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The effects of stocking density in physiological parameters and growth of the endangered teleost species piabanha, *Brycon insignis* (Steindachner, 1877)

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ABSTRACT

This study investigated the effects of stocking density on the growth and fatty acid (FA) of *Brycon insignis* metabolism. Fingerlings (360) were distributed into eight ponds at two stocking densities (105 and 210 g/m^3). The analysis of growth showed that the condition factor (*K*) and the coefficient of variation (CV) for body mass were not affected by stocking density. However, final body mass and length, specific growth rate (SGR), and weight gain (WG) were higher in the low stocking density group, which also presented a higher feed efficiency (FE) and survival (S). By contrast, muscle protein levels were higher in the high stocking density group. The plasma and muscle lipid content were not affected by stocking density, but fish reared at lower stocking density presented higher lipid concentration in the liver, with no differences in hepatosomatic index values. Even with the differences observed in metabolic and growth parameters, plasma cortisol was not affected by stocking density. The FA profile in the muscle and liver neutral fraction were not affected by stocking density, but the FA in the polar fractions differed between the two stocking densities. In the liver, total polyunsaturated fatty acids (PUFA) and PUFA n − 3 increased in higher stocking density, mainly due to an increase in docosahexaenoic acid (DHA). In addition, PUFA n − 6 were also increased in the higher stocking density group, mainly due to an increase in arachidonic acid (AA) and docosadienoic acid (22:2n − 6). In the muscle polar fraction, the saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) decreased in the animals from the higher stocking density group, and this reduction was compensated by an increase in PUFA n − 3 and PUFA n − 6, mainly the FA with 20–22 carbons (20:4n − 6; 22:4:n − 6; 22:5n − 6, 22:5n − 3, and 22:6n − 3). A different profile was observed for the C18 PUFAs, mainly 18:2n − 6 and 18:4n − 6, which were higher in the lower density stocking group. The data suggest that when living in high stocking density, *B. insignis* differentially utilizes the hepatic lipids as energy source and remodels the membrane fatty acids, with higher amounts of DHA in the polar muscle fraction compensated for by a decrease in MUFA. The zootechnical and physiological indices reveal that the lower stocking density group achieve overall better performance.

1. Introduction

The stocking density is one of the most important parameters affecting the growth, performance, and productivity in fish farming activities. A specific stocking density can have either positive or negative effects on fish growth, and this interaction seems to be species-specific (Merino et al., 2007).

Crowding is a factor involved in physiological stress (Barton, 2002). The increase in stocking densities can alter the immunological responses and physiological processes, mainly those related to metabolism and behavior (Vijayan et al., 1990; Irwin et al., 1999; Barcellos et al., 2004; Kristiansen et al., 2004; Schram et al., 2006). It has been noticed that inappropriate stocking densities can alter lipid metabolism, mainly of triglycerides, in brook charr (*Salvelinus fontinalis*) (Vijayan et al., 1990). In gilthead sea bream, *Sparus aurata*, different stocking densities altered fatty acid metabolism, with a decrease in hepatic oleic acid, a monounsaturated fatty acid important as energy source, mainly in higher stocking densities (Montero et al., 1999). Also, crowding is responsible for the increase in plasma cortisol, which plays an important role in the low efficiency of immunological responses under these conditions (Mommsen et al., 1999; Di Marco et al., 2008).

Abbreviations: K, condition factor; Wt, total weight; Wi, initial weight; Lt, total length; SGR, specific growth rate; WG, weight gain; CV, coefficient of variation; FE, feed efficiency; S, survival; HIS, hepatossomatic index; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; AA, arachidonic acid.

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Different studies have examined the effects of stocking density on the growth of different aquaculture species: turbot (*Scophthalmus maximus*) (Carro-Anzalota and McGuinty, 1986), arctic charr (*Salvelinus alpinus*) (Christiansen et al., 1992), African catfish (*Clarias gariepinus*) (Kaiser et al., 1995), summer flounder (*Paralichthys dentatus*) (King et al., 1998), Dover sole (*Solea solea*) (Schram et al., 2006), and California halibut (*Paralichthys californicus*) (Merino et al., 2007). However, few studies have analyzed the effect of crowding within the context of the physiological alterations in fishes (Di Marco et al., 2008).

Brycon insignis Steindachner, 1877, commonly named piabanha, dwell in drainages of southeastern Brazil (Hilsdorf et al., 2010; Matsumoto and Hilsdorf, 2009). This species was an important fishery resource with a commercial catch of 24 tons/year in 1951 (Machado and Abreu, 1952). Today, stocks of piabanha are depleted, and the species is listed as critically endangered on the Brazilian list of threatened species (Hilsdorf et al., 2008). As a result, restocking programs have been undertaken by artificial induced reproduction and fingerling production (Andrade-Talmelli et al., 2002).

Understanding the physiological responses of this species under different stocking densities is imperative to maintain broodstocks and fingerlings in healthy conditions for reintroduction operations. This study investigates the effects of stocking density on the piabanha growth and examines its possible effects on lipids levels and also in the metabolism of fatty acids.

2. Materials and methods

2.1. Experimental animals

Piabanha fingerlings used in the present study were obtained from the hydroelectric power plant hatchery of the São Paulo State Power Company (CESP) (23°24′50.65″S and 45°36′04.87″W), and transferred to the Ponte Nova Fish Farm (23°35′33.8″S and 45°58′09.1″W), where the trials were carried out.

2.2. Experimental conditions

The animals were acclimated to the fish farm conditions for 2 months, where they were initially maintained in one earthen pond (120 m^3). After this acclimation period, 360 fish with an initial body weight of ~35 g were randomly distributed into two stocking densities (3 and 6 fish/m^3; 105 g/m^3 and 210 g/m^3 respectively, at the beginning of the experiment) and redistributed among eight 10 m^3 ponds protected with pond cover nets, with 4 repetitions (4 ponds for each stocking density). Even considering that the higher density is just twice the lower density, both stocking densities were initially considered satisfactory to keep the animals in normal physiological conditions. These stocking densities were chosen after the review of the literature on tropical fish species with the same body size, mainly with the data for *Rhamdia quelen* (Baldisserotto and Radünz- Neto, 2005). To maintain the water quality, the ponds were supplied with a continuous water flow (1800 l h^{-1}). The fish were maintained under a natural photoperiod.

During the experimental period, the fish were manually fed with commercial feed (PURINA; moisture, 13%; crude protein, 36%; crude lipid, 4%; ash, 14%; crude fiber, 7%; calcium, 2.5%; phosphorous, 0.6%; and pellet diameter, 3–4 mm) twice a day during the light period. Fish were not fed on the sample day to minimize handling stress. The experiment was carried out for 16 months (from September 2005 to December 2006). The amount of food consumed was monitored daily, and the body weight and length changes in each tank were measured at 2-month intervals (except when the water temperature was below 20 °C). The biomass data were used to adjust the feeding rate at 2-month intervals and the amount of food was calculated to be 5% of the biomass/day, offered to the animals in 1 daily portion. The initial food consumption was 20 g/tank/day in the 105 g/m^3 group and 40 g/tank/day in the 210 g/m^3 group. At the end of the experiment, the lower density group intake was 45 g/tank/day and the higher density group was 60 g/tank/day. The use of extruded diet was important to assess the amount of food eaten and after 4–6 h of manual feeding, the remaining pellets were weighed to discount the amount of food not eaten by the fish. The amount of food presented herein already considered the non-consumed pellets. Temperature and dissolved oxygen were monitored daily with an oximeter (model 55; YSI, Yellow Springs, OH). Ammonia was monitored by the colorimetric method described by Clesceri et al. (1998).

2.3. Growth parameters

To evaluate the growth and performance of the experimental animals, some parameters were considered. Condition factor (K) was calculated using the formula: total weight/total length^3 × 100; Weight gain (WG): (final weight − initial weight) × 100/initial weight; the feed efficiency (FE): biomass increment × 100/food consumed; specific growth rate (SGR): 100 × [(ln(Wf) − ln(Wi))/T], where ln (Wf) and ln (Wi) are the natural logarithms of the weight at the beginning (Wi) and end (Wf) of the experiment and T is the time interval in days; and the survival (S): final number of fish × 100/initial number of fish. The coefficient of variation for weight (CV, %) within tanks was calculated with the formula: weight standard deviation/mean weight × 100, and it was used to assess size variation.

2.4. Fish sampling and metabolic analyses

Twenty animals from each experimental group (five from each pond) were sampled at the end of the experimental period to evaluate several physiological parameters, many of which related to lipid metabolism. Fish were captured and quickly anesthetized with tricaine methanesulfonate (MS-222; Sigma Diagnostics INS, St. Louis, MO) (1 g MS-222:10 l water) neutralized with sodium bicarbonate (1 g:10 l water). Within 1 min, blood samples were taken from the caudal vein using heparinized syringes. The plasma was then separated by centrifugation (655.1 *g*) and stored frozen at −80 °C until it was assayed for cortisol, protein, and lipid concentration. After blood collection, the fish were sacrificed by decapitation (according to the institutional animal care protocols and approval) and total length (Lt, in cm) and total body weight (Wt, in g) for each animal were recorded.

Biometrical parameters were recorded (Table 1). The animals were dissected and had their liver removed and weighed to calculate the hepatosomatic index (HSI)—the percentage of body weight

Table 1
Growth parameters from *Brycon insignis* reared at two stocking densities (105 g/m^3 and 210 g/m^3) in experimental ponds for 16 months.

Stocking densities		
	105 g/m^3	210 g/m^3
Initial total length (cm)	15.5 ± 0.04	15.6 ± 0.01
Final total length (cm)	23.2 ± 0.08[a]	22.2 ± 0.01[b]
Initial body weight (g)	35.2 ± 0.46	35.7 ± 0.10
Final body weight (g)	118.4 ± 1.64 [a]	100.3 ± 0.64[b]
K	0.90 ± 0.004	1.07 ± 0.004
SGR (%)	0.3 ± 0.01[a]	0.2 ± 0.02[b]
WG (g)	235.8 ± 1.85 [a]	181.4 ± 0.57[b]
CV initial (%)	27.3 ± 0.66	23.1 ± 0.08
CV final (%)	17.5 ± 0.28	17.0 ± 0.11
(FE)	53.7 ± 0.57 [a]	34.1 ± 0.20[b]
S (%)	100.0 [a]	88.0 ± 0.21[b]

A different letter in a row indicates significant differences between the stocking densities (P<0.05). Data are presented as mean ± standard error of the mean (SEM). K, condition factor; SGR, specific growth rate; WG, weight gain; CV, coefficient of variation; FE, feed efficiency; S, survival.

represented by the liver [HSI = (liver weight/total weight) ×100] (Table 1). The liver and a sample of epaxial white muscle were stored at −80 °C.

Plasma total proteins were measured according to Lowry et al. (1951). Tissue proteins were measured using the same method, after precipitation and solubilization according to the method suggested by Milligan and Girard (1993). Total protein was determined with a spectrophotometer at 660 nm using a series of bovine serum albumin (Sigma Diagnostics INS, St. Louis, MO) standard curve.

Total lipids from liver and muscle were extracted macerating the tissues with a chloroform–methanol–water (2:1:0.5) solution (Folch et al., 1957) adapted from Parrish (1999). Lipid determination was performed according to Frings et al. (1972). Total lipids were determined with a spectrophotometer at 540 nm using a cod liver oil methyl esters (Sigma Diagnostics INS, St. Louis, MO) standard curve. Plasma lipids were quantified with the same method, without previous extraction.

The lipid extracts (as described above) were separated into polar lipids (phospholipids) and neutral lipids (triglycerides) using an activated silica column (Yang, 1995). Methylation of each fraction was performed with acetyl chloride (5% HCl in methanol) (Christie, 2003), and fatty acid composition was determined as methyl esters using a Varian Model 3900 Gas Chromatograph (GC) coupled to flame ionization detection (FID). Fatty acids were identified by comparing their retention times with those of known methyl esters standards (FAME) (Supelco, 37 components, Sigma-Aldrich).

The FAME were analyzed on a capillary column CP Wax 52 CB, 0.25-μm thickness, inside diameter of 0.25 mm, and 30-m length. Hydrogen was used as carrier gas at a linear velocity of 22 cm/s. The temperature program was 170 °C for 1 min followed by a 25 °C/min ramp to 240 °C, and a final hold time of 5 min. Injector and flame ionization detector (FID) temperatures were 250 and 260 °C, respectively.

Plasma cortisol concentration was measured using a commercially available ELISA (enzyme-linked immune sorbent assay) kit (Cortisol ELISA, RE52061, IBL, Hamburg GmbH).

2.5. Data analysis

All values were expressed as the mean ± standard error of the mean (M ± SEM). Comparisons of physiological and zootechnical data between both stocking densities were made using the Nested Design ANOVA (Sokal and Rohlf, 1995). In all analyses, the differences were considered to be significant when $P<0.05$. These analyses were performed using the statistical software STATISTICA 6.0 Copyright © StatSoft, Inc. 1984–2001.

3. Results

3.1. Water quality and temperature profile

Dissolved oxygen and ammonia concentration and pH values were maintained within the ideal parameters for most teleost species. However, water temperatures varied throughout the year, directly interfering with *B. insignis*' growth. The data presented in Fig. 1 show that during the higher temperature period (from December to April), the body mass increased in both groups (slightly higher in the low stocking density group) and then stop increasing from the end of fall until the beginning of spring, i.e., during winter.

3.2. Growth in different stocking densities

The growth parameters are presented in Table 1. The condition factor (K) and variation coefficient (CV) for body mass were not affected by stocking density. However, final body mass and length, specific growth rate (SGR), and weight gain were higher in the low stocking density group. The low stocking density group also presented higher feed efficiency (FE) and survival (S) rates.

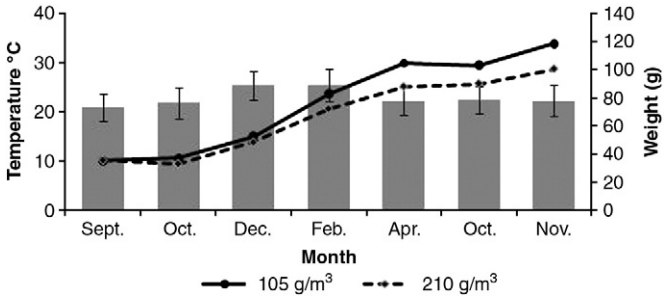

Fig. 1. Body mass (g) and temperature (°C) variation over time of *B. insignis* reared in both stocking densities (105 and 210 g/m^3). The temperature data coincide with the months when the growth data were registered.

3.3. Metabolic and cortisol analyses

Table 2 shows the results with regard to the influence of stocking density on metabolism and cortisol levels. Plasma and hepatic proteins were not affected by stocking density; however, muscle proteins were higher in the high-density stocking density group. Plasma and muscle lipid content were not affected by stocking density. Fish subjected to lower stocking density presented higher lipid concentration in the liver, though the HSI values did not change. Despite the differences observed in metabolic and growth parameters, plasma cortisol was not affected by stocking density.

Considering the fatty acids profile of the analyzed tissues, there were no differences in neutral lipid fractions of liver and muscle, considering the different stocking densities (Tables 3 and 4). However, considerable changes occurred in the polar lipids of both tissues.

In the liver polar lipid fatty acids, the saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) profiles were not affected by stocking density. Total polyunsaturated fatty acids (PUFA), PUFA n-3 and the ratio PUFA Σ n-3/Σ n-6 were higher in the higher stocking density group. The main fatty acid altered was docosahexaenoic acid, 22:6n-3 (DHA). In contrast, the PUFA n − 3, eicosapentaenoic acid, 20:5n-3 (EPA), and dodecatetraenoic acid, 22:4n − 3 were lower in polar hepatic fraction of the higher stocking density group (Table 5). PUFA n − 6 was also higher in the higher stocking density group, mainly due to an increase in 20:4n − 6 (ARA) and 22:2n − 6 (Table 5).

In the polar fraction of the white muscle, total SFA was lower in the higher stocking density, due to a lower percentage of palmitic acid (C16:0). The same profile was observed in the MUFA, with a lower percentage of 18:1n − 9, 20:1n − 9 and 24:1 in the higher stocking density group. This lower SFA and MUFA content in the higher stocking density group was compensated for higher amounts of total PUFA, PUFA n-3, due to an increase in docosapentaenoic acid, 22:5n-3 (DPA) and DHA from omega 3 series and arachidonic acid, 20:4 n-6

Table 2
Effect of fish density on some metabolic parameters in *Brycon insignis*.

	105 g/m^3	210 g/m^3
HSI (%)	0.7 ± 0.07	0.5 ± 0.06
Liver protein (mg/g)	43.6 ± 1.98	39.2 ± 1.98
Plasma protein (mg/ml)	27.8 ± 2.05	25.2 ± 3.17
Muscle protein (mg/g)	47.2 ± 5.49[a]	59.3 ± 3.44[b]
Liver lipid (mg/g)	27.6 ± 2.49[a]	18.6 ± 2.01[b]
Muscle lipid (mg/g)	7.5 ± 0.74	5.5 ± 0.65
Plasma lipid (mg/dl)	494.9 ± 16.75	412.7 ± 26.08
Plasma cortisol (ng/ml)	83.4 ± 2.57	96.0 ± 8.47

A different letter in the row indicates significant difference between the stocking densities ($P<0.05$). Data are presented as mean ± standard error of the mean (SEM). Hepatossomatic index (HSI).

Table 3
Fatty acid profile (%) of neutral lipids fraction in liver of *Brycon insignis* in different stock densities (mean ± SEM).

Fatty acid (%)	105 g/m^3	210 g/m^3
C14:0	0.7 ± 0.06	0.7 ± 0.07
C16:0	15.4 ± 1.44	17.3 ± 0.52
C18:0	11.4 ± 0.88	12.1 ± 1.23
C20:0	0.4 ± 0.06	0.5 ± 0.06
C24:0	0.6 ± 0.08	0.4 ± 0.06
Σ SFA	28.7 ± 1.97	32.3 ± 3.48
C16:1	2.3 ± 0.24	2.3 ± 0.19
C18:1n-9	28.4 ± 1.83	25.4 ± 1.07
C20:1n-9	1.6 ± 0.17	1.3 ± 0.09
Σ MUFA	33.1 ± 2.42	30.0 ± 0.05
C18:3n-3	0.9 ± 0.08	0.9 ± 0.11
C20:5n-3	0.4 ± 0.05	0.3 ± 0.07
C22:4n-3	0.5 ± 0.06	0.6 ± 0.11
C22:5n-3	0.7 ± 0.10	0.6 ± 0.07
C22:6n-3	2.9 ± 0.49	4.7 ± 0.61
Σ n3 PUFA	7.3 ± 0.71	7.5 ± 0.93
C18:2n-6	11.8 ± 1.16	12.3 ± 1.38
C18:4n-6	0.5 ± 0.05	1.5 ± 0.23
C20:3n-6	0.4 ± 0.05	2.2 ± 0.20
C20:4n-6	1.3 ± 0.12	3.7 ± 0.61
C22:2n-6	0.7 ± 0.11	0.7 ± 0.12
C22:4n-6	2.7 ± 0.28	0.5 ± 0.04
C22:5n-6	2.6 ± 0.38	0.8 ± 0.11
Σ n-6 PUFA	21.9 ± 2.63	21.4 ± 1.82
Σ PUFA	32.1 ± 2.83	39.6 ± 0.94
Σ n-3/ Σ n-6	0.1 ± 0.02	0.1 ± 0.08

Values below 0.5% were not presented in the table. Σ Sat, Σ MUFA, Σ PUFA, Σ PUFA n − 6, and Σ PUFA n − 3 are the sum of saturated, monounsaturated, polyunsaturated, polyunsaturated n-6, and n-3, respectively.

(ARA), 22:4n-6 and 22:5n-6 from omega 6 series. This alteration resulted in an increase in the $\Sigma\ n-3/\Sigma\ n-6$ in the 210 g/m^3 group. On the other hand, the C18 PUFAs 18:2n-6 and 18:4n − 6 were higher in the lower stocking density group (Table 6).

Table 4
Fatty acid profile (%) of neutral lipids fraction in white muscle of *Brycon insignis* in different stock densities (mean ± SEM).

Fatty acid (%)	105 g/m^3	210 g/m^3
C16:0	16.8 ± 0.83	14.5 ± 3.74
C18:0	8.0 ± 0.30	8.0 ± 0.30
C20:0	0.4 ± 0.04	0.2 ± 0.04
C24:0	0.9 ± 0.04	0.2 ± 0.04
Σ SFA	27.2 ± 1.11	24.4 ± 1.13
C16:1	1.7 ± 0.10	1.4 ± 0.10
C18:1n-9	27.1 ± 1.07	26.7 ± 1.21
C20:1n-9	0.8 ± 0.04	0.2 ± 0.04
Σ MUFA	29.3 ± 0.22	29.1 ± 1.52
C18:3n-3	1.1 ± 0.04	0.2 ± 0.04
C18:4n-3	0.7 ± 0.06	0.3 ± 0.06
C20:4n-3	1.0 ± 0.19	0.2 ± 0.01
C20:5n-3	0.6 ± 0.04	0.6 ± 0.04
C22:4n-3	1.2 ± 0.02	0.2 ± 0.02
C22:5n-3	1.1 ± 0.04	0.2 ± 0.04
C22:6n-3	3.6 ± 0.50	6.3 ± 0.50
Σ n 3 PUFA	9.4 ± 1.03	9.0 ± 0.63
C18:2n-6	18.1 ± 0.23	20.0 ± 1.27
C18:4n-6	2.1 ± 0.04	0.2 ± 0.04
C20:3n-6	1.0 ± 0.06	2.3 ± 0.02
C20:4n-6	2.3 ± 0.25	0.2 ± 0.01
C22:4n-6	0.4 ± 0.04	0.5 ± 0.12
C22:5n-6	2.2 ± 0.58	1.7 ± 0.19
Σ n-6 PUFA	28.7 ± 0.52	31.2 ± 0.91
Σ PUFA	35.7 ± 1.11	36.9 ± 1.19
Σ n-3/Σ n-6	1.0 ± 0.14	0.4 ± 0.01

Values below 0.5% were not presented in the table. Σ Sat, Σ MUFA, Σ PUFA, Σ PUFA n − 6, and Σ PUFA n − 3 are the sum of saturated, monounsaturated, polyunsaturated, polyunsaturated n-6, and n-3, respectively.

Table 5
Fatty acid profile (%) of polar lipids fraction in liver of *Brycon insignis* in different stock densities (mean ± SEM).

Fatty acid (%)	105 g/m^3	210 g/m^3
C14:0	0.5 ± 0.05	0.7 ± 0.08
C15:0	0.5 ± 0.13	0.9 ± 0.10
C16:0	12.3 ± 1.10	17.3 ± 1.18
C18:0	11.6 ± 0.82	17.1 ± 1.29
C20:0	0.5 ± 0.07	0.4 ± 0.05
C22:0	1.2 ± 0.24	0.74 ± 0.10
C24:0	0.9 ± 0.10	1.4 ± 0.21
Σ SFA	27.6 ± 1.50	26.8 ± 2.51
C16:1	1.7 ± 0.21	1.9 ± 0.15
C18:1n-9	20.4 ± 2.65	19.4 ± 1.76
C20:1n-9	2.1 ± 0.21	2.1 ± 0.12
C20:1n-11	0.6 ± 0.11	0.3 ± 0.01
C24:1	0.8 ± 0.09	1.0 ± 0.14
Σ MUFA	22.5 ± 2.49	18.4 ± 2.68
C18:3n-3	0.6 ± 0.08	0.4 ± 0.06
C20:4n-3	0.5 ± 0.06	0.2 ± 0.01
C20:5n-3	0.7 ± 0.07^a	0.4 ± 0.05^b
C22:4n-3	2.6 ± 0.08^a	0.3 ± 0.02^b
C22:5n-3	1.1 ± 0.06	1.0 ± 0.12
C22:6n-3	11.6 ± 1.10^a	22.1 ± 1.37^b
Σ n 3 PUFA	16.4 ± 2.33^a	24.8 ± 1.50^b
C18:2n-6	8.5 ± 0.98	6.9 ± 0.71
C18:4n-6	0.8 ± 0.09	1.4 ± 0.25
C20:4n-6	7.1 ± 0.53^a	10.3 ± 0.31^b
C22:2n-6	0.4 ± 0.04^a	1.7 ± 0.06^b
C22:4n-6	0.5 ± 0.06	0.4 ± 0.03
C22:5n-6	4.4 ± 0.43	4.0 ± 0.47
Σ n-6 PUFA	22.0 ± 2.66^a	29.5 ± 1.50^b
Σ PUFA	42.1 ± 3.01^a	54.5 ± 3.71^b
Σ n-3/ Σ n-6	0.6 ± 0.02^a	0.9 ± 0.03^b

Values below 0.5% were not presented in the table. Σ Sat, Σ MUFA, Σ PUFA, Σ PUFA n − 6, and Σ PUFA n − 3 are the sum of saturated, monounsaturated, polyunsaturated, polyunsaturated n − 6, and n − 3, respectively. ^{ab} Different letters indicate statistical differences ($P<0.05$) between groups.

4. Discussion

The results of the present study clearly indicate that the stocking density has a marked effect on the lipid metabolism of *Brycon insignis*

Table 6
Fatty acid profile (%) of polar lipids fraction in white muscle of *Brycon insignis* in different stock densities (mean ± SEM).

Fatty acid (%)	105 g/m^3	210 g/m^3
C16:0	16.8 ± 5.42^a	9.9 ± 2.32^b
C18:0	11.5 ± 0.95	10.0 ± 0.75
Σ SFA	29.0 ± 2.05^a	20.7 ± 0.97^b
C16:1	0.5 ± 0.05	0.3 ± 0.02
C18:1n-9	12.4 ± 0.90^a	8.2 ± 0.52^b
C20:1n-9	0.6 ± 0.04^a	0.4 ± 0.02^b
C24:1	0.5 ± 0.06^a	0.3 ± 0.01^b
Σ MUFA	13.9 ± 0.84^a	9.2 ± 0.50^b
C20:5n-3	1.0 ± 0.06	1.1 ± 0.06
C22:5n-3	2.3 ± 0.17^a	3.3 ± 0.16^b
C22:6n-3	21.5 ± 1.63^a	28.1 ± 1.54^b
Σ n3 PUFA	26.9 ± 2.00^a	34.7 ± 1.94^b
C18:2n-6	8.5 ± 0.31^a	7.0 ± 0.39^b
C18:4n-6	2.0 ± 0.31^a	1.0 ± 0.09^b
C20:3n-6	3.9 ± 0.15	4.1 ± 0.12
C20:4n-6	6.8 ± 0.49^a	9.0 ± 0.41^b
C22:4n-6	6.0 ± 0.60^a	9.0 ± 0.46^b
C22:5n-6	5.5 ± 0.49^a	7.7 ± 0.41^b
Σ n-6 PUFA	28.8 ± 1.10	28.6 ± 0.93
Σ PUFA	55.3 ± 2.51^a	62.6 ± 1.93^b
Σ n-3/ Σ n-6	0.9 ± 0.07 ^{a}	1.2 ± 0.09 ^{b}

Values below 0.5% were not presented in the table. Σ Sat, Σ MUFA, Σ PUFA, Σ PUFA n − 6, and Σ PUFA n − 3 are the sum of saturated, monounsaturated, polyunsaturated, polyunsaturated n − 6, and n − 3 respectively. ^{ab} Different letters indicate statistical differences ($P<0.05$) between groups.

and the density of 105 g/m^3 resulted in a better growth and performance.

To evaluate a satisfactory fish stocking density, many additional parameters must be analyzed, such as tank dimensions, feeding parameters, and water quality and temperature (Ellis et al., 2002; Håstein et al., 2005; Di Marco et al., 2008). Temperature is considered one of the most important biotic factors affecting growth, food consumption, and feed efficiency in fish (Azaza et al., 2007; Martinez et al., 1996). Therefore, identifying the range of temperatures tolerated by a species is important to determine the viability of its growth. Given its importance, this has been ascertained for many fish species, including the tropical freshwater species *Oreochromis niloticus* (Azaza et al., 2007), *Oreochromis mossambicus* (El-Sayed et al., 1996; Campinho et al., 2004), *Piaractus mesopotamicus* (Anelli et al., 2004), and *R. quelen* (Chippari-Gomes et al., 1999).

The data showed that temperatures negatively affected *B. insignis* growth during the fall–winter period, independently of the stocking density (Fig. 1). The water temperature registered in the present study can be considered an important parameter to predict growth of the species in the southeast of the country because it affects weight gain, independently of the food ingested, feed efficiency, condition factor, and stocking density (Wang et al., 2009). It is important to point out that in the region where *B. insignis* is endemic, the annual water temperature ranges from 18 to 24.1 °C (CETESB, 2009), which is similar to that supplied for this trial.

The stocking density of 105 g/m^3 resulted in higher values of length, weight, specific growth rate (SGR), weight gain (WG), and feed efficiency (FE). However, no changes were found in the condition factor (*K*), an indicator of general fish well-being. *K* varied from 0.90 to 1.07, values that classify the fish as healthy, according to Fulton's condition, as previously found for halibut (*P. californicus*) (Merino et al., 2007). Another important point to consider is the survival rate, which was higher in the lower stocking density group, which presented null mortality along the experiment. These results are consistent with what has been previously found in *Puntius sarana* in different stocking densities (Chakraborty et al., 2003).

It is also worth noting that, although some growth parameters could be considered better for the low-density group, there was no difference in the coefficient of variation (CV) for body mass between both groups, suggesting a homogeneous growth. The increase in stocking density can cause higher variations in body mass, increasing the CV, mainly due to the establishment of hierarchical groups (Lambert and Dutil, 2001), as observed in turbot, *S. maximus* (Irwin et al., 1999). Some species such as African catfish, vundu catfish (Toko et al., 2007), European sea bass (Di Marco et al., 2008), California halibut (*P. californicus*) (Merino et al., 2007), also showed homogeneous CV in different stocking densities. Most authors conceived that this depends on many factors, mainly the species behavior and the stocking density considered. Thus, this suggests that the stocking densities used for *B. insignis* in this study were not enough to alter the species behavior and led to a disproportional food acquisition in a significant way. On the other hand, feed efficiency (FE) was better for the lower density group, as also observed for the California halibut (*P. californicus*) (Merino et al., 2007). Some authors consider that differences in FE are related to an increase in competition for food and space (Haque et al., 1994; Islam, 2002) that can also lead to an increase in stress and a consequent increase in metabolic rate (Purser and Hart, 1991; Canario et al., 1998). In this condition, the energy required for growth is deviated to supply this increase in metabolic rate.

4.1. Metabolic and cortisol analyses

B. insignis in higher stocking density accumulates lower amounts of lipids in the liver, but the HSI did not significantly change due to this accumulation, showing just a slightly decrease, which was not considered to be statistically different from the lower stocking density group. This HSI profile was observed in brook charr (Vijayan et al., 1990) due to reductions in hepatic glycogen and in gilthead sea bream (Montero et al., 1999) because of a decrease in hepatic lipids.

In *B. insignis*, the tendency for HSI to decrease in the higher stocking density group was followed by a decrease in hepatic lipids, suggesting a higher utilization of hepatic lipids in a condition that can be more stressful for the animals. In *Salmo salar*, crowding reduces the HSI by 15%, indicating that stressed fish redirect the energy to cope with the crowding stressor (Basrur et al., 2010). In brook charr, the animals stocked in higher density (120 kg/m^3) had an increase in the activity of hydroxyacyl CoA dehydrogenase (HOAD), an enzyme involved in β-oxidation, and a decrease in glucose-6-phosphate dehydrogenase (G6PDH), when compared with the lower stocking density group (30 kg/m^3), suggesting a decrease in lipid synthesis (Vijayan et al., 1990). This alteration in the rate of synthesis/oxidation of lipids could be the reason for the lower growth rate suggested for the higher density group during the months with warmer-than-average temperatures (Fig. 1). Furthermore, this is considered one of the main causes of the low growth rates found for many fish species in captivity (Clutter and Brascamp, 1998).

Plasma and muscle lipids concentration were not affected by higher stocking density, and data showed that *B. insignis* presented lower amounts of white muscle lipids, when compared to the liver. This suggests that during the juvenile period, this species uses the liver as a lipid depot organ, as observed for *Salminus brasiliensis* (Moreira et al., 2002), *Perca fluviatilis*, and *S. salar* (Henderson and Tocher, 1987). On the other hand, in *Pleuronectes platessa* (Dawson and Grimm, 1980), *Pagrus major*, and *Seriola lalandi* (Ando et al., 1993), the muscle lipid concentration is higher than the hepatic concentration. These differences can be interspecific, but also intraspecific in the case of adults, depending on age, growth rate, diet composition, and maturation stage (Sheridan, 1994; Jobling et al., 1998; Tobin et al., 2006).

Analyzing the protein data, the results showed that the increase in stocking density had no effect on hepatic and plasma protein levels. However, muscle protein levels were higher in the higher density group. Considering that the growth rate was lower in the higher stocking density group and that the metabolic rate is inversely proportional to body mass, it can be assumed that the protein synthesis in the higher stocking density group was higher, leading to this increase in muscle protein.

The analysis of the fatty acids (FA) of the neutral (triglycerides) and polar (phospholipids) lipids showed that the increase in stocking density had no effects on the profile of FA in the triglycerides (TG) but altered the FA composition in the phospholipids (PL), the FA that are esterified into membrane PLs in muscle and liver cells (Tables 5 and 6). In the liver membranes, the higher stocking density caused an increase in the percentage of polyunsaturated fatty acids (PUFA), specifically the n-3-PUFA, due to an increase in the HUFA (highly unsaturated fatty acid) 22:6n-3 (DHA, docosahexaenoic acid). On the other hand, the lower stocking density group presented liver membranes with higher amounts of the less unsaturated HUFAs, 20:5n − 3 (EPA, eicosapentaenoic acid) and 22:4n − 3 (dodecatetraenoic acid). In *S. aurata* (Montero et al., 1999), the results found in hepatic phospholipids were different, showing that the higher stocking density group (40.8 kg/m^3) exhibited a decrease in DHA when compared with the lower stocking density group (10.56 kg/m^3).

The FA from the n-3 family, mainly DHA and EPA, are related with many physiological processes and are present in higher amounts in marine fish species (Person-Le Ruyet et al., 2004; Sargent et al., 2002). The percentage of PUFA n − 3 found in hepatic phospholipids in *B. insignis* (16.4 to 24.8%) is considerably lower when compared with the same tissue in *S. aurata* (a marine species) juveniles. In this latter group, polar lipids in the liver contain ~40% of PUFA n − 3. Both FA (DHA and EPA) are the main phospholipid components in fish membranes (Sargent et al., 2002; Skalli et al., 2006) and can be released from the

PL due to the action of phospholipases, lipoxygenases, and cyclooxygenases to produce leukotrienes, prostaglandins, and thromboxanes and other compounds that are important in the immune system (Ganga et al., 2005; Uhing et al., 1990).

The higher percentage of DHA in the muscle and liver membranes of the higher stocking density group could be ascribed to an increase in the amount and/or activity of the desaturase and/or elongase enzymes (hepatic enzymes that elongate and desaturate FA acids chains). In addition, this could also be the result of a physiological response against a stressor (the increase in stocking density), considering that these animals presented a lower growth. DHA can directly modulate the cellular metabolism. The "membrane pacemaker theory of metabolism" states that the metabolic rate of a given species is determined by the activity of the membrane-bound proteins associated with the energy-consuming processes of cells and that the lipid composition of the cellular membranes alters the physical properties of these membranes, directly modulating changes in metabolic rates (Hulbert and Else, 1999). According to this theory, DHA is the fatty acid primarily responsible for modulating the metabolic rate in the animals. Studies with mammals with different body mass showed that smaller animals have a higher amount of DHA in membrane phospholipids than bigger animals (Couture and Hulbert, 1995), and this profile is found in every body tissue, except for the brain (Hulbert, 2008).

In the present work, a higher DHA percentage in the polar fraction of the analyzed tissues of the higher density group (the smaller animals) was encountered. In addition, animals from this group also presented a higher amount of protein in the white muscle. These results suggest that in *B. insignis*, the stocking of 210 g/m^3 can change the metabolic activity remodeling the membrane fatty acids. This alteration in DHA content in tissue membranes due to allometry can be compensated by C18 PUFA n−3 fatty acids (as linolenic acid) or PUFA n−6 (in heart) and/or MUFA (skeletal muscle and kidney) (Hulbert, 2008). In *B. insignis*, this tendency was observed because the higher amount of DHA in the polar muscle fraction was compensated by a decrease in MUFA (Table 6).

Many authors have shown that stocking density interferes with the metabolism of many fish species (Montero et al., 1999, 2001; Schreck et al., 1985; Vijayan et al., 1990); however, the muscle fatty acid profile is rarely investigated. According to Henderson and Tocher (1987), the muscle fatty acid composition should be carefully interpreted, due to the possibility of elongation and desaturation of C18:2n−6 and C18:3n−3 into their biological active homologues, C20:4n−6 (ARA), C20:5n−3 (EPA), and C22:6n−3 (DHA) being accumulated in white muscle, as observed in freshwater species. As a result, it was suggested that the higher amounts of DHA and other C20-22 PUFA in the higher stocking density group could be attributed to an alteration in the activity of the elongases and desaturases.

PUFA n−3 can alter the gene expression of the enzymes involved in fatty acid oxidation (Hsu and Ding, 2003). In turn, these fatty acids can also alter the activity of the enzymes implicated in the triglycerides synthesis in mitochondria and peroxisomes (Willumsen et al., 1993). This can cause a decrease in SFA, as observed for *B. insignis*, which presented an increase in PUFA n−3 and a decrease in SFA in the muscle membranes of the animals from the higher stocking density group.

Additionally, crowding increases the eutrophication in aquatic environments due to the increase in nitrogen compounds originated from fish metabolism. However, no differences in water quality parameters (mainly dissolved oxygen) were detected, and it is known that an increase in polar PUFA n−3 due to an increase in algae is not common in freshwater ecosystems, where the main PUFA belong to n−6 fatty acids family (Olsen, 1999).

The stocking density did not alter plasma cortisol in *B. insignis*. Similar findings have been previously described for *Dicentrarchus labrax* (Sammouth et al., 2008) and *C. gariepinus* (van de Nieuwegiessen et al., 2008). The plasma cortisol concentration observed in *B. insignis* is under the basal concentration (83.4 ng/ml in the high-density group and 96 ng/ml in the low-density group) detected in another congeneric species *Brycon cephalus* (110 ng/ml) (Carneiro and Urbinati, 2001). However, alterations in cortisol concentration were found in salmonids (Mazur and Iwama, 1993), sea bream *D. labrax* (Barton et al., 2005; Di Marco et al., 2008), *Plecoglossus altivelis* (Iguchi et al., 2003), and *S. aurata* (Montero et al., 1999) under different stocking densities. The plasma cortisol concentration in *B. insignis* is high compared to fish species living in temperate regions, such as *S. aurata* (3.9 ng/ml; Montero et al., 1999), *Oncorhynchus mykiss* (2.5 ng/ml; Bleau et al., 1996), *S. salar* (17.1 ng/ml; Basrur et al., 2010), and also when compared to fish species living in warmer regions, such as *O. mossambicus* (20 ng/ml; Nokaido et al., 2010), *Cyprinus carpio* (20 ng/ml; Ruane and Komen, 2003), *Pseudoplatystoma corruscans* (12 ng/ml; Fagundes and Urbinati, 2008), and *R. quelen* (34.8 ng/ml Barcellos et al., 2010). This finding suggests that *Brycon* species have a basal cortisol plasma level higher than most of the fish species analyzed so far, probably because the acclimation to experimental conditions in this group is more stressful than the change in the stocking density. Consequently, the alteration in cortisol concentration is low, and these animals do not return to basal cortisol levels under experimental conditions.

Some studies focusing on conservation physiology induce animals to mild stress to assess the capacity of an individual to react to environmental stress (Wikelski and Cooke, 2006). However, not all environmental conditions are able to induce a detectable increase in glucocorticoids concentration (Rich and Romero, 2005; Wikelski and Cooke, 2006). Therefore, when an external event that would be viewed as a stressor fails to activate the hypothalamus–pituitary–interrenal (HPI) axis, this could be interpreted either as an event not actually being perceived as stressful or as evidence of some mechanism that prevented the activation of the axis (Wingfield and Sapolsky, 2003). Additionally, Basrur et al. (2010) suggested that fish can initially respond to a stress condition by increasing glucocorticoids, but in time, habituation to the stressor can result in a reduction in physiological alterations, i.e., plasma cortisol, which is the situation, proposed in the present study because of the long-term experimental period.

High stocking densities can generate physiological and social effects on fish, and these alterations seem to be species-specific (Barton, 2002; Mommsen et al., 1999). As observed in *B. insignis*, plasma cortisol was not altered by the increase in stocking density; however, alterations in lipid metabolism and in specific essential fatty acids were observed. The metabolic parameters measured, together with higher feed efficiencies, survival, and growth rates, which are considered good indicators to evaluate chronic stress (Kristiansen et al., 2004; Schram et al., 2006), indicate that the lower stocking density group had better growth. However, it is important to emphasize that the goal of this work was not to test crowding stocking situations for *B. insignis*, since no previous information is available for this species, so even the higher stocking density can be considered low when compared with studies with other fish species. Therefore, additional experiments with increasing stocking densities should be done in order to evaluate these physiological parameters in actually crowding situations.

The data obtained in the present study suggest that *B. insignis* reared in a stocking density of 105 g/m^3 show better zootechnical and physiological indices. However, even during the months with higher than average temperatures, when the lower density group presented higher growth, SGR was low, evidencing that further experiments with this species may be required. The continuance of the study with this species is important to provide data and gain knowledge to optimize the production, improving the management of this species in captivity, and consequently the fish restocking program in the Paraiba do Sul Basin.

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