetically packed in black plastic bags, and stored at −18 ºC until use. Throughout the study, the diets were provided until apparent satiation twice a day (08.00 and 16.00 h). Each day, the tanks were siphoned 30 min after the end of the first feeding.

The experiment was conducted in accordance with ethical and animal welfare guidelines, and was carried out in Lavras, Minas Gerais, Brazil (−21°14'43" latitude, −44°59'59" longitude, and 919 m altitude). Forty males of *P. mesopotamicus* were obtained from commercial fish farm, each weighing 864.0±75.0 g and with approximately one year of age. They were maintained in an experimental station containing 40 glass tanks of 100 L volume each, arranged in a water recirculation system equipped with sand, ultraviolet, and biologic filters. Water quality parameters, including pH (6.92±0.18), dissolved oxygen (5.73±0.49 mg L^{-1}), ammonia (0.0007±0.0002 mg L^{-1}), nitrite (0.089±0.007 mg L^{-1}), and temperature (27.6±0.2 ºC), were monitored regularly and remained within acceptable values for pacu (Urbinati and Gonçalves, 2005). Fish were maintained under photoperiod of 12 h light:12 h dark.

The survival rate was calculated considering the number of fish that died during the experimental period. Apparent feed intake for each pacu was recorded daily, in a dry matter basis, subtracting the weight of the container with feed before and after feeding. For weight gain calculations, the fish were weighed in the first and last experimental days. Feed conversion ratio was calculated by dividing feed intake by body weight gain.

At the end of the experimental period, the fish were fasted for 24 h, captured from the tanks using a trawl, and then blood samples were collected by puncture of the tail vein using Vacutainer® tubes without anticoagulant. After blood collection, the pacu were anesthetized with benzocaine (Pharmasys, Piracicaba, SP, Brazil) (150 mg L^{-1}),

Table 1 - Ingredients and nutritional composition of the basal diet without ractopamine

Item	
Ingredient (g kg^{-1} as fed basis)	
Soybean meal (45% CP)	405.0
Corn	348.8
Fish meal	80.0
Wheat meal	80.0
Soybean oil	35.2
Bicalcium phosphate	38.0
Ascorbic acid	0.6
Vitamin and mineral mix[1]	5.0
Butylated hydroxytoluene	0.2
Salt	2.0
L-lysine HCL	1.9
DL-methionine	2.8
L-threonine	0.5
Nutritional composition (as-fed basis)	
Gross energy (MJ kg^{-1})	17.2
Crude protein (g kg^{-1})	312.8
Ether extract (g kg^{-1})	70.8

CP - crude protein.
[1] Vitamin and mineral mix (Total Alimentos Ltda®; Três Corações, MG, Brazil). Quantity per kg of diet: vitamin A, 1500 IU; vitamin B3, 1000 IU; vitamin B1, 20 mg; vitamin B2, 15 mg; vitamin B12, 10 mcg; vitamin E, 25 mg; vitamin PP, 120 mg; choline, 2000 mg; calcium pantothenate, 80 mg; folic acid, 2 mg; Mn, 80 mg; iodine, 3 mg; Fe, 24 mg; Zn, 50 mg; Cu, 8 mg; Se, 0.10 mg; butylated hydroxytoluene, 170 mg.

slaughtered, eviscerated discarding the skin with scales and the head, and weighed. During the evisceration procedure, the liver was collected and immediately frozen in liquid nitrogen. Additionally, the fillets were collected, weighed for calculation of the fillet yield (weight fillet/fish weight × 100), and stored at −20 °C until chemical analysis.

The blood samples were centrifuged (1372 × *g* for 15 min) and the serum was collected to determine cortisol level (μg dL^{-1}) by an enzyme immunoassay kit (Diagnostics Biochem Canada Inc., Canada, Catalog number CAN-C-270) as well as the levels of triacylglycerols (mg dL^{-1}) and total protein (g dL^{-1}) using standard commercial colorimetric kits (Labtest Diagnóstica SA, Lagoa Santa, Minas Gerais, Brazil, Catalog numbers of 87 for triacylglycerols and 99 for total protein).

Hepatic extracts (0.1 g of liver for 1.2 mL of 25 mM HEPES-KOH buffer; pH 7.2) were obtained according to methodology described by Ribeiro et al. (2013) and were used to determine the catalytic activity of the glucose-6-phosphate dehydrogenase (EC1.1.1.49) and malic enzymes (EC1.1.1.40). The protein contents in the hepatic extracts (g mL^{-1}) were measured according to the method of Bradford (1976) using bovine serum albumin as a standard to allow the calculation of the specific activity of the evaluated enzymes. Glucose-6-phosphate dehydrogenase activity was measured according to Graeve (1994) and expressed as U mg^{-1} protein (since 1 U corresponds to 1 μmol of 6-phospho-D-gluconate released during 1 s of reaction at pH 7.2 and temperature of 25 ºC). Malic enzyme activity was determined according to Spina Junior et al. (1970) and expressed as U mg^{-1} protein (since 1 U corresponds to 1 μmol of pyruvate released during 1 s of reaction at pH 7.2 and temperature of 25 ºC).

Fillet samples were used to determine the levels of crude protein (Kjeldahl method; N x 6.25), ether extract (Soxhlet method), ash (gravimetric method), and moisture (gravimetric method) according to standard procedures of the AOAC (1990). In addition, white muscle aliquots of the dorsal region (8 × 4 × 4 mm) of each fish were collected and immediately fixed in modified Karnovsky's solution during 24 h, without any previous freezing of the tissues. Karnovsky's solution consisted of 2.5% glutaraldehyde and 2% formaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. After fixation, the samples were dehydrated and encased in plastic resin. Resin blocks were then cut in sections of 4 μm using a microtome (Leica Biossystem, Germany). Histological cuts were stained with hematoxylin and eosin and utilized to verify the type of muscle growth (hyperplasia and/or hypertrophy) and the amount of adipose tissue deposited between muscle fibers. Stained sections were analyzed via optical microscopy (model CX31, Olympus, Japan), at 10x magnification, coupled to a digital camera (Altramodel SC30, Olympus, Japan). Diameters of 200 muscle fibers were measured for each cut, according to Dubowitz et al. (2013), using the software of morphometric analysis SIS Cell B (Olympus, Japan). Afterwards, the muscle fibers were classified according their diameters (<30 μm, between 30 and 50 μm, >50 μm) (Almeida et al., 2008) to aid in understanding the muscle growth. For determination of adipose tissue deposited between muscle fibers, 1564 intersections for each histological cut were evaluated using the software Image J (Wayne Rasband, National Institutes of Health, Bethesda, MD).

Initially, all data were checked by a normality test (Shapiro-Wilk). Normality of the data was confirmed for the apparent performance, survival rate, serum parameters, and specific activity of the hepatic enzymes, in addition to yield and chemical composition of fillets. These data were then subjected to analysis of variance using the General Linear Model of the statistical software SAS (Statistical Analysis System, version 9.4), and when significant, the means were compared with each other by the Student-Newman-Keuls test ($P<0.05$). However, data related to muscle growth and deposition of adipose tissue between muscle fibers showed non-normal distribution. Therefore, they were subjected to the non-parametric Kruskal-Wallis test ($P<0.05$), and when significant, the data were subjected to the multiple comparison Nemenyi test ($P<0.05$).

Results

Two fish that received the feed supplemented with 45.00 mg kg^{-1} ractopamine died and, therefore, this treatment resulted in the smaller survival rate (Table 2). All the experimental diets were well accepted by the fish. The five diets evaluated did not change ($P>0.05$) the apparent feed intake measured during the 60 experimental days (632.08±12.68 g). However, the higher level of ractopamine supplementation evaluated in this research impaired the weight gain and the feed conversion.

The blood parameters and the catalytic activity of the hepatic lipogenic enzymes were influenced ($P<0.05$) by the dietary ractopamine level (Table 3). In general, serum cortisol content gradually increased ($P<0.05$) with the increasing concentrations of dietary ractopamine. When compared with the animals fed basal diet without ractopamine, the fish fed the diet supplemented with 45.00 mg kg^{-1} ractopamine showed a relative increase of 124% in the circulating cortisol content. Furthermore, the fish fed diet containing 45.00 mg ractopamine per kg showed higher serum concentrations of

triacylglycerols and total protein, representing a relative increase of approximately 97 and 36%, respectively, compared with pacu fed the control diet. The maximum concentration of ractopamine evaluated in this study also reduced the specific activity of the glucose-6-phosphate dehydrogenase and malic enzymes in approximately 28 and 62%, respectively.

The dietary ractopamine concentration did not affect (P>0.05) the fillet yield (456.7±7.1 g kg^{-1}) or (P>0.05) the levels of crude protein (230.7±14.2 g kg^{-1}) and ash (13.3±0.4 g kg^{-1}) determined in the fillets (Table 4). However, the lowest (P<0.05) ether extract content and highest (P<0.05) moisture content were measured in fillet from pacu fed diet containing 11.25 mg kg^{-1} ractopamine.

The frequency of relative distribution of the muscle fibers in pacu, according to their diameter, was similar (P>0.05) for all dietary ractopamine concentrations. In general, there was a predominance of muscle fibers with diameters greater than 50 µm (64.6±2.7%), followed by fibers with diameters between 30 and 50 µm (25.8±1.6%), with the fewest fibers having diameters less than 30 µm (9.6±1.9%) (Table 5). The diet supplemented with 11.25 mg kg^{-1} ractopamine resulted in reduced (P<0.05) fat deposition between the muscle fibers of *P. mesopotamicus* in finishing phase, which can be observed in the photographic images referring to the histological sections of muscle tissue (Figure 1).

Discussion

The two fish that died were of the dietary treatment containing 45.00 mg of ractopamine per kg of feed. In addition, this dietary treatment impaired the weight gain and the feed conversion of the fish, representing an important indication that this level of ractopamine inclusion is very high and should not be used for pacu.

Table 2 - Performance and survival rates of *Piaractus mesopotamicus* fed diets supplemented with increasing concentrations of ractopamine during the 60 experimental days

Dietary ractopamine supplementation (mg kg^{-1})	Survival rate (%)[1]	Apparent feed intake (g per fish)	Weight gain (g per fish)	Feed conversion (g:g)
0.00	100a	648.63a	108.25a	5.99b
11.25	100a	626.88a	107.25a	5.85b
22.50	100a	625.25a	108.13a	5.78b
33.75	100a	639.50a	110.25a	5.80b
45.00	75b	630.15a	94.63b	6.66a
Coefficient of variation (%)	13.34	8.74	7.66	7.98

[1] Eight fish were evaluated for each experimental diet.
Means with different letters in the same column are significantly different from each other (P<0.05) by the Student-Newman-Keuls test.

Table 3 - Serum levels of cortisol, triacylglycerol, and total protein and activity of lipogenic enzymes in liver extracts of *Piaractus mesopotamicus* fed diets supplemented with increasing concentrations of ractopamine

Dietary ractopamine supplementation (mg kg^{-1})	Cortisol (µg dL^{-1})	Triacylglycerol (mg dL^{-1})	Total protein (g dL^{-1})	G6PD (U mg^{-1} protein)[1]	Malic enzyme (U mg^{-1} protein)[2]
0.00	3.27c	307.41b	4.78b	1.74a	0.26a
11.25	3.60c	262.96b	5.05b	1.82a	0.24a
22.50	4.52bc	329.01b	5.23b	1.71a	0.21a
33.75	5.17b	493.21ab	5.01b	2.00a	0.25a
45.00	7.33a	606.17a	6.52a	1.27b	0.10b
Coefficient of variation (%)	16.12	20.96	10.00	14.08	11.82

[1] Glucose-6-phosphate dehydrogenase activity was expressed as U mg^{-1} protein, in which 1 U corresponds to 1 µmol of 6-phospho-D-gluconate released during 1 s of reaction.
[2] Malic enzyme activity was expressed as U mg^{-1} protein, in which 1 U corresponds to 1 µmol of pyruvate released during 1 s of reaction.
Means with different letters in the same column are significantly different from each other (P<0.05) by the Student-Newman-Keuls test.

Table 4 - Yield and chemical composition determined in the fillets of *Piaractus mesopotamicus* fed diets supplemented with ractopamine at increasing concentrations

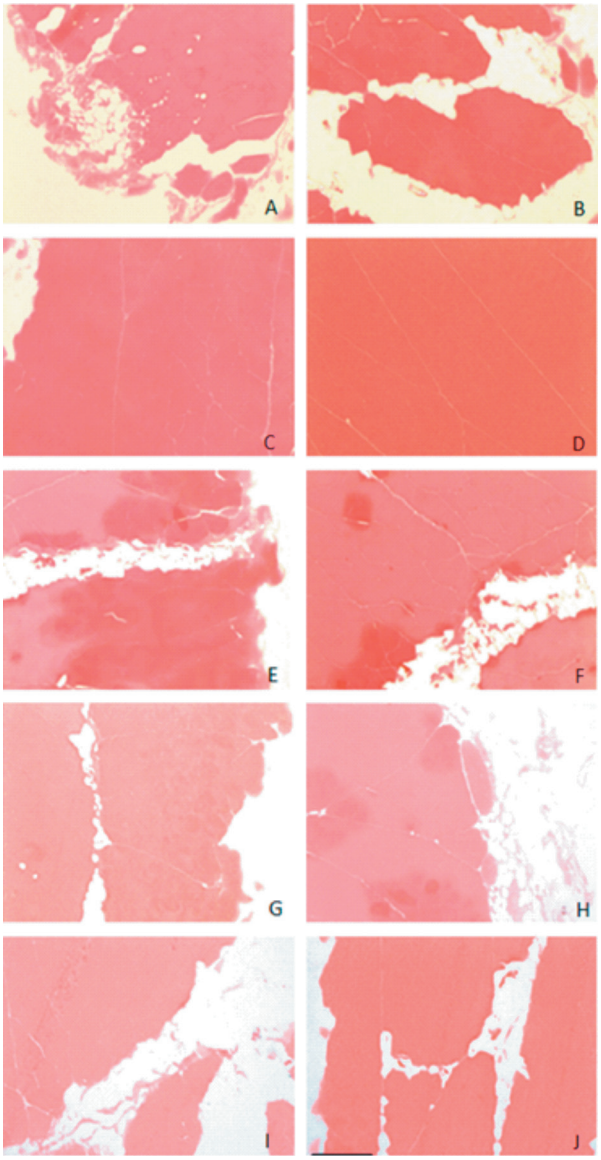
Dietary ractopamine supplementation (mg kg^{-1})	Fillet yield (g kg^{-1})	Crude protein (g kg^{-1})	Ash (g kg^{-1})	Ether extract (g kg^{-1})	Moisture (g kg^{-1})
0.00	44.84a	25.37a	1.29a	65.10a	668.30b
11.25	44.98a	21.92a	1.35a	34.00b	733.30a
22.50	45.98a	22.96a	1.32a	55.20a	702.00ab
33.75	46.32a	23.25a	1.40a	52.50a	701.00ab
45.00	46.24a	21.87a	1.31a	61.90a	706.30ab
Coefficient of variation (%)	3.90	19.50	17.30	19.67	2.87

Means with different letters in the same column are significantly different from each other (P<0.05) by the Student-Newman-Keuls test.

Table 5 - Frequency of relative distribution of the white muscle fibers and deposition of adipose tissue between muscle fibers of *Piaractus mesopotamicus* fed diets supplemented with increasing concentrations of ractopamine

Dietary ractopamine supplementation (mg kg^{-1})	% of fibers according to their diameter			Adipose tissue determined in the histological cuts (%)[1]	Muscle tissue determined in the histological cuts (%)[1]
	<30 µm	30 a 50 µm	>50 µm		
0.00	9.2a	24.3a	66.5a	18.95	81.05
11.25	6.9a	27.0a	66.1a	4.41[2]	95.59[2]
22.50	11.9a	27.9a	60.2a	17.87	82.13
33.75	10.7a	25.3a	64.0a	11.50	88.50
45.00	9.1a	24.5a	66.4a	16.16	83.84

[1] For relative determination of the adipose and muscle tissues, 1564 intersections for each histological cut were evaluated using the software Image J (Wayne Rasband, National Institutes of Health, Bethesda, MD).
[2] Differs from the other four dietary treatments described in the column (P<0.05) by the multiple comparison Nemenyi test.
Means with same letter in the same column are significantly equal to each other (P>0.05) by the non-parametric Kruskal-Wallis test.



Dietary ractopamine supplementation (mg kg^{-1}): 0.00 for A and B, 11.25 for C and D, 22.50 for E and F, 33.75 for G and H, and 45.00 for I and J.
Hematoxylin and eosin; x10. Scale bar = 100 µm.
Cells stained in pink-red color correspond to muscle cells. Places that were not stained correspond to adipose tissue.

Figure 1 - Transverse sections of white skeletal muscle of *Piaractus mesopotamicus* fed diets supplemented with increasing concentrations of ractopamine.

It is important to emphasize that the results observed in the present experiment can be discussed based on the increasing concentration of dietary ractopamine because all fish showed similar feed intake. The elevation of the serum cortisol level in *P. mesopotamicus* can be considered a primary biologic response to a stressful situation (Abreu et al., 2009). In turn, this important hormone triggers a series of secondary responses that may alter physiologic and metabolic parameters in pacu (Biller et al., 2008). Therefore, the results of this study indicated that ractopamine can act as a stressor agent culminating in the activation of the hypothalamic-pituitary-interrenal-axis in pacu when the feed is supplemented with β-adrenergic in concentrations higher than 11.25 mg kg^{-1} and is provided to animals in the finishing phase during 60 days. Similarly, increased circulating cortisol levels were not noted in finishing pigs fed diets containing 10 mg kg^{-1} or less of ractopamine (Athayde et al., 2013). There are no scientific reports that explain how and why chronic exposure to high concentrations of dietary ractopamine is recognized by the hypothalamus of teleost fish as a stressor agent and, therefore, this warrants further investigation.

Cortisol is an important steroid hormone, secondary to stress, that increases the availability of oxidizable substrates (Tort, 2011). In relation to lipid metabolism, it has been long known that cortisol along with epinephrine can stimulate lipolysis in adipose tissue (Mommsen et al., 1999). Therefore, in this study, it is likely that the increased level of blood cortisol stimulated lipolysis in the adipose tissue of pacu, resulting in hydrolysis of triacylglycerols into glycerol and fatty acids. Normally, most of the glycerol arising from degradation of acylglycerols stored in adipose tissue is directed through the bloodstream to the liver, where, for example, it can be used as a gluconeogenic precursor (Rui, 2014). It is important to consider that the colorimetric method used in this study to determine the serum triacylglycerol content has glycerol as an

intermediary substrate. Thus, the largest triacylglycerol value observed in fish fed the diet containing 45.00 mg kg^{-1} ractopamine may be related to the major release of cortisol, which, in turn, possibly stimulated release of epinephrine. Epinephrine may then have induced lipolysis in the adipose tissue resulting in increase of blood glycerol content, which was quantified colorimetrically.

Although it is ideal to establish comparisons between the same species at the same age, there is no sufficient number of studies on ractopamine use in feeds for pacu, making this degree of comparison difficult at this moment. In this context, Bicudo et al. (2012) reported that juveniles of *P. mesopotamicus* fed diets containing ractopamine concentrations ranging between 10 and 40 mg kg^{-1} for 60 days did not show any changes in plasma triacylglycerol level. This reinforces the hypothesis that the variation in serum triacylglycerol level in this study is one of the biologic responses triggered by elevation of circulating cortisol level. Furthermore, part of this increase in triacylglycerol level may also be related to lipogenesis inhibition in the liver (indicated by reduction of hepatic lipogenic enzyme activities), which is also triggered by the higher blood cortisol content (Sunny et al., 2002). Thus, since the hepatic lipogenic pathway is inhibited, a higher serum concentration of triacylglycerols is expected.

Although cortisol may have stimulated lipolysis in the adipose tissue (even indirectly by an increased epinephrine concentration), it seems that this effect does not occur for triacylglycerols deposited between the white muscle fibers of pacu because the diet that resulted in higher circulating cortisol level did not decrease fat content in the muscle tissue. However, to have a tissue response to cortisol, it is essential that there are specific intracellular glucocorticoid receptors (Tort, 2011). Thus, a possible explanation for the fact that cortisol stimulates lipolysis in adipose tissue but not in muscle tissue may be that there are distinct distributions of glucocorticoid receptors among cells located in different tissues (Bury et al., 2003; Nesan and Vijayan, 2013).

Most fatty acids originating from triacylglycerol hydrolysis in adipose tissue are associated with the albumin protein for transport in the bloodstream to the liver, where the fatty acids can be metabolized and used according to the needs of the animals (Mersmann, 2002). Therefore, the higher total protein level in pacu fed the diet containing 45.00 mg kg^{-1} ractopamine may be an indication that these fish had an increased serum albumin concentration in response to the higher content of fatty acid released from lipolysis that was stimulated by cortisol. Moreover, because of its lipophilic character, cortisol needs to bind with proteins such as corticosteroid-binding globulins to be carried within the bloodstream (Lin et al., 2010). This may also have contributed to the increased serum total protein level measured in the *P. mesopotamicus* in this study.

The NADPH + H^+ coenzyme is an indispensable reducing agent in lipid biosynthesis (lipogenesis). Therefore, it is possible that the speed of the lipogenic pathway in the liver is reduced whenever the NADPH + H^+ concentration in the hepatocyte is lower. The reduced form of this coenzyme is produced from the oxidative step of the pentose-phosphate pathway and from reactions catalyzed by malic enzyme (or NADP-dependent malate dehydrogenase). The glucose 6-phosphate dehydrogenase enzyme catalyzes the irreversible oxidation reaction of glucose 6-phosphate + $NADP^+$ to 6-phosphogluconolactone + NADPH + H^+ and represents an important regulation point of the pentose-phosphate pathway, while malic enzyme produces NADPH + H^+ by oxidation of malate to pyruvate. That is why these two enzymes are recognized as lipogenic (Rui, 2014). In this study, the hypothesis that the addition of ractopamine at 45.00 mg kg^{-1} level contributed directly, in some way, to the reduction of hepatic lipogenic enzyme activity cannot be ruled out. However, when all evaluated parameters are simultaneously analyzed, the results indicate that the reduction in catalytic activity of the glucose 6-phosphate dehydrogenase and malic enzymes was not due to a direct effect of the β-adrenergic effect, but instead to the blood cortisol elevation, which also inhibited lipogenesis.

Although there are indications that cortisol inhibited hepatic lipogenesis, it seems that this hepatic anabolic pathway is not directly related to the rate of fat deposition between the muscle fibers, since the diet that reduced hepatic lipogenesis did not result in a lower ether extract content in fish fillets. It is accepted that the main tissue for fat reserves in vertebrates is adipose tissue (Peirce et al., 2014). Therefore, it is logical that hepatic lipogenesis inhibition might be more related to triacylglycerol deposition in adipose tissue than in muscle. In this study, the amount of fat stored as adipose tissue (visceral fat, for example) was not measured, because the main objective of this study was to determine which ractopamine concentration promoted a lower ether extract in fillets, which are a cut with great marketing potential as a product for human consumption.

The average ash content determined in pacu fillets in this study was similar to the values reported by Bicudo et al. (2010) and Oliveira et al. (2014), who also determined the chemical composition of *P. mesopotamicus* fillets. Similarly, Bicudo et al., (2012) did not observe differences in crude protein and ash contents in fillet of pacu juveniles fed diets supplemented with ractopamine in concentrations

of up to 40.00 mg kg^{-1}. In another study, the addition of up to 21 mg kg^{-1} ractopamine to diets provided to juvenile carp (*Cyprinus carpio*) also had no effect on protein deposition (Devens et al., 2012). Associating the results observed for fillet yield, crude protein content in the fillet, and the frequency of relative distribution of the muscle fibers, it is possible to infer that the ractopamine levels evaluated did not promote hyperplasic growth (recruitment of new muscle fibers) or hypertrophic growth (increase in muscle fiber diameter) in the fish. For other farm animals in the finishing phase, such as pigs, there are reports that dietary ractopamine improves animal performance (Brumm et al., 2004; Mimbs et al., 2005). However, it is important to emphasize that the growth rate in fish is different from the rate observed for other farm animal groups. While pigs have large weight gain in the finishing phase, most of the fish in the same phase have a minimal growth rate (Johnston, 1999). Other researchers have also reported that the use of dietary ractopamine does not improve the performance of *P. mesopotamicus* juveniles (Bicudo et al., 2012) or other fish species such as *Cyprinus carpio* juveniles (Devens et al., 2012) and *Oncorhynchus mykiss* (Moccia et al., 1998).

Typically, the carcass water content is inversely proportional to the fat deposition in the muscle tissue (Garbossa et al., 2013), which supports the fact that the lowest ether extract level and the highest moisture content were concurrently determined in the fillets of pacu fed dietary ractopamine at 11.25 mg kg^{-1}. Moreover, it was observed in this study that fillets from fish fed dietary ractopamine at 11.25 mg kg^{-1} had the lowest amount of ether extract as well as less fat deposition between the muscle fibers. These results are important because the current consumer market requires the provision of high-protein, low-fat meat (Font-i-Furnols and Guerrero, 2014). These beneficial effects can be attributed to the direct action of the β-adrenergic used in the diet, because the serum cortisol level in the fish fed diet containing 11.25 mg kg^{-1} ractopamine was similar to that determined in fish fed the basal diet without ractopamine. Haji-abadi et al. (2010) also observed a reduction of the fat level in the fillet of *Oncorhynchus mykiss* fed diet containing 10 mg kg^{-1} ractopamine and the authors attributed this result to the lipolytic action of ractopamine in the muscle tissue.

When ractopamine binds to the β-adrenergic receptor, a series of intracellular signaling events that use cyclic AMP as a second messenger occurs and culminates in various cellular responses, which, in turn, promote tissue-specific metabolic and physiologic effects (Mersmann, 1998). An important effect of ractopamine is triggering the phosphorylation process (activation) of hormone-sensitive lipase, which is a key enzyme of the lipolytic pathway (Mersmann, 2002). Dietary 11.25 mg kg^{-1} ractopamine may have stimulated lipolysis in the adipose tissue in intensity lower than required to increase the serum glycerol content, and for this reason, the serum triacylglycerol of this experimental group did not increase (because the colorimetric method of analysis for triacylglycerol used in this work also quantifies glycerol). However, this hypothesis cannot be confirmed because lipolytic parameters were not evaluated in the adipose tissue of pacu in this study.

Another important result observed in this study was that when ractopamine was added to the feed in concentrations higher than 11.25 mg kg^{-1}, there was no reduction in fat deposition in *P. mesopotamicus* fillets. This probably occurred because, with prolonged exposure to high concentrations of β-adrenergic agonists, cyclic AMP activates a protein kinase that phosphorylates the membrane receptor specific for ractopamine, rendering it inactive. Downregulation of β-adrenergic receptors in the muscle tissue due to chronic exposure to β-adrenergic agonists has also been reported in previous studies (Smith, 1989; Spurlock et al., 1994; Ferreira et al., 2013). Therefore, supplementation of the diet for 60 days with ractopamine in concentrations higher than 11.25 mg kg^{-1} is not recommended for pacu in the finishing phase because it probably triggers a downregulation mechanism (or β-adrenergic receptor desensitization) that affects the ability of the fish to reduce the fat content in muscle tissue.

Conclusions

Dietary ractopamine promotes metabolic and physiologic responses in a tissue-specific manner in *Piaractus mesopotamicus*. Muscle growth is not affected by dietary ractopamine; however, the fillet fat content is reduced when fish feed a diet supplemented with 11.25 mg kg^{-1} ractopamine during the last 60 days of the finishing phase. Moreover, when the supplementation levels of dietary ractopamine are above 11.25 mg kg^{-1}, the serum cortisol level is increased, indicating that high concentration of dietary ractopamine can act as a stressful situation for the pacu.

Acknowledgments

The authors are grateful to the Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), for their financial support.

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