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ORIGINAL ARTICLE

Effect of density of fingerling and juvenile pirarucu during transportation on water quality and physiological parameters

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ABSTRACT

We assessed the effect of stocking density on physiological parameters (blood lactate, glucose, cortisol, hematocrit), water quality (temperature, dissolved oxygen, pH, unionized ammonia, carbon dioxide), and survival during the transportation of fingerling (24.5 ± 4.7 g) and juvenile (615.8 ± 122.2 g) pirarucu (*Arapaima gigas*) for six hours in plastic bags. The tested densities were 65, 80, 95, 110 and 125 g L^{-1} for fingerlings, and 50, 80, 110, 140 and 170 g L^{-1} for juveniles (three replicates each). Parameters were measured prior to and immediately after transportation, and at 24 and 96 hours recovery after transportation. No mortality was observed, except for fingerlings (< 3%) at densities of 110 and 125 g L^{-1} during recovery. All the water quality parameters were significantly altered after the transportation of fingerlings and juveniles. Water temperature, dissolved oxygen, carbon dioxide and unionized ammonia increased, but pH decreased. Only carbon dioxide and unionized ammonia differed among densities. Cortisol levels did not increase over time, except for the juveniles at 170 g L^{-1}, which still had high cortisol after 96 hours. Glucose significantly increased after transportation for all the treatments and returned to the initial values during the recovery period. Conversely, the lactate values were still high after 96 hours. Hematocrit was assessed only for juveniles and was significantly lower after transportation. We conclude that fingerling and juvenile pirarucu can be safely transported at densities up to 95 g L^{-1} and 140 g L^{-1}, respectively.

KEYWORDS: *Arapaima gigas*, closed system, lactate, cortisol, glucose

Efeito da densidade de transporte de alevinos e juvenis de pirarucu sobre a qualidade da água e parâmetros fisiológicos

RESUMO

Este estudo avaliou o efeito da densidade de estocagem sobre parâmetros fisiológicos (lactato, glicose, cortisol e hematócrito), qualidade de água (temperatura, oxigênio dissolvido, pH, amônia não ionizada e gás carbônico) e sobrevivência no transporte de alevinos (24.5 ± 4.7 g) e juvenis (615.83 ± 122.19 g) de pirarucu (*Arapaima gigas*) em sacos plásticos por seis horas. Avaliamos densidades de 65, 80, 95, 110 e 125 g L^{-1} para alevinos, e de 50, 80, 110, 140 e 170 g L^{-1} para juvenis (três réplicas cada). Os parâmetros de resposta foram medidos antes e logo após o transporte, e após 24 e 96 horas de recuperação. Não foi observada mortalidade, exceto para alevinos (< 3%) das densidades de 110 e 125 g L^{-1} durante o período de recuperação. Foram observadas alterações significativas em todos os parâmetros de qualidade de água após o transporte de alevinos e juvenis. Houve elevação da temperatura, oxigênio dissolvido, gás carbônico e amônia não ionizada e decréscimo do pH, com diferenças entre as densidades apenas para gás carbônico e amônia não ionizada. Não houve elevação dos níveis de cortisol, exceto para juvenis na densidade de 170 g L^{-1} que ainda permaneciam com nível alto de cortisol às 96 horas. A glicose se elevou após o transporte em todos os tratamentos, retornando aos valores iniciais na recuperação, ao contrário do lactato, que se elevou e permaneceu alto até as 96 horas. O hematócrito foi avaliado apenas para juvenis e diminuiu significativamente após o transporte. Concluímos que alevinos de pirarucu podem ser transportados com segurança em densidades de até 95 g L^{-1} e juvenis em densidades de até 140 g L^{-1}.

PALAVRAS-CHAVE: *Arapaima gigas*, sistema fechado, lactato, cortisol, glicose

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INTRODUCTION

Live fish transportation is a routine practice in fish farming that allows fish to be moved between production chain links. During transportation, fish are simultaneously subject to several stressors, mainly long exposure to poor water quality and inappropriate stocking densities, and to poor handling, confinement and acclimation (Harmon 2009). These stressors, in conjunction with fish health status and the level of blood chemical changes, are the main factors to affect post-transportation survival (Berka 1986; Portz *et al.* 2006).

Fish may be transported in closed systems and all conditions for survival must be provided while fish are confined (Berka 1986). As closed systems offer no air or water exchanges, loading capacity is an important determinant for fish safety during transportation. Water quality is one of the main factors to determine loading capacity during transportation (Portz *et al.* 2006) and is a limiting factor of stocking density during transportation as water deterioration increases with fish density because metabolic residue accumulates (Lim *et al.* 2003). High densities may also cause injuries to fish (King 2009; Ross and Ross 2008). Moreover, there is pressure from the production sector for higher stocking densities to improve economic viability (Portz *et al.* 2006; Carneiro *et al.* 2009).

With increasing pirarucu, *Arapaima gigas* (Schinz 1822) farming in Brazil (IBGE 2018), transporting fish of different size classes to connect production sector links has intensified, mainly between hatcheries and grow-out farms, which frequently demand fish as long as 20 cm. Fish larger than 20 cm are generally demanded by producers whose production structures are deficient (Rebelatto *et al.* 2015). Nevertheless, very few studies have assessed transportation variables. Brandão *et al.* (2006) assessed the physiological effects of transporting pirarucu (33 g) in plastic bags at a density of 20 g L^{-1} for three hours, and reported a rise in glucose levels and lower lactate levels after transportation, but no mortality. Gomes *et al.* (2006) reported no fish mortality after transporting pirarucu (33 g) in plastic bags at a density of 40 g L^{-1} for three hours at different NaCl concentrations (0, 1, 3, 5 g L^{-1}), but Na^+, Cl^- and Ca^{2+} net fluxes increased and salt levels were not recommended. Gomes *et al.* (2003) assessed the transportation of pirarucu (1.1 kg, 1 fish per bag) in plastic bags (10 L water) for six hours with and without oxygen supply. They reported no mortality, although glucose levels were higher, which indicates stress. Lima and Oliveira (2018) evaluated three densities (80, 120, 160 kg m^{-3}) for the transportation of pirarucu (9 kg) in an open system. They did not reach the maximum loading capacity, noted no mortality. However, hematocrit values were higher, lactate concentrations were lower, and they observed alterations in water quality parameters (higher ammonia and carbon dioxide (CO_2) concentrations, and less dissolved oxygen).

The gap in information on adequate pirarucu transportation still exists, mainly concerning basic aspects such as the effect of density and how it directly affects costs. Therefore, the present study aimed to assess the effect of different stocking densities on water quality, physiological stress parameters and mortality while transporting fingerlings and juveniles pirarucu in plastic bags for six hours.

MATERIAL AND METHODS

Experimental design

The present study was conducted by performing two transport trials for fingerling and juvenile pirarucu, respectively. Prior to transportation, fish from both size classes were kept in 300 L tanks (1-2 g L^{-1}) and fed *ad libitum* a commercial extruded feed for carnivorous fish species (40% crude protein) three times a day. The fish transportation route included paved roads and back roads, and lasted 6 hours (h), considered a short transportation time (Sampaio and Freire 2016). This time allows the producer to sell fish in cities at a distance of up to some 400 km away, which allows providing a service to at least an entire state in north Brazil.

Fingerlings had mean ± SD for weight and length of 24.5 ± 4.7 g and 15.6 ± 1.0 cm, respectively. The trial was conducted according to a completely randomized design with five stocking densities (65, 80, 95, 110, 125 g L^{-1}) and three replicates, which totaled 15 experimental units. Fish were transported in 60-liter plastic bags containing 5 L of water, completely filled with pure oxygen after fish were stocked in bags.

Juveniles had mean ± SD weight and length of 615.8 ± 122.2 g and 47.5 ± 6.3 cm, respectively. The trial was conducted according to a completely randomized design with five stocking densities (50, 80, 110, 140, 170 gL^{-1}) and three replicates, which gave 15 experimental units. Fish were transported in 60-liter plastic bags containing 10 L of water, completely filled with pure oxygen after fish were stocked in bags.

To minimize the influence of handling stress on transportation, two days before the trials began, fish were captured and immobilized for biometric measurements (weight and total length) (Cupp *et al.* 2017). Then they were distributed in the 300 L tanks in the densities informed above at a constant water flow (4.5 L min^{-1}) until they were packed for shipping. On the day of transportation, fish were captured from tanks with a dip net, blood was sampled from the caudal vein and fish were transferred to plastic bags. After transportation, fish were placed in 15 separate recovery tanks (300 L). Feeding was suspended for 24 h before and after transportation to empty the fish digestive tract. Then fish were fed for the following 72 h, when they were fasted again for blood sampling 96 h after transportation. Accumulated

mortality was recorded immediately after transportation and during the 96-hour recovery period.

For the physiological parameters in the fingerling trial, blood from two fish per experimental unit (six per treatment) was sampled before, immediately after, 24 h and 96 h after transportation. Blood samples were collected from the caudal vasculature with EDTA-treated syringes in less than 1 minute without anesthesia. The fish used for blood sampling were not returned to the experimental units. Due to the density-determined lower number of juveniles per treatment, blood was sampled from all the fish for the physiological parameters and fish were returned to the experimental units.

Water analysis

Water quality parameters were measured immediately before and after transportation. As the same water source was used to fill the bags in each experiment, only one sample of source water was analyzed, as a control for water condition immediately before transportation. After transportation, one water sample was taken per experimental unit (three per treatment). Water pH, dissolved oxygen, unionized ammonia and temperature were measured by a probe (YSI, Yellow Springs, USA). Carbon dioxide was measured with a commercial colorimetric kit (Alfakit, Florianópolis, SC, Brazil).

Physiological analysis

Fish physiological parameters were assessed by measuring the following blood indicators: cortisol (ng dL^{-1}), glucose (mg dL^{-1}) and lactate (mg dL^{-1}) for both trials and hematocrit (%) only in juveniles due to insufficient blood volume sampled in fingerlings. Hematocrit was determined after centrifuging blood (1387 g, 5 min) in microcapillary tubes. The microplate enzyme immune assay (Monobind, Lake Forest, CA, USA) was used to determine the blood cortisol concentration. Blood glucose and lactate were determined by the enzymatic method (Labtest, Lagoa Santa, MG, Brazil). The analyses were performed at Empresa Brasileira de Pesquisa Agropecuária (Palmas, Tocantins, Brazil).

Statistical analysis

The same statistical analyses were performed for the fingerling and juvenile data. Mortality and water quality parameters after transport were compared by a one-way ANOVA, followed by a Tukey test ($p < 0.05$). Differences between the control and water quality parameters after transportation were compared by a Student's t-test ($p < 0.05$). The physiological stress indicators were compared among treatments by a two-way ANOVA, with density and transport period as factors, followed by a Tukey test ($p < 0.05$). To evaluate the homogeneity of variance and normality premises, the Bartlett and the Shapiro-Wilk tests were respectively performed. When premises were not met, data were Box-Cox-transformed prior to the analysis. Statistical analyses were performed in R (R Core Team 2016).

Ethics and legal aspects

The study complied with Brazilian guidelines for the care and use of animals for scientific and educational purposes being approved by the Ethics Committee for the Use of Animals–CEUA of the National Research Center on Fisheries, Aquaculture and Agricultural Systems–CNPASA (protocol nos. 34/2017 and 40/2018).

RESULTS

Fingerling trial

No mortality was observed immediately after transportation. However by the end of the recovery period, mortality was recorded at the higher densities of 110 and 125 g L^{-1} (Table 1). Temperature, dissolved oxygen, unionized ammonia and CO_2 increased after transportation, but pH decreased. Water pH, temperature, dissolved oxygen and CO_2 concentration did not vary significantly among densities

Table 1. Overall mortality (96 h after transportation) of fingerlings of pirarucu, *Arapaima gigas* and water quality parameters immediately before (control) and after fingerling transportation for six hours in plastic bags at densities of 65, 80, 95,110 and 125 g L^{-1}. Values are the mean ± standard deviation of three replicates.

Water parameter	Control	After transportation				
		65 gL^{-1}	80 gL^{-1}	95 gL^{-1}	110 gL^{-1}	125 gL^{-1}
Temperature (°C)	27.1 A	31.87 ± 0.46 Ba	31.63 ± 0.85 Ba	31.23 ± 0.25 Ba	31,27 ±0.35 Ba	33.90 ± 4.31 Aa
Dissolved oxygen (mgL^{-1})	2.37 A	6.85 ± 0.55 Ba	6.05 ±0.68 Ba	6.53 ± 0.21 Ba	6.59 ± 0.42 Ba	6.26 ± 0.59 Aa
pH	7.31 A	6.62 ± 0.13 Ba	6.51 ± 0.13 Ba	6.43 ± 0.04 Ba	6.42 ± 0.01 Ba	6.42 ± 0.11 Ba
Unionized ammonia (mgL^{-1})	0.05 A	1.62 ± 0.26 Bd	1.94 ± 0.11 Bd	2.39 ± 0.12 Bc	3.08 ± 0.02 Bb	3.67 ± 0.17 Ba
Carbon dioxide (mgL^{-1})	2.0 A	12.67 ± 6.43 Aa	10.67 ± 1.15 Ba	10.33 ± 4.04 Aa	14.33 ± 6.03 Aa	13.67 ± 5.13 Aa
Overall mortality (%)	-	0.00 b	0.00 b	0.00 b	2.67 ± 3.06 a	0.33 ± 0.58 ab

Different lowercase letters indicate significant differences among treatments (one-way ANOVA, followed by a Tukey test at $p < 0.05$). Uppercase letters denote significant differences between the control and values after transportation (Student's t-test at $p < 0.05$).

immediately after transportation, but unionized ammonia rose with increased density (Table 1).

The blood glucose concentration was influenced significantly by density and time (immediately before, after, and at 24 h and 96 h after transportation). Higher concentrations were observed immediately after transportation, which returned to the initial values by the end of the recovery period for all the tested densities (Figure 1; Table 2).

The blood lactate concentration did not differ significantly between densities. With time however, i.e., significantly higher values were observed immediately after transportation that decreased during the recovery period, but did not return to the initial values. The blood cortisol levels did not increase with either time or density.

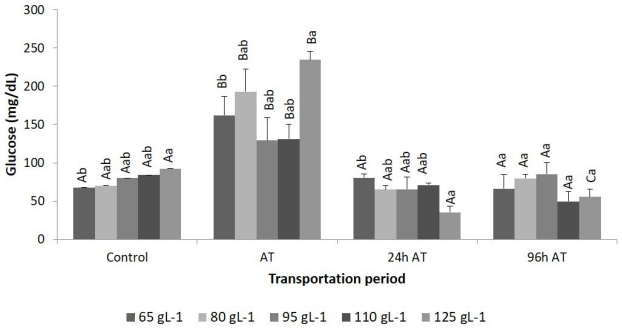

Figure 1. Mean plasma glucose concentration (mg dL^{-1}) during the transportation procedure of pirarucu, *Arapaima gigas* fingerlings at different densities. Control – before transport; AT – immediately after transport, 24 h AT – 24 h after transport; 96 h AT – 96 h after transport. Different lowercase superscript letters indicate significant differences between densities. Uppercase letters denote differences between transportation periods (Tukey, $p < 0.05$).

Table 2. Cortisol, lactate and glucose levels in pirarucu *Arapaima gigas* fingerlings at different densities and at different times during the transport process. Control – before transport; AT – immediately after transport, 24 h AT – 24 hours after transport; 96 h AT – 96 hours after transport. Values are the mean ± standard deviation of three replicates. The significance of the two-way ANOVA for each dependent variable is also shown.

Density ($g\ L^{-1}$)	Transportation period	Cortisol ($\mu g\ dL^{-1}$)	Lactate ($mg\ dL^{-1}$)	Glucose ($mg\ dL^{-1}$)
65	Control	4.89 ± 4.58	9.74 ± 0.73 Ca	67.54 ± 11.69 Ab
	AT	7.55 ± 5.26	24.44 ± 2.08 Aa	161.90 ± 24.71 Bb
	24 h AT	2.73 ± 0.75	25.81 ± 10.56 Aa	80.31 ± 4.95 Ab
	96 h AT	4.86 ± 4.17	20.17 ± 10.18 Ba	65.99 ± 18.75 Aa
80	Control	6.55 ± 0.27	8.21 ± 1.45 Ca	69.96 ± 26.52 Aab
	AT	8.75 ± 6.28	25.98 ± 3.41 Aa	193.15 ± 29.37 Bab
	24 h AT	8.12 ± 0.81	27.35 ± 8.34 Aa	65.12 ± 5.54 Aab
	96 h AT	5.69 ± 3.79	14.02 ± 10.87 Ba	79.70 ± 4.76 Aa
95	Control	7.71 ± 2.60	9.23 ± 2.90 Ca	80.04 ± 6.56 Aab
	AT	11.04 ± 8.25	30.26 ± 4.89 Aa	129.03 ± 30.22 Bab
	24 h AT	7.95 ± 2.52	22.05 ± 3.12 Aa	65.39 ± 16.21 Aab
	96 h AT	7.34 ± 3.27	19.32 ± 3.49 Ba	85.28 ± 15.14 Aa
110	Control	4.92 ± 6.93	6.67 ± 5.08 Ca	83.87 ± 11.40 Aab
	AT	11.92 ± 10.22	29.57 ± 0.78 Aa	131.12 ± 19.25 Bab
	24 h AT	13.08 ± 8.05	23.25 ± 9.82 Aa	70.97 ± 2.71 Aab
	96 h AT	4.58 ± 0.15	16.58 ± 6.92 Ba	49.40 ± 12.69 Aa
125	Control	5.50 ± 2.87	5.64 ± 3.63 Ca	92.14 ± 8.27 Aa
	AT	12.05 ± 5.98	18.29 ± 8.34 Aa	234.74 ± 11.13 Ba
	24 h AT	2.13 ± 1.07	10.43 ± 0.36 Aa	35.08 ± 7.98 Aa
	96 h AT	6.87 ± 4.96	13.16 ± 9.21 Ba	55.38 ± 9.98 Ca
Density (D)		ns	ns	$p < 0.01$
Transportation period (P)		ns	$p < 0.001$	$p < 0.001$
D x P interaction		ns	ns	$p < 0.01$

Different lowercase letters indicate significant differences between densities and uppercase letters denote differences between transportation periods (Tukey test, $p < 0.05$); ns (not significant).

Juvenile trial

No mortality was observed immediately after transportation or during the 96-hour recovery period. Temperature, dissolved oxygen, unionized ammonia and CO_2 increased significantly after transportation, but pH decreased. Water dissolved oxygen concentration, pH and temperature did not differ significantly among densities immediately after transportation, whereas unionized ammonia and CO_2 increased with density (Table 3).

Juvenile pirarucu physiological responses were significantly influenced by the density/time interaction (immediately before, after, and 24 h and 96 h after transportation), except the blood lactate levels (Table 4).

Blood lactate was higher after transportation at higher densities and did not return to the initial levels after the recovery period. Cortisol levels did not increase significantly with transportation, except for the 170 g L^{-1} density, which increased after transportation and did not return to the initial values after the recovery period (Figure 2). Glucose levels increased after transportation for most densities and returned to the initial values during the recovery period (24 h and 96 h) (Figure 3a). Hematocrit levels decreased significantly after transportation and during the recovery period for all the densities, and were lowest for the 140 and 170 g L^{-1} densities (Figure 3b).

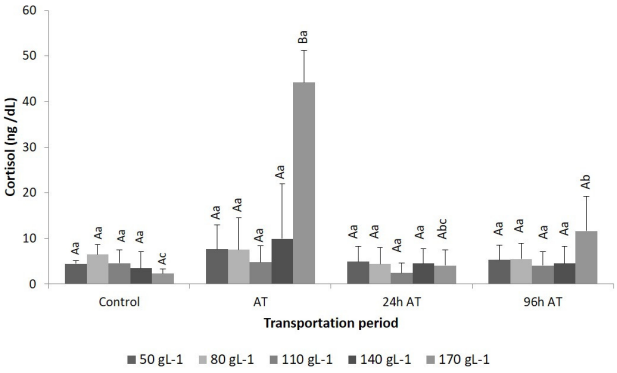

Figure 2. Mean plasma cortisol concentration (ng dL^{-1}) during the transportation procedure of pirarucu, *Arapaima gigas* juveniles at different densities. Control – before transport; AT – immediately after transport, 24 h AT – 24 h after transport; 96 h AT – 96 h after transport. Different uppercase superscript letters indicate significant differences between densities. Lowercase letters denote difference between transportation periods (Tukey, $p < 0.05$).

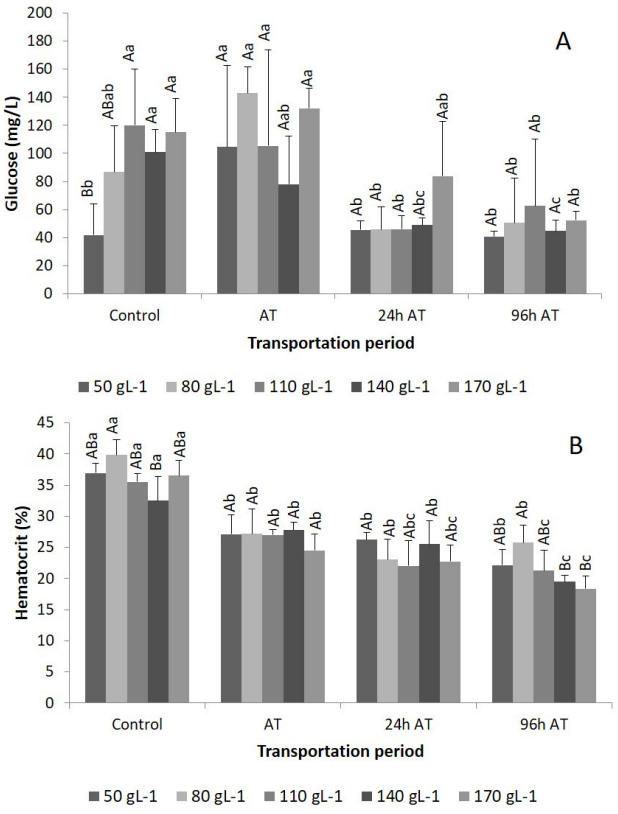

Figure 3. Mean plasma glucose concentrations (mg dL^{-1}) (A) and hematocrit (%) (B) during the transportation procedure of pirarucu, *Arapaima gigas* juveniles at different densities. Control – before transport; AT – immediately after transport, 24 h AT – 24 h after transport; 96 h AT – 96 h after transport. Different uppercase superscript letters indicate significant differences between densities. Lowercase letters denote differences between transportation periods (Tukey, $p < 0.05$).

Table 3. Water quality parameters immediately before (control) and after juvenile of pirarucu *Arapaima gigas* transportation for six hours in plastic bags at densities of 65, 80, 95,110 and 125 g L^{-1}. Values are the mean ± standard deviation of three replicates.

Water parameter	Control	After transportation				
		50 g L^{-1}	80 g L^{-1}	110 g L^{-1}	140 g L^{-1}	170 g L^{-1}
Temperature (°C)	27.8 A	31.75 ± 0.15 Ba	31.97 ± 0.17 Ba	31.55 ± 0.13 Ba	31.4 ± 0.08 Ba	31.42 ± 0.05 Ba
Dissolved oxygen (mg L^{-1})	5.55 A	16.92 ± 1.93 Ba	15.7 ± 0.74 Ba	16.23 ± 2.89 Ba	14.91 ± 2.48 Ba	15.24 ± 1.98 Ba
pH	6.79 A	6.26 ± 0.10 Ba	6.17 ± 0.10 Ba	6.12 ± 0.07 Ba	6.15 ± 0.05 Ba	6.16 ± 0.07 Ba
Unionized ammonia (mg L^{-1})	0.05 A	0.32 ± 0.09 Bc	0.50 ± 0.10 Bc	0.80 ± 0.10 Bb	0.94 ± 0.07 Bb	1.34 ± 0.17 Ba
Carbon dioxide (mg L^{-1})	2.0 A	3.88 ± 0.63 Bc	4.08 ± 0.00 Bc	8.25 ± 1.94 Bb	7.87 ± 1.44 Bb	11.7 ± 0.48 Ba

Different lowercase letters indicate significant differences among treatments (one-way ANOVA, followed by Tukey test $p<0.05$). Uppercase letters denote significant differences between the control and after the transportation values, Student's t-test ($p<0.05$).

Table 4. Cortisol, lactate, glucose and hematocrit levels in pirarucu *Arapaima gigas* juvenile at different densities and for distinct transport process periods. Control – before transport; AT – immediately after transport, 24 h AT – 24 h after transport; 96 h AT – 96 h after transport. Values are the mean ± standard deviation of three replicates. The significance of the two-way ANOVA for each dependent variable is also shown.

Density (g L^{-1})	Transportation period	Cortisol (µg dL^{-1})	Lactate (mg dL^{-1})	Glucose (mg dL^{-1})	Hematocrit (%)
50	Control	4.45 ± 0.76 Aa	44.06 ± 29.25 Ab	41.90 ± 22.03 Bb	36.96 ± 1.53 ABa
	AT	7.67 ± 5.26 Aa	80.98 ± 16.44 Aa	104.59 ± 58.17 Aa	27.08 ± 3.17 Ab
	24 h AT	4.95 ± 3.28 Aa	34.59 ± 12.34 Aa	45.48 ± 6.28 Ab	26.22 ± 1.17 Ab
	96 h AT	5.33 ± 3.23 Aa	104.58 ± 20.35 Aa	40.82 ± 3.97 Ab	22.17 ± 2.50 ABb
80	Control	6.48 ± 2.22 Aa	34.01 ± 16.98 Ab	86.72 ± 32.95 ABab	39.83 ± 2.44 Aa
	AT	7.53 ± 7.04 Aa	98.76 ± 43.48 Aa	142.94 ± 18.65 Aa	27.17 ± 4.02 Ab
	24 h AT	4.42 ± 3.56 Aa	50.62 ± 33.70 Aa	45.83 ± 16.28 Ab	23.00 ± 3.30 Ab
	96 h AT	5.53 ± 3.45 Aa	77.45 ± 27.71 Aa	50.49 ± 32.09 Ab	25.75 ± 2.77 Ab
110	Control	4.59 ± 2.90 Aa	36.45 ± 40.39 Ab	120.06 ± 39.85 Aa	35.50 ± 1.29 ABa
	AT	4.78 ± 3.58 Aa	75.62 ± 23.46 Aa	105.37 ± 68.17 Aa	27.00 ± 0.82 Ab
	24 h AT	2.47 ± 2.20 Aa	82.61 ± 30.04 Aa	46.12 ± 9.68 Ab	22.00 ± 4.08 Abc
	96 h AT	4.07 ± 3.01 Aa	85.64 ± 34.46 Aa	62.43 ± 47.59 Ab	21.25 ± 3.30 ABc
140	Control	3.47 ± 3.64 Aa	50.54 ± 38.11 Ab	100.99 ± 16.21 Aa	32.50 ± 3.87 Ba
	AT	9.96 ± 12.07 Aa	71.70 ± 22.66 Aa	78.15 ± 34.17 Aab	27.75 ± 1.26 Ab
	24 h AT	4.56 ± 3.21 Aa	93.94 ± 37.64 Aa	48.87 ± 4.96 Abc	25.50 ± 3.79 Ab
	96 h AT	4.53 ± 3.72 Aa	66.30 ± 16.40 Aa	44.77 ± 7.45 Ac	19.50 ± 1.00 Bc
170	Control	2.33 ± 1.00 Ac	66.54 ± 42.66 Ab	115.11 ± 23.90 Aa	36.50 ± 2.52 ABa
	AT	44.18 ± 7.03 Ba	133.31 ± 13.90 Aa	132.13 ± 14.12 Aa	24.50 ± 2.65 Ab
	24 h AT	4.10 ± 3.36 Abc	105.59 ± 17.75 Aa	83.90 ± 39.06 Aab	22.75 ± 2.63 Abc
	96 h AT	11.64 ± 7.59 Ab	79.00 ± 49.64 Aa	52.26 ± 6.33 Ab	18.33 ± 2.08 Bc
Density (D)		$p < 0.05$	$p < 0.05$	$p < 0.01$	$p < 0.001$
Transportation period (P)		$p < 0.01$	$p < 0.001$	$p < 0.001$	$p < 0.001$
D x P Interaction		$p < 0.05$	ns	$p < 0.05$	$p < 0.01$

Different uppercase superscript letters indicate significant differences between densities. Lowercase letters denote difference between transportation periods (Tukey, $p < 0.05$); ns (not significant).

DISCUSSION

The mortality of fingerlings at higher densities demonstrated that transportation at such densities had irreversible stress effects or osmoregulatory dysfunction on the fish, which Lim *et al.* (2003) stated as the most frequent cause of post-shipment fish mortality. In general, small fish are not affected by transportation stress and most mortality occurs during recovery, possibly due to additional stress from acclimation (Froese 1988), which results in immune suppression (Harmon 2009) and even death. Although no mortality was observed for juveniles, cortisol increased after transportation and had not returned to the initial values by the end of the recovery period. This finding indicates that the loading capacity in closed systems (plastic bags) came very close to the maximum at 170 g L^{-1}. Our results are consistent with those reported by Lima and Oliveira (2018) in juvenile pirarucu (9.0 kg) transported at a density of 160 g L^{-1} in open systems with no mortality. This demonstrates that pirarucu can withstand higher stocking densities as they grow.

As expected, water quality deteriorated after transportation. Higher water temperature after transporting fingerlings or juveniles was due to the plastic bags being exposed to air temperature, which is frequently higher than the water temperature in the study area. The same trend has been observed for pirarucu transportation in an open system (Lima and Oliveira 2018).

A higher water oxygen concentration after transportation is commonly reported for fish transported in plastic bags as pure oxygen is injected into water (Carneiro *et al.* 2009; Zeppenfeld *et al.* 2014). Our oxygen concentrations after transporting both fingerlings and juveniles were at the levels recommended for fish (Boyd 1979), which indicates that the water oxygen was not a limiting factor for transportation (Harmon 2009). The higher oxygen levels after transportation in the juvenile bags as compared to fingerlings, can be related

to the characteristic of this species to become obligate air-breathing as they grow (1 kg), whereas fingerling (24.5 g) respiration is aquatic (Brauner *et al*. 2004).

The decrease in water pH after transporting in both fingerlings and juveniles was similar to what was reported by Treasurer (2012) for the transportation of *Gadus morhua* Linnaeus 1758, and by Carneiro *et al*. (2009) for *Rhamdia quelen* (Quoy and Gaimard 1824). The drop in water pH is probably related to the increased CO_2 levels observed at the end of transport, which result from animal metabolism (Harmon 2009). As the drop in pH was similar for all the tested densities, it is apparently not related to juvenile mortality. As pirarucu is a native fish species to the Amazon basin, where water is naturally acidic (Gomes *et al*. 2003), it tolerates pH values below 7.0.

The unionized ammonia levels increased at higher densities in both fingerlings and juveniles, even after the 24-hour fasting period recommended prior to fish transportation (Conte 2004; Harmon 2009), as also reported by Carneiro *et al*. (2009) with *Rhamdia quelen.* During transportation of the juveniles, the unionized ammonia levels were below 2.0 mg L^{-1}, which was the maximum concentration reported by Cavero *et al*. (2004) and did not cause mortality in the 2.6 kg juvenile pirarucu (Cavero *et al.* 2004). Unionized ammonia concentration during transportation was 2.39 mg L^{-1} for fingerlings at 95 g L^{-1} with no recorded mortality, which demonstrates that it was within the limits for pirarucu of 24 g at this density. At the densities for which mortality occurred, concentrations went above 3.0 mg L^{-1}, a high value, but still below those reported by Lima and Oliveira (2018) for transportation water of juvenile pirarucu (9 kg), with no reported mortality. Nevertheless, younger individuals are frequently more sensitive to ammonia (Salin and Williot 1991), which indicates that unionized ammonia may be one of the factors that contributed to the mortality that we observed during the transportation of fingerlings.

An increase in CO_2 during transportation was expected as a result of fish metabolism in a closed system. Although CO_2 accumulation in water may be more critical for fish than ammonia (Wedemeyer 1996; Portz *et al*. 2006), it occurred uniformly at all fingerling densities. Although CO_2 levels increased at higher juvenile densities, they generally did not exceed the values recommended by Wedemeyer (1996) as being safe for freshwater fish, i.e., below 20-30 mg L^{-1}, so that we suggest that it was not responsible for the observed fingerling mortalitiy. Additionally, a sharp rise in water CO_2 levels may result in blood acidification and higher hematocrit values (Paterson *et al*. 2003), which was not observed in our juveniles. Yet the low hematocrit values after transportation and during the recovery period demonstrate that the CO_2 values were at the limit tolerated by the species.

Although longer fasting periods are recommended for larger fish (Froese 1998), ammonia excretion was greater in the fingerling pirarucu than in the juveniles. Furthermore, fingerling oxygen consumption and CO_2 production were higher than in the juveniles, even though they were handled similarly and at lower densities. This scenario suggests that younger pirarucu have higher metabolic rates which, in turn, result in faster water deterioration and, thus, less carrying capacity, as reported by Berka (1986) and Portz *et al*. (2006), who recommend lower densities for smaller individuals.

Physiological parameters are good indicators of the fish stress response and can be influenced by water quality deterioration, a critical factor in fish transportation (Sampaio and Freire 2016), as observed in all our treatments. Of all the measured parameters, lactate increased significantly after transportation for both fingerlings and juveniles, and did not returned to the initial values until the end of the recovery period, as also reported by Gomes *et al*. (2006) in pirarucu transportation at different salinities. Similar to that observed by Gomes *et al.* (2006), lactate was the strongest physiological response in pirarucu because it had not returned to the initial values after the recovery period. Higher post-transportation lactate levels indicate lactic acid accumulation due to increased physical activity (Barton *et al*. 2002; Dobsikova *et al*. 2009), which is possibly related to capture, handling and confinement stress during transportation (Harmon 2009; Iversen *et al*. 2005; Portz *et al.* 2006). This further stresses the need to use anaerobic metabolism when the aerobic metabolism cannot provide fish with enough energy (Oliveira *et al*. 2018). Additionally, increased unionized ammonia in the water may result from fish agitation (Portz *et al*. 2006), and, consequently, higher lactate levels after transportation. A change in lactate levels is a frequent stress response in fish (Ross and Ross 2008) and may be quite variable. Atlantic salmon (Iversen *et al*. 2005) and *Paralichthys olivaceus* (Temminck and Schlegel, 1846) (Hur *et al.* 2006) displayed similar behavior after transportation, whereas Brandão *et al*. (2006) and Lima and Oliveira (2018) reported lower lactate levels after the transportation of larger pirarucu. Finally, fish in stressful situations may use lactate to produce glucose (Mommsem *et al*. 1999), which is an important energy source in the metabolic processes of pirarucu that face stress (Gomes *et al*. 2006).

As observed in most of our treatments, rising levels of glucose, but not of cortisol, were reported for pirarucu (34 g) by Brandão *et al*. (2006), who attributed this increase to catecholamines. Catecholamines are responsible for the immediate increase in blood glucose levels, whereas cortisol maintains high levels for longer (Barton *et al*. 2002), which may explain the return of the blood glucose levels to the initial values during the recovery period. Catecholamines release is also responsible for higher blood lactate levels (Ross and Ross 2008), which may explain the increase in lactate we observed. In general, a rise in blood glucose levels is attributed to fish

needing to maintain the energy levels required for metabolic activities and, with a startle response, to provide the sudden high demand from muscles (Brandão *et al*. 2006). Gomes *et al*. (2003) have attributed high glucose levels in pirarucu (1.1 kg), even during the recovery period, to continuous stress from handling to perform blood sampling. Conversely, Lima and Oliveira (2018) did not observe any alterations in blood glucose during pirarucu transportation. Generally no pattern appears in the variation of blood glucose levels as a fish response to stressors, not even in the same experimental group (Mommsem *et al*. 1999; Ross and Ross 2008). The different responses of glucose levels to transportation stress in pirarucu may be related to both nutritional status and developmental stage, and should be analyzed together with other physiological parameters (Mommsem *et al*. 1999; Barton *et al*. 2002).

As observed in our juvenile treatments, Carneiro *et al*. (2009) found that the cortisol level peaked at the highest density of *Rhamdia quelen*, but with no mortality. Nevertheless, higher cortisol levels in our juveniles at 170 g L^{-1} and changes in water quality parameters, especially unionized ammonia, indicate that the system carrying capacity came close to its maximum. As in our juvenile densities up to 140 g L^{-1}, Gomes *et al*. (2003) did not report any cortisol level alteration in pirarucu (1.1 kg) transported in plastic bags either with or without oxygen addition. Hence pirarucu may be capable of maintaining low cortisol levels when they are exposed to stressors for short times (Gomes *et al*. 2003; Lima and Oliveira 2018).

Brandão *et al*. (2006) and Lima and Oliveira (2018) reported increased hematocrit in pirarucu after transportation, and related this hemoconcentration to increased oxygen demand to recover from exposure to a stressor. However we transported pirarucu in plastic bags with supersaturated oxygen and, as changes in cortisol levels were minor, fish may not have activated such a route to increase oxygen availability in blood. Furthermore, pirarucu is a freshwater species and, as transportation took place without adding salt to water, water uptake by fish took place to maintain osmoregulation (Harmon 2009; Silveira *et al*. 2009). This caused hemodilution, which could explain the drop in hematocrit in our study.

CONCLUSIONS

As pirarucu had increased blood glucose and lactate levels in all our treatments, but without high cortisol levels in most treatments, a secondary stress response was observed after transportation. However, the blood glucose levels returned to the initial values by 96 h, and the lower cortisol and lactate levels demonstrated how fast pirarucu can recover from a stressful situation. Densities up to 95 g L^{-1} can be used safely to transport fingerling pirarucu. The 110 g L^{-1} density presented low mortality, but cortisol levels did not increase, and physiological parameters did not differ from fish at lower densities, save ammonia levels, and should be used with caution, preferably with longer fasting periods. The 140 g L^{-1} density is not recommended for transporting fingerling pirarucu, but densities up to 140 g L^{-1} may be safely used to transport juveniles. The 170 g L^{-1} density presented no mortality for juveniles, but increased the cortisol which remained higehr than initial values by the end of the recovery period. These findings indicate that the system carrying capacity at this density came close to its maximum and is, thus, not recommended for transporting juvenile pirarucu. Finally, as the lactate and hematocrit values had not returned to the initial values after the recovery period, we recommend avoiding fish being exposed to other stressors during this period.

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