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DIGESTIVE ENZYMATIC ACTIVITY OF PIRARUCU "AMAZON COD" USING LIVE FOOD DURING INITIAL FEEDING TRAINING

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ABSTRACT

The objective of this work was to verify the digestive enzymatic activity of pirarucu (Arapaima gigas) using live food during initial feeding training. A total of 804 juveniles of pirarucu were homogeneously distributed in six circular PVC tanks with a capacity of 500 L (400 L water volume) and a flow rate of 10 L h-1. The experiment was conducted in three phases, four days for each phase. First phase: providing live food diluted in water; Second phase: providing live food diluted in water with gradual introduction of feed in the proportion of 1%, 2%, and 3% of total fish biomass; Third phase: fishes were fed only with feed until they were satiated. During the feeding period, fishes were fed six times a day: 08:00, 10:00, 12:00, 14:00, 16:00 and 18:00 h. Two types of live prey were tested in triplicate: T1 = nauplii of *Artemia* sp.; T2 = mixture of native zooplankton. At the end of experiments, the results of weight gain, the percentage of animals that ate and survival did not present any significant statistical differences between treatments ($p > 0.05$). *Artemia* sp. and Amazon zooplankton mix are efficient for the initial feeding training of pirarucu.

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INTRODUÇÃO

The pirarucu (*Arapaima gigas*), also known as the "Amazon cod", is a large-sized species that is carnivorous, native to the Amazon basin, and can reach, in natural conditions, 3 meters in length and attain a weight of 200 kilograms (Vital and Tejerina-Garro 2018). The average yield of fillets is 57% with very few intramuscular bones, and the fillet meat is clear, low in fat, and very tender, making this fish command a high market value (Rodrigues *et al* 2019). The production of fry depends exclusively on spontaneous and continuous reproduction in large nursery ponds where the animals form couples and spawn up to 7 times a year (Saint-Paul 2017). Because it is a carnivorous fish, pirarucu cannot be raised in an initial intensive regime because it does not voluntarily accept balanced feed mixtures, as is common for other carnivorous fish such as the speckled catfish (*Pseudoplatystoma coruscans*), the peacock bass (*Cichla*sp) and the trahira (*Hopliaslacerdae*) whichalso present similar difficulties when raised in captivity (Soares *et al*. 2007; Barbosa *et al*. 2011; Luz and Portella 2015). In general, the initial phases of growth related to feeding transition periods for carnivorous fish are critical due to the difficulty that these animals present in accepting a dry feed diet (Souza *et al*. 2015). For pirarucu, this phase is of fundamental importance because during this period diseases often appear that can reduce fry survival (Marinho *et al*. 2013). The information about the profile of digestive enzymes during the period of initial feeding training is of great importance for full understanding of nutritional physiology of pirarucu. From knowledge about the enzymatic profile of this species, the use of feeds with exogenous enzymes can be tested, thus improving zootechnical results through better absorption efficiency of ingredients and nutrients in commercial feeds for carnivorous fish (Cavero *et al*. 2019). Therefore, the objective of this research was to analyze and compare the enzymatic profile present in the digestive tract of pirarucu during initial feeding training with two types of live prey.

MATERIALS AND METHODS

Study site, experimental units and treatments: This research was conducted at the Coordination for Research in Aquaculture (CPAQ) at the National Institute for Research in the Amazon (INPA), Manaus –

Amazonas – Brazil. A total of 804 male and female pirarucu with an average weight of 1.5 ± 0.1 g, total average length of 5.0 ± 0.1 cm and 20 days of age were received and immediately distributed ($p>0.05$) in six circular PVC tanks with a capacity of 500 L (water volume was 400 L) and a flow rate of 10 L h^{-1}. The fish were fed six times a day at 08:00, 10:00, 12:00, 14:00, 16:00 and 18:00 h. Two types of live prey were tested in triplicate (we supplied the prey according to the item "Types of live food"): T1 = nauplii of *Artemia* sp.; T2 = mixture of Amazon native zooplankton. This experiment was realized in agreement with the Ethical Principles for Animal Research established by the National Council for Control of Animal Experimentation (CONCEA). and was approved by the institutional Commission for Ethics in the Use of Animals of Universidade Federal do Oeste do Pará (Certificate nº 0620190077).

Types of live food: The mixture of Amazon native zooplankton was collected in a 120 m^2 nursery pond with a clay bottom using a plankton net with a 60 µm mesh size. During collection the predominance of Copepod and Cladocera plankton species were noted. The fish were fed with zooplankton diluted in 5 L of water in the following numbers of live prey: Copepod = 670,000 per feeding; Cladocera = 130,000 per feeding. The nauplii of *Artemia* sp. were provided at a proportion of 665,000 individuals per feeding.

Experimental phases: The experiment was conducted in three phases, four days for each phase (12 days of experiment). The experiment begun with the first phase that consisted of providing live food diluted in water. The second phase, together with the live food diluted in water, consisted of the gradual introduction of feed in the proportion of 1%, 2%, and 3% of total fish biomass. And in the third phase, the fish were fed only with feed until they were satiated. The feed used in this experiment was a commercial feed specific for carnivorous fish (Nutripeixe™ by Purina Co.; Ribeirão Preto, São Paulo, Brazil) that was extruded and ground, with 45% total protein and 3,000 kcal of energykg^{-1}. The size of the feed particles in both treatments was approximately 100 µm. After the end of experiment, we made biometric measurements of all fishes and the rates of eaters (ER), survival (SR) and weight gain (WG) were quantified. Additionally, observations were conducted of fish behavior with respect to aggression and competition for food during all three experimental phases.

Monitoring of water quality: Water quality was monitored daily at 09:00 through measurement of the following parameters: dissolved oxygen (mg L^{-1}), pH, temperature (ºC), total ammonia (NH_3 + NH_4) (mg L^{-1}), electrical conductivity (µS cm^{-2}), alkalinity ($CaCO_3$ mg L^{-1}), hardness (CaCO3 mg L^{-1}) and CO2 (mg L^{-1}).

Enzymatic assays: In order to determine the profile of digestive enzymes twelve fishes were collected on an empty stomach from each treatment during the initial feeding trials, every-other-day, totaling 72 animals (24 from each of the three phases). Each fish was anesthetized before the necropsy through immersion in a dilute solution of eugenol (Souza *et al*. 2012). After euthanasia, the digestives tracts were maintained on ice (0-4 ºC) for subsequent identification until the moment of the enzymatic assays. The parts of the digestive tract were divided into the stomach (determination of acid protease) and the pyloric ceca + intestine (determination of alkaline protease, amylase and lipase). For one minute, each portion of tissue (0.5 g) was homogenized in 2 mL of phosphate buffer (10 mM)-tris (20 mM) (neutral pH buffer 7.0) at 4 ºC using a tissue homogenizer. The extract clarification was made using a bench-top centrifuge in 12,000 g for three minutes at 4 ºC. Casein (Caseína™, Merck Co.; Catalog number: 102244; Cotia, São Paulo, Brazil), diluted 1:1000 in water, was used as the substrate solution of the reaction for the determination of proteolytic activity. For the pH assays, 1 mL of glycine/HCl 0.1 M (acid pH buffer 3.0) and 1 ml of tris-HCl 0.1 M (alkaline pH buffer 8.0) were used. The variation in the concentration of the enzyme in vitro was conducted using endogenous protease contained in the cellular homogenate. After incubation at 30 ºC the mixture of the reaction was interrupted by the addition of 250 µl of TCA (trichloroacetic acid) at 8% and centrifuged at 3,000 g for 10 minutes. The absorbance of the supernatant was measured at 280 nm (Hidalgo *et al*. 1999). The protease activity was calculated by creating a standard tyrosine curve and determining using a relationship between the value of Upro (1.0 µmol of tyrosine released per minute) and the wet weight from the stomach tissue (organ responsible for the production of acid protease) and intestinal tissue (organ responsible for the production of alkaline protease). Enzymatic activity of lipase of the cellular homogenate was measured according to Gawlicka *et al*. (2000). The homogenate was incubated at 30 ºC in a buffer of ammonium bicarbonate 24 mM, pH 7.8, containing 0.4 mM of substrate (p-nitrophenyl myristate, Sigma N2502) diluted in 0.5% Triton X-100. Incubation time was 30 minutes, and the sample absorbance reading was done at 405 nm. Lipase activity was calculated by creating a standard curve of p-nitrophenol and determined using a relationship between the value of Ulip (1.0 µmol of p-nitrophenol released per minute) and the wet weight from the intestinal tissue. Amylo-hydrolytic activity was estimated using a modification of the method of Bernfeld (1955). The reaction was incubated in 1.0 mL of buffer of sodium/phosphate citrate of mono-basic sodium 0.2M pH 8.0, and 1 mL 0.5% starch solution in buffer with addition of 100 µL of the cellular homogenate. This reaction mixture was incubated for 30 minutes at 30 °C. Subsequently, 250 µL of 5% zinc sulfate was added, followed by 250 µL of barium hydroxide 0.3 N, and the reaction mixture was centrifuged at 12,000 g for 3 minutes. Glycose concentration was measured in the supernatant using the method of Park-Johnson (1949). Amylase activity was calculated using a relationship between the value of Uamy (1.0 µmol of reduction of starch in glucose released per minute) and the wet weight from the intestinal tissue. The determination of the activities of protease, lipase and amylase in the extracts from the digestive tracts of the juvenile pirarucu was done using end point spectrophotometry (Worthington Enzyme Manual, 1982) and were expressed in IU (inhibition unit) per milligram of protein. The homogenized protein contents were obtained using the Lowry method (Villela *et al*. 1973).

Theory/Calculation

Growth, survival and quantity of animals that fed were calculated by:

Eaters rate (ER) = (fish that accepted feed / total fish) * 100
Survival rate (SR) = (number of fishes at the end / number of fishes at the start) *100
Weight gain (WG) = average final weight (g) - average initial weight (g)

Enzymatic activity was calculated by:

Acid protease activity (ACPA) = Upro / stomach tissue (mg)
Alkaline protease activity (ALPA) = Upro / intestinal tissue (mg)
Lipase activity (LA) = Ulip / intestinal tissue (mg)
Amylo-hydrolytic activity (AA) = Uamy / intestinal tissue (mg)
Upro = 1.0 µmol of tyrosine released per minute
Ulip = 1.0 µmol of p-nitrophenol released per minute
Uamy = 1.0 µmol of reduction of starch in glucose released per minute

Statistical analysis: The treatment averages for the water quality, eaters rate (ER), survival rate (SR), and weight gain (WG) were compared using a Student's t-test at a level of α=5% probability. Before being submitted to statistical tests, the values of SR and ER, expressed as a percentage, were transformed using the arc-sine transformation (Bhujel 2009).

RESULTS

Water quality: The water physicochemical parameters measured during the experimental period did not present any variation that could have interfered with the development of fish in both treatments (Table 1).

Table 1. Water quality parameters (average ± standard deviation) during initial feedingof juvenile pirarucu, *Arapaima gigas*, with live prey as initial diet [(1)]

Observed parameter	Treatments			
	Artemia sp.	Amazon zooplankton	"t"	DF
pH	6.1 ± 0.4 a	6.2 ± 0.4 a	1.23	35
Temperature (ºC)	26.3 ± 0.4 a	26.4 ± 0.5 a	1.26	35
Electrical conductivity (μS cm^{-2})	33.9 ± 4.6 a	39.4 ± 5.0 a	1.30	35
Dissolved oxygen (mg L^{-1})	6.1 ± 0.3 a	5.9 ± 0.2 a	1.26	35
Total ammonia ($NH_3 + NH_4$)	3.10^{-3}± 10^{-3} a	2.10^{-3}± 10^{-3} a	1.77	35
Alkalinity (mg $CaCO_3$ L^{-1})	17.8 ± 3.9 a	18.9 ± 2.9 a	1.36	35
Hardness (mg $CaCO_3$ L^{-1})	8.5 ± 2.6 a	9.1 ± 2.8 a	1.36	35
Carbon dioxide (mg L^{-1})	0.6 ± 0.2 a	0.5 ± 0.1 a	1.57	35

Legend: (DF) = Degrees of freedom
[(1)] Averages in rows followed by the same letter are not different by the Students t-test at 5% probability.

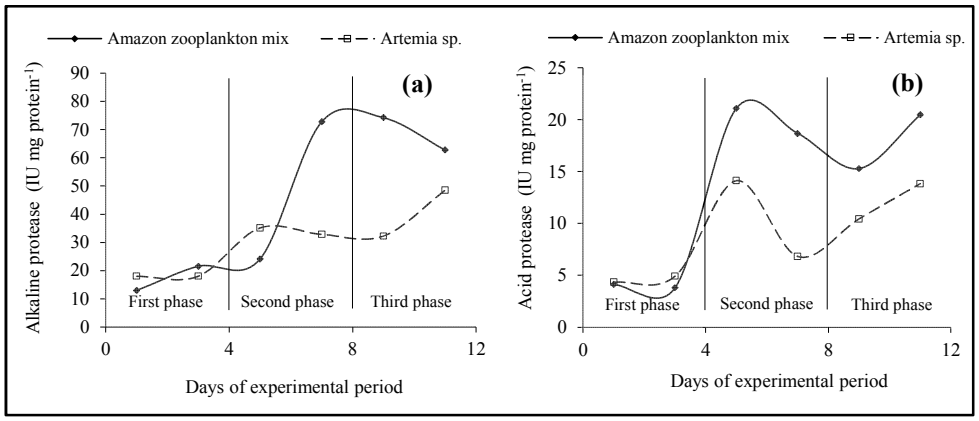

Figure 1. Profile of enzymatic activity, alkaline protease (pH 8.0) (a) and acidic protease (pH 3.0) (b) of juvenile pirarucu during feed training

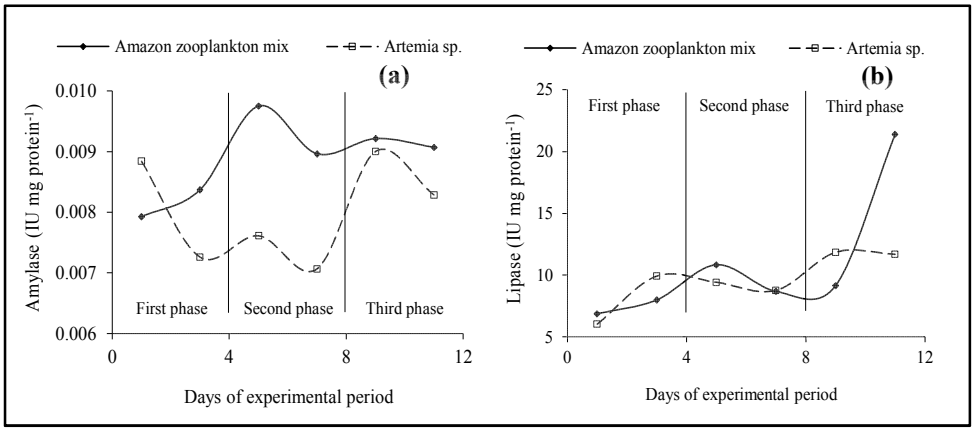

Figure 2. Specific activity of amylase (a) and lipase (b), of juvenile pirarucu during feed training

Table 2. Survival, percentage of eaters and weight gain of juvenile pirarucu, *Arapaima gigas*, submitted to initial feeding with live prey as the initial diet [(1)]

Initial diet	SR (%)	ER (%)	WG (g)
Artemia sp.	99.0 ± 0.4 a	99.0 ± 0.4 a	1.0 ± 0.1 a
Zooplankton	99.8 ± 0.4 a	99.8 ± 0.4 a	1.0 ± 0.1 a
Degrees of freedom	2	2	2
"t"	4.03	4.03	4.27

Legend: (SR) = Survival Rate; (ER) = Eaters Rate; (WG) = Weight Gain
[(1)] Averages in rows followed by the same letter are not different by the Students t-test at 5% probability. Values are average ± standard deviation.

Digestive enzymatic activity: Figures 01 and 02 show that juvenile pirarucu had an increase in enzymatic activity after the initial period of initial feeding, demonstrating that there is a natural protease, lipase and amylase response after the initial use of commercial feed during initial feeding. The acidic and alkaline proteolytic activity showed differences between the treatments (Figure 1). In the treatment that used Amazon zooplankton as live prey during initial feeding the juvenile pirarucu had greater enzyme activity. With respect to amylolytic activity, the animals from the treatment that used zooplankton as live prey had a more intense enzymatic response in comparison to the treatment with *Artemia* sp., and this difference was more pronounced in the first days after the initial feeding with commercial feed. Lipolytic activity showed a tendency of enzymatic response that was similar between treatments during the first days after feeding with commercial feed, but at the end of the initial feeding period the animals from the zooplankton treatment had a superior enzymatic response (Figure 2). These results for lipolytic and amylolytic activity were lower than those for proteolytic (acidic and alkaline) activity.

Growth, eaters, survival and behavior: With respect to the eaters rate (ER), survival rate (SR) and weight gain (WG), the treatments did not present statistically significant differences ($p<0.05$). The rates for FR and SR showed results near 100% using zooplankton and *Artemia*

sp. as the initial diet (Table 2). In relation to behavior, the animals displayed aggressiveness by biting the dorsal fins of competitor fish.

DISCUSSION

The physicochemical data for water from the experimental units are in agreement with those suggested for pirarucu raised in captivity (Oliveira *et al*. 2012; Cavero *et al*. 2019). During the experiment the temperature of the water in the tanks was stable (Table 1) indicating that this factor did not interfere in the enzymatic activity of the fish during the different phases of initial feeding. According to Hill and Lawson (2015), the optimum temperature for pirarucu varies between 24 and 31 °C. In comparison with fish from temperate waters, tropical fish have more rapid body development due to the effect of temperature on their metabolism (Castro-Ruiz *et al*. 2019). Since these are poikilothermic animals, water temperature will also influence enzymatic activity, since on cold days these fish will eat less, causing less mechanical stimulation of the digestive tract and a decrease in the secretion of cholecystokinin, a gastrointestinal hormone that is important for the activation of pancreatic enzymatic secretion in Teleostei (Navarro-Guillén *et al*. 2017). In order to classify fish as omnivores, herbivores, or carnivores, morphometric parameters are commonly used and residual food in the digestive tract of these animals is analyzed. However, it is also possible to classify fish by relating feeding habits with the profile of digestive tract enzymes (Hani *et al*. 2018). In the case of pirarucu, proteolytic activity has been shown to be superior to lipolytic and amylolytic activities (Figures 01 and 02).

Other species of carnivores, such as sturgeon (*Acipenser naccarii*) and trout (*Oncorhynchus mykiss),* have enzymatic behavior where lipolytic and amylolytic activities are less than proteolytic activity. Phylogenetic and ontogenetic characteristics are determining factors for these aspects, because even when these carnivorous species are fed with diets that are predominately composed of lipids or carbohydrates, the quantity of protease (acidic and alkaline) secreted is greater in comparison to amylase and lipase (Sanz *et al*. 2015). With respect to differences in lipolytic activity, of the juvenile pirarucu fed with zooplankton and *Artemia* sp., Sontakke *et al*. (2019) reported a statistical difference in lipase produced by the carnivorous osteoglossum*Notopteruschitala* that was influenced by the profile of fatty acids of the different types of live prey (*Artemia* sp., *Moina* sp., Tubifex) used in the experimental diets. Overall, enzymatic activity of proteases (acidic and alkaline), amylase and lipase was greater (only in the third phase) when the feeding transition occurred first using native zooplankton (Figures 1 and 2). This relationship is probably due to the fact that these microorganisms represent the natural food source of pirarucu during the first phases of life, although in both treatments there was an increase in enzymatic activity from the moment when the feed was offered. It is important to state that carnivorous fish naturally produce very little amylase in comparison to other enzymes (Figure 02). In general, carnivorous fish, as pirarucu, have a tendency to produce a greater quantity of amylase only during the first days of life, and this volume is considerably reduced over time (Castro-Ruiz *et al*. 2019).

Amylolytic activity of pirarucu was not influenced by the introduction of feed, even by the commercial feed that contains pre-gelatinized starch, a nutrient that is obtained after the process of making the extruded feed. The term "pre-gelatinized" is used to explain structural transformations of starch that occur during the production of making the extruded feed. The process of extrusion is conducted using high temperatures that cause the degradation of the most insoluble crystalline parts of starch, increasing the solubility and absorbability of the starch by fish, besides conferring greater stability and durability of the feed pellets (Romano *et al*. 2018). Souza *et al*. (2015) analyzed juvenile pirarucu submitted to periods of initial feed training wherein there was a progressive substitution of highly palatable humid feed (a pâté or pasta of ground fish) for a diet composed of dry feed and found no statistical differences between treatments. This means that the duration of the period of transition between humid and dry feeds did not influence the zootechnical responses of the animals. The survival rate obtained in this experiment was superior to that reported by Luz and Portella (2015), who conducted initial feed training with trahira (*Hopliaslacerdae*), and observed survival above 95% when using only nauplii of *Artemia* sp. at different densities. Additionally, these authors reported a rate of more than 88% from the moment that commercial feed began to be progressively introduced into the diet. Luz *et al*. (2011), using nauplii of *Artemia* sp. and a Scott emulsion for initial feed training of pacamã (*Lophiosilurus alexandri*), found survival rates that oscillated between 60% and 73%. However, there was a statistical difference only for the treatment that used commercial feed during the entire period of initial feed training. Agadjihouèdé *et al*. (2012) submitted two species of catfish native to the African continent, *Clariasgariepinus* and *Heterobranchuslongifilis*, to initial feed training using *Artemia* sp. and freshwater zooplankton during the larval phase.

After 10 days of the experiment, survival rates were more than 97% for the larvae of *C. gariepinus*, and more than 78% for the larvae of *H. longifilis*, with no statistical differences between the treatments using *Artemia* sp. and zooplankton. The eaters rate (%) did not present statistical differences between treatments, and the values were very near the total number of fishes used in the experiment, which is an important result because it is a signal that the juvenile pirarucu trained with live prey do not need any extra stimulus for acceptance of dry feed. Since the nutrients in extruded feed are subject to leaching losses, the use of live prey during initial feed training of pirarucu is crucial for water quality maintenance and success of intensive cultivation systems (Santos *et al*. 2013). The description of the ontogeny of digestive enzymatic activity, for marine as well as freshwater species, is important because changes in digestive capacity will determine the types of nutrients that can be absorbed by fish, and this can be an indicator that can be used for the determination of larval development and the degree of maturity of the digestive tract (Castro-Ruiz *et al*. 2019). Knowledge of the exact quantity and the specificity of each enzyme that exists in a digestive system, and the conditions in which enzymatic hydrolysis occurs allows for the estimation of the digestibility of food with greater precision. This will enable the production of feeds that include supplementary quantities of exogenous enzymes in order to improve results related to zootechnical performance and economic viability (Cavero *et al*. 2019). The aggressive behavior observed in this study can be associated with the progressive substitution of live prey for the diet of dry feed, and this could have negatively affected fish behavior. However, juvenile pirarucu are gregarious in nature (Cavero *et al*. 2003) and can be influenced by conditions that favor the establishment of hierarchical classes based on size, since aggressive and cannibalistic behavior is more common between individuals of different size classes. In fish, cannibalism can be understood as being the act of partially or totally eating an individual of the same species, independent of age or growth stage (Pereira *et al*. 2017). Perera *et al*. (2016) cite elevated levels of cannibalism during initial feed training of *Channa striata*, as well as Agadjihouèdé *et al*. (2012) for *H. longifilis* and *C. gariepinus*, Luz and Portela (2015) for trahira (*H. lacerdae*), and Torres *et al*. (2017) for pacamã (*L. alexandri*). These results demonstrate that aggressive behavior is inherent to individuals that have carnivorous feeding habits, a phenomenon that is amply described in the scientific literature. Perera *et al*. (2016) affirm that the production of carnivorous fish can become difficult in the absence of well-developed strategies for the use of live prey; however, an initial diet of live prey in feed training trials of carnivorous fish is widely accepted. The inclusion of live prey during the initial feeding period of carnivorous fish is a feeding strategy that is considered viable in the attempt to gain acceptance of dry feed by juvenile pirarucu, since live prey is a food that is naturally consumed. This also provides the advantage of training smaller-sized individuals without the necessity of using other food sources to attract the fish. Lipolytic and proteolytic activity of pirarucu juveniles presented a positive gradient during initial feeding. Amazon zooplankton as live prey stimulated greater enzyme activity in comparison with *Artemia* sp. for the initial feeding of pirarucu.

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